Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development

Amanda L. Evans2 and Philip J. Gage1,2,3,*

1Department of Ophthalmology and Visual Sciences, 2Department of Cell and Developmental Biology and 3Department of Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

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Heterozygous mutations in the homeobox gene, PITX2, result in ocular anterior segment defects and a high incidence of early-onset glaucoma. Pitx2 is expressed in both the neural crest and the mesoderm-derived precursors of the periocular mesenchyme. Complete loss of function in mice results in agenesis or severe disruption of periocular mesenchyme structures and extrinsic defects in early optic nerve development. However, the specific requirements for Pitx2 in neural crest versus mesoderm could not be determined using these mice, and only roles in the initial stages of eye development could be assessed due to early embryonic lethality. To determine the specific roles of Pitx2 in the neural crest precursor pool, we generated neural crest-specific Pitx2 knockout mice (Pitx2-ncko). Because Pitx2-ncko mice are viable, we also analyzed gene function in later eye development. Pitx2 is intrinsically required in neural crest for specification of corneal endothelium, corneal stroma and the sclera. Pitx2 function in neural crest is also required for normal development of ocular blood vessels. Pitx2-ncko mice exhibit a unique optic nerve phenotype in which the eyes are progressively displaced towards the midline until they are directly attached to the ventral hypothalamus. As Pitx2 is not expressed in the optic stalk, an essential function of PITX2 protein in neural crest is to regulate an extrinsic factor(s) required for development of the optic nerve. We propose a revised model of optic nerve development and new mechanisms that may underlie the etiology of glaucoma in Axenfeld–Rieger patients.

INTRODUCTION

Glaucoma is a constellation of disease processes that result in vision loss due to changes in the optic nerve head and accompanying death of retinal ganglion cells (RGCs). Glaucoma often occurs in association with elevated intraocular pressure (IOP) due to physical or functional changes within the anterior segment of the eye, but its relationship to IOP is not absolute. The most common form of glaucoma in the USA is primary open angle glaucoma, in which elevated IOP is associated with optic nerve damage. In contrast, the most common form in Japan is normal tension glaucoma, in which IOP is unchanged, but optic nerve damage is associated with defects in blood flow (1). Additionally, elevated IOP does not always lead to glaucoma (2). The underlying molecular mechanisms leading to initiation and progression of most forms of glaucoma are largely unknown.

Developmental glaucomas provide a unique entry into studying the disease; patients generally develop more severe glaucoma at younger ages, which facilitates the mapping of gene loci. In cases in which genetic mutations are identified, the study of animal models is possible. Heterozygous mutations in the human homeobox gene, PITX2, underlie Axenfeld–Rieger syndrome (ARS) in the subset of patients mapping to chromosome 4q25 (3). This autosomal-dominant condition results in developmental defects within the ocular anterior segment in structures derived from the periocular mesenchyme, including the cornea, iris and outflow tract (3–7). In addition, 50% of affected individuals develop early-onset glaucoma. PITX2 encodes a homeodomain transcription factor that regulates expression of downstream
target genes (8–15). Interestingly, both gain- and loss-of-function mutations in PITX2 have been functionally identified (16–21). Murine Pitx2 is expressed throughout the periocular mesenchyme, including not only the structures of the anterior segment but also the sclera, ocular vasculature and extraocular muscles (3,22). It is widely assumed that glaucoma in these patients is the result of elevated IOP due to the anterior segment defects (6). However, the underlying molecular mechanisms and the reason(s) why only 50% of affected individuals develop glaucoma are not known. Collectively, these observations suggest that more complex factors than simply elevated IOP contribute to the etiology of glaucoma in affected patients.

Animal models of glaucoma have been very powerful in identifying its underlying causes (23). Previously, an animal model of ARS was created by targeted deletion of Pitx2 in mice in order to further analyze the functions of this important regulatory gene (24–27). These global knockout mice die at e14.5 of heart defects, but this model has revealed several important roles for Pitx2 in early eye development. The eye defects include the agenesis of the corneal endothelium and stroma and loss of extraocular muscles, which derive from periocular mesenchyme (24,25,27). Additionally, development of the optic nerve from neural ectoderm is abnormal, despite the fact that Pitx2 is not expressed there (25). These results suggest that Pitx2 has both intrinsic and extrinsic roles in eye development.

Fate mapping experiments in chick provided the first evidence that both neural crest and mesoderm precursors contribute to the periocular mesenchyme (7,28–31). The optic cup, optic stalk and lens interact with both precursor pools early in eye development. We recently used binary transgenic systems to establish that neural crest and mesoderm fates in mice are similar to those in chick, but there is a greater contribution of mesoderm to anterior segment structures, including the corneal endothelium and stroma. Interestingly, Pitx2 is expressed in both neural crest and mesoderm precursors beginning early in eye development (22). Collectively, these data indicate that the ocular defects of the global Pitx2 knockout mice could arise from a requirement for Pitx2 function in neural crest or mesoderm or both. The neural crest precursors are of particular interest because it has been suggested that deficiencies in neural crest function underlie many human anterior segment disorders (32).

To determine the role of Pitx2 in the neural crest lineages of the ocular mesenchyme, we created a neural crest-specific knockout of Pitx2 (Pitx2-ncko) by mating mice carrying the Pitx2flox allele with mice carrying a Cre transgene driven by the Wnt1 promoter (33). Studying these mice enabled us to determine the roles of Pitx2 expression in the neural crest during early eye development. In addition, post-natal survival of Pitx2-ncko mice allowed identification of novel requirements for Pitx2 function in the later stages of eye development. We discovered defects in both the mesenchymal tissues that normally express Pitx2, as well as in neural tissues that do not, demonstrating that Pitx2 expression in the neural crest cells has both intrinsic and extrinsic functions. On the basis of the data, we propose a new model for development of the optic nerve from the optic stalk and new mechanisms that may contribute to the etiology of glaucoma in ARS.

RESULTS

Tissue-specific targeting of Pitx2 in neural crest

We previously described generation of a conditional Pitx2 allele (Pitx2flox) by introduction of loxP sites into introns flanking exon 5, which encodes the homeodomain essential for DNA binding and protein function (25). We included the R26R Cre reporter allele in our crosses so that we could readily detect cells in which Cre-mediated excision occurred (34). To obtain Pitx2-ncko mice, mice homozygous for Pitx2flox and the Cre reporter allele R26RloxP (Pitx2flox/flox R26RloxPloxP mice) were mated with Pitx2 null heterozygotes carrying the Wnt1-Cre transgene. The endogenous Wnt1 gene is expressed in the midbrain and along the entire length of the dorsal neural tube, which contains the pre-migratory neural crest (35). Wnt1-Cre is active by e8.5 in the pre-migratory neural crest (33,35), 2 days before the expression of Pitx2 in the ocular neural crest at day e10.5 (36), thereby allowing sufficient time for efficient conversion of Pitx2flox to the null allele. Wnt1-Cre has also been used successfully to study the role of β-catenin in the craniofacial neural crest (37). In our current experiments, excision of the homeodomain results in conversion of the fully functional Pitx2flox to the non-functional Pitx2null allele in the cells expressing Wnt1-Cre, creating pups that are homozygous null for Pitx2 in the neural crest population, but heterozygous elsewhere. Control and experimental mice were identified by PCR-based genotyping. To confirm that expression of Wnt1-Cre resulted in specific and efficient silencing of Pitx2 in neural crest, sections from control and Pitx2-ncko embryo were co-immunostained for β-gal, to detect marked neural crest, and PITX2. In control embryos at e12.5, PITX2 is expressed in both neural crest and mesoderm surrounding the eye primordia (Fig. 1A, C and E). In contrast, in Pitx2-ncko embryos, PITX2 expression is observed only in mesoderm, even though neural crest cells are present (Fig. 1B, D and F). These results establish that Cre-mediated excision of Pitx2 was highly efficient and specific for neural crest in the mutant embryos. In mice that were identified as mutants, 20 of 22
mice examined exhibited the consistent mutant phenotype described subsequently. The remaining two mice had one mutant eye and one eye with similar but less severe defects. These observations confirm that the Pitx2-ncko phenotype is highly consistent and fully penetrant. In contrast to global Pitx2 knock out animals, Pitx2-ncko mice are viable and survive at least until weaning, suggesting that Pitx2 function in neural crest is not strictly required for development of the cardiovascular system or other essential organs (data not shown).

Pitx2-ncko mice are clinically anophthalmic

To examine the cumulative phenotype, as well as to gain insight into the role of Pitx2 in later eye development, Pitx2-ncko mice and their control littermates were harvested at e16.5 and e18.5. The eyes of control littermates are visible through the fused eyelids (Fig. 2A), whereas the eyes are not visible externally in the Pitx2-ncko mutants (Fig. 2B). Dissection of the mutant head revealed that two eyes are present, but buried within the skull, near the midline, directly beneath the brain (Fig. 2C and D). The presence of globes that are not externally visible indicates that Pitx2-ncko mice are clinically anophthalmic (38). Mutant eyes are generally devoid of pigment except for a cone-shaped region at the anterior segment (Fig. 2C and D). The lens and retina are present in each mutant eye (Fig. 2C–H).

Examination of histological sections revealed that the mutant eyes are attached directly to the ventral hypothalamus rather than connected through an extended optic nerve (Fig. 2E and F). Multiple histologically distinct layers are apparent in the retinas of mutant animals, as are RGCs that extend axons, which enter the ventral hypothalamus and form a structure resembling an optic chiasm (Figs 2H and 6J). Lens blebbing is also observed, which is a common feature in many eye mutants (39). The control retinal-pigmented epithelium (RPE) is fully pigmented at e16.5 (Fig. 7E). In contrast, the mutant RPE is devoid of pigment at this stage, but it remains a columnar epithelium (Fig. 2C and H). The developing anterior segment remains heavily pigmented (Fig. 2D and H). Both the optic stalk and the RPE arise from the neural ectoderm where Pitx2 is not expressed (Fig. 1E) (36). Therefore, these phenotypes indicate that one essential function of Pitx2 in the neural crest is to regulate expression of extrinsic factors that influence development of the neural ectoderm.

Multiple defects in structures derived partially or totally from neural crest are also apparent in Pitx2-ncko eyes. The corneal endothelium and stroma, which receive contributions from neural crest and mesoderm, are absent in mutant eyes (Fig. 2F and H) (22). The sclera surrounding the eye is derived from the neural crest and is completely absent in the mutant eyes (Fig. 2G and H) (22). Hyaloid blood vessels, composed of a mesoderm-derived endothelial lining and neural crest-derived pericytes, are present but appear hypomorphic relative to wild-type eyes (Fig. 2G and H). Muscle bundles, which contain mesoderm-derived myocytes and neural crest-derived fascia cells, are present adjacent to the dysmorphic anterior segment in Pitx2-ncko eyes (Fig. 2H).

We hypothesized that these muscles may be the extraocular muscles. Collectively, these results indicate that a second essential function of PITX2 is to regulate expression of intrinsic factors within neural crest precursor cells that are required for their subsequent specification into mature lineages.

Defects in Pitx2-ncko eye primordial begin at e11.5

To identify the origins of the accumulated eye defects, we examined Pitx2-ncko mice beginning at e10.5, when Pitx2

Figure 2. Accumulated morphological defects in late gestation Pitx2-ncko mice. (A) Control embryo at e16.5. (B) Clinical anophthalmia (arrow) in e16.5 Pitx2-ncko embryo. (C and D) Low and high magnification views of dissected e16.5 Pitx2-ncko head. Note the visibility of lens in mutant eyes due to lack of pigment except in the anterior segment (arrow). (E and F) H&E-stained transverse sections of control and Pitx2-ncko heads at e16.5. Note the attachment of mutant eyes directly to the ventral hypothalamus. (G and H) Close-up of control and mutant eyes at e16.5. Note that the extraocular muscle condensations (closed arrowheads) are shifted in reference to the optic cup. The cornea (arrow), anterior segment and sclera (open arrowhead) are absent in the mutant eye. L, lens; H, hypothalamus; R, retina; V, hyaloid vessels; ON, optic nerve.
expression is initially activated in the ocular neural crest. Control and mutant mice are indistinguishable at this time point (Fig. 3A and E). At e10.5, the optic stalk in both control and mutant eyes consists of a 10–12-cell thick neuroblastic cell layer. By e11.5 in control eyes, the optic stalk has begun to extend and thin, reflecting the increased distance between the optic cup and the ventral diencephalon (Fig. 3B). By e12.5, the wild-type stalk consists of a 1–2-cell thick neural epithelium, and the distance between the optic cup and the brain is further increased (Fig. 3C). In contrast, the optic stalk in Pitx2-ncko mice remains a thick, neuroblastic structure throughout development and does not extend laterally, but remains very short (Fig. 3F and G). The mutant eyes remain closely associated with the ventral diencephalon and become directly attached to the diencephalon by e14.5 (Fig. 3F). There is also an increase in distance between the optic cup and the surface ectoderm as the head grows rapidly in size during this period (Fig. 3F and G). We examined markers of cell proliferation (Ki67 and phospho-Histone H3) and apoptosis (TUNEL) but did not find any changes that could account for the optic stalk phenotype (data not shown).

Defects in the development of structures derived from periocular mesenchyme are also apparent in Pitx2-ncko mice by e11.5. In wild-type eyes, neural crest and mesoderm cells migrate into the anterior segment beneath the surface ectoderm immediately after formation of the lens vesicle (Fig. 3B) (22). By e12.5, compaction of the mesenchyme to form the corneal endothelium is evident, and by e16.5, the corneal stroma is also present (Fig. 3C and D). In Pitx2-ncko eyes, mesenchyme is present within the anterior segment by e11.5 (Fig. 3F). However, the mesenchymal layer is noticeably thickened at this time point, and there is no subsequent formation of either the corneal endothelium or the stroma layers (Fig. 3G and H). Proliferation is not increased in the ocular mesenchyme of Pitx2-ncko mice (data not shown). Therefore, the increasing thickness of the anterior segment in mutant eyes is not due to increased proliferation caused by the loss of Pitx2 in this tissue, but instead appears to be secondary to the displacement of the optic cup.

**Molecular analysis of periocular mesenchyme fates in Pitx2-ncko mice**

Several of the structures defective in the Pitx2-ncko eye contain neural crest cells that normally express Pitx2, so we examined molecular markers to determine how the loss of Pitx2 affects cell fate. To confirm our initial finding that the mutant vasculature is reduced in the Pitx2-ncko eye, we performed immunostaining for collagen IV, a vascular endothelial marker (40). We found that there were fewer hyaloid vessels in the mutant eye (Fig. 4A and B). Although it is unknown whether the hyaloid vasculature forms by vasculogenesis or angiogenesis, in either case, the vessels are initially formed as primitive tubes by endothelial cells of mesodermal origin. Platelet-derived growth factor released by the endothelial tubes recruits mural cells, which in turn stabilize the new vessels, possibly by secreting vascular endothelial growth factor or angiopoietin-1 (41–45). The mural cells of the eye vessels are pericytes, which are neural crest-derived (22,46). We hypothesized that the reduced vasculature phenotype seen in the Pitx2-ncko could be due to a deficiency in the migration of neural crest-derived pericytes to the primitive endothelial tubes. We assayed this by examining the presence of NG2, a marker for differentiated pericytes and their precursors (47–49) and found that pericytes were present by immunofluorescence, although they may not be fully functional (Fig. 4C and D). Defects in the ability of Pitx2-negative pericytes to differentiate and stabilize the developing vessels could
account for the reduction in the number of hyaloid blood vessels.

Although extraocular muscles are absent in global Pitx2 knockout mice, our initial histological examination of the Pitx2-ncko eye suggested that extraocular muscles were present, but in an abnormal orientation to the optic cup. To confirm, we analyzed the expression of several muscle-related markers during extraocular muscle development. Extraocular muscles were specified normally as indicated by the presence of the transcription factors PITX1 and myogenin via immunofluorescence at e12.5 (Fig. 5C and D). These populations of cells did not migrate from their original locations in the eye field, despite the displacement of the optic cup towards the midline. By e14.5, the muscles had differentiated, as indicated by the presence of developmental myosin heavy chain immunofluorescence (Fig. 5G and H).

**Loss of Pitx2 in the neural crest lineage results in severe optic nerve defects**

The optic nerve defects in the Pitx2-ncko mice are striking, and to our knowledge unique, so we pursued the underlying molecular aspects further. On the basis of the early and severe optic stalk phenotype in the Pitx2-ncko mice, we considered the possibility that the optic stalk was never specified properly. To test this hypothesis, we examined the expression of Pax2, an early marker for optic stalk specification that is required for normal development (50–52). We found that Pax2 protein is expressed in the optic stalk of both control and mutant animals at e12.5, establishing that a lack of initial specification cannot account for the optic stalk defects in Pitx2-ncko mice (Fig. 6A and B). Consistent with the previous reports, Pax2 expression in control eyes at e12.5 is limited to the optic stalk and does not enter the optic cup (Fig. 6A) (50,51). In contrast, Pax2 expression in e12.5 Pitx2-ncko eyes extends into the posterior presumptive RPE and outer layer of the optic stalk (Fig. 6B and D). The expansion of Pax2 into the posterior RPE becomes progressively more extensive until ~50% of the RPE is expressing Pax2 by e16.5 (Fig. 7E and F). We also found that Vax1, another homeobox transcription factor required for optic stalk specification, is present and expanded in the same manner as Pax2 (Fig. 6E and F) (53–55). Because Pax2 and Vax1 are both activated by Sonic hedgehog (Shh) signaling (56,57), we examined whether increased midline Shh expression could account for the expanded expression of these genes. We found that the spatial and temporal pattern of Shh mRNA expression is unchanged in the mutant mice (Fig. 6G and H) (data not shown).

After the initial specification of the optic stalk by Pax2, the stalk attracts the axons of the RGCs while undergoing significant morphogenetic movements (50,51). Defects in routing of the RGCs axons are often associated with defects in optic nerve development (50,53–55). Initial histological examination suggested that RGC axons entered the optic disk in Pitx2-ncko eyes (Fig. 3G and H). We used immunostaining for β-tubulin to confirm that RGC axons exit the eye at the optic disk and subsequently associate with Pax2-expressing cells of the optic stalk at e12.5 (Fig. 6A–D). Pax2-positive cells of the optic stalk are ultimately fated to delaminate and migrate among the axons in the optic nerve, where they differentiate as astrocytes (Fig. 6I) (50,58). In the mutant at e16.5, only a small percentage of Pax2-positive cells of the stalk have invaded the axons of the optic nerve, whereas the majority remain localized at the periphery (Fig. 6J). The axons exiting the mutant eyes enter directly into the ventral hypothalamus where they form a primitive optic chiasm (Fig. 6K and L).

**RPE development is disrupted in Pitx2-ncko eyes**

Shortly after the optic vesicle invaginates to form the optic cup, signals from the periocular mesenchyme specify the outer layer of the optic cup as the RPE (59). This initial specification appears to occur normally in Pitx2-ncko mice, because the mutant RPE is indistinguishable from the control in both morphology and gene expression at e10.5 (Fig. 7A and B) (data not shown). However, the progressive loss of pigment beginning at e12.5 suggests that subsequent expansion or maintenance of the RPE is blocked in the mutant eyes (Fig. 7C–J). Pax2 is known to inhibit the expression of Pax6 as the boundary is established between the Pax2-expressing optic stalk and the Pax6-expressing RPE layer (60). Pax6 is subsequently required to activate expression of the downstream transcription factor Mitf, which is required for RPE specification (61–65). Otx1 and Otx2 also encode transcription factors required for RPE specification, but their expression does not depend on Pax6 (66–68). We hypothesized that suppression of RPE-specifying genes, including Pax6, Mitf and Otx2, may account for the progressive block in RPE differentiation or expansion in Pitx2-ncko eyes. At e10.5, both control and mutant presumptive RPE express Pax6, Mitf and Otx2 (Fig. 7A and B) (data not shown). In Pitx2-ncko eyes, expression of Pax6 H.}

**Figure 4. Reduction of hyaloid vasculature.** (A and B) Immunofluorescence for the blood vessel endothelial cell marker, collagen IV (green), at e12.5 in control and Pitx2-ncko eyes. (C and D) Immunofluorescence for the pericyte marker, NG2 (green), in control and mutant eyes at e12.5. Insets are representative high magnification views. Red blood cells appear orange due to autofluorescence.
protein is excluded from cells expressing PAX2 (Fig. 7A–F). As expected, pigment is similarly excluded from cells expressing PAX2 (Fig. 7C–F). Mitf and Otx2 expression are also lost in the presumptive RPE cells that do not express pigment at e12.5 (Fig. 7G–J).

**DISCUSSION**

Sophisticated experimental genetics in mice provides a powerful approach for understanding human development and disease by allowing for precise molecular, cellular and temporal dissection of gene function. Tissue-specific knockouts can be particularly useful when trying to understand the function of a gene that is expressed in multiple precursor pools in developing organs. In the present study, we used conditional targeting to determine the processes in early eye development that require function of the homeobox gene, Pitx2, in the neural crest. We also identified critical new roles for Pitx2 in later eye development, which was previously impossible to study due to the embryonic death of Pitx2 global knockout mice. Furthermore, we propose two potential mechanisms for the early development of glaucoma in human PITX2 patients and identify a previously unrecognized regulatory pathway in optic nerve development that may have implications for other eye diseases.

**Intrinsic requirements for Pitx2 in tissues derived from the periocular mesenchyme**

Given the corneal defects seen in ARS patients with PITX2 mutations (69), Pitx2 was predicted to play an important role in corneal development. The global Pitx2 knockout mice lack a corneal endothelium and corneal stroma, confirming an essential role for the gene in corneal development (24,25,27). On the basis of our current results, specification of corneal endothelium and stroma requires Pitx2 function in neural crest precursors, which are the primary contributors to these tissues (22). Pitx2 is also expressed in the subset of mesoderm cells that contribute to the cornea, but this small number of cells is not able to rescue corneal formation in Pitx2-ncko mice (22). Although the phenotype of global Pitx2 knockout mice suggested that the mutant anterior segment was hypercellular (24,25,27), we are unable to find evidence of increased proliferation in the periocular mesenchyme of Pitx2 mutant mice (data not shown). Thus, the thickened appearance of the presumptive cornea is likely secondary to the displacement of the optic cup.

Corneal differentiation requires inductive signals from the lens (70,71), and lens defects result in cornea defects in human disease (72–75). Pitx2 expression is activated in neural crest cells as they migrate into the anterior segment (22) (P.J.G., unpublished data). On the basis of these observations and our current results, we propose that Pitx2 is normally a direct downstream effector that is induced in response to signals from lens. Activation of Pitx2 in neural crest within the presumptive anterior segment is required to initiate a cascade of genes required for corneal differentiation. In the absence of Pitx2 expression, neural crest cells are not competent to respond appropriately to the signals from the lens, and corneal differentiation fails to occur. It is also possible that corneal agenesis is secondary to the displacement of the lens in Pitx2-ncko mice, but we favor our model of a primary role for Pitx2 because human PITX2 patients have corneal defects without eye displacement (69). The role of Pitx2 in cornea formation is an important question that must be answered in the future.
Scleral agenesis is a second example of an intrinsic defect in the Pitx2-ncko eye. The sclera, the white outermost coat of the eye, is derived from the neural crest lineage of the periocular mesenchyme, which expresses Pitx2 (22). Although signals from the RPE have been shown to induce sclera formation, only part of the RPE is disrupted in the Pitx2-ncko eye (76). Because the sclera is completely absent in the Pitx2-ncko mice, we conclude that Pitx2 expression is required for neural crest cells to adopt scleral fates. Pitx2 may confer competence on neural crest cells to respond to the signals from the RPE. Currently, little is known about the development of the sclera; our data provide the first clues about its genetic origins. Recent data about the causes of nanophthalmos (extreme hyperopia) demonstrate that scleral development is involved in controlling the shape of the eye, which is critical, given the precise optics required for vision (77).

Other tissues that are derived from the periocular mesenchyme do not show an absolute requirement for Pitx2 in the neural crest lineage. Although the hyaloid vasculature of the Pitx2-ncko mice is hypoplastic, the mutant pericyte precursors are still recruited to the primitive endothelial vessels. This suggests that Pitx2 expression is not strictly required for neural crest cells to adopt the pericyte fate in the eye, as judged by the commonly used marker NG2 (48,49). However, the mutant pericytes may have compromised function that could cause defects in vessel formation and growth. This idea comes from the knowledge that pericytes play a particularly crucial role in the formation of the vascular plexus of the eye (78,79). We hypothesize that the Pitx2-null pericytes do not express signaling factors required to stimulate the proliferation of vascular endothelial cells and induce the remodeling associated with the formation of additional vessels. The absence of Pitx2 in the neural crest population results in abnormal development of the hyaloid vasculature, so the retinal vasculature, which replaces the hyaloid vasculature after birth, may also be hypoplastic.

Expression of Pitx2 is not required for the adoption of all neural crest fates in the developing eye. Although we cannot exclude the possibility of subtle defects, the expression of the muscle-specification genes, myogenin and Pitx1, and the differentiated muscle marker, myosin heavy chain, in the muscle primordia indicates that muscle formation is relatively normal in Pitx2-ncko mice. Neural crest cells are present in the extraocular muscles of mutant eyes (data not shown), where they form the connective fascia and satellite cells (22). Therefore, we conclude that Pitx2 expression in neural crest cells is not required for extraocular muscle formation. Because extraocular muscles are completely lost in the global Pitx2null mice, this implies that the expression of Pitx2 in the

Figure 6. Optic stalk specification and morphogenesis. (A and B) Expression of PAX2 protein (red) in control and mutant eyes at e12.5. Staining for β-tubulin labels RGC axons (green). (C and D) PAX2 expression (red) and staining for RGC axons (green) in a cross-section (sagittal) of the optic stalk at e12.5. White arrowheads denote the limits of PAX2 expression, and a white arrow indicates the optic fissure. (E and F) Vax1 expression (purple) in the control and mutant optic stalk at e12.5. Arrowheads denote the limits of expression. Pigmented RPE appears black. (G and H) Sonic Hedgehog expression in the ventral diencephalon (future hypothalamus) in the control and mutant brain at e12.5. Infundibulum is marked with an arrowhead. (I and J) Cross-section of the optic nerve at e16.5, showing the PAX2-expressing astrocyte precursors (red) and the axons (green) in control and mutant mice. The unlabeled blue cells in the mutant are retina. (K and L) Transverse H&E sections of e16.5 embryos showing control optic chiasm (K) formed by the axons of the RGCs at the midline near the ventral hypothalamus. In the mutant, a primitive chiasm (L) is formed by the RGC axons as they exit the eyes. R, retina.
Our current data support a two-step model for optic stalk development (Fig. 8). During the initial formation of the eye beginning at e8.5, Shh diffuses from the midline and activates Pax2, Vax1 and Vax2 expression in the optic stalk (56,57). These transcription factors act synergistically to repress Pax6 and define the optic stalk as separate from the optic cup (50,53–55,60). This initial specification of the optic stalk takes place normally in Pitx2-ncko mice, as indicated by normal expression of Shh, Pax2 and Vax1 (Fig. 6). Later, beginning at e10.5, Pitx2 expression in the periorcular mesenchyme activates signals that cause the morphogenetic extension of the optic stalk. This step fails to occur in Pitx2-ncko mice, resulting in a foreshortened optic stalk. To the best of our knowledge, this is the first example of the neural crest population patterning the neuroectoderm from which it is originally derived, which raises the possibility that this process occurs in other neural tube derivatives.

The progressive RPE defects we observed in the Pitx2-ncko eye are likely secondary to the defects in optic nerve development and the displacement of the eyes. The close proximity of the mutant eyes to the midline source of Shh is probably sufficient to drive increased Pax2 expression, which shifts the boundary between Pax2 and Pax6 expression distally. The reciprocal changes in Pax2 and Pax6 expression are likely to be the cause of the loss of pigment in large portions of the RPE in the Pitx2-ncko mice. Pax6 initially activates Mitf, which is required for normal RPE development and is lost in the unpigmented portions of the RPE (59,61,62,64,83). Expression of Otx2, another gene required for RPE development that does not depend on Pax6, is also absent in the regions of pigment loss. Mitf expression may be required for the maintenance of Otx2 expression, as proposed by Martinez-Morales et al. (66), or Pax2 may repress Otx2 expression. Overall, our data are consistent with the

Extrinsic effects on neural ectoderm

Our results have identified a previously unknown role for Pitx2 function in the neural crest during optic stalk development. One of the ongoing processes of early eye development is the partitioning of the neural ectoderm-derived optic vesicle into the regions that will form the optic stalk, neural retina and RPE by signaling, gene activation and gene repression. In Pitx2-ncko mice, early partitioning of the optic vesicle occurs normally, but subsequent morphogenesis of the optic stalk is abnormal. This results in a foreshortened optic stalk and eyes that are displaced towards the midline. Although the end phenotype superficially resembles cyclopia, the presence of two eye fields and the absence of other midline defects allowed us to rule out this line of inquiry. Because Pitx2 is never expressed in the optic stalk, this indicates that the defect in optic stalk development is extrinsic (non-cell autonomous). Pitx2 expression in the neural crest mesenchyme must be activating an extracellular signal that is required for optic stalk development. The periorcular mesenchyme is known to signal other tissues of the eye. For example, an activin-like signal from the mesenchyme is required to define the RPE, and FGF10 and BMP7 cause the development and morphogenesis of the lacrimal gland from the surface ectoderm (59,80–82). Our finding that the Pitx2-dependent signaling function is localized to the neural crest component of the mesenchyme raises the possibility that it acts as a form of signaling center.

Our results have identified a previously unknown role for Pitx2 function in the neural crest during optic stalk development, a hypothesis that could be tested in mesoderm-specific Pitx2 knockout mice.

**Figure 7.** Expansion of PAX2 results in RPE-specification defects. (A and B) Co-immunostaining shows expression pattern of PAX2 (red) and PAX6 (green) at e10.5 in control and Pitx2-ncko mice. (C–F) Expression of PAX2 (red), PAX6 (green) and pigment (white) at e12.5 (C and D) and e16.5 (E and F) in control and mutant mice. Yellow arrowheads mark the sharp demarcation between PAX2 and pigment expression. To show pigment expression, brightfield images were inverted. (G–J) *In situ* hybridization for Mitf (G and H) and Otx2 (I and J) at e12.5 in control and Pitx2-ncko mice. Inset shows the expression of Otx2 (purple) in cells that express pigment (brown) on their apical surface. Arrowheads indicate the extent of pigment expression in the posterior optic cup.
role of Pax2 as a repressor of RPE development proposed by Martinez-Morales et al. (61).

Implications for human health

In all, our results suggest several new possible mechanisms by which early-onset glaucoma can occur in ARS patients with PITX2 mutations. It is widely assumed that these patients have anterior segment defects that cause increased IOP, which leads to the development of glaucoma relatively early in life (6). Although we do not discount the role elevated IOP plays in glaucoma, we propose that human PITX2 patients may have additional developmental eye defects that lead to the accelerated development of glaucoma. Although the human patients clearly do not have the severe optic nerve defects seen in the Pitx2-ncko mice, they may have more subtle defects in optic nerve development that render the optic nerve more susceptible to damage. The reduced number of Pax2-positive astrocyte precursors that invade the axons of the RGCs in the Pitx2-ncko mice is particularly noteworthy. Astrocytes are critical for the maintenance and survival of the RGC axons and they have been implicated in the pathogenesis of glaucoma (84–86). If human PITX2 patients have reduced number of astrocytes or altered astrocyte function, it could make their optic nerves more vulnerable to the effects of other factors like elevated IOP and lead to the RGC axon damage and death seen in glaucoma.

Reduced ocular blood flow has long been associated with normal tension glaucoma (87) and has also been proposed to contribute to other forms of glaucoma (88,89). Retinal blood vessel development cannot be critically assessed in Pitx2-ncko mice due to the severely dysmorphic ocular growth. However, the clear defect in hyaloid vessel formation implies that normal retinal blood vessel development or function may also depend on PITX2 function in neural crest because the two vasculature systems are developmentally related (90). The observed deficiency in astrocytes in Pitx2-ncko eyes provides additional evidence for defective retinal vessel development because astrocytes guide retinal vessel formation (91,92). It has not been clear whether defects in ocular blood flow are a cause or effect of glaucoma (93,94). Our results with Pitx2-ncko mice raise the possibility that PITX2 patients may have fewer ocular vessels, which would be another risk factor for developing glaucoma.

The pronounced internal displacement of the eyes in Pitx2-ncko mice is reminiscent of other human eye diseases, including anophthalmia and septo-optic dysplasia, and suggests potential underlying genetic mechanisms in these diseases. Although we cannot formally exclude the possibility, it seems unlikely that mutations in PITX2 itself will be identified in these conditions because mice globally deficient in Pitx2 die during development (24–27). However, genes for the extrinsic signaling factor(s) regulated by PITX2 in neural crest or the downstream effectors in the neural epithelium are strong candidate genes for these diseases.

Mutations in SOX2 (95), SIX6 (96), PAX6 (97), RAX/RX (98) and OTX2 (99) have been associated with anophthalmia, but the underlying genetic defects in many cases of anophthalmia remain to be identified. These genes are only expressed in the neural ectoderm and are associated with early defects in formation or survival of the optic vesicle (66,100–103). Our work suggests that cases of clinical anophthalmia may be associated with genes expressed in the pericocular mesenchyme as well. Mutations in HESX1 have been identified in septo-optic dysplasia, a disease which affects the midline structures of the brain and optic nerves (104). Mice with mutations in Hesx1 have a ‘buried’ eye phenotype, which bears similarities to the phenotype described here (104). This suggests that Hesx1 may be part of the same pathway as Pitx2, thus it is possible that mutations in PITX2 or its downstream targets may underlie some cases of septo-optic dysplasia.

Our findings raise the possibility that these disorders may also result from defects occurring later in eye development, and the underlying molecular defects could affect genes expressed in either the neural epithelium or the surrounding neural crest. These findings demonstrate that understanding the underlying genes and pathways is critical to understanding the disorders that result when development goes awry.

MATERIALS AND METHODS

Generation of Pitx2-ncko mice

The generation of Pitx2<sup>lox</sup> mouse has been previously described (25). Mice carrying the R26R Cre reporter allele (34) were obtained from the Jackson Laboratories. Wnt1-Cre mice, which carry a transgene containing a Cre cassette under the control of the Wnt1 promoter, were obtained from A. McMahon (33). The Pitx2<sup>lox/lox</sup>;R26R/R26R parental line was generated by serial mating of Pitx2<sup>lox</sup> and R26R mice and their progeny. The Wnt1-Cre;Pitx2<sup>+/−</sup> parental line was generated by mating Wnt1-Cre and Pitx2<sup>mut</sup> mice. All procedures involving mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

The Wnt1-Cre;Pitx2<sup>+/−</sup> mice were mated with the Pitx2<sup>lox/lox</sup>;R26R/R26R mice to generate timed pregnancies.
The morning after mating was designated as embryonic day 0.5. Embryos were collected by C-section after euthanasia of the mother. The resulting embryos were genotyped for Cre (105) and Pitx2 (25) using PCR-based methods. Embryos with a Wnt1-Cre;Pitx2\textsuperscript{lox/++} genotype were considered mutant, whereas Wnt1-Cre;Pitx2\textsuperscript{lox/-} embryos were used as controls.

**Histology**

All embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline, washed, dehydrated, embedded in paraffin and sectioned at 7 μm. Mounted sections for morphological analysis were dewaxed, rehydrated and stained with hematoxylin and eosin (H&E).

**Immunofluorescence**

Paraffin sections were dewaxed and treated for antigen retrieval by boiling for 10 min in 10 mM citrate buffer (pH 6.0). Sections were treated with Image-iT FX signal enhancer (Molecular Probes) or biotinylated (Jackson Immunoresearch) species-specific secondary antibodies. When biotinylated secondary antibodies were used, signals were detected using tyramide signal amplification kits (Molecular Probes).

**In Situ hybridization**

Antisense riboprobes to Otx2 (J. Rubenstein) (106), Mitf (C. Hodgkinson) (107), Pax1 (P. Mathers) and Shh (A. McMahon) (108) were generated and labeled with digoxigenin according to standard procedures. Paraffin sections were processed for in situ hybridization as previously described (109). All probes were incubated at 57°C.

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**Conflict of Interest statement.** The authors state that they have no conflicts of interest.

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