This is the published version of a paper published in *Investigative Ophthalmology and Visual Science*.

Citation for the original published paper (version of record):

Vicente, A., Byström, B., Domellöf, F P. (2018)
Altered Signaling Pathways in Aniridia-Related Keratopathy
https://doi.org/10.1167/iovs.18-25175

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:
http://urn.kb.se/resolve?urn=urn:nbn:se:umu:diva-155119
Altered Signaling Pathways in Aniridia-Related Keratopathy

André Vicente,1 Berit Byström,1 and Fátima Pedrosa Domellöf1,2

1Department of Clinical Science, Ophthalmology, Umeå University, Umeå, Sweden
2Department of Integrative Medical Biology, Section for Anatomy, Umeå University, Umeå, Sweden

Correspondence: Fátima Pedrosa Domellöf, Department of Clinical Science, Ophthalmology, Umeå University, S-901 85 Umeå, Sweden; fatima.pedrosa-domellof@umu.se.

Submitted: July 2, 2018
Accepted: October 18, 2018
Citation: Vicente A, Byström B, Pedrosa Domellöf F. Altered signaling pathways in aniridia-related keratopathy. Invest Ophthalmol Vis Sci. 2018;59:5531–5541. https://doi.org/10.1167/iovs.18-25175

PURPOSE. To study the Notch1, Wnt/beta-catenin, sonic hedgehog (SHH), and mammalian target of rapamycin (mTOR) cell signaling pathways in naïve and surgically treated corneas of aniridia cases with advanced aniridia-related keratopathy (ARK).

METHODS. Two naïve corneal buttons from patients with advanced ARK submitted to penetrating keratoplasty for the first time, one corneal button from an ARK patient that had undergone a keratolimbal allograft (KLAL), two corneal buttons from ARK patients who had previously undergone centered or decentered transplantation, and two adult healthy control corneas were processed for immunohistochemistry in this descriptive study. Antibodies specific against elements of the Notch1 (Notch1; Dlk1; Numb), Wnt/beta-catenin (Wnt5a; Wnt7a; beta-catenin), SHH (glioma-associated oncogene homolog [Gli1]; Hes1), and mTOR (mTOR1; ribosomal protein S6 [rpS6]) signaling pathways were used as well as antibodies against PAX6 and keratin 13 (Krt13).

RESULTS. All ARK corneas presented signs of conjunctivalization and analogous signaling pathway changes in the subepithelial pannus and epithelium, with decreased detection of the Notch1 signaling pathway and an increased presence of the Notch1 inhibitors Numb and Dlk1. Increased detections of Wnt/beta-catenin (enhanced presence of Wnt5a, Wnt7a, and beta-catenin), SHH (detection of Gli1 and Hes1), and mTOR (identification of mTOR and rpS6) signaling pathways were found in the subepithelial pannus and epithelium of all ARK corneas, when compared with normal controls.

CONCLUSIONS. The similarity in pathway alterations found in all ARK corneas, irrespective of limbal stem cell transplantation, further supports the discussion on the role of host-specific factors and limbal stem cell deficiency in ARK.

Keywords: aniridia-related keratopathy, signaling pathways, keratoplasty, keratolimbal allograft, limbal stem cell deficiency, PAX6, sonic hedgehog, mTOR, Wnt, Notch1
includes the transplantation of two to three crescents of corneoscleral rim to the host eye in order to completely surround the limbus of the patient. It typically requires triple immunosuppression to be successful. Chronic stromal inflammation is associated with limbal stem cell deficiency and can interfere with the capacity of limbal stem cell grafts to originate healthy corneal epithelium and is, therefore, a threat to the success of transplanted limbal epithelial stem cells in different pathologies. It is very difficult to acquire corneal button samples of eyes with ARK, as aniridia is a rare condition and surgical treatments are generally reserved as the last therapeutic option. A few highly conserved signaling pathways, including Notch1, sonic hedgehog (SHH), mTOR, and Wnt/beta-catenin, are responsible for the control of cell proliferation and homeostasis in mammals and are vital for eye development. The Notch1 and Wnt/beta-catenin signaling pathways have been identified in the fate determination of limbal stem cells, and the loss of PAX6 induces the conversion of these stem cells into epidermal stem cells. The Notch1 signaling pathway is involved in the regulation of cell fate, both during development and in adult tissue homeostasis, and it is critical for the regulation of proliferation and vertical migration of basal corneal epithelial cells and corneal epithelial repair. Mice corneal progenitor cells lacking Notch1 originate a hyperplastic, keratinized, skin-like epithelium in response to damage. In addition, a switch of cell fate in Notch1−/− epithelium results in an abnormal stroma with neovascularization and a novel extra layer of stroma (pannus), located between the epithelium and the well-organized normal stroma. Dlk1, a transmembrane protein related to developmental processes, is required to maintain cells in the progenitor mode and acts as an inhibitor of Notch1 signaling. Numb is an endocytic adaptor protein that functions as a Notch1 pathway inhibitor. A delicate tuning between the Wnt/beta-catenin and Notch1 signaling pathways is essential during normal tissue development and renewal. The Wnt/beta-catenin signaling has been shown to contribute to augmented proliferation and colony-forming capacity of primary human corneal limbal stem cells and has both Wnt5a and Wnt7a as ligands. Wnt7a is associated with corneal epithelial differentiation control through PAX6. Wnt5a signals mainly via the noncanonical Wnt pathway, and its role in canonical signaling is not clear as it can activate or inhibit the canonical Wnt signaling pathway, depending on receptor availability. The inhibition of Wnt7a or PAX6 transforms limbal stem cells into skin-like epithelium. The ablation of beta-catenin in corneal stromal cells leads to precocious stratification of the cornea epithelium in a mouse model, and a decisive function in epithelial stratification has been therefore attributed to the Wnt/beta-catenin signaling pathway. In small-eye mice, which have lower levels of PAX6, beta-catenin has been shown to be reduced. In PAX6 heterozygotic mice, epithelial cell loss occurs at a higher rate, and if it exceeds cell production capacity, it can lead to the failure of corneal homeostasis.

The SHH pathway is associated with the regulation of cell differentiation, proliferation, and maintenance of tissue polarity. Debridement of the corneal epithelium leads to transient upregulation of SHH expression. SHH activation leads to triggering of the glioma-associated oncogene homolog (Gli1) transcription factor that regulates cell growth and survival. It directly influences Hes1 in the cell nucleus, which is also regulated by the Notch1 signaling pathway, that is crucial to mammalian eye development and homeostatic function of corneal epithelial stem and progenitor cells. The serine/threonine kinase mammalian target of rapamycin (mTOR) signaling pathway regulates proliferation and growth. The activation of this pathway leads to phosphorylation and activation of the ribosomal protein S6 (rpS6). Knockout mice for tuberous sclerosis complex, an inhibitor for the mTOR signaling pathway, present a similar phenotype to anterior segment dysgenesis and aniridia. A loss of mTOR signaling in a mouse model leads to an antiapoptotic corneal environment in which injured and dying cells can contribute to additional tissue destruction. mTOR signaling might be an upstream regulator of PAX6, as disturbed mTOR complex 1 signaling led to reduction in the number of cells that expressed PAX6, BMP4, and Msh homeobox 1. The precise regulation of this signaling pathway seems to be of value in the context of ARK.

The present descriptive study is the first to evaluate some of the most important cell signaling pathways (Notch1, Wnt/beta-catenin, SHH, and mTOR) in both naïve and retransplanted human corneal samples. In our previous study, we established that all these ARK corneas had analogous histopathologic changes with a disruption or absence of epithelial basal membrane, irregular epithelium, loss of the organized pattern of collagen lamellae, lack of collagen I and, in addition, vascularization.

**Materials and Methods**

**Patients**

Aniridia is a very rare disease, and corneal surgery is the last therapeutic option for ARK. This study comprised five corneal buttons from patients with advanced ARK, including two naïve corneal buttons with advanced ARK from patients that underwent their first penetrating keratoplasty (cases A and B), a corneal button from an ARK patient who had been previously submitted to a KLAL (case C), a corneal button from an ARK patient who had received a centered corneal transplantation (case D), and another who had received a decentered corneal transplantation (case E). The diagnosis of aniridia was based on classic clinical features and confirmed by several ophthalmologists in different clinics. In cases D and E, genetic analysis was also done and identified a heterozygous mutation in the PAX6 gene. Case A (Fig. 1a) was a 29-year-old female with irregular epithelium, recurrent erosions, a cloudy cornea, and vision lower than 20/200. Corneal epithelial debridement was performed several times without success. The patient refused systemic immunosuppression and could not, therefore, undergo a keratolimbal stem cell transplantation. A centered penetrating keratoplasty was then performed, even though it had a considerable risk for pannus overgrowth and failure, in an attempt to tackle the recurrent erosions. Case B (Fig. 1b) was a 65-year-old male who was aphakic after cataract extraction and had been unsuccessfully submitted to phototherapeutic keratectomy because of recurrent corneal erosions. The patient continued with severe corneal surface problems and had an opaque cornea. A Boston keratoprosthesis was implanted following the penetrating keratoplasty, as immunosuppression was not possible due to his general health condition. Case C (Fig. 1c) was a 38-year-old female, who two years previously had had a KLAL transplantation, following the technique described by Holland et al. Nevertheless, cataract developed, the cornea did not clear, and visual acuity remained extremely low. The patient was then submitted to a centered penetrating keratoplasty combined with cataract extraction.

Case D was a 35-year-old female who underwent first a centered corneal transplantation and then a decentered retransplantation due to the recurrence of ARK with severe ocular surface disease and persistent corneal erosions. Case E was a
37-year-old female who was submitted to a second decentered retransplantation after a first decentered transplantation that presented persistent cloudiness and recurrent corneal erosions. This study included also two adult control healthy corneas, one from an 82-year-old male donor who had never undergone eye surgery and another from a 74-year-old male donor who had previously been submitted to cataract surgery. All samples were collected with the approval of the Regional Ethical Committee in Umeå and Gothenburg. Informed consent was obtained in accordance with the principles of the Declaration of Helsinki.

**Cornea Samples**

The corneal buttons from patients and the two normal corneas were fixed in formalin and embedded into paraffin wax after removal. Serial sections, 4 µm thick, were collected on Superfrost Plus slides (Thermo Scientific, Rockford, IL, USA). The slides were dried overnight at 60°C in a vertical position and then deposited at 4°C in a slide box.

**Immunofluorescence Staining**

Sections were prepared and treated according to well-established protocols and handled with Tissue-Clear (1466; Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) to be dewaxed and then rehydrated. A water bath incubation (95°C, 30 minutes) in prewarmed citrate buffer (10 mM citric acid, 0.05% Tween 20; pH 6.0) or Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20; pH 9.0) was performed for antigen retrieval, and the sections were then cooled down at room temperature (20 minutes). The slides were rinsed for 5 minutes in running water and then immersed in 0.01 M PBS.

The slides were incubated for 15 minutes with 5% normal goat or donkey serum at room temperature before the incubations with both the primary and secondary antibodies to block unspecific binding. Monoclonal (mAb) and polyclonal (pAbs) primary antibodies against Notch1, Dlk1, Numb, Hes1, Gli1, mTOR, phosphorylated rpS6, Wnt5a, Wnt7a, beta-catenin, PAX6, and keratin 13 (Krt13) were used (Table 1). The sections were incubated with the primary antibodies at 4°C overnight, incubated with the secondary antibodies (Table 2) at 37°C for 30 minutes, and then washed 3 times for 5 minutes in 0.01 M PBS containing 0.01% NaN₃. Vectashield mounting medium or Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; H-1000 and H-1200; Vector Laboratories, Inc., Burlingame, CA, USA) was used to assemble the sections.

In control sections, the primary antibody was omitted to examine potential unspecific binding. Hematoxylin-eosin

**TABLE 1. Primary Antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX6</td>
<td>A B528427</td>
<td>1:25</td>
<td>Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA</td>
</tr>
<tr>
<td>Krt13</td>
<td>MA1-35542</td>
<td>1:250</td>
<td>Thermo Fisher Scientific, Rockford, IL, USA</td>
</tr>
<tr>
<td>Dlk1</td>
<td>Ab21682</td>
<td>1:1000</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Notch1</td>
<td>Ab52627</td>
<td>1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>Ab32572</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>Wnt7a</td>
<td>Bs-66495</td>
<td>1:100</td>
<td>Bios. Inc., Woburn, MA, USA</td>
</tr>
<tr>
<td>Beta-catenin</td>
<td>Ab32572</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>Numb</td>
<td>Ab14140</td>
<td>1:250</td>
<td>Abcam</td>
</tr>
<tr>
<td>Gli1</td>
<td>Ab 92611</td>
<td>1:800</td>
<td>Abcam</td>
</tr>
<tr>
<td>mTOR</td>
<td>(7C10) 2983</td>
<td>1:500</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Hes1</td>
<td>GTX 108356</td>
<td>1:500</td>
<td>Genetex, Irvine, CA, USA</td>
</tr>
<tr>
<td>rpS6</td>
<td>GTX 60800</td>
<td>1:800</td>
<td>Genetex</td>
</tr>
</tbody>
</table>
sections were also made for assessment of the general morphology.

**Microscopy**

A Leica DM 6000 B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a motorized stage and a digital high-speed fluorescence charge-coupled device camera (Leica DFC360 FX; Leica Microsystems) was used to evaluate and photograph the slides. The images were processed with Adobe Photoshop CS6 software (Adobe Systems, Inc., San Jose, CA, USA). Immunostaining was performed under identical conditions for all samples; images were acquired under identical fluorescence microscopy settings and were thereafter processed to reflect the observations under the microscope so that comparisons between different samples treated with the same antibody could be performed. It should be noted that comparisons in staining intensity cannot be made between samples treated with different antibodies, as differences in labeling intensity may reflect differences in antibody affinity or epitope availability rather than differences in protein amount.

**Results**

All patients had been diagnosed with aniridia many years previously, and the diagnoses were confirmed by several pediatric ophthalmologists and cornea specialists in different clinics, but because genetic analysis was only available in two patients (cases D and E), we investigated the immunoreactivity of all samples with an antibody specific against Pax6. In the controls, all nuclei present in the different layers of the cornea epithelium were strongly labeled with the Pax6 antibody. In contrast, in all ARK cases, immunolabeling with Pax6 was weak and not present in all nuclei. There were clusters of stronger Pax6 nuclei in the KLAL ARK cornea (case C). The labeling was weakest in the naïve (case A and B), centered (case D), and decentered (case E) ARK corneas. These findings give further support to the clinical diagnoses of aniridia.

In the cross sections stained with hematoxylin-eosin (Figs. 1A–F), all ARK corneas presented irregular epithelium with varying thickness throughout consecutive sections. The epithelial basement membrane was fragmented and absent in some segments in which the Bowman’s layer was also absent. In contrast, in the control corneas, the epithelium presented regular morphology and thickness as well as epithelial basement membrane (Fig. 1F). In the control healthy corneas, the stroma comprised approximately 90% of the corneal thickness (Fig. 1F), whereas in all ARK corneas, a subepithelial pannus of variable thickness was present (Fig. 1A–E, double-headed arrows). The pannus was present in both central and peripheral parts of the corneal buttons of all ARK corneas, and it contained blood vessels. Both the endothelium and Descemet’s membrane had a normal and similar appearance in the ARK and the control corneas. Immunolabeling with the antibody against Krt13 was present in the epithelium of all ARK corneas, whereas it was completely absent in the control corneas (Figs. 2F–J), indicating conjunctivalization of the ARK corneas. Islands of labeled cells were present among parts with less labeling, in the epithelium of the KLAL cornea (case C), indicating heterogeneity among the epithelial cells.

In normal corneas, immunolabeling for Notch1 was present around basal epithelial cells, where the transient amplifying cells are known to reside, and in a few streaks in the stroma (Fig. 3A). In all ARK corneas, Notch1 labeling was absent in the epithelium (Figs. 3B–E). The epithelium, pannus, and blood vessels in the anterior pannus in all ARK corneas were strongly labeled with pAbs against Dlk1 (Figs. 3G–J). In contrast, in the control corneas, discrete immunostaining was present only in the epithelium (Fig. 3F). Labeling with the pAbs against Numb was also noted in the epithelium, pannus, and blood vessels of all aniridia cases (Figs. 3L–O), as described above for Dlk1. The centered transplanted ARK had a more discrete labeling in the basal epithelial layer (Fig. 3N). In contrast, these pAbs labeled mainly the epithelial cell membranes and did not label the stroma in the control corneas (Fig. 3K).

Immunostaining with the pAbs against Hes1 was absent in control corneas (Fig. 3P). In all ARK corneas, there was strong labeling with these pAbs in the epithelium and in streaks in the pannus, with regional variation, as well as in the blood vessels (Figs. 3Q–T).

The epithelium and the subepithelial pannus were strongly labeled by the pAbs against Gli1 in all aniridia cases (Figs. 4B–E), whereas these pAbs did not label the normal corneas (Fig. 4A). All ARK corneas were labeled with the mAb against mTOR in the epithelium, subepithelial pannus, and blood vessels (Figs. 4G–J). In contrast, no immunostaining was detected with the mAb against mTOR in the normal corneas (Fig. 4F). Immunostaining with mAb against the phosphorylated rpS6 was absent from the stroma and only scarcely present in the surface of the epithelium of normal corneas, in a pattern suggesting sticky adherence to the epithelial surface (Fig. 4K). In contrast, the mAb against rpS6 labeled the epithelium and abundant streaks in the anterior subepithelial pannus of the aniridia cases (Figs. 4L–O). Blood vessels in the anterior pannus were also labeled with the mAb against rpS6 in all ARK corneas (Figs. 4L–O).

The epithelium of normal control corneas and all ARK corneas was labeled with pAbs against Wnt5a (Figs. 5A–E). In all ARK corneas, the anterior subepithelial pannus was also labeled with the pAbs against Wnt5a (Figs. 5B–E). Immunostaining against Wnt7a was detected in the epithelium as well as in sparse streaks in the stroma in the control corneas (Fig. 5F). In all aniridia corneas, pAbs against Wnt7a labeled the epithelium and the pannus, with regional variation. These pAbs also labeled the posterior stroma of the aniridia corneas (not shown). The blood vessels in the subepithelial pannus were also labeled with the pAbs against Wnt5a and Wnt7a in all aniridia corneas (Figs. 5B–E, G–J). The mAb against beta-catenin strongly labeled the contours of the epithelial cells, blood vessels, and streaks in the subepithelial pannus of all aniridia corneas (Figs. 5L–O). In control corneas, the contours of the epithelial cells were labeled with the mAb against beta-catenin, whereas the corneal stroma was not labeled with this antibody (Fig. 5K).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-mouse Dylight 488</td>
<td>715-485-150</td>
<td>1:100</td>
<td>Molecular Probes, Life Technologies, Darmstadt, Germany</td>
</tr>
<tr>
<td>Goat anti-mouse Alexa 488</td>
<td>A21121</td>
<td>1:300</td>
<td>Jackson ImmunoResearch Europe Ltd</td>
</tr>
<tr>
<td>Donkey anti-rabbit FITC</td>
<td>715-095-152</td>
<td>1:50</td>
<td>Jackson ImmunoResearch Europe Ltd</td>
</tr>
<tr>
<td>Goat anti-rabbit Alexa 488</td>
<td>A11054</td>
<td>1:300</td>
<td>Molecular Probes, Life Technologies</td>
</tr>
</tbody>
</table>

Table 2. Secondary Antibodies
DISCUSSION

This is the first study evaluating components of some of the most important cell signaling pathways (Notch1, Wnt/beta-catenin, SHH, and mTOR) in cornea buttons from patients with ARK, including cases of ARK recurrence after transplantation. Even though genetic analysis was not available in all patients, the clinical diagnoses were confirmed by several ophthalmologists, and the pattern of immunolabeling with the antibody against PAX6 supports the aniridia diagnosis. Furthermore, the low levels of PAX6 immunoreactivity in the epithelium of all ARK cases suggested that these were host-derived cells, even in the cases where limbal stem cells were included in the transplant (cases C and E). However, downregulation of PAX6 has also been associated with abnormal epidermal differentiation in other severe ocular surface diseases, such as Stevens-Johnson syndrome, chemical burns, and recurrent pterygium.54 The presence of Krt13 indicated the presence of conjunctival epithelial cells in all ARK cases, which fitted nicely with the clinical characteristics of these patients.

The study has limitations inherent to the very small number of cases used and the availability of paraffin sections only. Although we could not perform RNA-based studies to evaluate up- or downregulation of the components of these pathways, the use of immunohistochemistry allows the comparison between controls and aniridia. Immunohistochemistry is not a quantitative method, but differences in staining levels and patterns among different sections treated with a given antibody, under identical conditions, reveal differences in epitope availability that most likely translate into differences in protein abundance between the sections studied.

The major new findings of the present study were the following:

1. Decreased detection of the Notch1 signaling pathway in all ARK corneas, with increased presence of the Notch1 inhibitors Numb and Dlk1 in the subepithelial pannus and epithelium.
2. Increased detection of the Wnt/beta-catenin signaling pathway with an augmented presence of Wnt5a, Wnt7a, and beta-catenin in the subepithelial pannus and epithelium of all ARK corneas.
3. The lack of Wnt7a did not seem to be the cause of keratinization in ARK corneas.
4. There was an increased detection of elements of the SHH signaling pathway, as demonstrated by the detection of Gli1 and Hes1 in the epithelium and the subepithelial pannus of all ARK corneas.
5. The mTOR signaling pathway was detected, with the presence of mTOR and rpS6 in all ARK corneas, in the epithelium and the subepithelial pannus.
6. All ARK corneas, irrespective of being naive ARK, KLAL, and transplanted ARK corneas, were both centered or decentered and showed similar signaling pathways changes and conjunctival phenotypes in the epithelium.

ARK has been classically attributed to a primary limbal stem cell deficiency55 based on the corneal histopathologic characteristics of the disease, including vascularization and the presence of goblet cells.56 However, more recent evidence suggests that ARK might be due both to intrinsic deficiency of limbal stem cells and to a deficient stem cell niche, with factors such as abnormal wound healing response, defective corneal epithelial migration, impaired epithelial differentiation, and...
FIGURE 3. Cross sections of normal corneas (A, F, K, P) and naïve ARK corneas (B, G, L, Q). KLAL ARK corneas (C, H, M, R), centered transplanted ARK corneas (D, I, N, S), and decentered transplanted corneas (E, J, O, T) labeled with antibodies (green) against Notch1 (A–E), Dlk1 (F–J), Numb (K–O), and Hes1 (P–T). Cell nuclei are labeled blue by DAPI in A–T. Labeling for Notch1, a marker for a signaling pathway involved in the regulation of cell proliferation, was present scattered around basal cells and in some scarce streaks in the stroma in the normal corneas (A). In all ARK corneas, Notch1 labeling was absent in the epithelium and stroma (B–E). In normal corneas, Dlk1, a Notch1 inhibitor, presented a discrete epithelial staining pattern and was completely absent in the stroma (F). The staining pattern in all ARK corneas was different, with strong labeling of the epithelium, subepithelial pannus, and blood vessels (G–J, arrows label the blood vessels). Numb, another Notch1 inhibitor, presented a similar staining pattern to Dlk1, with labeling of the epithelium, blood vessels, and subepithelial pannus of all the aniridia cases (L–O, arrows label the blood vessels). Numb was absent in the stroma of normal corneas (K). The labeling with pAbs against Hes1, a downstream effector of both Notch1 and SHH signaling pathways, was absent in the normal corneas (P). In all ARK corneas, strong labeling of the epithelium, blood vessels, and streaks in the subepithelial pannus, with regional variations, was found with this antibody (Q–T, arrows label the blood vessels). Bars: 100 μm.
conjunctival changes contributing to the pathogenesis of the condition.4,10,52 The present findings in aniridia patients, although obtained at the end stage of ARK, may be interpreted as suggesting that both epithelium and stroma were involved, as similar signaling changes were found in the epithelium and pannus and the epithelial basal membrane was disrupted,5,7 suggesting the possibility of cell signaling crosstalk between these structures. The pathogenesis of ARK can be considered in two different ARK settings in the samples studied, with respect to the presence or absence of donor limbal cells. In cases A, B, and D, donor limbal cells were absent (naïve ARK and centered transplanted ARK corneas), whereas in cases C and E (KLAL and decentered transplantation), donor limbal stem cells were initially present. Given that all corneas had low levels of immunoreactivity to PAX6, the cells in the cornea epithelium appeared to be of host origin, and a possible explanation for the loss of donor limbal stem cells in cases C and E would be inflammation and immune reaction. Furthermore, evidence of conjunctivalization was found, as the epithelial cells of all ARK cases were labeled by the antibody against Krt13. The signaling changes detected in the present study mimic the previously reported mRNA expression characteristic of the conjunctival epithelium regarding Notch1, Numb, beta-catenin, Wnt5a and Wnt7a, Hes1, Gli1, mTOR, and rpS6.57 However, even though the KLAL cornea clearly displayed islands of conjunctivalization among what we assume were cornea epithelial cells, no heterogeneity in the distribution of immunoreactivity to any signaling pathway elements was noticed, which suggested that conjunctivalization may not be the only explanation for the changes found.

**Figure 4.** Cross sections of normal corneas (A, F, K) and naïve ARK corneas (B, G, L), KLAL ARK corneas (C, H, M), centered transplanted ARK corneas (D, I, N), and decentered transplanted ARK corneas (E, J, O) labeled with antibodies (green) against Gli1 (A–E), mTOR (F–J), and rpS6 (K–O). Cell nuclei are labeled blue by DAPI in A–O. PAs against Gli1, a downstream effector of the SHH signaling pathway that regulates maintenance of tissue polarity, proliferation, and cell differentiation, did not immunolabel the control corneas (A), whereas in all aniridia cases, it strongly labeled the epithelium and subepithelial pannus (B–E). It also labeled the blood vessels that were present in the subepithelial pannus (B–D, arrows). In all ARK corneas, the mAb against mTOR, part of a signaling pathway related with the control of cell proliferation, growth, transcription, protein synthesis, ribosomal biogenesis, and cytoskeletal organization, labeled the epithelium, subepithelial pannus, and blood vessels in the anterior subepithelial pannus (G–J, arrows label the blood vessels). The normal corneas were not labeled (F). A mAb against the phosphorylated rpS6, a downstream element of the mTOR signaling pathway, scarcely labeled the surface of the epithelium in normal corneas, in a pattern suggesting sticky adherence to the epithelial surface (K). In all ARK corneas, this mAb labeled the epithelium and abundant streaks in the subepithelial pannus, as well as the blood vessels (L–O, arrows label the blood vessels). Bars: 100 μm.
We cannot exclude the possibility of an altered stroma that could contribute to the ARK pathophysiology and influence the corneal epithelium through a disrupted epithelial basal membrane. In the cases where the corneal stroma has been replaced by donor tissue (transplanted ARK corneas) the keratocytes in the stroma are, at least initially, donor cells without PAX6 mutation. Following corneal transplantation, donor epithelial cells tend to be rapidly replaced, but a high percentage of donor stromal cells can survive for long periods of time, even over 30 years. Taken together, this might suggest a more important role for the epithelium in the pathogenesis of ARK.

The Notch1 signaling pathway has been shown to be important for correct corneal epithelial repair. The Notch1 inhibitors Numb and Dlk1 were present in the epithelium and subepithelial pannus in the anterior stroma as well as in blood vessels centrally located in the ARK corneas, whereas they were practically not detected in the control corneas. Dlk1 is a known inhibitor of Notch1 and is thought to keep cells in an immature status, maintaining the progenitor cell populations. These data fit our findings of Dlk1 restricted to the limbal corneal stroma in normal adults. Numb inactivates Notch1 and was also identified in abundant streaks in all the ARK corneas. Labeling with the mAb against beta-catenin, a downstream effector of the Wnt/beta-catenin signaling pathway specifically associated with growth regulation, revealed a strong labeling of the contours of the epithelial cells, blood vessels, and streaks in the subepithelial pannus in all ARK corneas. In the normal corneas, this mAb labeled the epithelial cells only and was totally absent from the corneal stroma. Bars: 100 μm.
Notch1 in the cornea was found to be a contributing factor for differentiation in the corneal epithelium in earlier studies.\(^\text{54,62}\) The corneas of mice lacking Notch1 heal with a hyperplastic, keratinized, skin-like epithelium and present an abnormal stroma with neovascularization and a novel extra layer of stroma.\(^\text{53,56}\) The aniridia cases included in this study showed a clinical status of cloudy, almost opaque, corneas with a “skin-like” appearance, as shown in Figure 1a-c, which might result from the scarcity of Notch1 in ARK. This subepithelial fibrosis/pannus is also present in cases with total limbal stem cell deficiency.\(^\text{54}\)

The equilibrium between Notch1 and Wnt/beta-catenin signaling pathways, essential to tissue development and homeostasis, seemed to be disturbed in all ARK corneas. Antibodies against Wnt5a and beta-catenin labeled the subepithelial pannus strongly and were scarce in the posterior, healthier stroma. This immunostaining pattern would fit former reports of Wnt5a being expressed in human inflammatory diseases\(^\text{50}\); nevertheless, Wnt5a has never been linked to inflammation in the cornea. It is unclear whether Wnt5a works through the canonical pathway\(^\text{50}\) but similar immunostaining patterns for Wnt5a and beta-catenin found in the present study favor the canonical pathway in ARK corneas. Transforming growth factor-beta and Wnt/beta-catenin signaling can collaborate to drive tissue fibrosis,\(^\text{59}\) which were both present in ARK.

Wnt7a influences corneal epithelial differentiation through PAX6, and downregulation of Wnt7a or PAX6 have been associated with the transformation of limbal stem cells into keratinized epithelium.\(^\text{40}\) Nevertheless, in all the ARK corneas included in this study, Wnt7a was present both in the epithelium, similar to control corneas, and in the subepithelial pannus. Therefore, a deficiency of Wnt7a does not seem to be the underlying cause for keratinization in ARK corneas, leaving a reduced level of PAX6 as a probable cause.\(^\text{40}\)

SHH pathway augmented detection, revealed by Gl1 and Hes1, was present in all ARK corneas. This cell signaling pathway influences cell differentiation, proliferation, and tissue polarity.\(^\text{43}\) and one of the most important downstream effectors of this pathway is Gl1,\(^\text{65}\) which was detected in the ARK but not in the control corneas. Our study suggests there is an upregulation of the SHH cell signaling pathway and, therefore, raises the question whether drugs that inhibit downstream effectors in the SHH pathway could be of interest in ARK.\(^\text{40}\) Hes1 is associated with increased DNA synthesis and proliferation capacity and maintenance of progenitor cells by preventing differentiation during development.\(^\text{57}\) The detection of Hes1 in ARK corneas and the absence in controls correlated with the cell proliferation changes present in the epithelium and subepithelial pannus of ARK corneas.

The mTOR cell signaling pathway has been assigned a crucial role as a growth and cell proliferation regulator.\(^\text{49}\) In the present study, both mTOR and its downstream effector rpS6 were detected in all ARK corneas but not in controls.

PAX6 is a central molecular switch, as it directly regulates upstream and downstream transduction pathways.\(^\text{67,68}\) These regulatory events may not occur in all the ocular cell types or at the same time. PAX6 regulates and maintains human corneal epithelium cell identity, as it has an important role in controlling cell differentiation.\(^\text{69}\) PAX6 is known to be able to reduce Wnt signaling through Sfrp1/2 and to stimulate the Notch cell signaling pathway through Notch2.\(^\text{67,70}–\text{72}\) Even though it has been proven that PAX6 controls corneal wound healing, the specific gene regulation provided by PAX6 in corneal tissues has not been studied, with the exceptions of Aldh3a1\(^\text{74}\) and VEGF receptor-1.\(^\text{75}\) The augmented detection of the Wnt, SHH, and mTOR signaling pathways and the decreased detection of the Notch1 signaling pathway in corneal tissues of patients with ARK are likely related to reduced PAX6 levels.

Stem cell insufficiency has been considered to be the cause of ARK but this view has been challenged as limbal epithelial stem cells may survive in aniridia patients.\(^\text{10}\) In the present study, all ARK corneas, irrespective of limbal stem cell transplantation, presented similar disturbed cell signaling pathways that further supports the ongoing discussion on the role of host-specific factors, including the microenvironment surrounding the limbal stem cells, the corneal epithelium and stroma, in addition to the role of limbal stem cell deficiency, in the pathophysiology of ARK.

Acknowledgments

The authors thank Ulf Stenevi, MD, PhD (Department of Ophthalmology, University of Gothenburg, Sweden) for providing some of the rare samples and Mona Lindström, PhD (Department of Integrative Medical Biology, Section for Anatomy, Umeå University) for excellent technical assistance.

Supported by grants from Swedish Research Council (2015-02438; Stockholm, Sweden), County Council of Västerbotten (Umeå, Sweden), Kronprinsessan Margaretas Arbetsnämnd för Sjukskadade (Valdemarsvik, Sweden), The Medical Faculty, Umeå University (Umeå, Sweden), and Carmen and Bertil Règners Stiftelsen (Stockholm, Sweden).

Disclosure: A. Vicente, None; B. Byström, None; F. Pedrosa Domellöf, None.

References


27. Tsai TH, Sun MH, Ho TC, Ma HI, Liu MY, Tsao YP. Notch prevents transforming growth factor-beta-assisted epithelial-mesenchymal transition in cultured limbal progenitor cells through the induction of Smad7. **Mol Vis.** 2014;20:522–534.


