Reduced Corneal Thickness and Enlarged Anterior Chamber in a Novel ColVIIIa2<sup>G257D</sup> Mutant Mouse

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PURPOSE. The purpose of this study was the morphologic and genetic characterization of the novel eye size mutant Aca23 in the mouse.

METHODS. The eyes of the mutants were characterized in vivo by optical low-coherence interferometry, Scheimpflug imaging, and funduscopy. Visual acuity was examined using a virtual optomotor system. Morphology was studied by histology, in situ hybridization, and immunohistochemistry. Linkage analysis was performed using genomewide scans with single nucleotide polymorphisms and microsatellite markers.

RESULTS. Aca23 is a new semidominant eye size mutant that was discovered in an ENU mutagenesis screen. The phenotype includes increased anterior chamber depths, extended axial lengths, and reduced thickness of corneal layers. Aca23 was mapped to chromosome 4. A G→A point mutation was identified at cDNA position 770 of Col8a2 encoding collagen VIII α2. The transition results in a G257D amino acid exchange affecting a highly conserved glycine residue in the collagenous domain. Proliferation of corneal endothelium, eye fundus, and visual acuity are not affected.

CONCLUSIONS. The mouse mutant Aca23 described here offers the first point mutation of the Col8a2 gene in the mouse. The results of this study suggest that a functional collagen VIII α2 is essential for the correct assembly of the Descemet’s membrane and for corneal stability. Aca23 might be used as a novel model for keratoglobus. (Invest Ophthalmol Vis Sci. 2009;50:5653–5661) DOI:10.1167/iovs.09-3550

More than 161 million people worldwide are blind or suffer from severe visual disorders. In most cases, pathologies of ocular tissues negatively affect visual properties. This includes irregularities of different eye size parameters such as axial length of the entire eye globe (microphthalmia/macrophthalmia), lens size (microphakia/macrophakia), or anterior chamber depth. Congenital eye size diseases very often occur in addition to further systemic abnormalities inside and outside the eye rather than as isolated pathologic phenotypes.

The eye size-related disease with the highest frequency is microphthalmia (incidence 0.6–2.3 per 10,000 births). A broad range of different syndromes associated with microphthalmia have already been described. However, only few genes have currently been identified to play an essential role in the development of this phenotype. SOX2 was found to be mutated in approximately 10% of patients with microphthalmia/anophthalmia. Other causative transcription factor genes include MITF, PAX6, RAX, CHX10, and Otx2 (for a recent review, see Verma and FitzPatrick).

In contrast to microphthalmia, lens size abnormalities are usually rare diseases with an incidence between 1 per 10,000 births and a few dozen patients worldwide. Microphakia is part of the Warburg micro syndrome (WARBM1). Marfan’s syndrome (MFS), rhizomelic chondrodysplasia punctata (RCDP), and ectopia lentis et pupillae (ELP)6–8. The causative gene for each of these syndromes has been identified. RAB3GAP, encoding RAB3 GTPase activating protein, is affected in patients with WARBM1. MFS cases result from alterations of the fibrillin-1 gene (FBN1). Furthermore, RCPD is caused by mutations in the PEX7 gene,13–15 which codes for the peroxisomal type 2 targeting signal (PTS2) receptor.

Congolet anterior chamber depth pathologies are typically observed in glaucomatous syndromes and corneal diseases. Abnormally large anterior chambers were described in patients with primary congenital glaucoma (buphthalmos). Buphthalmos was demonstrated to be caused by mutations of the human cytochrome P4501B1 gene (CYP1B1). A shallow anterior chamber is characteristic for autosomal dominant and recessive cornea plana (CNA1/CNA2) in which the cornea is opaque, flattened, and of a low refractive power. CNA1, but not CNA1, was identified to be caused by mutations in the keratanase-encoding gene KERA. Furthermore, keratoglobus was identified in patients with posterior polymorphous corneal dystrophy (PPCD), a rare corneal endothelial disease caused by mutations in the transcription factor genes TCP9 and Zeb1 and in the collagen type VIII gene COL8A2. Even though various eye size-associated mutations have already been described, linkage studies point to a broad genetic heterogeneity with additional, not yet identified, loci and alleles. The fact that causative mutations are still completely unknown in many eye size associated-syndromes further indicates the necessity to search for additional mutations. To learn more about causative genes and alleles, we are currently screening for novel abnormal eye size mouse models within a dominant ENU (N-Ethyl-N-nitrosourea) mutagenesis program. In this study, we present the first characterized eye size mutant line Aca23, which exhibits significantly larger anterior chamber depths, longer axes, and thinner corneas than determined for wild-type mice. The causative mutation maps to chromosome 4 and represents a new missense mutation in Col8a2, encoding collagen type VIII. Funduscopic parameters and visual properties are not altered.
Males. (phenotypic deviations were tested for a dominant mode of inheritance. at the age of 11 weeks for abnormalities of eye size. Mice with C57BL/6J mice.34 The offspring of the ENU-treated mice were screened with an intraperitoneal injection of ketamine (137 mg/kg) and xylazine (6.6 mg/kg). The anesthetized mouse was placed on a platform and LEDs arranged in a circle that had to be placed in the center of the pupil. Central measurements of corneal thickness, anterior chamber depth, lens thickness, and axial length as well as data evaluation were performed essentially as described.35,37

**Figure 1.** Eye size analysis. Mean anterior chamber depth and axial length were enlarged in Aca23/-/+ (A/+: hatched columns) and Aca23/-/- (A/A; white columns) compared with the C57BL/6J control (wild-type [WT]; black columns). Corneal thickness was reduced in the mutants. (a) Males. (b) Females. Significant differences are marked by asterisks (*P < 0.001; t-test).

**Materials and Methods**

**Mice**
Mice were kept under specific pathogen-free conditions at the Helmholtz Center Munich. The use of animals was in accordance with the German Law of Animal Protection and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male C57BL/6J mice were treated with ENU (80 mg/kg body weight applied by intraperitoneal injection in three weekly intervals) at the age of 10 to 12 weeks according to Ehling et al.36 and were mated to untreated female C57BL/6J mice.34 The offspring of the ENU-treated mice were screened at the age of 11 weeks for abnormalities of eye size. Mice with phenotypic deviations were tested for a dominant mode of inheritance.

**Eye Size Determination**
The eye sizes of the Aca23 mutants were examined at the age of 11 weeks using optical low-coherence interferometry (ACMaster; Meditec, Carl Zeiss, Jena, Germany). Briefly, each mouse was anesthetized with an intraperitoneal injection of ketamine (137 mg/kg) and xylazine (6.6 mg/kg). The anesthetized mouse was placed on a platform and oriented in an appropriate position using light signals from six infrared LEDs arranged in a circle that had to be placed in the center of the pupil. Central measurements of corneal thickness, anterior chamber depth, lens thickness, and axial length as well as data evaluation were performed essentially as described.35,37

**Funduscopy**
Mice at the age of 9 months were administered 1% atropine to each eye to dilate their pupils. The fundus examination was performed with an indirect ophthalmoscope (Heine Sigma 150K; Haag-Streit GmbH, Wedel, Germany) and a super field lens (Volk 90D; Haag-Streit GmbH). Digital fundus images were taken with an indirect ophthalmoscope (Heine Video Omega 2C; Haag-Streit GmbH) connected to a video grabber (VRmAVC; Dieter Mann GmbH, Mainaschaff, Germany) and a 40-D or 60-D lens (Volk; Fronhäuser GmbH, Unterhaching, Germany). The images were imported in an image-processing program (Photoshop 10.0; Adobe, Unterschleissheim, Germany).

**Scheimpflug Imaging**
Scheimpflug images were taken from 3-month-old Aca23 mice using a digital camera system (Pentacam; Oculus GmbH, Wetzlar, Germany). Each mouse was anesthetized with an intraperitoneal injection of ketamine (137 mg/kg) and xylazine (6.6 mg/kg). For imaging, mice were positioned on a platform such that the vertical light slit (light source: LEDs; 475 nm) was orientated in the middle of the eyeball. Fine adjustment of the distance between eye and camera was undertaken.
with the help of the provided software to guarantee optimal focus. Subsequently, measurements were taken manually.

**Virtual Vision Test**

Vision tests were performed between 9 AM and 4 PM using a virtual optomotor system (Cerebral Mechanics, Lethbridge, AB, Canada), as described previously. Briefly, a rotating cylinder covered with a vertical sine wave grating was calculated and drawn in virtual three-dimensional space on four computer monitors facing to form a square. Visually unimpaired mice track the grating with reflexive head and neck movements (head-tracking). Visual acuity of the tested mice was quantified by a stepwise increase of the spatial frequency (0.3, 0.319, 0.342, 0.364, 0.386, 0.414, 0.439, 0.469, and 0.5 cyc/deg) until no optomotor response could be detected. The highest spatial frequency that the tested mouse could track was identified as the threshold for visual acuity. To avoid habituation, rotation direction was changed that the tested mouse could track was identified as the threshold for visual acuity. To avoid habituation, rotation direction was changed.

**Histology**

Eyes of 3-month-old mice were histologically analyzed for cornea, retina, and optic nerve head pathologies. The eyes were fixed for 7 days in Davidson solution and embedded in plastic medium (JB-4; Polyscience Inc., Eppelheim, Germany) according to the manufacturer’s protocol. Sectioning was performed with an ultramicrotome (OMU3; Reichert-Jung, Walldorf, Germany). Serial transverse 3-μm sections were cut with a glass knife and stained with methylene blue and basic fuchsin.

**In Situ Hybridization**

In situ hybridization of sections from postnatal day (P) 7 was performed essentially as described by Grimm et al. Briefly, the embryos were fixed in 4% paraformaldehyde and embedded (Jung Histowax; Cambridge Instruments, Nussloch, Germany). Sections (7–10 μm) were cut with a microtome (RM-2065; Leica, Nussloch, Germany) and mounted onto slides. The sections were evaluated with a light microscope (Axioplan; Carl Zeiss). Images were acquired by means of a scanning camera (AxioCam; Jenoptik, Jena, Germany) and imported into an image-processing program (Photoshop 10.0; Adobe, Unterschleishheim, Germany).

**Immunohistochemistry**

Immunohistochemistry was performed with 2-μm paraffin sections from eyes of 3-month-old mice on an automated immunostainer, as described previously. Sections were incubated with a 1:800 diluted primary keratin 12 polyclonal antibody (TransGenic Inc., Kobe, Japan) and were counterstained with hematoxylin. Anti-rabbit biotinylated IgG (Vector, Burlingame, Canada) was used as the secondary antibody.

**Mapping**

Heterozygous carriers (first generation) were mated to wild-type C3HeB/FeJ mice, and the offspring (second generation) were backcrossed to wild-type C3HeB/FeJ mice. DNA was prepared from tail tips of affected offspring of the third generation (G3), as described previously. For genomewide linkage analysis, genotyping of a genomewide mapping panel consisting of 149 single nucleotide polymorphisms was performed using a MALDETOF (matrix-assisted laser desorption ionization, time of flight analyzer) mass spectrometry high-throughput genotyping system supplied by Sequenom (MassExtend; San Diego, CA). Fine mapping of the Aca23 mutation was performed.
with the microsatellite markers D4Mit279, D4Mit338, D4Mit249, D4Mit73, D4Mit336, and D4Mit293.

**PCR and Sequencing**

For the molecular analysis, RNA was isolated at embryonic day (E) 12.5 from heads of Aca23/H11002/H11002 or wild-type embryos (C57BL/6J) with the RNA-Bee kit (AMS Biotechnology, Abingdon, UK). RNA samples were reverse transcribed to cDNA using a T-primed first-strand kit (ReadytoGo; GE Healthcare, Freiburg, Germany). Genomic DNA was isolated from the tail tips of wild-type C57BL/6J mice or homozygous/heterozygous mutants according to standard procedures.

PCR of the mutated site was performed with the primers Col8aIII-1 (5′-CAGGGCCTCCAGGATTTAG-3′) and Col8aIII-2 (5′-CTCTCCTGGTACCTCTGT-3′) using a PTC-225 thermocycler (MJ Research, Waltham, MA). Products (491 bp) were analyzed by electrophoresis on a 1.5% agarose gel. Sequencing was performed commercially (GATC Biotech, Konstanz, Germany) after direct purification of the PCR products (Nucleospin Extract II; Macherey-Nagel, Düren, Germany). For restriction site genotyping, PCR products were digested by Hpy188III. Cleavage into two subfragments (240 bp and 251 bp) indicated the Aca23-specific G→A exchange.

**RESULTS**

**Phenotype of Aca23**

The Aca23 mutant was detected in a dominant ENU mutagenesis screen because of its significantly enlarged anterior chamber depths, longer axes, and reduced corneal thicknesses. The mutation has complete penetrance. The litter size in the matings of heterozygotes and homozygotes indicated normal fertility and viability of the mutants.

Further eye size measurements indicated a semidominant mode of inheritance. In Aca23/H11002/H11002 animals, values for anterior chamber depth and corneal thickness were significantly less affected (P < 0.001) than in homozygous animals but were significantly altered in comparison with the wild types (P < 0.001; Fig. 1a, b). In both cases, the cornea does not develop opacities and remains transparent at least until 15 months of age. Scheimpflug investigations identified a globoid, keratoglobus-like protrusion of the anterior chamber in Aca23 mice (Fig. 2). More detailed analysis demonstrated clearly that the observed enlarged axis is a consequence of this protrusion. The increased axial length reflects exactly the increase of the anterior chamber depth, and the distance between anterior lens and retinal pigment epithelium was not significantly altered in Aca23/H11002/H11002 mice (males, 2.992 ± 0.031 mm; females, 2.986 ± 0.024 mm) compared with wild-type mice (males, 2.999 ± 0.035 mm; females, 2.950 ± 0.030 mm).
the mutants. Scale bars, 20 μm. 

**FIGURE 5.** Histology of the cornea and in situ hybridization. (a) In 3-month-old mice, thickness of corneal epithelium (Ep), stroma (S), and Descemet’s membrane (DM) is reduced in Aca23<sup>+/−</sup> (A/++; middle) and Aca23<sup>+/−</sup> (A/A; right) compared with the C57BL/6j control (wild-type [WT]; left). En, endothelium. Scale bars, 10 μm. (b) The expression pattern of Col8a2 at P7 in the posterior cornea (arrow) of Aca23<sup>+/−</sup> (A/+) and Aca23<sup>+/−</sup> (A/A) is comparable to that in C57BL/6j control (wild-type [WT]). Signal intensity appears weaker in the mutants. Scale bars, 20 μm.

**Genotype of Aca23**

We performed a genomewide linkage analysis by crossing homozygous mutants on a C57BL/6j background (G1) to wild-type C3HeB/FjJ mice. Heterozygous mutants (G2) were backcrossed to C3HeB/FjJ mice. The SNP-based analysis of 73 G3 offspring revealed linkage of the Aca23 phenotype to the distal part of chromosome 4 within an interval spanning more than 30 Mb (between rs3022989 and rs3023011; cytoband C6-D3). Further fine-mapping using 203 G3 mice located the Aca23 mutation between the flanking microsatellite markers D4Mit249 and D4Mit73 (Fig. 3). Based on these data, the genetic order was calculated (genetic distance ± SD in parenthesis): D4Mit249 (0.49 ± 0.49 cm), Aca23 (1.48 ± 0.85 cm), D4Mit73. The critical interval of approximately 1.0 Mb includes 35 genes. Eighteen of them are predicted only because of the existence of ESTs, but they are not yet fully annotated.

Sequence analysis of the candidate gene Col8a2 in a heterozygous Aca23 mouse detected a G→A exchange at cDNA position 770 (counting the starting ATG as position 1; Fig. 4a). This transition generates an Hpy188III restriction site (TC-NNGA), which was confirmed in the genomic DNA of all tested homozygous Aca23 mutants (n = 5). In contrast, all tested wild-type strains (C3H, C57BL/6, BALB/c, DBA, and CFW) lacked this restriction site, demonstrating that the observed exchange does not represent a general polymorphism (Fig. 4b).

The 770 G→A transition is predicted to cause an exchange of Gly to Asp at amino acid position 257 of the ColVIIIa2 protein. This residue lies within the highly conserved collagenous domain of ColVIIIa2 (position 137–532), which consists of 136 (Gly-X-Y) units (X and Y = any amino acid). It represents the first position of unit 45.

**Histologic Consequences of Aca23**

To investigate pathologic mechanisms initiated by the Aca23 mutation, we performed histologic studies of the eyes of 3-month-old mice. Structural alterations at the cellular level of iris, trabecular meshwork, and lens were not found in Aca23 mice (data not shown). In the cornea, we observed a reduced size of epithelium, stroma, and Descemet’s membrane (Fig. 5a). This was more pronounced in Aca23<sup>+/−</sup> than in Aca23<sup>+/−</sup>. The corneal endothelium remained single-layered in all tested eyes. In situ hybridizations revealed the expression of Col8a2 in the Descemet’s membrane and the endothelial layer of the wild-type cornea (Fig. 5b). The Col8a2 expression pattern was not altered in heterozygous or homozygous individuals.

To investigate the integrity of the corneal endothelium, we performed keratin12 immunohistochemistry. In both wild types and mutants, expression of this epithelial marker was limited to the superficial and suprabasal epithelial layers of the anterior cornea (Fig. 6). These data excluded a transformation of corneal endothelium into multilayered epithelial like cells because it is associated with particular mutations in the human COL8A2 gene resulting in glaucoma from blocking of the trabecular meshwork.

Detailed histology findings of the retina and optic nerve head, pathologies of which are often associated with glaucoma, were normal. The inner retinal layers were present, and damage of the optic nerve fibers was not detectable in the retinal sections of the mutants (Fig 7a). Furthermore, a characteristic thick nerve fiber layer entered the optic nerve head (Fig. 7b). These findings were confirmed by ophthalmoscopic investigations of 9-month-old Aca23<sup>+/−</sup> and Aca23<sup>+/−</sup> animals. All tested mutants revealed a homogenously pigmented...
fundus with a well-defined vessel pattern comparable to the wild-type fundus. Enlargement of the optic disc, typical for glaucoma, was not found (Fig. 7c).

Visual Acuity of Aca23

In humans, corneal protrusion is generally associated with strong myopia. To study consequences of the anterior protrusion on visual properties of Aca23 mice, vision tests with the virtual optomotor system were performed with 2- to 8-month-old mice. The response of Aca23−/− and Aca23+/+ mice clearly demonstrated that visual properties were not affected. All tested mice responded normally to the moving stripe pattern. A median spatial frequency threshold of $x_{0.5}=0.414$ cyc/deg, a 25% quantile of $x_{0.25}=0.414$ cyc/deg, and a 75% quantile of $x_{0.75}=0.439$ cyc/deg was observed for Aca23−/−. Aca23−/− individuals revealed a median threshold of $x_{0.5}=0.414$ cyc/deg, a 25% quantile of $x_{0.25}=0.386$ cyc/deg, and a 75% quantile of $x_{0.75}=0.414$ cyc/deg. These data did not differ significantly from the visual acuity of the C57BL/6J control ($x_{0.5}=0.414$ cyc/deg; $x_{0.25}=0.439$ cyc/deg; Fig. 8).
Discussion

We have established and characterized a novel mouse mutant, Aca23, with abnormalities of the anterior eye segment. The phenotype includes larger anterior chambers and axes and thinner corneas. Histologic analysis confirmed a reduced size of corneal epithelium, stroma, and Descemet’s membrane. This putatively affects the resistance of the cornea to the natural intraocular pressure, which might result in the observed anterior protrusion.

Genomewide linkage analysis and fine mapping placed the Aca23 mutation on chromosome 4 within a 1.0-Mb interval containing 35 coding genes. Two of them were described to be responsible for ocular disorders: Col8a2 coding for a collagen type VIII protein and the neurochondrin gene Ncdn. The latter was excluded as a candidate because of its close distance of approximately 0.05 Mb to the flanking marker D4Mit73, which did not fit to the calculated distance of 1.48 ± 0.85 cM between D4Mit73 and Aca23. Concerning Col8a2, various missense mutations of the human COL8A2 gene were described to initiate posterior polymorphous corneal dystrophies. Furthermore, targeted inactivation of Col8a1/Col8a2 has been reported to cause a thinning of corneal stroma and Descemet’s membrane in the mouse. Considering these data, it is likely that the observed missense mutation at cDNA-position 770 of Col8a2 represents the causative mutation of the Aca23 phenotype. Consequently, Aca23 is the first described pathogenic point mutation in the murine Col8a2 gene. The affected glycine residue is highly conserved and homologous to position 261 of the human COL8A2. Missense mutations at this position have not yet been identified in humans with corneal endothelial dysstrophies. Therefore, Aca23 demonstrates for the first time that this residue is essential for the functionality of Col8a2. Moreover, this is the first described pathogenic mutation affecting the first position of a (Gly-X-Y) collagen triple helix repeat subunit.

Type VIII collagen is a major component of specialized extracellular matrices such as sclera and skin for a review, see Shuttleworth). However, malformations seem to be restricted to the corneal layers in Aca23 animals. In the wild-type Descemet’s membrane, the collagen type VIII molecules are assembled into a characteristic hexagonal lattice structure, which is formed by two distinct polypeptides, α1(VIII) and α2(VIII), encoded by the genes COL8A2 and COL8A1. A putative misfolding of Col8a2Col8a1 could disrupt the lattice structure by preventing coordinated coassembly of both building blocks. The result would be an irregular mosaic, in which Col8a1 putatively predominates. Furthermore, incorporation of Col8a2Col8a1 could be completely inhibited. The observed structural effects might be potentiated by an altered expression of the mutated Col8a2, which seems to be reduced in Aca23.

Various point mutations in the human collagen type VIII A2 gene are associated with two distinct corneal dystrophies, FECD (OMIM 136800) and PPCD (OMIM 122000). Both forms share many features including a thickened Descemet’s membrane caused by the secretion of a pathologic collagenous layer and an altered morphology of the corneal endothelium. In particular, endothelial wartlike guttate are typical for FECD. This was not found in Aca23. In PPCD, corneal endothelium is replaced by aberrant epithelial-like cells, which might initiate secondary glaucoma by extending into the anterior chamber and blocking the trabecular meshwork. However, our immunohistochemical and histologic data further excluded these specific symptoms. Aca23, therefore, does not seem to represent a model for classical human FECD or PPCD. Rather, it combines clinical features of keratoglobus, which is generally defined by bilateral protrusions, with a yet undescribed corneal pathology. A comparable combination has been reported for a human patient with keratoglobus with posterior polymorphous dystrophy.

The Aca23 phenotype described in this study resembles ocular pathologies of a Col8a2Col8a1 null mutant, which was also reported to develop keratoglobus-like protrusions and irregularly thin corneal stromas and Descemet’s membranes. Stromal thinning was suggested to be initiated by an abnormal migration of precursor cells during corneal development because of the lacking collagen VIII α1 and α2. The fact that Col8a2 causes comparable effects on stromal thickness demonstrates an essential role of Col8a2 in these yet unknown mechanisms. Concerning the Descemet’s membrane, the reduced thickness observed in both mouse mutants is in contrast to the basal membrane thickening of human corneal dystrophies. Obviously, structural consequences of altered collagen VIII are not the same in the corneas of mice and humans. This might point to interspecies differences in collagen function and corneal development. Further experiments are required to understand these putatively alternative pathways.

Because the cornea contributes most of the focusing power in the human eye, myopia is the most common refractive disorder associated with anterior segment enlargement. However, anterior chamber protrusion does not influence visual properties in Aca23 mice. Even homozygous individuals responded well in the virtual vision test and were still able to resolve spatial frequencies up to 0.469 cycles/deg. This further demonstrates that a refractive error of a few diopters, as is expected for Aca23, is less relevant for the vision of the mouse because of a generally high refractive power of murine cornea and lens of approximately 500 diopters. Consequently, Aca23 would not have been detected as an eye-related mutant in a screening approach based on a vision test. This emphasizes the benefits of the fast and reliable low-coherence interferometry technique for the establishment of eye size-associated disease models.

Conclusion

We identified a new mouse mutant that represents a model for keratoglobus. It is further characterized by a pathologic phenotype of the corneal Descemet’s membrane. Molecular analysis characterized the first point mutation in the murine Col8a2 gene encoding collagen type VIII α2. This mutation might influence the assembly of the corneal basement membrane. Visual acuity is not altered by the resulting anterior globoid protrusion.

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References


