Developmental Biology 388 (2014) 192–204

Contents lists available at ScienceDirect

Developmental Biology

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

Sfrp1a and Sfrp5 function as positive regulators of Wnt and BMP signaling during early retinal development

Vanessa L. Holly [1], Sonya A. Widen [1], Jakub K. Famulski [1], Andrew J. Waskiewicz *

Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada

ARTICLE INFO

Article history:
Received 7 December 2012
Received in revised form
16 December 2013
Accepted 13 January 2014
Available online 20 January 2014

Keywords:
Retina
Wnt
BMP
Sfrp1
Sfrp5
Coloboma
Gdf6

ABSTRACT

Axial patterning of the developing eye is critically important for proper axonal pathfinding as well as for key morphogenetic events, such as closure of the optic fissure. The dorsal retina is initially specified by the actions of Bone Morphogenetic Protein (BMP) signaling, with such identity subsequently maintained by the Wnt-β catenin pathway. Using zebrafish as a model system, we demonstrate that Secreted frizzled-related protein 1a (Sfrp1a) and Sfrp5 work cooperatively to pattern the retina along the dorso-ventral axis. Sfrp1a/5 depleted embryos display a reduction in dorsal marker gene expression that is consistent with defects in BMP- and Wnt-dependent dorsal retina identity. In accord with this finding, we observe a marked reduction in transgenic reporters of BMP and Wnt signaling within the dorsal retina of Sfrp1a/5 depleted embryos. In contrast to studies in which canonical Wnt signaling is blocked, we note an increase in BMP ligand expression in Sfrp1a/5 depleted embryos, a phenotype similar to that seen in embryos with inhibited BMP signaling. Overexpression of a low dose of *sfrp5* mRNA causes an increase in dorsal retina marker gene expression. We propose a model in which Sfrp proteins function as facilitators of both BMP and Wnt signaling within the dorsal retina.

Introduction

A functional visual system requires precise axonal connections between the retina and brain. Retinal ganglion cell (RGC) projections create a topographic (or retinotopic) map of the visual field in the brain's visual processing center. Patterning of the developing retina defines dorso-ventral and naso-temporal axes, thereby specifying expression domains of axon guidance molecules in RGCs. Axes are initiated by coordinated actions of extracellular signaling molecules, establishing the expression domains of transcription factors in the dorsal, ventral, nasal, and temporal quadrants (reviewed in (Lemke and Reber, 2005)). Retinal transcription factors subsequently activate expression of the guidance cue molecules, Eph and Ephrin, receptor–ligand pairs that mediate changes in the cytoskeleton of RGC growth cones during axon guidance (Scicolone et al., 2009).

Sonic Hedgehog (Shh) signaling from the midline and optic stalk establishes ventral retina identity (Ekker et al., 1995; Sasagawa et al., 2002; Zhang and Yang, 2001). Opposing this signal, extraocular Bone Morphogenetic Proteins (BMPs), which are located lateral to the evaginating optic vesicle, initiate dorsal identity (Adler and Belecky-Adams, 2002; French et al., 2009; Gosse and Baier, 2009). Initiation of dorsal retina identity is apparent just after evagination of the optic vesicle in zebrafish, with markers such as *t-box 5a* (*tbx5a*) and *BMP and activin membrane bound inhibitor a* (*bambia*) expressed at 12 hours post fertilization (hpf) in zebrafish. Embryos lacking either of two BMPs, *gdf6a* (*growth differentiation factor 6a*) or *bmp2b*, display profound defects in dorsal retina patterning, including decreased expression of *tbx5a* and *bambia* (French et al., 2009; Kruse-Bend et al., 2012). Dorsal and ventral retina identities mutually inhibit each other to ensure neither tissue encompasses the entire retina. Ectopic expression of ventral genes expands ventral identity at the expense of dorsal retina; *ventral anterior homeobox 2* (*vax2*) mRNA overexpression in *Xenopus* causes a marked expansion of other ventral markers such as *pax2* and a reduction in the dorsal marker *vent2* (Barbieri et al., 1999). Ventralization of the retina is also associated with aberrant RGC projection, where nearly all dorsal RGCs misproject (Sakuta et al., 2001; Schulte et al., 1999). Alterations of dorsal markers can also influence the ventral retina. Reduction in the dorsally expressed *gdf6a* causes ventralization, with a complete expansion of *vax2* into the dorsal-most region of the retina (French et al., 2009; Gosse and Baier, 2009). Conversely, overexpression of the dorsal retina gene *bmp4* in mouse dorsalizes the retina and reduces the expression domain of *vax2* (Behesti et al., 2006). Overexpression of chick *Tbx5* mRNA also dorsalizes the retina, though with more subtle changes in retinotopic mapping than *vax2*-induced retina ventralization (Koshiba-Takeuchi et al., 2000).

* Correspondence to: University of Alberta, Department of Biological Sciences, CW405 Biological Sciences Building, 11455 Saskatchewan Drive, Edmonton, AB, Canada T6G2E9. Fax: +1 780 492 4403.
E-mail address: aw@ualberta.ca (A.J. Waskiewicz).
[1] These authors contributed equally to this work.

0012-1606/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2014.01.012

Wnt signaling, likely emanating from the RPE (Retinal Pigmented Epithelium), has been implicated in the maintenance of dorsal retina identity. Initiation of ocular Wnt signaling in zebrafish, as determined by the Wnt signaling transgenic, *Tg(TOP:dGFP)*^{w25} (Dorsky et al., 2002), occurs after optic vesicle formation and initiation of dorsal–ventral eye patterning (Veien et al., 2008). To date, only three *wnt* genes have documented expression in the developing zebrafish eye. *Wnt11r* has lens-specific expression and *wnt2* and *wnt8b* are expressed in the RPE between 14 and 16 hpf. Overexpression of the Wnt inhibitors *dickkopf 1b* (*dkk1b*) or dominant negative *tcf7la* (the zebrafish ortholog of mouse *Tcf3*) inhibits canonical Wnt signaling. In congruence with canonical Wnt activity appearing subsequent to dorsal–ventral retina axis initiation, blockade of Wnt signaling does not affect expression of early dorsal or ventral retina markers (Veien et al., 2008). However, embryos at later stages display severe defects in dorsal retina gene maintenance including loss of *aldh1a2*, *tbx5a*, and *bambia* and the ablation of the dorsally expressed BMP ligands, *bmp2b*, *bmp4*, and *gdf6a*. Dorsal eye phenotypes caused by loss of Wnt signaling are rescued by overexpressing *bmp4*, a member of the Bone Morphogenic Protein (BMP) family. This leads to the model that Wnts act upstream of BMP signaling during the retinal maintenance phase to establish proper dorso-ventral patterning in the retina (Veien et al., 2008). In support of this, mutation of the mouse Frizzled (Fz) co-receptor, *Lrp6*, causes ocular defects including an expansion of ventral markers (*Vax2*) at the expense of dorsal ones (*Tbx5* and *Bmp4*) (Zhou et al., 2008,2010).

Robust specification of axial pattern typically requires both morphogens and opposing antagonists. We have previously shown that BMP signaling in the dorsal retina antagonizes and constrains the ventral domain (French et al., 2009). However, a ventral antagonist of the dorsal BMP and Wnt signaling has yet to be identified. As the dorsal retina is specified by BMP and Wnt signaling, researchers searched for ventral retina-specific molecules known to inhibit the activities of these growth factors. BMP signaling is opposed by the *chordin*, *noggin*, and *follistatin* gene families (reviewed in (Thomsen, 1997)). A chordin-related chick gene, *ventroptin*, specifies ventral retina identity by antagonizing dorsal BMP ligands (Sakuta et al., 2001). However, *ventroptin* expression has thus far only been identified in the chick leaving the identity of a zebrafish ventral antagonist unclear. Our goal was therefore to identify molecules that modulate retinal BMP or Wnt signaling during retinal patterning.

Wnt signaling is modulated by Secreted frizzled-related proteins (Sfrps), a family of extracellular proteins that contain a Cysteine-Rich Domain (CRD) that is homologous to the extracellular portion of the Wnt-binding receptor, Fz. Based on their structural similarity to the CRD of the Fz receptor, Sfrps were classically assumed to be Wnt inhibitors. In support of this model of Sfrp function, original experiments investigating the relationship between Sfrps and Wnts showed that *Xenopus sfrp3* (zebrafish *frzb*) mRNA rescues the ventralization phenotypes resulting from *wnt8* mRNA overexpression (Leyns et al., 1997; Wang et al., 1997). Similar conclusions were also obtained in zebrafish following rescue of *wnt8b* ventralization phenotypes with *sfrp1a* mRNA overexpression (Kim et al., 2007). Furthermore, overexpression of *wnt11* can rescue foregut defects caused by *sfrp5* overexpression in *Xenopus* (Li et al., 2008). Recent experiments, however, have led to an emerging model whereby Sfrp function is dependent on both dose and context (Esteve et al., 2011; Lopez-Rios et al., 2008). The simple model that Sfrp proteins target only Wnt signaling has also been questioned, with evidence that the *sfrp*-related *sizzled* functions to oppose embryonic BMP signaling (Muraoka et al., 2006).

To identify potential modulators of retinal BMP and Wnt signaling, we cloned *sfrp* genes that are expressed in the early zebrafish retina. The zebrafish genome contains seven known *sfrp* genes: *sfrp1a*, *sfrp1b*, *sfrp2*, *sfrp3* (*frzb*), *sfrp5*, *sizzled*, and *tlc*. On the basis of phylogenetic analysis, *sfrp1a, sfrp1b*, *sfrp2*, and *sfrp5* are more closely related (Tendeng and Houart, 2006). In this study, we focused on *sfrp1a* and *sfrp5*, which are both expressed in the developing retina at the time of retinal patterning. Using morpholino knockdown, we unexpectedly determined that *sfrp1a* and *sfrp5* function as Wnt and BMP signaling facilitators during retinal development. Loss of *sfrp1a* and *sfrp5* leads to reduction in the expression domain of dorsal patterning markers as well as BMP and Wnt signaling reporters. Furthermore, we show that Sfrp1a and Sfrp5 are also involved in choroid fissure fusion, a morphological process known to require proper retinal patterning. On the basis of careful analysis of dorsal retina gene expression we propose a model whereby *sfrp1a* and *sfrp5* act to facilitate BMP signaling during initiation of dorsal retina identity, while during the maintenance phase *sfrp1a* and *sfrp5* enhance activation of Wnt signaling therefore maintaining dorso-ventral axis patterning.

Materials and methods

Zebrafish strains and morpholinos

Animal protocols were consistent with guidelines by the Canadian Council of Animal Care and approved by the University of Alberta's Animal Care and Use Committee (Protocol #427). All experiments were performed using the wildtype AB zebrafish strain, with the exception of experiments utilizing the *Tg*(*Olrx3:eGFP*) (generated using *oryzias latipes rx3* promoter (Rembold et al., 2006)), *Tg(BMPRE-AAV.Mlp:eGFP)* (Collery and Link, 2011), *Tg*(*TOP:dGFP*)^{w25} (Dorsky et al., 2002), or *Tg*(*hsp701:dkk1b-GFP*)^{w32} (Stoick-Cooper et al., 2007) transgenic strains. Embryos were grown at 25.5–33 °C and staged according to developmental hallmarks (Kimmel et al., 1995). Morpholino oligonucleotides (MO; GeneTools) were heated at 65 °C for 10 min and allowed to cool before injecting into 1–4 cell stage zebrafish embryos. Splice-blocking MOs targeting *sfrp1a* (*sfrp1a*-MO1: TAGTCATTTAGACTTACCGTTGGGT) and *sfrp5* (*sfrp5*-MO1: TGAGTGCTGTAGATAGAACAAAA-GA) were co-injected, each at 3 ng doses. Morpholino specificity was confirmed through the use of RT-PCR, which showed reduced levels of correctly spliced transcript (data not shown). Morphological phenotypes were also recapitulated by injecting non-overlapping splice-blocking and translation-blocking MOs: *sfrp1a* (non-overlapping splice-block)-MO2: TGTCCTGAAAGAGAGAAAATGCTGT; *sfrp1a* (translation-block)-MO3: GGACAAAGATGCAAGGGACTTCATT; *sfrp5* (translation-block)-MO2: ACACCTGCCTCTTCAGCTCCGC CAT. *Gdf6a* MOs were previously shown to effectively and specifically knockdown Gdf6a protein and recapitulate the phenotype of *gdf6a*$^{-/-s327}$ mutants (French et al., 2009).

Live imaging

Prior to imaging, embryos were injected with 3 ng each of *sfrp 1a*-MO1 and *sfrp5*-MO1 into the *Tg(Olrx3:GFP)* strain of zebrafish. Dechorionated wildtype (uninjected) and *sfrp1a/sfrp5* morpholino injected embryos (18 hpf) were laterally mounted in low melting point agarose (1.5%) and submerged in embryo media. Images were taken using a Zeiss Axio Imager Z1, Zeiss LSM700 laser confocal scanner, and a 20× water-emersion objective lens. The Zeiss Zen software was programmed to image every 10 min over a span of 24 h, with ambient temperature maintained at 28 °C.

Whole mount in situ hybridization and immunofluorescence

Analysis of mRNA expression by whole mount in situ hybridization was performed as previously described (French et al., 2009; Gongal and Waskiewicz, 2008). RT-PCR was used to generate 600–1000 bp templates for direct probe synthesis or sub-cloned