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Down-regulation of *Pax6* is associated with abnormal differentiation of corneal epithelial cells in severe ocular surface diseases

W Li^{1,3}, Y-T Chen¹, Y Hayashida¹, G Blanco¹, A Kheirkah¹, H He¹, S-Y Chen¹, C-Y Liu², and SCG Tseng^{1,*}

¹Ocular Surface Center and TissueTech Inc., Miami, FL 33173, USA

²Department of Ophthalmology, University of Cincinnati, OH 45267, USA

³Eye Institute and Xiamen Eye Center, Xiamen University School of Medicine, Xiamen, Fujian 316005, People's Republic of China

Abstract

Pax6 is the universal master control gene for eye morphogenesis. Other than retina and lens, *Pax6* also expressed in the ocular surface epithelium from early gestation until the postnatal stage, in which little is known about the function of *Pax6*. In this study, corneal pannus tissues from patients with ocular surface diseases such as Stevens–Johnson syndrome (SJS), chemical burn, aniridia and recurrent pterygium were investigated. Our results showed that normal ocular surface epithelial cells expressed *Pax6*. However, corneal pannus epithelial cells from the above patients showed a decline or absence of *Pax6* expression, accompanied by a decline or absence of K12 keratin but an increase of K10 keratin and filaggrin expression. Pannus basal epithelial cells maintained nuclear p63 expression and showed activated proliferation, evidenced by positive Ki67 and K16 keratin staining. On 3T3 fibroblast feeder layers, *Pax6* immunostaining was negative in clones generated from epithelial cells harvested from corneal pannus from SJS or aniridia, but positive in those from the normal limbal epithelium; whereas western blots showed that some epithelial clones expanded from pannus retained *Pax6* expression. Transient transfection of an adenoviral vector carrying *EGFP–Pax6* transgenes into these *Pax6*[−] clones increased both *Pax6* and K12 keratin expression. These results indicate that *Pax6* helps to maintain the normal corneal epithelial phenotype postnatally, and that down-regulation of *Pax6* is associated with abnormal epidermal differentiation in severe ocular surface diseases. Reintroduction of activation of the *Pax6* gene might be useful in treating squamous metaplasia of the ocular surface epithelium.

Keywords

Pax6; squamous metaplasia; cornea; epithelium

Introduction

Paired box homeotic gene 6 (*Pax6*) functions as an early ectodermal gene expressed during optic vesicle formation for eye development. In this capacity, *Pax6* regulates cell proliferation, determines differentiation and mediates apoptosis [1–3]. Mutation of the *Pax6* homologue *eyeless* in *Drosophila* causes severe defects in eye development [4]. Heterozygous null mutations in the *Pax6* gene result in a distinct small eye syndrome in mice and rats [5,6] and a spectrum of eye abnormalities in humans, including aniridia, Peter's anomaly and autosomal dominant keratitis [1,7,8]. During eye morphogenesis, *Pax6* is expressed by most cell types including neuroectodermally-derived neural and pigment layers of the retina, iris and ciliary body and in the epithelially-derived lens and cornea [9–11]. Functional *Pax6* is required for achieving normal development of the corneal epithelium [12]; corneal epithelia of *Pax6*^{+/-} mice failed to undergo proper differentiation and maturation during development [13,14]. Correct *Pax6* dosage is necessary for normal clonal epithelial growth during corneal development, normal limbal stem cell activity and correct corneal epithelial cell migration [15]. Postnatally, *Pax6* expression is restricted to corneal, conjunctival, lens and iris epithelia and amacrine cells of the retina [11,16]. However, besides the evidence showing that *Pax6* expression is linked with the control of EGF-induced cultured corneal epithelial cell proliferation [17,18] and *K12* keratin gene expression [19,20], little is known about how *Pax6* functions in the ocular surface epithelium in the postnatal stage.

The ocular surface is covered by corneal, limbal and conjunctival epithelia, which are all originated from the surface ectoderm but remain stratified and non-keratinized. Their normal epithelial phenotypes contribute to the health of the ocular surface. However, in severe ocular surface diseases, such as Stevens–Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP), chemical/thermal burns, dry eye, Sjögren's syndrome and vitamin A deficiency [21–23], the epithelial phenotype may turn into a non-secretory, keratinized epithelium [24,25] via a pathological process termed squamous metaplasia, leading to severe visual loss or blindness.

Squamous metaplasia of the ocular surface epithelia is accompanied by the loss of goblet cells, increased cellular stratification, enlargement of superficial cells and keratinization [25–29]. Histopathologically, squamous metaplasia is correlated with chronic inflammation or scarring of the stroma [29]. Ultrastructurally, squamous metaplasia is characterized by the presence of stratum corneum with cornified envelopes and keratohyalin granules [23]. Biochemically, squamous metaplasia is correlated with loss of expression of cornea-specific keratins K3 and K12 [23,30,31] and emergence of epidermis-specific keratins K1 and K10 [23,30], and such cornified envelope-specific proteins as transglutaminase I [32], involucrin, filaggrin and loricrin [30]. Besides xerophthalmia caused by systemic vitamin A deficiency, which is known to cause squamous metaplasia of the ocular surface epithelia in experimental animals [23,26] and human patients [33,34], little is known about the pathogenesis of squamous metaplasia found in other ocular surface diseases.

Since *Pax6* is restricted to ocular surface epithelia but not other stratified epithelia, such as epidermis, we are prompted to hypothesize that *Pax6* may be a key player to control the phenotype of ocular surface epithelia. In this study, we indeed found that in patients with severe ocular surface diseases *Pax6* was down-regulated or depleted in ocular surface epithelial cells, which showed abnormal epidermal differentiation. Furthermore, *Pax6* expression was lost in clones generated from the pannus epithelium with squamous metaplasia, and transient reintroduction of *Pax6* up-regulated keratin K12 expression. The significance of this finding is further discussed.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's/F12 medium, fetal bovine serum (FBS) and human recombinant epidermal growth factor (h-EGF) were purchased from Invitrogen (Carlsbad, CA, USA). Dispase II was obtained from Roche (Indianapolis, IN, USA). Hydrocortisone, dimethyl sulphoxide, cholera toxin, insulin–transferrin–sodium selenite media supplement, Hoechst-33 342 dye, mouse anti- β -actin antibody and FITC-conjugated goat anti-mouse IgG were from Sigma (St. Louis, MO, USA). Texas Red-conjugated donkey anti-goat IgG was from Jackson ImmunoResearch (West Grove, PA, USA). Mouse anti-Pax6, rabbit anti-filaggrin, and goat anti-keratin 12 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-K10 keratin, K16 keratin and ABCG-2 antibodies were from Chemicon (Temecula, CA, USA). Mouse anti-Ki67, p63 (Clone 4A4), K19 keratin antibodies and diaminobenzidine (DAB) were from DakoCytomation (Carpinteria, CA, USA). ABC kit and anti-fade mounting solution with DAPI were from Vector Laboratories (Burlingame, CA, USA).

Human samples

Written informed consent was obtained from all patients. Patient tissues were handled according to the Declaration of Helsinki. This study was in compliance with the Health Insurance Portability and Accountability Act (HIPAA) and was approved by the Institutional Review Board of the Baptist Hospital of Miami/South Miami Hospital Inc. (Miami, FL, USA). Ten patients were included, with the underlying aetiology of Stevens–Johnson syndrome ($n = 5$), chemical burn ($n = 3$), aniridia ($n = 1$) and recurrent pterygium ($n = 1$). All patients were seen at the Ocular Surface Center and their blinding corneal pannus tissues were surgically removed by one surgeon (SCGT) during July 2004–October 2006. Corneoscleral tissues from human donor eyes obtained from the Florida Lions Eye Bank (Miami, FL, USA) and oral mucosa tissue and conjunctival tissue from human donors obtained from the Baptist Hospital were used as controls.

Isolation and clonal culturing of epithelial cells from the corneal pannus

Epithelial sheets were isolated from tissues, using our previously described methods [35]. A part of the epithelial sheet was extracted by RIPA buffer as described below, and the rest was rendered into single cells by further digestion with 0.25% trypsin/0.53 mM EDTA at 37 °C for 10 min. Clonal cultures were created by seeding 2×10^4 cells/60 mm dish with mitomycin C-pretreated 3T3 cells as feeder layers, and cultured in supplemented hormonal epithelial medium (SHEM) [35] for 8–10 days.

Immunostaining

Immunofluorescence or immunohistochemical staining of cryo-sections or cultured cells were performed as previously described [35] and scored by two of us, regarding the intensity of K10, K12, Pax6 staining as + + + + (in normal control, respectively), + + + (majority of the cells were positive), ++ (about 50% of cells were positive), + (<50% of cells were positive) and – (all the cells were negative).

Western blot analysis

At the end of epithelial cultivation, residual 3T3 feeder layer cells were removed by gentle pipetting, and epithelial clones were extracted in cold RIPA buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail). Equal amounts of protein extracts (20 μ g) were compared to those from

epithelial sheets harvested directly from pannus tissues and normal conjunctival tissues by subjecting to western blot, using Pax6 and β -actin antibodies.

EGFP–Pax6 adenoviral transfection

EGFP–Pax6 adenoviral shuttle plasmid was generated as previously reported [18]. At the end of pannus epithelial clonal culturing, epithelial cells were harvested and seeded in eight-well chamber slides at the density of 1×10^4 /well in SHEM. After attachment overnight, the cells were transfected with EGFP–Pax6 adenovirus at the multiplicity of infection (MOI) of 100, using EGFP adenovirus as control. After transfection, the cells were incubated for 72 h and terminated by fixation in 4% paraformaldehyde for immunostaining to Pax6 and K12 keratin.

Statistical analysis

Summary data are reported as means $\pm$ SD. Group means were compared using the appropriate version of Student's unpaired *t*-test. The test results were two-tailed, with $p < 0.05$ considered statistically significant.

Results

Expression of keratins and Pax6 by the normal ocular surface epithelia

Consistent with previous reports [36–40], positive expression of K12 keratin was noted in the full thickness of the normal corneal epithelium (Figure 1A), but only in limbal suprabasal cell layers, leaving negative staining in limbal basal epithelial cells (Figure 1D). K12 keratin was totally negative in the conjunctival epithelium (Figure 1G). Expression of K10 keratin was negative in the corneal (Figure 1A), limbal (Figure 1D), and conjunctival (Figure 1G) epithelia. Consistent with previous reports [41–43], nuclear expression of Pax6 was also present in the full thickness of the normal corneal epithelium (Figure 1B), but was negative in the corneal stromal keratocytes, as shown by Hoechst counterstaining, whose intensity was markedly quenched in the Pax6-positive nuclei of corneal epithelial cells (Figure 1C). In the limbus and the conjunctiva, nuclear expression of Pax6 was also present in nearly all epithelial cell layers (Figure 1E, H, respectively) except for some basal cells, which were Pax6-negative when compared to the Hoechst nuclear counterstaining (Figure 1F, I, respectively). These Pax6-negative cells were positive for vimentin but negative for pancytokeratin, suggesting that they might be either Langerhans cells or melanocytes (data not shown).

Abnormal epidermal differentiation in the corneal pannus from severe ocular surface diseases

To investigate whether the corneal epithelial phenotype was still maintained in severe ocular surface diseases, we studied 10 corneal pannus tissues removed from the eyes of patients with such ocular surface diseases as SJS, chemical burn, aniridia and recurrent pterygium. All 10 patients developed fibrovascular pannus tissues on the corneal surface, resulting in a severe loss of vision, with the visual acuity ranging from light perception (LP) to 20/400. Table 1 summarizes the key clinical characteristics and laboratory investigation of these patients. Double staining for K12 and K10 keratins revealed that there was a notable decrease of K12 keratin expression in the epithelium of all pannus samples, and that the majority of the K12-positive cells were located in the central corneal area, while only sporadic or small groups of K12-positive cells were in peripheral corneal and limbal areas. In two samples, one with SJS (Patient 2) and the other with aniridia (Patient 6), K12 keratin expression was totally lost in the pannus epithelium. In contrast, K10 keratin expression was present in all pannus samples, where there was absence of the normal corneal epithelium as shown above. In some samples (Patients 4, 6 and 7), K10 keratin was expressed in most suprabasal epithelial cells in peripheral corneal and limbal regions, and as patches or sporadically in other samples. Some pannus

suprabasal cells (Patients 4 and 8) expressed both K12 and K10 keratins. Notably, basal epithelial cells exhibited negative staining to both K12 and K10 keratins in the limbal area of all pannus tissues, indicating that they remained undifferentiated. These results indicated abnormal epidermal differentiation in all corneal pannus epithelia taken from these severe ocular surface diseases.

To investigate whether Pax6 was still maintained in these abnormal differentiated epithelia, immunohistochemical and Hoechst staining were performed. To our surprise, all samples exhibited decreased Pax6 expression in pannus epithelia at variable levels (Table 1). A representative result is shown in Figure 2, taken from Patient 4. In the central corneal region of the pannus, where positive K12 keratin expression was noted (Figure 2A), we also found positive Pax6 nuclear expression (Figure 2B, C), suggesting that the corneal epithelial phenotype was preserved when Pax6 was expressed. However, Pax6 nuclear staining was markedly attenuated in the peripheral corneal region (Figure 2E, F), and became totally negative in the limbal region of the pannus (Figure 2H, I), where K12 keratin expression decreased but K10 keratin expression increased (Figure 2D, G, respectively). These results indicated that abnormal epidermal differentiation was highly correlated with down-regulation of Pax6 expression.

To further characterize the phenotypic change of corneal pannus epithelial cells, we performed immunostaining to p63, Ki67, ABCG-2, K16 keratin, K19 keratin and filaggrin. A representative result from Patient 4 (Figure 3) showed strong nuclear Ki67 staining, indicative of proliferation, in basal and suprabasal cells of the peripheral and limbal regions of the pannus tissue, where there was significant epithelial stratification. Positive nuclear staining to p63, a marker originally thought to be expressed by basal epithelial progenitor cells of all stratified epithelia [44–47] but now recognized as a marker of cells with proliferation potential, was found in almost all basal and some suprabasal epithelial cells. Positive membranous expression of ABCG-2, thought to be a limbal epithelial stem cell marker [48,49], was found in suprabasal pannus epithelial cells instead of basal cells. Expression of K16 keratin, indicative of an alternative pathway of keratinocyte proliferation [50], was found in almost all suprabasal pannus epithelial cells. Expression of epidermal terminal differentiation markers, such as filaggrin, was found in the suprabasal and superficial epithelial cells of the pannus. In contrast, expression of K19 keratin, indicative of conjunctival and limbal basal epithelial cells [51], was negative in these pannus epithelia. Taken together, these results indicated that the pannus epithelium exhibited squamous metaplasia with hyper-proliferation.

Pax6 loss might begin at the progenitor cell level

To investigate whether or not the loss of Pax6 expression happened at the progenitor cell level, we harvested epithelial cells directly from corneal pannus and seeded them on 3T3 feeder layers for 10–12 days in SHEM. Epithelial clones generated from the normal limbus as a positive control maintained Pax6 expression (Figure 4A), while those from the oral mucosa as a negative control did not show Pax6 expression (Figure 4B). Interestingly, epithelial clones generated from the pannus removed from Patient 6 with aniridia (Figure 4C) and from Patient 1 with SJS (Figure 4D) also did not show Pax6 expression. Double staining confirmed that these clones also did not express K12 and K10 keratins (data not shown), indicating that they were not differentiated. Western blot analyses showed that Pax6 was expressed by normal limbal epithelial cells expanded both on plastic (Figure 5, lane 1) and on 3T3 feeder layers (Figure 5, lane 2), and by normal conjunctival epithelial cells directly isolated *in vivo* (Figure 5, lane 3). In contrast, Pax6 was weakly positive in pannus epithelial cells directly isolated from Patient 1 with SJS (lane 4), but negative in pannus epithelial cells expanded on 3T3 feeder layers from Patient 7 with chemical burn (lane 5). However, Pax6 was still maintained in pannus epithelial cells expanded on 3T3 fibroblast feeder layers from Patient 9 with SJS (lane 6).

Transfection of Pax6 up-regulated Pax6 and keratin 12 expression in Pax6-negative corneal pannus epithelial cells

Pax6 is a positive transcription factor essential for K12 keratin expression [19] and up-regulates the activity of K12 promoter in cultured corneal epithelial cells [20]. We thus wondered whether over-expression of Pax6 might induce K12 expression in those K12-negative progenitor cells expanded from corneal pannus specimens. To answer this question, clonally expanded epithelial cells on 3T3 fibroblast feeder layers from pannus epithelia (Patients 1 with SJS), which had been confirmed to be devoid of Pax6 expression, were transfected with EGFP-adenovirus or EGFP-Pax6-adenovirus for 72 h. The results showed that the negative control transfected with EGFP-adenovirus expressed negligible Pax6 (Figure 6A) and K12 keratin (Figure 6C). However, EGFP-Pax6-adenovirus-transfected cells showed a dramatic increase of Pax6 (Figure 6B) and K12 keratin expression (Figure 6D), and the difference was statistically significant (Figure 6E; both $p < 0.001$). The same result was obtained using clonally expanded epithelial cells from Patient 8 with chemical burn (data not shown). These results collectively indicated that Pax6 over-expression also up-regulated K12 expression by these corneal pannus epithelial cells that had lost Pax6 expression.

Discussion

Squamous metaplasia is a common pathological process, which occurs in almost all epithelial tissues and is a hallmark of a number of severe ocular surface diseases [25]. Herein, we confirm that squamous metaplasia, as evidenced by expression of K10 keratin and filaggrin, is accompanied by the loss of K12 keratin expression in all vascularized pannus tissues removed from corneal surfaces inflicted with SJS, chemical burn, aniridia and recurrent pterygium (Figure 2, Figure 3). In the model of urothelial squamous metaplasia, Liang *et al* [52] proposed five models explaining how squamous metaplasia might occur: transdifferentiation at the terminal differentiated cell level; dedifferentiation and redifferentiation; pluripotency or plasticity of stem cells; selected expansion of different stem cells; and expansion and replacement by a different lineage. In our study, although expression of K12 and K10 keratins were mutually exclusive in most cases, they were co-expressed by some cells (Figure 2D, G, arrowheads), indicating that abnormal epidermal differentiation of the corneal epithelium might occur as a result of direct transdifferentiation at the level of terminal differentiated cells.

However, we cannot rule out the likelihood that there is a change at the level of progenitor cells, because we also found that squamous metaplasia correlated well with the loss of Pax6 (Figure 2). During eye morphogenesis, ectodermal cells expressing Pax6 develop into ocular surface epithelia, while those which do not express Pax6 take a 'default differentiation pathway' and develop into epidermis [53]. Our study clearly showed a consistent loss of Pax6 in corneal pannus epithelia of severe ocular surface diseases. The trend of such Pax6 loss in the corneal pannus was to start from the basal layer and progress toward the suprabasal layers, and to start from the limbus and spread toward the central cornea (Figure 2), matching well with the normal differentiation pathways of corneal epithelial cells. This prompted us to speculate that corneal epithelial stem cells in the limbal area might have started with the loss of Pax6 expression and differentiated into epidermal stem cells. This interpretation was further supported by the finding that epithelial clones derived from the corneal pannus and expanded on 3T3 fibroblast feeder layers did not express Pax6 (Figure 4C, D), while those of the normal limbal epithelial pro-genitors did (Figure 4A). These Pax6-negative clones did not express K10 and K12 keratins, indicating that they were undifferentiated (not shown). Because K10 keratin was expressed by suprabasal cells in the limbal and peripheral corneal regions of pannus tissues, we speculated that these K10-positive cells might have been the progeny of the Pax6-negative basal cells. If this interpretation were correct, we further surmised that Pax6 may control the plasticity of limbal epithelial stem cells and that its loss might be responsible for

transdifferentiation at the stem cell level in squamous metaplasia. In line with the finding that Pax6 binding sites are identified in the K12 promoter, and overexpression of Pax6 stimulates K12 promoter activity in cultured corneal epithelial cells [20], we demonstrated that transient up-regulation of *Pax6* gene expression via adenoviral plasmids significantly increased K12 keratin expression in those *Pax6*[−] epithelial clones generated from pannus epithelia (Figure 6). Therefore, strategies to bring back Pax6 expression could be incorporated to treat squamous metaplasia in the future.

Cumulative evidence indicates that limbal epithelial stem cells are regulated by their surrounding micro-environment, the so-called limbal stem cell niche (for review, see [54]). The loss of Pax6 in corneal pannus tissues exhibiting the pathological process of squamous metaplasia resembled ‘dedifferentiation and transdifferentiation’ of adult rabbit central corneal transit amplifying cells when reprogrammed into epidermal differentiation after engraftment to murine embryonic dermis [55]. In the latter model, nuclear expression of Pax6 undergoes translocation to the cytoplasm before turning into negative expression after interaction with the embryonic dermal cues [56]. Therefore, squamous metaplasia observed herein might be triggered by signals from the pathological limbal stroma to down-regulate *Pax6* expression. Mouse null expression of *Dkk2*, a *Dickkopf* (*Dkk*) family member that functions as a Wnt pathway inhibitor, deprives the ocular surface epithelium of the corneal fate decision with the loss of Pax6 and K12 keratin expression, thus giving rise to epidermal differentiation [57]. Further studies are needed to investigate the epithelial–mesenchymal interaction in these severe ocular surface diseases, in which the limbal stroma is known to be chronically inflamed and scarred [29].

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Figure 1.

Expression of K10 and K12 keratins and Pax6 in normal cornea, limbal and conjunctival epithelia. Double staining of K12 (red) and K10 (green) keratins showed full-thickness expression of K12 keratin in the normal corneal epithelium (A), negative staining in limbal basal epithelial cells (D) and totally negative staining in the conjunctival epithelium (G). K10 keratin was not expressed by normal corneal (A), limbal (D) and conjunctival (G) epithelia. Pax6 was expressed in the nuclei of full-thickness corneal epithelium (B), as confirmed by Hoechst counterstaining (C). In the limbus (B) and the conjunctiva (E), Pax6 was expressed in nearly all epithelial cells except for some basal cells, in which Hoechst double staining was not quenched by Pax6 nuclear staining (F and I, respectively, above the dotted lines). Bar, 100 μ m

Figure 2.

Abnormal epidermal differentiation in the corneal pannus of severe ocular surface disease (Patient 4). The majority of epithelial cells of the central corneal region of the pannus still expressed K12 keratin (red), while few suprabasal and superficial cells expressed K10 keratin (green) (A). In contrast, fewer suprabasal epithelial cells in the peripheral corneal (D) and limbal (G) regions of the pannus expressed K12 keratin, but a majority of suprabasal cells expressed K10 keratin. Some cells expressed both K12 and K10 keratins (arrowheads in D, G). Pax6 and Hoechst double staining showed that most epithelial cells in the central corneal region expressed Pax6 (B, C), while Pax6 nuclear staining was markedly attenuated in the

peripheral corneal region (E, F) and became totally negative in the limbal region of the pannus (H, I). Bar, 100 μ m

Figure 3.

Abnormal epidermal differentiation in pannus (Patient 4). There was a significant increase in epithelial stratification. Basal and suprabasal cells showed strong nuclear Ki67 and p63 staining. ABCG-2 was expressed in suprabasal pannus epithelial cells. K16 keratin was strongly expressed in almost all suprabasal epithelial cells. K19 keratin was negative. Filaggrin was expressed in suprabasal and superficial epithelial cells. Bar, 100 μ m

Figure 4.

Pax6 expression in epithelial clones expanded by 3T3 feeder layers. Nuclear Pax6 staining was positive in nearly all cells of the clone generated from normal limbal epithelial cells (A) but negative in those generated by normal oral mucosa epithelial cells (B). Interestingly, Pax6 was also negative in all cells in the clone generated from the pannus removed from Patient 6 with aniridia (C) and from Patient 1 with SJS (D). Bar, 100 μ m

Figure 5.

Western blot analysis of Pax6, which was expressed by normal limbal epithelial cells expanded on plastic (lane 1) or on 3T3 feeder layers (lane 2), and by normal tarsal conjunctival epithelial cells isolated directly *in vivo* (lane 3). In contrast, Pax6 was weakly expressed by pannus epithelial cells directly isolated from Patient 1 with SJS (lane 4), and not expressed by pannus epithelial cells expanded on 3T3 feeder layers from Patient 7 with a chemical burn (lane 5). However, Pax6 was still expressed by pannus epithelial cells expanded on 3T3 fibroblast feeder layers from Patient 9 with SJS (lane 6). β-actin was used as reference protein

Figure 6.

Transfection of *Pax6* up-regulates Pax6 and K12 keratin. Clonally expanded pannus epithelial cells from Patient 1 with SJS were transfected with EGFP-adenovirus (A, C) or EGFP-Pax6-adenovirus (B, D) for 72 h. EGFP-adenovirus-transfected cells expressed negligible Pax6 (A) and K12 keratin (C), while EGFP-Pax6-adenovirus-transfected cells showed a dramatic increase of Pax6 (B) and K12 keratin expression (D). The difference was significant between the two (E). Bar, 100 μ m

Table 1

Clinical characteristics and laboratory findings

Immunostaining						
Patient	Age/Sex	Diagnosis	K12	K10	Pax6	Epithelial culture Protein extract
1	36/F	SJS	++	++	++	Y
2	36/F	SJS	-	++	+	N
3	51/M	Recurrent pterygium	+	++	+	N
4	10/M	SJS	+	+++	+	N
5	8/M	SJS	+	+	+	N
6	16/F	Aniridia	-	+++	+	N
7	56/M	Chemical burn	+	+++	+	Y(Clone)
8	68/M	Chemical burn	+	++	+	N
9	28/F	SJS	+	++	+	Y(Clone)
10	54/M	Chemical burn	+	++	+	Y
11	35/M	Normal control	+++	-	+++	Y

F, female; M, male; SJS, Stevens–Johnson syndrome; Y, performed; N, not performed; clone, proteins were extracted from cultured epithelial clones and submitted to western blot analysis.