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Analysis of expression of transcription factors in early human retina

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ABSTRACT

The retina originates in the central nervous system. Due to its accessibility and simplicity, the retina has become an invaluable model for studying the basic mechanisms involved in development. To date, considerable knowledge regarding the interactions among genes that coordinate retinal development has been gained from extensive research in model animals. However, our understanding of retinal development in humans remains undeveloped. Here, we analyze the expression of transcription factors that are involved in the early development of the retina in human embryos at 6–12 weeks post-conception. Our work demonstrates that early developing neural retinas can be divided into two layers, the outer and inner neuroblast layers. Eye-field transcription factors and those related to the early development of the retina have distinct expression patterns in the two layers. Cell-type-specific transcription factors emerge at 8 weeks. These data provide clear and systemic structures for early retinal development in human.

1. Introduction

The mature retina is a tissue showing laminar organization and containing highly specialized photoreceptors, interneurons and projection neurons. The rod and cone photoreceptors form the outer nuclear layer (ONL), while the horizontal, bipolar and amacrine interneurons form the majority of the inner nuclear layer (INL). The retinal ganglion cells (RGCs) are projection neurons that make up the ganglion cell layer (GCL). All these cell types are precisely arranged to be an elaborate neural network.

The process of retinal development has become quite clear through extensive research on model animals. Derived from the neuroectoderm, the eye primordia evaginate to form optic vesicles. Then, the optic vesicle interacts with the prospective lens in the surface ectoderm. The optic vesicle gives rise to pigmented and ciliary epithelia, as well as the neural retina (NR). By coordinating extrinsic and intrinsic factor, the retinal progenitor cells (RPCs) in the NR generate ganglion cells, horizontal interneurons, cone photoreceptors, amacrine interneurons, rod photoreceptors, bipolar interneurons and Müller glia (Harris, 1997; Livesey and Cepko, 2001; Marquardt and Gruss, 2002). These processes are controlled by multiple homeodomain and basic helix-loop-helix (bHLH) genes (Cepko, 1999; Hutcheson and Vetter, 2001; Hatakeyama et al., 2001).

During early eye development, the eye field which can be distinguished by eye-field transcription factors (EFTFs), forms before the evagination (Zuber et al., 2003). Genetic evidence demonstrates that EFTFs are crucial for eye formation (Chow et al., 1999; Lagutin et al., 2001; Loosli et al., 1999; Mathers et al., 1997; Porter et al., 1997; Bernier et al., 2000; Carl et al., 2002). In mammals, the EFTFs include Pax6, Rax, Six3 and Lhx2. In particular, the homeodomain gene Pax6 is considered a 'master gene' in the development of the eye (Gehring and Ikeo, 1999). Mutations in Pax6 are associated with abnormalities in all the ocular tissues: retina, iris, lens and cornea (Hever et al., 2006). Pax6 is also required for RPC multipotency, and directly controls the transcriptional activation of bHLH transcription factors (TFs), which task RPCs with generating the different retinal cell fates (Marquardt et al., 2001). The chronological cell birth sequence in retinal neurogenesis is largely a result of the cooperation of homeodomain (Pax6, Rax, Six3, Otx2, Chx10, Crx, and Prox1) (Mathers et al., 1997; Philips et al., 2005; Furukawa et al., 1997a; Nishida et al., 2003; Liu et al., 1994; Chen et al., 1997; Dyer et al., 2003) and bHLH genes (Mash1, Math3, Math5, and NeuroD) (Tomita et al., 1996; Inoue et al., 2002; Brown et al., 1998; Morrow et al., 1999). These genes are highly conserved across various species.

Although the mechanisms of specification and morphogenesis of diverse cell types are relatively well investigated in model animals, to date, we still lack adequate information about the expression and function of cell markers in the development of human retinas. Here we

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analyze the expression patterns of TFs, including EFTFs that are involved in retinal neurogenesis in humans. We can observe that retinas at early developmental stages show two layers. The number and shape of nuclei as well as the expression of Ki67 divides the early retina into two layers: the outer and the inner neuroblast layer. The expression patterns found for most of the analyzed TFs in the two layers are complementary, with the EFTFs Rax and Lhx2 in the outer-layer NR, while Pax6 and Six3 are predominantly located in the inner-layer NR. Sox2 and Chx10 are gradually confined to the outer-layer NR. This expression pattern echoes their functions in RPCs. The cell-type-specific TFs appear at gestation week 8, indicating the start of retinal neurogenesis. These data provide new insight into the early development of the human retina.

2. Materials and methods

2.1. Tissue collection

The human fetal tissues used here were from patients requesting termination of pregnancy. All the procedures were approved by the Ethics Committee of Institutes of Biomedical Sciences Fudan University and with the informed consent of the patients. And all human material was handled with special care following the requirements and regulations set by the ethics committee. Fetal tissues were obtained within 1 h after abortion and the developmental stages of these fetus specimens were identified according to the postconceptional weeks. The tissues were cut into 6 μm frozen sections for immunohistochemistry.

2.2. Immunohistochemistry and microscope

Tissues were rinsed with 0.01 M phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 4 h. Dehydrate these samples with 15% and 30% sucrose, then embedded with OCT and frozen sectioned. Sections were washed with 0.01 M PBS then treated with 0.2% Triton X-100 and 10% normal donkey serum for 1 h at room temperature before they were incubated 4 °C overnight in primary antibodies (Table S1). Correspondence secondary fluorescent antibodies (Table S2) and DAPI were used at 1:2000 for 2 h at room temperature. Slides were mounted with Fluoromount-G (SouthernBiotech). Images were taken with confocal laser-scanning microscope (TCS SP8, Leica Microsystems, Wetzlar, Germany) and fluorescence microscope (ECLIPSE TE2000-S, Nikon, Tokyo, Japan), and processed with Photoshop CS2 software (Adobe Systems, San Jose, CA, USA). A diagram was drawn to indicate the site of retina that we studied (Fig. S1).

3. Results

3.1. The neural retina starts to stratify during embryonic weeks 6–8

The human retina folds into a two-walled optic cup (OC) at 6 weeks. At this developmental stage, Ki67, a cellular marker for proliferation, was detected in a few cells within the NR close to the apical surface. The neuronal class III β-tubulin (Tuj1) is expressed in prospective RPE and NR at this developmental stage (Fig. 1A1–C1). The morphology of the OC is barely changed at the later developmental stage studied (8–12 weeks). Early developing neural retinas can be divided into two layers, the outer and inner neuroblast layer, based on the cell number and morphology, as well as the expression of Ki67 and Tuj1. The division between these two layers is highly distinct (Fig. 1A2–C4, dotted lines). At 8–12 weeks, Ki67$^+$ cells were scattered within the outer-layer NR near the apical surface. Cell density in the outer-layer NR was much higher than in the inner-layer NR (Fig. 1A2–A4). The expression of Tuj1 is complementary to that of Ki67. Tuj1 was detected exclusively in the inner-layer NR, indicating the start of neurogenesis in the NR (Fig. 1B2–B4). Further, nuclei that were located in the inner-layer NR were more elongated and sparser than those in the outer-layer NR (Fig. 1C2–C4).

Developing RPE was observed to contain some Ki67$^+$ cells (Fig. 1A2–A4). All these data indicate that 6–8 weeks is the start of human retinal neurogenesis. RPCs in the NR undergo symmetric and asymmetric cell division to give rise to postmitotic neurons (Tuj1$^+$ cells) as well as progenitor cells to create a laminar retina. The scattered Ki67$^+$ cells (Fig. 1A1–A4) probably result from interkinetic nuclear migration.

3.2. EFTFs have distinct expression patterns during retinal neurogenesis

The mammalian EFTFs Pax6, Rax, Six3 and Lhx2 are not only eye-field-specific transcription factors but also participate in later developmental stages (Fig. 2A1–A4, B1–B4, D1–D4 and E1–E4). In 6-week-old retinas, Rax, Six3 and Lhx2 were detected evenly in the whole NR (Fig. 2B1, D1 and E1), while the expression of Pax6 was stronger near the peripheral OC (ciliary epithelium) (Fig. 2A1). Pax6, Six3 and Lhx2 were also detected in the RPE (Fig. 2D1 and E1).

In 8–12-week-old NR, Rax and Lhx2 are exclusively expressed in the outer-layer NR while Six3 is expressed strongly in the inner-layer NR, displaying complementary expression (Fig. 2B2–B4, D2–D4 and E2–E4). Pax6 is detected throughout the NR, with stronger expression in the inner-layer NR (Fig. 2A2–A4).

3.3. Transcription factors that are critical in retinogenesis also participate in retinal neurogenesis

In addition to EFTFs, several other TFs are essential for retinogenesis during early developmental stages. These TFs include Chx10, Sox2 and Otx2 (Liu et al., 1994; Burmeister et al., 1996; Kamachi et al., 1998; Bovolenta et al., 1997; Taranova et al., 2006; Matsushima et al., 2011) and are mainly located in the outer-layer NR. Chx10 was detected in the whole NR in 6-week-old specimens. It was much stronger in the central NR (Fig. 3B1), contrary to the expression pattern of Pax6. In retinas at 8–12 weeks, Chx10 was limited to the outer-layer NR (Fig. 3B2–B4, dotted lines). Interestingly, Chx10 is restricted to the RPCs, which remain multipotent in the outer layer NR. The newly generated photoreceptor precursors (Fig. 3B4 blue dotted lines) and amacrine precursors (Fig. 3B4, solid blue lines), which are Pax6$^+$ (Fig. 3A4 solid blue lines) and NeuroD$^+$ (Fig. 4A4), are both Chx10$^-$.

Sox1 and Sox2 have entirely different functions in the development of the retina, which can be inferred from their expression patterns. Sox2 was detected throughout the entire OC in 6-weeks-retina (Fig. 3E1) and was located exclusively in the outer-layer NR at the later developmental stages (Fig. 3E2–E4), while Sox1 was not expressed in the retinas at either developmental stage (Fig. S2 B1–B4).

In 6-week-old retinas, the Otx2 is strongly expressed in the RPE, whereas its expression is relatively weak in the entire NR. Like the expression pattern of Pax6 in the NR at 6 weeks, Otx2 expression is much stronger near the peripheral OC (Fig. 6B1, Arrows). In 8–12-week-old retinas, Otx2 is located in the RPE and the outer-layer NR. However, unlike other TFs, Otx2 expression is scattered throughout the outer-layer NR and then gradually collects near the apical surface (Fig. 4B2–B4).

3.4. Cell-type-specific transcription factors begin to participate in the neural retina's stratification at 8 weeks

During human retinal neurogenesis, the specific TFs that define each progenitor in chronological sequence of cell birth emerge at 8 weeks. Otx2, NeuroD and Crx are generally considered to be significant TFs in photoreceptor differentiation (Nishida et al., 2003; Chen et al., 1997; Morrow et al., 1999). In 8-week-old retina, NeuroD expression is scattered throughout the outer-layer NR, with sporadic NeuroD$^+$ cells present in the inner-layer NR (Fig. 4A1). Almost all the Otx2$^+$ cells are