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Human aniridia limbal epithelial cells lack expression of keratins K3 and K12

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ABSTRACT

Aniridia is a rare disease of the eye that affects the iris, lens and the cornea. In about 90% of the cases, patients showed a loss of PAX6 function. Patients with aniridia often develop aniridia-related keratopathy (ARK), due to limbal stem cell insufficiency.

The aim of this study was to determine the differentiation status of limbal epithelial cells (LECs) in patients with ARK.

Epithelial cells were isolated from the limbus region of two patients with aniridia and cultured in KSFM medium supplemented with EGF and BPE. Normal cells were obtained from limbus region of cadaveric control patients. Cells were analyzed with RT-PCR, qPCR and Western blot to evaluate expression of the developmental transcription factor, PAX6, potential stem cell markers, ΔNp63α and ABCG2, and corneal differentiation markers, keratin 12 (K12) and K3. Conjunctival differentiation markers, keratin 13 (K13) and K19 were also investigated. Cells were immunostained to evaluate K3, PAX6, and p63α protein expression. Protein coding sequence of PAX6 from patient LEC-cDNA was cloned and sequenced.

RT-PCR showed that K3 and K12 transcripts were absent from patient cells, but present in healthy control preparations. Transcription levels of PAX6, ABCG2, and p63α of aniridia patients show no differences compared to normal control cells. Western blot showed reduced PAX6, protein levels in aniridia-LECs compared to control-LECs. Immunostaining also showed reduced PAX6 and K3 expression in aniridia-LECs compared to control-LECs. One aniridia patient showed a loss of stop codon in half of the cloned transcripts. In the second aniridia patient mRNA degradation through nonsense mediated decay seems to be very likely since we could not identify the mutation c.174C > T (Refseq. NM_000280), or misspliced transcripts in cDNA.

We identified decreased PAX6 protein levels in aniridia patients in addition to decreased K12 mRNA levels compared to control cells. This result indicates an altered differentiation of limbal epithelial cells of aniridia patients. Further studies are necessary to evaluate the mechanism of differentiation of limbal epithelial cells in aniridia.

1. Introduction

Aniridia is frequently caused by *PAX6* haploinsufficiency, due to several mutations in the *PAX6* gene locus (Neuhaus et al., 2014; Prosser and van Heyningen, 1998; Tzoulaki et al., 2005). PAX6 is an embryonic transcription factor expressed in conjunctival and corneal epithelium (Koroma et al., 1997). Aniridia causes a loss of the stem cell niche in the palisades of Vogt, and it progresses to aniridia-related keratopathy (ARK) (Lagali et al., 2013; Le et al., 2013; Nishida et al., 1995). It is often argued that, in aniridia, there is also a loss of limbal epithelial stem cells (LESCs). The limbus region prevents conjunctiva and blood vessels from growing onto the clear corneal surface (Chen and Tseng, 1991). Thus, the loss of LESCs would facilitate conjunctival cell invasion into the cornea (Dua and Azuara-Blanco, 2000; Nishida et al., 1995).

The corneal epithelium provides a barrier to protect the underlying stroma from pathogens, and it supports the nutrition of corneal stromal cells. Permanent loss of corneal epithelial cells is compensated by a proliferation of LESCs (Sangwan, 2001; Secker and Daniels, 2008). The LESC population is estimated to comprise 0.5%–10% of the total limbal cell population (Ebrahimi et al., 2009; Romano et al., 2003). There is no unique marker for LESCs; however, it is widely accepted that LESCs are

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located in the limbus region, proliferate slowly, lack most differentiation markers (Cotsarelis et al., 1989; Dua and Azuara-Blanco, 2000; Schermer et al., 1986). Although stem cells proliferate slowly, their daughter cells, transient amplifying cells (TACs), become more differentiated and proliferate rapidly (Tseng, 1989). TACs migrate into the central cornea and from the basal to the apical surfaces, then differentiate. This differentiation coincides with the appearance of the corneal keratins, K3 and K12 (Ahmad et al., 2006; Kolli et al., 2008). Because limbal stem cells are known to express elevated levels of ΔNp63α and ABCG2 (an ABC transporter), these molecules are referred to as limbal stem cell markers (Budak et al., 2005; Pellegrini et al., 2001).

Destruction of the limbus region, or the LESCs it harbors, leads to recurrent epithelial defects, and conjunctiva and blood vessel invasions cause keratopathy (Huang and Tseng, 1991). There is evidence that the PAX6 gene is involved in ARK progression (Mort et al., 2011; Nishida et al., 1995), the mechanism remains unknown, but oxidative stress and perpetual wound healing state is a hallmark of $Pax6^{+/-}$ mouse corneal epithelial cells (Ou et al., 2008, 2010). Based on the different pathways of ARK development, several hypotheses have been proposed to explain how PAX6 might be involved in processes that influence pathogenesis.

One hypothesis is based on the lack of a stem cell niche in patients with ARK. This observation was linked to stem cell insufficiency, which is also observed in other diseases that affect the limbus (Lagali et al., 2013; Le et al., 2013; Nishida et al., 1995). Therefore, it is commonly believed that patients with aniridia lack LECs. However, $Pax6^{+/-}$ mice showed reduced corneal epithelial cell layers (Davis et al., 2003; Ramaesh et al., 2003), but unaltered expression of p63α, an established LESC proliferation maker (Pellegrini et al., 2001) and an enhanced overall LESC proliferation rate (Douvaras et al., 2013). Thus, it was hypothesized that, in humans, PAX6 could accelerate LESC proliferation, and this may lead to stem cell depletion after several years (Rama et al., 2015).

A second hypothesis holds that PAX6 might be involved in aniridia pathogenesis by binding to the *K12* promoter (Liu et al., 1999). K12 is expressed in the central corneal epithelium, and it is important for maintaining epithelial stability. Previous studies in patients with Meesmann dystrophy showed that several mutations in the *K3* and *K12* genes led to “unstable” epithelium (Corden et al., 2000; Irvine et al., 1997). Other studies showed that PAX6 influenced the structural proteins desmoglein and γ-catenin, which suggested that, in aniridia, corneal epithelial cell differentiation was disturbed. This disturbance resulted in insufficient adherence and migration of the cell layer formed by TACs (Davis et al., 2003).

A third hypothesis was based on the observation that, in $PAX6^{+/-}$ mice, conjunctival and goblet cells localized to the central corneal region, before conjunctival cell incursion (Ramaesh et al., 2005b). Ramaesh et al. hypothesized that alterations in the basement membrane or stromal keratocytes might influence corneal epithelial cell differentiation. Indeed, $Pax6^{+/-}$ mice showed intracellular vacuoles in non-epithelial cell types, like endothelial and stromal cells (Mort et al., 2011). Thus, it was hypothesized that PAX6-mediated alterations in neighboring cells might influence LESC differentiation. However, those observations prompted the question of whether conjunctival and corneal progenitor cells arise from the same origin. Some groups claimed that adjacent cells could influence the fates of corneal and conjunctival progenitor cells. However, it is widely accepted that conjunctiva and corneal epithelial cells arise from distinct stem cell lineages (Wei et al., 1996; Wu et al., 1994) (Review: (Sangwan, 2001)).

Those extensive studies have shown that the mechanism of ARK progression, though controversial, appears to be more complex than a simple loss of stem cells. Moreover, the patients with aniridia that we examined typically exhibited corneal pannus.

The present study aimed to investigate whether epithelial cells existed in the limbus region of patients with aniridia, and how these cells were affected by the aniridia phenotype. To that end, we isolated and characterized epithelial cells from the limbal region of these patients. Our findings were expected to advance our understanding of the mechanisms of ARK progression in humans.

2. Materials and methods

2.1. Ethical considerations

All experiments were conducted according to the principles expressed in the Declaration of Helsinki. The use of corneal scleral donor rims for an aniridia research project was approved by the Ethics Committee of the Saarland (Number 226/15). Informed consent was obtained from the two patients with aniridia, before keratoplasty.

2.2. Surgery and isolation of aniridic epithelial cells

Two patients with aniridia underwent pannus removal before a perforating keratoplasty. During this surgery, 2–3 mm length of the limbal region beneath the pannus was biopsied. The biopsy was taken with a spring scissor at the corneal limbus, after removal the fibrous pannus with the hockey knife. The pannus overgrows the Limbus from conjunctiva side and covered stem cell niche. It was gently peeled off. The degenerated avascular parts were removed using a hokey knife. The limbus region was dissected with this procedure. Since the limbal epithelial cells are known to reside in deeper layers of the limbus and it is very likely that there will be epithelial cell clusters, which have not been removed during this procedure. For controls, we acquired corneoscleral rings from patients without aniridia. For qPCR experiments, an additional control was acquired from donor cornea prior organ culture to exclude effects of organ culture. Therefore, a 2 mm Ø Biopsy of limbus region was taken and treated similar than aniridia samples. In Table 1 all relevant information's regarding patients and controls and passaging number used for experiments are listed.

2.3. Cell culture

Control and aniridia LECs were extracted in a similar manner, as described previously (Chen et al., 2011; Gonzalez and Deng, 2013). Briefly, limbal tissue was digested in collagenase A solution [4 mg/ml] diluted in keratinocyte serum-free medium (KSFM; Roche Life Science, Cat. No. 10103578001). After 20-h incubations at 37 °C, tissues were dissociated with pipetting, and incubated again for 1 h. Tissue suspensions were filtered through a 20-μm CellTricks filter (Sysmex Partec,

Table 1
Information about donors, and culture conditions.

Name/Age/Sex	PMT	Organ Culture[a]	P0 (24 Well)	P1 (2 × 6Well)	P2 (3 × 6 Well)
Ctrl1/84/m	15 h	22 Days (4 Days Medium II)	13 days	+4 days	+3 days
Ctrl2/61/m	13 h	17 Days	9 days	+4 days	+4 days
Ctrl3/54/m	12 h	no	11 days	+3 days	+4 days
AN1/49/f	no	no	7 days	+3 days	+3 days
AN2/51/f	no	no	8 days	+4 days	+4 days

PMT = Post mortem Time, m = male, f = female, P = Passage, no = Step or value was not applicable.
[a] 34 °C, Medium I (MEM-Earle's + 2% FCS) purchased from Biochrome AG, Medium II additional contains 6% (w/v) Dextran.