**Review**

**Neural Crest Derivatives in Ocular Development: Discerning the Eye of the Storm**

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Neural crest cells (NCCs) are vertebrate-specific transient, multipotent, migratory stem cells that play a crucial role in many aspects of embryonic development. These cells emerge from the dorsal neural tube and subsequently migrate to different regions of the body, contributing to the formation of diverse cell lineages and structures, including much of the peripheral nervous system, craniofacial skeleton, smooth muscle, skin pigmentation, and multiple ocular and periocular structures. Indeed, abnormalities in neural crest development cause craniofacial defects and ocular anomalies, such as Axenfeld-Rieger syndrome and primary congenital glaucoma. Thus, understanding the molecular regulation of neural crest development is important to enhance our knowledge of the basis for congenital eye diseases, reflecting the contributions of these progenitors to multiple cell lineages. Particularly, understanding the underpinnings of neural crest formation will help to discern the complexities of eye development, as these NCCs are involved in every aspect of this process. In this review, we summarize the role of ocular NCCs in eye development, particularly focusing on congenital eye diseases associated with anterior segment defects and the interplay between three prominent molecules, *PITX2*, *CYP1B1*, and retinoic acid, which act in concert to specify a population of neural crest-derived mesenchymal progenitors for migration and differentiation, to give rise to distinct anterior segment tissues. We also describe recent findings implicating this stem cell population in ocular coloboma formation, and introduce recent evidence suggesting the involvement of NCCs in optic fissure closure and vascular development.

**Key words:** neural crest; congenital eye diseases; Axenfeld Rieger syndrome; congenital glaucoma; eye development

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**Introduction**

Neural crest cells (NCCs) are a population of multipotent embryonic stem cells that give rise to a wide range of cell and tissue types throughout the body. During gastrulation, NCCs originate at the neural plate border and migrate from folds of the neural ectoderm as the neuroepithelium closes to form the neural tube (Beebe and Coats, 2000; Creuzet et al., 2005; Whikehart, 2010). These cells subsequently migrate, pervading different regions of the embryo and yielding a broad range of tissues (from myofibroblasts, melanocytes, endocrine cells, neurons, and glial cells to cartilage and bone) (Beebe and Coats, 2000; Creuzet et al., 2005; Whikehart, 2010). Both migratory routes and derivatives of neural crest vary with rostrocaudal position along the neural tube. The vagal crest, derived from the caudal hindbrain, contributes to the heart, and together with the sacral neural crest, also form the enteric nervous system that innervates the gut (Creuzet et al., 2005). At the cephalic level, NCCs form the mesectoderm, which subsequently gives rise to craniofacial connective, dermal and skeletal tissues, neurons, and the cranial ganglia (Creuzet et al., 2005; Gage et al., 2005; Kish et al., 2011) (Fig. 1). With respect to ocular development, the NCCs migrating to the eye are primarily derived from the prosencephalon (developing forebrain) and mesencephalon (developing midbrain) (Whikehart, 2010). These cells give rise to portions of the corneal endothelium and stroma, iris stroma, ciliary body stroma and muscles, and trabecular meshwork of the eye (Hay, 1980; Beebe and Coats, 2000; Cvekl and Tamm, 2004; Gage et al., 2005; Whikehart, 2010; Kish et al., 2011).

While the NCCs are migrating from the edge of the neural tube, the neuroectodermal-derived optic sulci appear as shallow pits along the neural plate and form the optic vesicles that protrude laterally from the prosencephalon until apposed to the surface ectodermal-derived optic placode (Creuzet et al., 2005) (Fig. 2). Concomitant with surface ectoderm thickening for the differentiation of the lens, morphogenetic movements involving the invagination of the optic vesicles leads to formation of a bilayered optic cup (Beebe and Coats, 2000; Creuzet et al., 2005; Harada et al., 2007; Whikehart, 2010; Kish et al., 2011).

A loose array of neuroectoderm-derived cranial NCCs, termed the periorbital mesenchyme (POM), migrate around the posterior of the optic cup. In humans, the NCC migrates in three waves, while in mice and chick there appears to be only two waves (Johnston et al., 1979; Hay, 1980; Gage et al., 2005). In humans, the first wave of NCCs migrates into the space between the anterior surface...
of the lens and the surface ectoderm destined to form the corneal epithelium to form the corneal endothelium (Gage et al., 2005; Whikehart, 2010) (Fig. 3). A second wave of cells migrates between the corneal epithelium and endothelium to become the keratinocytes of the corneal stroma. The corneal epithelium synthesizes components of the extracellular matrix for the formation of primary stroma when the lens detaches from the surface ectoderm, while a third wave of NCCs migrates to the angle between the posterior cornea (endothelium) and the anterior edge of the optic cup, eventually contributing to the ciliary body and iris stroma (Gage et al., 2005; Whikehart, 2010) (Fig. 3). The POM located in the tissues anterior to the chamber angle between the anterior edge of the eye cup and the endothelium initially remains undifferentiated at this stage, but subsequently develops into flat endothelial-like cells, comprising the trabecular meshwork and Schlemm’s canal, respectively (Gage et al., 2005; Whikehart, 2010) (Fig. 3).

Further development of the optic cup involves distinct regionalized differentiation into at least four different structures, including the retina, the retinal pigment epithelium (RPE), the iris epithelium, and the ciliary epithelium (Fig. 3). Notably, a second essential function of the POM during ocular development is to provide essential signals for the patterning of ocular ectoderm primordia, which not only includes the specification of the RPE from the optic cup, but also the induction of lacrimal glands from the surface ectoderm and the differentiation of the optic stalk from the neural ectoderm (Fuhrmann et al., 2000; Gage et al., 2005; Kao et al., 2013). Moreover, cells originating from the surface epithelium interact with the POM for proper eyelid development (Le Lievre and Le Douarin, 1975).

Over the last decade, studies have highlighted the contributions of the NCCs to ocular and periocular development, emphasizing the importance of these cells in vertebrate ocular evolution. Indeed, defects in neural crest formation lead to severe craniofacial defects and ocular anomalies, and a comprehensive understanding of the interactions involved in the molecular regulation of the neural crest would provide insight into the complexities underlying congenital eye diseases. Herein, we discuss the role of ocular NCCs in eye development, particularly focusing on congenital eye diseases associated with anterior segment defects and the interplay between three prominent molecules, PITX2, CYP1B1, and retinoic acid (RA), which act in concert to specify a population of neural crest-derived mesenchymal progenitors for migration and differentiation to give rise to distinct anterior segment tissues. We also describe recent findings suggesting a role for NCCs in ocular fissure closure and blood vessel formation and introduce recent evidence suggesting the involvement of NCCs in optic fissure closure and vascular angiogenesis.
Anterior Segment Dysgenesis: Ocular Anomalies Associated With NCC Defects

The abnormal migration of NCCs and subsequent disruption of their derivative structures has been implicated in congenital eye diseases affecting the anterior segment of the eye. Anterior segment dysgenesis (ASD) encompasses a group of developmental disorders affecting the function of structures in front of the vitreous surface of the eye, including the cornea, iris, lens, ciliary body, trabecular meshwork, Schlemm’s canal, and sclera (Sowden, 2007). Increasing evidence has shown that transcriptional events are critical for many aspects of neural crest development, and the pathogenesis of several anterior segment disorders has been associated with defects in these genes.

Axenfeld-Rieger Syndrome and Pitx2

Axenfeld-Rieger syndrome (ARS) describes a rare (1 in 200,000) group of genetically and phenotypically heterologous disorders that primarily affect the eye and are also associated with systemic issues, including cardiovascular outflow malformations, craniofacial and dental defects, umbilical abnormalities, and pituitary anomalies with endocrine sequelae (MacDonald et al., 2004; Evans and Gage, 2005; Bohnsack et al., 2012). Ophthalmic manifestations of ARS are typically limited to varying degrees of ASD and include posterior embryotoxon (anteriorization of the angle structures into peripheral cornea) and iris hypoplasia (MacDonald et al., 2004; Evans and Gage, 2005; Sowden, 2007; Bohnsack et al., 2012). Maldevelopment of the iris results in corectopia (pupil displacement) and polyopia (multiple pupils) (Sowden, 2007). Further, abnormal iris strands can extend from the iris to the posterior embryotoxon, thereby covering the trabecular meshwork in the iridocorneal angle and resulting in glaucoma in ~50% of affected individuals (Gage et al., 1999; MacDonald et al., 2004; Sowden, 2007; Bohnsack et al., 2011). Molecular genetics studies have identified specific gene mutations, and one of the most commonly affected genes is paired-like homeodomain 2 (PITX2) on chromosome 4q25 (Vaux et al., 1992; Semina et al., 1996, 1997). More than 45 point and chromosomal mutations, including both gain- and loss-of-function of the PITX2 gene, have been identified. ARS is typically inherited in an autosomal dominant pattern, indicating that eye development is highly sensitive to alterations in PITX2 expression and function.

PITX2, a member of the homeobox protein family, is a transcription factor that plays a critical role in early development, particularly in the formation of structures in the anterior segment of the eye. In mice, Pitx2 is expressed in the cranial neural crest-derived POM, but not in ocular tissues derived from the neural and surface ectoderm (Gage et al., 1999; Evans and Gage, 2005). Pitx2 knockout mice die early in embryonic development, as a result of heart defects and display severe ocular defects, loss of extraocular muscles (EOMs), and jaw and pharyngeal arch abnormalities (Gage et al., 1999). Due to this severe lethal phenotype, conditional knockout studies in mice were used to demonstrate the specific requirement of Pitx2 in the cranial neural crest for optic stalk formation and development of the corneal endothelium and stroma and sclera (Evans and Gage, 2005). Similarly, in zebrafish, we have found that knockdown of Pitx2 or expression of a dominant negative mutant form of the gene disrupted neural crest migration into the craniofacial region (unpublished data) and caused malformation of the jaw, pharyngeal arches, corneal endothelium, and iris stroma (Bohnsack et al., 2012). Thus, Pitx2 is critical in the cranial neural crest, and disruption in gene function or expression results in craniofacial and ocular malformations.

The regulation of PITX2 expression in the cranial neural crest is critical for ocular development. The essential morphogen, RA has been shown in both mice and zebrafish to regulate Pitx2 expression in the POM (Duester, 2009). RA is derived from vitamin A (retinal) through a series of enzymatic reactions and exists as locally regulated gradients that control numerous embryonic processes (Schier and Needleman, 2009; Bohnsack et al., 2012; Bohnsack and Kahana, 2013). In the craniofacial region, the developing eye produces RA in a specific spatial and temporal pattern (Reijntjes et al., 2004). In mice, chick, and zebrafish, RA synthesis enzymes (e.g., RALDH2, RALDH3, RALDH4) are expressed in the developing dorsal and ventral retina, while RA degradation enzymes (e.g., CYP26A1 and CYP26C1) are localized to the cranial and caudal retina; thus, creating RA gradients that are centered around the dorsal and ventral axis of the eye (Molotkov et al., 2006; Cvekl and Wang, 2009; Duester, 2009) (Fig. 4). While systemic disruption of RA levels are embryonic lethal and cause severe pan-ocular malformations, localized alterations of RA signaling show
that the POM is the primary target that regulates anterior segment development (Kumar and Duester, 2010). In the POM, RA binds to RA receptors (RARs $\beta$ and $\gamma$ subunits) which then heterodimerize with the retinoid X receptor (RXR) $\alpha$ subunit. This nuclear hormone receptor complex then upregulates the expression of Pitx2 in the POM (Evans and Gage, 2005; Matt et al., 2005; Diehl et al., 2006; Kumar and Duester, 2010; Lupo et al., 2011). In zebrafish, neural crest-derived corneal endothelial and iris stromal malformations induced by decreased RA synthesis were rescued by knockdown of Cyp1b1 in zebrafish embryos, early $cyp1b1$ expression in the retina occurs independently of the toxin-induced activation of the aryl hydrocarbon receptor, suggesting an as yet unidentified endogenous substrate for $cyp1b1$ in the eye (Yin et al., 2008). In vitro studies have demonstrated that Cyp1b1 alone is sufficient to efficiently oxidize retinol to retinal and subsequently to RA in a dehydrogenase-independent pathway (Chambers et al., 2007). Further, expression of the $Cyp1b1$ ortholog in chick is associated with RA activity during early development (Chambers et al., 2007). In zebrafish, we have found that $cyp1b1$ was expressed in a specific spatial and temporal pattern in the dorsal and ventral retina in areas that overlapped with the RA synthesis enzymes, raldh2 and raldh3 (Figs. 4 and 5A–C). Further, we found that alterations in $Cyp1b1$ levels correlated with response of a RA reporter in the periocular tissues (Figs. 5D–H). Despite these findings, knockdown of $Cyp1b1$ in zebrafish embryos showed an early delay in neural crest-derived iris stromal formation (Figs. 5I–L) and retinal development that recovered by the larval stage. $Cyp1b1$ deficient mice also did not exhibit a consistent phenotype as intraocular pressures were normal, despite mild angle abnormalities involving Schlemm's canal, the trabecular meshwork, cornea, and iris. Thus, additional studies are required to determine whether RA mediates the molecular effects of $CYP1B1$ in neural crest and eye development.

**CONGENITAL OCULAR COLOBOMA AND THE NEURAL CREST**

During the early stages of eye formation, invagination of the optic vesicle generates a bilayered optic cup with a
groove along the ventral margin. This groove, named the optic fissure (also known as choroidal fissure or embryonic fissure), is essential for the entry of mesenchyme cells that give rise to blood vessels that nourish the eye throughout the life of the organism (Harada et al., 2007). Subsequently, the margins of the optic cup close around these vessels. In humans, the optic fissure closes during the 6–7th weeks of gestation (Barishak, 1992), while in mice and zebrafish, the fissure closes between 11 and 13 days post fertilization (dpf) and 1.5–2.5 dpf, respectively (Chang et al., 2006). Failure of fissure closure results in colobomas that affect the iris, lens, zonules, ciliary body, retina, choroid, and optic nerve of the eye, and occur in ~1 in 10,000 births (Shah et al., 2011, 2012). As this condition also affects the major tissues of the anterior segment of the developing eye, it is appropriate to discuss coloboma formation with respect to ASD, specifically highlighting the implications for the involvement of NCCs in choroid fissure closure.

Ocular coloboma can be seen in isolation or as part of a multisystem syndrome associated with other ocular abnormalities, such as microphthalmia (small, disorganized globe), glaucoma, Peters anomaly, and cataract, in the absence of systemic findings (Chang et al., 2006; Shah et al., 2011, 2012). CHARGE, which is characterized by defects affecting the eyes, heart, brain, craniofacial structure, and genitourinary system, is the most common syndrome associated with colobomas (Porges et al., 1992; Eccles and Schimmenti, 1999; Morrison et al., 2000, 2002b; Guirgis and Lueder, 2003; Gregory-Evans et al., 2004; Chang et al., 2006). However, there are numerous chromosomal abnormalities and syndromes with unknown genetic causes that also exhibit colobomas. In these syndromic cases, the eye phenotype is observed concomitant with craniofacial abnormalities, further supporting the idea that NCCs play a role in the pathophysiology of colobomas (Morrison et al., 2002a; Gregory-Evans et al., 2004).

Studies in model organisms, primarily mice and zebrafish, suggest that genetic lesions or toxic exposures can lead to abnormal dorsoventral patterning of the optic vesicle, reduced expression of ventral eye and POM genes, excessive cell proliferation, and tissue fusion defects (Mui et al., 2005; Kim et al., 2007; McLaughlin et al., 2007; See and Clagett-Dame, 2009; Lupo et al., 2011; Weiss et al., 2012). Hence, multiple mechanisms are involved in optic fissure closure, underscoring the complexity of this process. However, a mechanistic understanding of the process of optic fissure closure is still missing, and it is poorly understood why this process sometimes fails. As the neural crest gives rise to many of the tissues in the developed eye, it is reasonable to propose that these cells play an important and critical role in the cellular and molecular mechanisms underlying this step of ocular development. Recent studies indicate that POM cells play a critical role in choroid fissure closure (Etchevers et al., 2001; Weiss.
et al., 2012). However, what POM cells do, where POM cells go, or how POM cells function during this process remains unknown. To provide insight into this process, we must first consider the development of coloboma from the perspective of the potential role of NCCs in this ocular defect.

RA AND Pitx2 DEFECTS LEAD TO FAILURES IN OPTIC FISSURE CLOSURE

As previously described, RA has long been implicated in the development of the vertebrate eye. The requirement for RA signaling in eye development was first described in reports of severe congenital eye defects in vitamin A deficient and/or RA-deficient human, pig, and rat fetuses (Warkany and Schraffenberger, 1946). Indeed, the ASD prominently observed in these individuals included agenesis of the lens, iris and corneal stroma, corneal endothelium, the absence of the anterior chamber, and optic nerve coloboma. These effects are phenocopied in mice, where various combinations of RA-synthesis enzymes or RARs are deleted, indicating that RA is required during choroid fissure closure (See and Clagett-Dame, 2009). However, tight control of RA levels appears to be critical for this process as both RA deficiency and excess during embryogenesis induces colobomas in mice and zebrafish. In mice, treatment of pregnant dams with RA changes gene expression along the proximal–distal axis of the fissure, thereby altering specification of retina, retinal pigment epithelial, choroid, and optic nerve. Further, in zebrafish, local delivery of RA to the eye not only resulted in coloboma, but also induced ectopic fissure formation elsewhere in the retina (Bohnsack and Kahana, 2013). As previously described, RA targets the neural crest-derived POM that surrounds the developing eye (Matt et al., 2005). Interestingly, neural crest-specific knockout of all RAR genes in mice causes colobomas and eversion of the ventral optic cup, suggesting that RA acts via the POM to guide the morphogenesis of the optic cup and closure of the choroid fissure (Lohnes et al., 1994; Matt et al., 2008). In addition, the known RA-target in the POM, PITX2, has also been identified as playing a role in optic fissure closure (Gage et al., 1999). Optic fissure closure is defective or significantly delayed in Pitx2 null mutant mouse embryos (See and Clagett-Dame, 2009; Kumar and Duester, 2010). Further, conditional knockout of Pitx2 specifically in the neural crest also gives rise to colobomas (Gage et al., 2005; Sclafani et al., 2006). Thus, the effects of RA and PITX2 on not only anterior segment development, but also optic cup morphogenesis and specifically ocular fissure closure are via the neural crest.

Conclusions and Future perspectives

The most dynamic event in the morphogenesis of the developing eye is the migration and differentiation of neural crest-derived pericocular mesenchymal cells to provide multiple mature cell lineages necessary for normal ocular development and vision, including the corneal endothelium and stroma, trabecular meshwork, ciliary body muscles, and iris stroma (Hay, 1980; Cvekl and Tamm, 2004). The POM also provides the essential signals for the patterning of the neural ectoderm, including specification of the RPE from the optic cup and differentiation of the optic stalk from the neural ectoderm (Hay, 1980; Cvekl and Tamm, 2004). The derivation of these structures requires complex interactions between the neural crest, neural ectoderm, and surface ectoderm (Chow and Lang, 2001). Genetic or acquired defects in the development or function of the POM lead to debilitating vision loss and ocular disease, emphasizing the importance of these cells in every aspect of ocular development.

The use of animal models, particularly mice, chick, and zebrafish, has improved our understanding of neural crest development and importantly the pathogenesis of congenital eye diseases, such as ARS, congenital glaucoma, and colobomas. The next step is to use this information to create gene therapies or use stem cell technology to reverse or bypass the molecular defects to restore vision in children affected with these blinding diseases. Further, developmental studies will provide important insight into regenerative treatments for degenerative diseases of neural crest-derived tissues such as keratoconus, Fuchs dystrophy, and adult-onset primary open-angle glaucoma.

Our understanding of neural crest contributions to the eye should continue to be expanded. Through ophthalmic genetics and congenital eye diseases, the knowledge regarding the molecular regulation of neural crest contributions to the anterior segment is growing. Another highly important area of research regarding ocular neural crest involves blood vessel formation. Studies in mice and quail have demonstrated that pericytes and vascular smooth muscle cells, which surround endothelial cells in the hyaloid, retinal, choroidal, and optic nerve vasculature, are in part, derived from NCCs (Etchevers et al., 2001; Gage et al., 2005; Trost et al., 2013). Impaired blood vessel development during eye development can lead to congenital eye diseases, including colobomas and microphthalmia. These vascular support cells play a key role in tissue homeostasis by stabilizing blood vessels, participating in blood flow regulation, and mediating the formation of the blood retina barrier (Saint-Geniez and D’Amore, 2004; Gariano and Gardner, 2005; Kim et al., 2006; Jeong et al., 2008; Hyoung Kim et al, 2011 ). In adults, loss of vascular pericytes in retinal blood vessels is a key element in the pathogenesis of diabetic retinopathy (Hammes et al., 2002; Hammes, 2005; Beltramo and Porta, 2013). Understanding the contributions of the neural crest to blood vessels in the developing eye holds promise for the treatment of both congenital and degenerative eye diseases.

The neural crest is a dynamic embryonic stem cell population that plays a critical role in ocular development. The neural crest interacts with the surrounding neural
ectoderm, surface ectoderm, and mesoderm and gives rise to numerous components of the anterior segment of the eye. Studies delineating neural crest development are key for understanding the pathogenesis of congenital eye diseases, such as those outlined in this review. Elucidating these molecular signals will unlock the potential for novel treatments for congenital diseases. Further, these pathways also have implications in regenerative therapies for adult degenerative diseases of neural crest-derivatives. Additional studies using animal models and genetic techniques will expand our knowledge of this unique population of stem cells and pioneer treatments.

References


