Mechanisms of Development 123 (2006) 793–800

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Determinative role of Wnt signals in dorsal iris-derived lens regeneration in newt eye

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Received 2 August 2006; received in revised form 24 August 2006; accepted 27 August 2006
Available online 1 September 2006

Abstract

We have previously shown that lens regeneration from the pigmented epithelium of the dorsal iris in the adult newt eye proceeds in two steps after lens removal or intraocular FGF2 injection. The FGF2-dependent proliferation of iris pigmented epithelium and activation of early lens genes that occur over the entire circumference of the iris comprise the first step, while subsequent dorsally confined lens development marks the second step. Here, we investigated the expression of *Wnt* and Wnt receptor *Frizzled* genes in lens-regenerating iris tissues. *Wnt2b* and *Frizzled4* were activated only in the dorsal half of the iris in synchrony with the occurrence of the second step, whereas *Wnt5a* and *Frizzled2* were activated in both halves throughout the period of the first and second steps. Cultured explants of the iris-derived pigmented epithelium in the presence of FGF2 underwent dorsal-specific lens development fully recapitulating the in vivo lens regeneration process. Under these conditions, Wnt inhibitors Dkk1, which specifically inhibits the canonical signal pathway, and/or sFRP1 repressed the lens development, while exogenous Wnt3a, which generally activates the canonical pathway like Wnt2b, stimulated lens development from the dorsal iris epithelium and even caused lens development from the ventral iris epithelium, albeit at a reduced rate. Wnt5a did not elicit lens development from the ventral epithelium. These observations indicate that dorsal-specific activation of *Wnt2b* determines the dorsally limited development of lens from the iris pigmented epithelium.
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Keywords: Newt; Lens regeneration; Iris; FGF2; *Wnt2b*; *Wnt5a*; *Frizzled2*; *Frizzled4*

1. Introduction

Total regeneration of experimentally excised lens from the dorsal part of the iris pigmented epithelium of newts has been a key model of tissue regeneration via cells originating from a foreign tissue, and a number of experimental approaches have been taken to characterize this regeneration process (Mikami, 1941; Reyer, 1954; Yamada, 1966; Eguchi, 1967). An important conclusion was the tissue autonomy of the pigmented epithelium of dorsal iris, namely even after grafting iris fragments in an eye chamber (Reyer, 1954), or placing pigmented epithelial explants under tissue culture conditions (Okamoto et al., 1998; Hayashi et al., 2002), only dorsally derived pigmented epithelial tissues develop lentoids. Due to this strict spatial restriction of the lens origin in the newt iris, it has often been assumed that all cellular processes to initiate lens regeneration occur only in the dorsal iris.

However, reinvestigation of the process of lens regeneration in the newt eye, with examination of the expression of lens differentiation-associated transcription factors and signaling molecules in addition to morphological alterations and BrdU incorporation, clearly indicated that lens regeneration from the newt iris proceeds through two major

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0925-4773/$ - see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mod.2006.08.009

steps after lens removal (Mizuno et al., 1999; Hayashi et al., 2004). The first step is cell proliferation at the pupillary margin, thickening of the iris tissue, and augmentation of the expression of early lens transcription factors Pax6, Sox2, and MafB. The response of cells to the lens removal in this first step occurs over the entire circumference of the iris tissue. The second step is development of the transparent lens from the iris, accompanied by the expression of late lens transcription factors Sox1 and Prox1, and followed by β-crystallin expression. It is this second step that occurs exclusively in the dorsal iris.

We further demonstrated that the first step of the lens regeneration process is initiated by the action of FGF2, based on the following evidence (Hayashi et al., 2004): (1) after lens removal, expression of *Fgf2* is uniquely augmented among various *Fgf* genes; (2) intraocular injection of a soluble form of FGF receptor (FGFR2(IIIc)/Fc) interfering with FGF2 action inhibited all responses to lens removal characteristic of the first step of lens regeneration; and (3) injection of FGF2 into an eye chamber of a normal eye specifically elicited all the molecular and cellular reactions involved in the first step, including the activation of endogenous *Fgf2* expression in the entire iris tissue. None of the other FGFs tested had this activity. This response to exogenous FGF2 caused the development of a second lens from the dorsal iris, perfectly mimicking the lens regeneration process. In addition to this ability of FGF2 to initiate the first step of lens regeneration, FGF activity is also required for later events of the lens regeneration process (Del Rio-Tsonis et al., 1997; McDevitt et al., 1997; Del Rio-Tsonis et al., 1998; Hayashi et al., 2002, 2004).

It was also noted that the first step responses elicited by exogenous FGF2 were indistinguishable between the dorsal and ventral iris, in contrast to the somewhat stronger reactions in the dorsal iris after lens removal. This suggests that after lens removal the source of FGF2 to initiate the first step of lens regeneration is localized to the dorsal side of the eye. However, this cannot be the mechanism by which lens regeneration occurs only from the dorsal pupillary margin of the iris, because even under the condition of the uniformly activated first step process after FGF2 injection, the second lens developed only from the dorsal iris (Hayashi et al., 2004).

In the present study, we investigated the mechanism underlying dorsal iris-specific lens development in the second step of lens regeneration from the pigmented epithelium. We speculated that signaling molecules that can only act over a short range are involved in this dorsal-specific lens-regenerating reaction. Wnt proteins were strong candidates for such molecules, as previous studies indicated that inactivation of the Wnt co-receptor LPR5 impairs lens development (Stump et al., 2003), and Wnt signaling promotes lens maturation (Lyu and Joo, 2004). We thus investigated the expression of *Wnt* genes and Wnt receptor *Frizzled* genes in iris tissue after lens removal or intraocular FGF2 injection.

We show here that activation of the canonical Wnt signal activates the lens developmental program in the pigmented epithelium of the iris after FGF2 treatment, and even allows lens development from the ventral iris-derived pigmented epithelium. The results indicate that dorsal iris-specific activation of Wnt2b is likely to be the major cause of dorsal iris-specific derivation of the regenerating lens.

2. Results

2.1. Expression of Wnt and Frizzled genes in the newt eye and lens-regenerating iris

From the total RNA of tail-bud stage newt embryos, *Wnt2b*, *Wnt3*, *Wnt3a*, *Wnt4*, *Wnt5a*, and *Wnt5b* cDNAs were amplified by RT-PCR using degenerate primers. The identity of these cDNA clones was confirmed by nucleotide sequence determination. *Frizzled2*, *4*, and *10* cDNAs of newt were also cloned similarly.

Iris tissues were excised from eyes at various times after the removal of the lens or intraocular injection of FGF2, cut into dorsal and ventral halves. RNAs were extracted from pools of these iris fragments and used for examination of gene expression by RT-PCR, comparing the dorsal and ventral halves (Fig. 1A). As the entire regeneration process proceeds 2 days sooner after FGF2 injection compared with after lens removal (Hayashi et al., 2004), specimens were collected considering this 2-day difference. Among the *Wnt* genes examined, *Wnt2b* (which acts through the canonical Wnt signaling pathway) and *Wnt5a* (which acts through non-canonical signaling pathways) were the only *Wnt* transcripts detected in the iris tissues of lens-removed or FGF2-injected eyes (Fig. 1Ba).

It was remarkable that *Wnt2b* expression was activated only after removal of the lens (after 8 days) or after FGF2 injection (after 6 days), and predominantly in the dorsal half of the iris tissue (Fig. 1B). In contrast, *Wnt5a* was already expressed at a low level in both halves of the iris tissue of the normal eye. After the lens removal or intraocular FGF2 injection, the expression level of *Wnt5a* was augmented very quickly (by 4 days after lens removal and 2 days after FGF2 injection) and equally in both the dorsal and ventral halves, and this augmented *Wnt5a* expression level was maintained throughout the period of the lens regeneration process.

Not only these *Wnt* genes, but also Wnt receptor *Frizzled* genes were regulated in a manner responding to the removal of the lens or intraocular FGF2 injection (Fig. 1Bb). In particular, *Frizzled4* was activated only after lens removal/FGF2 injection and was confined to the dorsal half of the iris, similar to the activation pattern of *Wnt2b* but occurring slightly later, paralleling lens differentiation as indicated by *βB1-crystallin* expression (12 days after lens removal). In contrast, *Frizzled2* was expressed at a low level in the normal iris in both the dorsal and ventral halves, and its expression level gradually increased in

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