Strain-Dependent Anterior Segment Dysgenesis and Progression to Glaucoma in Col4a1 Mutant Mice

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目的。突变基因编码的Ⅳ型胶原α1（COL4A1）导致多系统疾病，包括前段结构发育不全（ASD）和视神经变性。渗透性和严重性是不同表型的个体特征，取决于遗传背景。在这里，我们测试了COL4A1突变在两种不同遗传背景下的效果，以比较遗传背景是如何影响眼发育不全、眼压和视神经的。

方法。Col4a1突变小鼠在C57BL/6J背景上培养，与CAST/EiJ和F1杂交后，通过裂隙灯显微镜检查和光学相干断层成像测量眼内压和比较眼组织切片的视神经轴突丢失和视神经的崩溃。

结果。我们发现CAST/EiJ近交系小鼠的表型特征是相对均匀和程度深的，突变CASTB6F1近交系小鼠一般影响非常轻微。与其他小鼠相比，129B6F1小鼠的ASD和眼压异常较严重，与伴有视神经损伤的视神经轴突丢失和视神经的崩溃。

结论。Col4a1突变小鼠的表型特征与ASD和视神经损伤有关，且不同近交系小鼠对基因的反应程度不同。这些数据表明遗传背景差异在ASD和视神经损伤的可变性中起着一定作用。

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and, accordingly, mutations in either gene cause multisystem disorders.\(^5\,^6\) Although often cited for their involvement in cerebrovascular disease including porencephaly and hemorrhagic stroke, \textit{Col4a1} and \textit{Col4a2} point mutations were first identified in independent forward genetic screens in mice with cataracts and ocular dysgenesis.\(^7\,^9\) Homozygous mutant mice are rarely viable,\(^7,^8,^10\) and complete \textit{COL4A1C}OL4A2 deficiency is lethal.\(^11\) Moreover, the absence of an obvious phenotype in mice that are heterozygous for null mutations of both genes suggests that mutations typically act via a dominant negative mechanism.\(^11\) More recently, patients with \textit{COL4A1} mutations were reported with a variety of ASD phenotypes including microphthalmia, cataracts, Axenfeld-Rieger malformations and glaucoma.\(^5,^6,^12\,^13\)

The disease spectrum in patients is broad and severity varies, as does the presence or absence of extraocular findings. For example, while most patients both have ocular and nonocular findings, one large family was reported with only isolated, nonsyndromic congenital cataracts in all 15 affected members.\(^9\) It is clear that both allelic heterogeneity and genetic context influence the penetrance and severity of phenotypes caused by \textit{Col4a1} and \textit{Col4a2} mutations.\(^7\,^9,^24,^25\) In an earlier study, we described ASD and high IOP in mice with the \textit{Col4a1}	extsubscript{ex41} mutation (a splice acceptor mutation that skips exon 41) on a C57BL/6J (B6) genetic background.\(^9,^10\) However, \textit{Col4a1}	extsubscript{+/−} mice on a B6 background also had optic nerve hypoplasia (which is also reported in patients with \textit{COL4A1} mutations)\(^5\), and so we could not conclude whether or not these mice developed glaucomatous optic nerve damage. In \textit{Col4a1}	extsubscript{+/−} mice, both ASD and optic nerve hypoplasia are suppressed when the mice were crossed for a single generation (F1) to 129S6/SvEvTac or to CAST/EiJ (referred to as 129B6F1 and CASTB6F1, respectively).\(^9\) In another study of \textit{Col4a1} mutant mice ASD and optic nerve head cupping were reported but IOPs were not measured and the genetic context was not stated.\(^9\) In the present study, we aged 129B6F1 and CASTB6F1 mice and evaluated them for ASD severity, IOP elevation, and hallmarks of glaucoma. These strains were selected because they are genetically distinct from the original strain (B6) and highly divergent from each other.\(^26\) We made few notable exceptions, pathology in CASTB6F1 mice was uniformly and nearly completely suppressed, even in mice aged for over 30 months. In contrast, \textit{Col4a1}	extsubscript{+/−} 129B6F1 mice had highly variable ASD and hallmarks of glaucoma including age-related IOP elevation, loss and damage to retinal ganglion cell axons, and optic nerve head cupping. These findings indicate that \textit{Col4a1}	extsubscript{+/−} 129B6F1 mice represent a genetic model for age-related glaucoma and will be useful for understanding both anterior segment development and how ASD can progress to glaucoma.

**Materials and Methods**

**Animals**

The \textit{Col4a1}	extsubscript{+/−} mice were originally identified in a mutagenesis screen conducted at The Jackson Laboratory (Bar Harbor, ME, USA) and the ocular phenotypes have been previously described.\(^9,^10\) \textit{Col4a1}	extsubscript{+/−} mice that had been backcrossed to C56BL/6J (B6) mice for at least six generations were mated with CAST/EiJ (CAST) or 129S6/SvEvTac (129) mice to produce CASTB6F1 and 129B6F1 mice, respectively. All animals were maintained in full-barrier facilities free of specific pathogens on a 12-hour light/dark cycle with food and water ad libitum. All experiments were compliant with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco (CA, USA).

**Slit-Lamp Biomicroscopy and IOP Measurement**

Ocular anterior segment examinations were performed on \textit{Col4a1}	extsubscript{+/−} and \textit{Col4a1}	extsubscript{+/−} mice by observers masked to the genotypes using a slit-lamp biomicroscope (Topcon SL-D7; Topcon Medical Systems, Oakland, NJ, USA) attached to a digital SLR camera (Nikon D200; Nikon, Melville, NY, USA). Intraocular pressures were measured using the microneedle method as previously described.\(^27,^28\) Briefly, mice were anesthetized with ketamine (99 mg/kg) and xylazine (9 mg/kg) and placed on the measurement platform. Intraocular pressures were measured by inserting a microneedle into the anterior chamber within 3 to 4 minutes after sufficient anesthesia. All measurements were performed during the light cycle of the room and both male and female mice were used for each age group. We detected an effect of sex in 5- to 7-month-old 129B6F1 mice (2-way ANOVA; sex, \(P = 0.0002\); genotype, \(P = 0.4144\); sex and genotype interaction, 0.7316) in both \textit{Col4a1}	extsubscript{+/−} and \textit{Col4a1}	extsubscript{+/−} mice (post hoc Tukey’s multiple comparison test; \(P = 0.011\) and 0.018, respectively). We did not detect a sex effect for other groups and so we combined the data from both sexes.

**Histologic Analysis**

**Iridocorneal Angles and Optic Nerve Heads.** Eyes were harvested at appropriate ages and fixed in situ with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.2), dehydrated in graded ethanol and embedded in fresh Historesin (Leica, Heidelberg, Germany). Embedded tissues were sectioned and stained with hematoxylin and eosin (H&E). The extent of damage is indicated using a 4-tiered scale as described previously.\(^29\) Briefly, three slides, each containing eight sections (\(n = 24\) sections), were analyzed from different regions of the eye and the assigned grades were averaged. Scoring was done without knowledge of the mouse genotype and used a numerical scheme (0, 1, 2, or 3).

**Optic Nerves.** Postorbital, intracranial portions of optic nerves were processed and analyzed as previously described.\(^30\) Briefly, the top of the skull and most of the brain overlying the optic nerves were removed and the remaining tissue was fixed overnight with 0.8% PFA and 1.2% glutaraldehyde in 0.1 M phosphate buffer. The tissue was embedded in Embed 812 resin (Electron Microscopy Sciences, Ft. Washington, PA, USA). One-micron cross sections of postorbital optic nerve were stained with paraphenylenediamine (PPD). Paraphenylenediamine differentially stains the axoplasm of sick or dying axons darkly, thus permitting detection of axon injury. An optic nerve grading system was used as previously described\(^31\) to determine the level of glaucomatous damage. The damage level accounts for several factors including the number of healthy axons remaining, the number of damaged axons, and the amount of scarring associated with gliosis. All nerves were scored by at least two investigators masked for the age and genotype of the mouse. Both investigators were unaware of the grade assigned by the other investigator. When the grades from the two investigators did not agree, a third investigator graded the nerves and the most common grade was used as the final grade for the optic nerve.

**Funduscopy and Optical Coherence Tomography**

Mice were anesthetized with a steady flow of 1.5% to 3% isoflurane. The eyes were topicaly anesthetized with one drop of proparacaine, diluted with one drop of a 1:1 mixture of
analyzed by slit-lamp biomicroscopy. On the CASTB6F1 background, ASD in Col4a1 elevation.

eyes), and pigment on the lens (11/21 eyes). Frequency of severely enlargement of anterior chamber increases with age suggesting age-related IOP elevation. Not all phenotypes could be assessed in all eyes. Similar subphenotypes were also present in the 22-month-old cohort where we observed enlarged and tortuous iris vasculature (21/24 eyes), cataracts (4/27 eyes), enlarged anterior chambers (27/29 eyes; arrowheads in [F, J, L]), persistent pupillary membranes (25/26 eyes) and pigment on the lens (17/27 eyes). Note: sample sizes vary because not all phenotypes could be assessed in all eyes. Similar subphenotypes were also present in the 22-month-old cohort where Col4a1 exacerbated mice had abnormal iris vasculature (18/18 eyes), cataracts (3/21 eyes), enlarged anterior chambers (20/20 eyes), persistent pupillary membranes (13/21 eyes), and pigment on the lens (11/21 eyes). Frequency of severely enlargement of anterior chamber increases with age suggesting age-related IOP elevation.

Results

Clinical Phenotypes Are Dependent Upon Genetic Context

Our prior studies showed that both CASTB6F1 and 129B6F1 genetic contexts suppressed severe ASD and optic nerve hypoplasia typically seen in Col4a1+/loxP mice on the B6 background. However, even mild developmental defects might still be sufficient to contribute to progressive pathology as mice age. To determine if CASTB6F1 and 129B6F1 mutant mice developed glaucoma, we aged and analyzed cohorts of Col4a1+/+ and Col4a1+/loxP mice. Slit-lamp biomicroscopy of Col4a1+/+ and Col4a1+/loxP CASTB6F1 revealed relatively normal appearance of anterior ocular structures, yet mildly enlarged anterior chambers, in mutant eyes at all ages (Figs. 1A–D). While ASD in this genetic context was generally very mild, a few exceptions were observed. Out of 22 eyes analyzed at 30 months of age, one had severe cataract and enlarged anterior chamber, a second had iris coloboma and a third had corneal neovascularization (Supplementary Fig. S1). In the 129B6F1 genetic context, Col4a1+/loxP mice had much more variable and severe ASD at all ages (Figs. 1E–L). The most highly penetrant phenotypes were abnormally tortuous iris vasculature, persistent pupillary membrane, and pigment on the anterior surface of the lens. The frequencies of these phenotypes were similar regardless of age and enlargement of the anterior chamber was the most variable phenotype. Although almost all eyes had enlarged anterior chambers some mice had severely enlarged chambers (compare Figs. 1J and 1L). The frequency of severely enlarged eyes tended to increase with age suggesting high IOP may develop with age.

Iridocorneal Angles Show Variable Abnormalities of the Anterior Segment That Increase With Age

To better assess the effects of CASTB6F1 and 129B6F1 genetic contexts on the extent of pathology, we performed histologic analyses of the iridocorneal angles in eyes from Col4a1+/+ and Col4a1+/loxP mice (Fig. 2). Despite the relatively normal clinical appearance of the eyes from CASTB6F1 mutant mice, histologic analyses revealed pathology including anterior synchia and hypoplastic or absent Schlemm’s canal and trabecular meshwork (Figs. 2A–D). Consistent with our observations with slit-lamp biomicroscopy, the morphologic defects in Col4a1+/loxP 129B6F1 mice were more severe and included hypoplastic or absent Schlemm’s canal, hypoplastic trabecular meshwork, and hypoplastic ciliary body (Figs. 2E–H). Iridocorneal angles were also closed to varying extents and anterior synchia were present (Figs. 2F, 2H). The histologic phenotypes found in individual mice and aged cohorts demonstrate that significant variation was observed between individual eyes. Notably, although generally considered to be ‘developmental defects,’ by 22 months these pathologies not only persisted but also increased in severity indicating the occurrence of disease-related remodeling that is unrelated to development (Figs. 2I–K).

Aged 129B6F1 Mutant Mice Have Elevated IOP

The CASTB6F1 and 129B6F1 genetic contexts suppress ASD compared with Col4a1+/loxP on a B6 background. Despite this, mutant mice from both strains had histologic abnormalities in the iridocorneal angles and enlarged anterior chambers...
suggesting that they may also have ocular hypertension. To address this possibility we measured IOPs of Col4a1<sup>+/+</sup> and Col4a1<sup>+/Dex41</sup> mice from both genetic backgrounds over multiple ages (Figs. 3A, 3B). Consistent with the rescuing effect of the CAST background, CASTB6F1 mutant mice had IOP values and distributions that were nearly indistinguishable from control animals even when aged to 22 months (Mann-Whitney U test, $P = 0.928$) with the exceptions of one Col4a1<sup>+/Dex41</sup> eye with very low IOP and one with mildly elevated IOP (22.4 mm Hg). Overall the mean IOPs for both Col4a1<sup>+/+</sup> and Col4a1<sup>+/Dex41</sup> CASTB6F1 mice significantly decreased with age. In 129B6F1 mice, mean IOP values also decreased with age in Col4a1<sup>+/+</sup> mice. In contrast, an age-related IOP decrease did not occur in Col4a1<sup>+/Dex41</sup> mice, and the IOPs of Col4a1<sup>+/Dex41</sup> mice were higher than Col4a1<sup>+/+</sup> mice at 12 to 15 and 18 to 22 months of age ($P = 0.032$ and 0.039, respectively). In addition, the ranges of IOPs in eyes from Col4a1<sup>+/Dex41</sup> mice increased with age (5.5–24.1, 3.0–24.6, and 4.9–36.7 mm Hg for 5–7, 12–15, and 18–23 months of age, respectively). Importantly, we identified a number of Col4a1<sup>+/Dex41</sup> eyes that had elevated IOPs. At each age group, respectively, there were 2 (7.1%), 10 (23.8%), and 10 (17.2%) mutant eyes with IOPs that were greater than the highest measured age-matched control IOP (22.5, 20.2, and 19.8 mm Hg for control mice at 5–7, 12–15, and 18–23 months of age, respectively).

**Glaucoma Hallmarks in Col4a1<sup>+/Dex41</sup> Mice on the 129B6F1 Background**

Loss of, and damage to, retinal ganglion cell axons in the optic nerve and subsequent excavation of the optic nerve head are hallmarks of glaucoma. Col4a1<sup>+/Dex41</sup> on a B6 background had optic nerve hypoplasia that precluded assessment of glaucomatous optic nerve damage in these animals. However,
indistinguishable from those from their CASTB6F1 littermate controls (Figs. 4B vs. 4E); however, Col4a1+/ex41 129B6F1 mice were deemed to have optic nerve head excavation by OCT (Figs. 4K vs. 4H).

**DISCUSSION**

Here, we show that a Col4a1 mutation can cause ASD, age-related IOP elevation and glaucomatous neurodegeneration with characteristic retinal ganglion cell axon loss and optic nerve damage. To do this we sectioned optic nerves from CASTB6F1 and 129B6F1 mice and stained them with PPD, which differentially stains the axoplasm of sick or dying axons. Stained optic nerve sections were then graded for the extent of damage by at least two independent investigators that were masked to the genotypes (Figs. 3C–G). Although we observed moderately damaged optic nerves in both Col4a1+/ex41 and Col4a1+/ex41 mice aged for over 2 years we did not detect severely damaged nerves from CASTB6F1 mice of either genotype (Figs. 3C, 3D, 3G). In 129B6F1 mice we did not detect differences between optic nerves from Col4a1+/+ and Col4a1+/ex41 animals up to 5 months of age; however, we identified a portion of severely damaged optic nerves in older Col4a1+/ex41 mice. The numbers of damaged optic nerves were significantly different between Col4a1+/+ and Col4a1+/ex41 mice at 12 to 15 months and 18 to 23 months of age (Fisher’s exact test, P = 0.002 and P = 0.052, respectively; Figs. 3E–G).

Next, we tested whether optic nerve damage in Col4a1+/ex41 129B6F1 mice was accompanied by optic nerve head excavation. While the optic nerve heads of Col4a1+/+ and Col4a1+/ex41 CASTB6F1 mice (Figs. 4A–F) and Col4a1+/+ 129B6F1 mice (Figs. 4G–I) appeared normal, eyes from aged Col4a1+/ex41 129B6F1 mice revealed optic nerve head excavation (Figs. 4J–L). Hematoxylin and cosin-stained sections showed clearly visible loss of both the retinal ganglion cells and the nerve fiber layer and the optic nerve head was cupped and disorganized in 3/5 mutant 129B6F1 eyes. Finally, we used OCT to screen additional mice from both backgrounds for evidence of optic nerve head excavation. The optic nerve heads of Col4a1+/ex41 CASTB6F1 mice were indistinguishable from those from their Col4a1+/+ littermate controls (Figs. 4B vs. 4E); however, Col4a1+/ex41 129B6F1 mice were deemed to have optic nerve head excavation by OCT (Figs. 4K vs. 4H).
nerve head cupping in a subset of eyes. Genetic context modifies these age-related phenotypes, whereby 129B6F1 mutant mice had highly variable pathology including some eyes with relatively severe ASD. Although the majority of these mice had some level of ASD, only a portion of the 129B6F1 mice develops glaucoma. In the CASTB6F1 genetic context, ASD was strongly suppressed in the vast majority of Col4a1+/Dex41 mice. Notably however, at least three eyes had very severe phenotypes suggesting that even in genetically identical animals other factors can determine the phenotypic outcome. Our data also suggest that despite the obvious presence of developmental defects, iridocorneal angle malformations, and IOP elevation both progress with age. If this holds true in patients, then there may indeed be a therapeutic window for intervention to prevent or delay vision loss in patients with COL4A1 mutations.

To date, there are over 70 COL4A1 mutations identified in patients and approximately one-third of these are reported to have ASD or cataracts.3,6,15,17,20,23,52 In contrast, all 13 mutations in mice were identified primarily because of this phenotype. Both observations could reflect ascertainment biases. COL4A1 is often considered for its role in cerebrovascular disease and is only recently being considered as an ASD candidate gene. However, in mice, ASD is readily identified in forward genetic mutagenesis screens and a role in cerebrovascular disease could have more easily gone undetected. Given that COL4A1 and COL4A2 mutations are very likely to contribute to multisystemic disorders that include ASD (e.g., Muscle-Eye-Brain disease32) and not just isolated ASD, it is likely that patients with COL4A1 and COL4A2 mutations may be selected against by exclusion criteria in studies focused solely on identifying genes underlying more typical Axenfeld-

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**Figure 4.** Optic nerve head excavation in Col4a1+/Dex41 129B6F1 mice. Analyses of optic nerve heads from both Col4a1+/+ (A–C) and Col4a1+/Dex41 CASTB6F1 (D–F) revealed well-defined ganglion cells (arrow) and robust nerve fiber layers (arrowheads). In contrast, while control 129B6F1 eyes (G–I) had morphologically normal optic nerve heads in three of five Col4a1+/+ 129B6F1 eyes at 22 months (J–L), both the ganglion cell and nerve fiber layers were absent and the optic nerve head was cupped, a sign of severe glaucoma. Optical coherence tomography imaging is able to detect optic nerve cupping in vivo and, using OCT, we identified cupping in a portion of Col4a1+/Dex41 129B6F1 eyes. Among the 10 Col4a1+/Dex41 129B6F1 eyes independently examined by three investigators, six were unanimously considered as having optic nerve head cupping. In contrast, all Col4a1+/Dex41 CASTB6F1 eyes (n = 8) and eyes from Col4a1+/+ mice on either background (n = 8 and 6 for CASTB6F1 and 129B6F1, respectively) had normal optic nerve head morphology. Horizontal bars in fundus images (A, D, G, J) indicate the position of OCT scanning beams.
Rieger types of dysgenesis. With the ongoing shift toward analyzing all genes through exome or genome sequencing, rather than selecting candidates, we expect that the number of ASD patients with COL4A1 or COL4A2 mutations will continue to increase. Our data predict that patients with COL4A1 and COL4A2 mutations may range from having severe ASD and optic nerve hypoplasia to age-related ocular hypertension with or without RGC loss, or perhaps to no ocular pathology at all. However, both genes should be considered as candidate genes in patients with ASD, especially those where ASD is part of a multisystem disorder. PITX2 and FOXC1 mutations are the most common genetic causes of ASD identified. While proteins with which PITX2 and FOXC1 interact have been identified and cellular pathways have been implicated, the mechanisms underlying ASD are largely unknown. Thus, it remains unknown if COL4A1 mutations interact with Pitx2 and Foxc1 or if they represent a mechanistically distinct form of ASD. Like COL4A1 and COL4A2, the roles of PITX2 and FOXC1 in normal ocular development and function are still being fully understood, as are the mechanisms by which mutations in either gene cause ASD and glaucoma. PITX2 and FOXC1 are transcription factors that are expressed in overlapping subsets of periscleral mesenchyme cells that differentiate into the ocular anterior segment structures. In contrast, COL4A1 and COL4A2 are extracellular matrix proteins that are present in ocular basement membranes through development. Collagen processing enzymes, procollagen lysyl hydroxylases, which hydroxylate lysines in collagens, and thus creating carbohydrate binding sites and promoting the stability of the collagen network, have been demonstrated to be PITX2 transcriptional targets, and it is possible that the pathogenic pathways are shared and that the pathogenic effects of COL4A1 and COL4A2 mutations are genetically downstream of PITX2 and FOXC1. Alternatively, extracellular matrix molecules can act as instructive signals or regulators for many developmental processes and it is possible that COL4A1 and COL4A2 are genetically upstream of PITX2 and FOXC1. Furthermore, multiple additional extracellular matrix proteins and their modifying enzymes also contribute to ASD in mice and patients, including FBN2, LAMB2, heparin sulfate proteoglycans (AGRN), and heparan sulfate synthetase enzymes. Perhaps the most relevant is the recent identification that mutations in peroxidase, a key enzyme in extracellular organization of COL4A1 and COL4A2, cause congenital cataracts, ASD and developmental glaucoma in patients and in mice. The extents to which the pathogenic mechanisms involved in these cases are distinct or overlapping need to be explored.

The partial and nearly complete suppression of ASD and glaucoma in 129B6F1 and CASTB6F1 mice, respectively, demonstrates reproducible differential effects of the two backgrounds. One possibility is that both inbred strains carry the same genetic modifier but that CAST has additional properties that confer more profound suppression. A second possibility is that each strain has a different modifier locus (or loci). Interestingly, we have recently shown that the CASTB6F1 background suppresses intracerebral hemorrhages yet 129B6F1 had no effect, indicating that the capacity for genetic modification between the two strains differs for this particular phenotype. Current efforts are underway to identify the pathway(s) by which the genetic modifier(s) suppress pathology. Once identified, we can test the relevance of this pathway to ASD in other matrix and matrix-associated genes. Moreover, pharmacologic manipulation of this same pathway may represent a therapeutic alternative to prevent vision loss in ASD patients that do not respond to current treatments.

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References

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