Gene Expression Patterns 23-24 (2017) 32–44

Contents lists available at ScienceDirect

Gene Expression Patterns

journal homepage: http://www.elsevier.com/locate/gep

Temporal profiling of photoreceptor lineage gene expression during murine retinal development

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ARTICLE INFO

Article history:
Received 9 November 2016
Received in revised form
3 February 2017
Accepted 7 March 2017
Available online 10 March 2017

Keywords:
Retinal progenitor cells
Rod and cone photoreceptors
Transcription factors
Temporal sequence
Mouse

ABSTRACT

Rod and cone photoreceptors are photosensitive cells in the retina that convert light to electrical signals that are transmitted to visual processing centres in the brain. During development, cones and rods are generated from a common pool of multipotent retinal progenitor cells (RPCs) that also give rise to other retinal cell types. Cones and rods differentiate in two distinct waves, peaking in mid-embryogenesis and the early postnatal period, respectively. As RPCs transition from making cones to generating rods, there are changes in the expression profiles of genes involved in photoreceptor cell fate specification and differentiation. To better understand the temporal transition from cone to rod genesis, we assessed the timing of onset and offset of expression of a panel of 11 transcription factors and 7 non-transcription factors known to function in photoreceptor development, examining their expression between embryonic day (E) 12.5 and postnatal day (P) 60. Transcription factor expression in the photoreceptor layer was observed as early as E12.5, beginning with *Crx, Otx2, Rorb, Neurod1* and *Prdm1* expression, followed at E15.5 with the expression of *Thrb, Neurog1, Sall3* and *Rxrg* expression, and at P0 by *Nrl* and *Nr2e3* expression. Of the non-transcription factors, peanut agglutinin lectin staining and cone arrestin protein were observed as early as E15.5 in the developing outer nuclear layer, while transcripts for the cone opsins *Opn1mw* and *Opn1sw* and Recoverin protein were detected in photoreceptors by P0. In contrast, Opn1mw and Opn1sw protein were not observed in cones until P7, when rod-specific *Gnat1* transcripts and rhodopsin protein were also detected. We have thus identified four transitory stages during murine retina photoreceptor differentiation marked by the period of onset of expression of new photoreceptor lineage genes. By characterizing these stages, we have clarified the dynamic nature of gene expression during the period when photoreceptor identities are progressively acquired during development.
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1. Introduction

The neural retina is a laminar structure that is comprised of one glial and six neuronal cell types that are organized into three cellular layers. The outer nuclear layer (ONL), which lies on the apical side of the retina, contains the cell bodies of cone and rod photoreceptors. The inner nuclear layer (INL), which is in the center of the retina, is populated by amacrine, horizontal and bipolar cell interneurons as well as Müller glia, which are the only glial cells generated in the retina. Finally, the ganglion cell layer (GCL), on the basal side of the retina, contains ganglion cells and displaced amacrine cells. In addition, the retinal pigment epithelium (RPE) is a non-neural epithelial cell layer that overlies the ONL.

Eye formation begins with an evagination of the diencephalon at E8.5 in mice, leading to the development of bilateral optic pits that contact the head ectoderm to give rise to optic vesicles by E9.0 (Fuhrmann, 2010; Wawersik and Maas, 2000). Inductive signals

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http://dx.doi.org/10.1016/j.gep.2017.03.001
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from the optic vesicles cause the formation of lens placodes in the overlying ectoderm by E9.5. By E10.0, there is a coordinated invagination of the lens placode and the optic vesicle, resulting in the formation of a lens pit and double-layered optic cup (Fuhrmann, 2010; Wawersik and Maas, 2000). The inner layer of the optic cup adjacent to the lens gives rise to the neural retina, while the outer layer gives rise to the RPE.

In the murine retina, cellular differentiation begins at E10 and lasts until P5-6 in the central retina and P11 in the periphery, followed by eye opening at P14 (Cepko, 1996; Young, 1985). Lineage tracing experiments using retroviral gene transfer, DNA or dye labelling, epitope tagging, or clonal density cultures demonstrated that early RPCs are multipotent, giving rise to all seven retinal cell types (Alexiades and Cepko, 1997; Fekete et al., 1994; Holt et al., 1988; Jensen and Raff, 1997; Moody et al., 2000; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). Retinal cells are generated in a sequential albeit overlapping order that is conserved among vertebrates (Marquardt and Gruss, 2002; Wassle and Boycott, 1991). RGCs are the first cells to differentiate, followed closely and in an overlapping manner by horizontal cells, cone photoreceptors and amacrine cells, all of which are primarily generated during the embryonic period in mouse (Cepko, 1996, 1999; Young, 1985). Rod photoreceptors, bipolar cells and Müller glia cells are the next cell types to be generated, also in a highly overlapping fashion, peaking during the early postnatal period (Cepko, 1999; Cepko et al., 1996; Young, 1985).

While lineage tracing experiments support the existence of multipotent RPCs, committed precursors that give rise to specific retinal cell types also exist (Pearson and Doe, 2004). RPCs give rise to committed photoreceptor precursors that progress to fully differentiated photoreceptors expressing genes involved in phototransduction and morphogenesis (Swaroop et al., 2010). Rod development occurs in an early and late phase, with some rod precursors generated as early as E14, but most generated in the late phase beginning after E19, peaking at P4 and extending to P10 (Cepko et al., 1996; Morrow et al., 1998; Rapaport et al., 2004). Notably, the onset of rhodopsin expression, a terminal differentiation marker of a rod fate, does not occur until 5.5–6.5 days after rod precursors exit the cell cycle (or 8.5–12.5 days for early-born rods) (Morrow et al., 1998). In contrast, cone precursors are generated as early as E11, peaking at E13-E16, and completing their genesis by P0 (Carter-Dawson and LaVail, 1979; Rapaport et al., 2004). Similar to rods, the expression of mature cone markers is delayed, with *Opn1sw* transcripts detected at around P0, several days after most cones have already exited the cell cycle (Swaroop et al., 2010).

Several transcription factors have been identified that control photoreceptor fate specification and differentiation, but a comprehensive comparison of the onset and offset of their expression has not been reported. A temporal analysis of photoreceptor gene expression is warranted as current attempts to generate induced photoreceptors by cellular reprogramming requires a detailed understanding of normal lineage progression. We therefore characterized the temporal expression patterns of a panel of 11 transcription factors and 7 non-transcription factors previously implicated in the development of photoreceptor lineages.

2. Results and discussion

2.1. Onset of gene expression in photoreceptor lineages at earliest embryonic stage (E12.5)

We set out to perform a temporal analysis of gene expression in murine photoreceptor lineages at seven different stages of retinal development (E12.5, E15.5, P0, P7, P14, P21, P60), encompassing the period of cone (E12.5-P0) and rod (E15.5-P14) genesis and photoreceptor differentiation, maturation and maintenance (P14-P60). The eleven transcription factors we examined included members of the homeodomain (*Cone-rod homeobox* (*Crx*), *Orthodenticle homeobox 2* (*Otx2*)), nuclear hormone receptor (*RAR-related orphan receptor beta* (*Rorb*), *Thyroid hormone receptor beta* (*Thrb*), *Retinoid X receptor gamma* (*Rxrg*), *Nuclear receptor subfamily 2, group E, member 3* (*Nr2e3*)), basic-helix-loop-helix (bHLH) (*Neurogenic differentiation 1* (*Neurod1*), *Neurogenin 1* (*Neurog1*)), zinc finger (*Spalt like transcription factor 3* (*Sall3*), *PR domain containing 1, with ZNF domain* (*Prdm1*)) and neural zipper (*Neural retina leucine zipper gene* (*Nrl*)) families. In addition, we examined seven non-transcription factor markers, including those labeling cones (Arrestin 3 (Arr3), *Opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan)* (*Opn1mw*), *Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan)* (*Opn1sw*), peanut agglutinin (PNA – a lectin), rods (*Guanine nucleotide binding protein, alpha transducin 1* (*Gnat1*), Rhodopsin (Rho)) and both rods and cones (Recoverin (Rcvrn)).

We began our analysis at E12.5, just after the first cone photoreceptor precursors have exited the cell cycle. RNA *in situ* hybridization on tissue sections revealed that at E12.5, transcripts for *Crx* (Fig. 1A,A′), *Otx2* (Fig. 1H,H′), *Rorb* (Fig. 1O,O′), *Neurod1* (Fig. 1V,V′) and *Prdm1* (Fig. 1CC,CC′) were detected in the upper outer neuroblast layer (ONBL), which we have designated the presumptive ONL (pONL). In contrast, none of the other genes that we analysed were expressed at this time point (Figs. S1–3).

Between E12.5 and P0, *Crx* expression was detected in the pONL and in scattered cells throughout the ONBL, where dividing RPCs are giving rise to committed precursors for different neuronal lineages (Fig. 1A–C,A′-C′). By P7, when differentiation is complete in the central retina, and up to P60, *Crx* transcripts were primarily localized to the ONL, with an additional narrow band of Crx^+ cells also detected in the upper INL, in a subset of bipolar cells (Fig. 1D–G,D′-G′), as previously described (Samson et al., 2009). *Crx* is a transcriptional target of *Otx2* and is required for both cone and rod development (Chen et al., 1997; Furukawa et al., 1997; Nishida et al., 2003). While photoreceptors are properly specified in *Crx* null mutants, these cells do not differentiate normally; outer segments do not form properly, synaptic terminals and phototransduction pathways are defective, mature markers are downregulated (e.g., Rho, Arr) and photoreceptor degeneration ensues (Chen et al., 1997; Furukawa et al., 1999; Livesey et al., 2000; Morrow et al., 2005).

Otx2 transcripts were similarly detected in the pONL and in scattered cells throughout the ONBL as early as E12.5, peaking at P0 of development, with additional expression also detected in the developing inner neuroblast layer (INBL), where differentiating INL and GCL cells accumulate, and in the RPE (Fig. 1H–J,H′-J′). However, by P7, *Otx2* was expressed in only a few scattered cells in the ONL and RPE, with predominant expression instead detected in bipolar cells in the INL, as previously shown (Koike et al., 2007), a pattern that was maintained between P14-P60 (Fig. 1K–N,K′-N′). *Otx2* is thought to be the earliest acting transcription factor in the photoreceptor lineage, similar to its Drosophila ortholog, *orthodentricle* (*otd*) (Finkelstein and Boncinelli, 1994), which is also an ortholog of *Crx* (Plouhinec et al., 2003; Tahayato et al., 2003). Conditional retinal deletion of *Otx2* results in a complete loss of both rod and cone photoreceptors, and a reduction in expression of several key genes involved in photoreceptor development and survival, including *Crx*, *Nrl*, *Nr2e3* and *Neurod1* (Nishida et al., 2003; Omori et al., 2011). In contrast, overexpression of *Otx2* promotes ectopic photoreceptor cell genesis (Nishida et al., 2003). *Otx2* specifies a photoreceptor fate in part by inducing the expression of *Crx* (Beby and Lamonerie, 2013; Chen et al., 1997; Furukawa et al., 1997; Hennig et al., 2008). Additional genes that function downstream of *Otx2* are *Vsx2* and *Prdm1*, with the loss of *Prdm1* also leading to a

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