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MECHANISMS OF DEVELOPMENT

Sonic hedgehog is involved in formation of the ventral optic cup by limiting *Bmp4* expression to the dorsal domain

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ABSTRACT

Accumulating evidence suggests that Sonic hedgehog (Shh) signaling plays a crucial role in eye vesicle patterning in vertebrates. Shh promotes expression of Pax2 in the optic stalk and represses expression of Pax6 in the optic cup. Shh signaling contributes to establishment of both proximal–distal and dorsal–ventral axes by activating Vax1, Vax2, and Pax2. In the dorsal part of the developing retina, *Bmp4* is expressed and antagonizes the ventralizing effects of Shh signaling through the activation of *Tbx5* expression in chick and Xenopus. To examine the roles of Shh signaling in optic cup formation and optic stalk development, we utilized the *Smoothened* (*Smo*) conditional knockout (*CKO*) mouse line. Smo is a membrane protein which mediates Shh signaling into inside of cells. Cre expression was driven by *Fgf15* enhancer. The ventral evagination of the optic cup deteriorated from E10 in the *Smo-CKO*, whereas the dorsal optic cup and optic stalk develop normally until E11. We analyzed expression of various genes such as *Pax* family (*Pax2/Pax6*), *Vax* family (*Vax1/Vax2*) and *Bmp4*. *Bmp4* expression was greatly upregulated in the optic vesicle by the 21-somite stage. Then *Vax1/2* expression was decreased at the 20- to 24-somite stages. *Pax2/6* expression was affected at the 27- to 32-somite stages. Our data suggest that the effects of the absence of Shh signaling on *Vax1/Vax2* are mediated through increased *Bmp4* expression throughout the optic cup. Also unchanged patterns of *Raldh2* and *Raldh3* suggest that retinoic acid is not the downstream to Shh signaling to control the ventral optic cup morphology.

1. Introduction

In vertebrates, there are three members of the hedgehog family: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) (Ingham and McMahon, 2001). Shh is required for multiple aspects of development in a wide range of tissue types (reviewed in McMahon et al., 2003). Smo is a membrane protein which medicates hedgehog (Hh) signal into the cells (Taipale et al., 2002). In the absence of Hh, Patched (Ptc) represses Smo. Hh binding to Ptc releases Smo,

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which then transduces the signal intracellularly. Downstream of Smo, a multimolecular network, through interactions with microtubules, transduces the Hh signal to modify the activity of Gli proteins. These zinc-finger motif transcription factors, Gli1, Gli2 and Gli3, play critical roles in the mediation and interpretation of Hh signals through the activation and repression of Hh target genes (Amato et al., 2004).

The eye develops from the optic vesicle which arises as an optic eminence of the neuroepithelium of ventrolateral forebrain at embryonic day (E) 8–8.5 in mice (Rugh, 1968; Pei and Rhodin, 1970). As the optic vesicle expands distally, its proximo-distal (P–D) axis is established. Next the distal-most region invaginates to form the optic cup while the proximal region gives rise to the optic stalk. Shh, secreted from the ventral midline, plays important roles in this process. Genetic ablation of Shh in mice leads to severe defects in the anterior neural tube and cyclopia (i.e. the presence of an unseparated optic vesicle) (Chiang et al., 1996). Gain-of-function experiments led to the conclusion that Shh promotes proximal fate and represses distal fate by regulating the expression of Pax genes. In zebrafish and Xenopus, Shh overexpression promotes expression of Pax2, a marker of optic stalk, and represses expression of Pax6, a marker of the retina (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). In addition, these two genes transcriptionally repress each other, forming a precise boundary between the retina and the optic stalk (Schwarz et al., 2000).

Studies in mice suggest that Shh is also involved in the establishment of eye dorsal–ventral (D–V) axis. Previous studies implicate the paired homeodomain transcription factors, Pax6 and Pax2, and the secreted Shh in dorsal–ventral patterning of the optic vesicle. Soon after the evagination of the optic vesicle, the expression of Pax6 becomes restricted to the cells of the developing optic cup, which include progenitors of the pigment epithelium and the retina (Grindley et al., 1995). The expression domain of Pax2 first overlaps that of Pax6 in the ventral retinal cells surrounding the choroid fissure. Later, Pax2 expression is complementary to Pax6 expression with a sharp boundary between the retina and the optic stalk (Nornes et al., 1990; Schwarz et al., 2000). Loss of Pax6 function in the small eye (Sey) mouse and rat leads to the absence of the eyes (Grindley et al., 1995; Osumi-Yamashita et al., 1997) while loss of Pax2 results in defects of the optic tract and chiasm (Torres et al., 1996). Furthermore, Pax6 and Pax2 expression in the optic vesicle is regulated by Shh. Alterations in Shh activity in zebrafish have been shown to perturb Pax6 and Pax2 expression, leading to anomalies of eye development (Macdonald et al., 1995; Ekker et al., 1995).

The optic vesicles receive two antagonistic signals: Shh from the ventral midline and BMP4 from the dorsal part of the optic vesicle. These molecules act in a coordinated manner to pattern the eye along the D–V axis, repressing each other (Ohkubo et al., 2002). It is likely that this mutual repression is achieved by their target genes, *Vax2* and *Tbx5*. *Vax2* is activated in the ventral part of the optic vesicle by Shh (Sasagawa et al., 2002). *Tbx5* is activated in the dorsal part of the optic vesicle by *Bmp4* (Sasagawa et al., 2002; Koshiba-Takeuchi et al., 2000). Their misexpression affects the D–V axis of the eye (Barbieri et al., 1999; Koshiba-Takeuchi et al., 2000). Vax2 drives development of the ventral tissue by inhibiting development of the dorsal tissue (Mui et al., 2005).

Vax1 and *Vax2* are homeobox genes and expressed in the retina primordium. The two genes share the same gene organization (Ohsaki et al., 1999). At E9.5, both *Vax* genes were expressed in the ventral optic vesicles. Between E11.5 and E14.5, *Vax1* became restricted to the optic stalk while *Vax2* was expressed in the ventral half of the neural retina anlagen. At E9.5, the optic vesicle had already been patterned along the dorsal–ventral axis through the action of *Shh* (Mui et al., 2005). By the study of *Vax1* homozygous mutants, it has been indicated that *Vax1* and *Pax2* expression in the optic stalk requires midline signals, such as Shh (Hallonet et al., 1999). Also, Shh overexpression leads to dorsal expansion of the *Vax2* expression domain (Sasagawa et al., 2002). *Vax2* has been thought to play an important role in eye development because of both its expression patterns and functional studies carried out in frog and chicken (Barbieri et al., 1999; Schulte et al., 1999). In another report, the analysis of *Vax2* mutant mice demonstrates that *Vax2* is essential for normal eye formation and pathfinding of retinal ganglion cell axons (Barbieri et al., 2002).

The previous studies have demonstrated that Shh signaling regulates the above genes during eye development. However, it has not been elucidated whether these genes are the direct targets of Shh signaling. In this study, we examined expression patterns of these genes in *Smo*-conditional knock-out mice. We identified the temporal and spatial changes of expression of these genes. At least at early stages, the effects of Shh signaling on *Vax1/Vax2* expression were mediated through Bmp4, but not through Pax6 and Pax2. It is also possible that Shh signaling does not directly regulate *Vax1/Vax2* expression in the eye field at all stages. Furthermore, Shh signaling is critical for the ventral retinal cell proliferation and survival. Our data also suggest Shh activity is required to maintain the dorsal part of the developing optic cup.

2. Results

2.1. *Generation of Smo-conditional knock-out mice*

To examine the roles of Shh signaling in optic cup development, we utilized the *Smo* conditional allele line (Zhang et al., 2001). To remove the *Smo* conditional allele in the developing optic cup cells, we used the Cre transgenic mice in which Cre expression is driven by the *Fgf15* enhancer (Saitsu et al., 2005). To gain *Fgf15nCre; Smo c/−* mice (*Smo-CKO*), *Fgf15nCre* mice were mated with *Smo +/− mice*. *Fgf15nCre; Smo +/−* mice were mated with *Smo c/c* to produce *Fgf15nCre; Smo c/−* (*Smo-CKO*). *Smo-CKOs* were considered as conditional mutant embryos. *Smo c/+* and *Fgf15nCre; Smo c/+* were considered as Control 1 and 2, respectively.

To clarify Cre expression, coronal sections of eyes were immunostained at the 26-somite stage (E9.75) (Fig. 1A–C) and the 36-somite stage (E10.5) (Fig. 1D–F). Cre expression was observed in the distal to ventral walls of the optic vesicle (Fig. 1B and C) at the 26-somite stage (E9.75). At the 36-somite stage (E10.5), *Smo-CKOs* and Control 2 expressed Cre (brown) in the dorsal and middle domains of neural retina (Fig. 1E and F). Cre expression in these embryos corresponded to *Fgf15* expression at the same stage. Cre staining was not