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Functional mode of FoxD1/CBF2 for the establishment of temporal retinal specificity in the developing chick retina

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ABSTRACT

Two winged-helix transcription factors, *FoxG1* (previously called *chick brain factor1*, *CBF1*) and *FoxD1* (*chick brain factor2*, *CBF2*), are expressed specifically in the nasal and temporal regions of the developing chick retina, respectively. We previously demonstrated that *FoxG1* controls the expression of topographic molecules including *FoxD1*, and determines the regional specificity of the nasal retina. *FoxD1* is known to prescribe temporal specificity, however, molecular mechanisms and downstream targets have not been elucidated. Here we addressed the genetic mechanisms for establishing temporal specificity in the developing retina using an *in ovo* electroporation technique. Fibroblast growth factor (Fgf) and Wnt first play pivotal roles in inducing the region-specific expression of *FoxG1* and *FoxD1* in the optic vesicle. Misexpression of *FoxD1* represses the expression of *FoxG1*, *GH6*, *SOHo1*, and *ephrin-A5*, and induces that of *EphA3* in the retina. *GH6* and *SOHo1* repress the expression of *FoxD1*. In contrast to the inhibitory effect of FoxG1 on bone morphogenic protein (BMP) signaling, *FoxD1* does not alter the expression of *BMP4* or *BMP2*. Studies with chimeric mutants of FoxD1 showed that FoxD1 acts as a transcription repressor in controlling its downstream targets in the retina. Taken together with previous findings, our data suggest that *FoxG1* and *FoxD1* are located at the top of the gene cascade for regional specification along the nasotemporal (anteroposterior) axis in the retina, and *FoxD1* determines temporal specificity.

Introduction

Topographic maps with a defined spatial ordering of neuronal connections are a key feature of the brain's organization. The retinotectal projection is a good model for studies on the formation of topographic maps. During development, retinal ganglion cell axons grow from the eye to the optic tectum, the primary visual center in lower vertebrates, or the superior colliculus (SC) in mammals. Retinal ganglion cell axons from the nasal (anterior) retina project to the posterior region of the tectum (or SC), while axons from the temporal (posterior) retina project to the anterior tectum/SC. The dorsal and ventral retinal axons are connected to the ventral (lateral) and dorsal (medial) tectum/SC, respectively. During the last two decades, many molecules with a topographic expression have been identified in the retina (McLaughlin et al., 2003). Among them, the Eph family of receptor tyrosine kinases and their ligands, the ephrins, have been shown to be involved in sculpturing topographic connections (McLaughlin and O'Leary, 2005).

Eph receptors are classified into two subfamilies, EphA and EphB, according to their preference for either glycosyl–phosphatidyl–inositol-anchored ephrin-A ligands or transmembrane ephrin-B ligands (Eph Nomenclature Committee, 1997). In chicks, the EphA3 receptor is expressed in a temporal high-nasal low gradient in the retinal ganglion cells (Cheng and Flanagan, 1994), and *ephrin-A2* and *ephrin-A5* are expressed in posterior high-anterior low gradients in the tectum (Cheng and Flanagan, 1994; Drescher et al., 1995; Feldheim et al., 1998; Frisén et al., 1998). Several lines of evidence suggest that EphA receptors and ephrin-A ligands in both the retina and the tectum/SC mediate axonal navigation by controlling repulsion/affinity and branching of the retinal axons along the anteroposterior (A–P) axis on the tectum/SC (Nakamoto et al., 1996; Monschau et al., 1997; Brown et al., 2000; Feldheim et al., 2000; Feldheim et al., 2004; Yates et al., 2001). In addition to EphA3, four EphA receptors (EphA4, EphA5, EphA6, and EphA7) are uniformly expressed in the chick retina (Monschau et al., 1997; Connor et al., 1998). On the other hand, their ligands, *ephrin-A2* and *ephrin-A5*, are expressed with a nasal high-temporal low gradient in the retina (Marcus et al., 1996; Connor et al., 1998; Hornberger et al., 1999). Overexpression of *ephrin-As* in temporal axons leads to errors in the topographic targeting of temporal axons (Dütting et al., 1999; Hornberger et al., 1999), suggesting a role for retinal ephrin-As in the formation of topographic

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projections. Thus, EphAs uniformly expressed in the retina are also thought to be involved in the topographic projections along the A–P axis, with the help of ephrin-As expressed with a gradient in the retina.

The graded distributions of these cell surface molecules are achieved by topographically expressed morphogens and transcription factors in the retina. A large number of molecules show asymmetrical expression patterns along the two axes in the early developmental stages of the retina (Shintani et al., 2004). Two winged-helix (WH) transcription factors, *FoxG1* (previously called *CBF1*) and *FoxD1* (*CBF2*), begin to be expressed specifically in the nasal and temporal regions of the developing chick retina, respectively, prior to Hamburger–Hamilton (HH) stage 10 (Yuasa et al., 1996). Misexpression of *FoxG1* and *FoxD1* reversed the topographic map in the retinotectal system along the A–P axis (Yuasa et al., 1996), indicating that they play a key role in the retinal regional specification along the A–P axis. Subsequently, we showed that *FoxG1* controls all of the asymmetrically distributed molecules along the A–P axis, and determines the nasal specificity in the retina through multiple mechanisms (Takahashi et al., 2003). In this process, *FoxG1* controls *ephrin-A5* via a DNA binding-dependent mechanism, *ephrin-A2* via a DNA binding-independent mechanism, and *FoxD1*, *GH6*, *SOHo1*, and *EphA3*, via dual mechanisms. In contrast, although *FoxD1* plays an essential role in determining temporal regional specificity in the retina (Yuasa et al., 1996; Yamagata et al., 1999), details regarding its downstream target genes and mode of action remain unclear.

We previously identified a novel secretory molecule, Ventroptin, in the chick retina, which is an antagonist of bone morphogenic proteins (BMPs) (Sakuta et al., 2001). At the early developmental stages, *Ventroptin* is specifically expressed in the ventral retina, with a complementary pattern to the dorsal-specific expression of *BMP4*. The counteraction between BMP4 and Ventroptin governs the regional specification along the dorsoventral (D–V) axis in the retina by regulating the expression of downstream target genes, such as *Tbx5* and *cVax* (Sakuta et al., 2001). From E6 onward, *Ventroptin* begins to be expressed in not only a ventral high-dorsal low gradient but also a nasal high-temporal low gradient in the retina (oblique-gradiently). Concomitantly, instead of *BMP4*, *BMP2* begins to be expressed in an oblique-gradient fashion, complementary to that of *Ventroptin* to counteract it (Takahashi et al., 2003). Recently, we further revealed that the topographic molecules so far reported to have a gradient only along the D–V axis and *ephrin-A2* with a gradient only along the A–P axis are both controlled by the BMP2 signal, and that they are expressed in an oblique-gradient manner from E6 onward in the chick retina (Sakuta et al., 2006). As a result, BMP2 signaling controls retinotectal projections along the two axes (Sakuta et al., 2006). The inhibitory effect of FoxG1 on BMP signaling is attributable to the turning of the expression of *Ventroptin* and *BMP2* into the oblique-gradient pattern (Takahashi et al., 2003). However, it is not clear whether *FoxD1* affects BMP signaling.

In the present study, to gain insight into downstream target genes of *FoxD1*, we examined the effects of the misexpression of *FoxD1* on the expression of the topographic molecules in the developing chick retina by electroporation of a retroviral vector carrying the *FoxD1* gene into the optic vesicle. Here we show that *FoxG1* and *FoxD1* repress each other's expression. Misexpression of *FoxD1* inhibits the expression of *GH6*, *SOHo1*, and *ephrin-A5*, and induces *EphA3* expression. Conversely, *GH6* and *SOHo1* repress the expression of *FoxD1*. In contrast to the inhibitory effect of FoxG1 on BMP signaling, FoxD1 does not alter the expression of *BMP4*, *BMP2*, *Ventroptin*, or *ephrin-A2*. *FoxD1* has dual functions as a transcriptional repressor and activator in cultured cells, but acts as a transcriptional repressor to control its downstream target genes in the developing chick retina.

Materials and methods

Plasmids

The coding regions of *fibroblast growth factor 8a* (*Fgf8a*), *Wnt3a*, *Sonic hedgehog* (*Shh*), *FoxD1*, *GH6*, and *SOHo1* were cloned into the RCAS-NS retroviral vector (Suzuki et al., 2000) via the SLAX-NS shuttle vector (Suzuki et al., 2000) to yield Fgf8a/RCAS, Wnt3a/RCAS, Shh/RCAS, FoxD1/RCAS, GH6/RCAS, and SOHo1/RCAS, respectively. A dominant negative form of chick *fibroblast growth factor receptor 1* (*DN-Fgfr1*) lacking the entire tyrosine kinase domain (amino acid residues 1 to 399) was generated by PCR, as the dominant negative mutant *Xenopus Fgfr1* (Amaya et al., 1991), and subcloned into RCAS-NS via SLAX-NS to yield DN-Fgfr1/RCAS.

To make FoxD1–eve/RCAS and VP16–FoxD1/RCAS, the repression domain of the *Drosophila* even-skipped protein (Han and Manley, 1993) or the activation domain of the herpes simplex virus VP16 protein (Triezenberg et al., 1988) was first fused with the FoxD1 DNA-binding domain (amino acid residues 139 to 245). They were inserted once into SLAX-NS myc (Takahashi et al., 2003), and then subcloned into the vector RCAS-NS.

The coding region of chick *FoxD1* was inserted into pcDNA3.1(+) (Invitrogen) and pCMV-BD (Stratagene) to yield FoxD1/pcDNA and FoxD1/pCMV-BD, respectively. To prepare mFoxD1/pcDNA, xFoxD1/pcDNA, mFoxD1/pCMV-BD, and xFoxD1/pCMV-BD, the coding regions of mouse *FoxD1* (mFoxD1) and *Xenopus FoxD1* (xFoxD1) were cloned from a P0 mouse retina cDNA and a stage 40 *Xenopus* eye cDNA by PCR, respectively. They were subcloned into pcDNA3.1(+) and pCMV-BD. To make EnRD/pCMV-BD and VP16AD/pCMV-BD, the repression domain of the *Drosophila* Engrailed protein (Badiani et al., 1994) or the activation domain of the VP16 protein was cloned into pCMV-BD.

In situ hybridization and riboprobes

Sectioning and whole-mount *in situ* hybridization were carried out as described previously (Suzuki et al., 2000). All the samples were treated in the same way throughout the study. Image acquisition and figure processing of the sections and whole-mount samples were performed as reported (Suzuki et al., 2000). The electroporated experimental side was compared with the non-electroporated control side of the same embryo.

Templates used for digoxigenin-labeled RNA probes were as follows: a 646-bp fragment of chick *Fgf8* (nucleotide residues 1–645; GenBank accession number U55189), a 1197-bp fragment of chick *Fgfr1* (56–1252; MN_205510), a 920-bp fragment of chick *Wnt1* (21–940; AY753286), a 1131-bp fragment of chick *Wnt3* (16–1146; NM_204675), a 1278-bp fragment of chick *Shh* (211–1488; MN_204821) and a 947-bp fragment of chick *Six3* (206–1152; MN_204364). The templates for the probes for *FoxG1*, *FoxD1*, *GH6*, *SOHo1*, *EphA3*, *ephrinA5*, *ephrinA2*, *BMP2*, *BMP4*, *cVax*, and *Ventroptin* were described previously (Sakuta et al., 2001; Takahashi et al., 2003).

In ovo electroporation

In ovo electroporation was performed as detailed previously (Sakuta et al., 2008). Retroviral expression constructs for electroporation were suspended in phosphate-buffered saline containing 0.05% Fast Green (Sigma, St. Louis, MO) together with pEGFP-N1 (Clonetech). Chick embryos sensitive to the RCAS-NS retrovirus were used for electroporation at HH stage 8 and incubated in a humidified incubator. The transfection efficiency was checked by the expression of GFP (Supplementary Fig. 1) (see also Sakuta et al., 2008). After electroporation, the virus particles are produced from the introduced retroviral vector DNA and infect neighboring cells. As a result, the whole right retina was homogeneously infected. Embryos of normal size and morphology, in which the infection was fairly limited to the eye region, were used for assays.