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Molecular Control of Retinal Ganglion Cell Specification and Differentiation

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1. Introduction

The mammalian retina is a laminated sensorineural epithelium composed of six classes of neurons that include the rod, cone, bipolar, horizontal, amacrine, and ganglion cells (RGCs), and one type of glia, the Müller cells. RGCs are the sole output neurons in the retina whose projecting axons form the optic nerve essential for conveying light signals to the higher visual system in the brain. Glaucoma causes degeneration of the optic nerve and RGCs that leads to impaired vision and blindness. Recent advances in stem cell-based therapy to restore functional retinal circuits in the damaged retina have made it promising to develop an effective treatment of glaucoma in the future. It is conceivable that, in the future, RGCs derived through controlled differentiation of stem cells by various retinogenic factors may provide renewable sources of replacement RGCs for glaucoma patients. Because differentiation of stem cells normally recapitulates the events that occur during embryogenesis, it will be important to understand the molecular basis of RGC development and identify intrinsic and extrinsic factors involved in RGC fate determination and differentiation.

In this chapter, we will summarize and discuss recent molecular genetic studies on intrinsic and extrinsic factors required for RGC development, and how they act to establish RGC competence, determine RGC fate and facilitate RGC differentiation (Fig. 1). We will also discuss gene regulatory networks governing RGC development as well as safeguard mechanisms ensuring RGC differentiation from multipotent retinal precursors. Finally, we will discuss how our knowledge about the intrinsic and extrinsic retinogenic factors may contribute to devising efficient means to generate RGCs from stem cells. Some of our own work will be highlighted throughout the chapter.

2. Intrinsic factors

2.1 Transcription factors involved in RGC fate and differentiation

Pou4f/Brn3 factors. Our molecular cloning of the POU-domain transcription factor Pou4f2/Brn3b and demonstration of its expression in RGCs about two decades ago (Xiang et al., 1993) provided an entry point for the ensuing explosion of studies on the genetic

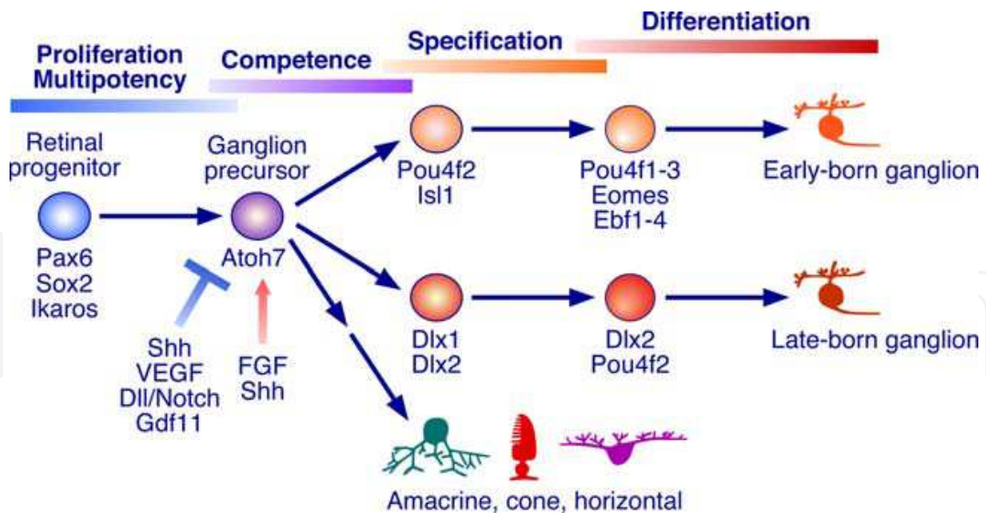

Fig. 1. Schematic illustration of developmental stages and intrinsic and extrinsic factors leading to RGC production from progenitors. Multiple transcription factors and signaling molecules are involved to drive progression from multipotent retinal progenitors to RGC competent precursors and to eventually specified and differentiated RGCs. The Atoh7-expressing RGC precursors are also competent to generate amacrine, horizontal and cone cells

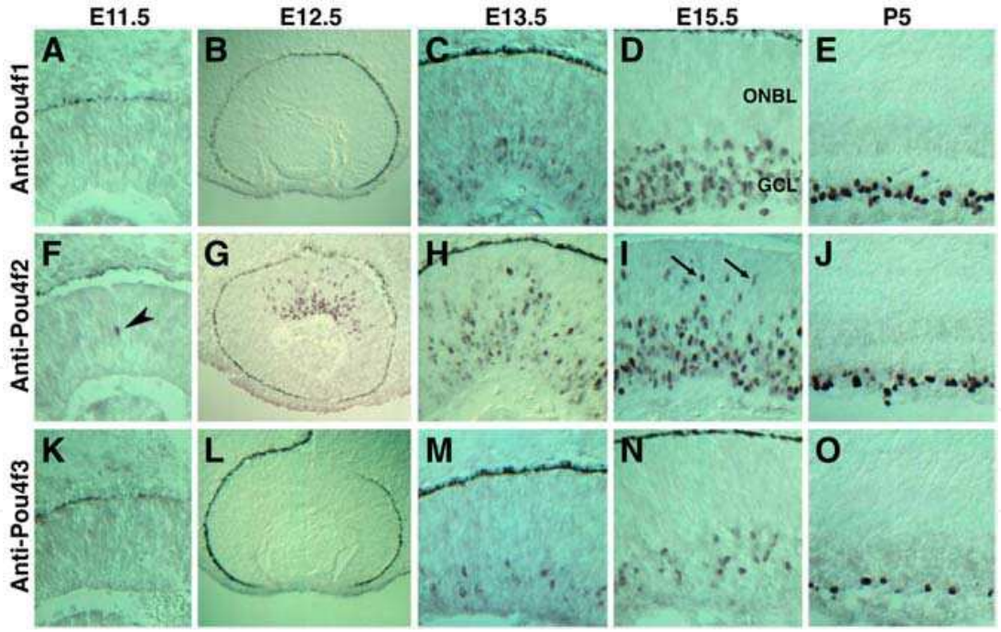

Fig. 2. Expression patterns of Pou4f proteins during mouse retinogenesis. Retinal sections from the indicated stages were immunostained with anti-Pou4f1, anti-Pou4f2 and anti-Pou4f3 antibodies. Pou4f2 commences its expression in occasional cells (indicated by the arrow head) of E11.5 retinas. At E12.5-E18.5, it is localized in a large number of cells in the ganglion cell layer (GCL), as well as in scattered cells (indicated by arrows) within the outer neuroblastic layer (ONBL). In postnatal retinas, Pou4f2 expression is restricted to the RGCs in the GCL. Pou4f1 and 3 are found only in cells of the GCL beginning at E13.5

pathway and gene regulatory network of RGC development. In vertebrates (from zebrafish to human), there exist three family members of Pou4f factors (Pou4f1-3 or Brn3a-c) and within the retina their expression is all confined to RGCs (DeCarvalho et al., 2004; Gerrero et al., 1993; Hutcheson and Vetter, 2001; Turner et al., 1994; Xiang et al., 1995; Xiang et al., 1993). In the adult mouse retina, Pou4f1 and 2 are estimated to be localized in more than 70% of RGCs whereas Pou4f3 in ~ 30% of total RGCs (Xiang et al., 1995). During mouse retinogenesis, the three Pou4f factors exhibit overlapping but spatiotemporally distinct expression patterns, with only Pou4f2 sporting a pattern characteristic of the dynamic profile of RGC genesis (Gan et al., 1996; Xiang, 1998). At prenatal stages, Pou4f2-immunoreactive cells are seen at the time of first RGC birth at embryonic day 11.5 (E11.5) (Young, 1985), and in newborn migrating RGCs in the outer neuroblastic layer (Fig. 2F-J) (Gan et al., 1996; Xiang, 1998). The onset of Pou4f1 and 3 expression occurs two days later only in differentiated RGCs within the ganglion cell layer (Fig. 2A-E, K-O) (Xiang, 1998). Interestingly, there seems to be always one Pou4f factor whose spatiotemporal expression pattern follows closely with the timing of RGC generation in other vertebrates, such as cPou4f3/cBrn3c and XBrn3d, in the case of chicken and *Xenopus*, respectively (Hutcheson and Vetter, 2001; Liu et al., 2000a). This correlative early Pou4f expression suggested to us that Pou4f function might not only be required for RGC differentiation but also for an earlier role (Xiang, 1998), which we later showed to be in RGC fate specification (Qiu et al., 2008).

All three *Pou4f* genes were deleted in mice by gene targeting to study their developmental function (Erkman et al., 1996; Gan et al., 1999; Gan et al., 1996; McEvilly et al., 1996; Xiang et al., 1997; Xiang et al., 1996). *Pou4f2* inactivation leads to optic nerve hypoplasia as well as thinner retinas with reduced thickness of the ganglion cell, nerve fiber and inner plexiform layers (Fig. 3A-C) (Gan et al., 1996). *Pou4f2* mutant optic nerves are diminished in cross-sectional area by approximately 5-fold and have a significantly reduced density of axons (Fig. 3D,E). The mutant retinas lose ~ 70-80% of total RGCs and exhibit a dramatic decrease in the number of Pou4f1- and Pou4f3-positive cells and Thy1- and SMI-32-immunoreactive processes (Fig. 3F-I) (Gan et al., 1996). These RGC defects occur early in development in the mutant (Fig. 3J-Q). Optic nerve hypoplasia, nerve fiber defasciculation, and diminished Pou4f1, Pou4f2, Ebf1, and Pou6f2/RPF-1 expression are visible as early as E12.5 (Gan et al., 1999; Xiang, 1998). RGC axon guidance errors are also present at multiple intraocular and extraocular points along their projection pathways in developing and adult *Pou4f2* null mutant mice (Erkman et al., 2000). Meanwhile, inactivating *Pou4f2* appears to cause many presumptive RGC precursors to switch to amacrine or horizontal cell fates (Qiu et al., 2008). These improperly differentiated cells are likely to degenerate by apoptosis as cell death significantly increases in the mutant retina (Gan et al., 1999; Xiang, 1998). Thus, Pou4f2 plays an essential role in RGC differentiation as well as their fate specification. It was thought that Pou4f2 might be required only for RGC differentiation and survival because in *Pou4f2*$^{lacZ/lacZ}$ knockin retinas, normal number of β-gal(galactosidase)$^{+}$ cells is initially produced that migrate into the inner neuroblastic layer (Gan et al., 1999). All of these β-gal^{+} cells were assumed to be RGCs but they were not confirmed as such using molecular markers. In fact, many cells within the inner neuroblastic layer of *Pou4f2* null retinas extend short, microtubule-rich and nonfasciculated neurites characteristic of dendrites rather than axons (Gan et al., 1999; Wang et al., 2000). This phenotype may manifest a switch from RGCs to dendrite-bearing amacrine and horizontal cells even though a change of RGC cell polarity cannot be completely ruled out.

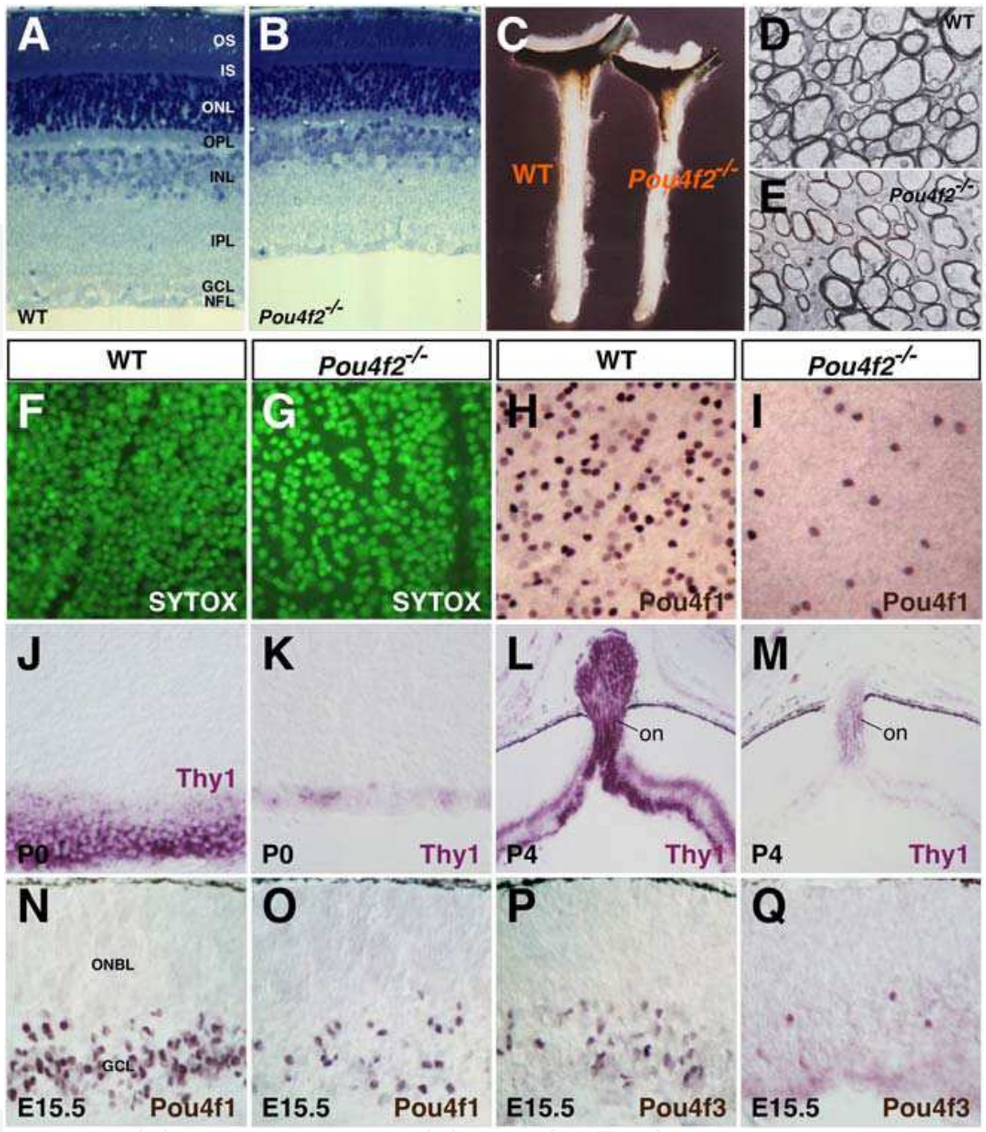

Fig. 3. Loss of RGCs in *Pou4f2*$^{-/-}$ retinas. (A,B) Semi-thin sections of wild-type (WT) and mutant retinas stained with toluidine blue. (C) Optic nerves of the wild-type and mutant mice. (D,E) Electron micrographs of wild-type and mutant optic nerves. (F-I) In wholemount retinas, nuclei in the ganglion cell layer were labeled with SYTOX (F,G) or immunostained with an anti-Pou4f1 antibody (H,I). (J-M) Thy1 immunoreactivity in sections of retinas from the indicated stages. (N-Q) E15.5 retinal sections were immunolabeled with anti-Pou4f1 (N,O) or anti-Pou4f3 (P,Q) antibodies. In the adult *Pou4f2*$^{-/-}$ retina, there is a dramatic loss of RGCs as indicated by the reduced retinal thickness (A,B), diminished optic nerve diameter (C), decreased axon density within the optic nerve (D,E), and reduced cell number in the ganglion cell layer (F-I). The loss of RGCs immunoreactive for Thy1, Pou4f1 or Pou4f3 occur early in development in the mutant retina (J-Q). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment; NFL, nerve fiber layer; ONBL, outer neuroblastic layer; on, optic nerve; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment

Given that the three Pou4f factors are 95% identical in protein sequence of the DNA-binding POU domain and have similar DNA-binding specificity and transcriptional activity (Gruber et al., 1997; Liu et al., 2000a; Xiang et al., 1995), it is unsurprising that they display a significant degree of functional redundancy in RGC development. Thus, no obvious RGC defects are found in *Pou4f1* and *3* conventional knockout mice despite the presence of other severe sensory deficiency (Xiang et al., 1997; Xiang et al., 1996). Moreover, there are more severe RGC loss and axon growth defects in *Pou4f2* and *3* compound mutant mice (Wang et al., 2002). We speculate that Pou4f2 can largely take over the function of Pou4f1 and 3 due to its early expression whereas the converse is not true because the belated expression of Pou4f1 and 3 precludes them from compensating an early specification/differentiation role of Pou4f2 (Xiang, 1998). Consistent with this notion, when *Pou4f1* was knocked in the *Pou4f2* locus to ensure its early expression, all phenotypes associated with *Pou4f2* inactivation were apparently completely rescued (Pan et al., 2005). In spite of the extensive overlap in expression and function, however, individual Pou4f factors have distinct roles during RGC development. For instance, conditional deletion of *Pou4f1* changes dendritic morphology and stratification of RGCs, increases the ratio of bistratified to monostratified RGCs, and causes modest RGC loss (Badea et al., 2009; Badea and Nathans, 2011). Although conditional inactivation of *Pou4f2* results in similar defects, it causes no alteration in RGC dendritic stratification but additionally leads to RGC transdifferentitation and central projection defects (Badea et al., 2009; Badea and Nathans, 2011). However, conditional *Pou4f3* mutants lack any of these RGC phenotypes (Badea and Nathans, 2011).

Pou4f2 is not only necessary but also sufficient to promote RGC differentiation from retinal progenitors. In mouse embryonic retinas, misexpressed Pou4f2 dramatically increased Pou4f1-positive RGCs (Qiu et al., 2008). When misexpressed in chick retinal progenitors by the replication-competent RCAS retroviral vector, mouse Pou4f2 increased cells immunoreactive for Isl1 and NF200, two RGC markers, by 20-50% (Fig. 4C,D,L) (Liu et al., 2000a). Forced expression of mouse Pou4f1 and Pou4f3 as well as chicken Pou4f3 (cPou4f3) all similarly promoted RGC differentiation (Fig. 4M,N) (Liu et al., 2000a), suggesting that all Pou4f factors have a similar potential to promote RGC development but this potential may be limited by their order of expression in vivo. Moreover, misexpression of each of the three mouse Pou4f factors induced many cells to express cPou4f3 in the outer neuroblastic layer (Fig. 4G-I) (Liu et al., 2001). No cPou4f3-positive cells were induced when a mutant Pou4f3 protein was ectopically expressed that contained a deletion in the POU-homeodomain (Fig. 4J) (Liu et al., 2001). Therefore, *Pou4f* gene expression may be cross-activated and auto-activated to help maintain their expression necessary for RGC specification and differentiation (Fig. 4O). Nevertheless, other mechanisms must operate to maintain *Pou4f1* and *2* expression since inactivating either gene in mice does not completely abolish the expression the other in RGCs (Badea et al., 2009; Gan et al., 1996; Xiang et al., 1996). In this regard, the Isl1 LIM-homeodomain transcription factor appears to play a role in the maintenance of *Pou4f2* expression (Mu et al., 2008; Pan et al., 2008).

Pou4f2 regulates a large set of downstream genes to fulfill its crucial function in RGC specification and differentiation. Microarray gene expression profiling of *Pou4f2* wild-type and mutant retinas has revealed hundreds of genes whose expression is altered in the mutant (Mu et al., 2004; Qiu et al., 2008). Among the downregulated genes are those encoding transcription factors and proteins involved in morphogenesis, nervous system development, neuronal cell projection, synaptic vesicle, and neurofilament, consistent with the role of Pou4f2 in RGC development. In particular, Pou4f2 activates the expression of Shh

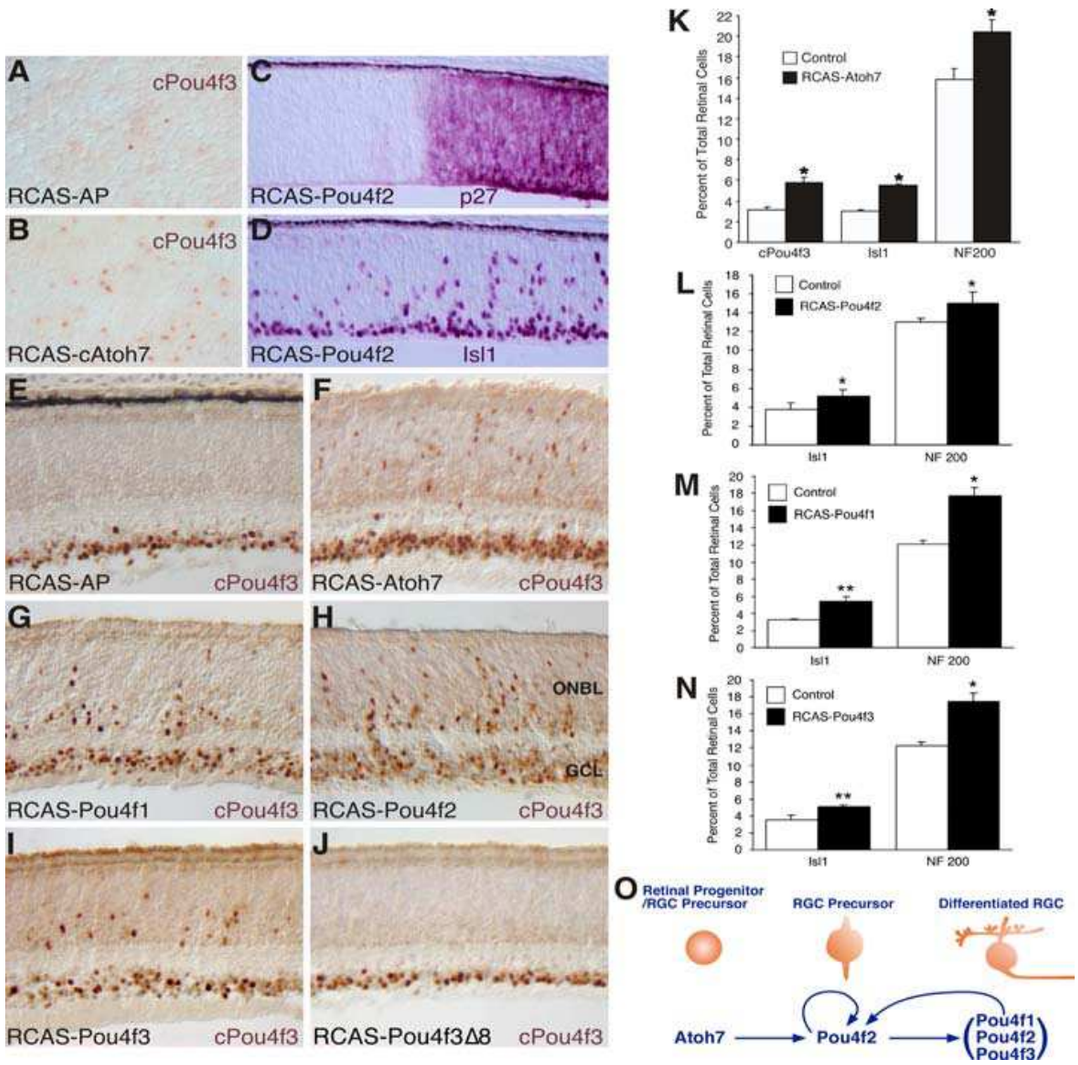

Fig. 4. Regulatory relationship between Atoh7 and Pou4f factors and effects of misexpressed Atoh7 and Pou4f factors on RGC production in the chick retina. (A,B) In the outer neuroblastic layer (ONBL) of E11.5 flat-mount retinas infected with RCAS-cAtoh7 viruses, there was a significant increase of cPou4f3-immunoreactive cells compared to the control (RCAS-AP). (C,D) Adjacent sections from an E7.5 retina infected with RCAS-Pou4f2 viruses were immunostained with anti-p27 gag (C) or anti-Isl1 (D) antibodies. More Isl1-immunoreactive cells were found in the patch stained with the anti-p27 gag antibody than in the patch negative for it. (E-J) Sections from intermediate regions of E11.5 control retinas and retinas infected with RCAS-Atoh7, RCAS-Pou4f1, RCAS-Pou4f2, RCAS-Pou4f3, or RCAS-Pou4f3Δ8 viruses were immunostained with an anti-cPou4f3 antibody. Misexpressed Atoh7, Pou4f1, Pou4f2, and Pou4f3 caused a significant increase of cPou4f3 immunoreactive cells in the ONBL, whereas misexpressed Pou4f3Δ8 had no effect. (K-N) Quantitation of cPou4f3-, Isl1- or NF200-positive RGCs in retinas infected with RCAS-Atoh7, RCAS-Pou4f2, RCAS-Pou4f1, or RCAS-Pou4f3 viruses. (O) Schematic illustrating regulatory relationship between Atoh7 and Pou4f factors during RGC development. Atoh7 may directly activate *Pou4f2* expression which in turn may activate the expression of *Pou4f1* and *Pou4f3*. *Pou4f* gene expression may be cross-activated and auto-activated to help maintain their expression. GCL, ganglion cell layer

(sonic hedgehog) signal pathway genes (Mu et al., 2004). On the other hand, Pou4f2 is also required to repress many genes involved in non-RGC generation to ensure faithful differentiation of RGCs (Qiu et al., 2008).

Isl1. Isl1 resembles Pou4f2 in many ways in controlling RGC development. During mouse retinogenesis, it is co-expressed with Pou4f2 in migrating newborn RGCs beginning at E11.5 (Pan et al., 2008). Conditional inactivation of *Isl1* results in optic nerve hypoplasia, a loss of ~70% of RGCs, delayed RGC axon growth, and RGC axon pathfinding errors and fiber defasciculation (Mu et al., 2008; Pan et al., 2008). Further RGC loss is seen in *Pou4f2* and *Isl1* compound null mice (Pan et al., 2008), implicating distinct as well as redundant functions between Pou4f2 and Isl1 during RGC development. Indeed, these two factors regulate overlapping but distinct groups of genes and they co-occupy the promoters of several shared RGC genes (Mu et al., 2008; Pan et al., 2008). Isl1 does not act genetically upstream of Pou4f2 since its absence does not alter *Pou4f2* expression in early embryonic retinas (Mu et al., 2008). However, it is unclear whether Isl1 acts only in parallel with Pou4f2 or additionally downstream of it to control RGC differentiation.

Dlx1 & Dlx2. The Dlx1 and 2 homeodomain transcription factors are also co-expressed with Pou4f2 in developing RGCs during mouse retinal development (de Melo et al., 2003). Their compound mutants display a mild optic nerve hypoplasia, a loss of ~ 30-40% of RGCs, and increased apoptosis (de Melo et al., 2005). The RGC loss in double mutants results from decreased generation of late born RGCs, thereby indicating a critical role for Dlx1 and 2 in differentiation of late-born RGCs (de Melo et al., 2005). Because these two factors normally repress the expression of a photoreceptor marker *Crx* (de Melo et al., 2005), it is possible that they may also have a role in specifying late-born RGCs.

Eomes & Ebf factors. These factors act as Pou4f2 effectors to mediate part of Pou4f2 function during RGC development. Their genes are among the most downregulated in *Pou4f2* null retinas as determined by microarray analysis (Qiu et al., 2008). Eomes is a T-box transcription factor that similar to Pou4f1 and 3, is expressed in developing RGCs only upon their arrival into the RGC layer (Mao et al., 2008). Its expression is directly activated by Pou4f2 through a 5′ enhancer and is completely downregulated in *Pou4f2* null retinas (Mao et al., 2008). Conditional inactivation of *Eomes* causes a 30% decrease in RGC number and optic nerve size, reduced and disorganized RGC axon myelination, and increased apoptosis (Mao et al., 2008). Ebf1-4 belong to a small family of HLH (helix-loop-helix) transcription factors that are selectively expressed in RGCs in mouse embryonic retinas (Fig. 5A) (Jin et al., 2010). Their RGC expression is dramatically downregulated in *Pou4f2* null mutants (Fig. A-D). It appears that Pou4f2 can bind to the promoter of *Ebf3* to directly activate its expression (Jin et al., 2010). To investigate a possible role for Ebfs during RGC development, we used a replication-incompetent retroviral vector that carries a GFP reporter to misexpress Ebf1 and Ebf-EnR, a dominant-negative form of Ebf constructed by fusing the repressor domain of the *Drosophila*-engrailed protein to the Ebf1 N-terminus. We infected mouse retinal explants with Control-GFP, Ebf1-GFP or Ebf-EnR-GFP viruses at E13.5 when progenitors are still competent for producing RGCs. The infected retinas were harvested after 4 days in culture to analyze RGC production. We found that forced Ebf-EnR expression reduced the proportion of Pou4f1- and Pou4f2-immunoreactive RGCs by ~40-50%, whereas Ebf1 exerted no effect on them (Fig. 5E-K) (Jin et al., 2010). Thus, Ebfs are necessary but insufficient to promote RGC differentiation. It is unknown whether Eomes is sufficient to promote RGC formation as there is yet no report of pertinent gain-of-function analysis.

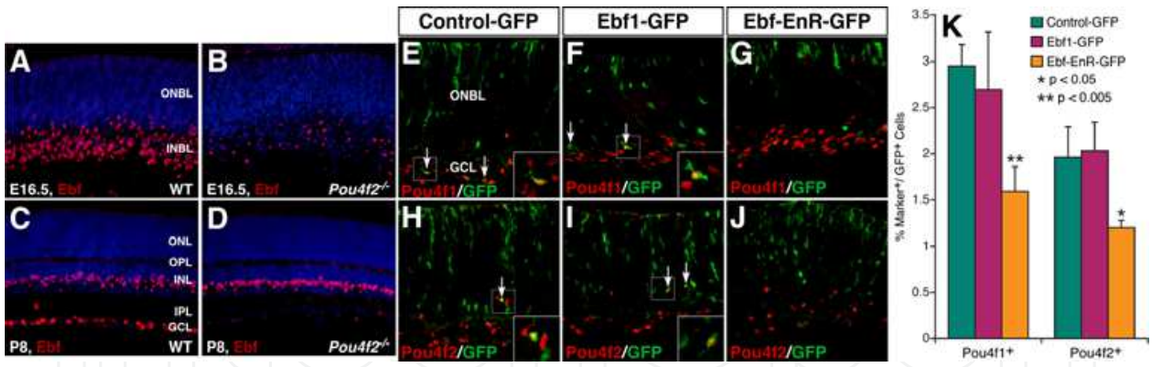

Fig. 5. Requirement for Ebf factors in RGC differentiation. (A-D) Retinal sections from wild-type (WT) and *Pou4f2*$^{-/-}$ mice at the indicated stages were immunostained with a pan-Ebf antibody and weakly counterstained with nuclear DAPI. In the mutant retina, there is a dramatic decrease of Ebf-immunoreactive cells within the INBL or GCL. (E-K) Effect of Ebf1 and a dominant-negative Ebf misexpressed in E13.5 mouse retinal explants on the formation of RGCs. Sections from retinas infected with Control-GFP, Ebf1-GFP or Ebf-EnR-GFP viruses were double immunostained with an anti-GFP antibody and antibodies against Pou4f1 or Pou4f2 (E-J). Virus-transduced retinal cells that became immunoreactive for Pou4f1 or Pou4f2 were then quantified (K, each histogram represents the mean±SD for three retinas). Misexpressed wild-type Ebf1 does not change the number of RGCs immunoreactive for Pou4f1 or Pou4f2; whereas, the dominant-negative form diminishes cells immunoreactive for them. Arrows point to representative colocalized cells, and insets show corresponding outlined regions at a higher magnification. GCL, ganglion cell layer; INL, inner nuclear layer; INBL, inner neuroblastic layer; IPL, inner plexiform layer; ONBL, outer neuroblastic layer; ONL, outer nuclear layer; OPL, outer plexiform layer

2.2 Transcription factors involved in RGC competence

Prior to the initiation of retinogenesis, neuroepithelial cells in the optic vesicle must acquire multipotency and establish competence for the generation of the full range of retinal cell types. Pax6, a paired homeodomain transcription factor, and Sox2, a HMG-box transcription factor, appear to coordinately regulate multipotency of retinal progenitor cells including the potency to generate RGCs. Conditional ablation of either gene in mouse retinal progenitors results in a failure to produce RGCs and other non-RGC cell types (Marquardt et al., 2001; Taranova et al., 2006). Retinal progenitors are postulated to undergo a series of successive stages of competence for the ordered generation of different cell types (Cepko, 1999; Cepko et al., 1996; Harris, 1997; Livesey and Cepko, 2001). It has been shown that the Ikaros zinc finger transcription factor plays a key role in establishing the early temporal competence stages responsible for generating early-born cell types including RGCs (Elliott et al., 2008). Inactivating *Ikaros* causes decreased numbers of early-born neurons without affecting late-born cell types whereas its misexpression in postnatal retinal progenitors is sufficient to confer them with prenatal competence to generate early-born cell types (Elliott et al., 2008). Pax6, Sox2 and Ikaros all directly and/or indirectly activate *Atoh7/Math5* expression to confer progenitors with the competence of RGC genesis (Fig. 1), as genetic ablation of any of these three genes leads to loss or decreased *Atoh7* expression (Elliott et al., 2008; Marquardt et al., 2001; Taranova et al., 2006).

Atoh7 is a proneural bHLH (basic helix-loop-helix) transcription factor expressed transiently in a subset of retinal progenitors/precursors (Brown et al., 1998; Kanekar et al., 1997; Liu et al., 2001). Misexpression of *Xenopus* Atoh7 (Xath5) in retinal progenitors promoted RGC differentiation by ~ 3-fold at the expense of amacrine, bipolar and Müller cells (Kanekar et al., 1997). We used the RCAS retroviral vector to overexpress chicken Atoh7 (cAtoh7/Cath5) in chick retinal progenitors and found that forced cAtoh7 expression significantly induced the expression of cPou4f3, a functional equivalent of Pou4f2 in the chick retina (Fig. 4A,B) (Liu et al., 2001). It increased RGCs immunoreactive for cPou4f3, Isl1 or NF200 by ~30-60% (Liu et al., 2001). Similarly, forced expression of mouse Atoh7 induced cPou4f3 expression and significantly promoted the differentiation of RGCs expressing Isl1 and NF200 in the developing chick retina (Fig. 4F,K). Furthermore, Atoh7 from both mouse and chicken was able to activate reporter gene expression through a *Pou4f2* promoter (Liu et al., 2001). Because all Pou4f factors have the ability to promote RGC formation (Liu et al., 2000a), our results demonstrate that Atoh7 can promote RGC differentiation by directly activating *Pou4f2/cPou4f3* expression, and further define an Atoh7-Pou4f2/cPou4f3 pathway underlying the specification and differentiation of RGCs (Fig. 4O) (Liu et al., 2001). This molecular pathway can be confirmed in *Atoh7* knockout mice by the near complete downregulation of *Pou4f2* expression in *Atoh7* mutant retinas (Wang et al., 2001).

Consistent with the gain-of-function studies, loss-of-function analyses in zebrafish and mice have confirmed an essential role for Atoh7 in RGC development. Its mutation in the zebrafish *lakritz* mutant causes a complete loss of RGCs and its deletion in mice results in the absence of optic nerve and a loss of ~95% of RGCs (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001). The virtual absence of Pou4f2-expressing cells in early *Atoh7* mutant retinas suggests that Atoh7 must have an early role during RGC development. However, a role for Atoh7 in RGC fate specification can be ruled out because genetically marked Atoh7-expressing precursors are found to give rise to multiple early-born retinal cell types including RGC, amacrine, horizontal and photoreceptor cells (Yang et al., 2003). Therefore, Atoh7-positive retinal precursors are multipotential and Atoh7 is required only for conferring these precursors with the competence of RGC generation. In agreement with this early function, microarray gene expression profiling analysis has revealed that Atoh7-regulated genes include the two branches of genes controlled by Pou4f2 and Isl1 and additionally contain many more that are not regulated by either factor (Mu et al., 2005). Moreover, *Atoh7* and *Pou4f2* double knockout mice display more severe loss of RGCs (Moshiri et al., 2008). Wt1, a zinc-finger transcription factor encoded by the Wilms' tumor gene, appears to regulate RGC differentiation also by directly activating *Pou4f2* expression (Wagner et al., 2002). However, it is unclear what is the relationship between Wt1 and Atoh7 and whether Wt1 is involved in conferring retinal precursors with RGC competence.

2.3 Safeguard mechanism by transcription factors to ensure RGC fidelity

As discussed above, RGC-competent Atoh7$^+$ precursors are able to generate RGC, amacrine, horizontal, and cone photoreceptor cells during mouse retinogenesis (Fig. 1) (Feng et al., 2006; Yang et al., 2003). Thus, in order to select the RGC fate from the multiple fates available for an RGC-competent precursor, it is conceivable that the commitment factor must suppress non-RGC fates while promoting the RGC fate to ensure the fidelity of RGC differentiation. In this regard, we have demonstrated that Pou4f2 has the ability to suppress amacrine, horizontal and late-born RGC fates to preferentially specify the early-born RGC fates (Qiu et al., 2008)

In E14.5 *Pou4f2* null retinas, our gene expression profiling study identified a large set of upregulated genes including *Th, Slc32a1/VIAAT*, *Slc6a1/GAT-1*, *Slc18a3/VAChT*, and *Gad1/GAD67*, which are normally expressed by amacrine and/or horizontal cells (Qiu et al., 2008). Consistent with this, immunostaining and RNA in situ hybridization revealed a marked increase in the expression of Th, GABA, GAT-1, calbindin D28K, and *Viaat* in the mutant retina (Fig. 6A-F) (Qiu et al., 2008), suggesting that GABAergic amacrine cells abnormally form in early embryonic retinas in the absence of *Pou4f2*. In *Pou4f2*$^{lacZ/lacZ}$ retinas, nearly all Th-immunoreactive cells were co-labeled for β-gal (Fig. 6B), indicating that these cells arise cell-autonomously due to a switch in fate of RGC precursors that would normally express Pou4f2. Double-immunostaining showed a significant increase of cells immunoreactive for both Lim1 and calbindin in *Pou4f2* null retinas (Fig. 6*E,F*), suggesting that loss of *Pou4f2* function causes increased horizontal cell differentiation. These supernumerary horizontal cells also arise cell-autonomously because in *Pou4f2*$^{lacZ/lacZ}$ retinas, many Lim1-immunoreactive cells co-expressed β-gal whereas these double-immunoreactive cells were absent from *Pou4f2*$^{+/lacZ}$ retinas (Qiu et al., 2008). The transcripts of *Dlx1* and *Dlx2* were among the most upregulated in *Pou4f2* null retinas as determined by microarray analysis. At E14.5, scattered in situ hybridization signals for *Dlx1* and *Dlx2* were seen in the outer neuroblastic layer but absent from the inner neuroblastic layer (INBL) of wild-type retinas (Fig. 6*Q,S*). In *Pou4f2*$^{-/-}$ retinas, however, both *Dlx1* and *Dlx2* were aberrantly expressed within the INBL (Fig. 6*R,T*), suggesting the generation of superfluous late-born RGCs within the INBL in the absence of *Pou4f2*. Thus, Pou4f2 has the activity to cell-autonomously inhibit differentiation of amacrine, horizontal and late-born RGCs. This inhibitory activity can be confirmed by overexpression experiments where we showed that misexpressed Pou4f2 suppressed the differentiation of all non-RGC cell types including amacrine and horizontal cells (Qiu et al., 2008).

Pou4f2 acts to inhibit the fates of non-RGCs by repressing the expression of transcription factor genes required for their specification and differentiation. By microarray analysis, in situ hybridization, qRT-PCR, and immunostaining, we were able to show that the expression of *Bhlhb5*, *Nr4a2/Nurr1*, *Neurod1*, *Math3*, and *Ptf1a* all exhibited significant increase in *Pou4f2* null retinas, especially within the INBL (Fig. 6G-L) (Qiu et al., 2008), consistent with the abnormal differentiation of most amacrine cells in this layer of the mutant retina. It has been shown that Neurod1, Math3 and Ptf1a are required for specifying amacrine cells and Bhlhb5 and Nr4a2 for differentiation of GABAergic amacrine cells (Feng et al., 2006; Fujitani et al., 2006; Inoue et al., 2002; Jiang and Xiang, 2009; Nakhai et al., 2007). Similarly, there was a significant increase in expression of *Prox1*, *Ptf1a*, *Lim1* and *Ngn2*, all transcription factor genes involved in horizontal cell development (Akagi et al., 2004; Dyer et al., 2003; Fujitani et al., 2006; Liu et al., 2000b; Nakhai et al., 2007; Poche et al., 2007), within the INBL of *Pou4f2* null retinas (Fig. 6E,F,M-P). In addition, we observed in *Pou4f2*$^{-/-}$ retinas a moderate but significant increase in the expression of *Otx2*, *Crx*, *Thrb2* and *Prdm1/Blimp1* (Qiu et al., 2008), which are all transcription factor genes involved in photoreceptor cell development (Brzezinski et al., 2010; Furukawa et al., 1997; Furukawa et al., 1999; Ng et al., 2001; Nishida et al., 2003; Wilm and Solnica-Krezel, 2005).These studies have led us to propose that while promoting the differentiation of early-born RGCs, Pou4f2 may actively suppress the differentiation of late-born RGC, amacrine, horizontal, and cone cells by repressing a network of transcription factor genes involved in their commitment and differentiation (Fig. 7*A*) (Qiu et al., 2008). This built-in negative regulatory program may serve as a safeguard mechanism to ensure the differentiation of all Pou4f2-expressing

precursors as early-born RGCs, thereby guaranteeing the fidelity of RGC differentiation. In the absence of *Pou4f2,* the release of the safeguard mechanism may cause RGC precursors to change their cell fates and abnormally generate amacrine, horizontal and late-born RGC cells that may ultimately degenerate by apoptosis (Fig. 7*B*). In spite of the apparent inhibition of late-born RGC fates, it is still possible that Pou4f2 may be involved in terminal differentiation of late-born RGCs given the crucial role of Pou4f2 in RGC differentiation and the co-expression between Pou4f2 and Dlx1/2 (de Melo et al., 2003).

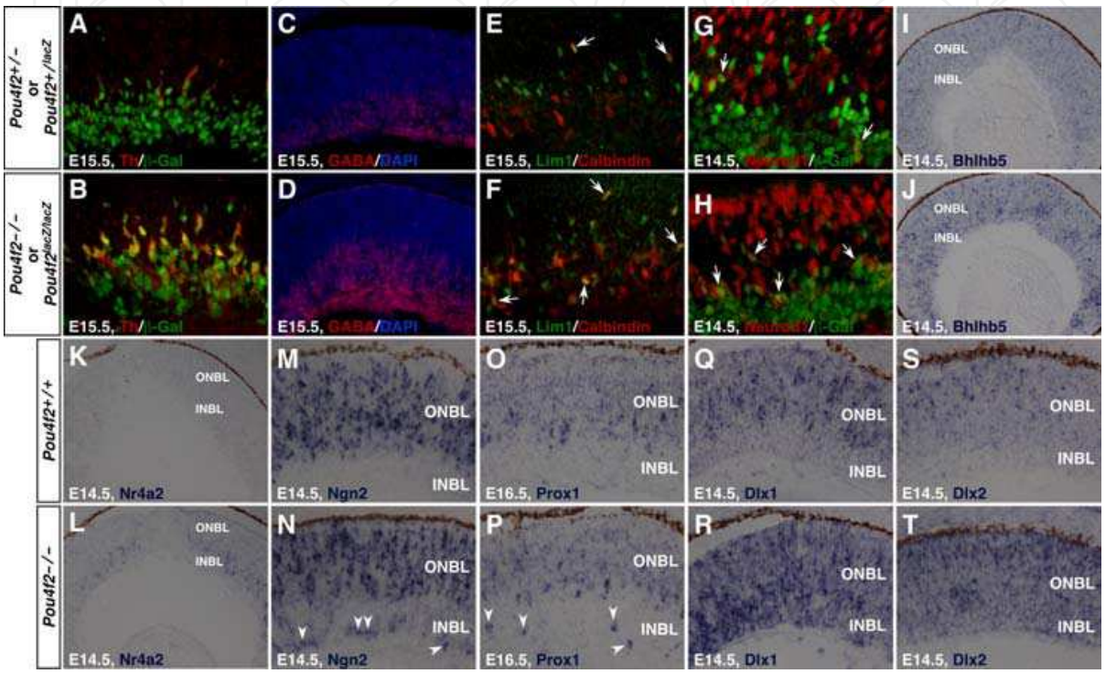

Fig. 6. Aberrant differentiation of amacrine, horizontal and ganglion cells in *Pou4f2* null mutant retinas. (A-H) Retinal sections at the indicated stages from *Pou4f2*$^{+/lacZ}$ (A,E,G), *Pou4f2*$^{lacZ/lacZ}$ (B,F,H), *Pou4f2*$^{+/-}$ (C), and *Pou4f2*$^{-/-}$ (D) embryos were immunostained with the indicated antibodies. Within the INBL of null retinas, there was a significant increase of GABA-immunoreactivity and cells double-immunoreactive for Lim1 and calbindin or β-gal and Th or Neurod1 (indicated by arrows). (I-T) Retinal sections at the indicated stages from *Pou4f2*$^{+/+}$ (K,M,O,Q,S), *Pou4f2*$^{+/-}$ (I), and *Pou4f2*$^{-/-}$ (J,L,N,P,R,T) embryos were in situ hybridized with the indicated RNA probes. Within the INBL of null retinas, there was a significant increase in expression of *Bhlhb5*, *Nr4a2*, *Ngn2* (indicated by arrowheads), *Prox1* (indicated by arrowheads), *Dlx1*, and *Dlx2*. INBL, inner neuroblastic layer; ONBL, outer neuroblastic layer

Despite the comprehensive negative regulatory program, Pou4f2 appears to suppress photoreceptor or glycinergic amacrine cell fates only weakly or not at all (Qiu et al., 2008), raising the possibility that it may have to cooperate with other regulatory factors to inhibit all other alternative cell fates available to a particular RGC precursor. Targeted *Atoh7* inactivation leads to increased cones and cholinergic amacrine cells (Brown et al., 2001; Wang et al., 2001), thereby indicating an inherent activity for Atoh7 to suppress cone and cholinergic amacrine cell fates even though the precursors expressing it are able to produce both cell types (Yang et al., 2003). Conceivably, Pou4f2 may cooperate with Atoh7 to ensure complete suppression of the amacrine and cone differentiation programs in RGC precursors.

Similar to Pou4f2, Atoh7 appears to repress the expression of *Neurod1*, *Math3*, *Bhlhb5*, and *Ngn2* to suppress the amacrine and/or horizontal cell fates (Feng et al., 2006; Mu et al., 2005). Although it remains to be determined whether Atoh7 also represses the expression of photoreceptor transcription factor genes, it seems that Pou4f2 and Atoh7, and likely other RGC transcription factors, may all have built-in safeguard mechanisms that only when working together can ensure the highest fidelity of RGC differentiation.

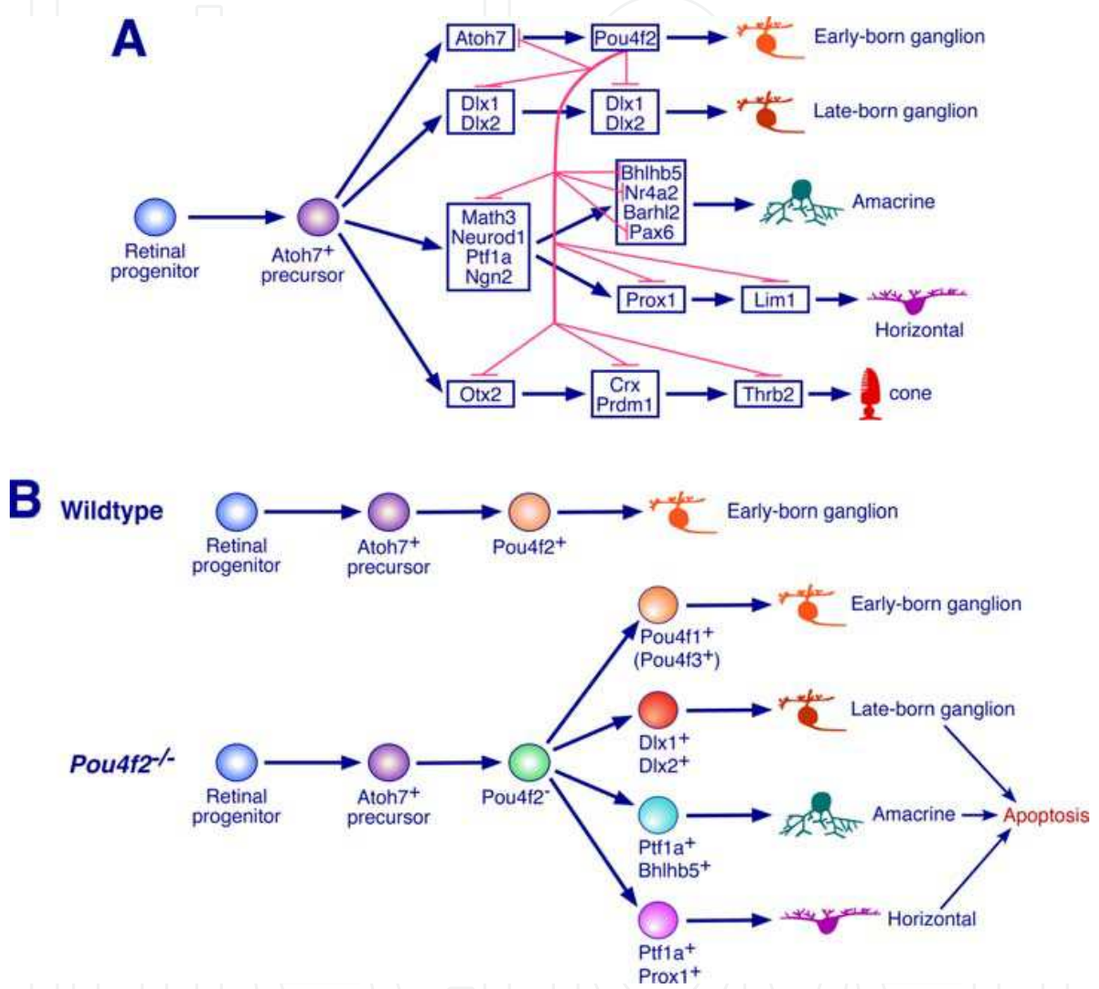

Fig. 7. Schematics illustrating the safeguard mechanism by which Pou4f2 specifies early-born RGCs. (A) RGC-competent Atoh7+ precursors are able to generate early-born and late-born RGCs and amacrine, horizontal and cone cells. When Pou4f2 is expressed in such a multipotential precursor, it commits it to an early-born RGC fate while preventing it from following other differentiation pathways by repressing the expression of a network of retinogenic transcription factor genes involved in fate commitment and differentiation of late-born RGC, amacrine, horizontal, and cone cells. (B) When expressed in RGC-competent precursors, Pou4f2 specifies early-born RGCs and promotes their differentiation in wild-type retinas. In the *Pou4f2* mutant, the safeguard mechanism is compromised and the precursors that would normally express Pou4f2 switch their fates to aberrantly produce late-born RGC, amacrine and horizontal cells that would quickly degenerate by apoptosis. A small portion of early-born RGCs may be produced due to functional compensation by Pou4f1 and Pou4f3

3. Extrinsic signaling

During retinal development, RGC generation initiates near the center of the developing retina and then propagates toward the periphery in a wave-like fashion (McCabe et al., 1999; Neumann and Nuesslein-Volhard, 2000). It has been shown that fibroblast growth factor 3 (FGF3) and FGF8 secreted from local organizing centers act together to induce the first wave of RGC differentiation from retinal progenitors (Martinez-Morales et al., 2005). FGF8 released from coated beads promotes *Atoh7* expression and triggers RGC differentiation whereas pharmacological inhibition of FGF signaling blocks *Atoh7* expression and RGC differentiation (Martinez-Morales et al., 2005). In zebrafish deficient for both *Fgf3* and *Fgf8*, *Atoh7* expression fails to initiate in the developing retina (Martinez-Morales et al., 2005). Indeed, FGF signaling activates *Xenopus Atoh7* gene expression through its 5′ regulatory sequence (Willardsen et al., 2009). FGF signaling may cooperate with Shh to coordinate the subsequent spread of RGC differentiation wave front. FGF19, via regulation of the *Pea3* and *Erm* Ets-domain transcription factor genes, together with Shh released from new born RGCs, are required for propagation of Shh own gene expression (McCabe et al., 2006; Vinothkumar et al., 2008). Mutations in Shh signaling components and cyclopamine treatment all disrupt normal propagation of *Shh* and *Atoh7* expression as well as RGC differentiation (Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003). FGF and Shh appear to activate the Ras-MAPK pathway to trigger and drive the wave of RGC differentiation (Neumann and Nuesslein-Volhard, 2000). Despite these compelling evidence in support of sequential induction of RGCs, other studies suggest that the progression of RGC differentiation wave may be governed by a dominant intrinsic mechanism pre-programmed into the progenitors (Kay et al., 2005).

In contrast to the crucial requirement of Shh signaling in RGC differentiation progression, Shh secreted by new born RGCs behind the neurogenesis wave front prevents progenitors from generating more RGCs, thereby ensuring a proper number of total RGCs to be produced in the retina. In the chick retina, Shh overexpression decreases RGC production whereas inhibiting endogenous Shh activity increases RGC differentiation (Zhang and Yang, 2001). Similarly, conditional ablation of *Shh* and *Smoothened* (*Smo*) in mice results in Atoh7-dependent overproduction of RGCs (Sakagami et al., 2009). VEGF (vascular endothelial growth factor) is also secreted by RGCs and other postmitotic neurons and can negatively regulate RGC differentiation (Hashimoto et al., 2006). Thus, RGC formation is reduced by enhanced VEGF signals but increased by disrupting VEGF signaling activity (Hashimoto et al., 2006). Besides these diffusible signals, Dll-Notch signaling mediated by cell-cell contacts also limits RGC production. Constitutively activated Notch and elevated Dll signal are shown to decrease RGC generation whereas inhibiting Notch signaling has the opposite effect (Ahmad et al., 1997; Austin et al., 1995; Dorsky et al., 1997; Dorsky et al., 1995; Nelson et al., 2007). Interestingly, both Shh and VEGF signaling activates expression of the Dll-Notch effector gene *Hes1*, which has an activity to suppress RGC differentiation (Hashimoto et al., 2006; Sakagami et al., 2009; Wang et al., 2005). Therefore, these divergent signaling pathways appear to converge on Hes1 to control proper RGC production during retinogenesis (Hashimoto et al., 2006).

In the mouse retina, inactivation of the *Gdf11* gene, which encodes a member of the TGF-β/activin protein family, causes an increase of RGC production from progenitors whereas inactivating the follistatin gene, its antagonist, reduces RGCs (Kim et al., 2005). On the other hand, treating embryonic retinal explants with Gdf11 in culture results in a decrease of

RGCs. Neither Gdf11 nor follistatin exert any effect on the proliferation of retinal progenitors (Kim et al., 2005). Therefore, follistatin appears to increase the number of progenitors competent for RGC generation whereas Gdf11 antagonizes this activity, thereby contributing to a balanced production of RGCs. Gdf11 and follistatin control retinal progenitor competence by shortening and elongating the duration of *Atoh7* expression, respectively (Kim et al., 2005).

4. Generation of RGCs from stem cells

As potential renewable sources of replacement cells for cell transplantation/replacement treatment, stem cells offer exciting future possibilities to restore functional retinal circuits in patients inflicted with retinal degeneration such as glaucoma (Dahlmann-Noor et al., 2010; Klassen et al., 2004; Lamba et al., 2008; MacLaren et al., 2006). It appears that the intrinsic and extrinsic mechanisms utilized to generate RGCs during normal development are recruited to stimulate RGC differentiation from stem cells both in vivo and in vitro. In the post-hatch chicken retina, FGF signaling is required to induce *Atoh7* expression and subsequent production of Pou4f1- and Isl1-immunoreactive RGCs from stem cells in the ciliary marginal zone (Fischer et al., 2002). Similarly, mouse embryonic stem (ES) cells can be induced to generate RGC-like cells that express Atoh7, Pou4f2 and Isl1 only when exposed to FGF2, and this RGC-inducing activity by FGF2 is further potentiated by Shh (Jagatha et al., 2009). In rat retinal progenitors, transient expression of the intrinsic factor Atoh7 led to *Pou4f2* upregulation and RGC induction (Yao et al., 2007). Pluripotent stem (iPS) cells induced from mouse fibroblasts can be also directed to form retinal progenitors that are capable of differentiating into RGC-like cells in the presence of suitable extrinsic and intrinsic cues. They produced RGCs expressing a variety of RGC-specific markers when cultured in retinal differentiation medium containing conditioned medium from E14 rat retinal cells (Parameswaran et al., 2010). These induced RGCs were able to extend processes toward superior collicular explants and exhibited typical neuronal activity (Parameswaran et al., 2010). Similarly, overexpression of Atoh7 could direct the iPS-derived retinal progenitors toward a RGC differentiation program, which could be further enhanced by inhibiting Notch signaling activity (Chen et al., 2010). Conceivably, application of a combination of stimulatory intrinsic and extrinsic factors while suppressing negative ones may lead to even greater induction of RGCs from stem cells. Transplanting in vitro induced retinal progenitors and RGCs intravitreally into postnatal rodent eyes resulted in only few cells that integrated into the RGC layer of the host retina (Chen et al., 2010; Jagatha et al., 2009), suggesting that postnatal retinas present a formidable barrier to cell migration and integration.

5. Summary and perspectives

Great strides have been made over the past two decades toward understanding both the intrinsic and extrinsic mechanisms of RGC specification and differentiation from retinal progenitors. Molecular genetic studies coupled with bioinformatic approaches have yielded a wealth of information about transcription factors and their regulatory gene networks as well as signaling events that lead to the establishment of RGC competence and eventual differentiation of RGCs (Fig. 1). Significant inroads have also been made toward understanding the molecular basis underlying the fidelity of RGC differentiation and

production of proper number of RGCs. Despite these exciting advances, however, there are still many questions remaining to be answered. For instance, how do intrinsic and extrinsic factors interact and cooperate at cellular and transcriptional levels to drive RGC development and ensure proper RGC number? How do Atoh7 and Pou4f2 activate RGC differentiation program genes while repressing non-RGC differentiation program genes at the molecular level and what are their direct targets? What factors are responsible for specifying the extremely diverse RGC subtypes? What factors are required to efficiently induce RGCs from stem cells and what prevents them from migrating and integrating into the intact retina? Progress in these areas will undoubtedly lead to many more exciting discoveries in years to come. Ultimately, understanding the molecular events and factors involved in RGC development may lay the groundwork to improve stem cell-mediated regeneration, leading to eventual development of effective treatments of glaucoma in the future.

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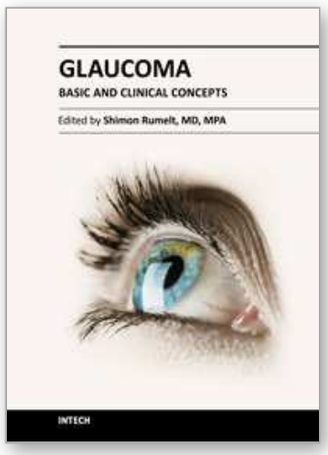

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This book addresses the basic and clinical science of glaucomas, a group of diseases that affect the optic nerve and visual fields and is usually accompanied by increased intraocular pressure. The book incorporates the latest development as well as future perspectives in glaucoma, since it has expedited publication. It is aimed for specialists in glaucoma, researchers, general ophthalmologists and trainees to increase knowledge and encourage further progress in understanding and managing these complicated diseases.

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