Mechanisms of Development 124 (2007) 543–550

www.elsevier.com/locate/modo

Compensational regulation of bHLH transcription factors in the postnatal development of *BETA2/NeuroD1*-null retina

Jang-Hyeon Cho [a,b,*], William H. Klein [a,d], Ming-Jer Tsai [b,c]

[a] *Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd. Unit 1000, Houston, TX 77030, USA*

[b] *Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*

[c] *Program of Development, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*

[d] *Graduate Training Program in Genes and Development, The University of Texas, Graduate School of Biomedical Sciences at Houston, Houston, TX 77030, USA*

Received 25 January 2007; received in revised form 24 May 2007; accepted 2 June 2007
Available online 12 June 2007

Abstract

The bHLH transcriptional factor *BETA2/NeuroD1* is essential for the survival of photoreceptor cells in the retina. Although this gene is expressed throughout the retina, *BETA2/NeuroD1* knockout mice show photoreceptor cell degeneration only in the outer nuclear layer of the retina; other retinal neurons are not affected. Previous studies on retina explants lacking three bHLH genes revealed that retinal neurons in the inner nuclear layer require multiple bHLH genes for their differentiation and survival. However, single- or double-gene mutations show no or a lesser degree of abnormalities during eye development, likely because of compensation or cooperative regulation among those genes. Because not all null mice survive until the retina is fully organized, no direct evidence of this concept has been reported. To understand the regulatory mechanisms between bHLH factors in retinal development, we performed a detailed analysis of *BETA2/NeuroD1* knockout mice. BETA2/NeuroD1 was expressed in all 3 layers of the mouse retina, including all major types of neurons. In addition, a null mutation of *BETA2/NeuroD1* resulted in up-regulation of other bHLH genes, *Mash1*, *Neurogenin2*, and *Math3*, in the inner nuclear layer. Our data suggest that compensatory and cross regulatory mechanisms exist among the bHLH factors during retinal development.

Keywords: *BETA2/NeuroD1*; *Mash1*; *Math3*; *Neurogenin2*; bHLH; Retina; Retinogenesis

1. Introduction

The retina is an excellent model system for studying the mechanism of neurogenesis because it is a relatively simple and laminated tissue. The retina is composed of distinct neuronal and glial cell populations arranged in three distinct layers: cone and rod photoreceptors are in the outer nuclear layer (ONL); bipolar cells, amacrine cells, horizontal cells, and Müller glial cells are in the inner nuclear layer (INL); and retinal ganglionic cells and displaced amacrine cells are in the ganglionic cell layer (GCL). Each cell type emerges from the retinal precursor population, and retinal precursor cell fate is influenced by both intrinsic and extrinsic factors (Cepko, 1999). Many *in vivo* and *in vitro* studies have suggested that basic helix–loop–helix (bHLH) transcription factors are intrinsic regulators for cell fate determination and differentiation (Cepko, 1999; Vetter and Brown, 2001; Hatakeyama and Kageyama, 2004; Akagi et al., 2004; Yan et al., 2005).

In developing mouse retina, bHLH genes such as *Mash1*, *Neurogenin2*, *Math3*, and *BETA2/NeuroD1* are expressed in the retinal progenitor cells or differentiating

* Corresponding author. Address: Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd. Unit 1000, Houston, TX 77030, USA. Tel.: +1 713 834 6264; fax: +1 713 834 6266.
E-mail address: JHCho@mdanderson.org (J.-H. Cho).

0925-4773/$ - see front matter
doi:10.1016/j.mod.2007.06.001

retinal neurons (Tomita et al., 1996; Morrow et al., 1999; Pennesi et al., 2003; Ma and Wang, 2006), whereas bHLH gene *Math5* is exclusively expressed in retinal ganglionic cells and their progenitors (Brown et al., 2001; Wang et al., 2001; Mu et al., 2005). Although these genes are often expressed in overlapping patterns and play critical roles in cell fate determination and differentiation, the molecular mechanisms underlying the postnatal differentiation of the diverse types of retinal neurons are largely unknown. In attempts to elucidate these mechanisms, mutational analyses have been performed using homologous recombination or conditional knockout strategies (Tomita et al., 2000; Inoue et al., 2002; Akagi et al., 2004; Ma and Wang, 2006). However, targeted mutations in any of these bHLH genes lead to perinatal lethality or result in no obvious phenotypes in the retina (Tomita et al., 1996, 2000; Ma and Wang, 2006). As examples, *Mash1* knockout mice die soon after birth and do not show any defects in the retina (Tomita et al., 1996), and only about 40% of *Math3*-null mutants are viable, although little abnormality has been observed in surviving mice (Tomita et al., 2000). These results raise the possibility that other bHLH genes could be functionally redundant in the retina; that is, a single bHLH gene deletion may not be sufficient to disrupt the whole process of retinogenesis.

To determine whether multiple bHLH factors are required for the differentiation and survival of distinct cell types, double- and triple-gene knockout mice have been generated (Tomita et al., 2000; Inoue et al., 2002; Akagi et al., 2004). In those studies, retinal explant experiments were used to overcome perinatal lethality. However, retinal explants do not survive long postnatally, making it difficult to examine possible cross regulation among bHLH genes during the late stages of retinogenesis and age-related degenerative processes of the adult retina. Thus, a useful *in vivo* model of surviving mutants is required for more definitive analysis.

BETA2/NeuroD1 is known to be expressed in the CNS and PNS as early as E8.5 and its expression persists through adulthood (Cho and Tsai, 2004). *BETA2/NeuroD1*-null mice exhibit a severe loss of cone and rod photoreceptors (Pennesi et al., 2003). In wild-type mice, a high level of *BETA2/NeuroD1* expression is observed in the outer half of the neuroblastic layer (NL) of the developing retina, and a lower expression level is observed in the developing INL around birth. A moderate expression level persists throughout the postnatal stages in the ONL and INL and remains at a stable level in the ONL of the adult retina (Morrow et al., 1999; Pennesi et al., 2003). In addition, in retinal explants, *BETA2/NeuroD1* is detected in the precursors of various lineages and plays essential roles in the specification of many distinct neuronal cell types in cooperation with other bHLH genes (Akagi et al., 2004). Although expression of *BETA2/NeuroD1* is detected in many neuronal lineages in the retina (Akagi et al., 2004), no other significant abnormalities have been found in the retina other than photoreceptor cell degeneration (Pennesi et al., 2003). It is possible that other bHLH factors may compensate for *BETA2/NeuroD* function in the differentiation and maintenance of these neurons. In this study, we characterized BETA2/NeuroD1 expression in major retinal neurons at various postnatal stages and found that the BETA2/NeuroD1 was expressed in all major neuronal cells of the retina. Furthermore, *BETA2/NeuroD1*-null mutation led to compensatory up-regulation of *Mash1, Math3,* and *Neurogenin2* in the INL.

2. Results

2.1. Expression of BETA2/NeuroD1 in the postnatal mouse retina

To address the importance of *BETA2/NeuroD1* in postnatal retinal development, we examined its spatiotemporal expression by immunohistochemically analyzing the postnatal stages from P0 to P30. We had previously demonstrated that *BETA2/NeuroD1* exhibits a dynamic expression pattern in the retina during embryonic stages (Pennesi et al., 2003). In that study, BETA2/NeuroD1 was expressed in the outer half of the NL and in a certain populations of cells in the developing INL and GCL (Morrow et al., 1999; Pennesi et al., 2003). In our current study, at P0 and P3, BETA2/NeuroD1 expression was observed in the outermost 3–5 layers of the NL, and a few faintly expressing retinal cells were also observed in the middle part of the NL as well as in the GCL (Figs. 1A and B). At P5, in addition to the strong immunoreactivity in the presumptive ONL where differentiating photoreceptors are localized, BETA2/NeuroD1 expression was observed in the outer half of the developing INL where differentiating horizontal, bipolar, and amacrine cells are localized (Fig. 1C). Notably, BETA2/NeuroD1 delineated the boundary between the outer and inner halves of the INL by its preferential expression. The expression patterns were persistently high in the ONL and outer half of the INL at P10 (Fig. 1D) and P15 (Fig. 1E) and remained at a stable level in the adult retina at P30 (Fig. 1F). In addition, punctate staining was observed in the developing inner half of the INL and GCL at all stages examined (Figs. 1C–E). This spatiotemporal expression pattern of BETA2/NeuroD1 in the INL during postnatal development raises the possibility that *BETA2/NeuroD1* has functions in cell type specification in the INL, the GCL, or both.

2.2. Characterization of BETA2/NeuroD1-expressing neurons in the retina

To define the subpopulations of BETA2/NeuroD1-expressing neurons in the retina, especially in the INL and GCL, we performed double immunofluorescence with a series of markers for retinal neurons and the anti-NeuroD1 antibodies on P15 wild-type retinas. The expression of BETA2/NeuroD1 was detected mainly in the