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Eya1 acts upstream of Tbx1, Neurogenin 1, NeuroD and the neurotrophins BDNF and NT-3 during inner ear development

Rick A. Friedman[a,*], Linna Makmura[a], Elzbieta Biesiada[a], Xiaobo Wang[b], Elizabeth M. Keithley[b]

[a]*Gonda Department of Cell and Molecular Biology, House Ear Institute, 2100 W. Third Street, Los Angeles, CA 90057, USA*
[b]*Division of Otolaryngology, UCSD School of Medicine, 9500 Gilman Dr, La Jolla, CA 92093, USA*

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Abstract

Cell fate specification during inner ear development is dependent upon regional gene expression within the otic vesicle. One of the earliest cell fate determination steps in this system is the specification of neural precursors, and regulators of this process include the Atonal-related basic helix-loop-helix genes, *Ngn1* and *NeuroD* and the T-box gene, *Tbx1*. In this study we demonstrate that Eya1 signaling is critical to the normal expression patterns of *Tbx1*, *Ngn1*, and *NeuroD* in the developing mouse otocyst. We discuss a potential mechanism for the absence of neural precursors in the $Eya1^{-/-}$ inner ears and the primary and secondary mechanisms for the loss of cochleovestibular ganglion cells in the $Eya1^{bor/bor}$ hypomorphic mutant.

Keywords: *Eya1*; *Ngn1*; *NeuroD*; *BDNF*; *NT-3*; *Tbx1*; Otic vesicle; Cochleovestibular ganglion; Neurogenesis

1. Introduction

The development of the mammalian ear is directed by a series of tissue movements and inductive interactions that promote spatial patterning and cellular differentiation (Noden and Van de Water, 1992). The identification of genes involved in these processes is facilitating our understanding of the complexity of ear development and the molecular pathogenesis of some forms of genetic hearing loss. Genetic hearing loss can be divided into two broad classes: non-syndromic (occurring in isolation) and syndromic (associated with other abnormalities). A number of forms of syndromic hearing loss demonstrate anomalies of one or more compartments of the ear (outer, middle, and inner), suggesting an early developmental effect (Kalatzis et al., 1998). One such autosomal dominant form, Branchio-Oto-Renal (BOR) syndrome (MIM 113650), has been well described clinically and genetically.

Although the association of branchial arch anomalies and hearing loss has been recognized for nearly a century, it has only been in the last 25 years that BOR syndrome has been defined. Melnick et al. reported a father and three of six children with mixed hearing loss, abnormally cupped pinnae, preauricular pits, branchial cleft fistulae and renal anomalies (Melnick et al., 1975). BOR syndrome is an autosomal dominant disorder with incomplete penetrance and variable expressivity.

Recently, mutations in the human homologue of the *Drosophila* eyes absent gene (*EYA1*) were identified in BOR patients (Abdelhak et al., 1997). *EYA* genes encode a family of proteins (*EYA1–4*) that are highly conserved in the animal kingdom. The EYA proteins are characterized by a carboxy-terminal domain (ED), necessary for cooperative co-factor binding, and a PST rich carboxy-terminal trans-activating domain (Xu et al., 1997). In *Drosophila*, *eyes absent* (*eya*), has been shown to interact synergistically, in a regulatory network including *eyeless*, *sine oculis* and *dachshund*, during compound eye morphogenesis (for reviews see Desplan (1997), and Wawersik and Maas (2000)). The vertebrate homologues of *ey*, *eya*, *so*, and *dac*, *Pax*, *Eya*, *Six*,

* Corresponding author. Tel.: +1 213 273 8078; fax: +1 213 273 8088.
E-mail address: rfriedman@hei.org (R.A. Friedman).

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and *Dach*, have been characterized and several studies have demonstrated that this regulatory network is conserved in vertebrate eye, limb, kidney, and ear development (Heanue et al., 1999, 2002; Torres et al., 1996; Xu et al., 1999). It has recently been demonstrated that Eya possesses intrinsic phosphatase activity that, when complexed with Dach, Six1 and CBP, permits the expression of target genes essential for precursor cell proliferation and survival (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003).

The effects of mutations in the mouse orthologue, *Eya1*, on inner ear development have been described by us and others (Johnson et al., 1999; Xu et al., 1999). Targeted deletion of *Eya1* in mice suggests that it is involved in the early inductive events of inner ear morphogenesis, including cochleovestibular ganglion (cvg) neurogenesis (Xu et al., 1999). We have described a hypomorphic allele ($Eya1^{bor/bor}$) in which the adult homozygous phenotype consists of severe cochlear dysmorphogenesis and absence of the cochlear and vestibular nerves (Johnson et al., 1999).

Neural progenitor specification is one of the earliest fate determination steps in the developing ear. The Atonal-related basic helix-loop-helix genes, *Neurogenin1* (*Ngn1*) and *NeuroD* are essential positive regulators of cvg neurogenesis (Ma et al., 2000). Despite our increasing understanding of the genes governing regional and cellular specification in the developing inner ear, little is known of the genetic mechanisms underlying the regulation of these factors. Targeted and naturally occurring mutations in *Eya1* provide excellent model systems for the study of these early genetic events in the developing ear, particularly the process of neural precursor specification. In this manuscript, utilizing the null and hypomorphic *Eya1* alleles, we demonstrate that Eya1 influences cvg neurogenesis throughout development through its effects on the expression of *Tbx1*, *Ngn1*, *NeuroD*, *BDNF* and *NT-3*.

2. Results

2.1. Eya1 mutant mice display alterations in the expression of proneural genes Ngn1 and NeuroD in a dose dependent fashion

Recent evidence suggests that both Eya1 and Six1, as part of a conserved genetic network, are involved in cell fate specification within the otocyst epithelium (Zheng et al., 2003). Early in inner ear development, *Eya1* is expressed in the otic placode (E9) and is regionalized to the ventral otocyst (E10.5) (Kalatzis et al., 1998). Evidence from our laboratory and others supports a role for Eya1 in inner ear development. Targeted deletion of *Eya1* led to absence of cvg morphogenesis and inner ear developmental arrest at the otocyst stage (E10.5) (Xu et al., 1999). Deficient levels of Eya1 ($Eya1^{bor/bor}$) led to a number of morphogenetic abnormalities (Johnson et al., 1999). Whole mount preparations of the hypomorphic ear revealed subtle abnormalities of the *pars superior*, or vestibular portion. Specifically, the lateral semicircular canal, the last to appear developmentally, is foreshortened with a much narrower diameter than that of the wild-type. Several of the postnatal inner ears studied also revealed an incomplete common crus, the region of the joined non-ampullated ends of the superior and posterior semicircular canals. The abnormalities of the *pars inferior*, or cochlear portion of the inner ear, were the most severe and constant. All but the most basal one-quarter of the cochlea was absent in the adult mutant inner. Histological analysis demonstrated the rudimentary basal portion of the mutant cochlea with a spiral ligament and no overlying *stria vascularis*. Additionally, there was complete absence of the organ of Corti, and cochlear and vestibular nerves in the adult hypomorphs.

To explore the mechanisms underlying failed neurogenesis in the inner ears of the *Eya1* mutants we examined the expression of *Ngn1* and *NeuroD* in *Eya1* null ($Eya1^{-/-}$) and deficient ($Eya1^{bor/bor}$) mice. *Ngn1* (Fig. 1B) and *NeuroD* (Fig. 1E) transcripts were completely absent in the otic vesicles of E10.5 $Eya1^{-/-}$ embryos in contrast to the their expression in the ventral otic epithelium and delaminating neuroblasts of the wild-type mice (Fig. 1A,D). These data suggest that Eya1, either directly or through the action of another control gene(s), regulates the expression of these proneural genes and/or the specification of the neural precursors in the otocyst that normally express them.

As mentioned above, *Eya1* expression begins early in the otic placode stage (E8.5–E9). Its expression persists in the otic epithelium and cvg throughout development suggesting an ongoing effect of Eya1 on spiral and vestibular ganglion survival (Kalatzis et al., 1998). We have shown that mice homozygous for a hypomorphic allele of *Eya1* ($Eya1^{bor/bor}$) have a less severe inner ear defect than that present in the null. Cochlear morphogenetic arrest occurs at approximately E12, and the spiral and vestibular ganglion cells are absent in the adult. We hypothesized that cvg developmental arrest would occur at a later stage in the $Eya1^{bor/bor}$ mice than in the $Eya1^{-/-}$, and that the $Eya1^{bor/bor}$ mice would provide insights into the ongoing effects of Eya1 on cvg development.

To investigate the developmental basis for the reduction of the cvg in the $Eya1^{bor/bor}$ adult mice, we surveyed the expression of these same proneural markers at the time of neuroblast specification and delamination (E10.5). *Ngn1* expression in the $Eya1^{bor/bor}$ otic vesicle was reduced in the otic epithelium (Fig. 1C) in comparison to the wild-type (Fig. 1A). Similarly, *NeuroD* expression in the $Eya1^{bor/bor}$ inner ears was detected at reduced levels overall in comparison to the wild-type. Furthermore, much of the *NeuroD* expression in the hypomorph was confined to the otic epithelium at this stage, with few *NeuroD*-positive delaminated cells in the cvg rudiment (Fig. 1F) compared to the wild-type (Fig. 1D).