Eb1f1 deficiency causes increase of Müller cells in the retina and abnormal topographic projection at the optic chiasm

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ABSTRACT

The Eb1 transcription factors play important roles in the developmental processes of many tissues. We have shown previously that four members of the Eb1 family are expressed during mouse retinal development and are both necessary and sufficient to specify multiple retinal cell fates. Here we describe the changes in cell differentiation and retinal ganglion cell (RGC) projection in Eb1f1 knockout mice. Analysis of marker expression in Eb1f1 null mutant retinas reveals that loss of Eb1f1 function causes a significant increase of Müller cells. Moreover, there is an obvious decrease of ipsilateral and retinoretinal projections of RGC axons at the optic chiasm, whereas the contralateral projection significantly increases in the mutant mice. These data together suggest that Eb1f1 is required for suppressing the Müller cell fate during retinogenesis and important for the correct topographic projection of RGC axons at the optic chiasm.

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1. Introduction

The mouse retina is composed of six classes of neuronal cells, including the ganglion, amacrine, horizontal, bipolar, rod and cone cells, and one class of glia cells, the Müller cells. The seven classes of cells can be further divided into more than 50 subgroups with distinct morphologies and functions [1,2]. All retinal cell types arise from the same group of multipotent progenitors during retinogenesis. The process of retinal development is under the tight and delicate controls of both intrinsic and extrinsic factors [3,4]. The rod and cone photoreceptors reside in the outer nuclear layer and are responsible for collecting the light signal from the environment. The horizontal, bipolar and amacrine cells are interneurons located in the inner nuclear layer and are responsible for integrating and relaying the signal from the photoreceptors. The retinal ganglion cells (RGCs) integrate the signals from amacrine and bipolar cells, and then transmit them through their long exons, which bundle into the optic nerve and cross the optic chiasm before finally projecting into the brain.

Visual signals from each retina are transmitted to the thalamus and cerebral cortex on both sides of the brain. One of the crucial steps to establish the binocular and 3-dimensional vision is the proper RGC axon projection at the optic chiasm region. In the wild type mouse, there are about 3–5% RGC axons projecting ipsilaterally at the optic chiasm, while more than 95% RGC axons project contralaterally to the other side of the brain [5,6]. Interestingly, there is a small portion of RGC axons originated from the nasal part of the retina that project through the chiasm into the contralateral optic nerve, which is called the retinoretinal projection [7–10]. The retinoretinal projection normally disappears soon after birth, and its mechanism and developmental significance is still not clear. Whether RGC axons would extend to cross the midline at the optic chiasm, and at which direction they will further project into the brain, are under the dynamic control of interactions between a group of factors, such as EphB1/Ephrin B2 [11,12], NrCAM [13], Slit1/Slit2 [10], and transcription factors Zic2 [14,15], Foxd1 [16], etc.

The Eb1/Olf (Early B-Cell Factors) family of proteins are helix-loop-helix (HLH) transcription factors including four members, Eb1f1 through Eb4, that are important for neural development [7,17–19], adipogenesis [20,21], and lymphocyte development [22,23]. Previously we reported that all four members of the Eb1 family are involved in retinal cell specification [24]. In the retinas, Ebfs are expressed in horizontal, ganglion, type 2 OFF-cone bipolar, and non-All glycinergic amacrine cells [24]. However, the loss-of-function study with the knockout mice has not been done yet. Here we report the increase of Müller cell in the retina and abnormal RGC axon projection at the optic chiasm in the Eb1f1 null mouse, and reveal an essential role of Eb1f1 in regulating these developmental events.
2. Materials and methods

2.1. Animals

All experiments with mice were performed in compliance with the IACUC protocols approved by the University of Medicine and Dentistry of New Jersey. The animals were housed and bred at the university facility. The C57BL/6J mice were obtained from the Jackson Laboratory. The Ebf1 knockout mice were reported previously [22] and maintained by breeding with C57BL/6J mice.

2.2. In vitro retinal explant culture and immunostaining

In vitro retinal explant culture, immunohistochemistry, and whole-mount immunostaining were performed as described previously [24–26]. The following primary antibodies were used: mouse anti-Brn3a (Millipore); goat anti-Bhlhb5 (Santa Cruz Biotechnology); rabbit anti-calbindin D-28 k (Swant); sheep anti-Chx10 (Exalpha); rabbit anti-GABA (Sigma); mouse anti-glutamine synthetase (Millipore); goat anti-GLYT1 (Millipore); rabbit anti-NF150 (Millipore); rabbit anti-Pax6 (Millipore); rabbit anti-recoverin (Millipore); and rabbit anti-RxRg/RxRc (Santa Cruz Biotechnology). Images were captured with either the Nikon Eclipse 80i Microscope or Leica TCS-SP2 Confocal microscope.

2.3. Dil labeling and tracing

DilC18(3) (1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate) crystal was purchased from Invitrogen (Lot 454239). P0 mice were decapitated and fixed in fresh 4% PFA overnight. After the removal of lens and retina, a DilC18(3) crystal was placed onto the spot of the optic nerve. The heads were kept at 37°C for 2 weeks in PBS with 0.05% sodium azide in a sealed cell culture plate. They were then dissected and the optic chiasm was exposed for imaging. All images were taken with the same parameters from the Nikon Eclipse 80i microscope.

2.4. Quantification

Retinas from the same litter were used for quantification analysis. Confocal images from matched regions of wild type and mutant retinas were captured with the same scanning thickness. At least 6 regions from each retina and 3 retinas from each genotype were captured. Marker-positive cells were scored from the images.
All data were tested for significance using two sample Student’s t test with unequal variances.

The Dil labeling images were quantified using the NIH ImageJ software. First, use the freehand tool to select the region (ipsilateral, contralateral, or retinoretinal), then choose plugins → analyze → measure RGB. Record the value of the area and the average intensity of the red signal in the area. The percentage of contralateral, ipsilateral and retinoretinal projections was calculated by the relative value of (area / intensity).

3. Results

3.1. Early Ebf1 mutant retinas show no obvious cell fate changes

Ebf1 heterozygotes were crossed to obtain the homozygous mutant mice, which usually die soon after birth. To investigate whether loss of Ebf1 would affect cell fates in early stage retinas, E13.5 and P0 retinas were examined. At E13.5, the size and morphology between wild type and mutant retinas are indistinguishable, and ganglion cells are unchanged in the mutants (data not shown). At P0, retinas were examined for any defects by immunostaining with antibodies against several retina cell markers, which include Pax6 for RGCs in the ganglion cell layer, amacrine and horizontal cells in the neuroblastic layer (Fig. 1A and B), Chx10 for bipolar and progenitor cells (Fig. 1C and D), Brn3a for RGCs (Fig. 1E and F), calbindin for horizontal cells and starburst and other amacrine cells (Fig. 1G and H), recoverin and RxRg (also called RxRy) for rod and cone photoreceptors, respectively, in the outer neuroblastic layer (Fig. 1I-L); NF150 for horizontal cells in the inner neuroblastic layer and RGC axon fibers (Fig. 1M–P). None of the markers exhibited any obvious difference between wild type and Ebf1 mutant retinas, suggesting that the absence of Ebf1 does not affect the fates and differentiation of early retinal cell types. The normal nerve fiber patterns revealed by NF150 labeling imply that there are no intraocular RGC axon projection defects in the Ebf1−/− retinas.

3.2. Müller cells increase in the Ebf1−/− retina

Since Ebf1 homozygous mutants die perinatally, we are unable to assess in vivo the development of late-born cell types in the mutant retina as most of them are not generated. To circumvent this problem, in vitro retinal explant culture was employed to examine any changes in cell fates and differentiation. P0 Ebf1+/+ and Ebf1−/− retinal explants were cultured in vitro for 8 days and their sections were then immunolabeled with the indicated antibodies. (A and B) Anti-Pax6 stains amacrine, horizontal cells and RGCs. (C and D) Anti-GLYT1 stains glycineric amacrine cells. (E and F) Anti-GABA stains GABAergic amacrine cells. (G and H) Anti-calbindin stains horizontal and some amacrine cells. (I and J) Anti-Bhlhb5 stains type 2 OFF-cone bipolar cells and GABAergic amacrine cells. (K and L) Anti-Chx10 stains bipolar cells. (M and N) Anti-glutamine synthetase (G.S.) stains Müller cells. There are no obvious changes in these markers except the increase of G.S.-immunoreactive Müller cells labeled by G.S. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: J (for A–N), 50 μm.
retinas were dissected and cultured on filters for 8 days. We examined markers for major cell types, for instances, Pax6 (Fig. 2A and B) for ganglion, horizontal and amacrine cells, GABA (Fig. 2E and F) for GABAergic amacrine cells, calbindin (Fig. 2G and H) for horizontal and some amacrine cells, and Chx10 (Fig. 2K and L) for bipolar cells. There was no significant difference in the number of cells immunoreactive for these markers between wild type and mutant retinas, except that G.S. Müller cells increase in the mutant. *p < 0.05.

3.3. Ebf1 inactivation causes aberrant RGC axon projection at the optic chiasm

At the optic chiasm region, the majority of RGC axons cross the midline and project contralaterally to the other side of the brain; the remaining RGC axons (about 3–5% in the mouse) project ipsilaterally to the same side. Since Ebf1 and other Ebfs are involved in the axon guidance and projection in other tissues [27–30], it’s possible that they may play similar roles in regulating the RGC axon projections in the retina and optic chiasm. As aforementioned, the intraocular projection is normal at P0 (Fig. 1O and P), suggesting that Ebf1 normally suppresses the differentiation of Müller glial cells.

4. Discussion

4.1. Ebf1 influences cell specification and differentiation in the mouse retina

Ebfs have been reported to influence cell differentiation and cell fates in lymphocyte genesis [22,23,31], in adipogenesis [20,21,32] and in neuronal differentiation [7,17,29,33], including specification of multiple retinal cell types and subtypes in the retinal development [24]. In the adult mouse retinas, Ebfs are expressed strongly in the RGCs, glycinergic amacrine cells, type 2 OFF-cone bipolar cells, and weakly in the horizontal cells; but not in GABAergic amacrine cells, photoreceptors, or Müller cells. Overexpression of Ebf1, Ebf2, or Ebf3 is sufficient to induce the glycinergic amacrine, type 2 OFF-cone bipolar, and horizontal cells in the mouse retina. Such effect can be successfully suppressed by the overexpression of a dominant-negative form Ebf1EnR [24].

In our current work, however, we found no obvious changes in glycinergic amacrine, bipolar and horizontal cells in Ebf1/C0 mutants, which is not surprising. First, Ebf1, Ebf2, Ebf3 and Ebf4 have a near identical expression pattern and act redundantly in the retina [24], and it is highly possible that other Ebfs could compensate for the complete loss of Ebf1 in the Ebf1 null retina. Such a redundant role is evidenced by the observations in the brain. While there are olfactory neuron projection defects in Ebf2 and Ebf3 double heterozygous mice, the Ebf2 or Ebf3 single heterozygous mouse shows no such defects [27], since partial loss of Ebf2 can be compensated by Ebf3, and vice versa. Another evidence from the opposite angle is that, Ebf1/C0 embryos show specific defects in the embryonic striatum, where Ebf1 is the only Ebf gene expressed [34]. Therefore, the loss of Ebf1 function in homozygous mutant retinas is very likely to be partly compensated by other Ebf factors in the neuronal differentiation process. Second, due to the limitation of the in vitro explant culture, there are a lot of cell deaths that would easily cover the minor differences between wild type and mutants. Using conditional knockout mouse of Ebf1 should overcome the limitation. To avert the redundant effect and fully understand the role of Ebfs in retinal cell development, mouse with compound knockouts of two or more Ebfs should be examined for any possible specification defects.

In the cultured Ebf1/C0 retinal explant, there is a 27% increase of Müller cells compared to the wild type, indicating that Ebf1 normally inhibits the Müller cell fate during retinogenesis. This is consistent with our previous gain-of-function analysis showing that misexpressed Ebf1 has a potent activity to suppress Müller cell differentiation [24]. In addition, the role of Ebf1 to bias a neuronal versus a glial cell fate is also shared by many other bHLH factors, for examples, Mash1 [25], NeuroD [35,36], Math3 [35,36], Math5 [37], Ngn1 and Ngn2 [38], etc.

4.2. Ebf1 regulates RGC axon guidance and projection

In various neural tissues, Ebfs have been shown to play an important role in controlling axon guidance and pathfinding. In the C. elegans, mutants of the Ebf homolog unc-3 have motor neuron axon pathfinding deficiency and display moving
abnormality [30]. In the mouse olfactory bulb, knockout of Ebf2 or Ebf3 causes defects in olfactory axon projection [27]. In the cerebellum, null mutation of Ebf2 leads to Purkinje cell migration defect [33]. Ebf5s are expressed in newborn RGCs and continue their expression throughout the adult stage, suggesting that Ebf5s may be involved in RGC axon guidance and projection, and other RGC routine functions. Our Dil tracing experiment demonstrated that Ebf1 does regulate the RGC axon projection.

In the Ebf1 null retina, the RGC axon fibers form normally, as seen by NF150 staining of P0 retinas (Fig. 1P). There are no obvious defects in the optic fissure or optic disk, and optic nerves exit the eyes normally. However, abnormal RGC axon projections do occur at the optic chiasm in Ebf1 null mice. Compared to the wild type, Ebf1 mutants exhibit more contralateral projection of RGC axons but less ipsilateral projection. The retinoretinal projection is also greatly reduced in the Ebf1 mutant, a phenotype contrary to Slit1/Slit2 double knockout mice [10], and EXT1 null mice [39], suggesting Ebf1 has an opposing role against Slit1/Slit2 and EXT1 in regulating RGC axon projection. On the other hand, transcription factors Zic2 [14,15], Foxd1 [16] and Foxg1 [40,41] are the crucial proteins in regulating the ipsilateral and contralateral projections. Microarray analysis may give clues to how these factors are affected in the Ebf1 mutants.

Conflict of interest statement

The authors declare that they have no competing financial interests.

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