Molecular Vision 2007; 13:337-44 <http://www.molvis.org/molvis/v13/a37/>
Received 5 October 2006 | Accepted 28 February 2007 | Published 1 March 2007

Identification of Notch-1 expression in the limbal basal epithelium

Padmaja B. Thomas,[1] Yi-Hsin Liu,[2,3] Feng Feng Zhuang,[3] Shivaram Selvam,[1,4] Sang W. Song,[1] Ronald E. Smith,[1,2] Melvin D. Trousdale,[1,2] Samuel C. Yiu[1,2]

[1]Ocular Surface Center, Doheny Eye Institute; [2]Department of Ophthalmology, Keck School of Medicine; [3]Center for Craniofacial Molecular Biology; [4]Mork Family Department of Chemical Engineering and Materials Science, Viterbi School of Engineering, University of Southern California, Los Angeles, CA

Purpose: To determine whether Notch-1, a ligand-activated transmembrane receptor known to maintain cells in an undifferentiated state, primarily progenitor cells in other systems, could be used as a stem cell marker in human limbal epithelium.

Methods: Human corneoscleral tissues obtained from the Doheny Eye & Tissue Transplant Bank were prepared for cross section and whole mount analysis. Tissue for whole mount was incubated in dispase; the epithelial sheet was removed and fixed in 4% paraformaldehyde. Sections and whole mount were stained with antibodies against Notch-1, Notch-2, β-1 integrin, α-6, and the G2 subtype member of the ATP binding cassette transporter (ABCG2). Specificity of the Notch-1 antibody was determined by western blot analysis with Cos-7 cells transfected with Notch-1. Explant culture was performed and only primary cultures were used in this experiment.

Results: Notch-1 was found to be expressed in the limbal basal region where stem cells reside. Notch-1 antigenicity was more pronounced in cell clusters, mainly in the palisades of Vogt. The central cornea was almost devoid of Notch-1. The intensity of Notch-1 staining in cultured cells from the limbal explants was high in only a few cells. The Notch-1 signal was diminished in dividing cells. Expression in cultured cells was more cytoplasmic; few cells showed additional nuclear staining. The Notch-1-stained whole mount showed only a few cells in the limbal region. A 300 kDa and a 110 kDa band confirmed the specificity of the antibody in Cos-7 cells transfected with Notch-1. Double staining for ABCG2 and Notch-1 showed some ABCG2-positive cells co-expressing Notch-1 in the limbal basal epithelium, indicating that Notch-1-expressing cells might be a unique subpopulation of cells with stem cell properties.

Conclusions: Immunofluorescence data shows that Notch-1 could be a possible marker for the stem cells in the limbal basal epithelium. Further studies and characterization of the Notch pathway in corneal development will provide valuable clues for the identification of stem cells.

The cornea is a complex, multilayered, multifunctional structure that provides photo protection, refraction, and transparency and helps protect internal ocular structures. Its outermost layer is the corneal epithelium, which plays a significant role in maintaining transparency. Corneal epithelium undergoes continuous self-renewal, through the actions of the stem cells in the palisades of Vogt in the corneal limbus [1-3]. These slow cycling, relatively undifferentiated, limbal stem cells have high proliferative potential [4,5]. The limbal stem cells generate transient amplifying cells (TAC) that migrate to the central cornea, which is devoid of stem cells [6]. The TAC, with only limited proliferative capacity, subsequently enter into a terminal differentiation pathway.

A deficiency of limbal stem cells, caused by chemical trauma (chemical injury) or by eye diseases such as Stevens-Johnson syndrome or ocular cicatricial pemphigoid, is a major cause of visual impairment in humans. In persons with limbal stem cell deficiency, the adjacent conjunctival cells migrate to the cornea, causing corneal opacification and subsequent functional blindness. Since limbal stem cells are required to restore the corneal epithelium, conventional corneal transplantation is unsuccessful for the above diseases. Recent therapeutic interventions demonstrate that stem cell allograft transplantation can restore vision in patients with limbal stem cell deficiency [7-9]. The ex vivo expansion of limbal stem cells was a significant milestone for restoration of corneal epithelial integrity through transplantation [10-12]. However, since the limbal epithelium consists of three cell types: stem cells, TAC, and differentiated cells, isolating a pure population of stem cells is difficult [6].

Although a wide variety of stem cell markers have been proposed, no known marker or method is available to differentiate stem cells from TAC in the corneal epithelium. However, advances in stem cell research in hematopoietic, neural, and epidermal systems help us to borrow some of the stem/progenitor cell markers and might also be usable as limbal stem cell markers. In a hematopoietic system, stem cells can be isolated using specific markers and with side scatter as the main parameter [13]. Beta-1 integrin showed a two- to three-fold increase in intensity in epidermal stem cells, compared with the epidermal TAC [14]. This same phenomenon is observed in human hair follicles [15-17]. Because corneal epithelium and epidermal keratinocytes have similar characteristics, we undertook an immunohistochemical characterization of the β-1 integrin and α-6 integrin. Some reports suggest that

Correspondence to: Samuel C. Yiu, M.D., Ph.D., Doheny Eye Institute, 1450 San Pablo Street, DEI5704, Los Angeles, CA, 90033; Phone: (323) 442-6504; FAX: (323) 442-6402; email: syiu@doheny.org

the G2 subtype member of the ATP binding cassette transporter (ABCG2) may act as a stem cell marker since it is expressed exclusively in the limbal zone and believed to be responsible for the side population phenotype of the ocular surface epithelium [18,19] which exhibits stem cell properties. We also analyzed the expression pattern of ABCG2 in the limbal and central corneal epithelium.

Previous reports suggest the role played by Notch family members in maintaining stem cells in neural and hematopoietic systems might also apply to corneal epithelial stem cells. Notch transmembrane receptor 1 (Notch 1) and the other family members are highly conserved, and play crucial roles in determining cell fates and developmental processes through cell to cell interactions [20,21]. In the presence of the ligand, notch receptors undergo sequential proteolytic cleavage steps that ultimately release the intracellular domain from the membrane. Modulation of the Notch pathway revealed its role in differentiation, proliferation, and self-renewal mainly in the neural and hematopoietic systems [22]. In the hematopoietic system, Notch receptors are found in early stem cells and their ligands are found in the bone marrow stroma [23]. Notch members are reportedly essential for maintenance, but not for the generation, of neural stem cells in mammals [24]. In mice, the Notch signals have a profound effect on the proliferative potential of the progenitors in the intestine [25]. Inactivation of Notch-1 in young mice caused the development of opaque plaques in the cornea, leading to blindness [26]. Impaired differentiation of prostatic epithelial cells was found in Notch-1 conditional knockout mice [27]. Because Notch-1 plays an active role in maintaining progenitor cells in an undifferentiated manner in several systems, we decided to evaluate its role in limbal stem cells to determine whether it could be a marker for isolating and enriching stem cells to improve transplantation.

METHODS

Reagents: Epilife corneal growth medium was purchased from Cascade Biologicals (Portland, OR). Notch-1 and Notch-2 antibodies were purchased from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA), and α-6 integrin (GOH3) from Santa Cruz Biotechnology Inc (Santa Cruz, CA). GAPDH antibody, β-1 integrin antibody, and ABCG2 antibody were purchased from Chemicon (Temecula, CA).The secondary antibodies for immunofluorescence and the HRP conjugated secondary antibody for western blotting were purchased from Jackson Immunoresearch (West Grove, PA). The research grade chemicals for western blot were obtained from Roche Applied Sciences (Indianapolis, IN).

Cell culture: Research grade human corneoscleral tissues were obtained from the Doheny Eye & Tissue Transplant Bank (Los Angeles, CA). All tissues used for this study were stored for less than 4 days in preservative (Optisol-GS; Baush and Lomb, Rochester, NY). Human tissue was handled according to the tenets of the Declaration of Helsinki. Explant culture was done according to established protocols [28,29] after minor modifications. Briefly, the central portions of cornea were removed by careful trephination. The excess sclera, conjunctiva, and iris were also removed. The limbal rim was cut into pieces of about 2x2 mm^2. Each piece was put on a cover slip inside a culture dish and covered with a drop of Epilife growth media with human corneal growth supplement (HCGS). The explants were then cultured in the same media. Only primary cultures were used in this experiment.

Immunofluorescence: Immunofluorescence staining was performed according to previously reported methods [30]. In brief, cells were washed once with phosphate-buffered saline, fixed in 4% paraformaldehyde for 10 min, and then washed and blocked with 5% bovine serum albumin for 15 min. The cells were incubated in primary antibody (α-6 1:50, β-1 1:100, Notch-1 1:10, and ABCG2 1:20) for 1 h at 37 °C, then washed with phosphate-buffered saline 3 times for 5 min each. The cells were then incubated in secondary antibody at a dilution of 1:40 for 45 min, washed 3 times, and mounted with antifade mounting medium (Vectashield, Vector Laboratories, Burlingame, CA). Cryosectioned corneal buttons were stained with the antibodies using a similar protocol and analyzed by confocal microscope (Carl Zeiss Meditec, Oberkochen, Germany). The epithelial sheet was removed from the stroma by dispase digestion using the established protocol and processed for whole mount [31]. Briefly, after the rims were rubbed off, the endothelium and uvea were put in dispase 50 mg/ml for 18 h at 4 °C with moderate shaking. Under a dissecting microscope, the already loose epithelial sheet was separated with a flat-edged spatula. The whole mount was stained for β-1 integrin, Notch-1, and ABCG2. All immunofluorescence was performed with negative controls by incubating the sections with PBS in place of the primary antibody.

Western blot analysis: Notch-1 cDNA, obtained as a gift from Dr. Spyros Artavanis-Tsakonas, was transfected into the Cos-7 cell line. The Cos-7 cells were plated one day before transfection. When the cells attained 60-80% confluence, they were transfected with DNA-TransFectin (Bio Rad, Hercules, CA) complex (a reaction mixture consisted of 2 ug of Notch 1 plasmid DNA in 100 μl of the media and 8 μl of the TransFectin reagent). Twenty-four h after transfection, cells were collected and lysed for protein extraction and used for western blotting. Protein concentration was determined using the Bradford assay (BioRad). Identical amounts of protein were separated on 10% SDS/PAGE and electroblotted onto polyvinylidene fluoride membrane (Pall Life Sciences, Ann Arbor, MI). The transfer was monitored by a prestained protein molecular weight marker (Invitrogen, Carlsbad, CA). After transfer, blots were probed with hybridoma supernatant for Notch-1 at a dilution of 1:10, then incubated with a 1:10,000 dilution of horseradish peroxidase conjugated anti-rat antibody for Notch-1. Immunodetection was performed using the chemiluminescence method according to manufacture's reccomendations (Roche Applied Science, Indianapolis, IN). Membranes were stripped and reprobed with GAPDH antibody to visualize equal protein loading.

RESULTS

Intensity of the β-1 integrin staining was increased in the limbal region where limbal stem cells reside. Integrin-bright

and -dull patterns of cellular staining were seen in the limbus and corneal zones, respectively (Figure 1). In tissue sections stained with β-1 integrin, limbal basal cells showed a brighter pattern than corneal basal cells. α-6 antibody stained the suprabasal cells of the limbus and cornea. α-6 integrin strongly stained a group of cells in the limbal basal region in some sections (Figure 1). When the limbal whole mount was stained with β-1 integrin, a cluster of cells was strongly labeled in a region of palisades of Vogt (Figure 2).

The immunofluorescence data using Notch-1 monoclonal antibody revealed Notch-1 expression in limbal basal cells (Figure 1). Expression in the limbal basal region was not continuous, and Notch-1 antigenicity was more pronounced in clusters of cells that were mainly located in the palisades of Vogt, the area believed to contain highest number of stem cells in the human limbus. The corneal zone was almost devoid of Notch-1-expressing cells. Notch-2 showed no specific staining in the limbal or corneal zones (data not shown). In tissue sections, the expression pattern of Notch-1 was clearly in the membrane.

Immunofluorescence detected the expression of ABCG2 protein in the limbal epithelia, but the central cornea was devoid of cells expressing ABCG2. The antigenicity was not only in the basal cells in the limbus, some of the proximal cells also showed positive staining. Interestingly, double staining with Notch-1 and ABCG2 demonstrated that some of the ABCG2-positive cells also expressed Notch-1. The number of cells positive for both was limited and all Notch-1 positive cells coexpressed ABCG2 (Figure 3).

The cells from the limbal explants stained with Notch-1.The staining intensity was high in only a few cells compared to the large number of cells in the confluent culture. The Notch-1 signal disappeared in dividing cells (Figure 4). Notch-1 expression in cultured cells was more prominent in the cytoplasm, (Figure 4) but additional nuclear staining was detectable in a few other cases (data not shown). In the Notch-

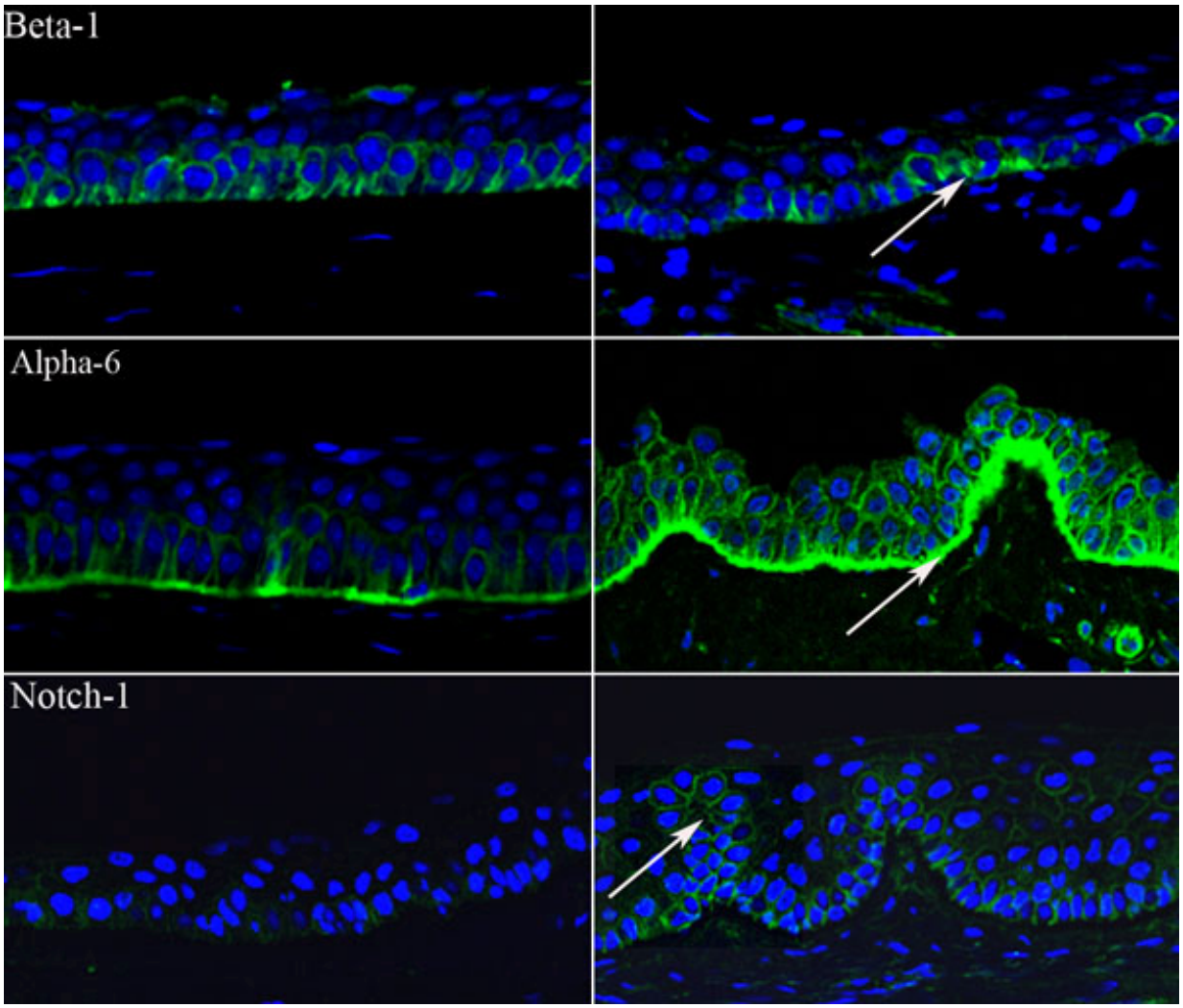

Figure 1. Differential expression of integrin β1, integrin α-6, and Notch-1 in the human limbo-corneal epithelium. Immunofluorescent staining of integrin β1, integrin α-6, and Notch-1 on frozen sections of human cornea (left panels) and limbus (right panels). The arrow points to the positively labeled basal cells at the limbus (green fluorescence indicated FITC conjugated anti mouse/rat IgG and blue fluorescence demonstrates nuclear counterstain with DAPI).

1-stained whole mount, only a few cells were stained in the limbal region (Figure 2).

Western blot analysis was performed to determine the specificity of the Notch-1 antibody, using human myeloma cell line ARH 77 (expresses endogenous Notch-1) as the positive control and U266 (which is devoid of endogenous Notch-1) as the negative control. Western blot analysis revealed a band of approximately 300 kDa that might be full-length Notch-1 expressed in ARH77. Because the ARH 77 showed some processed forms of Notch other than the 110 kDa (data not shown), we transfected the Notch-1 into the Cos-7 cell line. The protein extract was used as a positive control. Notch-1-transfected Cos-7 showed the unprocessed 300-kDa band and the activated 110-kDa band (Figure 5).

DISCUSSION

Corneal epithelium is continually renewed by cells in the basal limbal epithelium, which is thought to be the site where stems cells reside [2,4]. We sought to separate a true stem cell population from the limbal epithelium, which may not include the TAC. β-1 and α-6 integrins are known to show an intensity difference in the staining of epidermal keratinocytes, with bright cells in the basal region. We observed a similar staining pattern with brightly stained β-1 and α-6 cells in limbal basal

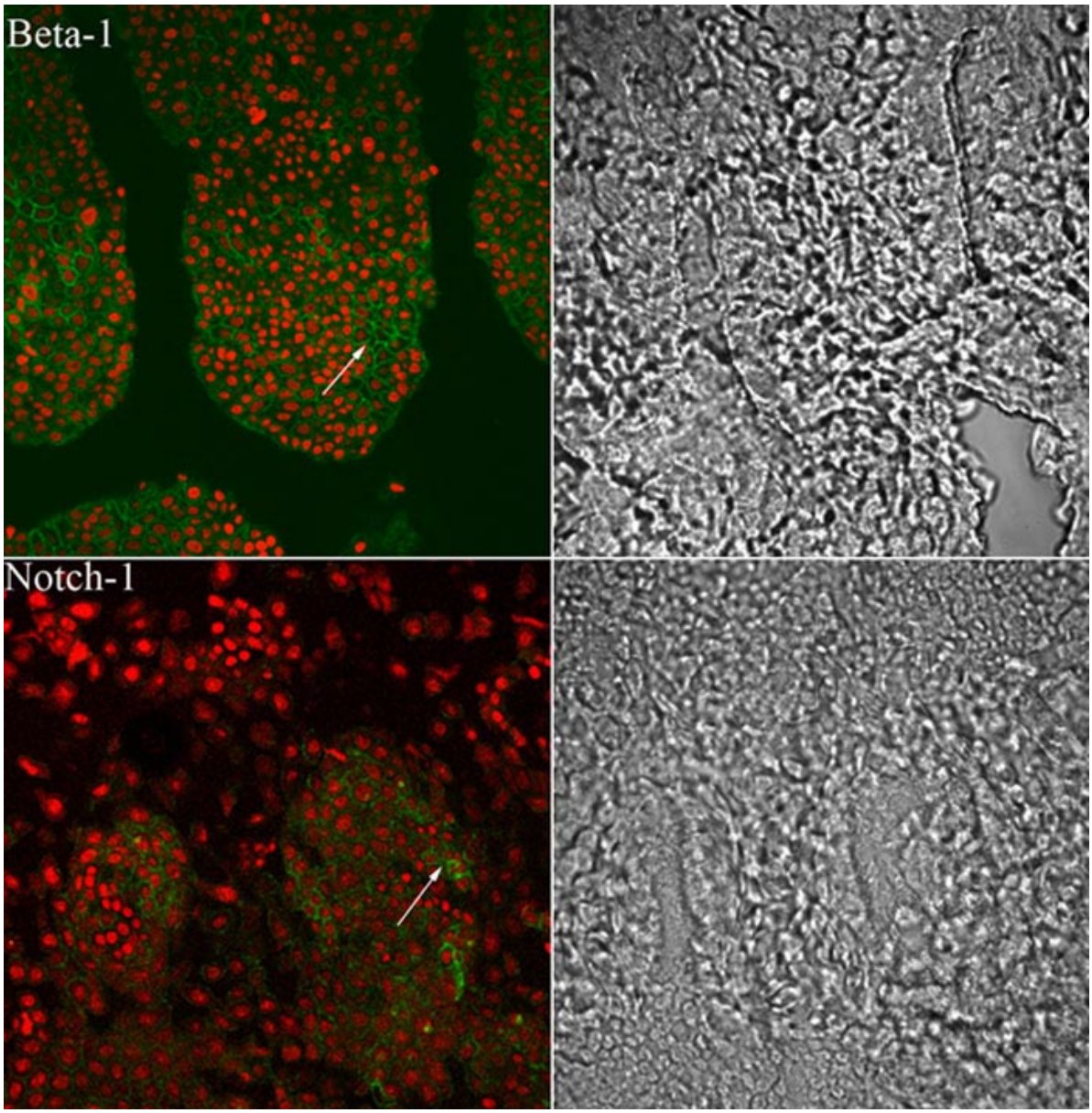

Figure 2. Localization of integrin β1 and Notch-1 in the whole mount. The immunoflurescence staining showing the limbal area with the palisade of Vogt environment in the left panel. Immunostaning for integrin β1 showed a group of bright cells in the limbal area (see arrow). Notch-1 expression was prominent only in the putative stem cells in the palisades of Vogt. (In the panels, green fluorescence represents FITC conjugated anti mouse/rat IgG and the red fluorescence shows nuclear counter stain with propidium iodide). The phase contrast micrographs in the right panels show the limbal architecture.

cells compared to the basal or suprabasal corneal cells. Our results with β-1 integrin were analogous to Jensen's epidermal stem cell findings of a two- to threefold increase in β-1 intensity compared to that seen in TAC [14]. A recent study of limbal stem cells revealed abundant integrin beta-1 expression by cell membranes of corneal and limbal epithelia with a much higher level of expression by the limbal basal cells [32]. Earlier, α-6 integrin was also proposed as a stem cell marker since epidermal keratinocytes, which have a high level of α-6 integrin expression, showed stem cell characteristics [33,34]. α-6 and β-1 bright cells had much more specific properties of stem cells when compared to β-1 positive and α-6 marginal cells, indicating that α-6 integrin was a potential epidermal stem cell marker. However, the alpha-6 integrin in corneal epithelial stem cells was found more in the suprabasal cells of the limbus than in the limbal basal cells [32]. Our corneal epithelium study showed a different pattern, comparable to β-1 integrin staining, with brighter cells in the limbal basal region rather than in the suprabasal region. Since integrins mediate cell matrix adhesion, the abundance of β-1 and α-6 integrin in the limbal basal cells may help explain the strong adhesion of the limbal basal cells to the extra cellular matrix. This is evident in the resistance of the limbus to shear forces compared to the central corneal cells. At this stage it is difficult to differentiate between the stem cells and the TAC in the corneal epithelium. All markers we have tried have been inconclusive.

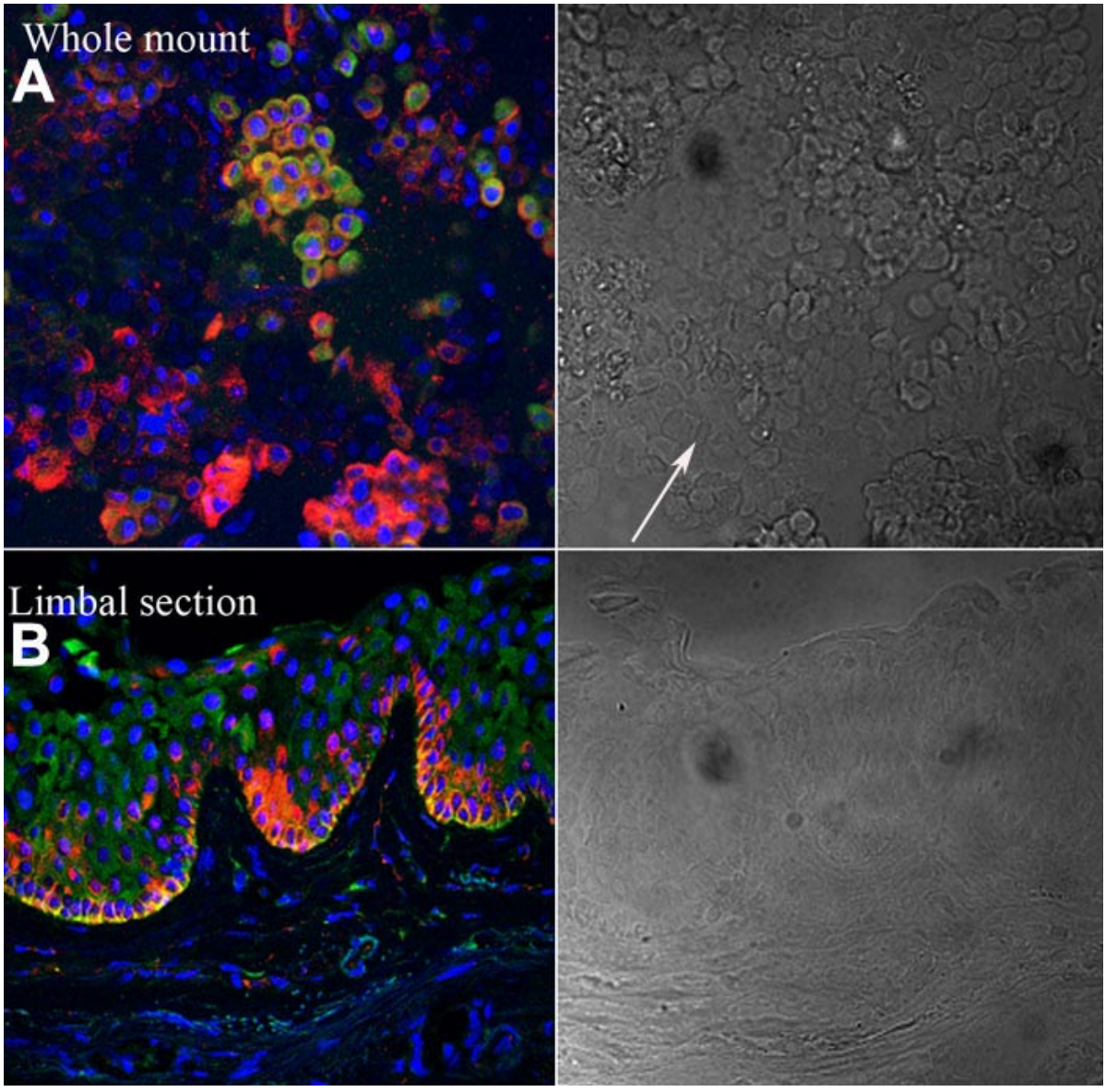

Figure 3. Colocalization of ABCG2 and Notch-1 in the human Limbo-corneal epithelium. **A**: When the whole mount was double stained with ABCG2 and Notch-1, subsets of cells were positive for both. Note that most Notch-1 positive cells (green fluorescence: FITC conjugated anti rat IgG) coexpressed ABCG2 (red fluorescence: rhodamine red X conjugated anti mouse IgG and nuclear counter stain with DAPI). **B**: When frozen sections were double stained they showed colocalization of ABCG2 and Notch-1 in a subset of cells in the limbal basal region.The right panels show representative phase contrast micrographs for **A** and **B**.

Notch-1 is known to play an important role in maintenance of undifferentiated cells in neural and hematopoietic systems. We hypothesized that Notch-1 may be present only in the limbal basal cells, especially in the stem cells of the corneal epithelium. It is likely that Notch-1 plays a role in keeping corneal stem cells in an undifferentiated manner in the limbal epithelium, as it was expressed only in a small group of cells in the limbal basal region and was undetected in the central corneal cells. Our findings demonstrate that Notch-1 antibody is specific, It showed a full-length 300 kDA band with Notch-1-transfected COS 7 cells. Our results also showed additional bands, other than 300 kDa, in western blot analysis, a finding that has also been reported in baby hamster kidney cells transfected with Notch-1. The most prominent among the processed forms was 110 kDa, and a precursor/product relationship was found between full-length Notch and the 110 kDa Notch fragment [30]. A similar pattern of expression was seen in rat retina [35].

The Notch-1 signal identified in the cytoplasm and nucleus in cultured cells might be an activated form of Notch-1. The activated form is usually found in differentiated cells. Immunohistochemical analysis has regularly failed to reveal the nuclear translocation of Notch-1, [36] but, in rare instances, Notch-1 was expressed in the nucleus in differentiated human cervical epithelium and in some human cervical neoplasms [30]. There are also reports of the Notch-1 signal in the nucleus in differentiated neurons in the rat retina [35]. In our studies, the staining pattern was similar in sections of corneal limbal region and in whole mount. In both cases the signal was entirely found in a few cells in the limbal basal region, and very specifically membrane staining. When the Notch signal in the culture is compared with the sections of limbus and whole mount, the cells with Notch -1 expression in the limbal basal region might be undifferentiated cells with stem cell properties. We have identified cells which coexpress ABCG2 and Notch-1. Expression of ABCG2 was previously suggested as a corneal stem cell marker [18] and the side population cells over expressed the ABCG2 [19]. Prostatic stem cells also expressed ABCG2 [37], but the prostatic transient amplifying cells stained negative [38]. Several studies identified an ABCG2-dependent side population in a wide variety of adult

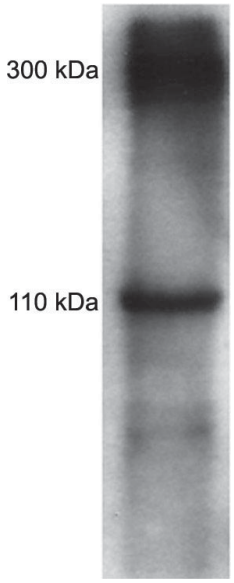

Figure 5. Western blot for Notch-1 antibody specificity. COS 7 cells were transfected with Notch-1 cDNA. After 24 h the cells were harvested, lysed, and protein was extracted. The western blot was probed with monoclonal anti mouse Notch-1 antibody (DSHB) Immunodetection was done by chemilumniscence.

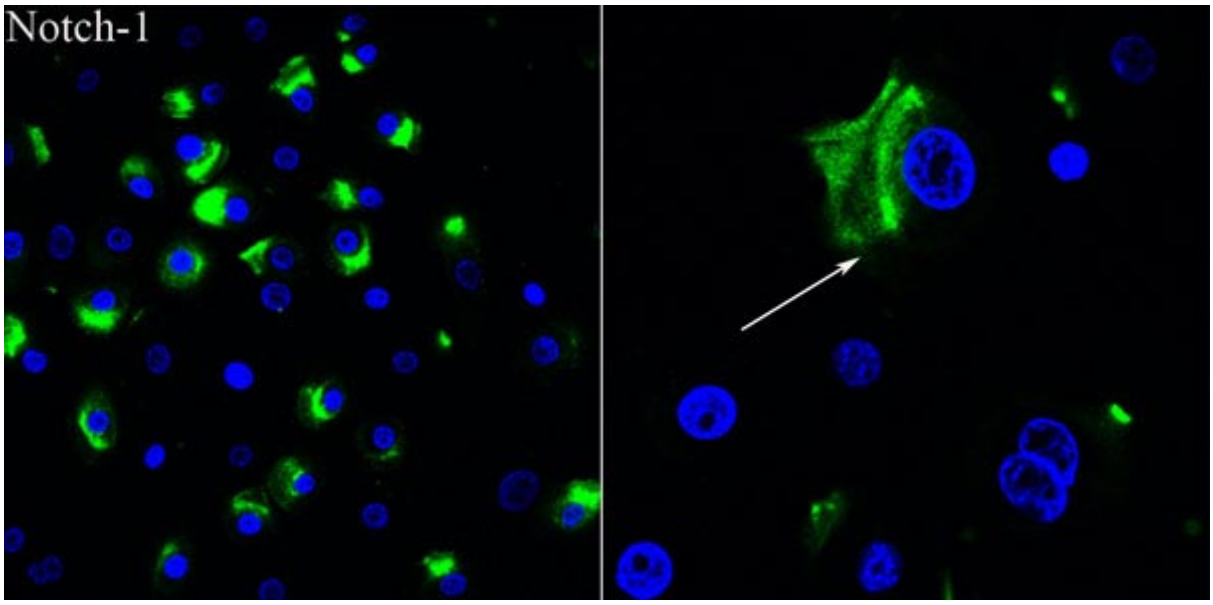

Figure 4. Characterization of epithelial outgrowth from limbal explants. Notch-1 showed expression in the cytoplasm while the signal disappeared in dividing cells. The arrow points to a cell with positive staining. In the panels, the green fluorescence is FITC conjugated anti mouse/rat IgG, and the blue fluorescence is nuclei counterstained with DAPI. The right panel is an enlarged view of the left panel.

tissues in different species [37,39,40]. A recent report demonstrated the presence of an ABCG2-dependent side population of cells in the human periodontal ligaments [41]. In limbal basal cells the ABCG2 expression was found to be high [32] and considered to be stem cell-associated marker. Connexin 43 (Cx43) marginal cells from the limbal basal region expressed more ABCG2-positive cells compared to Cx43 positive cells, with little to no positive staining [42]. One critical question that needs to be addressed is whether TAC stain negative for ABCG2. In the present study we found that ABCG2-positive cells were found more in the limbal region compared to Notch-1 positive cells. Given the limited number of Notch-1 and ABCG2-double positive cells, Notch-1 may be a potential stem cell marker

Notch keeps cells in their proliferative state by inhibiting differentiation, as was found with the crypts of intestinal cells in the mice [25]. The Notch family members and their ligands maintain a balance between cell proliferation and differentiation. Notch receptors are expressed in hematopoietic cells, and they control stem cell induction and lineage cell fate decisions [43]. The Notch signaling pathway is active in adult hematopoietic stem cells and is down regulated in terminally differentiated progeny [44]. Our study found the same phenomenon, with Notch-1 expressed in the limbal basal cells but barely detected in the terminally differentiated corneal epithelial layer. Notch pathway activation inhibits myogenic differentiation [45,46]. Notch is also thought to contribute to neural precursor cell maintenance, inhibiting the differentiation of these cells to neurons and oligodendrocytes [47,48]. It is likely that Notch-1 plays a similar role in keeping corneal stem cells in an undifferentiated manner in the limbal epithelium. Our data provide a rationale for future work to focus on the role of Notch members in the development and maintenance of corneal stem cells. Manipulating the Notch pathway may provide a way to enrich stem cells, thus providing new therapeutic strategies to treat corneal stem cell deficiencies. Further studies are underway to examine the role of Notch-1 in corneal development.

ACKNOWLEDGEMENTS

We thank Dr. Spyros Artavanis-Tsakonas (Harvard Medical School, Massachusetts General Hospital Cancer Center, Charlestown, MA) for kindly providing the Notch cDNA and Dr Alan L Epstein (University of Southern California, Los Angeles, CA) for providing the myeloma cell lines. We also thank the expert technical help received from Antony Rodreguiz and Ernesto Barren (Specialized Microscopic Core-Lab). This study was supported by National Eye Institute grants EY014392 and EY03040 and by an unrestricted grant from Research to Prevent Blindness and a Baxter Foundation Junior Faculty Award to Dr. Yiu. The Notch-1 monoclonal antibody developed by Spyros Artavanis-Tsakonas was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NIHCD and maintained by The University of IOWA, Department of Biological Sciences, Iowa City, IA, 52242.

REFERENCES

1. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 1971; 229:560-1.
2. Dua HS, Azuara-Blanco A. Limbal stem cells of the corneal epithelium. Surv Ophthalmol 2000; 44:415-25.
3. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 1986; 103:49-62.
4. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 1989; 57:201-9.
5. Lavker RM, Dong G, Cheng SZ, Kudoh K, Cotsarelis G, Sun TT. Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. Invest Ophthalmol Vis Sci 1991; 32:1864-75.
6. Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. J Cell Sci 1998; 111:2867-75.
7. Dua HS, Azuara-Blanco A. Corneal allograft rejection: risk factors, diagnosis, prevention, and treatment. Indian J Ophthalmol 1999; 47:3-9.
8. Holland EJ, Schwartz GS. The evolution of epithelial transplantation for severe ocular surface disease and a proposed classification system. Cornea 1996; 15:549-56.
9. Tsai RJ, Tseng SC. Human allograft limbal transplantation for corneal surface reconstruction. Cornea 1994; 13:389-400.
10. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet 1997; 349:990-3.
11. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. Cornea 2000; 19:421-6.
12. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. N Engl J Med 2000; 343:86-93.
13. Johnsen HE, Knudsen LM. Nordic flow cytometry standards for CD34+ cell enumeration in blood and leukapheresis products: report from the second Nordic Workshop. Nordic Stem Cell Laboratory Group (NSCL-G). J Hematother 1996; 5:237-45.
14. Jensen UB, Lowell S, Watt FM. The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. Development 1999; 126:2409-18.
15. Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis. Cell 1995; 80:83-93.
16. Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G. The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. J Cell Sci 1998; 111:3179-88.
17. Moll I. Proliferative potential of different keratinocytes of plucked human hair follicles. J Invest Dermatol 1995; 105:14-21.
18. Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. J Cell Sci 2005; 118:1715-24.
19. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of

the side-population phenotype. Nat Med 2001; 7:1028-34.
20. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999; 284:770-6.
21. Mumm JS, Kopan R. Notch signaling: from the outside in. Dev Biol 2000; 228:151-65.
22. Varnum-Finney B, Xu L, Brashem-Stein C, Nourigat C, Flowers D, Bakkour S, Pear WS, Bernstein ID. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 2000; 6:1278-81.
23. Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S, Bhatia M. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. J Exp Med 2000; 192:1365-72.
24. Hitoshi S, Alexson T, Tropepe V, Donoviel D, Elia AJ, Nye JS, Conlon RA, Mak TW, Bernstein A, van der Kooy D. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev 2002; 16:846-58.
25. Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S. Notch signals control the fate of immature progenitor cells in the intestine. Nature 2005; 435:964-8.
26. Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP, Radtke F. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003; 33:416-21.
27. Wang XD, Leow CC, Zha J, Tang Z, Modrusan Z, Radtke F, Aguet M, de Sauvage FJ, Gao WQ. Notch signaling is required for normal prostatic epithelial cell proliferation and differentiation. Dev Biol 2006; 290:66-80.
28. Joseph A, Powell-Richards AO, Shanmuganathan VA, Dua HS. Epithelial cell characteristics of cultured human limbal explants. Br J Ophthalmol 2004; 88:393-8.
29. Kim HS, Jun Song X, de Paiva CS, Chen Z, Pflugfelder SC, Li DQ. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. Exp Eye Res 2004; 79:41-9.
30. Zagouras P, Stifani S, Blaumueller CM, Carcangiu ML, Artavanis-Tsakonas S. Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc Natl Acad Sci U S A 1995; 92:6414-8.
31. Espana EM, Romano AC, Kawakita T, Di Pascuale M, Smiddy R, Tseng SC. Novel enzymatic isolation of an entire viable human limbal epithelial sheet. Invest Ophthalmol Vis Sci 2003; 44:4275-81.
32. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells 2004; 22:355-66.
33. Kaur P, Li A. Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells. J Invest Dermatol 2000; 114:413-20.
34. Ohishi K, Katayama N, Shiku H, Varnum-Finney B, Bernstein ID. Notch signalling in hematopoiesis. Semin Cell Dev Biol 2003; 14:143-50.
35. Ahmad I, Zaqouras P, Artavanis-Tsakonas S. Involvement of Notch-1 in mammalian retinal neurogenesis: association of Notch-1 activity with both immature and terminally differentiated cells. Mech Dev 1995; 53:73-85.
36. Kidd S, Baylies MK, Gasic GP, Young MW. Structure and distribution of the Notch protein in developing Drosophila. Genes Dev 1989; 3:1113-29. Erratum in: Genes Dev 1989; 3:2020.
37. Huss WJ, Gray DR, Greenberg NM, Mohler JL, Smith GJ. Breast cancer resistance protein-mediated efflux of androgen in putative benign and malignant prostate stem cells. Cancer Res 2005; 65:6640-50.
38. Litvinov IV, Vander Griend DJ, Xu Y, Antony L, Dalrymple SL, Isaacs JT. Low-calcium serum-free defined medium selects for growth of normal prostatic epithelial stem cells. Cancer Res 2006; 66:8598-607.
39. Kurita T, Medina RT, Mills AA, Cunha GR. Role of p63 and basal cells in the prostate. Development 2004; 131:4955-64.
40. Litvinov IV, De Marzo AM, Isaacs JT. Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling? J Clin Endocrinol Metab 2003; 88:2972-82.
41. Kawanabe N, Murakami K, Takano-Yamamoto T. The presence of ABCG2-dependent side population cells in human periodontal ligaments. Biochem Biophys Res Commun 2006; 344:1278-83.
42. Chen Z, Evans WH, Pflugfelder SC, Li DQ. Gap junction protein connexin 43 serves as a negative marker for a stem cell-containing population of human limbal epithelial cells. Stem Cells 2006; 24:1265-73.
43. Duncan AW, Rattis FM, DiMascio LN, Congdon KL, Pazianos G, Zhao C, Yoon K, Cook JM, Willert K, Gaiano N, Reya T. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol 2005; 6:314-22.
44. Shawber C, Nofziger D, Hsieh JJ, Lindsell C, Bogler O, Hayward D, Weinmaster G. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development 1996; 122:3765-73.
45. Gaiano N, Fishell G. The role of notch in promoting glial and neural stem cell fates. Annu Rev Neurosci 2002; 25:471-90.
46. Nofziger D, Miyamoto A, Lyons KM, Weinmaster G. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. Development 1999; 126:1689-702.
47. Fehon RG, Johansen K, Rebay I, Artavanis-Tsakonas S. Complex cellular and subcellular regulation of notch expression during embryonic and imaginal development of Drosophila: implications for notch function. J Cell Biol 1991; 113:657-69.
48. Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J 1999; 18:2196-207.

The print version of this article was created on 1 Mar 2007. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.