Developmental Biology 371 (2012) 57–65

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Extraocular ectoderm triggers dorsal retinal fate during optic vesicle evagination in zebrafish

Renee Kruse-Bend [a], Jude Rosenthal [a], Tyler S. Quist [a], Eric S. Veien [a], Sabine Fuhrmann [b], Richard I. Dorsky [a,*], Chi-Bin Chien [a]

[a] Department of Neurobiology and Anatomy, 20 North 1900 East, Room 401 MREB, University of Utah, Salt Lake City, UT 84132, USA
[b] Department of Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah, Salt Lake City, UT 84132, USA

ARTICLE INFO

Article history:
Received 11 April 2012
Received in revised form
10 July 2012
Accepted 9 August 2012
Available online 17 August 2012

Keywords:
Bmp
Dorsal
Retina
Initiation
Gdf6a
Morphogenesis

ABSTRACT

Dorsal retinal fate is established early in eye development, via expression of spatially restricted dorsal-specific transcription factors in the optic vesicle; yet the events leading to initiation of dorsal fate are not clear. We hypothesized that induction of dorsal fate would require an extraocular signal arising from a neighboring tissue to pattern the prospective dorsal retina, however no such signal has been identified. We used the zebrafish embryo to determine the source, timing, and identity of the dorsal retina-inducing signal.

Extensive cell movements occur during zebrafish optic vesicle morphogenesis, however the location of prospective dorsal cells within the early optic vesicle and their spatial relationship to early dorsal markers is currently unknown. Our mRNA expression and fate mapping analyses demonstrate that the dorsolateral optic vesicle is the earliest region to express dorsal specific markers, and cells from this domain contribute to the dorsal retinal pole at 24 hpf.

We show that three *bmp* genes marking dorsal retina at 25 hpf are also expressed extraocularly before retinal patterning begins. We identified *gdf6a* as a dorsal initiation signal acting from the extraocular non-neural ectoderm during optic vesicle evagination. We find that *bmp2b* is involved in dorsal retina initiation, acting upstream of *gdf6a*. Together, this work has identified the nature and source of extraocular signals required to pattern the dorsal retina.

Introduction

Achieving precise retinotopic axon targeting during development requires the patterning of projecting (retina) and target (brain) tissues along anterior–posterior and dorsal–ventral axes, such that individual cells of both tissues acquire a molecularly specified positional identity (McLaughlin et al., 2003; McLaughlin and O'Leary, 2005). Regarding the first step in fate specification, we hypothesized that the dorsal retinal initiating signal would be a diffusible molecule, providing asymmetric positional information to prospective dorsal retina, from a location external to the eye field. However an extraocular initiator of dorsal retinal fate has not yet been identified.

Past research has identified several genes necessary for establishing dorsal retinal fate (Asai-Coakwell et al., 2007; Behesti et al., 2006; French et al., 2007; French et al., 2009; Gosse and Baier, 2009; McLaughlin et al., 2003; Murali et al., 2005; Plas et al., 2008; Sakuta et al., 2006) but has focused on expression and regulation within the retinal field. Historically, a simple model was set forth in which dorsal expression of *bmp4* initiates dorsal *tbx5* and downregulates ventral *vax2* (McLaughlin et al., 2003). In support of this model, *bmp4* knockout mice never initiate expression of *tbx5* (Murali et al., 2005). Bmp4 gain of function experiments, and loss of function of receptors, also suggest Bmp-mediated regulation of *tbx5* in the dorsal retina (Behesti et al., 2006; Murali et al., 2005). However extraocular factors for initiating *bmp4* expression are unknown, and *bmp4* null mice die shortly after E9.5, precluding further analysis of gene expression and retinotectal topography in this model (Murali et al., 2005). Furthermore, recent work in zebrafish suggested that *bmp4* does not initiate dorsal-specific gene expression, whereas the *Bmp* family gene *gdf6a* plays a critical role (French et al., 2009). Gdf6a is a Bmp family member known to affect eye development in humans and mouse (Asai-Coakwell et al., 2007; Asai-Coakwell et al., 2009), yet its role in dorsal retinal patterning in these species has not yet been analyzed. In zebrafish, *gdf6a* is expressed extraocularly during optic vesicle evagination, and in the prospective dorsal retina from 15–48 hpf. *gdf6a* is critical for dorsal initiation, yet it has not been determined what time, and from what tissue, *gdf6a* acts. It is unknown whether *gdf6a* is an extraocular initiator of dorsal retinal fate, or acts only within the retinal field, suggesting another extraocular signal necessary for initiating *gdf6a* within the retina. We set out to determine

* Corresponding author. Fax: +1 801 581 4233.
E-mail address: richard.dorsky@neuro.utah.edu (R.I. Dorsky).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.08.004

the identity, time of action, and tissue source of an extraocular initiator, as well as the domain of the optic vesicle first initiated with dorsal-specific markers.

The first known manifestation of retinal dorsal identity in zebrafish is the expression of the T-box transcription factor genes, *tbx2a* and *tbx5a*, in restricted optic vesicle domains (Veien et al., 2008). The primary candidates for regulation of *tbx* genes, and thus initiation of dorsal identity, are the morphogens of the bone morphogenetic protein family (Bmps). Five *bmp* genes mark the dorsal domain of the zebrafish eye at 24 hpf—*bmp2a, bmp2b*, *bmp4*, *bmp7b* and *gdf6a* (Shawi and Serluca, 2008; Thisse et al., 2001; Thisse and Thisse, 2005). The continuing expression of all known retinal patterning genes from early to late stages of eye development led us hypothesize that these *bmps* were likely candidates for dorsal initiation signals. However it is unknown which of these candidate morphogens are expressed early, during optic vesicle evagination, in extraocular spatiotemporal domains where they could initiate the prospective dorsal retina transcription factors. Furthermore, it is not known which region of the early optic vesicle is poised to receive the dorsal initiation signal. Recent research analyzed the 24 hpf fates of early optic vesicle cells (Kwan et al., 2012), and found extensive cell movements during this time period. We theorized that extraocular dorsal initiation signals should be located adjacent to the region of the earliest polarized expression of dorsal fate within the retina. Tissues adjacent to the early optic vesicle that could give rise to the initiation signal include the neural tube (medial), non-neural ectoderm (dorsal and lateral), and prechordal plate mesoderm (anterior). In this study, we show that the lateral region of the early optic vesicle, which first expresses dorsal specific transcription factors, and is adjacent to non-neural ectoderm, is fated to give rise to the dorsal retina.

We analyzed the precise timing of dorsal initiation using a pharmacological inhibitor of Bmp signaling, and found that Bmps are required for initiating dorsal retinal markers prior to their expression within the optic vesicle, indicating that bmps are initiating dorsal retina by signaling from an extraocular tissue. We analyzed the spatiotemporal domains of genes encoding candidate initiators—*gdf6a*, *bmp2b*, and *bmp4*, and using mutants we determined the necessity of these genes for initiating dorsal fate in zebrafish.

Our work shows that dorsal retinal fate is initiated by extraocular *gdf6a*, arising from the non-neural head ectoderm at 11.5 hpf. Adjacent tissue of the dorsolateral optic vesicle leaflet receives this signal and upregulates T-box transcription factors in this domain. Additionally, we find that *bmp2b* is necessary for dorsal retinal initiation, acting upstream of *gdf6a*, possibly to establish its expression in the extraocular ectoderm.

Materials and methods

Animals

We maintained adult zebrafish (*Danio rerio*) on a 14-hour light, 10-hour dark cycle. We raised embryos in E3 embryo medium with methylene blue at 28.5 °C, anesthetized with 0.2 mg/ml tricaine and fixed in 4% paraformaldehyde unless otherwise noted. We staged embryos according to Kimmel et al. (1995), by counting somites (morphologically) and/or by *in situ* using the somite marker *myoD*. We used Tübingen strain embryos for Kaede fate map and Bmp inhibitor (LDN 193189) experiments.

Mutants

We used four mutant lines for this work: $bmp2b^{tc300}$ (Mullins et al., 1996), $bmp4^{st72}$ (Stickney et al., 2007), $gdf6a^{s327}$ (Muto et al., 2005) and oep^{m134} (Schier et al., 1997; Zhang et al., 1998). The $bmp2b^{tc300}$ line was generated in the Tübingen strain background; $bmp4^{st72}$, and $gdf6a^{s327}$ were generated in Tupfel long fin (TL) wild type strain and the oep^{m134} line was generated in the AB wild type strain. We maintained all mutant lines on their original backgrounds.

Genotyping

We genotyped adult fin clips and whole mount individual *in situ* embryos using derived Cleaved Amplified Polymorphic Sequences (dCAPS) PCR. PCR primers for $gdf6a^{s327}$ and $bmp4^{st72}$ were previously described by (Gosse and Baier, 2009; Stickney et al., 2007) respectively. We generated additional primers for genotyping $bmp4^{st72}$ and $bmp2b^{tc300}$ using the web-based program dCAPS Finder 2.0 (Neff et al., 2002).

$bmp4^{st72}$ Fwd: TGGTGAGGCACAACACCTCCAACTAG, Rev: CCGAGTCAGCGGGTGACTTTTGCCGTC. SpeI cuts mutant band. $bmp2b^{tc300}$ Fwd: GAAGTATCCGAGGAGGCTGA Rev: CCTCCACCACCATGTCCT. HaeIII cuts mutant band.

In situ hybridization

We performed whole mount *in situ* hybridization to analyze mRNA expression as described by Thisse and Thisse (2008) with the following modifications: we incubated and washed some of our samples using a Biolane HTI *in situ* machine (Huller and Huttner AG, Tübingen, Germany). We synthesized labeled riboprobes (fluorescein-UTP-labeled for *rx3*, all others labeled with Digoxigenin-UTP) using *in vitro* transcription RNA labeling kits from Roche. Probes were as follows: *tbx2a* (Dheen et al., 1999), *tbx5a* (Ruvinsky et al., 2000), *bmp4* (gift, M. Mullins, University of Pennsylvania), *tbx4*, *bmp2b* (Nikaido et al., 1997), *vax2* (Take-uchi et al., 2003), *pax2a*, *rx3*, *gdf6a* (Veien et al., 2008), *pitx3*, *ephrinB2a* (Durbin et al., 1998), *myoD* (Weinberg et al., 1996) and *isl1* (Inoue et al., 1994). Digoxigenin probes were developed with BM-Purple, and the fluorescein *rx3* probe was developed using INT-BCIP (Roche Applied Science). We cleared whole mount embryos in 50–80% glycerol and imaged them using an Olympus SZX 12 stereomicroscope, an Olympus SN1H045411-H camera, and Picture Frame™ imaging software version 2.3. Sectioned embryos were embedded in plastic according to (Sullivan-Brown et al., 2011), and sectioned transversely at 12 μM thickness using a Reichert-Jung 2050 microtome. All sections were imaged on an Olympus BX51WI inverted microscope using the same camera and imaging software as for whole mount images. In cases where a particular mutant phenotype could not be identified, we imaged and genotyped individual embryos to confirm that the pictures presented contained the correct genotypes.

Kaede fate map

We prepared capped *NLS-Kaede* mRNA using the mMessage mMachine kit from Ambion. We injected 2 ng (500 ng/μL) *NLS-Kaede* mRNA into 1-cell stage zebrafish embryos according to (Hatta et al., 2006), and raised them in E2 with gentamycin (E2/GN). At 11 hpf we dechorionated and mounted embryos live in 1.5% low melting temperature agarose to image them from the dorsal side on an FV1000-XY Olympus IX81 confocal microscope. Images shown are maximum intensity projections rendered using ImageJ.

Bmp inhibitor LDN 193189

LDN 193189 (Stemgent) was dissolved in 100% DMSO. We incubated dechorionated embryos in 1 μM LDN 193189, 1% DMSO in E2/GN or equal volume 1% DMSO/E2/GN as a control. We began