The Ocular Surface

Pathophysiology of aniridia-associated keratopathy: developmental aspects and unanswered questions

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Pathophysiology of aniridia-associated keratopathy: developmental aspects and unanswered questions

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Abstract

Aniridia, a rare congenital disease, is often characterized by a progressive, pronounced limbal insufficiency and ocular surface pathology termed aniridia-associated keratopathy (AAK). Due to the characteristics of AAK and its bilateral nature, clinical management is challenging and complicated by the multiple coexisting ocular and systemic morbidities in aniridia. Although it is primarily assumed that AAK originates from a congenital limbal stem cell deficiency, in recent years AAK and its pathogenesis has been questioned in the light of new evidence and a refined understanding of ocular development and the biology of limbal stem cells (LSCs) and their niche. Here, by consolidating and comparing the latest clinical and preclinical evidence, we discuss key unanswered questions regarding ocular developmental aspects crucial to AAK~~, while~~. We also ~~highlighting~~highlight hypotheses on the potential role of LSCs and the ocular surface microenvironment in AAK. The insights thus gained lead to a greater appreciation for the role of developmental and cellular processes in the emergence of AAK~~, and~~. They also highlight areas for future research to enable a deeper understanding of aniridia, and thereby the potential to develop new treatments for this rare but blinding ocular surface disease.

~~Inhalt~~

Table contents

Introduction

Aniridia is a rare, pan-ocular, bilateral and congenital disease affecting the normal development and function of almost all eye structures. ~~A variable degree of iris hypoplasia or total absence of iris is the hallmark of the disease.~~ Aniridia is inherited in an autosomal dominant manner, with high penetrance and yet with variable phenotypic expressivity even within the same family. A variable degree of iris hypoplasia or total absence of iris is the hallmark of the disease. ~~Over 90% of~~Over 90% of cases of aniridia involve haploinsufficiency of the *PAX6* gene, commonly due to heterozygous non-sense mutations on one copy of the gene. Well over ~~1000~~500 *PAX6* unique mutations have been identified in patients with familial or sporadic aniridia (http://LOVD.nl/PAX6) occurring in all exons and in non-coding regions of the gene~~, and~~. They include point mutations leading to amino acid substitution, deletions, insertions, premature termination, splicing defects or loss of the entire gene. Aniridia can occur either as an isolated malformation or as part of a syndrome such as WAGR ~~syndrome caused by large deletions that affect both *PAX6* and the adjacent *WT1* gene,~~ (also known as WAGR complex, Wilms tumour-aniridia syndrome, aniridia-Wilms tumour syndrome~~),~~) caused by large deletions that affect both *PAX6* and the adjacent *WT1* gene. WAGR is characterized by **W**ilms tumor, **A**niridia, **G**enitourinary anomalies and developmental delay (formerly 'mental **R**etardation') [1, 2~~] or~~]. Aniridia may also occur in Gillespie syndrome, caused by mutation in a different gene, *ITPR1*, ~~-~~consisting of aniridia, cerebellar ataxia and intellectual impairment [3]. Although nearly all P*AX6*-aniridia patients develop progressive opacification of the cornea termed aniridia-associated-keratopathy (AAK), glaucoma and cataract, the underlying molecular mechanisms and physiological causes of the many pathological features of the disease have not yet been elucidated. A main reason for this is that ocular development, maintenance and regeneration ~~involves~~involve crosstalk between different tissues, and complex ~~interaction~~interactions with the immune, nervous, and metabolic systems.

~~In particular, the~~ AAK ~~accompanying~~, also sometimes termed aniridia-related keratopathy (ARK) or aniridic keratopathy, is a highly prevalent~~,~~ condition in *PAX6*-heterozygotes that is potentially painful and severely limits functional vision [4]. As the cornea is readily accessible and can be partly or fully replaced, AAK is a prime target for therapies to improve vision in aniridia. Even a small reduction in the severity of AAK can translate into significant benefits in vision and ocular surface symptoms. Moreover, AAK is progressive, so it is worthwhile to concentrate efforts into understanding its pathogenesis and pathophysiology, because the ~~prospect~~potential may exist for slowing, altering, or even arresting its progression ~~or severity~~ in younger subjects.

Symptoms of AAK include breakdown of the corneal surface, with epithelial thinning or loss, inflammation with immune cell infiltration, vascularisation and chronic progressive opactification. These symptoms overlap partly or wholly with those that arise when the stem cells at the periphery of the cornea – limbal stem cells (LSCs) – are deficient due to disease or injury. Traditionally, it has been widely believed that AAK is a consequence of a progressive limbal stem cell deficiency (LSCD) [5, 6], although to date, there is no definitive proof of loss or degradation of limbal stem cells (LSCs) or their niche as the causal event triggering AAK. Given the complexity of the pathology present in AAK [7], it is more likely that *PAX6* controls multiple physiological and biological factors that act together, and that their dysregulation in aniridia leads to AAK development.

Identifying the possible underlying pathogenic mechanisms leading to AAK development and progression holds the potential for establishing new therapeutic options for the single greatest unmet need of patients severely affected by aniridia. In this review, we highlight key questions of importance – many still unresolved – concerning developmental aspects and the emergence of AAK. We show that to regard AAK as purely a limbal stem cell deficiency is to ignore the

multiple developmental and pathogenic events in aniridic eyes, affecting multiple tissues, that may contribute to the onset or progression of the disease. We highlight some potential therapeutic strategies that may arise from a fuller understanding of the developmental basis of AAK. The topics discussed are also relevant more generally to diverse types of environmental or congenital corneal pathologies that involve LSCD, corneal neovascularization and opacification. Finally, we present recent findings on translation of results from animal models to humans as a critical step in developing future therapies to treat AAK and understanding their relevance to other corneal diseases.

1 Are developmental deficiencies involved in the emergence of AAK?

PAX6[1] codes for a key transcription factor that is essential for eye development and maintenance. *PAX6* is expressed at the earliest stages of eye development and in multiple eye tissues throughout life. ~~Multiple~~Several *Pax6*-heterozygous mutant murine strains ~~(~~(collectively 'small eye' or 'Sey' mice) have been ~~characterised~~characterized and used as *in vivo* models of aniridia, to study the roles of the gene and the developmental and ~~degenerative~~pathological aspects of the disorder [8]. The mouse models represent a spectrum of mutations. Some such as the Harwell allele $Pax6^{Sey\text{-}H}$ and 'Dickies small eye' $Pax6^{Sey\text{-}Dey}$ are large deletions affecting *Pax6* and surrounding genes, and show severe phenotypes that are perhaps not ideal for studying human aniridia and AAK [9-12]. Others, including $Pax6^{Sey}$ (=$Pax6^{SeyMH}$), $Pax6^{Sey\text{-}Neu}$ (=$Pax6^{Sey\text{-}Neu1}$), $Pax6^{ADD4802}$, $Pax6^{Coop}$, $Pax6^{Aey11}$, $Pax6^{AEY18}$ and the allelic series $Pax6^{2Neu}$ to $Pax6^{10Neu}$ and $Pax6^{Leca1–4}$ include point mutations leading to premature termination, frameshifts or splice defects [9, 13-16]. There are also engineered deletions ($Pax6^{LacZ}$) and floxed alleles ($Pax6^{flox}$) that yield large deletions upon action of Cre recombinase [17-19]. With the exception of some hypomorph alleles ($Pax6^{4Neu}$, $Pax6^{7Neu}$ and $Pax6^{Coop}$) and a gain of function ($Pax6^{ADD4802}$), most alleles listed above are thought or known on the basis of nonsense-mediated RNA decay, phenotypes, and/or allelic complementation studies, to be null for *Pax6*. Unless stated otherwise, all *Pax6* mouse mutants discussed below represent null alleles.

Pax6-knockout results in early failure in lens placode development and anophthalmia (eye absence) but heterozygotes display a phenotype that ~~closely~~ resembles human aniridia, ~~with degenerative~~including a progressive AAK [14, 28]. Although the phenotype of *Pax6* mouse mutants is affected by genetic background and there is individual variation in severity even within litters, overall they are remarkably consistent models of AAK. ~~With very high penetrance~~Concordance with human aniridia, however, is not complete. For example, $Pax6^{+/-}$ mice ~~show~~exhibit microphthalmia with a 10% reduction in eye diameter and reduced lens size; while microphthalmia can ~~often~~also occur in human aniridia, most patients have a relatively normal eye and lens size [20]. The extent to which microphthalmia in mice may modulate the anterior segment dysgenesis associated with *Pax6* is not known, but it should be noted that the transgenic 'PAX77' mouse that overexpresses 5-6 copies of human *PAX6* exhibits microphthalmia (including microcornea) without an AAK phenotype [21~~]~~. , 22].

Typically, the ~~cornea of~~ *Pax6* heterozygous newborn mice ~~presents~~exhibit morphological ~~and anatomical phenotypes~~alterations in the corneal epithelium ~~as well as~~ . Separation of the lens from the cornea during development is delayed in ~~the posterior layers of the cornea consisting of~~*Pax6*-mutant mice and iridocorneal and/or lens-cornea adhesions similar to Peters' anomaly ~~[22-25].~~may be observed at birth, dependent on mouse strain and the *Pax6* allele [23-26]. The

[1] Human nomenclature is used whenever applicable. If findings are species-related the gene/protein nomenclature of the species is used according to the literature source.

gross abnormalities ~~that~~these mice ~~show~~can exhibit at birth are small eyes and ~~usually~~/or an opacity of the central cornea due to delayed or failed detachment of the lens from the cornea ~~(i.e. Peters anomaly).~~. In contrast to the murine models, the ocular surface of the majority of aniridia patients does not exhibit any apparent gross abnormality at birth. Exceptionally, rare cases of Peters anomaly are caused by *PAX6* mutations [~~26]. Additionally,~~27] but in nearly all cases of congenital aniridia, a proper separation of the cornea and lens is observed~~, contrary to the eye phenotype of *Pax6* heterozyogous mouse pup [27].~~ [28]. Despite ~~the~~a seemingly normal cornea observed early in life, however, closer inspection of the central cornea in a 4-year-old children with congenital aniridia indicated reduced sensitivity to mechanical touch, reduced sub-basal nerve density and elevated presence of antigen-presenting dendritic cells [~~28~~29]. Moreover, the corneal thickness is known to be pathologically increased in aniridia, even in childhood [~~28~~29]. As outlined below, *PAX6* is expressed in many eye structures during development~~.~~ (section 1.1). Therefore, it is probable that AAK is at least partly influenced by developmental defects and not solely due to postnatal emergence of pathological corneal function. AAK penetrance is full, but its phenotypic expressivity is highly variable between individuals, even between siblings [~~29~~30]. This suggests that strong environmental and stochastic components, and/or modifier genes that act in concert with *PAX6* and vary between individuals can influence the expression of the disease. This could also be true for epigenetic differences between individuals ~~modulating~~that may modulate AAK severity. It would moreover be important to understand how the different eye structures influence each other during corneal development, and the role of PAX6 levels in influencing the onset and severity of AAK.

PAX6/*Pax6*, the mammalian orthologue of the Eyeless gene (Ey) in Drosophila, is a paired and homeodomain transcription factor which is essential for eye~~,~~ development. The activity of PAX6 in eye development is evolutionarily conserved as the human or mouse *Pax6* genes can induce ectopic formation of the compound eye from Drosophila imaginal discs as well as in Xenopus embryos [31, 32]. *PAX6*, however is also important for brain, gut and pancreas development [~~30-32~~33-35]; see review [~~33~~36]. This multiple organ involvement partially explains why haploinsufficiency of *PAX6* causes in adults, not only aniridia phenotype, but also several non-ocular conditions such as obesity, glucose intolerance and diabetes, and anosmia [~~34, 35]. The activity of PAX6 in eye development is evolutionarily conserved as the human or mouse Pax6 genes can induce ectopic formation of the compound eye from Drosophila imaginal discs as well as in Xenopus embryos [36, 37].~~37, 38]. Sleep disorders are also reported in patients and *PAX6* may impact brain structures such as the pineal gland [39].

The importance of *PAX6* for different structures of the eye in particular is highlighted by the fact that aniridia patients suffer from multiple eye abnormalities in both anterior and posterior ~~tissues~~segments of the eye. *PAX6* is expressed in the ~~majority of the~~multiple ocular cell types ~~and~~ from the earliest stages of eye development and throughout life (***Figure 1-3***). The exact role of PAX6 in lens and optic cup derivatives has been systematically investigated using conditional mutagenesis (reviewed in [~~38~~40]). By contrast, the impact of *Pax6* on corneal development ~~remains~~continues to be further explored, requiring efficient genetic deletion in the various corneal cell types [~~39~~41].

1.1 How is the anterior segment of the eye formed?

In vertebrate eye development, *Pax6* expression is detected in the anterior neural plate in the eye field region [~~40~~42] and subsequently in both the neuroectoderm and surface ectoderm progenitors of the eye [18, ~~30, 40, 41~~33, 42, 43]. The surface ectoderm gives rise to the lens

and corneal ~~anterior~~ epithelial ~~structures (~~layers (lens, limbus and corneal epithelium), while the neuroectoderm populates the optic vesicles that undergo morphogenesis to form the optic cups. The outer layer of the optic cups is populated by the ~~pigmented epithelium progenitors (iris and ciliary body pigmented epithelia and~~ retinal pigmented epithelium progenitors (RPE~~)),~~), while the inner layers of the optic cup differentiate to form all of the retinal neurons and the Müller glia cells~~(~~. The anterior optic cup rim gives rise to the iris and ciliary body pigmented epithelia ***Figure 1***). The ocular mesenchyme surrounding the optic cup rim eventually contributes to the iris and corneal stroma. The high and continuous expression of *Pax6* in cells that derive from surface ectoderm and optic cup (lens, corneal epithelium, iris and ciliary epithelium) is required for the expression of genes encoding ~~for~~ transcription factors, structural and signaling molecules, which are critical for the morphogenesis and differentiation of the neuronal, pigmented and the transparent cornea and lens eye lineages. The role of Pax6 in each of these structures and the signaling cues that mediate their coordinated development during the formation of the anterior segment of the eye, as relevant to the onset of AAK, is briefly summarized in the next section.

~~1.1 How is the anterior segment of the eye formed?~~

The anterior segment of the eye includes the cornea, conjunctiva anterior chamber, iris, lens and ~~lens~~associated structures. In development of these structures, the neural and surface ectodermal cells interact with mesenchymal cells of neural crest and mesodermal origin. These various and complex interactions are briefly described below. For a detailed description of the associated processes, see reviews [~~42-45~~44-47]. The putative influence of PAX6 dosage on these different processes in development of the corneal phenotype is discussed in separate sections.

1.1.1 Early development – Surface ectoderm and optic vesicle

Morphologically, the development of the eye is evident with the formation of the optic vesicles.

The optic vesicles are *PAX6*-expressing bilateral evaginations from the diencephalon that give rise to the optic cup through morphogenesis (reviewed in [48, 49]). The optic cup interacts with surface ectoderm, lens and migrating mesenchymal cells. Disruption of developmental processes has been described by manipulating signaling pathways. A saucer-shaped optic cup, ventral coloboma, or a deficiency of periocular mesenchyme were observed by manipulating either Wnt-, Lrp6-, or retinoic acid signaling [50]. The peripheral rim of the optic cup contain progenitors that will give rise, during post natal stages to the pigmented and non-pigmented layers of the iris and ciliary body (reviewed in [51]). Wnt ligands and BMP signaling from the surface ectoderm elicit Wnt/GSK3β-response in retinal pigment epithelium progenitors (RPE) and are crucial for production of the correct number of RPE cells and proper curvature of the optic cup [50, 52].

~~1.1.1~~1.1.2 Lens

As soon as the optic vesicle forms, in mice at E8.5, *Pax6* is expressed in neuronal and surface ectoderm progenitors of the eye (See E8.5 ***Figure 1***). The lens derives from Pax6-expressing lens-competent facial ectoderm which is contacted by the optic vesicle [53].

After the lens placode has been ~~. In both regions~~induced, it invaginates to form the lens vesicle. The detailed mechanisms of lens induction and genetic and signaling networks are reviewed in [54]. In the lens and optic cup, *Pax6* expression is specified independently, ~~through distance gene regulatory networks and~~ by cis-regulatory elements [~~38, 46~~40, 54]. *Pax6* autoregulates its own expression and the *Pax6* surface ectodermal enhancer element driving PAX6 autoregulation interacts with another transcription factor, SOX2 [~~47~~55]. *Sox2* expression is induced in the surface ectoderm upon an inductive signal from the optic vesicle and determines lens placode formation (See E9.5 ***Figure 1***) [~~48~~56].

~~Notably in adult mice, SOX2 is also involved in the regulation of TP63 and the stem/progenitor cell state in corneal epithelium [49]. Therefore, it is likely that similar or modified PAX6-dependent regulatory networks or a reduced strength of inductive signals could impact the LSC fate determination (Discussed further in section 1.4).~~

~~After the lens placode has been~~ specified, it invaginates to form the lens vesicle. ~~The detailed mechanisms of lens induction and genetic and signaling networks are reviewed in [46].~~ The cells from the anterior pole of the lens vesicle give rise to the lens epithelial cells, whereas the posterior cells differentiate into lens fiber cells. PAX6 ~~is involved from prior~~continues to ~~lens placode formation (being required for lens competence [50]) and subsequently~~be expressed during lens ~~placode formation and~~ invagination ~~as well as in lens fiber~~ and differentiation [19, ~~51~~57] as reviewed by Cvekl A *et al.*[~~52].~~58] and is maintained in the lens epithelium throughout life.

1.1.3 Corneal Stroma and endothelium

After the lens, optic cup and ocular surface ectoderm have been specified, neural crest cells and mesodermal cells migrate between these structures. The corneal stroma is formed by neural crest-derived cells from the periocular mesenchyme, a population of mesenchymal cells located near the optic cup and presumptive lens. Fate mapping of mesoderm-derived cells showed a contribution to the corneal endothelium and stroma [59, 60].

In humans (and birds), three waves of neural crest cells (NCCs) migration are reported in contrast to two waves in mice (Note that Figure 1 refers to mouse development). A first wave of NCCs migrate between the lens and the surface ectoderm to form the corneal endothelium. A second wave forms the stromal keratocytes. The third wave of NCCs contributes to the ciliary body and iris structures (see Section 1.1.4). In mice a single wave of neural crest migration gives rise to both endothelium and stroma.

Studies isolating primary stromal cells from transgenic mouse corneas revealed similarities in mRNA expression profiles (*Twist*, *snail, Slug and Sox9*) between neural crest-derived precursors and the isolated corneal precursor cells. This finding was regarded as evidence for a neural crest origin of these cells, which are important for the turnover of stromal tissue [61].

The corneal endothelium provides an important pump function, actively maintaining a fluid and electrolyte balance between the anterior chamber and corneal stroma to prevent corneal swelling, thus maintaining corneal transparency. The cellular density of this single endothelial cell layer is critical for maintaining an adequate pump function. From a histologic analysis of human fetuses, it has been shown that the cellularity of the endothelium rapidly decreases in the prenatal period from 16 weeks of gestation to term, at the same time the cornea grows in size [62]. The reduction in endothelial cell density during this period is about 50%, while the density reduces further by a third during the first two years of life. A putative effect of PAX6 levels on stromal, limbal stromal and endothelial development and how this could influence AAK is discussed in Section 1.6.

1.1.4 Iris and anterior chamber angle

The anterior chamber angle is the angle between the iris and the corneal endothelium in the limbal region. The iridocorneal angle contains important aqueous humor drainage structures such as the trabecular meshwork and Schlemm's canal.

The contiguous iris and ciliary body epithelia derive from the rim of the optic cup while the stromal layers derive from the ocular mesenchyme [26]. A *Pax6* expression gradient is observed in the optic cup with the highest level from the distal (close to the lens) to proximal side (close to the optic nerve [33] (Figure 1, E 15.5).

For the iridocorneal angle, lineage tracing experiments in mice revealed the contribution of NCCs to the ciliary muscles, ciliary blood vessels, anterior iris, trabecular meshwork and Schlemm's canal in the iridocorneal angle [59, 63, 64]. Mesodermal cells also contribute to structures such as the lining of Schlemm's canal and the iris stroma, but not to the ciliary muscles [59]. Pax6 may participate in regulation of the factors required for the migration of NCC into the eye [65] (see also reviews on neural crest of the eye [44, 66]).

In mouse embryos (E 15.5), iris and ciliary body progenitor cells can be molecularly distinguished from cells which form the presumptive neural retina. Thus the proper development of the iris relies on the correct compartmentalization of the optic cup (See review in [26] for molecular details).

Although the molecular mechanism responsible for the normal development of the iridocorneal angle has not yet been elucidated, a series of developmental steps are described in the mouse where final maturation of Schlemm's canal and the trabecular meshwork extends postnatally to P42 [67]. In humans, the developmental processes are similar, with all rudimentary structures developed at birth [68]. Further maturation and reorganization take place, likely due to mechanical stress and aqueous humor flow and this process could last 1-8 years postnatally [69].

The impact of changing PAX6 levels on anterior chamber development and possible impact on the ocular surface is discussed in Section 1.5.

~~1.1.2~~1.1.5 Limbus and limbal stem cells

After the lens has formed, the PAX6-positive cells ~~maintained in~~of the surface ectoderm ~~later~~ segregate and give rise to the lineages of the anterior ocular surface epithelia, including ~~the~~ conjunctival and limbal/corneal lineages [19] (***Figure 1***, E8.5-E15.5).). In humans (but not mice), specialized anatomical structures, the 'palisades of Vogt', develop at the limbus – the boundary ring around the periphery of the cornea where the stem cells reside in adult life. The developmental aspects of their formation is of utmost interest, as they are important for stem cell homeostasis and are affected in AAK. To make the overview easier to understand, a comparison of the key events of the anterior chamber development in different animal models is shown (***Figure* 2**).

At 8.5 WG (week of gestation), the human fetal cornea is still continuous with the surface ectoderm. At 12-22 WG, in turn, individual components such as the conjunctiva, cornea and limbus can be distinguished ~~[53].~~by gene expression [70]. In 12 WG fetal corneas, the presumptive limbus ~~in~~ is observed as ~~a "ridge like"~~'ridge like' feature or ~~dimpling~~a 'dimpling' in the epithelium [~~54, 55~~71, 72]. In human fetal corneas, the ~~unique limbal niche structures that are known as the '~~palisades of ~~Vogt'~~Vogt are not yet detected and are probably formed postnatally. During 8-22 WG, the limbal epithelium starts to become thicker than in the surrounding cornea and conjunctiva. Single cell RNA-seq studies of the developing human cornea indicate the presence of a proliferating epithelial progenitor cluster with highly expressed corneal epithelial stem and progenitor markers *TP63, CLDN1, CLDN4* and *TXNIP*

at 18 WG, indicating the first presence of "a peripheral limbal-like region" harboring the limbal stem and/or progenitor cells [5370].

FunctionalDue to the lack of detailed molecular and mechanistic studies have beenof cell fate determination in the mammalian limbus, corneal epithelium and conjunctiva, here we refer also to functional studies done in chickens, to elucidate the time pointpoints when the cornea and conjunctiva become spatially separated by a limbus.

In this animal modelthe chickens, a diffusion barrier between the corneal epithelium and conjunctiva is established at embryonic day 8 (See ***Figure 2*** for species comparison) [5673]. This observation is also supported byconsistent with recent transcriptional data infrom the developing human cornea at 12 WG, where there are two separate clusters of ocular surface and conjunctival epithelium are observed [53].epithelial cells [70]. This separation in chicken may be achieved through the differential expression of Cx43a connexin, CX43, or in response to other events allowing differential responses to, as yet unknown, inductive signals [56-58].73-75]. CX43 is present in the conjunctiva and is also strongly expressed in the corneal epithelium, but is absent at the limbal border region. This feature is thought to isolate limbal cells from signals in the surrounding tissues and to play a role in their maintenance in an undifferentiated state [73]. The detailed signaling pathways underlying these processes, however, remain to be further elucidated. Interestingly, several markers of adult LSCs are initially expressed throughout the entire corneal epithelium and it is only after stratification that their labeling pattern becomes restricted to the limbal epithelium. In common with other adult stem cell systems therefore, it thus appears that the adult stem cells are a spatially restricted subpopulation of a progenitor population that is specified during embryogenesis. Although the limbus is specified prenatally, there may be some overlap in stratification of corneal epithelium and further maturation of limbal structures. Thus, it could thus be hypothesized that signals from the stratifying corneal epithelium are also necessary for further maturation of the limbus (See Section 1.3), as could influence underlying corneal stromal cells (See Section 1.6).

1.1.3 Corneal epithelial specification and further specification of the limbus

1.1.6 In mice, further specification of the developing limbus and differentiation of the corneal epithelium as reflected by upregulation of tissue specific keratins or other markers, happens even postnatally.Specification of conjunctival-, corneal and limbal epithelia

Despite their proximity within a contiguous ocular surface epithelium and their common and persistent expression of *Pax6* during development and throughout life, the corneal epithelial cells and the conjunctival epithelial cells belong to two distinct lineages [76, 77] arising from different populations [78]. These two lineages arise simultaneously from *Pax6* positive ectodermal cells that remain on the embryonic ectodermal surface of the developing eye once the lens vesicle has formed [79, 80].

In vivo studies with ocular epithelial cells isolated from rabbits and transplanted into mice have shown that limbal and corneal epithelial cell-derived cysts contained only stratified squamous-type epithelial cells. In contrast, conjunctival epithelial cell-derived cysts contained stratified columnar-type epithelial cells interspersed with Periodic Acid Schiff (PAS) staining-positive cells with goblet-like structure [78]. Despite the fact that such isolated cells might not contain stem cells or may be influenced by differentiated co-transplanted cells, such findings would support the hypothesis that corneal and limbal epithelial cells originate from a different embryonic lineage than conjunctival epithelial cells, and that goblet cells originate from the conjunctival compartment and not following external modulation, as originally proposed [78]. Nevertheless, lineage tracing of these hypothesized progenitor populations will be needed to

unequivocally address this issue in wild type mice and furthermore, explore the ocular lineage specification in *PAX6*-mutated mice.

In spite of their importance, relatively little is known about the factors regulating conjunctival goblet cell development. Conjunctival epithelial cells and goblet cells derive from a common bipotent progenitor [81, 82]. SAM Pointed Domain-Containing ETS Transcription Factor (SPDEF) has been described as a crucial transcription factor for goblet cell differentiation [83]. Goblet cell differentiation and mucin secretion appear to be directly related to the eyelid opening. In humans, the eyelids are fused until the 5^{th}-6^{th} month of intrauterine life, and goblet cells appear in the fornix extending toward the palpebral and bulbar regions from the 8^{th} to 9^{th} week of gestational age [84, 85]. Studies are currently underway to evaluate when the neural regulation of goblet cell secretion becomes functional [86] and the hierarchical network of transcription factors regulating goblet cell development in healthy and pathophysiological conditions (reviewed in [87]).

Some signals which prevent the conjunctival phenotype in the central cornea epithelium are known [88, 89]. The extent of *PAX6* involvement in these processes will be discussed further in Section 1.2. In contrast to the poorly known mechanisms determining cell fates, the tissue specific markers (mostly cytokeratins) for corneal limbal and conjunctival epithelia are well described, although species-specific differences in some of these markers exist.

The conjunctival, limbal and corneal epithelium are composed of one to two cell layers before eyelid opening, after which the thickness of these cell layers increases to 4 – 5 cell layers (see **Figure 2** for animal model comparison). Cells become stratified and distinguishable by their morphology, with basal cells having a cuboidal shape and being attached to the epithelial basement membrane. The intermediate wing cells (anterior to the basal cells) are present, and are in turn covered by flattened superficial squamous epithelial cells. In the fetus, only minor keratin expression is visible in the superficial shedding cells [72, 90]. These processes are similar in different model organisms and have been previously summarized [91].

Early murine corneal epithelial differentiation takes place at embryonic day 15 (E15), as expression of the Pax6-target gene *Krt12* becomes apparent and is a specific marker of the corneal epithelium throughout life [92, 93]. This is likely also true in humans, as the neutral counterpart of KRT12, KRT3, becomes visible in human fetal corneas from 12 WG to 17 WG in superficial cells. By 20 WG, KRT3 can be detected in the superficial cells of limbus and cornea, similar to the adult cornea [71]. Notably, the sequential appearance of the KRT3 and KRT12 pair is different in chicken and rabbit animal models [94].

In mice the developing limbus matures upon corneal epithelial stratification that is associated with eyelid opening around postnatal day 10-14 (P10-14).

~~After that time, KRT12 is strongly expressed in basal epithelial cells, and the corneal epithelium continues to mature even until 3 to 6 months postnatally [59]. Transcriptional changes in the corneal epithelium upon eye opening in mice, which are similar to humans, are summarized by Zieske JD [60].~~

~~Early murine corneal epithelial differentiation takes place at embryonic day 15 (E15), as *Krt12* expression becomes apparent [61].~~ KRT12 is expressed in the superficial epithelial cells and extends to all suprabasal cells and in later embryonic stages to all layers of the epithelium upon maturation [~~62, 63~~95, 96]. The corneal epithelium continues to mature up to 3 to 6 months postnatally [92]. Transcriptional changes in the corneal epithelium upon eye opening in mice, which are similar to humans, have been summarized [91].

One week after birth, α9 integrin, which marks the limbal region in humans and in mice [6497], is equally distributed across the entire corneal epithelium and becomes restricted to the limbus in mice and rats at 8 weeks postnatally [65, 6690, 98]. Similarly, keratins can be used to observe limbal maturation. KRT19 is found to be expressed throughout the entire murine corneal epithelium prior to eyelid opening, butthough after stratification, the expression of this keratin becomes restricted to the mouse limbal epithelium [6799]. KRT19 is also reported to be concentrated in human limbal epithelium but species differences during maturation of corneal epithelium are likely [68]. In line with this, expression of the differentiation-associated KRT12 marker is markedly enhanced after corneal epithelial stratification postnatally [59].100].

In early human development (8-14 WG), KRT15 is expressed across the entire ocular surface including conjunctiva, limbal and corneal epithelia, but becomes restricted to the limbal epithelium from 17 WG, prior to eye opening (See ***Figure 2*** C_2) [5471].

These data seem to suggest that, before stratification of the ocular surface epithelial stratificationepithelium, stem cells are not restricted to the limbus. but rather distributed throughout the entire limbal/corneal epithelium. (This would also point to the important role of stromal stem cell niche in maintaining stem cell capacity in adults as described in 2.1 **Controversy on limbal stem cells**). Additional support for this assertionhypothesis came from the use of a X-chromosomal *LacZ* transgene, which allows random and irreversible labeling of embryonic blastocyst cells in mice.female mice based on X-chromosome inactivation mosaicism at early embryonic blastocyst stage. Interestingly, in the first 3-4 weeks after birth, a disorganized mosaic pattern of $LacZ^+$-labelled patches of cells appeared dispersed throughout the entire basal epithelial layer consistent with disorganized growth of the corneal epithelium driven by a uniform population of proliferative progenitors or stem cells. limbal/corneal epithelia. .However, from P30 onward, a typical radial stripe pattern became gradually evident of cell migration into the cornea became evident as the stem cells became restricted to the limbus [101]. These elegant studies [101, 102] together with other studies mentioned above, indicate that the corneal epithelium is self-sustained by its own pool of stem cells, these stem cells probably differentiate before P30 and was continuously observed from 6-8 weeks age and throughout life [69].

KRT3that stage, the limbus becomes visible in human fetal corneas from 12 WG to 17 WG in superficial cells. By 20 WG, KRT3 could be detected in the superficial cells of limbus and cornea, similar to the adult cornea [54]. Conjunctival epithelial cells and gobletthe unique stem cell location. Notably, the original 'XLacZ' mouse model has limitations due to the fact that tracing is of blastocyst-stage (E3-4) cells derive from a common bipotent progenitor [70, 71]. SAM Pointed Domain-Containing ETS Transcription Factor (SPDEF) has been described as a transcription factor crucial for goblet cell differentiation [72]. The signals which specify corneal or conjunctivaland because it does not allow cell type-specific tracing in a clonal, temporal manner by vital microscopy. However, advanced quantitative lineage are not fully understood. Dkk2 signaling from the mesenchyme to ocular surface ectoderm where Wnt signaling is inhibited, is likely to prevent conjunctival phenotype in the central cornea [73, 74]. The extent of PAX6 involvement in these processes is tracing studies has further shown that this concept is correct, revealing additional aspects of limbal stem cell biology (discussed in section 1.2.2.1 below, [103-108])

1.1.41.1.1 Corneal Stroma and endothelium

The corneal stroma is formed by neural crest-derived cells from the periocular mesenchyme, a population of mesenchymal cells located near the optic cup and presumptive lens. Fate mapping of mesoderm-derived cells showed a contribution to the corneal endothelium and stroma [75, 76].

In humans (and avians), three waves of neural crest cell (NCC) migration are reported in contrast to two waves in mice (Note that Figure 1 refers to mouse development). A first wave of NCCs migrate between the lens and the surface ectoderm to form the corneal endothelium. A second wave forms the stromal keratocytes. The third wave of NCCs contributes to the ciliary body and iris structures (see Section 1.1.6). In mice a single wave of neural crest migration gives rise to both endothelium and stroma.

Studies in transgenic mice isolating primary stromal cells from mouse corneas revealed similarities in mRNA expression profiles (*Twist*, *snail, Slug and Sox9*) between neural crest-derived precursors and the isolated corneal precursor cells, which was regarded as evidence for a neural crest origin of these cells, which are important for turnover of stromal tissue [77].

The corneal endothelium provides an important pump function, actively maintaining a fluid and electrolyte balance between the anterior chamber and corneal stroma to prevent corneal swelling, thus maintaining corneal transparency. The cellular density of this single endothelial cell layer is critical for maintaining an adequate pump function. From a histologic analysis of human fetuses, it has been shown that the cellularity of the endothelium rapidly decreases in the prenatal period from 16 WG to term, at the same time the cornea grows in size [78]. The reduction in endothelial cell density during this period is about 50%, while the density reduces further by a third during the first two years of life. A putative effect of PAX6 levels on stromal, limbal stromal and endothelial development and how this could influence AAK is discussed in Section 1.6.

1.1.5 1.1.7 Corneal nerves

In mice, branching nerve bundles cover the entire corneal stroma by E16.5. The corneal epithelium is first innervated at E16.5 and nerves subsequently form a swirl pattern in the subbasal nerve plexus at about three weeks postnatally [79 109]. The ophthalmic nerves arise from the trigeminal ganglion which is both derived from neural crest and ectodermal cells (see the review in [43 45]. At least in chickens, the cornea is innervated solely by the neural crest derived neurons of the trigeminal ganglion [80 110].

1.1.6 1.1.1 Iris and anterior chamber angle

The iris and ciliary body (CB) epithelium is derived from the tips of the optic cup while the stromal layers are from the ocular mesenchyme [25]. A *Pax6* expression gradient is observed in the optic cup with the highest level from the distal (close to the lens) to proximal side (close to the optic nerve [30] (Figure 1, E 15.5). In mouse embryos (E 15.5), iris and CB progenitor cells can be molecularly distinguished from neuronal cells which form the presumptive retina. Thus the proper development of the iris relies on the correct compartmentalization of the optic cup (See review in [25] for molecular details).

The iris structure is not exclusively formed by the optic cup, but also by neural crest and mesodermal cells contributing to the iris stroma and ciliary body. Pax6 may also participate in regulation of the factors required for the migration of NCC into the eye [81] (see also reviews on neural crest of the eye [42, 82]).

The anterior chamber angle is the angle between the iris and the corneal endothelium in the limbal region. The iridocorneal angle contains important aqueous humor drainage structures such as the trabecular meshwork and Schlemm's canal.

Although the molecular mechanism responsible for the normal development of the iridiocorneal angle has not yet been elucidated, a series of developmental steps are described in the mouse where final maturation of Schlemm's canal and the trabecular meshwork extends to

P42 postnatally [83]. In humans, the developmental processes are similar, with all rudimentary structures developed at birth [84]. Further maturation and reorganization take place likely due to mechanical stress and aqueous humor flow and this process could last 1-8 years postnatally [85].

Lineage tracing experiments in mice have demonstrated that migrating NCC form the ciliary muscles, ciliary blood vessels, anterior iris, trabecular meshwork and Schlemm's canal in the iridocorneal angle [75, 86, 87]. Mesodermal cells also contribute to structures such as the lining of Schlemm's canal and the iris stroma, but not to the ciliary muscles [75].

The impact of changing PAX6 levels on anterior chamber development and whether this might impact the ocular surface is discussed in Section 1.5.

1.1.7 Optic cup

The optic vesicles are bilateral evaginations from the diencephalon that give rise to the optic cup through morphogenesis (reviewed in [88, 89]). The optic cup interacts with surface ectoderm, lens and migrating mesenchymal cells. So theoretically, developmental alterations disturbing these processes could also impact ocular surface development.

1.2 Could disturbed segregation and separation of corneal and conjunctival cell lineages contribute to AAK?

Lineage segregation of corneal epithelium from the conjunctiva in humans appears after sustained *PAX6* induction at the time of surface ectoderm specification. In mice, PAX6 is found in the ocular surface ectoderm at E8, and in humans at embryonic day 42 (***Figure 2***). Altered *PAX6* levels in the corneal and conjunctival epithelium during development could impact signals required to define borders and self-maintenance of corneal and conjunctival tissue identity. Deeper understanding of conjunctival and corneal differentiation is therefore essential to distinguish between the developmental and postnatal role of *PAX6*. In the mouse, the regulatory network of *Pitx2* and downstream *Dkk2* expressed in the mesenchyme inhibits Wnt/β-catenin signaling resulting in the inhibition of the conjunctival fate in the central cornea [~~74, 102~~88, 89, 123]. PITX2 is also described to integrate retinoic acid (RA) signaling from the surface ectoderm, optic cup and lens. Since RA signaling is altered in *Pax6*$^{Sey-/-/Sey}$ (*Pax6*$^{-/-}$) mouse eyes [~~103~~124], this could affect the crosstalk between the periocular mesenchyme and the surface ectoderm. In addition, transcriptional analysis in human subjects with aniridia indicates that RA metabolism could be altered in the ~~ocular surface epithelial~~conjunctival cells due to *PAX6* mutation [~~104~~125], but ~~this~~the mechanism requires further confirmation.

Whether ~~the~~ PAX6 protein levels could impact ~~the~~ proper segregation of corneal and conjunctival tissue during development, and therefore potentially influence the development of AAK, remains unknown; however, recent studies are beginning to address this question. A recent single-cell RNA-seq analysis of human corneal development [~~53~~70] encompassing 12-23 weeks of gestation indicated low overall *PAX6* expression throughout the developing cornea; however, in all cases the highest expression was observed in the epithelial layer. The same was also true for the adult cornea with highest *PAX6* expression observed in the corneal and conjunctival epithelium. Since several cell populations can be now identified by their transcriptional profile, it is now possible to investigate the extent to which *PAX6* is expressed ~~at the protein level~~ in these cell clusters or if some cells in these clusters exhibit higher or transient PAX6 expression. Another recent study has reported postnatal modification of PAX6 dosage in the *Pax6*$^{Sey-Neu/+}$ (*Pax6*$^{+/-}$) mouse model by ~~inhibition of~~inhibiting the mitogen-activated protein kinase kinase (MEK) pathway [~~105~~126]. In that study, pharmacologic MEK inhibition by ocular or systemic routes increased PAX6 protein expression in the basal epithelial layers to normal levels as in wild-type mice, resulting in ~~a~~ restoration of corneal anatomy and transparency ~~back~~ to a normal phenotype. This result provides evidence for a PAX6-dependent role in epithelial cell specification, even postnatally.

1.3 Does the limbus of AAK patients exhibit developmental defects?

Similarities between aniridia syndromes and LSCD may suggest that epithelial defects in AAK are the result of LSC failure. However, since pathological changes in aniridia are also observed in the nerves, inflammatory cells and corneal epithelium early in life, even before the limbus becomes overtly affected [29], it suggests that multiple pathological mechanisms may be involved and may precede and/or promote LSC insufficiency.

The exact location and differentiation characteristics of LSCs or their precursors during human development is not well studied at the molecular level, especially the postnatal formation of limbus niche structures which differ from those observed in mice [~~106~~127]. It has been previously hypothesized that the delayed formation of the limbus could be a cause for the late onset of AAK in aniridia [~~54~~71].

The ΔN isoform of Tp63α (ΔNp63α) is considered as a LSC marker [~~107~~128] and as a master inducer needed for the progression from an embryonic ectodermal monolayer epithelium into a dynamic, stratified epithelium found in the adult stage (e.g. in the corneal epithelium and epidermis) [~~108].~~129]. *PAX6* expression occurs developmentally before the expression of *TP63* [130, 131]. In contrast to *PAX6* that is widely expressed in eye tissues, *TP63* expression is limited to the ocular surface epithelia (conjunctiva, limbus, cornea) and is associated with ocular glands (lacrimal and meibomian) [132, 133]. Mutations in the *TP63* gene lead to Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate (EEC) and Ankyloblepharon-Ectodermal defects-Cleft lip/palate (AEC) syndromes, two syndromes with numerous tissues affected. These syndromes can also be associated with LSCD [~~109~~134]. TP63 was described to be equally expressed in the fetal limbal and central cornea [~~54~~71]. In ~~human adults~~adult humans, *TP63* expression is restricted to the limbus, and positive staining is only detected in central corneal epithelium when the corneal epithelium is regenerating after wounding [~~54, 110~~71, 135].

~~In the lens placode, SOX2 cooperates with PAX6 [111]. SOX2 is also involved in the regulation~~The occurrence of ~~*TP63* and the~~limbal stem~~/progenitor~~ cell ~~state in corneal epithelium, and both factors are linked to corneal opacification and neovascularization [49].~~

~~*PAX6* expression occurs developmentally before the expression of *TP63* [49, 112]. In contrast to *PAX6*~~dysgenesis shows that ~~is widely expressed in eye tissues, *TP63* expression is limited to the ocular surface epithelia (conjunctiva, limbus, cornea) and to associated epithelial glands (lacrimal and meibomian) [113, 114]. Similarities between aniridia syndromes and LSCD may suggest that epithelial defects in AAK are the result of LSC failure. However, since pathological changes in aniridia are also observed in the nerves, inflammatory~~stem cells ~~and corneal epithelium early in life, even before the limbus becomes overtly affected [28], it suggests that multiple pathological mechanisms may be involved and may precede and/or promote LSC insufficiency.~~

~~*PAX6* is important transcription factor~~are required for ~~neural differentiation [115, 116].~~corneal maintenance, however identifying the LSCs themselves is challenging [~~117~~136]. Two recent single-cell RNA-seq studies led to the identification of glycoprotein hormone subunit alpha 2 (GPHA2) as a novel ~~LSC~~ marker~~,~~ of an outer population of limbal stem cells. GPHA2, is a largely unexplored gene whose function appears to be essential for LSC self-renewal and differentiation [~~53, 118].~~70, 107]. *GPHA2*-overexpressing transgenic animals showed no gross phenotype alterations [137], but it would be interesting to examine the phenotype of these mice and generate a knockout mouse strain. GPHA2 expression was dramatically reduced to barely detectable levels following cultivation of human LSCs and in immunodeficient mice. This suggests that GPHA2 may be regulated by T cells critical for the adaptive immune response, and that may serve as an important contributor to the LSC niche [70, 107]. Noticeably, like the putative LSC marker KRT15, GPHA2 is not only expressed by basal limbal epithelial cells (i.e. LSCs), but it is also occasionally detected in limbal supra-basal cells [107], and therefore was also proposed to mark limbal committed or differentiated cells [138].

The processes, however, responsible for creating the limbal niche structure ~~and~~ secretion of its basement membrane, as well as association and recruitment of niche cells, are not sufficiently understood. It would be of interest to ~~test~~determine whether *GPHA2* expression is altered in AAK patients and/or in animal models of aniridia, to yield further evidence for the loss of LSC function, as is widely believed. In addition, lineage tracing in $Pax6^{+/-}$ mice ~~has allow detecting~~enabled the detection of pathogenic mechanisms associated with aniridia and the LSCs in light of developmental processes. ~~—~~For example, $Pax6^{+/-}$ LSCs in $Pax6^{+/-} \leftrightarrow Pax6^{+/+}$ chimeric mice are functional and produce streams of epithelial cells that migrate normally into the cornea, ~~though~~although these progeny are less likely to reach the center of the cornea than wild-type cells [~~119~~139]. This suggests that dosage deficiency of Pax6 does not

preclude normal specification of LSCs ~~and show that corneal epithelial defects may directly contribute to~~in a cell-autonomous manner. It remains unknown, however, if the ~~mutant phenotype~~relatively normal behavior of *Pax6*$^{+/-}$ cells in the chimeric mouse limbus represents a non-autonomous 'rescue' by secreted protein factors such as GPHA2 released from the wild-type cells.

1.4 Does lens development impact AAK?

In contrast to ~~humans with~~ aniridia patients, *Pax6*$^{Sey/+}$ (*Pax6*$^{+/-}$) mice have a more prominent anterior segment dysplasia and the lens often remains attached to the cornea. The mouse lens is larger in proportion to the rest of the eye, as compared to the human eye. This could result in a more severe lens and corneal phenotype in the mouse compared to the human eye [11]. In the chicken, it was shown that surgical removal of the lens affects multiple eye developmental processes~~,~~ including eye growth, and inhibits normal development of the peripheral retina, ciliary body, ~~growth of the~~ iris, and ~~the~~ migration of NCC into the cornea [~~120]. These NCC cells are however important to later stabilize corneal epithelium fate. It is reported that lens and corneal epithelium derive from the same nasal ectodermal precursors.~~140]. The corneal cell fate of surface ectoderm is stabilized by NCC migrating in the lens peripheral ectoderm. The formed stroma prevents PAX6 downregulation in ~~cornea~~the corneal epithelium. [~~121~~141].

~~The surgical ablation experiments described above could impact neighboring tissue making it difficult to draw conclusion from interactions between manipulated tissues. Using mouse models with controlled genetic knockdown, individual structures can be better manipulated during tissue-tissue interactions and more precise statements about possible interactions can be made.~~ The developing lens is a key signaling center ~~for~~during eye development. ~~Early on,~~ During formation of the lens placode, ligands of ~~the~~ Wnt, BMP and retinoic acid secreted from the surface ectoderm play a role in patterning of the optic cup [~~122, 123]. The~~50, 52]. Surgical removal of the lens from developing chicken eyes leads to downregulation of genes associated with retinoic acid, BMP and Wnt signaling in the peripheral retina, including the ciliary body, and some aspects of the lens-~~removed~~deficient phenotype (e.g. microphthalmia) can be recapitulated by inhibiting retinoic acid signaling, or rescued in lens-removed eyes by restoring retinoic acid [~~114~~142].

Pax6 is essential for early stages of lens induction ~~functioning in the lens lineage probably,~~ possibly through influencing modification and remodeling of chromatin ~~modifiers and remodelers~~ [19, ~~123]~~52] as reviewed in [~~46~~54]. In addition to its role within the lens lineage, Pax6 also ~~play~~plays a role in the adjacent optic vesicle ~~for triggering~~to trigger lens formation ~~as~~. When *Pax6* is knocked out ~~earlier~~at an early stage in the optic vesicle, the lens does not develop [113].

Consistent with the importance of *Pax6* ~~for onset of the~~ in lens formation ~~of the lens,~~ there is evidence that the lens is exquisitely sensitive to the correct *Pax6* gene dosage. In Mexican Cavefish *Astyanax mexicana*, it is the loss of *Pax6* expression specifically during lens development, that precipitates lens apoptosis which in turn leads to failure of retinal growth and the loss of ~~the~~ anterior segment structures [~~124~~143]. In mice, less than ~~about~~ 80% or more than ~~about~~ 120% of normal Pax6 activity is thought to result in lens defects that affect the rest of eye development, even though to adulthood [21]

~~-~~Moreover, results from experimental ~~genetic~~ inactivation ~~in mice of~~ one allele of *Pax6* in mice, specifically in the lens or in the optic cup ~~substantiate a key role for~~, indicate that *Pax6* expression in the lens is necessary for normal development of the anterior chamber [112].

Further evidence of the sensitivity of lens development to Pax6 levels was concluded based on mouse chimera experiments indicating that heterozygous *Pax6*$^{Sey\text{-}Neu/+}$ (*Pax6*$^{-/+}$) cells do not contribute to the developing embryonic lens, in contrast to their contribution to the other eye tissues [~~125~~144]. Additionally, in chimeric mice where the lens was wild-type, virtually all other aspects of anterior segment development ~~are~~were restored, including normal iris development, corneal epithelial morphology and limbal function [~~126, 127].~~ 145, 146].

~~In aniridia, PAX6 dosage reduction results in congenial cataract, however the impact of reduced PAX6 dosage on anterior segment remain to be determined as lens size and adhesion to the cornea is less prevalent in aniridia (as discussed at the beginning of this section 1.4).~~

~~Nevertheless, ~~A plausible working model for the developmental defects underlying the development of the aniridia phenotype is that lens signals regulated by the correct Pax6 dosage are required for normal development of other anterior segment structures. Identification of ~~those~~these lens signals ~~remains~~orchestrating anterior segment development should therefore be a high priority in eye research, irrespective of their roles in aniridia.

~~Congenital ~~An early onset of cataract is prevalent in aniridia [~~128~~147]. This is likely due to ~~anomalies in~~abnormality of the lens epithelium~~,~~ and lens fiber cells, and a thinning of the lens capsule [~~129-131~~148-150]. Non-cell-autonomous ~~mechanism~~mechanisms for cataract in aniridia have been also proposed as abnormal zonular fibers are reported to be associated with congenital cataract in aniridia [~~132, 133~~151, 152].

Anterior opacities in the lens and iris remnants in the anterior chamber ~~-~~are sometimes ~~been~~ observed in aniridic eyes (***Figure 4***), ~~which suggest~~suggesting that the separation of the lens and iris from the cornea may sometimes be arrested in human embryos [4]. Further detailed studies are necessary to investigate if the lens status affects the corneal endothelium. Since the keratopathy is mostly present in the anterior layers of the cornea, it is not obvious whether an incomplete separation of the lens or iris from the cornea could impact AAK. The putative crosstalk ~~to~~between the lens and other developing ocular tissues has been discussed ~~already~~ above. ~~For instance,~~ Similar to dysfunctional lens epithelial cells, an abnormally thin corneal epithelium in the heterozygous *Pax6*$^{+/-}$ mouse [~~27~~28] may represent developmentally immature epithelial cells (including limbal stem and progenitor cells) ~~that are unable of forming~~incapable to form the fully stratified multiple corneal epithelial layers~~,~~ that normally ~~arising~~arise postnatally in the mouse cornea [~~134~~87]. However, it must be noted that any apparent undifferentiated state may be a secondary consequence of the chronic abrasion and wound-healing physiology of the aniridic cornea [~~135, 136~~153, 154] .

1.5 Does ~~the malformation of the~~ anterior chamber and ~~the absence of~~ iris malformation impact AAK development?

The conditional inactivation of a single *Pax6* allele in mice from either the inner layer or the outer pigmented epithelium of the distal optic cup ~~resulted~~results in a profound iris hypoplasia~~.~~ [51]. The resulting reduction in Pax6 dosage ~~interrupted with~~interrupts different stages of iris development~~;~~: from reduction in the size of the progenitors, to delayed onset of muscle-specific markers and abrogated iris sphincter morphogenesis [~~137, 138~~51, 155]. Indeed several key factors for iris development are reduced in the developing iris of the *Pax6*$^{Sey\text{-}1Neu/+}$ mice including ~~-~~; *Pitx2*, *Igf2*, *Foxc1*, *TGFb2*, *Zic2* and *BMP4* [~~139~~156]. These ~~TFs~~transcription factors and ligands could impact the differentiation of the iris progenitors as well as the migration of NCC that populate the iris stroma and the cornea [~~140~~157].

The majority (50~~%~~ - % - 75~~-~~%) of aniridia patients develop glaucoma ~~probably~~most likely as a consequence of abnormal differentiation of the trabecular meshwork and/or complete absence

of Schlemm's canal~~) [141, 142~~ [158, 159]. Notably ~~the,~~ conditional haploinsufficiency of *Pax6* in the mouse lens and cornea - but not in the developing optic cup layers~~; -~~ disrupted trabecular meshwork and Schlemm's canal development and resulted in glaucoma [~~126~~145]. It is currently ~~not clear~~unclear, however, if this result is due to Pax6 activity in the lens and cornea ~~in~~ regulating factors required for the development of the drainage structures, or due to the abnormal morphology of the ~~mutant's eyes~~eye due to adhesion between lens, cornea and iris epithelium ~~[126~~in the model [145].

Another unsolved topic is how partial or complete loss of iris and the abnormal differentiation of the drainage structures in aniridia ~~impacts~~impact AAK progression. It should be considered that the positioning of the anterior chamber angle ~~is~~may be needed for signaling to LSC and their niche to develop correctly, as well as to ensure the proper flow of aqueous humor important to maintain the correct eye pressure and nutrition of the anterior segment structures ~~including the limbal niche.~~ .

In a recent study examining 87 eyes of aniridia patients, 21 of which had a partial iris, it was shown that the partial presence of an iris was strongly associated with a milder degree of AAK [~~28~~29]. Although this could support a connection between iris or chamber angle development and AAK, the mild keratopathy could also be caused by the common causative mutation itself.

1.6 Are mesenchymal structures ~~such as the (~~corneal stroma and endothelium~~)~~ affected ~~by altered signaling~~ during development?

A number of clinical studies have reported that the corneal stroma is abnormally thick in almost all cases of aniridia [6, 160, 161]. Although the causes of a thick stroma in aniridia are not yet clarified (as the corneal endothelium appears to function normally), it has been reported that, during normal human development, the corneal stroma is thicker *in utero* and progressively thins with increasing gestational age [162]. This has led to the hypothesis that the normal thinning of the corneal stroma in later developmental stages *in utero* is disrupted in aniridia [29]. A developmental origin for the thickened corneal stroma is supported by the lack of clinical signs of stromal edema and a sufficiently high endothelial cell density in aniridia for maintaining proper stromal hydration.

~~A~~ In the epithelia of the developing lens, retina, ciliary body, iris and cornea, *PAX6* is expressed at high levels, and this is easily detectable by *in situ* hybridization, Western blot and immunohistochemistry. In the mesenchymal component of some other ocular tissues, such as the corneal endothelium, corneal stromal keratocytes and trabecular meshwork mesenchyme, low and transient ~~expression of *Pax6* in the developing mouse eye plays~~levels of *PAX6* (at the limits of detection by the above techniques) have been reproducibly demonstrated during mid-late stages of development [163, 164]. These low levels of PAX6 have nevertheless been shown experimentally to represent a cell-autonomous requirement for contribution of cells to the corneal endothelium and stroma, and also play a role in the differentiation of trabecular meshwork ~~and in the formation of corneal endothelium and keratocytes [45, 50, 143]. PAX6 protein is detected in low and transient levels in mesenchymal-derived cells of the iridocorneal angle, corneal stroma and endothelium, iris stroma and prospective trabecular meshwork of wild-type mice [50, 143].~~[47, 163, 164]. Although *PAX6* is downregulated in the trabecular meshwork of normal adult eyes upon differentiation [163], recent single cell analysis has revealed the presence of *PAX6* transcripts in normal limbal corneal keratocytes, corneal stromal keratocytes as well as in corneal stromal stem cells, into adulthood [~~53~~70]. This correlates with the previous observation of PAX6 expression in a population of stromal stem cells [~~144~~165]. At the single-cell level in humans, *PAX6* mRNA is detected through all developmental stages at a low level [~~53]. The physiological significance of the low and transient levels of *PAX6* remain to be determined~~70]. (***Figure 5***).

~~The contribution of *PAX6* and the~~ Impaired development of the anterior chamber angle, iris and endothelium due to *PAX6* haploinsufficiency has been carefully studied and reviewed in a number of reports [~~27, 38, 45, 138, 143~~28, 40, 47, 155, 163], but the impact of PAX6-deficient corneal or limbal stromal cells on AAK remains elusive [~~145~~].

~~Still, one cannot exclude~~166]. PAX6 dosage is self-evidently crucial for normal eye development, so the ~~possibility~~molecular mechanism by which different tissues require either 'high' or 'low' levels of Pax6, and how dosage is controlled via regulatory DNA elements, still requires clarification. PAX6 directly interacts with multiple other proteins [167, 168] and the presence and stoichiometry of different binding partners in different cell types is expected to modulate PAX6 activity. Hundreds of genes are regulated, directly or indirectly, by PAX6 during eye development [169] and are affected to different degrees by changes in dosage. It is therefore expected that ~~the transient PAX6~~mesenchymal cells with 'low' levels of PAX6 will exhibit a different profile of downstream gene expression ~~during corneal stromal and endothelium development is needed to acquire the correct stromal/endothelial cell fates which can~~from epithelial cells with 'high' levels. The biological impact ~~the neighboring epithelium.~~

of *PAX6*-heterozygosity for low levels of expression in mesenchymal cells, if any, are unresolved. Taken together, the most parsimonious scenario is that *PAX6*, expressed at high levels, is functioning cell-autonomously in the optic cup, lens and corneal epithelium progenitors, and that these tissues have a non-autonomous influence on the surrounding anterior segmental neural crest and mesodermal lineages (See ***Figure 5***, blue arrows). ~~Nevertheless there is a cell-autonomous requirement for *Pax6* during development of both the corneal stroma and endothelium in mice [143].~~ As the Schlemm's canal lining and iris stroma are formed by mesodermal cells [~~75~~59] and are absent in *Pax6*$^{+/-}$ mice [~~81, 146] we need to consider the possibility~~65, 170], it may be possible that transient *PAX6* expression in these cells further contributes directly ~~also~~ to their formation.

~~PAX6 is transiently expressed in the mesenchymal neural crest derived cells of the anterior chamber angle. Later, PAX6 is downregulated in the trabecular meshwork of adult eyes [143]. Transient PAX6 expression could be important for differentiation of trabecular meshwork and the chamber angle, but more data are needed on the effects of transient PAX6 expression in mesenchymal cells in general.~~

~~Embryonic/fetal mesoderm and neural crest-derived cells that transiently express PAX6 during development could have an impact on the onset of AAK. However, these embryonic events cannot easily become a therapeutic target in postnatal patients.~~

~~A number of clinical studies have reported that the corneal stroma is abnormally thick in almost all cases of aniridia [6, 147, 148]. Although the causes of a thick stroma in aniridia are not yet known (as the corneal endothelium appears to function normally in aniridia), it is known that during normal human development, the corneal stroma is thicker *in utero* and progressively thins with increasing gestational age [149]. This has led to the hypothesis that the normal thinning of the corneal stroma that occurs in later developmental stages *in utero* is somehow disrupted in aniridia [28]. A developmental origin for the thickened corneal stroma is supported by the lack of clinical signs of stromal edema and a sufficiently high endothelial cell density in aniridia for maintaining proper stromal hydration.~~

~~Does~~

1.7 Do meibomian and lacrimal gland ~~development~~formation impact AAK development?

The ~~function~~functions of lacrimal and meibomian glands ~~is~~are essential for the production, stability and function of the tear film~~, and~~. Any factor disturbing the homeostasis of the ocular

surface unit may disrupt the stability of the tears, leading to ~~tissue~~ damage~~, including~~ of corneal and conjunctival epithelia and possibly impacting LSC function [~~150~~171]. These supportive glands are derived from the ocular surface ectoderm, but to date there is no evidence regarding the impact of these structures on other anterior structures during eye development. The morphogenic events necessary for the lacrimal gland development in *Pax6*$^{Sey/Sey}$ mice are defective [~~151~~172]. In *Pax6*$^{Sey/+}$at E19.5, the lacrimal bud becomes visible but its structure remains vestigial [~~152~~173]. In lacrimal gland organoid models, *Pax*6 is necessary ~~express~~for expression of the genes encoding the secretion machinery (~~Aquaporines~~aquaporins and ~~neurotransmitter genes~~neurotransmitters) but these analyses were performed ~~in~~with total *Pax6* knockout~~[153~~ model [174]. In addition, PAX6 is one of the transcription factors necessary to drive explant cultures or induced pluripotent stem cells (iPSCs) ~~to~~into a lacrimal gland cellular ~~phenotype [152, 154~~fate [173, 175].

The development of the meibomian glands requires proper eyelid closure and eyelid fusion during embryonic development~~, and~~. PAX6 could influence this process since it is expressed at low levels during eyelid development in a complex expression pattern [~~155, 156~~176, 177]. PAX6 is expressed during development in the acinar cells of meibomian glands, although its contribution to development of these glands requires further investigation [~~157~~178]. Protein composition of the tear film is altered in aniridia, and an elevation in inflammatory cytokine levels has been observed [~~158~~179]. Meibomian gland dysfunction has also been documented in aniridia patients [~~159-162~~180-183]. Still, it is unknown whether tear film and meibomian gland abnormalities arise from developmental defects or whether the function is impaired postnatally due to deficient ocular surface epithelia~~, as~~. AAK may underpin the inflammatory process and act as a possible trigger mechanism for dry eye and meibomian gland dysfunction ~~in aniridia patients.~~. Elevated interleukins ~~measured~~ in the tear film could be also caused by the chronic wound healing~~-~~ state of the corneal epithelium in aniridia or from inflammation in the limbus and corneal stroma [7]. It is also important to keep in mind that the developmental defects observed in mice may not necessarily be mirrored in humans. Longitudinally monitoring the morphology and function of the meibomian and lacrimal glands and tear film quality from birth to young adulthood may help to answer these questions and could provide important insights for patient care. Interestingly, similar to *PAX6*-related aniridia, *P63*-mutation in patients that suffer from ectodermal dysplasia (ED) also ~~displayed~~led to meibomian and lacrimal gland defects ~~as well as~~in addition to LSCD [~~109, 163~~134, 184].

~~Due to the complex processes involved in the development of the eye and the complex roles of *PAX6* in gene regulatory networks,~~ More precise information is needed about the spatiotemporal and functional importance of PAX6 expression in different tissues of the ocular surface during development and in adulthood, and critically, to determine the contribution of developmental abnormalities versus postnatal pathophysiological changes. Ongoing studies at the single-cell level in human corneas, supplemented with similar information ~~on~~in *Pax6*$^{+/-}$ mouse eyes, could provide the necessary platform for addressing these questions. ~~In addition, given the importance of PAX6 protein in ocular development and its deficiency in aniridia~~In addition, identifying the gene regulatory networks and molecular pathways leading to normal PAX6 production ~~can~~could possibly provide a ~~means~~molecular tool to modify ~~the~~ PAX6 levels ~~by molecular means [105~~[126], largely independent of the developmental stage and tissue type initially perturbed by *PAX6* mutation. Armed with this information, it may be possible to identify potential drug candidates with the ability to delay or prevent the development of AAK postnatally. Crucially, the impact of any treatment should be evaluated in terms of lacrimal and meibomian gland function, to assess the likelihood of maintaining a stable ocular surface environment in ~~the~~ longer term.

2 How aniridia AAK symptoms manifest postnatally

2.1 Is limbal stem cell deficiency a cause or a consequence of AAK?

~~Limbal stem cells:~~

The limbus (meaning '*border*' in latin) is the circumferential border defined by the corneoscleral transition and harbors the LSC niche~~:~~. In humans, the niche is typically located in the superior and inferior limbal regions where the 'Palisades of Vogt' structures are found [~~164, 165~~185, 186]. Accumulating evidence supports the hypothesis that the limbus is the sole niche of corneal epithelial stem cells, notwithstanding the impressive regenerative ability of the corneal epithelial cells themselves ~~at least in mice [106].~~[127]. The ~~crypt~~ shape and structure of the limbus ~~that is,~~ rich with blood and lymph vessels, suggests that it provides a protected microenvironment (LSC niche) that is enriched with nutrients and stem cell self-renewal factors [~~165, 166] [167~~186-188]. Slow-cycling nucleotide label-retaining cells have been specifically identified in the limbus while in the corneal periphery and center, only fast-cycling cells ~~were~~have been identified [~~168], and~~189]. Limbal epithelial cells have been shown to have high regenerative potential and long-term proliferation capacity *in vitro* and *in vivo* [~~169~~190].

LSCs produce transient amplifying cells which undergo further division and centripetal movement to replenish the basal corneal epithelium. A constant stream of these newly produced basal epithelial cells migrates towards the supra-basal epithelial layers and is accompanied by a loss of proliferation capacity leading to a terminally differentiated state [~~170].~~ 191]. Recent studies have identified two spatially discrete classes of stem cells in the limbus – an inner ring of active cells that are mostly responsible for repopulating the corneal surface during normal homeostasis, and an outer, more quiescent ring that may have a role after injury [107, 192]. It is this outer ring that expresses LSC marker *GPHA2* discussed earlier, while the inner ring is mostly responsible for the centripetal radial streams of corneal epithelial cells observed in mosaic reporter mice.

LSCD is a group of diseases that can be caused by environmental or genetic factors and is accompanied by conjunctival cell invasion, inflammation, corneal neovascularization, corneal opacification and blindness [~~171~~193]. It is commonly believed that dysfunction of LSCs and their stromal niche is the basis and cause for these pathologies [~~172~~194]. Accordingly, the inability of ~~LSC~~LSCs to replenish the corneal epithelium leads to recurrent ocular surface defects and abnormal healing of the corneal epithelium, often being replaced by conjunctival cells [~~173~~195]. This is often followed by neovascularization of the corneal stroma, compromised corneal transparency and consequently ~~the cornea becomes opaque that is associated with~~corneal opacification, leading progressively to poor vision. Typical changes in corneal epithelium-specific surface markers include the downregulation of corneal keratins KRT3/KRT12, and abnormal expression of conjunctival keratins KRT4/KRT13 or skin keratins KRT1/KRT10 [~~174]. Particularly~~196]. Also, goblet cells which are normally restricted to the conjunctiva, can be found in the cornea in cases of LSCD, and ~~they have~~their presence has also been used to diagnose LSCD [~~173~~195], although goblet cell presence can be variable in advanced stages of AAK [7]. Of note, PAX6 is also downregulated in severe ocular surface diseases including LSCD [~~174~~196].

Controversy: Accumulating experimental and clinical evidence supported the LSC dogma, namely that the LSCs are located in the limbal niches and are solely responsible for epithelial renewal. This notion subsequently became firmly accepted by the research community. Nevertheless, there was some conflicting evidence that had been overlooked or remains unresolved [~~175, 176~~197, 198]. Perhaps one of the most confusing observations was reported in the seminal and elegant study of Tsengs's group [~~177] that solidified~~199] which strengthens

the importance of the limbus. In that study, the entire limbal epithelium in 12 New Zealand White rabbits was surgically removed. Six-months later, the cornea in 8 of the 12 rabbits remained normal while the remaining four displayed only a mild phenotype of corneal neovascularization. However, when limbal epithelial depletion was coupled with epithelial debridement in the central cornea, corneas became vascularized. Likewise, in humans, a persistent central island of apparently normal corneal epithelium can persist for years, despite total limbal epithelial absence [~~176~~198]. Similarly, Majo and Barrandon reported that the ablation of limbal epithelium in mice did not induce LSCD [~~106~~127]. Limbal epithelial deletion is thus not necessarily sufficient to cause LSCD, however ~~'confetti'~~multiple lineage tracing studies in mice has shown the importance of LSCs in replenishing the corneal epithelium [~~178].~~ 103].

In a later experiment, Nasser and colleagues [~~179~~106] showed that ~~K15~~KRT15-GFP transgene specifically labels the limbal epithelium, and this model was used to validate limbal removal, ensuring total LSC depletion. Using triple transgenic animals and multiple "Confetti" lineage tracing, one-day post total limbal epithelial depletion, the Confetti^{+} corneal epithelial cells healed the denuded limbus, and after 7-10 additional days, these cells started to express limbal epithelial markers. These corneas were transparent, and the repaired limbus could successfully replenish the corneal epithelium for at least 6 months of experimental follow up. This study indicated that corneal epithelium-committed cells can dedifferentiate into apparent LSCs. When LSC depletion, however, is coupled with limbal stroma (niche) damage, dedifferentiation is inhibited and severe LSCD develops. Similar cell plasticity that allowed recovery from native stem cell loss has also been found in other epithelial tissues (reviewed in [~~180~~200]). Collectively, these studies suggest that the LSC niche (and not the LSCs alone) ~~are responsible~~is essential for maintenance of the transparent ~~corneal epithelium~~cornea, and that damage to the niche is most likely a key element of many LSCD pathologies. In light of this, gene~~-~~-linked LSCD (e.g., mutations in *PAX6* or *P63*), are likely to affect both LSCs and their niche. This underscores the important role of the limbal niche in corneal health and LSCD pathogenesis [~~181, 182~~201, 202]. Nevertheless, the intriguing possibility remains that a signaling program triggering corneal epithelial cell dedifferentiation to LSC-like cells could be exogenously applied even in the absence of a functioning limbal niche. Investigations into the signaling pathways responsible for triggering and maintenance of the dedifferentiation of corneal epithelial cells would therefore be of interest.

Aniridia~~-~~-related LSCD: The limbal niche likely plays a pivotal role in the specific form of progressive LSCD observed in AAK. *PAX6* is expressed in various tissues in adult eye (***Figure* 3**). *PAX6* mutation may lead to aberrant crosstalk between mutated LSC and mutated stromal and epithelial elements (corneal stromal mesenchymal stem cells, keratocytes, melanocytes, corneal nerves or dendritic cells~~), and~~). It may include an abnormal response to extracellular matrix (ECM) signals by mutated LSC, or dysregulated response of LSC to signals originating from blood or lymph cells. On the other hand, knockdown of *Pax6* in cultured LSC ~~was proposed to induce~~induces transdifferentiation into an epidermal-like fate [~~183~~203], suggesting that *Pax6* could also play a cell-autonomous role in maintaining LSC identity. Clinical evidence also points to an abnormal limbal niche in aniridia, that is either the result of abnormal developmental processes *in utero*, or ~~leads~~due to incomplete or aberrant postnatal development and/or maturation of the niche [~~184-187~~204-207]. In young children and adults with aniridia, abnormal limbal structures, inflammation, vascularization and a significant nerve deficit in the limbus have been observed throughout the range of AAK severity [4, ~~187~~207]. These changes are also accompanied in an early stage by islands of conjunctival tissue present within an otherwise transparent corneal epithelium. The evidence therefore seems to indicate that an early breakdown in the limbal niche may prevent a proper limbal niche environment from forming~~, thereby impacting~~. This would impact proper maturation of the limbal epithelium and the

sustenance of its associated stem cells and/or progenitors. ~~If~~ On the other hand, if the limbal niche in aniridia was fully functional but only the mutated LSCs were dysfunctional, corneal epithelial cells could presumably dedifferentiate and occupy the limbus as LSC-like cells~~, however~~. This is not in line with clinical observations, ~~and even corneal epithelial cells with~~suggesting that *PAX6* mutation ~~may not be capable of such~~could impair the dedifferentiation process. Still, the role of *PAX6* in LSC differentiation and epithelial cell dedifferentiation remains to be elucidated.

Comparison of AAK with other pathologies or genes causing ocular surface defects:

Many cellular and tissue properties are altered in aniridia, leading to pathologic changes associated with stem cell and ocular surface insufficiency that are also apparent in other rare disease entities such as severe dry eye, neurotrophic keratopathy and chronic inflammation. Several of these pathological processes are summarized in ***Figure 6***. Thus, a comparison ~~between~~of AAK to other eye diseases, which are not ~~present~~detectable at birth, but that could lead to stem cell deficiency~~, with AAK can~~ could bring new ~~data on aniridia~~insights. Several ocular surface diseases are associated with downregulation of *PAX6* in the corneal and conjunctival ~~epithelium [174, 188, 189].~~epithelia [196, 208, 209]. The limbus in aniridia may just be more sensitive to signals leading to pathology since the baseline level of the PAX6 protein is already low. LSCD can be induced by pathological ~~situations, like~~ triggers, such as chemical burns, infections, auto-immune disease ~~and~~[193]. The different signaling leading to epithelial-to-mesenchymal transition or fibrosis-like activation of transforming growth factor beta (TGF-β) ~~[171, 190~~by various cytokines as alreay hypothesied by Tseng et al. are summarized by Ljubimov et al. [210]. Thus, mechanisms uncovered in the context of aniridia may have bearing on other etiologies, and vice versa. Mutations in genes coding for key transcription factors and in stem cell-related genes (e.g. *TP63 [~~109~~134]*, *Klf4 [~~191, 192~~211, 212]*, *PITX2 [~~193~~213]*, *Sox2 [~~49~~131])* exhibit LSCD-like phenotypes in transgenic mice.

Interestingly, some of these transcription factors are ~~in addition~~also involved in neural crest and/or periocular mesenchyme specification. For example, *Pitx2,* a gene associated with anterior chamber dysgenesis if mutated, is mainly expressed in the developing corneal mesenchyme and later in the corneal stroma but not in surface ectoderm derived cells [~~73~~88]. Interestingly, *PITX2* mutation is associated with an iris hypoplasia similar to 'classical' aniridia [~~194~~214].

An inflamed and stressed environment has been reported in the *Pax6*$^{+/-}$ mouse epithelium. Elevated oxidative stress has been suggested as one important mechanism in developing AAK [~~135, 136~~153, 154]. Altered calcium waves lead to activation of ERK1/2 in mouse models [~~195~~215]. It has been reported that Desmoglein-1 (DSG1) together with Erbin1 can suppress ERK activation in epidermis [~~196~~216]. Notably, DSG1 is downregulated in the epithelium of *Pax6*$^{Sey/+}$ mice [~~23~~24] and therefore, together with elevated calcium, an activation of the ERK1/2 pathway could negatively impact differentiation through altered signaling in addition to oxidative stress. In line with this, pharmacologic inhibition of *ERK1/2* in human corneal limbal epithelial cells led to upregulation of *PAX6* and increased the production of PAX6 protein [~~105~~126].

Corneal-conjunctival boundary: Progressive infiltration of conjunctival goblet cells into the limbus is believed to be due to LSCD, but this assumption should be carefully studied. For instance, KRT12-positive goblet cells could migrate into the cornea ~~in cases of~~after large wounds [~~197~~217]. Also, in two clinical studies, only about 30% of aniridia cases had goblet cells ~~present and detectable~~ in the cornea [7, ~~28] upon examination~~29] detectable by *in vivo* confocal microscopy. The use of goblet cells for diagnosis of conjunctivalization and LSCD

may thus have drawbacks due to a heterogeneous distribution of goblet cells and potential presence of KRT12-positive goblet cells~~, as reported in mice [197]. Presently, there is a lack of specific markers to distinguish conjunctival cells from corneal progenitors [65, 198].~~ [217]. From experiments analyzing both the limbus and conjunctiva, it is known that conjunctival cells share some stem cell markers with LSCs (e.g., KRT15, KRT19 and TP63) [~~198]. This makes it~~218]. Thus, there is a lack of specific markers to distinguish conjunctival cells from corneal progenitors [98, 218]. It makes difficult to prove or disprove the presence of conjunctival progenitors in the limbus with existing markers; however, conjunctival cell migration patterns would differ as these cells are not typically mobile and conjunctival stem cells are enriched in the fornix region [~~199~~219]. If, however, the corneal epithelium or limbus is defective, the behavior of conjunctival cells ~~might~~could change.

Finally, it remains unanswered whether infiltration of conjunctival goblet cells into the corneal epithelium is a cause or a consequence of LSCD.

***PAX6* expression level and LSCD:** In humans, *PAX6* is expressed quite uniformly in both the limbal and corneal epithelial cells, regardless of the degree of differentiation [~~174~~196]. Alteration of the PAX6 protein level has an impact on different aspects of corneal homeostasis (stem cell survival, stem cell renewal, adhesion, migration, proper differentiation) [~~22,~~ 23, ~~27, 119~~24, 28, 139]. These properties were recently recapitulated *in vitro* in a *PAX6*-haploinsufficiency cellular model by genome editing of human limbal cells [~~200~~220]. Other alterations in the limbus have been reported, for example that epithelial cell proliferation is slightly enhanced in $Pax6^{+/-}$ mice [~~22~~23]. Hyperplasia in the limbus coupled to altered differentiation could explain why the thinning of the corneal epithelium occurs before the onset of conjunctivalization ~~occurs~~ in aniridia. Such morphological changes in aniridia are already detected at birth, although no apparent clinical consequences are observed at that early stage. ~~Therefore,~~ It is likely that abnormal development, as discussed above, impairs ~~LSCs~~LSC function and/or the LSC niche from birth in congenital aniridia.

$Pax6^{+/-}$ mice have limbal epithelial label~~--~~retaining (presumed) stem cells [~~201~~221]. In aniridia-like $Pax6^{Sey\text{-}Neu/+}$ mice, ~~an abnormal, distorted centripetal pattern of limbal strips was observed in~~ cell lineage tracing experiments ~~[119]. This~~revealed epithelial cell migration out of the limbus into the cornea (although the pattern of radial migration across the cornea was abnormal)[139]. These lines of evidence, and the wide expression pattern of PAX6, suggests that $Pax6^{+/-}$ mice do in fact have functional stem cells~~, but that~~. However, *Pax6* haploinsufficiency may reduce the number of LSC, or affect their ability to self-renew, undergo asymmetric cell division and/or diminish the potency of short-lived corneal epithelial progenitors. Such disturbance of proliferative capacity has been also described in ~~EEC~~Ectrodactyly-Ectodermal Dysplasia-Clefting Syndrome, where ~~in that condition~~ the epithelium is also thin [~~202]~~222]. It is also currently not known whether the spatial distribution of outer, Gpha2-positive, quiescent LSCs and inner, Gpha2-negative LSCs described above for wild-type mice and humans is disrupted in *Pax6*-mutants. In chimeric mice (~~with alleles for *LacZ* and *Pax6* present or absent in various combinations~~mixtures of wild-type and $Pax6^{+/-}$ cells formed by aggregation of eight-cell embryos), the contribution of $Pax6^{+/-}$ cells to the limbus was slightly but significantly reduced compared to wild-type, suggesting a mild deficiency of maintenance or proliferation of LSCs. However, relatively normal radial stripes of corneal epithelial cells emerged from the limbus to populate the cornea in these chimeras, [~~119~~139] Furthermore, corneal epithelial thickness and stratification was normal, suggesting that other tissues (lens, keratocytes or unaffected corneal epithelial cells) could rescue, by paracrine effects, the murine AAK phenotype [~~119~~139]. Further work has provided additional

evidence that the *Pax6*$^{+/-}$ cells themselves are unable to respond to guidance cues originating from the corneal stroma [~~203~~223].

Corneal neovascularization: Neovascularization of the cornea is not a specific phenomenon of AAK but a consequence of LSCD and chronic inflammation and wound healing in general. When the ocular surface is inflamed, ~~as witnessed by the presence of~~with high levels of inflammatory cytokines in tears and migration of inflammatory cells into the central cornea, new vessels sprout and migrate towards the central cornea along with conjunctival tissue~~, that~~. In advanced AAK ~~forms~~ a thick, opaque pannus forms that replaces the epithelium. How is the cornea normally maintained in an avascular state? Maintenance of an avascular transparent state has been attributed to a tightly regulated balance between pro- and anti-angiogenic factors ~~(and their associated signaling)~~ in the corneal epithelium and stroma [~~204~~224]. If the endogenous signaling networks preventing angiogenesis are impaired, a favorable environment for blood vessel growth will ensue~~, thereby promoting AAK progression.~~ .

For example in the corneal epithelium, soluble VEGF receptor-1 (sVEGFR-1 or sflt-1) is required to maintain corneal avascularity and its expression has been reported to be impaired in aniridia [~~205~~225]. Restoring sflt-1 expression in the *Pax6*$^{+/-}$ mouse cornea leads to regression of blood vessels [~~205]~~225]. The microRNAs miR-204-5p and miR-184 have also been shown to be expressed in the cornea [~~206-208~~226-228], and are involved in both Pax6 signaling and regulation of angiogenic signaling [~~209, 210~~229, 230]. miR-204-5p is encoded by *MIR204* that is located in intron 9 of the human *TRPM3* gene on chromosome 9 [~~211~~231]. In animal models, *Pax6* binds directly to 5' regulatory sequences upstream of *Trpm3* to upregulate both *Trpm3* and *Mir204* transcripts [~~212-214~~232-234]. miR-204-5p impacts lens and retinal development [~~211, 212~~231, 232], but ~~was~~is also strongly downregulated in conjunctiva cells in aniridia [~~104~~125]. Moreover, a feedback loop exists whereby in the Japanese rice fish *medaka*, miR-204 represses the transcription factor *Meis2*, which in turn regulates *Pax6* expression [~~215~~235]. miR-184, also known for its roles in neovascularization and homeostasis in the cornea [~~216~~236], was shown to be essential for *Pax6* expression ~~of~~in embryonic stem cells that were differentiated into corneal epithelial-like cells~~, while it~~ [228]. It also repressed angiogenesis of cultivated LSCs by targeting proangiogenic factors [~~208~~228].

VEGF-C, another pro-angiogenic factor primarily involved in lymphangiogenesis, was found by proteomic analysis to be elevated in the tear film in aniridia, correlating with the presence of lymph vessels in the cornea in advanced-stage AAK [7, ~~161~~182]. Further pro-angiogenic cytokines were later found to be elevated in the tear film in aniridia, including basic fibroblast growth factor (FGF-2) and interleukin-1β [~~158~~179]. These and other pro-inflammatory cytokines, as well as inflammatory cell infiltration [7], represent an environment conducive to neovascularization. Interestingly, in a case report of long-term topical anti-VEGF treatment of corneal neovascularization secondary to aniridia, progression of AAK was seemingly halted and central vision was maintained during a 12-year period [~~217~~237]. This raises the possibility of long-term treatment of the pro-inflammatory and pro-angiogenic corneal environment as a ~~mean~~means to prevent, halt or potentially even reverse the progression of AAK. To investigate whether this could be a potential strategy for AAK management~~, it will~~ would require controlled studies, with participants carefully selected for mutational status, stage of AAK and rate of progression.

2.2 What is known about the limbal niche?

It is generally accepted that stem cells require a specific supportive environment to maintain *in situ* their undifferentiated state and properties of self-renewal and differentiation. As soon as daughter cells migrate into the peripheral or central cornea, local features of the limbal niche

environment and the factors they provide are no longer present, eventually leading to (terminal) differentiation of epithelial cells. This raises essential questions concerning the niche location and its molecular nature. Mesenchymal tissues are known to have a tight crosstalk with their neighboring epithelia and serve as a niche for epithelial stem cells, as has been shown for example with hair follicle bulge cells [~~218-220~~238-240]. Corneal stromal stem cells support limbal stem cells in cultured feeder layer systems, and are similarly believed to be important in LSC maintenance [~~221~~241]. Seminal grafting experiments show that the type of epithelium (epidermis versus corneal) is dictated by its underlying stroma [~~222~~242]. Corneal epithelium that was exposed to embryonic dermal signals underwent transdifferentiation into hair follicle forming epidermis. Such a niche should be composed of the surrounding cells and the secreted ECM deposited by the different cell types. Surrounding ~~LSC~~the LSCs are mesenchymal (neural crest or mesodermal derived; see ***Figure 2***) cells (keratocytes), blood vessels,, nerves, immune cells, melanocytes, and stromal stem cells, all located in the peripheral stroma. It has been proposed that mesenchymal cells physically interact with LSC via SDF-1/CXCR4 signaling to maintain stem cell function [~~223~~243]. This apparent link between LSC, the stromal niche and surrounding immune cells fits well with the presence of lymph/blood vessels around the limbus and the recent demonstration of lymphatic capillaries as critical SC-niche components in the skin [~~224~~244].

In addition, the basement membrane composition of the limbus is different from the basement membrane of the central cornea [~~225, 226~~245, 246]. The molecular composition of the niche-derived ECM has been identified with laser captured tissue by ~~Polisetti *et. al.*,~~ showing that LSCs are attached to the basement ~~the~~ membrane through laminin receptors, and intercellular contacts between LSCs and their niche cells are mediated mainly by cadherins [~~227~~247]. Binding between epithelial and stromal cells is mediated by N-cadherins [~~228~~248], and mesenchymal cells and melanocytes have been suggested to support stem cell properties and to enhance the clonal growth of LSCs *in vitro* [~~229, 230~~249, 250]. N-cadherins are also expressed in widely used 3T3-feeder cells and could be important for homogenic and heterogenic cell-to-cell binding with niche cells such as melanocytes [~~228, 231~~248, 251]. *In vitro*, melanocytes have been suggested to support the stemness of limbal cells [~~232].~~252].

In contrast, however, to surface ectodermal or neuroectodermal-derived structures, the limbal niche cells show only low or even transient *PAX6* expression, and the specific ablation of *PAX6* gene in these cells has never been achieved. It is thus, difficult to identify the direct contribution of *PAX6* to limbal niche function. Although PAX6 is typically not detected by stromal cells, it was reported that stromal cells in the limbus express *ABCG2* and *PAX6* [253], and the associated proteins encoded by these genes may support the limbal niche. It is not clear if PAX6 ~~plays a role in~~ influences limbal melanocytes, ~~nonetheless~~but it is involved in the melanogenesis of the retinal pigmented epithelium though activation of *MITF* [~~233~~254]. Interestingly, *MITF* mRNA is downregulated in the aniridic conjunctiva [~~104]. For~~ 125] but it is not known whether the limbus in aniridia is melanocyte deficient. It was shown that limbal mesenchymal cells~~, it was shown that they~~ could substitute 3T3-feeder cell function in a 3D culture model system [~~234~~255]. However, whether these positive effects are dependent on *PAX6* expression in mesenchymal cells has not yet been addressed~~.~~, nor is it known whether the limbal stromal mesenchymal cells are present or functional in aniridia.

~~Examination of corneal epithelial morphology in young children with aniridia indicates that abnormalities may already be present at birth [28]. *In vitro* experiments suggest that *PAX6* could play a direct role in maintaining stem cell properties of the neural crest-derived niche [235]. It is worth noting that in Hirschsprung disease, *PAX6*-related pathways are affected in NCCs due to alterations in methylation patterns of the *PAX6* promotor.~~

From clinical studies, it is known that patients with aniridia show a degradation of the limbal niche structures coinciding with a progressive ocular surface transformation [6, 7]. ~~Although we do not definitively know if limbal niche structures or the LSCs are already impaired at birth in aniridia, morphologically normal and intact LSC structures such as the Palisades of Vogt and focal stromal projections were reported in at least one subject with aniridia aged 54 years. In this case, no clinically apparent AAK was present and the proband did not have a mutation detected in the coding regions of *PAX6 [6]*.~~ Thus, it is possible that the limbal niche structure and function could be maintained despite the clinical diagnosis and absence of iris in congenital aniridia, depending on the type of *PAX6* mutation. However, as discussed above and in particular in the case of 'classical' *PAX6*-related aniridia, the limbal niche ~~supporting~~ environment is perturbed ~~since young age~~in infancy and possibly from birth, ~~possibly~~ leading to LSCD regardless of the early postnatal presence of ~~the limbal niche. In contrast, however, to surface ectodermal or neuroectodermal-derived structures, the limbal niche cells show only low or even transient PAX6 expression making it difficult to identify the direct contribution of *PAX6* to limbal niche function. Stromal cells in the limbus express *ABCG2* and *PAX6* [236], and the associated proteins encoded by these genes may support~~ the limbal niche.

The mechanical properties of the niche are also important to protect the LSCs [~~237~~256]. Altered mechanical properties could affect proliferation and differentiation of LSCs [~~238~~257] and may therefore impact AAK if mechanical properties in the niche are altered. Gouveia et al. have shown that LSCs can be regulated by modulation of biomechanical factors and that a mechanically compliant limbal niche substrate supports LSC properties at least in part through regulation of Hippo ~~signalling~~signaling. Migration of ~~transit~~transient amplifying cells into the corneal epithelium from the limbus may in part be driven by durotaxis – driving cells from areas of high substrate compliance (the limbal stroma) to low substrate compliance (the ~~crystalline~~ corneal stroma) [~~237~~256]. Moreover, increased stiffness at the limbus is present in LSCD and this may have important implications for our understanding of AAK, if it reduces the durotactic drive for cells to enter the corneal epithelium from the limbus [~~237~~256]. If confirmed in patients, an appropriate strategy for manipulating stiffness of the limbal niche could potentially restore its pliability and consequently its capacity to support LSC function as a *novel* therapy to prevent or even arrest the development of AAK in the future. Of note, the fibrovascular pannus that is observed to invade the limbus as AAK progresses is a mechanically more rigid and tough tissue than the original limbal epithelial and underlying stromal extracellular matrix tissue.

2.3 Do stromal cells influence aniridia?

In the normal adult corneal and limbal stroma, ~~only a few~~a very small proportion of cells ~~remain~~show a low level of *PAX6*~~-positive [144] and~~ expression [165] but these may represent stromal stem cell populations generally related to keratocytes. Since ~~these~~corneal stromal cells can differentiate (at least *in vitro*) into cells with a keratocyte phenotype when cultivated as neurospheres, it is intriguing to speculate a role for *PAX6* in early keratocyte differentiation which may be of relevance to corneal stromal changes occurring in aniridia [~~239~~258]. Haploinsufficiency and overexpression of *Pax6* both lead to alterations in the corneal stroma and endothelium in mice [~~240~~259]. *Pax6*$^{Sey\text{-}Neu/+}$ mice exhibit increased stromal cell apoptosis, abnormal wound-healing responses and reduced levels of MMP-9 secretion [~~241~~260]. Studies are currently underway to characterize stromal cells isolated from aniridic patient corneal buttons at the time of corneal graft transplantation surgery, which may shed light on the properties of *PAX6*-mutant stromal cells and their role in AAK.

Co-culture of normal corneal and limbal stroma with limbal and corneal epithelial sheets showed that limbal stroma modulates epithelial cell differentiation and proliferation whereas the corneal stroma promotes their differentiation [~~242~~261]. Corneal stromal signaling is important for stratification of the epithelium and involves the WNT/β-catenin signaling pathway [~~243~~262].

AAK coincides with major changes in signaling in the epithelium and derangement of the corneal stromal architecture, with a subepithelial pannus in the anterior stroma either lacking collagen I or only faintly labelled, but positive for collagen IV, fibronectin, tenascin-C, vimentin and α-SMA, in addition to displaying fibrotic markers and containing blood vessels [~~239, 244~~258, 263]. The remaining posterior stroma appears to have an almost normal morphology, including expression of type I collagen. The changes in the stroma could be relatively nonspecific and not related to mutation of stromal cells since they are also induced in the stroma of healthy corneal donor tissue which consists of non-mutated stromal cells, but it is known that AAK ~~redevelops~~recurs after standard corneal transplantation due to the ~~LSC insufficiency~~LSCD. The corneal endothelium is also of mesenchymal origin (See section 1.6). In humans, no alteration of corneal endothelium has been observed in aniridic patients ~~so far [28], compared to observations in mouse models.~~thus far [29]. Regular Descemet's membrane and normal to slightly elevated endothelial cell counts are usually observed in patients with aniridia [~~28, 245~~29, 264]. Cases with lower endothelial cell count have been found in older patients, but these are usually associated with morphological changes such as cornea guttata or with pleomorphism/polymegethism due to a long history of topical treatments for glaucoma or previous surgeries such as cataract extraction [~~246~~265].

Notably, it has been shown that PAX6 could be secreted, as already reported for other homeodomain proteins [~~247~~266]. This could suggest previously undetected nonautonomous effects for PAX6 in the cornea, and that PAX6 produced and secreted by epithelial cells could be internalized by the adjacent stromal cells. In ~~that~~this case, altered PAX6 dosage could have an indirect effect on stromal homeostasis. In zebrafish, antibodies capturing secreted PAX6 lead to developmental defects [~~248~~267]. In the future, it must be elucidated if such biological processes could be important not only for eye development in zebrafish, but also for corneal epithelial-stromal interactions. Experimental co-culture systems might be useful for addressing these questions.

2.4 Is limbal cell identity altered in aniridia?

In contrast to the spatially restricted LSCs, conjunctival stem cells can be found in all regions of the conjunctiva, but are predominantly located in the medial canthal and inferior forniceal areas [~~249~~268]. Since lineage segregation of corneal epithelium and conjunctiva appears after *PAX6* induction [~~30, 250~~33, 80], some functional overlap for *PAX6* function in corneal and conjunctiva should exist. The focus of *PAX6* on corneal epithelial phenotype might be misleading since sustained *PAX6* expression is also present in the conjunctiva and other factors may drive tissue-specific expression downstream of *PAX6*. For example, studies link severe ocular surface disease such as Sjogren's syndrome with a reduction of PAX6 expression in the conjunctiva [~~189~~209]. Therefore, the question of the role of *PAX6* in the conjunctiva and its contribution to AAK has been recently raised. Transcriptomic analysis of conjunctival cells was recently investigated in a cohort of 20 aniridia patients mostly showing AAK signs. Changes in gene expression and pathways related to proliferation and pro-angiogenic pathways (*FOSB, FOS, JUN, ATF3, FOSL1, EGR1, NR4A3, IL8*) suggested that conjunctiva is part of the pathology in aniridia ~~[104].~~where it is abnormally proangiogenic and proliferative [125]. The molecular link between angiogenesis and *PAX6*, however, requires further investigation. The

expression changes observed in patient-derived conjunctival cells could also result from secondary effects due to inflammation or severe dry eye.

Of interest, the anti-angiogenic miRNA-204-5p mentioned above is under the control of *PAX6*, and its expression is strongly reduced in the conjunctiva of aniridia patients [~~104, 214~~125, 234]. Notably, two of the ~~patients~~subjects in the cohort had mutations in regulatory elements of *PAX6* (located in the adjacent *ELP4* gene) and these mutations are thought to lead to a milder PAX6 reduction ~~than~~compared to the haploinsufficiency caused by mutations in coding regions of *PAX6* ~~which result in a "*de facto*" loss of function of one allele~~. miR-204-5p expression was suppressed to a lesser degree in these two subjects relative to those with *PAX6* coding mutations, and this correlated ~~to~~with a very mild AAK ~~[104]. Additionally, in the same two patients, several mRNAs similarly showed~~phenotype [125]. Likewise, only mild deregulation of genes ~~coding for~~involved in retinoic ~~acid related compounds~~pathway (*RBP1, ADH7, ALDH3A1, CYB1B1, PPARG~~), relative to those with~~) was found in these two subjects, in contrast to the major mis-expression found in classical loss-of-function *PAX6* ~~coding~~ mutations. ~~Accordingly~~In line with this, siRNA knockdown of *PAX6* in primary limbal epithelial cells reduced *ADH7* and *DSG1* expression [~~251~~269].

Comparing published transcriptomic data between ~~different epithelial cell types (~~limbal~~,~~ and conjunctival~~)~~ epithelia from aniridia patients *in vivo* and cellular models *in vitro*, reveals ~~only~~ a ~~few deregulated genes~~relatively minor overlap (unpublished data). ~~Thus~~This may reflect the absence of the niche from the *in vitro* context. Also, an important drawback is that much of the data described in the available literature was produced in a late stage disease. Given the complexity of the eye and the aniridia pathology, a blueprint of the transcriptome and proteome of different ocular epithelial cell types *in vivo*, including ~~different~~various differentiation states at early disease onset is desirable, to investigate how *PAX6* influences the genetic programs of these different cell types. ~~Likewise, quantitative data is lacking regarding PAX6 dosage, splice variants and non-PAX6 signaling that specifies the tissue identity that results in AAK.~~

2.5 What is the role of inflammation in aniridia?

Important inflammatory changes are observed in the cornea in aniridia. These have been documented in several patient cohorts and include an infiltration of mature antigen-presenting dendritic cells into the central cornea, infiltration of non-dendritic inflammatory cells, upregulation of inflammatory cytokines, and a deficit of corneal nerves [7, ~~28, 252~~29, 270]. The question arises as to whether these inflammatory changes precede or are a consequence of AAK. Clinical evidence supports the contention that inflammation precedes the development of AAK based on inflammatory cell infiltration of the limbal epithelium and central cornea during the first few years of life while the limbus is still intact [~~28~~29]. Because progression of AAK is associated with a chronic and worsening inflammatory status of the ocular surface [~~28~~29], it is possible that early subclinical inflammation may trigger AAK. The inflammatory environment may then be reinforced in a vicious circle, through inflammatory signaling induced by degradation of corneal limbal structures, infiltration of inflammatory cells from the conjunctiva and from the circulation via invading blood vessels, ~~and~~lack of supportive neurotrophic factors (see Section 2.6), and the presence of inflammatory cytokines in the tear film [7, ~~158, 161~~179, 182]. On the other hand, the inflammation ~~seen~~observed early in life prior to degradation of the limbus, may itself be triggered by incomplete limbal developmental and postnatal maturation processes. Features such as blood vessel sprouting from an incomplete cellular structure of the limbal palisades and the migration of dendritic cells from limbal and conjunctival regions into the central cornea [~~28~~29] may indicate that the limbal niche in aniridia is insufficient to prevent inflammatory cells ~~and their signaling~~ from entering the cornea.

~~It is known that inflammation can degrade the corneal nerves, and in aniridia an inverse relationship with corneal nerve presence and infiltration of inflammatory cells into the cornea has been observed in clinical studies [6, 7]. As inflammation persists, corneal nerves degenerate and AAK progresses. This raises the possibility of treating AAK by addressing the inflammation and nerve deficit, for instance by supplying neurotrophic factors. In one study, autologous serum eye drops were prospectively used in a case series to treat 13 subjects with AAK. The treatment instilled during a 3-month period, improved subjective symptoms and stabilized the corneal epithelium, tear film and vascular pannus. It is tempting to speculate that longer-term use of topical autologous serum in an early stage of AAK amenable to treatment may halt its progression. Notably, autologous serum drops are widely used by subjects with aniridia, and studies are needed to assess its long-term effect on the inflammatory status of the cornea, including the corneal nerves. In subjects without aniridia, it has been shown that autologous serum drops can promote corneal nerve regeneration [253], however this remains to be investigated in aniridia.~~

Ocular surface inflammation is always present in eyes with LSC deficiency, and presents a risk for stem cell transplantation, requiring therapeutic immunosuppression prior to limbal transplantation [~~254~~271]. Mesenchymal cells have been shown to modulate the immune response in several systems, and may therefore also play a role in limbal niche and inflammatory signaling [~~255, 256~~272, 273]. Interestingly, therapeutic mesenchymal cell transplantation in aniridia has shown promising early clinical results [~~257~~274], and may overcome the disadvantages of LSC transplantation in aniridia, possibly due to the enhanced immunomodulatory properties of mesenchymal stem cells. Different types of lymphocytes such as T-cells [~~258~~275] and dendritic cells [~~259, 260~~276, 277], reside at the limbus and in the cornea. The antigen-presenting dendritic cells (also known as Langerhans' cells) are normally resident in the limbal region and can be visualized using *in vivo* confocal microscopy [~~259~~276]. In healthy individuals, mostly immature, non-antigen-presenting dendritic cells are present in the central cornea in the basal epithelial layers. In aniridia subjects, however, there is an increased presence of mature, antigen-presenting dendritic cells, even at a young ~~ages~~age. A higher density of dendritic cells could be explained by a developmental delay, since the dendritic cell population reduces after birth in the central cornea [~~28~~29]. Since the limbus may ~~form~~mature postnatally [~~54, 261~~71, 278], a correct spatial isolation of antigen-presenting dendritic cells may not occur in aniridia, leading to persistent inflammation throughout the corneal epithelium.

Elevated inflammatory cytokine levels have been observed in the tear film of aniridia patients, and this may be a causal or exacerbating factor in the chronic wound healing response observed in mice [~~136, 158~~154, 179] and known to exist in human aniridia patients. Interleukins are expressed in healthy corneal epithelium and are released upon wounding. Although it is unclear whether inflammation triggers the initial AAK, it is important to control the ocular surface inflammation in aniridia as it may slow down the progression and consequently improve the prognosis of AAK. Interestingly, it was shown that in tears taken from subjects with aniridia, there is not only an increase in the pro-inflammatory cytokine IL-1β, but its anti-inflammatory counterpart IL-1RA is diminished, suggesting an imbalance in cytokines can promote or sustain the inflammation observed in aniridia [~~162], which~~183]. This is reminiscent of the altered pro/anti-angiogenic factor balance in the cornea in aniridia.

2.6 Do corneal nerves play a role in AAK development?

The cornea is ~~one of~~ the most densely innervated ~~tissues~~peripheral tissue in the ~~body, providing a high level of sensitivity to mechanical, chemical and temperature stimuli [262]. In addition to inducing reflex tear production, corneal nerves also stimulate blinking, and~~ human body. Most corneal nerve fibers are sensory in origin and are derived from the ophthalmic

branch of the trigeminal nerve. Corneal nerves and corneal epithelial cells release modulatory substances and growth factors that provide trophic support to ~~the entire cornea, facilitating its homeostasis, repair and regeneration. Damage or loss of corneal nerves as a result of disease, trauma or surgery~~each other, to maintain a healthy ocular surface [279, 280]. Local and systemic disease, however, can impair corneal ~~epithelial integrity and~~ innervation leading to decrease in tear production and impairment of wound healing~~, with consequent epithelial breakdown that can lead to stromal ulceration, scarring, melting, perforation and consequently permanent visual loss or blindness. An example of this is the rare corneal disorder known as neurotrophic keratopathy (NK). There are many different conditions that can cause damage to corneal nerves, including genetic, systemic (e.g., diabetes), central nervous system (e.g., tumours), and ocular (e.g., herpetic keratitis, burns, surgery) diseases.~~

~~Regarding systemic conditions, it is important to note that recent work has identified a pivotal role of metabolism in stem cell fate regulation [263], raising the question of whether LSCD may partially derive from known *PAX6*-associated metabolic changes [264]. Indeed, it has been shown in both humans and in mice that *PAX6* interferes with glucose metabolism and proinsulin processing via modulation of PC1/3 production [264]. Thus, given the fact that insulin receptor signaling is crucial for the maintenance of stem cell potency and lineage determination [265], new insights may be gained into the abnormal LSC fate caused by heterozygous *PAX6* mutations.~~

~~Corneal nerves are important mediators of corneal homeostasis, as they release neurotrophic factors capable of maintaining a healthy epithelium, tear film, stroma, and LSC niche [266-268]. In aniridia, corneal nerve deficit has been documented in both mouse models [269] and a number of human clinical studies [6, 7, 28]. The neural deficit can be severe. For instance, from clinical imaging studies even in young children, the density of corneal nerves observed is 50% of the normal level, and the age-related degeneration of corneal nerves in aniridia is five times the rate of nerve loss normally observed in healthy subjects due to normal aging [28]. Whether the remaining corneal nerves in aniridia are functionally normal remains to be investigated. Given the relevance of corneal nerves for homeostasis in the cornea, it follows that many of the observed clinical features in aniridia are likely linked to (or exacerbated by) the lack of neurotrophic support, for example dry eye, reduced blink reflex, altered epithelial healing, inflammation and impaired stem cell function. Importantly,~~ [279]. Neurotrophic factors released by corneal nerves, often in the context of inflammation and wound healing, include nerve growth factor (NGF), ~~a neurotrophic factor released by corneal epithelium and keratocytes, is constitutively expressed in the basal limbus with lower expression in the basal corneal epithelium. NGF has been shown to promote LSC proliferation *in vitro*, but also supports colony formation and expression of stem cell markers [270]. Therefore, NGF promotes "stemness" in corneal epithelium and these properties may be affected in AAK patients leading to LSCD. Furthermore, glial cell line~~epidermal growth factor (EGF), substance P (SP), calcitonin gene-related peptide (CGRP), acetylcholine, cholecystokinin, noradrenaline, serotonin, neuropeptide Y (NPY), brain-derived neurotrophic factor (~~GDNF), also expressed in the~~BDNF), and neurotrophin- (NT-) 3 [281]. SP and CGRP modulate corneal ~~epithelium, has been shown to stimulate~~ epithelial cell proliferation, migration, ~~colony formation, and proliferation while brain derived neurotrophic~~ adhesion, and stratification [279]. In addition, administration of SP combined with insulin-like growth factor ~~(BDNF) only enhanced colony formation [271]. In the *Pax6*$^{+/-}$ mouse, although projections of nociceptive axons into the corneal epithelium were badly~~ -1 (IGF-1) can increase the corneal healing rate and promote corneal epithelial cell adhesion [282]. Conditions such as AAK, where ocular surface homeostasis is disrupted, ~~no acute defects of neurotrophic support to the cornea were found (compared to wild-type) and the provision of neurotrophic support through addition of Substance P and NGF did not improve corneal wound healing [269]. It remains possible that~~

~~chronic reduction in neurotrophic support is a factor in human aniridia, therefore, further investigations are warranted to determine whether early interventions such as topical neurotrophic factors, for instance, may help to delay the progression of AAK.~~

~~A previous study by Lagali et al. [28] showed significantly reduced central corneal sensation in aniridia patients with *PAX6* coding mutations when compared with non-coding *PAX6* mutations. Corneal nerve density measurements using *in vivo* confocal microscopy indicated significant reduction in corneal nerve density in *PAX6* aniridia when compared with non-*PAX6*-coding cases. Reduced corneal nerve density has been reported not only in humans but also in a mouse model of aniridia [269]. In the same study, Lagali et al. also observed an order of magnitude increase in mature dendritic cells (DCs) in the central cornea in aniridia patients relative to the healthy cornea, and clear evidence of limbal damage even in early stages of aniridia. In a mouse model of keratoconjuntivitis sicca, CD4+ T-cells are involved in a mechanism leading to downregulation of PAX6 through an IL-1R1 dependent mechanism [188]. This resulted in a switch of the corneal-specific KRT12 epithelial maker to an epidermal-specific KRT10. This suggests an immune-mediated LSC functional breakdown linked to PAX6 levels. Other studies have reported an~~ can upset the close and important interaction between the ~~nervous and immune systems in the cornea [272] and there may also be a possible connection between tear cytokines and corneal DCs [162]. This neurogenic~~ nerves and the epithelium, impairing the normal corneal renewal process and leading to inflammation ~~of the cornea is defined as a bidirectional interaction between the nervous and immune systems that has been previously observed in other tissues and could also play a significant role in AAK development [273, 274]. Inflammation, a corneal nerve deficit and early conjunctivalisation as signs of LSCD are regularly observed in infants with aniridia as young as 9 months development, causing major brain defects and a wide range of neurodevelopmental disorders when mutated or deleted from developing embryos [275]. Interestingly, in addition to the aforementioned neurodevelopmental disorders associated with WAGR and Gillespie syndromes, heterozygous loss-of-function mutations in the *PAX6* gene are also associated with neurological and psychiatric conditions including nystagmus, impaired auditory processing and verbal working memory and autism [276]~~ and a wound healing state.

~~In this regard, it is not surprising that structural brain abnormalities and impaired cognition are some of the manifestations observed in aniridia patients. This may be partially related to the high prevalence of nystagmus in aniridia, observed in nearly 100% of all aniridia cases, besides known ophthalmic pathology [277, 278]. Curiously, the role of *Pax6* in neurodegeneration has also been investigated. A wide range of neurodegenerative markers was shown to be deregulated with *Pax6* knockdown, highlighting a *Pax6*-mediated control of cascades of proteins involved in growth, differentiation, maturation and survival of neural cells [279]. Indeed, this is totally in accordance with the progressive reduction in PAX6 protein levels observed during aging [280]. Interestingly, and linking the putative effects of *Pax6* on metabolism, *Pax6* was also shown to directly or indirectly regulate key genes for immunological surveillance and energy metabolism in the brain, genes that are also altered during aging [281].~~ A recent study by Lagali et al reported a significant corneal nerve density deficit in PAX6 aniridia subjects (n=46), that declined further with age. This was associated with significant corneal sensitivity reduction, reflecting a chronic impaired neurotrophic status [29]. The combination of limbal deficiency and reduced corneal sensation in subjects with *PAX6* aniridia would have a significant impact on the maintenance of the ocular surface, contributing to the development of an unstable corneal epithelium often associated with AAK. Perturbation of the neurotrophic function may also impact inflammation of the ocular surface, which is widespread in aniridia. The cornea normally enjoys an immune- privileged status, which plays an important role in corneal immune response, particularly in promoting corneal allograft survival. Corneal immune privilege is dependent on the interplay of several important factors such as: (i) an absence of blood vessels (limiting direct access of the immune system to the cornea) and lymphatic vessels (preventing efferent movement/delivery of antigens and

antigen-presenting cells (APC) to T cells located in the draining lymph nodes; (ii) a lack or low expression of major histocompatibility complex (MHC) class I and II antigens; (iii) presence of anti-inflammatory molecules, e.g., TGF-β, IL-10, IL-1RA; (iv) an Anterior Chamber-Associated Immune Deviation (ACAID) induced response, and finally (iv) presence of FasL, resulting in the apoptosis of infiltrating T cells. Many of these factors, however, are upset in AAK, leading to inflammatory cell invasion, expression of proinflammatory cytokines and a loss of immune privilege. Notably, different populations of immune cells have been reported in the cornea of subjects with AAK [7] and the pro/anti-inflammatory cytokine balance is perturbed in the tear film in aniridia [179].

Dendritic cells (MHC Class II positive mature antigen presenting cells capable of antigen presentation to naïve T-cells) are one type of inflammatory cell normally resident in the corneal stroma and epithelium, with highest density in the periphery. As described above, dendritic cells play an important role in corneal homeostasis as sentinels of both innate and adaptive immunity. Resident corneal dendritic cells also have a role in maintaining homeostasis of corneal nerves [283], suggesting a direct interaction between the immune system and peripheral nerves at the ocular surface level. As indicated above, a significantly elevated density of mature dendritic cells exists in aniridia subjects when compared with normal individuals [29]. This emphasizes the close relationship of dendritic cells, inflammation and neurotrophic status of the cornea in PAX6 aniridia patients. Macrophages, normally occupying only the corneal stroma, reside in close proximity to peripheral nerves [284, 285]. Macrophages express SP receptors, produce SP, and it is also known that neurotransmitters including SP can modulate immune activity, although more investigation is needed to completely elucidate whether SP is affected in aniridia. It is known, however, that the decrease in corneal nerve density in AAK is associated with simultaneous invasion of inflammatory cells reflecting disease progression and corroborating the interaction between the nerves and immune system [286].

It is not clear whether problems with innervation are partly responsible for insufficiency of the limbal niche in aniridia. In normal eyes, the basal side of the Palisades of Vogt is densely populated by corpuscular nerve endings, suggesting that neurotrophic factors may support stem cells not only directly, but indirectly by maintenance of the limbal niche [287, 288]. Human corneal-limbal organoids appear to maintain good niche function without innervation *in vitro* [289], but this does not preclude an *in vivo* role in niche development or maintenance. In mammals, though not in birds, *PAX6* is expressed transiently during early development of the trigeminal ganglion, in cells of both the OpV and mmV-derived components, and this correlates with delayed innervation of sensory structures such as whisker follicles in mutants (unpublished data). Putative links between *PAX6* mutation, disruption of limbal-corneal innervation, the degradation of palisade structure and [207, 270] deficiency of the limbal niche require further investigation.

As immune cell infiltration into the cornea facilitates (and is facilitated by) neovascularization, a deficit of corneal nerves may directly or indirectly play an important role in development of neovascularization in AAK. In experimental models of corneal neovascularization and trigeminal nerve ablation in the mouse, blood vessel invasion was shown to occur only in areas devoid of nerves, where inflammatory leukocytes and macrophage-lineage cells also infiltrated the cornea [290]. Conversely, where the nerve supply remained intact, the cornea remained vessel-free. A loss of antiangiogenic factors was noted in denervated corneas, in particular loss of vascular endothelial growth factor receptor-3 (VEGFR3) expression in the corneal epithelium and loss of pigment epithelium-derived factor (PEDF) expression in the corneal stroma. In a different mouse model of inflammation-induced dry eye disease, corneal nerves were diminished and expressed elevated levels of the proinflammatory neuropeptide SP [291]. Blockade of SP or its neurokinin-1 receptor effectively prevented vascular endothelial cell activation and reduced corneal neovascularization. In addition, VEGF has been shown to

mediate corneal repair in abrasion-induced corneal nerve damage models, where VEGF blockade effectively suppressed nerve regeneration [292, 293]. The source of VEGF in the corneal abrasion model is infiltrating T-cells and neutrophils [292]. Taken together, these studies indicate that corneal nerves, where damaged or accompanied by inflammation or when lost entirely, lose their ability to express angiostatic factors and instead express proinflammatory and regenerative factors, contributing to enhanced corneal neovascularization. These effects can be considered part of the normal wound healing response, and may thus explain why the cornea in AAK, which is in a chronically inflamed wound-like state, is characterized by a deficit of nerves and an abundance of blood vessels. Potential therapeutic approaches supplying deficient factors such as VEGFR3 or PEDF, or blocking factors aiding neovascular growth such as SP or VEGF, warrant further investigation in relevant *in vitro* and *in vivo* aniridia models.

Given the role of corneal nerves in maintaining avascularity and epithelial integrity, restoration of a healthy corneal nerve population may represent a viable therapeutic approach for AAK. As described above, autologous serum eye drops have been shown to relieve symptoms of neuropathic corneal pain and increase the abundance of corneal nerves in non-aniridia subjects [294]. In subjects with toxic corneal epitheliopathy induced by anti-glaucoma eye drops, a course of autologous serum drops significantly improved corneal sensitivity [295]. In a cohort of thirteen subjects with AAK, autologous serum eye drops instilled over an 8-week period resulted in subjective improvement in keratopathy symptoms and healed corneal epithelial defects in several cases; however, neovascularization and stromal scarring did not significantly improve during the course of treatment [296]. Longer-term treatment, careful patient selection (e.g., based on AAK grade) and/or use of molecules with specific nerve regenerative capacity may be of benefit in AAK. The recently approved recombinant human nerve growth factor (NGF) treatment (Cenegermin) may be of interest in this regard. Indicated for treatment of moderate to severe neurotrophic keratopathy (a rare disease characterized by diminished corneal innervation, non-healing epithelial wounds and corneal ulceration), NGF stimulates corneal epithelial cell growth and survival, aids in the maintenance of limbal stem cell function, promotes tear production and supports corneal re-innervation [297]. In clinical studies, 65 - 75% of patients receiving an 8-week course of Cenegermin eye drops exhibited complete corneal healing, although relapse occurred in about 20% while eye pain and reduced visual acuity were frequently reported adverse effects [297, 298]. Notably, it has also been shown that corneal subbasal nerve density significantly increased following an 8-week course of Cenegermin [299]. Whether these promising effects could be extended to AAK is unknown and would need to be explored; however, use of Cenegermin for conditions other than neurotrophic keratopathy is currently off-label. Here, animal models would be useful for initial investigations.

3 Molecular biology and genetics perspective

Due to the many observed *PAX6* mutations independently leading to aniridia and the heterogeneous clinical phenotype, it is difficult to associate specific mutations to AAK severity~~, but different mutation subtypes have been reported to have a strong association to~~ [4]. However, some classes of mutations (selected missense or non-coding mutations) are associated with reduced AAK progression and mild or absent LSCD [29]. Comparative OMICs of such patient samples could help elucidate correlations between specific mutations and degree of AAK severity ~~[4].~~ at the molecular level [125, 300]. Nonsense mediated decay (NMD) of mutant mRNA is the most common mechanism of PAX6 protein deficiency, but there is no evidence

~~that~~this is modulated by the position of mutations in the transcript ~~affects NMD~~. The effect of missense mutation is difficult to predict but some *in vitro* studies described the effect of the mutation in different binding domains. It might not be useful to compare different missense mutations with each other since they could have different properties. For *PAX6* run-on mutations (with a mutated stop codon), it is thought (but not yet demonstrated) that the mutated PAX6 protein is degraded or not produced. Mutations in regulatory domains of *PAX6* (also found as 3'-cis-regulatory region deletions) are interesting since PAX6 reduction could be less pronounced, and these mutations have been related to milder phenotypes [4, ~~186, 282]~~ .206, 300]. In rare cases, genes other than *PAX6* can putatively cause aniridia [~~283-285~~301-303]. Primary culture of patient cells (both epithelial and mesenchymal) should help to identify deregulated genes important for AAK development especially in cases of aniridia with mild AAK, to discriminate from *PAX6*-dependent expression changes that are not causative of AAK [~~104~~125]. Recent comprehensive reviews summarize in detail the genetics of congenital aniridia [1, 2, ~~286~~304]. Although the impact of *PAX6* levels and splice variants on iris and ciliary body development have been systematically studied [~~138~~155], we lack such detailed knowledge for LSCs and corneal epithelial cells. Also, PAX6 protein has been described to be located in the nucleus, cytosol or even in secreted form. It must be further evaluated ~~if~~how localization of PAX6 is controlled by post-translational modifications. *PAX6* mRNA expression may be not as dramatically altered as at the protein level, and thus mRNA expression and translation of *PAX6* need to be studied in detail in cells such as LSCs and differentiated epithelium. Interestingly, it has recently been shown that *Pax6* expression is negatively regulated by the microRNAs miR-7 and miR-135, and that protection of this inhibitory mechanism was capable of restoring PAX6 protein levels in isolated pancreatic islets in an aniridia mouse model [~~287~~305]. It remains to be determined whether a similar regulatory mechanism also occurs in LSCs. Nevertheless, these data, in addition to the putative target miR-204-5p discussed earlier, appear to suggest that RNA-based therapies could represent a potential ~~and~~ innovative therapeutic strategy for AAK.

4 Conclusions for further research strategies

Nearly all patients with aniridia suffer from AAK [4]. The onset of clinically apparent AAK differs between individuals even with the same mutations. ~~However, some classes of mutations (selected missense or non-coding mutations) are associated with reduced AAK progression and mild or absent LSCD [28]. Comparative OMICs of such patient samples could help elucidate correlations between specific mutations and degree of AAK severity at the molecular level [104, 282].~~ Based on our present level of knowledge, it is not clear if AAK is caused by LSCD while it seems likely that some loss of LSC function occurs, it is not necessary to invoke LSCD as the main cause of the phenotype seen in AAK eyes. Clinical microscopy findings suggest that a minimal degree of keratopathy is likely to exist in all cases of aniridia even before ocular surface changes become visible at the slit lamp. This ‘minimal keratopathy’ includes reduced mechanical touch sensitivity, a deficit of corneal nerves, and increased inflammatory cell presence in the central cornea [4]. LSCD may in fact be a consequence of the early minimal keratopathy in subjects where the specific *PAX6* mutation predisposes the cornea to a progressive AAK phenotype. The minimal keratopathy may in turn have a developmental origin. The chronic wound healing pathology of the corneal epithelium in AAK may overwhelm the $PAX6^{+/-}$ limbal regenerative potential during normal life [~~135, 136~~153, 154]. Essential knowledge, however, is still lacking concerning the critical factors needed to specify and maintain the limbal niche and how these relate to PAX6 expression both prenatally and postnatally; therefore, pathogenic mechanisms at the molecular level are still speculative. Early indications are that *PAX6* regulation of and by other genes and factors is complex, and multiple signaling pathways, molecular and cellular mechanisms and feedback loops appear to be active, resulting in the observed AAK phenotypes. Deciphering some of the key pathways and

mechanisms involved can provide insights that will be important for future and new therapies targeting AAK, keeping in mind that the complexity itself may provide multiple potential therapeutic targets.

Declaration of competing interest:
No conflicting relationship pertaining to this work exists for any author.

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We thank the two reviewers for their enthusiasm and very helpful comments and advice on our manuscript.
We have carefully replied to each point raised by the experts. Our answers are in boldface type. Changes made in the manuscript text are indicated in "red" or in the markup version of the manuscript where longer passages or complete sections were revised.

Reviewer #1:

This is a very well written and informative review article on aniridic keratopathy. It is likely to be highly cited as it is comprehensive, thoughtful, and does an excellent job identifying what is unknown about this condition, while exploring possible disease mechanism.

I just have a couple of small quibbles.
T1: Reference 20 describing the eye size in mice and humans many not be the most specific. There is a more recent paper that explicitly compares these issues see PMID: 33248069

We were not aware of this nice publication and are happy to cite this now as ref [20] in following context (page 2 / page 2 [Markup version]):

"For example, *Pax6*$^{+/-}$ mice exhibit microphthalmia with a 10% reduction in eye diameter and reduced lens size; while microphthalmia can also occur in human aniridia, most patients have a relatively normal eye and lens size [20]"

T2: on page 10, the manuscript says that congenital cataract is prevalent in aniridia.
However, while juvenile/early adult onset cataract is prevalent, I believe that most literature states that congenital (ie > present at birth) cataract in aniridia is rather uncommon

We thank the reviewer, and have corrected this erroneous and misleading statement (page 11/ page 15 [Markup version]).:
"An early onset of cataract is prevalent in aniridia [147]."

T3: on page 3, it would probably be good to mention pineal gland hypoplasia as it is a major tissue malformation and causes aniridic patients a great deal of problems PMID: 31943460

We have implemented the useful additions as follows (page 3 / page 3 [Markup version]).:
"…, and anosmia [37, 38]. Sleep disorders are also reported in patients and *PAX6* may impact brain structures such as the pineal gland [39]."

T4: on page 2, the manuscript likely overstates the phenotype of the cornea of Pax6 het mice. While some mouse mutants do have a high prevalence of peters anomaly, it is not common in many sey alleles.....

We have corrected the statement on corneal lens fusion according to the scientific literature (page 2/ page 2 [Markup version]).

"Separation of the lens from the cornea during development is delayed in *Pax6*-mutant mice and iridocorneal and/or lens-cornea adhesions similar to Peters' anomaly may be observed at birth, dependent on mouse strain and the *Pax6* allele [23-26]"

Reviewer #2

Referee report on *Pathophysiology of aniridia-associated keratopathy: developmental aspects and unanswered question*

This is very wide-ranging review of most of the current knowledge of corneal development and function in the context of early anterior segment development, and subsequent maturation of the possible tissues implicated in the development of aniridia-associated keratopathy (AAK). Throughout the article there is an exploration of whether AAK is caused by abnormal development arising as a result of aniridia-associated PAX6 mutations or whether early features of the ocular anomalies and aberrant maturation of the eye leads to cumulative malfunctioning. A huge number of references are cited. There is ample evidence presented that corneal stem cells are mislocalised and in some instances numbers are reduced from very early stages of aniridia. Similarly, parallel observations in the mouse model and other model systems are cited in places. Discussion of available evidence suggests that it is an over-simplification to suggest that AAK is simply the progressive consequence of limbal stem cell deficiency.

T5: The review is not an easy read! The sentences are often overly complex and include quite a few minor linguistic errors. Different sections seem to have been written by different authors further disturbing flow and uniformity, making it quite difficult to follow the narrative.

We thank the reviewer for this feedback, and we have now completely revised the manuscript and harmonized language were possible (all highlighted in red / tracked change).

T6: In section 1.1 the description of the development of the anterior segment jumps around without conveying the clear spatiotemporal scheme. Surely the optic cup should not come at the end. There is quite a lot of jumping from one species to another without clear distinction.

To address this, we have reorganized the developmental section to reduce redundancies and have distinguished the results in the different species from each other. Since not all findings on eye development have been carried out in all model organisms, we also wanted to deliberately point out the difficulties in interpreting and transferring them to another "model"; humans. We hope that we have now found a good balance between comprehensibility and actual complexity.

T7: The existence and origin of some components such as the conjunctiva are not defined clearly in Section 1.1.3.

To address this, we have now added several paragraphs and completely revised section 1.1.3 (Find it now as section 1.1.6) (page 7 / page 7 [Markup version]).

T8: The important details on genesis, migration and maturation of corneal stem cells in wild type and PAX6/Pax6 haploinsufficient mammals are not presented very clearly, despite the fact that much is written about disposition of these cells in wild type and haploinsufficient mouse and humans.

We thank Reviewer #2 for this useful comment. We tried to improve the understanding of these aspects in the manuscript. Also, we added the following new paragraph to Section 1.1.6 (Formely 1.1.3) (page 8 / page 9 [Markup version]):

"These data seem to suggest that, before stratification of the ocular surface epithelium, stem cells are not restricted to the limbus but rather distributed throughout the entire limbal/corneal epithelium.

(This would also point to the important role of stromal stem cell niche in maintaining stem cell capacity in adults as described in 2.1 **Controversy on limbal stem cells**) Additional support for this hypothesis came from the use of a X-chromosomal *LacZ* transgene, which allows random and irreversible labeling of embryonic blastocyst cells in female mice based on X-chromosome inactivation mosaicism at early embryonic blastocyst stage. Interestingly, in the first 3-4 weeks after birth, a disorganized mosaic pattern of $LacZ^{+}$-labelled patches of cells appeared dispersed throughout the entire limbal/corneal epithelia. .However, from P30 onward, a typical radial stripe pattern of cell migration into the cornea became evident as the stem cells became restricted to the limbus [101]. These elegant studies [101, 102] together with other studies mentioned above, indicate that the corneal epithelium is self-sustained by its own pool of stem cells, these stem cells probably differentiate before P30 and from that stage, the limbus becomes the unique stem cell location,. Notably, the original 'XLacZ' mouse model has limitations due to the fact that tracing is of blastocyst-stage (E3-4) cells and because it does not allow cell type-specific tracing in a clonal, temporal manner by vital microscopy. However, advanced quantitative lineage tracing studies has further shown that this concept is correct, revealing additional aspects of limbal stem cell biology (discussed in section 2.1 below, [103-108])"

Despite these improvements, many of these questions cannot be answered unambiguously from the point of view of developmental biology. A simple model of the specification and maintenance (self-renewal) of limbal stem cell populations is lacking. It is likely that entire corneal surface is progenitor/stem-like during embryogenesis and that in early life the stem cell phenotype becomes progressively restricted to the limbus, presumably because of stromal regionalisation. This would mimic the process of stem cell restriction in many other tissues. We know quite a bit about what the limbal niche needs to be and to secrete, and we can predict a bit from lineage tracing and modelling about the balance between limbal stem cell self-renewal, quiescence and production of TA cells in adults. But the information is scattered and there are still many questions open. All these limitations are discussed here.

T9: The discussion of the significance of low and transient PAX6 expression levels is confusing. Many studies have shown that normal PAX6 expression is spatiotemporally and quantitatively highly complex, but reproducible. The complex role of multiple enhancers within introns and also flanking both sides of the PAX6 gene have been explored in detail by many, including some of the authors of this review. Autoregulation and cross-regulation with other transcription factors (TFs) is mentioned, but the significance of these complex controls for orchestrating the diverse roles of PAX6 and for maintaining homeostasis of the developmental and maintenance functions is not emphasised. PAX6 has been shown to interact with many different transcriptional partners in space and time, though undoubtedly nowhere near all interactions have been established. Like all DNA-binding transcriptional regulators PAX6 most likely fulfils its roles in different complexes with other TFs. The exquisite and dynamic binding and tissue specificity of TFs is most likely achieved through the precise stoichiometry and timing of expression of different partner TFs determining oligomeric complex formation. Haploinsufficiency (=reduced expression) of a mutated TF will quantitatively alter a large number of these dynamic partnerships and therefore many developmental and maintenance processes.

Although no specific changes were requested, we have clarified this section (1.6) to articulate more precisely what is meant by high and low levels of Pax6. Extra information has been added as alluded to by the reviewer to describe that both Pax6 regulation and downstream activation are complex and influenced by presence and stoichiometry of binding partners. The consequences of this for gene expression in ocular mesenchymal cells is discussed briefly. The whole section has been reviewed,

and repetition removed so that the main message (that Pax6 is expressed in mesenchymal cells and has a role, but we do not know if they contribute directly to the aniridia phenotype) comes through more clearly.

"and the presence and stoichiometry of different binding partners in different cell types is expected to modulate PAX6 activity. Hundreds of genes are regulated, directly or indirectly, by PAX6 during eye development [169] and are affected to different degrees by changes in dosage." (page 13 / page 17 [Markup version])

One of the very interesting details of corneal function that I have not seen frequently discussed is the multiple roles of the normally very high density corneal nerve network. Reference 28 (authorship of which includes two of this review's authors) discusses the 50% reduction in sub-basal nerve density even in very early childhood in aniridia patients. The likely reduction in the important trophic neural factors is mentioned in this review, but wider exploration of this area could provide significant further insights into the aetiology of AAK and into its management.

It is a pity that this is discussed mostly at the end of the review.

We thank Reviewer #2 for this useful comment. We completely rearranged section 2.6 to provide the requested detailed exploration of neurotrophic factors and the consequences of a nerve deficit for AAK. We also refer to this important section earlier in the manuscript.

(T5) Some examples of linguistic anomalies

Pg 9 In addition, lineage tracing in Pax6+/- mice has allow detecting pathogenic mechanisms associated with aniridia and the LSCs in light of developmental processes.

Pg 10 …developmentally immature epithelial cells (including limbal stem and progenitor cells) that are unable of forming the fully stratified multiple corneal epithelial layers

"In addition to the role within the lens lineage, Pax6 also plays a role in adjacent optic vesicle for triggering lens formation as Pax6 is knocked out earlier in the optic vesicle, the lens does not develop"

Pg 12 "In lacrimal gland organoid models Pax6 is necessary to express the secretion machinery"

There are several other places elsewhere with prepositions missing

Thank you - we have now completed an extensive critical revision of language throughout the document by several native speakers.

T10

On Pg 9 there is another scientific point to make:

The "identification of novel LSC marker, GPHA2, whose function appears to be essential for LSC self-renewal and differentiation" [ref 53] is discussed. The acronym GPHA2 is not defined but I see it is glycoprotein hormone subunit alpha 2. The mouse chimaera experiment is described next:

“for example Pax6+/- LSCs in Pax6+/- ↔ Pax6+/+ chimeric mice are functional and produce streams of epithelial cells that migrate normally into the cornea, though are less likely to reach the center of the cornea than wild-type cells [119].

This suggests that dosage deficiency of Pax6 does not preclude normal specification of LSCs

and show that corneal epithelial defects may directly contribute to the mutant phenotype”

Pax6+/- cells might behave normally or near normally in a Pax6+/+ environment if diffusible factors, such as GHAP2 is likely to be, are present in the host tissue.

We clarify this point in the discussion as follows (page 10 / page 13 [Markup version]):

“This suggests that dosage deficiency of Pax6 does not preclude normal specification of LSCs in a cell-autonomous manner. It remains unknown, however, if the relatively normal behavior of *Pax6*$^{+/-}$ cells in the chimeric mouse limbus represents a non-autonomous ‘rescue’ by secreted protein factors such as GPHA2 released from the wild-type cells.”

In summary this review is a treasure trove of important information on the possible aetiology of AAK but greater writing clarity, more logical order of presentation, plus editing of the linguistic anomalies, would greatly improve the accessibility of the data presented

We thank Reviewer #2 for the enthusiasm. We hope that our revision matches the reviewer’s expectations and thank the reviewer in advance for these very helpful points which helped to substantially strengthen the text.

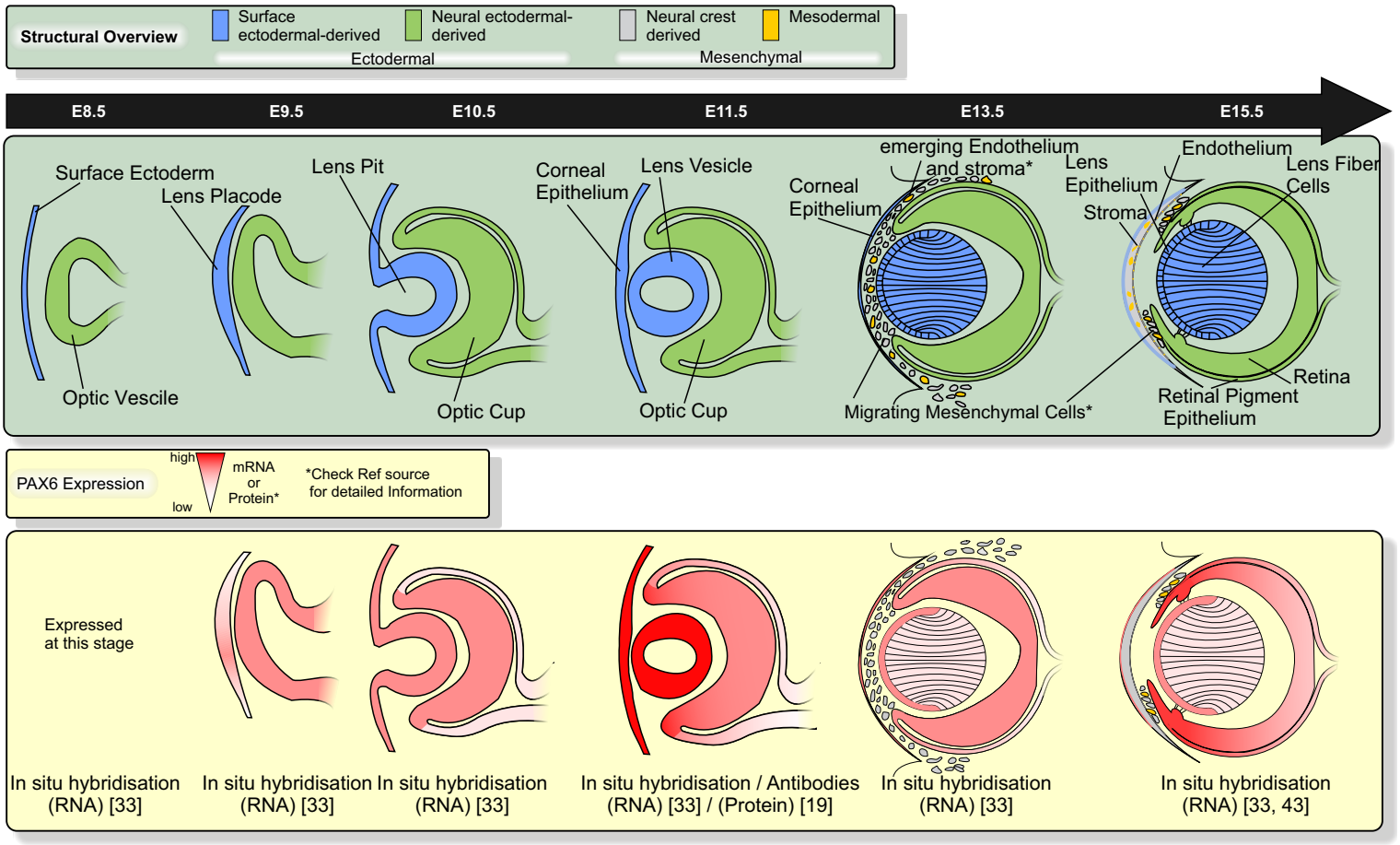

Figure 1: **Eye development and PAX6 expression in the mouse.** The green box shows the developing murine eye at different stages. The color code marks the embryonic origin of these tissues. (Blue: surface ectodermal, Green: Neural ectodermal; Grey: Neural crest; Yellow: Mesodermal). The yellow Box below shows the relative PAX6 expression in these different structures. The neural ectoderm forms the optic cup as a double-layered structure and is important for lens placode development of the surface ectoderm. The distal tips of the optic cup will develop iris structures, and this coincides with very high *PAX6* expression levels. The inner layer of the optic cup will form the neural retina the outer layer develops to retinal pigment epithelium. Upon separation of lens and surface ectoderm several waves of neural crest and mesodermal cells migrate into the anterior segment contributing to the corneal stroma, corneal endothelium, iris stroma and anterior chamber. Most of the data displayed derive from studies on murine development [19, 33, 43]. *Note that endothelium and stroma differentiation is displayed for mouse where endothelium and stroma differentiate from the same cell mass migrated in a first wave. However, it is unknown if endothelial cells are already specified prior migration [111]. A second migration wave later appears in the angle between future cornea and optic cup and differentiate into stroma of iris and ciliary body [47]. In human and birds three migration waves are observed. First endothelium is specified then mesenchyme migrates between epithelium and endothelium to differentiate to stroma and the third wave contributes to iris and ciliary body [46]. Graphics adapted from different sources [44, 47, 112].

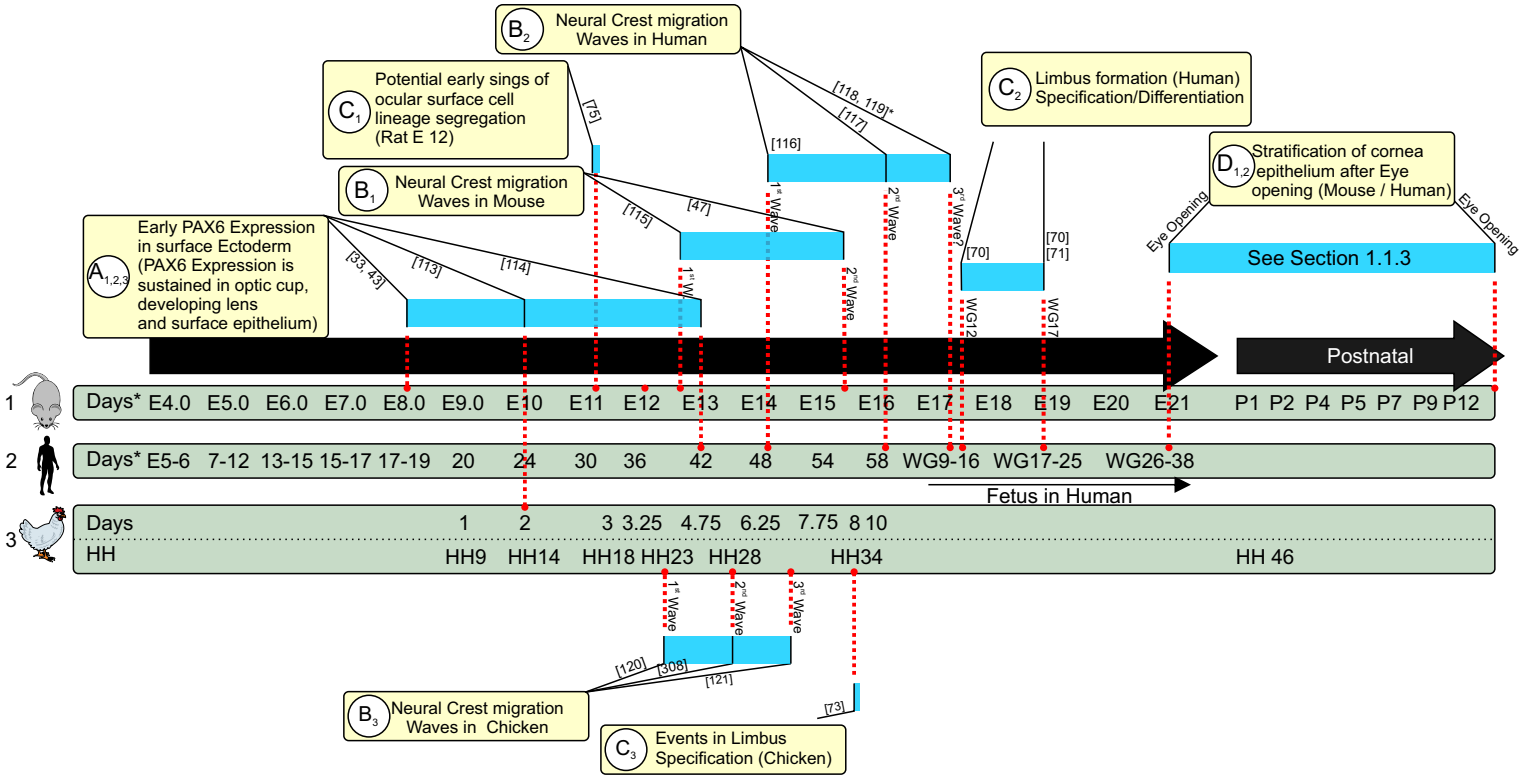

Figure 2: Aligned developmental stages of critical events in anterior eye development in several species.
* Embryonic days or weeks of gestation (WG). A1,2,3). Earliest expression of *PAX6* in surface ectoderm and neuroectoderm is further observed and sustained in several emerging structures such as optic vesicle, optic cup developing lens and surface epithelium [33, 43, 113, 114]. Note that given time points for each species showed the earliest observed expression in the cited studies. B) Neural crest migration is important for contributing to corneal structures such as endothelium and stroma as well as iris stroma. In mice two migration waves are observed and in avian and human there are three reported waves, although there are inconsistencies in the literature [44-47, 111]. B1) In mice neural crest cells increase at E13 [115]. In a second wave, mesenchymal cells migrate and contribute to stroma of the iris (E15.5) [47]. B2) In humans the first wave is in the 7th WG as no endothelial cells can be observed before [116]. In birds, the stroma is built up by mesenchymal cells migrating between epithelium and endothelium around the 8th WG [117]. Although there are descriptions of anterior chamber angle development, note that the migration path of these mesenchymal cells has not been addressed in those studies [118, 119]. B3) In chicken there are three waves of NCC. The first wave leads to formation of the endothelium [120]; the second wave develops into corneal keratocytes [120] and the third wave of NCC contributes to iris and mesodermal cells become distinguishable from endothelium at the seventh day [121]. C) The timing of limbus formation, specification and maturation at the molecular level is still enigmatic C1) Before keratin surface markers become distinguishable (See D), expression changes could lead to lineage segregation of corneal and conjunctival cells. In the rat, CX43 expression is lost at E12 and could explain a spatial separation of limbus and cornea epithelium [74]. C2) In the human, single cell analysis identified clusters of corneal, conjunctival and limbal cell lineages [70]. KRT15 starts to become restricted to limbal epithelium five weeks later at WG17 in humans [71]. At a similar timepoint, single cell analysis also identifies different stem cell and progenitor markers [70]. C3) In the chicken, a diffusion barrier is established in the limbal region before cell lines can be distinguished by cytokeratin expression changes. Interestingly, the limbal barrier is established prior to expression changes in CX43 [73]. D1,2) As apparent in the timeline, the changes in surface expression of keratins or integrins become clearer with further stratification. These processes occur much later than the first evidence of lineage segregation of conjunctiva, limbal and corneal epithelium (See Section 1.1.3). (Time points depicted in the graphics are obtained from cited literature. Stages are aligned with Carnie Stage comparison). Gestation Week (WG), E (Embryonic), P (Postnatal)

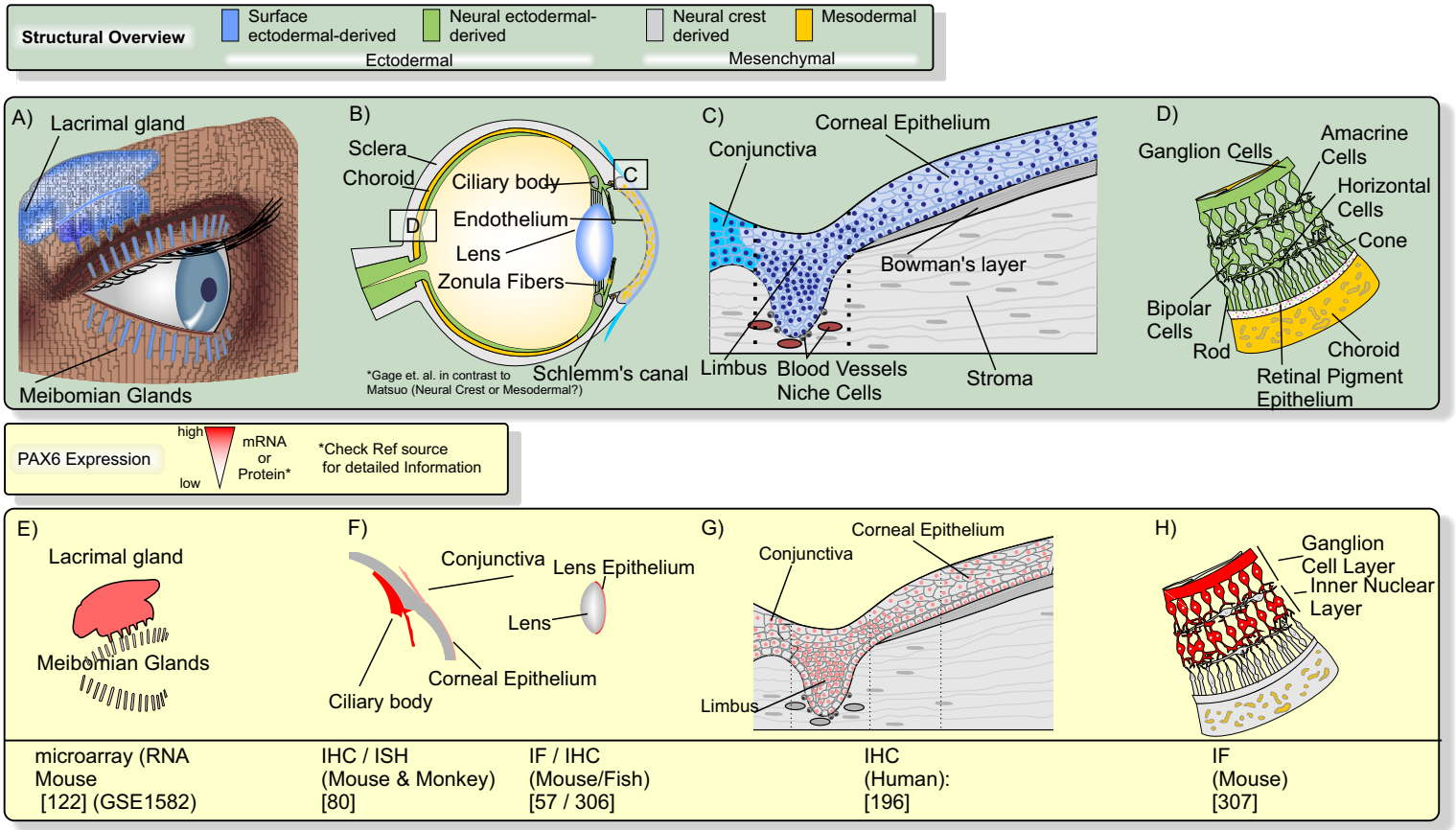

Figure 3: Postnatal PAX6 gene expression in humans.

The green Box (A-D) shows the different eye structures in human tissue. The color code marks the embryonic origin of these tissues. (Blue: surface ectodermal; Green: Neural ectodermal; Grey: Neural crest; Yellow: Mesodermal). The yellow box below shows the same ocular tissues which exhibit sustained *PAX6* expression during postnatal stages, thus likely also playing a role in adulthood. Note some Data from other model organisms are also transferred to the human structures shown here (E-H). There is evidence for *Pax6* expression in lacrimal and meibomian glands based on expression arrays [122] (A, E). *Pax6* is expressed in lens epithelium, ciliary body (B, F), limbal, corneal and conjunctival epithelium (C, G). In the adult retina, *Pax6* is expressed in the ganglion cell layer and the amacrine cells of the inner region of inner nuclear layer (D, H). Graphics freely adopted from different Internet sources.

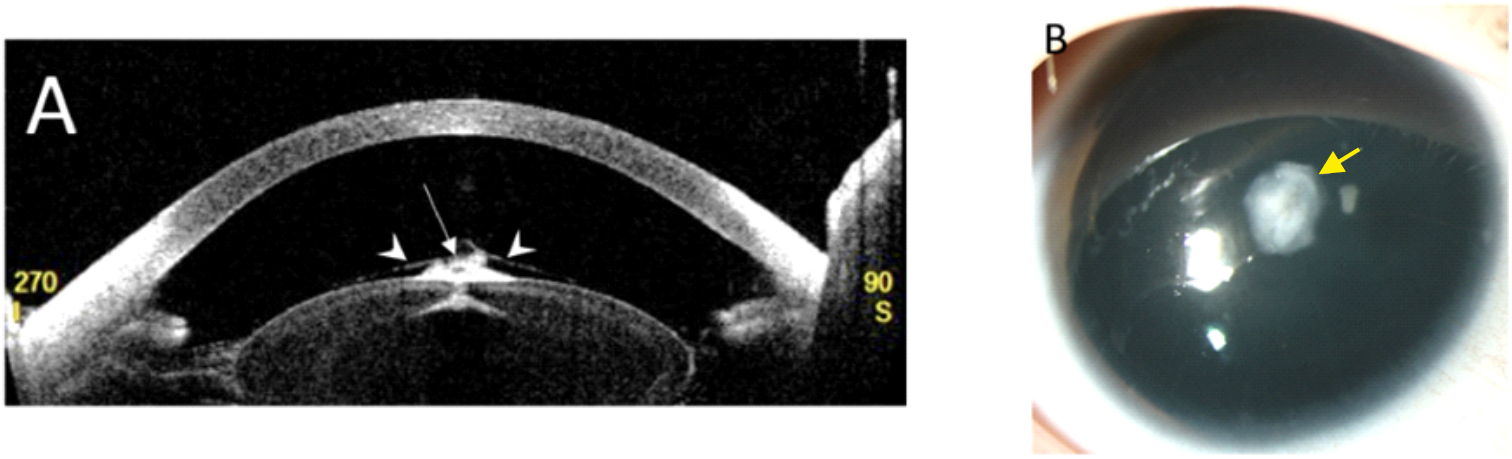

Figure 4: Right eye of a 10-year old female with aniridia and incomplete anterior chamber development. (A) Optical coherence tomography image of the lens epithelial congenital anomaly resulting in a fibrous structure protruding into the anterior chamber (arrow), along with iris remnants (arrowheads). (B) slit lamp photograph illustrating the superficial lens opacity in the center as a result of congenital fibrous structure (arrow). ads). (B) slit lamp photograph illustrating the superficial lens opacity in the center as a result of congenital fibrous structure (arrow).

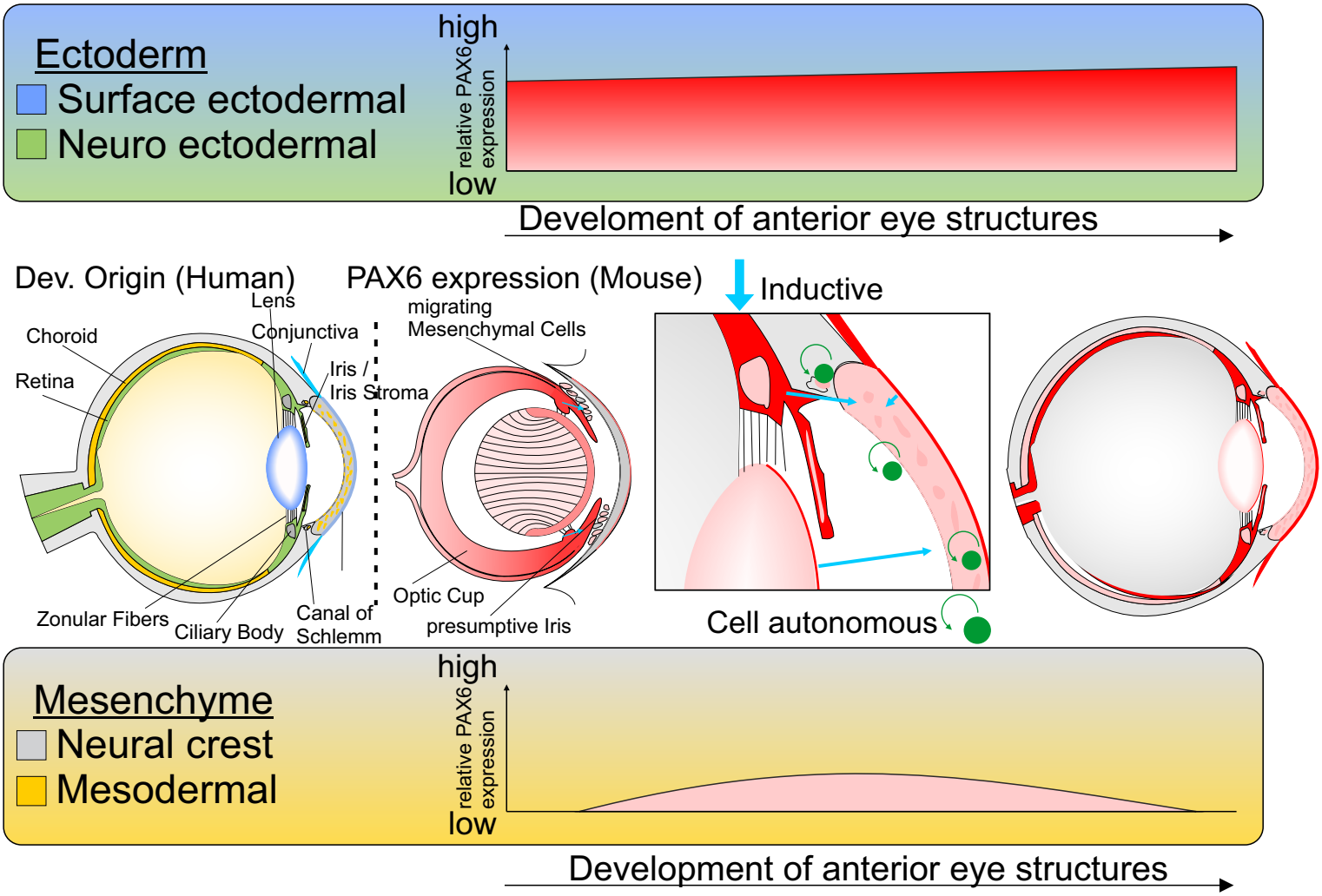

Figure 5: Sustained and transient PAX6 expression in different ocular tissues

Ectodermal-derived structures are indicated in blue and green color in the upper part of the figure. Their relative *PAX6* expression is high, illustrated by intensity of the red color. The mesenchymal derived cells (grey and yellow color) exhibit only a low or transient *PAX6* expression and contribute to corneal stroma, iris stroma, and ciliary body structures. *PAX6* function may be cell autonomous in the stroma, differentiation of trabecular meshwork and in the corneal endothelium (green circles). *PAX6* expression in ectodermal derived structures is high and continuous (green and blue colors in the left part of the central figure). This high expression is necessary for inductive events, for example for guidance of NCCs toward the cornea or anterior chamber angle (blue arrows) (See Review article Cvekl and Tamm [47] for further information). Note that the schematic images are based on both human structures (adult) and mice (development) and are mixed in this figure. *PAX6* expression is superimposed to human eye structures in the two images on the right. (adapted from Cvekl A and Tamm [47]).

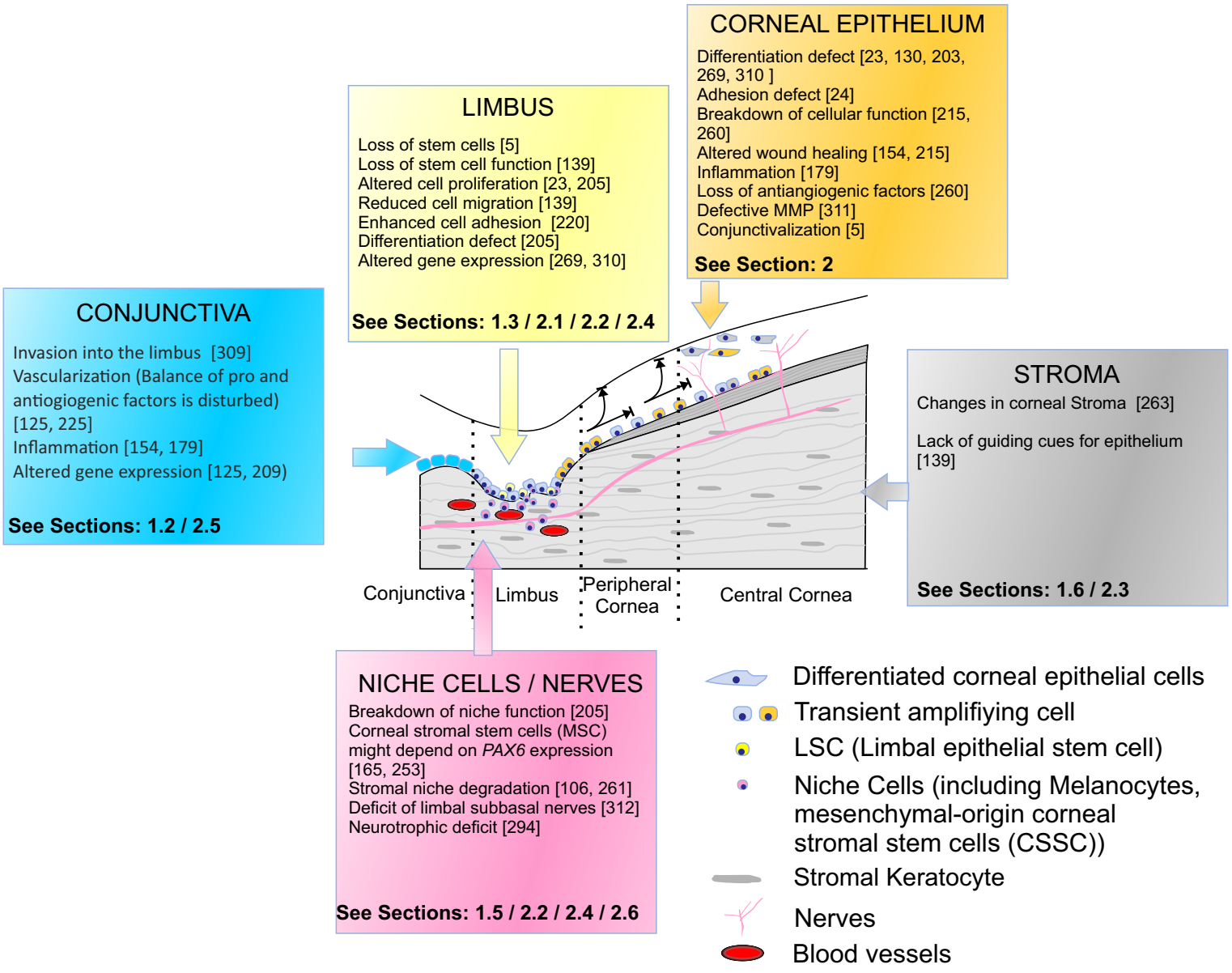

Figure 6: Molecular alterations responsible for AAK progression in animal models and humans.
The image is a compilation of in *vitro* and in *vivo* data of how observed alterations in the biological processes could lead to AAK if disturbed. The bottom of each box refers to Sections in the text where these hypotheses are further discussed.

Pathophysiology of aniridia-associated keratopathy: developmental aspects and unanswered questions

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Abstract

Aniridia, a rare congenital disease, is often characterized by a progressive, pronounced limbal insufficiency and ocular surface pathology termed aniridia-associated keratopathy (AAK). Due to the characteristics of AAK and its bilateral nature, clinical management is challenging and complicated by the multiple coexisting ocular and systemic morbidities in aniridia. Although it is primarily assumed that AAK originates from a congenital limbal stem cell deficiency, in recent years AAK and its pathogenesis has been questioned in the light of new evidence and a refined understanding of ocular development and the biology of limbal stem cells (LSCs) and their niche. Here, by consolidating and comparing the latest clinical and preclinical evidence, we discuss key unanswered questions regarding ocular developmental aspects crucial to AAK. We also highlight hypotheses on the potential role of LSCs and the ocular surface microenvironment in AAK. The insights thus gained lead to a greater appreciation for the role of developmental and cellular processes in the emergence of AAK. They also highlight areas for future research to enable a deeper understanding of aniridia, and thereby the potential to develop new treatments for this rare but blinding ocular surface disease.

Table contents

Introduction

Aniridia is a rare, pan-ocular, bilateral and congenital disease affecting the normal development and function of almost all eye structures. Aniridia is inherited in an autosomal dominant manner, with high penetrance and yet with variable phenotypic expressivity even within the same family. A variable degree of iris hypoplasia or total absence of iris is the hallmark of the disease. Over 90% of cases of aniridia involve haploinsufficiency of the *PAX6* gene, commonly due to heterozygous non-sense mutations on one copy of the gene. Well over 500 *PAX6* unique mutations have been identified in patients with familial or sporadic aniridia (http://LOVD.nl/PAX6) occurring in all exons and in non-coding regions of the gene. They include point mutations leading to amino acid substitution, deletions, insertions, premature termination, splicing defects or loss of the entire gene. Aniridia can occur either as an isolated malformation or as part of a syndrome such as WAGR (also known as WAGR complex, Wilms tumour-aniridia syndrome, aniridia-Wilms tumour syndrome) caused by large deletions that affect both *PAX6* and the adjacent *WT1* gene. WAGR is characterized by **W**ilms tumor, **A**niridia, **G**enitourinary anomalies and developmental delay (formerly 'mental **R**etardation') [1, 2]. Aniridia may also occur in Gillespie syndrome, caused by mutation in a different gene, *ITPR1*, consisting of aniridia, cerebellar ataxia and intellectual impairment [3]. Although nearly all P*AX6*-aniridia patients develop progressive opacification of the cornea termed aniridia-associated-keratopathy (AAK), glaucoma and cataract, the underlying molecular mechanisms and physiological causes of the many pathological features of the disease have not yet been elucidated. A main reason for this is that ocular development, maintenance and regeneration involve crosstalk between different tissues, and complex interactions with the immune, nervous, and metabolic systems.

AAK, also sometimes termed aniridia-related keratopathy (ARK) or aniridic keratopathy, is a highly prevalent condition in *PAX6*-heterozygotes that is potentially painful and severely limits functional vision [4]. As the cornea is readily accessible and can be partly or fully replaced, AAK is a prime target for therapies to improve vision in aniridia. Even a small reduction in the severity of AAK can translate into significant benefits in vision and ocular surface symptoms. Moreover, AAK is progressive, so it is worthwhile to concentrate efforts into understanding its pathogenesis and pathophysiology, because the potential may exist for slowing, altering, or even arresting its progression in younger subjects.

Symptoms of AAK include breakdown of the corneal surface, with epithelial thinning or loss, inflammation with immune cell infiltration, vascularisation and chronic progressive opactification. These symptoms overlap partly or wholly with those that arise when the stem cells at the periphery of the cornea – limbal stem cells (LSCs) – are deficient due to disease or injury. Traditionally, it has been widely believed that AAK is a consequence of a progressive limbal stem cell deficiency (LSCD) [5, 6], although to date, there is no definitive proof of loss or degradation of limbal stem cells (LSCs) or their niche as the causal event triggering AAK. Given the complexity of the pathology present in AAK [7], it is more likely that *PAX6* controls multiple physiological and biological factors that act together, and that their dysregulation in aniridia leads to AAK development.

Identifying the possible underlying pathogenic mechanisms leading to AAK development and progression holds the potential for establishing new therapeutic options for the single greatest unmet need of patients severely affected by aniridia. In this review, we highlight key questions of importance – many still unresolved – concerning developmental aspects and the emergence of AAK. We show that to regard AAK as purely a limbal stem cell deficiency is to ignore the multiple developmental and pathogenic events in aniridic eyes, affecting multiple tissues, that may contribute to the onset or progression of the disease. We highlight some potential therapeutic strategies that may arise from a fuller understanding of the developmental basis of

AAK. The topics discussed are also relevant more generally to diverse types of environmental or congenital corneal pathologies that involve LSCD, corneal neovascularization and opacification. Finally, we present recent findings on translation of results from animal models to humans as a critical step in developing future therapies to treat AAK and understanding their relevance to other corneal diseases.

1 Are developmental deficiencies involved in the emergence of AAK?

PAX6[1] codes for a key transcription factor that is essential for eye development and maintenance. *PAX6* is expressed at the earliest stages of eye development and in multiple eye tissues throughout life. Several *Pax6*-heterozygous mutant murine strains (collectively 'small eye' or 'Sey' mice) have been characterized and used as *in vivo* models of aniridia, to study the roles of the gene and the developmental and pathological aspects of the disorder [8]. The mouse models represent a spectrum of mutations. Some such as the Harwell allele $Pax6^{Sey\text{-}H}$ and 'Dickies small eye' $Pax6^{Sey\text{-}Dey}$ are large deletions affecting *Pax6* and surrounding genes, and show severe phenotypes that are perhaps not ideal for studying human aniridia and AAK [9-12]. Others, including $Pax6^{Sey}$ (=$Pax6^{SeyMH}$), $Pax6^{Sey\text{-}Neu}$ (=$Pax6^{Sey\text{-}Neu1}$), $Pax6^{ADD4802}$, $Pax6^{Coop}$, $Pax6^{Aey11}$, $Pax6^{AEY18}$ and the allelic series $Pax6^{2Neu}$ to $Pax6^{10Neu}$ and $Pax6^{Leca1–4}$ include point mutations leading to premature termination, frameshifts or splice defects [9, 13-16]. There are also engineered deletions ($Pax6^{LacZ}$) and floxed alleles ($Pax6^{flox}$) that yield large deletions upon action of Cre recombinase [17-19]. With the exception of some hypomorph alleles ($Pax6^{4Neu}$, $Pax6^{7Neu}$ and $Pax6^{Coop}$) and a gain of function ($Pax6^{ADD4802}$), most alleles listed above are thought or known on the basis of nonsense-mediated RNA decay, phenotypes, and/or allelic complementation studies, to be null for *Pax6*. Unless stated otherwise, all *Pax6* mouse mutants discussed below represent null alleles.

Pax6-knockout results in early failure in lens placode development and anophthalmia (eye absence) but heterozygotes display a phenotype that resembles human aniridia, including a progressive AAK [14, 28]. Although the phenotype of *Pax6* mouse mutants is affected by genetic background and there is individual variation in severity even within litters, overall they are remarkably consistent models of AAK. Concordance with human aniridia, however, is not complete. For example, $Pax6^{+/-}$ mice exhibit microphthalmia with a 10% reduction in eye diameter and reduced lens size; while microphthalmia can also occur in human aniridia, most patients have a relatively normal eye and lens size [20]. The extent to which microphthalmia in mice may modulate the anterior segment dysgenesis associated with *Pax6* is not known, but it should be noted that the transgenic 'PAX77' mouse that overexpresses 5-6 copies of human *PAX6* exhibits microphthalmia (including microcornea) without an AAK phenotype [21, 22].

Typically, the *Pax6* heterozygous newborn mice exhibit morphological alterations in the corneal epithelium. Separation of the lens from the cornea during development is delayed in *Pax6*-mutant mice and iridocorneal and/or lens-cornea adhesions similar to Peters' anomaly may be observed at birth, dependent on mouse strain and the *Pax6* allele [23-26]. The gross abnormalities these mice can exhibit at birth are small eyes and/or an opacity of the central cornea due to delayed or failed detachment of the lens from the cornea. In contrast to the murine models, the ocular surface of the majority of aniridia patients does not exhibit any apparent gross abnormality at birth. Exceptionally, rare cases of Peters anomaly are caused by *PAX6* mutations [27] but in nearly all cases of congenital aniridia, a proper separation of the cornea and lens is observed [28]. Despite a seemingly normal cornea observed early in life, however,

[1] Human nomenclature is used whenever applicable. If findings are species-related the gene/protein nomenclature of the species is used according to the literature source.

closer inspection of the central cornea in a 4-year-old children with congenital aniridia indicated reduced sensitivity to mechanical touch, reduced sub-basal nerve density and elevated presence of antigen-presenting dendritic cells [29]. Moreover, the corneal thickness is known to be pathologically increased in aniridia, even in childhood [29]. As outlined below, *PAX6* is expressed in many eye structures during development (section 1.1). Therefore, it is probable that AAK is at least partly influenced by developmental defects and not solely due to postnatal emergence of pathological corneal function. AAK penetrance is full, but its phenotypic expressivity is highly variable between individuals, even between siblings [30]. This suggests that strong environmental and stochastic components, and/or modifier genes that act in concert with *PAX6* and vary between individuals can influence the expression of the disease. This could also be true for epigenetic differences between individuals that may modulate AAK severity. It would moreover be important to understand how the different eye structures influence each other during corneal development, and the role of PAX6 levels in influencing the onset and severity of AAK.

PAX6/*Pax6*, the mammalian orthologue of the Eyeless gene (Ey) in Drosophila, is a paired and homeodomain transcription factor which is essential for eye development. The activity of PAX6 in eye development is evolutionarily conserved as the human or mouse *Pax6* genes can induce ectopic formation of the compound eye from Drosophila imaginal discs as well as in Xenopus embryos [31, 32]. *PAX6*, however is also important for brain, gut and pancreas development [33-35]; see review [36]. This multiple organ involvement partially explains why haploinsufficiency of *PAX6* causes in adults, not only aniridia phenotype, but also several non-ocular conditions such as obesity, glucose intolerance and diabetes, and anosmia [37, 38]. Sleep disorders are also reported in patients and *PAX6* may impact brain structures such as the pineal gland [39].

The importance of *PAX6* for different structures of the eye in particular is highlighted by the fact that aniridia patients suffer from multiple eye abnormalities in both anterior and posterior segments of the eye. *PAX6* is expressed in the multiple ocular cell types from the earliest stages of eye development and throughout life (***Figure 1-3***). The exact role of PAX6 in lens and optic cup derivatives has been systematically investigated using conditional mutagenesis (reviewed in [40]). By contrast, the impact of *Pax6* on corneal development continues to be further explored, requiring efficient genetic deletion in the various corneal cell types [41].

1.1 How is the anterior segment of the eye formed?

In vertebrate eye development, *Pax6* expression is detected in the anterior neural plate in the eye field region [42] and subsequently in both the neuroectoderm and surface ectoderm progenitors of the eye [18, 33, 42, 43]. The surface ectoderm gives rise to the lens and corneal epithelial layers (lens, limbus and corneal epithelium), while the neuroectoderm populates the optic vesicles that undergo morphogenesis to form the optic cups. The outer layer of the optic cups is populated by the retinal pigmented epithelium progenitors (RPE), while the inner layers of the optic cup differentiate to form all of the retinal neurons and the Müller glia cells. The anterior optic cup rim gives rise to the iris and ciliary body pigmented epithelia ***Figure 1***). The ocular mesenchyme surrounding the optic cup rim eventually contributes to the iris and corneal stroma. The high and continuous expression of *Pax6* in cells that derive from surface ectoderm and optic cup (lens, corneal epithelium, iris and ciliary epithelium) is required for the expression of genes encoding transcription factors, structural and signaling molecules, which are critical for the morphogenesis and differentiation of the neuronal, pigmented and the transparent cornea and lens eye lineages. The role of Pax6 in each of these structures and the signaling cues that

mediate their coordinated development during the formation of the anterior segment of the eye, as relevant to the onset of AAK, is briefly summarized in the next section.

The anterior segment of the eye includes the cornea, conjunctiva anterior chamber, iris, lens and associated structures. In development of these structures, the neural and surface ectodermal cells interact with mesenchymal cells of neural crest and mesodermal origin. These various and complex interactions are briefly described below. For a detailed description of the associated processes, see reviews [44-47]. The putative influence of PAX6 dosage on these different processes in development of the corneal phenotype is discussed in separate sections.

1.1.1 Early development – Surface ectoderm and optic vesicle

Morphologically, the development of the eye is evident with the formation of the optic vesicles.

The optic vesicles are *PAX6*-expressing bilateral evaginations from the diencephalon that give rise to the optic cup through morphogenesis (reviewed in [48, 49]). The optic cup interacts with surface ectoderm, lens and migrating mesenchymal cells. Disruption of developmental processes has been described by manipulating signaling pathways. A saucer-shaped optic cup, ventral coloboma, or a deficiency of periocular mesenchyme were observed by manipulating either Wnt-, Lrp6-, or retinoic acid signaling [50]. The peripheral rim of the optic cup contain progenitors that will give rise, during post natal stages to the pigmented and non-pigmented layers of the iris and ciliary body (reviewed in [51]). Wnt ligands and BMP signaling from the surface ectoderm elicit Wnt/GSK3β-response in retinal pigment epithelium progenitors (RPE) and are crucial for production of the correct number of RPE cells and proper curvature of the optic cup [50, 52].

1.1.2 Lens

As soon as the optic vesicle forms, in mice at E8.5, *Pax6* is expressed in neuronal and surface ectoderm progenitors of the eye (See E8.5 ***Figure 1***). The lens derives from Pax6-expressing lens-competent facial ectoderm which is contacted by the optic vesicle [53].

After the lens placode has been induced, it invaginates to form the lens vesicle. The detailed mechanisms of lens induction and genetic and signaling networks are reviewed in [54]. In the lens and optic cup, *Pax6* expression is specified independently, by cis-regulatory elements [40, 54]. *Pax6* autoregulates its own expression and the *Pax6* surface ectodermal enhancer element driving PAX6 autoregulation interacts with another transcription factor, SOX2 [55]. *Sox2* expression is induced in the surface ectoderm upon an inductive signal from the optic vesicle and determines lens placode formation (See E9.5 ***Figure 1***) [56].

The cells from the anterior pole of the lens vesicle give rise to the lens epithelial cells, whereas the posterior cells differentiate into lens fiber cells. PAX6 continues to be expressed during lens invagination and differentiation [19, 57] as reviewed by Cvekl A *et al.*[58] and is maintained in the lens epithelium throughout life.

1.1.3 Corneal Stroma and endothelium

After the lens, optic cup and ocular surface ectoderm have been specified, neural crest cells and mesodermal cells migrate between these structures. The corneal stroma is formed by neural crest-derived cells from the periocular mesenchyme, a population of mesenchymal cells located

near the optic cup and presumptive lens. Fate mapping of mesoderm-derived cells showed a contribution to the corneal endothelium and stroma [59, 60].

In humans (and birds), three waves of neural crest cells (NCCs) migration are reported in contrast to two waves in mice (Note that Figure 1 refers to mouse development). A first wave of NCCs migrate between the lens and the surface ectoderm to form the corneal endothelium. A second wave forms the stromal keratocytes. The third wave of NCCs contributes to the ciliary body and iris structures (see Section 1.1.4). In mice a single wave of neural crest migration gives rise to both endothelium and stroma.

Studies isolating primary stromal cells from transgenic mouse corneas revealed similarities in mRNA expression profiles (*Twist*, *snail, Slug and Sox9*) between neural crest-derived precursors and the isolated corneal precursor cells. This finding was regarded as evidence for a neural crest origin of these cells, which are important for the turnover of stromal tissue [61].

The corneal endothelium provides an important pump function, actively maintaining a fluid and electrolyte balance between the anterior chamber and corneal stroma to prevent corneal swelling, thus maintaining corneal transparency. The cellular density of this single endothelial cell layer is critical for maintaining an adequate pump function. From a histologic analysis of human fetuses, it has been shown that the cellularity of the endothelium rapidly decreases in the prenatal period from 16 weeks of gestation to term, at the same time the cornea grows in size [62]. The reduction in endothelial cell density during this period is about 50%, while the density reduces further by a third during the first two years of life. A putative effect of PAX6 levels on stromal, limbal stromal and endothelial development and how this could influence AAK is discussed in Section 1.6.

1.1.4 Iris and anterior chamber angle

The anterior chamber angle is the angle between the iris and the corneal endothelium in the limbal region. The iridocorneal angle contains important aqueous humor drainage structures such as the trabecular meshwork and Schlemm's canal.

The contiguous iris and ciliary body epithelia derive from the rim of the optic cup while the stromal layers derive from the ocular mesenchyme [26]. A *Pax6* expression gradient is observed in the optic cup with the highest level from the distal (close to the lens) to proximal side (close to the optic nerve [33] (Figure 1, E 15.5).

For the iridocorneal angle, lineage tracing experiments in mice revealed the contribution of NCCs to the ciliary muscles, ciliary blood vessels, anterior iris, trabecular meshwork and Schlemm's canal in the iridocorneal angle [59, 63, 64]. Mesodermal cells also contribute to structures such as the lining of Schlemm's canal and the iris stroma, but not to the ciliary muscles [59]. Pax6 may participate in regulation of the factors required for the migration of NCC into the eye [65] (see also reviews on neural crest of the eye [44, 66]).

In mouse embryos (E 15.5), iris and ciliary body progenitor cells can be molecularly distinguished from cells which form the presumptive neural retina. Thus the proper development of the iris relies on the correct compartmentalization of the optic cup (See review in [26] for molecular details).

Although the molecular mechanism responsible for the normal development of the iridocorneal angle has not yet been elucidated, a series of developmental steps are described in the mouse where final maturation of Schlemm's canal and the trabecular meshwork extends postnatally to P42 [67]. In humans, the developmental processes are similar, with all rudimentary structures developed at birth [68]. Further maturation and reorganization take place, likely due to mechanical stress and aqueous humor flow and this process could last 1-8 years postnatally [69].

The impact of changing PAX6 levels on anterior chamber development and possible impact on the ocular surface is discussed in Section 1.5.

1.1.5 Limbus and limbal stem cells

After the lens has formed, the PAX6-positive cells of the surface ectoderm segregate and give rise to the lineages of the anterior ocular surface epithelia, including conjunctival and limbal/corneal lineages [19] (***Figure 1***, E8.5-E15.5).). In humans (but not mice), specialized anatomical structures, the 'palisades of Vogt', develop at the limbus – the boundary ring around the periphery of the cornea where the stem cells reside in adult life. The developmental aspects of their formation is of utmost interest, as they are important for stem cell homeostasis and are affected in AAK. To make the overview easier to understand, a comparison of the key events of the anterior chamber development in different animal models is shown (***Figure* 2**).

At 8.5 WG (week of gestation), the human fetal cornea is still continuous with the surface ectoderm. At 12-22 WG, in turn, individual components such as the conjunctiva, cornea and limbus can be distinguished by gene expression [70]. In 12 WG fetal corneas, the presumptive limbus is observed as 'ridge like' feature or a 'dimpling' in the epithelium [71, 72]. In human fetal corneas, the palisades of Vogt are not yet detected and are probably formed postnatally. During 8-22 WG, the limbal epithelium starts to become thicker than in the surrounding cornea and conjunctiva. Single cell RNA-seq studies of the developing human cornea indicate the presence of a proliferating epithelial progenitor cluster with highly expressed corneal epithelial stem and progenitor markers *TP63, CLDN1, CLDN4* and *TXNIP* at 18 WG, indicating the first presence of "a peripheral limbal-like region" harboring the limbal stem and/or progenitor cells [70].

Due to the lack of detailed molecular and mechanistic studies of cell fate determination in the mammalian limbus, corneal epithelium and conjunctiva, here we refer also to functional studies done in chickens, to elucidate the time points when the cornea and conjunctiva become spatially separated by a limbus.

In the chickens, a diffusion barrier between the corneal epithelium and conjunctiva is established at embryonic day 8 (See ***Figure 2*** for species comparison) [73]. This observation is consistent with recent transcriptional data from the developing human cornea at 12 WG, where there are two separate clusters of ocular surface and conjunctival epithelial cells [70]. This separation in chicken may be achieved through the differential expression of a connexin, CX43, or in response to other events allowing differential responses to, as yet unknown, inductive signals [73-75]. CX43 is present in the conjunctiva and is also strongly expressed in the corneal epithelium, but is absent at the limbal border region. This feature is thought to isolate limbal cells from signals in the surrounding tissues and to play a role in their maintenance in an undifferentiated state [73]. The detailed signaling pathways underlying these processes, however, remain to be further elucidated. Interestingly, several markers of adult LSCs are initially expressed throughout the entire corneal epithelium and it is only after stratification that their labeling pattern becomes restricted to the limbal epithelium. In common with other adult stem cell systems, it thus appears that the adult stem cells are a spatially restricted subpopulation of a progenitor population that is specified during embryogenesis. Although the limbus is specified prenatally, there may be some overlap in stratification of corneal epithelium and further maturation of limbal structures. Thus, it could be hypothesized that signals from the stratifying corneal epithelium are also necessary for further maturation of the limbus (See Section 1.3), as could influence underlying corneal stromal cells (See Section 1.6).

1.1.6 Specification of conjunctival-, corneal and limbal epithelia

Despite their proximity within a contiguous ocular surface epithelium and their common and persistent expression of *Pax6* during development and throughout life, the corneal epithelial cells and the conjunctival epithelial cells belong to two distinct lineages [76, 77] arising from different populations [78]. These two lineages arise simultaneously from *Pax6* positive ectodermal cells that remain on the embryonic ectodermal surface of the developing eye once the lens vesicle has formed [79, 80].

In vivo studies with ocular epithelial cells isolated from rabbits and transplanted into mice have shown that limbal and corneal epithelial cell-derived cysts contained only stratified squamous-type epithelial cells. In contrast, conjunctival epithelial cell-derived cysts contained stratified columnar-type epithelial cells interspersed with Periodic Acid Schiff (PAS) staining-positive cells with goblet-like structure [78]. Despite the fact that such isolated cells might not contain stem cells or may be influenced by differentiated co-transplanted cells, such findings would support the hypothesis that corneal and limbal epithelial cells originate from a different embryonic lineage than conjunctival epithelial cells, and that goblet cells originate from the conjunctival compartment and not following external modulation, as originally proposed [78]. Nevertheless, lineage tracing of these hypothesized progenitor populations will be needed to unequivocally address this issue in wild type mice and furthermore, explore the ocular lineage specification in *PAX6*-mutated mice.

In spite of their importance, relatively little is known about the factors regulating conjunctival goblet cell development. Conjunctival epithelial cells and goblet cells derive from a common bipotent progenitor [81, 82]. SAM Pointed Domain-Containing ETS Transcription Factor (SPDEF) has been described as a crucial transcription factor for goblet cell differentiation [83]. Goblet cell differentiation and mucin secretion appear to be directly related to the eyelid opening. In humans, the eyelids are fused until the 5^{th}-6^{th} month of intrauterine life, and goblet cells appear in the fornix extending toward the palpebral and bulbar regions from the 8^{th} to 9^{th} week of gestational age [84, 85]. Studies are currently underway to evaluate when the neural regulation of goblet cell secretion becomes functional [86] and the hierarchical network of transcription factors regulating goblet cell development in healthy and pathophysiological conditions (reviewed in [87]).

Some signals which prevent the conjunctival phenotype in the central cornea epithelium are known [88, 89]. The extent of *PAX6* involvement in these processes will be discussed further in Section 1.2. In contrast to the poorly known mechanisms determining cell fates, the tissue specific markers (mostly cytokeratins) for corneal limbal and conjunctival epithelia are well described, although species-specific differences in some of these markers exist.

The conjunctival, limbal and corneal epithelium are composed of one to two cell layers before eyelid opening, after which the thickness of these cell layers increases to 4 – 5 cell layers (see **Figure 2** for animal model comparison). Cells become stratified and distinguishable by their morphology, with basal cells having a cuboidal shape and being attached to the epithelial basement membrane. The intermediate wing cells (anterior to the basal cells) are present, and are in turn covered by flattened superficial squamous epithelial cells. In the fetus, only minor keratin expression is visible in the superficial shedding cells [72, 90]. These processes are similar in different model organisms and have been previously summarized [91].

Early murine corneal epithelial differentiation takes place at embryonic day 15 (E15), as expression of the Pax6-target gene *Krt12* becomes apparent and is a specific marker of the corneal epithelium throughout life [92, 93]. This is likely also true in humans, as the neutral

counterpart of KRT12, KRT3, becomes visible in human fetal corneas from 12 WG to 17 WG in superficial cells. By 20 WG, KRT3 can be detected in the superficial cells of limbus and cornea, similar to the adult cornea [71]. Notably, the sequential appearance of the KRT3 and KRT12 pair is different in chicken and rabbit animal models [94].

In mice the developing limbus matures upon corneal epithelial stratification that is associated with eyelid opening around postnatal day 10-14 (P10-14). KRT12 is expressed in the superficial epithelial cells and extends to all suprabasal cells and in later embryonic stages to all layers of the epithelium upon maturation [95, 96]. The corneal epithelium continues to mature up to 3 to 6 months postnatally [92]. Transcriptional changes in the corneal epithelium upon eye opening in mice, which are similar to humans, have been summarized [91].

One week after birth, α9 integrin, which marks the limbal region in humans and in mice [97], is equally distributed across the entire corneal epithelium and becomes restricted to the limbus in mice and rats at 8 weeks postnatally [90, 98]. Similarly, keratins can be used to observe limbal maturation. KRT19 is found to be expressed throughout the entire murine corneal epithelium prior to eyelid opening, however after stratification, the expression of this keratin becomes restricted to the mouse limbal epithelium [99]. KRT19 is also reported to be concentrated in human limbal epithelium but species differences during maturation of corneal epithelium are likely [100].

In early human development (8-14 WG), KRT15 is expressed across the entire ocular surface including conjunctiva, limbal and corneal epithelia, but becomes restricted to the limbal epithelium from 17 WG, prior to eye opening (See ***Figure 2*** C_2) [71].

These data seem to suggest that, before stratification of the ocular surface epithelium, stem cells are not restricted to the limbus but rather distributed throughout the entire limbal/corneal epithelium. (This would also point to the important role of stromal stem cell niche in maintaining stem cell capacity in adults as described in 2.1 **Controversy on limbal stem cells**). Additional support for this hypothesis came from the use of a X-chromosomal *LacZ* transgene, which allows random and irreversible labeling of embryonic blastocyst cells in female mice based on X-chromosome inactivation mosaicism at early embryonic blastocyst stage. Interestingly, in the first 3-4 weeks after birth, a disorganized mosaic pattern of $LacZ^+$-labelled patches of cells appeared dispersed throughout the entire limbal/corneal epithelia. .However, from P30 onward, a typical radial stripe pattern of cell migration into the cornea became evident as the stem cells became restricted to the limbus [101]. These elegant studies [101, 102] together with other studies mentioned above, indicate that the corneal epithelium is self-sustained by its own pool of stem cells, these stem cells probably differentiate before P30 and from that stage, the limbus becomes the unique stem cell location. Notably, the original 'XLacZ' mouse model has limitations due to the fact that tracing is of blastocyst-stage (E3-4) cells and because it does not allow cell type-specific tracing in a clonal, temporal manner by vital microscopy. However, advanced quantitative lineage tracing studies has further shown that this concept is correct, revealing additional aspects of limbal stem cell biology (discussed in section 2.1 below, [103-108])

1.1.7 Corneal nerves

In mice, branching nerve bundles cover the entire corneal stroma by E16.5. The corneal epithelium is first innervated at E16.5 and nerves subsequently form a swirl pattern in the subbasal nerve plexus at about three weeks postnatally [109]. The ophthalmic nerves arise from the trigeminal ganglion which is both derived from neural crest and ectodermal cells (see the review in [45]. At least in chickens, the cornea is innervated solely by the neural crest derived neurons of the trigeminal ganglion [110].

1.2 Could disturbed segregation and separation of corneal and conjunctival cell lineages contribute to AAK?

Lineage segregation of corneal epithelium from the conjunctiva in humans appears after sustained *PAX6* induction at the time of surface ectoderm specification. In mice, PAX6 is found in the ocular surface ectoderm at E8, and in humans at embryonic day 42 (***Figure 2***). Altered *PAX6* levels in the corneal and conjunctival epithelium during development could impact signals required to define borders and self-maintenance of corneal and conjunctival tissue identity. Deeper understanding of conjunctival and corneal differentiation is therefore essential to distinguish between the developmental and postnatal role of *PAX6*. In the mouse, the regulatory network of *Pitx2* and downstream *Dkk2* expressed in the mesenchyme inhibits Wnt/β-catenin signaling resulting in the inhibition of the conjunctival fate in the central cornea [88, 89, 123]. PITX2 is also described to integrate retinoic acid (RA) signaling from the surface ectoderm, optic cup and lens. Since RA signaling is altered in $Pax6^{Sey/Sey}$ ($Pax6^{-/-}$) mouse eyes [124], this could affect the crosstalk between the periocular mesenchyme and the surface ectoderm. In addition, transcriptional analysis in human subjects with aniridia indicates that RA metabolism could be altered in the conjunctival cells due to *PAX6* mutation [125], but the mechanism requires further confirmation.

Whether PAX6 protein levels could impact proper segregation of corneal and conjunctival tissue during development, and therefore potentially influence the development of AAK, remains unknown; however, recent studies are beginning to address this question. A recent single-cell RNA-seq analysis of human corneal development [70] encompassing 12-23 weeks of gestation indicated low overall *PAX6* expression throughout the developing cornea; however, in all cases the highest expression was observed in the epithelial layer. The same was also true for the adult cornea with highest *PAX6* expression observed in the corneal and conjunctival epithelium. Since several cell populations can be now identified by their transcriptional profile, it is now possible to investigate the extent to which *PAX6* is expressed in these cell clusters or if some cells in these clusters exhibit higher or transient PAX6 expression. Another recent study has reported postnatal modification of PAX6 dosage in the $Pax6^{Sey\text{-}Neu/+}$ ($Pax6^{+/-}$) mouse model by inhibiting the mitogen-activated protein kinase kinase (MEK) pathway [126]. In that study, pharmacologic MEK inhibition by ocular or systemic routes increased PAX6 protein expression in the basal epithelial layers to normal levels as in wild-type mice, resulting in restoration of corneal anatomy and transparency to a normal phenotype. This result provides evidence for a PAX6-dependent role in epithelial cell specification, even postnatally.

1.3 Does the limbus of AAK patients exhibit developmental defects?

Similarities between aniridia syndromes and LSCD may suggest that epithelial defects in AAK are the result of LSC failure. However, since pathological changes in aniridia are also observed in the nerves, inflammatory cells and corneal epithelium early in life, even before the limbus becomes overtly affected [29], it suggests that multiple pathological mechanisms may be involved and may precede and/or promote LSC insufficiency.

The exact location and differentiation characteristics of LSCs or their precursors during human development is not well studied at the molecular level, especially the postnatal formation of limbus niche structures which differ from those observed in mice [127]. It has been previously hypothesized that the delayed formation of the limbus could be a cause for the late onset of AAK in aniridia [71].

The ΔN isoform of Tp63α (ΔNp63α) is considered as a LSC marker [128] and as a master inducer needed for the progression from an embryonic ectodermal monolayer epithelium into a dynamic, stratified epithelium found in the adult stage (e.g. in the corneal epithelium and epidermis) [129]. *PAX6* expression occurs developmentally before the expression of *TP63* [130, 131]. In contrast to *PAX6* that is widely expressed in eye tissues, *TP63* expression is limited to the ocular surface epithelia (conjunctiva, limbus, cornea) and is associated with ocular glands (lacrimal and meibomian) [132, 133]. Mutations in the *TP63* gene lead to Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate (EEC) and Ankyloblepharon-Ectodermal defects-Cleft lip/palate (AEC) syndromes, two syndromes with numerous tissues affected. These syndromes can also be associated with LSCD [134]. TP63 was described to be equally expressed in the fetal limbal and central cornea [71]. In adult humans, *TP63* expression is restricted to the limbus, and positive staining is only detected in central corneal epithelium when the corneal epithelium is regenerating after wounding [71, 135].

The occurrence of limbal stem cell dysgenesis shows that stem cells are required for corneal maintenance, however identifying the LSCs themselves is challenging [136]. Two recent single-cell RNA-seq studies led to the identification of glycoprotein hormone subunit alpha 2 (GPHA2) as a novel marker of an outer population of limbal stem cells. GPHA2, is a largely unexplored gene whose function appears to be essential for LSC self-renewal and differentiation [70, 107]. *GPHA2*-overexpressing transgenic animals showed no gross phenotype alterations [137], but it would be interesting to examine the phenotype of these mice and generate a knockout mouse strain. GPHA2 expression was dramatically reduced to barely detectable levels following cultivation of human LSCs and in immunodeficient mice. This suggests that GPHA2 may be regulated by T cells critical for the adaptive immune response, and that may serve as an important contributor to the LSC niche [70, 107]. Noticeably, like the putative LSC marker KRT15, GPHA2 is not only expressed by basal limbal epithelial cells (i.e. LSCs), but it is also occasionally detected in limbal supra-basal cells [107], and therefore was also proposed to mark limbal committed or differentiated cells [138].

The processes, however, responsible for creating the limbal niche structure secretion of its basement membrane, as well as association and recruitment of niche cells, are not sufficiently understood. It would be of interest to determine whether *GPHA2* expression is altered in AAK patients and/or in animal models of aniridia, to yield further evidence for the loss of LSC function, as is widely believed. In addition, lineage tracing in $Pax6^{+/-}$ mice enabled the detection of pathogenic mechanisms associated with aniridia and the LSCs in light of developmental processes. For example, $Pax6^{+/-}$ LSCs in $Pax6^{+/-} \leftrightarrow Pax6^{+/+}$ chimeric mice are functional and produce streams of epithelial cells that migrate normally into the cornea, although these progeny are less likely to reach the center of the cornea than wild-type cells [139]. This suggests that dosage deficiency of Pax6 does not preclude normal specification of LSCs in a cell-autonomous manner. It remains unknown, however, if the relatively normal behavior of $Pax6^{+/-}$ cells in the chimeric mouse limbus represents a non-autonomous 'rescue' by secreted protein factors such as GPHA2 released from the wild-type cells.

1.4 Does lens development impact AAK?

In contrast to aniridia patients, $Pax6^{Sey/+}$ ($Pax6^{+/-}$) mice have a more prominent anterior segment dysplasia and the lens often remains attached to the cornea. The mouse lens is larger in proportion to the rest of the eye, as compared to the human eye. This could result in a more severe lens and corneal phenotype in the mouse compared to the human eye [11]. In the chicken, it was shown that surgical removal of the lens affects multiple eye developmental processes including eye growth, and inhibits normal development of the peripheral retina, ciliary body,

iris, and migration of NCC into the cornea [140]. The corneal cell fate of surface ectoderm is stabilized by NCC migrating in the lens peripheral ectoderm. The formed stroma prevents PAX6 downregulation in the corneal epithelium. [141].

The developing lens is a key signaling center during eye development. During formation of the lens placode, ligands of Wnt, BMP and retinoic acid secreted from the surface ectoderm play a role in patterning of the optic cup [50, 52]. Surgical removal of the lens from developing chicken eyes leads to downregulation of genes associated with retinoic acid, BMP and Wnt signaling in the peripheral retina, including the ciliary body, and some aspects of the lens-deficient phenotype (e.g. microphthalmia) can be recapitulated by inhibiting retinoic acid signaling, or rescued in lens-removed eyes by restoring retinoic acid [142].

Pax6 is essential for early stages of lens induction, possibly through influencing modification and remodeling of chromatin [19, 52] as reviewed in [54]. In addition to its role within the lens lineage, Pax6 also plays a role in the adjacent optic vesicle to trigger lens formation. When *Pax6* is knocked out at an early stage in the optic vesicle, the lens does not develop [113].

Consistent with the importance of *Pax6* in lens formation, there is evidence that the lens is exquisitely sensitive to the correct *Pax6* gene dosage. In Mexican Cavefish *Astyanax mexicana*, it is the loss of *Pax6* expression specifically during lens development, that precipitates lens apoptosis which in turn leads to failure of retinal growth and the loss of anterior segment structures [143]. In mice, less than 80% or more than 120% of normal Pax6 activity is thought to result in lens defects that affect the rest of eye development, even though to adulthood [21]

Moreover, results from experimental inactivation one allele of *Pax6* in mice, specifically in the lens or in the optic cup, indicate that *Pax6* expression in the lens is necessary for normal development of the anterior chamber [112].

Further evidence of the sensitivity of lens development to Pax6 levels was concluded based on mouse chimera experiments indicating that heterozygous $Pax6^{Sey\text{-}Neu/+}$ ($Pax6^{-/+}$) cells do not contribute to the developing embryonic lens, in contrast to their contribution to the other eye tissues [144]. Additionally, in chimeric mice where the lens was wild-type, virtually all other aspects of anterior segment development were restored, including normal iris development, corneal epithelial morphology and limbal function [145, 146].

A plausible working model for the developmental defects underlying the development of the aniridia phenotype is that lens signals regulated by the correct Pax6 dosage are required for normal development of other anterior segment structures. Identification of these lens signals orchestrating anterior segment development should therefore be a high priority in eye research, irrespective of their roles in aniridia.

An early onset of cataract is prevalent in aniridia [147]. This is likely due to abnormality of the lens epithelium and lens fiber cells, and a thinning of the lens capsule [148-150]. Non-cell-autonomous mechanisms for cataract in aniridia have been also proposed as abnormal zonular fibers are reported to be associated with congenital cataract in aniridia [151, 152].

Anterior opacities in the lens and iris remnants in the anterior chamber are sometimes observed in aniridic eyes (***Figure 4***), suggesting that the separation of the lens and iris from the cornea may sometimes be arrested in human embryos [4]. Further detailed studies are necessary to investigate if the lens status affects the corneal endothelium. Since the keratopathy is mostly present in the anterior layers of the cornea, it is not obvious whether an incomplete separation of the lens or iris from the cornea could impact AAK. The putative crosstalk between the lens and other developing ocular tissues has been discussed above. Similar to dysfunctional lens epithelial cells, an abnormally thin corneal epithelium in the heterozygous $Pax6^{+/-}$ mouse [28] may represent developmentally immature epithelial cells (including limbal stem and progenitor cells) incapable to form the fully stratified multiple corneal epithelial layers that normally arise

postnatally in the mouse cornea [87]. However, it must be noted that any apparent undifferentiated state may be a secondary consequence of the chronic abrasion and wound-healing physiology of the aniridic cornea [153, 154] .

1.5 Does anterior chamber and iris malformation impact AAK development?

The conditional inactivation of a single *Pax6* allele in mice from either the inner layer or the outer pigmented epithelium of the distal optic cup results in a profound iris hypoplasia [51]. The resulting reduction in Pax6 dosage interrupts different stages of iris development: from reduction in the size of the progenitors, to delayed onset of muscle-specific markers and abrogated iris sphincter morphogenesis [51, 155]. Indeed several key factors for iris development are reduced in the developing iris of the $Pax6^{Sey\text{-}1Neu/+}$ mice including: *Pitx2*, *Igf2*, *Foxc1*, *TGFb2*, *Zic2* and *BMP4* [156]. These transcription factors and ligands could impact the differentiation of the iris progenitors as well as the migration of NCC that populate the iris stroma and the cornea [157].

The majority (50% - 75%) of aniridia patients develop glaucoma most likely as a consequence of abnormal differentiation of the trabecular meshwork and/or complete absence of Schlemm's canal [158, 159]. Notably, conditional haploinsufficiency of *Pax6* in the mouse lens and cornea - but not in the developing optic cup layers - disrupted trabecular meshwork and Schlemm's canal development and resulted in glaucoma [145]. It is currently unclear, however, if this result is due to Pax6 activity in the lens and cornea regulating factors required for the development of the drainage structures, or due to the abnormal morphology of the eye due to adhesion between lens, cornea and iris epithelium in the model [145].

Another unsolved topic is how partial or complete loss of iris and the abnormal differentiation of the drainage structures in aniridia impact AAK progression. It should be considered that the positioning of the anterior chamber angle may be needed for signaling to LSC and their niche to develop correctly, as well as to ensure the proper flow of aqueous humor important to maintain the correct eye pressure and nutrition of the anterior segment structures.

In a recent study examining 87 eyes of aniridia patients, 21 of which had a partial iris, it was shown that the partial presence of an iris was strongly associated with a milder degree of AAK [29]. Although this could support a connection between iris or chamber angle development and AAK, the mild keratopathy could also be caused by the common causative mutation itself.

1.6 Are mesenchymal structures (corneal stroma and endothelium) affected during development?

A number of clinical studies have reported that the corneal stroma is abnormally thick in almost all cases of aniridia [6, 160, 161]. Although the causes of a thick stroma in aniridia are not yet clarified (as the corneal endothelium appears to function normally), it has been reported that, during normal human development, the corneal stroma is thicker *in utero* and progressively thins with increasing gestational age [162]. This has led to the hypothesis that the normal thinning of the corneal stroma in later developmental stages *in utero* is disrupted in aniridia [29]. A developmental origin for the thickened corneal stroma is supported by the lack of clinical signs of stromal edema and a sufficiently high endothelial cell density in aniridia for maintaining proper stromal hydration.

In the epithelia of the developing lens, retina, ciliary body, iris and cornea, *PAX6* is expressed at high levels, and this is easily detectable by *in situ* hybridization, Western blot and immunohistochemistry. In the mesenchymal component of some other ocular tissues, such as

the corneal endothelium, corneal stromal keratocytes and trabecular meshwork mesenchyme, low and transient levels of *PAX6* (at the limits of detection by the above techniques) have been reproducibly demonstrated during mid-late stages of development [163, 164]. These low levels of PAX6 have nevertheless been shown experimentally to represent a cell-autonomous requirement for contribution of cells to the corneal endothelium and stroma, and also play a role in the differentiation of trabecular meshwork [47, 163, 164]. Although *PAX6* is downregulated in the trabecular meshwork of normal adult eyes upon differentiation [163], recent single cell analysis has revealed the presence of *PAX6* transcripts in normal limbal corneal keratocytes, corneal stromal keratocytes as well as in corneal stromal stem cells, into adulthood [70]. This correlates with the previous observation of PAX6 expression in a population of stromal stem cells [165]. At the single-cell level in humans, *PAX6* mRNA is detected through all developmental stages at a low level [70]. (***Figure 5***).

Impaired development of the anterior chamber angle, iris and endothelium due to *PAX6* haploinsufficiency has been carefully studied and reviewed in a number of reports [28, 40, 47, 155, 163], but the impact of PAX6-deficient corneal or limbal stromal cells on AAK remains elusive [166]. PAX6 dosage is self-evidently crucial for normal eye development, so the molecular mechanism by which different tissues require either 'high' or 'low' levels of Pax6, and how dosage is controlled via regulatory DNA elements, still requires clarification. PAX6 directly interacts with multiple other proteins [167, 168] and the presence and stoichiometry of different binding partners in different cell types is expected to modulate PAX6 activity. Hundreds of genes are regulated, directly or indirectly, by PAX6 during eye development [169] and are affected to different degrees by changes in dosage. It is therefore expected that mesenchymal cells with 'low' levels of PAX6 will exhibit a different profile of downstream gene expression from epithelial cells with 'high' levels. The biological impact of *PAX6*-heterozygosity for low levels of expression in mesenchymal cells, if any, are unresolved. Taken together, the most parsimonious scenario is that *PAX6*, expressed at high levels, is functioning cell-autonomously in the optic cup, lens and corneal epithelium progenitors, and that these tissues have a non-autonomous influence on the surrounding anterior segmental neural crest and mesodermal lineages (See ***Figure 5***, blue arrows). As the Schlemm's canal lining and iris stroma are formed by mesodermal cells [59] and are absent in $Pax6^{+/-}$ mice [65, 170], it may be possible that transient *PAX6* expression in these cells further contributes directly to their formation.

1.7 Do meibomian and lacrimal gland formation impact AAK development?

The functions of lacrimal and meibomian glands are essential for the production, stability and function of the tear film. Any factor disturbing the homeostasis of the ocular surface unit may disrupt the stability of the tears, leading to damage of corneal and conjunctival epithelia and possibly impacting LSC function [171]. These supportive glands are derived from the ocular surface ectoderm, but to date there is no evidence regarding the impact of these structures on other anterior structures during eye development. The morphogenic events necessary for the lacrimal gland development in $Pax6^{Sey/Sey}$ mice are defective [172]. In $Pax6^{Sey/+}$at E19.5, the lacrimal bud becomes visible but its structure remains vestigial [173]. In lacrimal gland organoid models, *Pax*6 is necessary for expression of the genes encoding the secretion machinery (aquaporins and neurotransmitters) but these analyses were performed with total *Pax6* knockout model [174]. In addition, PAX6 is one of the transcription factors necessary to drive explant cultures or induced pluripotent stem cells (iPSCs) into a lacrimal gland cellular fate [173, 175].

The development of the meibomian glands requires proper eyelid closure and eyelid fusion during embryonic development. PAX6 could influence this process since it is expressed at low levels during eyelid development in a complex expression pattern [176, 177]. PAX6 is expressed during development in the acinar cells of meibomian glands, although its contribution to development of these glands requires further investigation [178]. Protein composition of the tear film is altered in aniridia, and an elevation in inflammatory cytokine levels has been observed [179]. Meibomian gland dysfunction has also been documented in aniridia patients [180-183]. Still, it is unknown whether tear film and meibomian gland abnormalities arise from developmental defects or whether the function is impaired postnatally due to deficient ocular surface epithelia. AAK may underpin the inflammatory process and act as a possible trigger mechanism for dry eye and meibomian gland dysfunction. Elevated interleukins in the tear film could be also caused by the chronic wound healing state of the corneal epithelium in aniridia or from inflammation in the limbus and corneal stroma [7]. It is also important to keep in mind that the developmental defects observed in mice may not necessarily be mirrored in humans. Longitudinally monitoring the morphology and function of the meibomian and lacrimal glands and tear film quality from birth to young adulthood may help to answer these questions and could provide important insights for patient care. Interestingly, similar to *PAX6*-related aniridia, *P63*-mutation in patients that suffer from ectodermal dysplasia (ED) also led to meibomian and lacrimal gland defects in addition to LSCD [134, 184].

More precise information is needed about the spatiotemporal and functional importance of PAX6 expression in different tissues of the ocular surface during development and in adulthood, and critically, to determine the contribution of developmental abnormalities versus postnatal pathophysiological changes. Ongoing studies at the single-cell level in human corneas, supplemented with similar information in *Pax6*$^{+/-}$ mouse eyes, could provide the necessary platform for addressing these questions. In addition, identifying the gene regulatory networks and molecular pathways leading to normal PAX6 production could possibly provide a molecular tool to modify PAX6 levels [126], largely independent of the developmental stage and tissue type initially perturbed by *PAX6* mutation. Armed with this information, it may be possible to identify potential drug candidates with the ability to delay or prevent the development of AAK postnatally. Crucially, the impact of any treatment should be evaluated in terms of lacrimal and meibomian gland function, to assess the likelihood of maintaining a stable ocular surface environment in longer term.

2 How aniridia AAK symptoms manifest postnatally

2.1 Is limbal stem cell deficiency a cause or a consequence of AAK?

The limbus (meaning '*border'* in latin) is the circumferential border defined by the corneoscleral transition and harbors the LSC niche. In humans, the niche is typically located in the superior and inferior limbal regions where the 'Palisades of Vogt' structures are found [185, 186]. Accumulating evidence supports the hypothesis that the limbus is the sole niche of corneal epithelial stem cells, notwithstanding the impressive regenerative ability of the corneal epithelial cells themselves [127]. The shape and structure of the limbus, rich with blood and lymph vessels, suggests that it provides a protected microenvironment (LSC niche) that is enriched with nutrients and stem cell self-renewal factors [186-188]. Slow-cycling nucleotide label-retaining cells have been specifically identified in the limbus while in the corneal periphery and center, only fast-cycling cells have been identified [189]. Limbal epithelial cells have been shown to have high regenerative potential and long-term proliferation capacity *in vitro* and *in vivo* [190].

LSCs produce transient amplifying cells which undergo further division and centripetal movement to replenish the basal corneal epithelium. A constant stream of these newly produced

basal epithelial cells migrates towards the supra-basal epithelial layers and is accompanied by a loss of proliferation capacity leading to a terminally differentiated state [191]. Recent studies have identified two spatially discrete classes of stem cells in the limbus – an inner ring of active cells that are mostly responsible for repopulating the corneal surface during normal homeostasis, and an outer, more quiescent ring that may have a role after injury [107, 192]. It is this outer ring that expresses LSC marker *GPHA2* discussed earlier, while the inner ring is mostly responsible for the centripetal radial streams of corneal epithelial cells observed in mosaic reporter mice.

LSCD is a group of diseases that can be caused by environmental or genetic factors and is accompanied by conjunctival cell invasion, inflammation, corneal neovascularization, corneal opacification and blindness [193]. It is commonly believed that dysfunction of LSCs and their stromal niche is the basis and cause for these pathologies [194]. Accordingly, the inability of LSCs to replenish the corneal epithelium leads to recurrent ocular surface defects and abnormal healing of the corneal epithelium, often being replaced by conjunctival cells [195]. This is often followed by neovascularization of the corneal stroma, compromised corneal transparency and consequently corneal opacification, leading progressively to poor vision. Typical changes in corneal epithelium-specific surface markers include the downregulation of corneal keratins KRT3/KRT12, and abnormal expression of conjunctival keratins KRT4/KRT13 or skin keratins KRT1/KRT10 [196]. Also, goblet cells which are normally restricted to the conjunctiva, can be found in the cornea in cases of LSCD, and their presence has also been used to diagnose LSCD [195], although goblet cell presence can be variable in advanced stages of AAK [7]. Of note, PAX6 is also downregulated in severe ocular surface diseases including LSCD [196].

Controversy: Accumulating experimental and clinical evidence supported the LSC dogma, namely that the LSCs are located in the limbal niches and are solely responsible for epithelial renewal. This notion subsequently became firmly accepted by the research community. Nevertheless, there was some conflicting evidence that had been overlooked or remains unresolved [197, 198]. Perhaps one of the most confusing observations was reported in the seminal and elegant study of Tsengs's group [199] which strengthens the importance of the limbus. In that study, the entire limbal epithelium in 12 New Zealand White rabbits was surgically removed. Six-months later, the cornea in 8 of the 12 rabbits remained normal while the remaining four displayed only a mild phenotype of corneal neovascularization. However, when limbal epithelial depletion was coupled with epithelial debridement in the central cornea, corneas became vascularized. Likewise, in humans, a persistent central island of apparently normal corneal epithelium can persist for years, despite total limbal epithelial absence [198]. Similarly, Majo and Barrandon reported that the ablation of limbal epithelium in mice did not induce LSCD [127]. Limbal epithelial deletion is thus not necessarily sufficient to cause LSCD, however multiple lineage tracing studies in mice has shown the importance of LSCs in replenishing the corneal epithelium [103].

In a later experiment, Nasser and colleagues [106] showed that KRT15-GFP transgene specifically labels the limbal epithelium, and this model was used to validate limbal removal, ensuring total LSC depletion. Using triple transgenic animals and multiple "Confetti" lineage tracing, one-day post total limbal epithelial depletion, the Confetti^{+} corneal epithelial cells healed the denuded limbus, and after 7-10 additional days, these cells started to express limbal epithelial markers. These corneas were transparent, and the repaired limbus could successfully replenish the corneal epithelium for at least 6 months of experimental follow up. This study indicated that corneal epithelium-committed cells can dedifferentiate into apparent LSCs. When LSC depletion, however, is coupled with limbal stroma (niche) damage, dedifferentiation is inhibited and severe LSCD develops. Similar cell plasticity that allowed recovery from native stem cell loss has also been found in other epithelial tissues (reviewed in [200]). Collectively,

these studies suggest that the LSC niche (and not the LSCs alone) is essential for maintenance of the transparent cornea, and that damage to the niche is most likely a key element of many LSCD pathologies. In light of this, gene-linked LSCD (e.g., mutations in *PAX6* or *P63*), are likely to affect both LSCs and their niche. This underscores the important role of the limbal niche in corneal health and LSCD pathogenesis [201, 202]. Nevertheless, the intriguing possibility remains that a signaling program triggering corneal epithelial cell dedifferentiation to LSC-like cells could be exogenously applied even in the absence of a functioning limbal niche. Investigations into the signaling pathways responsible for triggering and maintenance of the dedifferentiation of corneal epithelial cells would therefore be of interest.

Aniridia-related LSCD: The limbal niche likely plays a pivotal role in the specific form of progressive LSCD observed in AAK. *PAX6* is expressed in various tissues in adult eye (***Figure* 3**). *PAX6* mutation may lead to aberrant crosstalk between mutated LSC and mutated stromal and epithelial elements (corneal stromal mesenchymal stem cells, keratocytes, melanocytes, corneal nerves or dendritic cells). It may include an abnormal response to extracellular matrix (ECM) signals by mutated LSC, or dysregulated response of LSC to signals originating from blood or lymph cells. On the other hand, knockdown of *Pax6* in cultured LSC induces transdifferentiation into an epidermal-like fate [203], suggesting that *Pax6* could also play a cell-autonomous role in maintaining LSC identity. Clinical evidence also points to an abnormal limbal niche in aniridia, that is either the result of abnormal developmental processes *in utero*, or due to incomplete or aberrant postnatal development and/or maturation of the niche [204-207]. In young children and adults with aniridia, abnormal limbal structures, inflammation, vascularization and a significant nerve deficit in the limbus have been observed throughout the range of AAK severity [4, 207]. These changes are also accompanied in an early stage by islands of conjunctival tissue present within an otherwise transparent corneal epithelium. The evidence therefore seems to indicate that an early breakdown in the limbal niche may prevent a proper limbal niche environment from forming. This would impact proper maturation of the limbal epithelium and the sustenance of its associated stem cells and/or progenitors. On the other hand, if the limbal niche in aniridia was fully functional but only the mutated LSCs were dysfunctional, corneal epithelial cells could presumably dedifferentiate and occupy the limbus as LSC-like cells. This is not in line with clinical observations, suggesting that *PAX6* mutation could impair the dedifferentiation process. Still, the role of *PAX6* in LSC differentiation and epithelial cell dedifferentiation remains to be elucidated.

Comparison of AAK with other pathologies or genes causing ocular surface defects:

Many cellular and tissue properties are altered in aniridia, leading to pathologic changes associated with stem cell and ocular surface insufficiency that are also apparent in other rare disease entities such as severe dry eye, neurotrophic keratopathy and chronic inflammation. Several of these pathological processes are summarized in ***Figure 6***. Thus, a comparison of AAK to other eye diseases, which are not detectable at birth, but that could lead to stem cell deficiency could bring new insights. Several ocular surface diseases are associated with downregulation of *PAX6* in the corneal and conjunctival epithelia [196, 208, 209]. The limbus in aniridia may just be more sensitive to signals leading to pathology since the baseline level of the PAX6 protein is already low. LSCD can be induced by pathological triggers, such as chemical burns, infections, auto-immune disease [193]. The different signaling leading to epithelial-to-mesenchymal transition or fibrosis-like activation of transforming growth factor beta (TGF-β) by various cytokines as alreay hypothesied by Tseng et al. are summarized by Ljubimov et al. [210]. Thus, mechanisms uncovered in the context of aniridia may have bearing on other etiologies, and vice versa. Mutations in genes coding for key transcription factors and

in stem cell-related genes (e.g. *TP63 [134]*, *Klf4 [211, 212]*, *PITX2 [213]*, *Sox2 [131])* exhibit LSCD-like phenotypes in transgenic mice.

Interestingly, some of these transcription factors are also involved in neural crest and/or periocular mesenchyme specification. For example, *Pitx2,* a gene associated with anterior chamber dysgenesis if mutated, is mainly expressed in the developing corneal mesenchyme and later in the corneal stroma but not in surface ectoderm derived cells [88]. Interestingly, *PITX2* mutation is associated with an iris hypoplasia similar to 'classical' aniridia [214].

An inflamed and stressed environment has been reported in the *Pax6*$^{+/-}$ mouse epithelium. Elevated oxidative stress has been suggested as one important mechanism in developing AAK [153, 154]. Altered calcium waves lead to activation of ERK1/2 in mouse models [215]. It has been reported that Desmoglein-1 (DSG1) together with Erbin1 can suppress ERK activation in epidermis [216]. Notably, DSG1 is downregulated in the epithelium of *Pax6*$^{Sey/+}$ mice [24] and therefore, together with elevated calcium, an activation of the ERK1/2 pathway could negatively impact differentiation through altered signaling in addition to oxidative stress. In line with this, pharmacologic inhibition of *ERK1/2* in human corneal limbal epithelial cells led to upregulation of *PAX6* and increased the production of PAX6 protein [126].

Corneal-conjunctival boundary: Progressive infiltration of conjunctival goblet cells into the limbus is believed to be due to LSCD, but this assumption should be carefully studied. For instance, KRT12-positive goblet cells could migrate into the cornea after large wounds [217]. Also, in two clinical studies, only about 30% of aniridia cases had goblet cells in the cornea [7, 29] detectable by *in vivo* confocal microscopy. The use of goblet cells for diagnosis of conjunctivalization and LSCD may thus have drawbacks due to a heterogeneous distribution of goblet cells and potential presence of KRT12-positive goblet cells [217]. From experiments analyzing both the limbus and conjunctiva, it is known that conjunctival cells share some stem cell markers with LSCs (e.g., KRT15, KRT19 and TP63) [218]. Thus, there is a lack of specific markers to distinguish conjunctival cells from corneal progenitors [98, 218]. It makes difficult to prove or disprove the presence of conjunctival progenitors in the limbus with existing markers; however, conjunctival cell migration patterns would differ as these cells are not typically mobile and conjunctival stem cells are enriched in the fornix region [219]. If, however, the corneal epithelium or limbus is defective, the behavior of conjunctival cells could change. Finally, it remains unanswered whether infiltration of conjunctival goblet cells into the corneal epithelium is a cause or a consequence of LSCD.

***PAX6* expression level and LSCD:** In humans, *PAX6* is expressed quite uniformly in both the limbal and corneal epithelial cells, regardless of the degree of differentiation [196]. Alteration of the PAX6 protein level has an impact on different aspects of corneal homeostasis (stem cell survival, stem cell renewal, adhesion, migration, proper differentiation) [23, 24, 28, 139]. These properties were recently recapitulated *in vitro* in a *PAX6*-haploinsufficiency cellular model by genome editing of human limbal cells [220]. Other alterations in the limbus have been reported, for example that epithelial cell proliferation is slightly enhanced in *Pax6*$^{+/-}$ mice [23]. Hyperplasia in the limbus coupled to altered differentiation could explain why the thinning of the corneal epithelium occurs before the onset of conjunctivalization in aniridia. Such morphological changes in aniridia are already detected at birth, although no apparent clinical consequences are observed at that early stage. It is likely that abnormal development, as discussed above, impairs LSC function and/or the LSC niche from birth in congenital aniridia.

Pax6$^{+/-}$ mice have limbal epithelial label-retaining (presumed) stem cells [221]. In aniridia-like *Pax6*$^{Sey\text{-}Neu/+}$ mice, cell lineage tracing experiments revealed epithelial cell migration out

of the limbus into the cornea (although the pattern of radial migration across the cornea was abnormal)[139]. These lines of evidence, and the wide expression pattern of PAX6, suggests that *Pax6*$^{+/-}$ mice do in fact have functional stem cells. However, *Pax6* haploinsufficiency may reduce the number of LSC, or affect their ability to self-renew, undergo asymmetric cell division and/or diminish the potency of short-lived corneal epithelial progenitors. Such disturbance of proliferative capacity has been also described in Ectrodactyly-Ectodermal Dysplasia-Clefting Syndrome, where the epithelium is also thin [222]. It is also currently not known whether the spatial distribution of outer, Gpha2-positive, quiescent LSCs and inner, Gpha2-negative LSCs described above for wild-type mice and humans is disrupted in *Pax6*-mutants. In chimeric mice (mixtures of wild-type and *Pax6*$^{+/-}$ cells formed by aggregation of eight-cell embryos), the contribution of *Pax6*$^{+/-}$ cells to the limbus was slightly but significantly reduced compared to wild-type, suggesting a mild deficiency of maintenance or proliferation of LSCs. However, relatively normal radial stripes of corneal epithelial cells emerged from the limbus to populate the cornea in these chimeras, [139] Furthermore, corneal epithelial thickness and stratification was normal, suggesting that other tissues (lens, keratocytes or unaffected corneal epithelial cells) could rescue, by paracrine effects, the murine AAK phenotype [139]. Further work has provided additional evidence that the *Pax6*$^{+/-}$ cells themselves are unable to respond to guidance cues originating from the corneal stroma [223].

Corneal neovascularization: Neovascularization of the cornea is not a specific phenomenon of AAK but a consequence of LSCD and chronic inflammation and wound healing in general. When the ocular surface is inflamed, with high levels of inflammatory cytokines in tears and migration of inflammatory cells into the central cornea, new vessels sprout and migrate towards the central cornea along with conjunctival tissue. In advanced AAK a thick, opaque pannus forms that replaces the epithelium. How is the cornea normally maintained in an avascular state? Maintenance of an avascular transparent state has been attributed to a tightly regulated balance between pro- and anti-angiogenic factors in the corneal epithelium and stroma [224]. If the endogenous signaling networks preventing angiogenesis are impaired, a favorable environment for blood vessel growth will ensue.

For example in the corneal epithelium, soluble VEGF receptor-1 (sVEGFR-1 or sflt-1) is required to maintain corneal avascularity and its expression has been reported to be impaired in aniridia [225]. Restoring sflt-1 expression in the *Pax6*$^{+/-}$ mouse cornea leads to regression of blood vessels [225]. The microRNAs miR-204-5p and miR-184 have also been shown to be expressed in the cornea [226-228], and are involved in both Pax6 signaling and regulation of angiogenic signaling [229, 230]. miR-204-5p is encoded by *MIR204* that is located in intron 9 of the human *TRPM3* gene on chromosome 9 [231]. In animal models, *Pax6* binds directly to 5' regulatory sequences upstream of *Trpm3* to upregulate both *Trpm3* and *Mir204* transcripts [232-234]. miR-204-5p impacts lens and retinal development [231, 232], but is also strongly downregulated in conjunctiva cells in aniridia [125]. Moreover, a feedback loop exists whereby in the Japanese rice fish *medaka*, miR-204 represses the transcription factor *Meis2*, which in turn regulates *Pax6* expression [235]. miR-184, also known for its roles in neovascularization and homeostasis in the cornea [236], was shown to be essential for *Pax6* expression in embryonic stem cells that were differentiated into corneal epithelial-like cells [228]. It also repressed angiogenesis of cultivated LSCs by targeting proangiogenic factors [228].

VEGF-C, another pro-angiogenic factor primarily involved in lymphangiogenesis, was found by proteomic analysis to be elevated in the tear film in aniridia, correlating with the presence of lymph vessels in the cornea in advanced-stage AAK [7, 182]. Further pro-angiogenic cytokines were later found to be elevated in the tear film in aniridia, including basic fibroblast growth factor (FGF-2) and interleukin-1β [179]. These and other pro-inflammatory cytokines, as well as inflammatory cell infiltration [7], represent an environment conducive to neovascularization. Interestingly, in a case report of long-term topical anti-VEGF treatment of

corneal neovascularization secondary to aniridia, progression of AAK was seemingly halted and central vision was maintained during a 12-year period [237]. This raises the possibility of long-term treatment of the pro-inflammatory and pro-angiogenic corneal environment as a means to prevent, halt or potentially even reverse the progression of AAK. To investigate whether this could be a potential strategy for AAK management would require controlled studies, with participants carefully selected for mutational status, stage of AAK and rate of progression.

2.2 What is known about the limbal niche?

It is generally accepted that stem cells require a specific supportive environment to maintain *in situ* their undifferentiated state and properties of self-renewal and differentiation. As soon as daughter cells migrate into the peripheral or central cornea, local features of the limbal niche environment and the factors they provide are no longer present, eventually leading to (terminal) differentiation of epithelial cells. This raises essential questions concerning the niche location and its molecular nature. Mesenchymal tissues are known to have a tight crosstalk with their neighboring epithelia and serve as a niche for epithelial stem cells, as has been shown for example with hair follicle bulge cells [238-240]. Corneal stromal stem cells support limbal stem cells in cultured feeder layer systems, and are similarly believed to be important in LSC maintenance [241]. Seminal grafting experiments show that the type of epithelium (epidermis versus corneal) is dictated by its underlying stroma [242]. Corneal epithelium that was exposed to embryonic dermal signals underwent transdifferentiation into hair follicle forming epidermis. Such a niche should be composed of the surrounding cells and the secreted ECM deposited by the different cell types. Surrounding the LSCs are mesenchymal (neural crest or mesodermal derived; see ***Figure 2***) cells (keratocytes), blood vessels,, nerves, immune cells, melanocytes, and stromal stem cells, all located in the peripheral stroma. It has been proposed that mesenchymal cells physically interact with LSC via SDF-1/CXCR4 signaling to maintain stem cell function [243]. This apparent link between LSC, the stromal niche and surrounding immune cells fits well with the presence of lymph/blood vessels around the limbus and the recent demonstration of lymphatic capillaries as critical SC-niche components in the skin [244].

In addition, the basement membrane composition of the limbus is different from the basement membrane of the central cornea [245, 246]. The molecular composition of the niche-derived ECM has been identified with laser captured tissue by, showing that LSCs are attached to the basement membrane through laminin receptors, and intercellular contacts between LSCs and their niche cells are mediated mainly by cadherins [247]. Binding between epithelial and stromal cells is mediated by N-cadherins [248], and mesenchymal cells and melanocytes have been suggested to support stem cell properties and to enhance the clonal growth of LSCs *in vitro* [249, 250]. N-cadherins are also expressed in widely used 3T3-feeder cells and could be important for homogenic and heterogenic cell-to-cell binding with niche cells such as melanocytes [248, 251]. *In vitro*, melanocytes have been suggested to support the stemness of limbal cells [252].

In contrast, however, to surface ectodermal or neuroectodermal-derived structures, the limbal niche cells show only low or even transient *PAX6* expression, and the specific ablation of *PAX6* gene in these cells has never been achieved. It is thus, difficult to identify the direct contribution of *PAX6* to limbal niche function. Although PAX6 is typically not detected by stromal cells, it was reported that stromal cells in the limbus express *ABCG2* and *PAX6* [253], and the associated proteins encoded by these genes may support the limbal niche. It is not clear if PAX6 influences limbal melanocytes, but it is involved in the melanogenesis of the retinal pigmented epithelium though activation of *MITF* [254]. Interestingly, *MITF* mRNA is downregulated in

the aniridic conjunctiva [125] but it is not known whether the limbus in aniridia is melanocyte deficient. It was shown that limbal mesenchymal cells could substitute 3T3-feeder cell function in a 3D culture model system [255]. However, whether these positive effects are dependent on *PAX6* expression in mesenchymal cells has not yet been addressed, nor is it known whether the limbal stromal mesenchymal cells are present or functional in aniridia.

From clinical studies, it is known that patients with aniridia show a degradation of the limbal niche structures coinciding with a progressive ocular surface transformation [6, 7]. Thus, it is possible that the limbal niche structure and function could be maintained despite the clinical diagnosis and absence of iris in congenital aniridia, depending on the type of *PAX6* mutation. However, as discussed above and in particular in the case of 'classical' *PAX6*-related aniridia, the limbal niche environment is perturbed in infancy and possibly from birth, leading to LSCD regardless of the early postnatal presence of the limbal niche.

The mechanical properties of the niche are also important to protect the LSCs [256]. Altered mechanical properties could affect proliferation and differentiation of LSCs [257] and may therefore impact AAK if mechanical properties in the niche are altered. Gouveia et al. have shown that LSCs can be regulated by modulation of biomechanical factors and that a mechanically compliant limbal niche substrate supports LSC properties at least in part through regulation of Hippo signaling. Migration of transient amplifying cells into the corneal epithelium from the limbus may in part be driven by durotaxis – driving cells from areas of high substrate compliance (the limbal stroma) to low substrate compliance (the corneal stroma) [256]. Moreover, increased stiffness at the limbus is present in LSCD and this may have important implications for our understanding of AAK, if it reduces the durotactic drive for cells to enter the corneal epithelium from the limbus [256]. If confirmed in patients, an appropriate strategy for manipulating stiffness of the limbal niche could potentially restore its pliability and consequently its capacity to support LSC function as a *novel* therapy to prevent or even arrest the development of AAK in the future. Of note, the fibrovascular pannus that is observed to invade the limbus as AAK progresses is a mechanically more rigid and tough tissue than the original limbal epithelial and underlying stromal extracellular matrix tissue.

2.3 Do stromal cells influence aniridia?

In the normal adult corneal and limbal stroma, a very small proportion of cells show a low level of *PAX6* expression [165] but these may represent stromal stem cell populations generally related to keratocytes. Since corneal stromal cells can differentiate (at least *in vitro*) into cells with a keratocyte phenotype when cultivated as neurospheres, it is intriguing to speculate a role for *PAX6* in early keratocyte differentiation which may be of relevance to corneal stromal changes occurring in aniridia [258]. Haploinsufficiency and overexpression of *Pax6* both lead to alterations in the corneal stroma and endothelium in mice [259]. *Pax6*$^{Sey\text{-}Neu/+}$ mice exhibit increased stromal cell apoptosis, abnormal wound-healing responses and reduced levels of MMP-9 secretion [260]. Studies are currently underway to characterize stromal cells isolated from aniridic patient corneal buttons at the time of corneal graft transplantation surgery, which may shed light on the properties of *PAX6*-mutant stromal cells and their role in AAK.

Co-culture of normal corneal and limbal stroma with limbal and corneal epithelial sheets showed that limbal stroma modulates epithelial cell differentiation and proliferation whereas the corneal stroma promotes their differentiation [261]. Corneal stromal signaling is important for stratification of the epithelium and involves the WNT/β-catenin signaling pathway [262].

AAK coincides with major changes in signaling in the epithelium and derangement of the corneal stromal architecture, with a subepithelial pannus in the anterior stroma either lacking

collagen I or only faintly labelled, but positive for collagen IV, fibronectin, tenascin-C, vimentin and α-SMA, in addition to displaying fibrotic markers and containing blood vessels [258, 263]. The remaining posterior stroma appears to have an almost normal morphology, including expression of type I collagen. The changes in the stroma could be relatively nonspecific and not related to mutation of stromal cells since they are also induced in the stroma of healthy corneal donor tissue which consists of non-mutated stromal cells, but it is known that AAK recurs after standard corneal transplantation due to the LSCD. The corneal endothelium is also of mesenchymal origin (See section 1.6). In humans, no alteration of corneal endothelium has been observed in aniridic patients thus far [29]. Regular Descemet's membrane and normal to slightly elevated endothelial cell counts are usually observed in patients with aniridia [29, 264]. Cases with lower endothelial cell count have been found in older patients, but these are usually associated with morphological changes such as cornea guttata or with pleomorphism/polymegethism due to a long history of topical treatments for glaucoma or previous surgeries such as cataract extraction [265].

Notably, it has been shown that PAX6 could be secreted, as already reported for other homeodomain proteins [266]. This could suggest previously undetected nonautonomous effects for PAX6 in the cornea, and that PAX6 produced and secreted by epithelial cells could be internalized by the adjacent stromal cells. In this case, altered PAX6 dosage could have an indirect effect on stromal homeostasis. In zebrafish, antibodies capturing secreted PAX6 lead to developmental defects [267]. In the future, it must be elucidated if such biological processes could be important not only for eye development in zebrafish, but also for corneal epithelial-stromal interactions. Experimental co-culture systems might be useful for addressing these questions.

2.4 Is limbal cell identity altered in aniridia?

In contrast to the spatially restricted LSCs, conjunctival stem cells can be found in all regions of the conjunctiva, but are predominantly located in the medial canthal and inferior forniceal areas [268]. Since lineage segregation of corneal epithelium and conjunctiva appears after *PAX6* induction [33, 80], some functional overlap for *PAX6* function in corneal and conjunctiva should exist. The focus of *PAX6* on corneal epithelial phenotype might be misleading since sustained *PAX6* expression is also present in the conjunctiva and other factors may drive tissue-specific expression downstream of *PAX6*. For example, studies link severe ocular surface disease such as Sjogren's syndrome with a reduction of PAX6 expression in the conjunctiva [209]. Therefore, the question of the role of *PAX6* in the conjunctiva and its contribution to AAK has been recently raised. Transcriptomic analysis of conjunctival cells was recently investigated in a cohort of 20 aniridia patients mostly showing AAK signs. Changes in gene expression and pathways related to proliferation and pro-angiogenic pathways (*FOSB, FOS, JUN, ATF3, FOSL1, EGR1, NR4A3, IL8*) suggested that conjunctiva is part of the pathology in aniridia where it is abnormally proangiogenic and proliferative [125]. The molecular link between angiogenesis and *PAX6*, however, requires further investigation. The expression changes observed in patient-derived conjunctival cells could also result from secondary effects due to inflammation or severe dry eye.

Of interest, the anti-angiogenic miRNA-204-5p mentioned above is under the control of *PAX6*, and its expression is strongly reduced in the conjunctiva of aniridia patients [125, 234]. Notably, two of the subjects in the cohort had mutations in regulatory elements of *PAX6* (located in the adjacent *ELP4* gene) and these mutations are thought to lead to a milder PAX6 reduction compared to the haploinsufficiency caused by mutations in coding regions of *PAX6*. miR-204-5p expression was suppressed to a lesser degree in these two subjects relative to those

with *PAX6* coding mutations, and this correlated with a very mild AAK phenotype [125]. Likewise, only mild deregulation of genes involved in retinoic pathway (*RBP1, ADH7, ALDH3A1, CYB1B1, PPARG*) was found in these two subjects, in contrast to the major mis-expression found in classical loss-of-function *PAX6* mutations. In line with this, siRNA knockdown of *PAX6* in primary limbal epithelial cells reduced *ADH7* and *DSG1* expression [269].

Comparing published transcriptomic data between limbal and conjunctival epithelia from aniridia patients *in vivo* and cellular models *in vitro*, reveals a relatively minor overlap (unpublished data). This may reflect the absence of the niche from the *in vitro* context. Also, an important drawback is that much of the data described in the available literature was produced in a late stage disease. Given the complexity of the eye and the aniridia pathology, a blueprint of the transcriptome and proteome of different ocular epithelial cell types *in vivo*, including various differentiation states at early disease onset is desirable, to investigate how *PAX6* influences the genetic programs of these different cell types.

2.5 What is the role of inflammation in aniridia?

Important inflammatory changes are observed in the cornea in aniridia. These have been documented in several patient cohorts and include an infiltration of mature antigen-presenting dendritic cells into the central cornea, infiltration of non-dendritic inflammatory cells, upregulation of inflammatory cytokines, and a deficit of corneal nerves [7, 29, 270]. The question arises as to whether these inflammatory changes precede or are a consequence of AAK. Clinical evidence supports the contention that inflammation precedes the development of AAK based on inflammatory cell infiltration of the limbal epithelium and central cornea during the first few years of life while the limbus is still intact [29]. Because progression of AAK is associated with a chronic and worsening inflammatory status of the ocular surface [29], it is possible that early subclinical inflammation may trigger AAK. The inflammatory environment may then be reinforced in a vicious circle, through inflammatory signaling induced by degradation of corneal limbal structures, infiltration of inflammatory cells from the conjunctiva and from the circulation via invading blood vessels, lack of supportive neurotrophic factors (see Section 2.6), and the presence of inflammatory cytokines in the tear film [7, 179, 182]. On the other hand, the inflammation observed early in life prior to degradation of the limbus, may itself be triggered by incomplete limbal developmental and postnatal maturation processes. Features such as blood vessel sprouting from an incomplete cellular structure of the limbal palisades and the migration of dendritic cells from limbal and conjunctival regions into the central cornea [29] may indicate that the limbal niche in aniridia is insufficient to prevent inflammatory cells from entering the cornea.

Ocular surface inflammation is always present in eyes with LSC deficiency, and presents a risk for stem cell transplantation, requiring therapeutic immunosuppression prior to limbal transplantation [271]. Mesenchymal cells have been shown to modulate the immune response in several systems, and may therefore also play a role in limbal niche and inflammatory signaling [272, 273]. Interestingly, therapeutic mesenchymal cell transplantation in aniridia has shown promising early clinical results [274], and may overcome the disadvantages of LSC transplantation in aniridia, possibly due to the enhanced immunomodulatory properties of mesenchymal stem cells. Different types of lymphocytes such as T-cells [275] and dendritic cells [276, 277], reside at the limbus and in the cornea. The antigen-presenting dendritic cells (also known as Langerhans' cells) are normally resident in the limbal region and can be visualized using *in vivo* confocal microscopy [276]. In healthy individuals, mostly immature, non-antigen-presenting dendritic cells are present in the central cornea in the basal epithelial layers. In aniridia subjects, however, there is an increased presence of mature, antigen-presenting dendritic cells, even at a young age. A higher density of dendritic cells could be

explained by a developmental delay, since the dendritic cell population reduces after birth in the central cornea [29]. Since the limbus may mature postnatally [71, 278], a correct spatial isolation of antigen-presenting dendritic cells may not occur in aniridia, leading to persistent inflammation throughout the corneal epithelium.

Elevated inflammatory cytokine levels have been observed in the tear film of aniridia patients, and this may be a causal or exacerbating factor in the chronic wound healing response observed in mice [154, 179] and known to exist in human aniridia patients. Interleukins are expressed in healthy corneal epithelium and are released upon wounding. Although it is unclear whether inflammation triggers the initial AAK, it is important to control the ocular surface inflammation in aniridia as it may slow down the progression and consequently improve the prognosis of AAK. Interestingly, it was shown that in tears taken from subjects with aniridia, there is not only an increase in the pro-inflammatory cytokine IL-1β, but its anti-inflammatory counterpart IL-1RA is diminished, suggesting an imbalance in cytokines can promote or sustain the inflammation observed in aniridia [183]. This is reminiscent of the altered pro/anti-angiogenic factor balance in the cornea in aniridia.

2.6 Do corneal nerves play a role in AAK development?

The cornea is the most densely innervated peripheral tissue in the human body. Most corneal nerve fibers are sensory in origin and are derived from the ophthalmic branch of the trigeminal nerve. Corneal nerves and corneal epithelial cells release modulatory substances and growth factors that provide trophic support to each other, to maintain a healthy ocular surface [279, 280]. Local and systemic disease, however, can impair corneal innervation leading to decrease in tear production and impairment of wound healing [279]. Neurotrophic factors released by corneal nerves, often in the context of inflammation and wound healing, include nerve growth factor (NGF), epidermal growth factor (EGF), substance P (SP), calcitonin gene-related peptide (CGRP), acetylcholine, cholecystokinin, noradrenaline, serotonin, neuropeptide Y (NPY), brain-derived neurotrophic factor (BDNF), and neurotrophin- (NT-) 3 [281]. SP and CGRP modulate corneal epithelial cell proliferation, migration, adhesion, and stratification [279]. In addition, administration of SP combined with insulin-like growth factor-1 (IGF-1) can increase the corneal healing rate and promote corneal epithelial cell adhesion [282]. Conditions such as AAK, where ocular surface homeostasis is disrupted, can upset the close and important interaction between the nerves and the epithelium, impairing the normal corneal renewal process and leading to inflammation and a wound healing state.

A recent study by Lagali et al reported a significant corneal nerve density deficit in PAX6 aniridia subjects (n=46), that declined further with age. This was associated with significant corneal sensitivity reduction, reflecting a chronic impaired neurotrophic status [29]. The combination of limbal deficiency and reduced corneal sensation in subjects with *PAX6* aniridia would have a significant impact on the maintenance of the ocular surface, contributing to the development of an unstable corneal epithelium often associated with AAK. Perturbation of the neurotrophic function may also impact inflammation of the ocular surface, which is widespread in aniridia. The cornea normally enjoys an immune- privileged status, which plays an important role in corneal immune response, particularly in promoting corneal allograft survival. Corneal immune privilege is dependent on the interplay of several important factors such as: (i) an absence of blood vessels (limiting direct access of the immune system to the cornea) and lymphatic vessels (preventing efferent movement/delivery of antigens and antigen-presenting cells (APC) to T cells located in the draining lymph nodes; (ii) a lack or low expression of major histocompatibility complex (MHC) class I and II antigens; (iii) presence of anti-inflammatory molecules, e.g., TGF-β, IL-10, IL-1RA; (iv) an Anterior Chamber-Associated Immune Deviation (ACAID) induced response, and finally (iv) presence of FasL, resulting in the

apoptosis of infiltrating T cells. Many of these factors, however, are upset in AAK, leading to inflammatory cell invasion, expression of proinflammatory cytokines and a loss of immune privilege. Notably, different populations of immune cells have been reported in the cornea of subjects with AAK [7] and the pro/anti-inflammatory cytokine balance is perturbed in the tear film in aniridia [179].

Dendritic cells (MHC Class II positive mature antigen presenting cells capable of antigen presentation to naïve T-cells) are one type of inflammatory cell normally resident in the corneal stroma and epithelium, with highest density in the periphery. As described above, dendritic cells play an important role in corneal homeostasis as sentinels of both innate and adaptive immunity. Resident corneal dendritic cells also have a role in maintaining homeostasis of corneal nerves [283], suggesting a direct interaction between the immune system and peripheral nerves at the ocular surface level. As indicated above, a significantly elevated density of mature dendritic cells exists in aniridia subjects when compared with normal individuals [29]. This emphasizes the close relationship of dendritic cells, inflammation and neurotrophic status of the cornea in PAX6 aniridia patients. Macrophages, normally occupying only the corneal stroma, reside in close proximity to peripheral nerves [284, 285]. Macrophages express SP receptors, produce SP, and it is also known that neurotransmitters including SP can modulate immune activity, although more investigation is needed to completely elucidate whether SP is affected in aniridia. It is known, however, that the decrease in corneal nerve density in AAK is associated with simultaneous invasion of inflammatory cells reflecting disease progression and corroborating the interaction between the nerves and immune system [286].

It is not clear whether problems with innervation are partly responsible for insufficiency of the limbal niche in aniridia. In normal eyes, the basal side of the Palisades of Vogt is densely populated by corpuscular nerve endings, suggesting that neurotrophic factors may support stem cells not only directly, but indirectly by maintenance of the limbal niche [287, 288]. Human corneal-limbal organoids appear to maintain good niche function without innervation *in vitro* [289], but this does not preclude an *in vivo* role in niche development or maintenance. In mammals, though not in birds, *PAX6* is expressed transiently during early development of the trigeminal ganglion, in cells of both the OpV and mmV-derived components, and this correlates with delayed innervation of sensory structures such as whisker follicles in mutants (unpublished data). Putative links between *PAX6* mutation, disruption of limbal-corneal innervation, the degradation of palisade structure and [207, 270] deficiency of the limbal niche require further investigation.

As immune cell infiltration into the cornea facilitates (and is facilitated by) neovascularization, a deficit of corneal nerves may directly or indirectly play an important role in development of neovascularization in AAK. In experimental models of corneal neovascularization and trigeminal nerve ablation in the mouse, blood vessel invasion was shown to occur only in areas devoid of nerves, where inflammatory leukocytes and macrophage-lineage cells also infiltrated the cornea [290]. Conversely, where the nerve supply remained intact, the cornea remained vessel-free. A loss of antiangiogenic factors was noted in denervated corneas, in particular loss of vascular endothelial growth factor receptor-3 (VEGFR3) expression in the corneal epithelium and loss of pigment epithelium-derived factor (PEDF) expression in the corneal stroma. In a different mouse model of inflammation-induced dry eye disease, corneal nerves were diminished and expressed elevated levels of the proinflammatory neuropeptide SP [291]. Blockade of SP or its neurokinin-1 receptor effectively prevented vascular endothelial cell activation and reduced corneal neovascularization. In addition, VEGF has been shown to mediate corneal repair in abrasion-induced corneal nerve damage models, where VEGF blockade effectively suppressed nerve regeneration [292, 293]. The source of VEGF in the corneal abrasion model is infiltrating T-cells and neutrophils [292]. Taken together, these studies indicate that corneal nerves, where damaged or accompanied by inflammation or when

lost entirely, lose their ability to express angiostatic factors and instead express proinflammatory and regenerative factors, contributing to enhanced corneal neovascularization. These effects can be considered part of the normal wound healing response, and may thus explain why the cornea in AAK, which is in a chronically inflamed wound-like state, is characterized by a deficit of nerves and an abundance of blood vessels. Potential therapeutic approaches supplying deficient factors such as VEGFR3 or PEDF, or blocking factors aiding neovascular growth such as SP or VEGF, warrant further investigation in relevant *in vitro* and *in vivo* aniridia models.

Given the role of corneal nerves in maintaining avascularity and epithelial integrity, restoration of a healthy corneal nerve population may represent a viable therapeutic approach for AAK. As described above, autologous serum eye drops have been shown to relieve symptoms of neuropathic corneal pain and increase the abundance of corneal nerves in non-aniridia subjects [294]. In subjects with toxic corneal epitheliopathy induced by anti-glaucoma eye drops, a course of autologous serum drops significantly improved corneal sensitivity [295]. In a cohort of thirteen subjects with AAK, autologous serum eye drops instilled over an 8-week period resulted in subjective improvement in keratopathy symptoms and healed corneal epithelial defects in several cases; however, neovascularization and stromal scarring did not significantly improve during the course of treatment [296]. Longer-term treatment, careful patient selection (e.g., based on AAK grade) and/or use of molecules with specific nerve regenerative capacity may be of benefit in AAK. The recently approved recombinant human nerve growth factor (NGF) treatment (Cenegermin) may be of interest in this regard. Indicated for treatment of moderate to severe neurotrophic keratopathy (a rare disease characterized by diminished corneal innervation, non-healing epithelial wounds and corneal ulceration), NGF stimulates corneal epithelial cell growth and survival, aids in the maintenance of limbal stem cell function, promotes tear production and supports corneal re-innervation [297]. In clinical studies, 65 - 75% of patients receiving an 8-week course of Cenegermin eye drops exhibited complete corneal healing, although relapse occurred in about 20% while eye pain and reduced visual acuity were frequently reported adverse effects [297, 298]. Notably, it has also been shown that corneal subbasal nerve density significantly increased following an 8-week course of Cenegermin [299]. Whether these promising effects could be extended to AAK is unknown and would need to be explored; however, use of Cenegermin for conditions other than neurotrophic keratopathy is currently off-label. Here, animal models would be useful for initial investigations.

3 Molecular biology and genetics perspective

Due to the many observed *PAX6* mutations independently leading to aniridia and the heterogeneous clinical phenotype, it is difficult to associate specific mutations to AAK severity [4]. However, some classes of mutations (selected missense or non-coding mutations) are associated with reduced AAK progression and mild or absent LSCD [29]. Comparative OMICs of such patient samples could help elucidate correlations between specific mutations and degree of AAK severity at the molecular level [125, 300]. Nonsense mediated decay (NMD) of mutant mRNA is the most common mechanism of PAX6 protein deficiency, but there is no evidence this is modulated by the position of mutations in the transcript. The effect of missense mutation is difficult to predict but some *in vitro* studies described the effect of the mutation in different binding domains. It might not be useful to compare different missense mutations with each other since they could have different properties. For *PAX6* run-on mutations (with a mutated stop codon), it is thought (but not yet demonstrated) that the mutated PAX6 protein is degraded

or not produced. Mutations in regulatory domains of *PAX6* (also found as 3'-cis-regulatory region deletions) are interesting since PAX6 reduction could be less pronounced, and these mutations have been related to milder phenotypes [4, 206, 300]. In rare cases, genes other than *PAX6* can putatively cause aniridia [301-303]. Primary culture of patient cells (both epithelial and mesenchymal) should help to identify deregulated genes important for AAK development especially in cases of aniridia with mild AAK, to discriminate from *PAX6*-dependent expression changes that are not causative of AAK [125]. Recent comprehensive reviews summarize in detail the genetics of congenital aniridia [1, 2, 304]. Although the impact of *PAX6* levels and splice variants on iris and ciliary body development have been systematically studied [155], we lack such detailed knowledge for LSCs and corneal epithelial cells. Also, PAX6 protein has been described to be located in the nucleus, cytosol or even in secreted form. It must be further evaluated how localization of PAX6 is controlled by post-translational modifications. *PAX6* mRNA expression may be not as dramatically altered as at the protein level, and thus mRNA expression and translation of *PAX6* need to be studied in detail in cells such as LSCs and differentiated epithelium. Interestingly, it has recently been shown that *Pax6* expression is negatively regulated by the microRNAs miR-7 and miR-135, and that protection of this inhibitory mechanism was capable of restoring PAX6 protein levels in isolated pancreatic islets in an aniridia mouse model [305]. It remains to be determined whether a similar regulatory mechanism also occurs in LSCs. Nevertheless, these data, in addition to the putative target miR-204-5p discussed earlier, appear to suggest that RNA-based therapies could represent a potential innovative therapeutic strategy for AAK.

4 Conclusions for further research strategies

Nearly all patients with aniridia suffer from AAK [4]. The onset of clinically apparent AAK differs between individuals even with the same mutations. Based on our present level of knowledge, it is not clear if AAK is caused by LSCD while it seems likely that some loss of LSC function occurs, it is not necessary to invoke LSCD as the main cause of the phenotype seen in AAK eyes. Clinical microscopy findings suggest that a minimal degree of keratopathy is likely to exist in all cases of aniridia even before ocular surface changes become visible at the slit lamp. This ‘minimal keratopathy’ includes reduced mechanical touch sensitivity, a deficit of corneal nerves, and increased inflammatory cell presence in the central cornea [4]. LSCD may in fact be a consequence of the early minimal keratopathy in subjects where the specific *PAX6* mutation predisposes the cornea to a progressive AAK phenotype. The minimal keratopathy may in turn have a developmental origin. The chronic wound healing pathology of the corneal epithelium in AAK may overwhelm the $PAX6^{+/-}$ limbal regenerative potential during normal life [153, 154]. Essential knowledge, however, is still lacking concerning the critical factors needed to specify and maintain the limbal niche and how these relate to PAX6 expression both prenatally and postnatally; therefore, pathogenic mechanisms at the molecular level are still speculative. Early indications are that *PAX6* regulation of and by other genes and factors is complex, and multiple signaling pathways, molecular and cellular mechanisms and feedback loops appear to be active, resulting in the observed AAK phenotypes. Deciphering some of the key pathways and mechanisms involved can provide insights that will be important for future and new therapies targeting AAK, keeping in mind that the complexity itself may provide multiple potential therapeutic targets.

Declaration of competing interest:
No conflicting relationship pertaining to this work exists for any author.

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