Foxn4 Controls the Genesis of Amacrine and Horizontal Cells by Retinal Progenitors

Shengguo Li,[1] Zeqian Mo,[1] Xuejie Yang,
Sandy M. Price, Michael M. Shen,
and Mengqing Xiang*
Center for Advanced Biotechnology
  and Medicine and
Department of Pediatrics
University of Medicine and Dentistry of New Jersey
Robert Wood Johnson Medical School
679 Hoes Lane
Piscataway, New Jersey 08854

Summary

During vertebrate retinogenesis, seven classes of cells are specified from multipotent progenitors. To date, the mechanisms underlying multipotent cell fate determination by retinal progenitors remain poorly understood. Here, we show that the Foxn4 winged helix/forkhead transcription factor is expressed in a subset of mitotic progenitors during mouse retinogenesis. Targeted disruption of Foxn4 largely eliminates amacrine neurons and completely abolishes horizontal cells, while overexpression of Foxn4 strongly promotes an amacrine cell fate. These results indicate that Foxn4 is both necessary and sufficient for commitment to the amacrine cell fate and is nonredundantly required for the genesis of horizontal cells. Furthermore, we provide evidence that Foxn4 controls the formation of amacrine and horizontal cells by activating the expression of the retinogenic factors Math3, NeuroD1, and Prox1. Our data suggest a model in which Foxn4 cooperates with other key retinogenic factors to mediate the multipotent differentiation of retinal progenitors.

Introduction

The vertebrate retina consists of six classes of neurons and one type of glial cells that are interconnected in a highly organized laminar structure. It develops from the optic vesicle, a protrusion of the diencephalic neuroepithelium of the neural tube. During retinogenesis, birthdating analyses have revealed a loose and yet fixed temporal order for the genesis of each cell type, whereas lineage tracing studies have clearly demonstrated that retinal progenitors are multipotent, since a given progenitor can give rise to more than one cell type (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988; Young, 1985). To accommodate these findings, a recent model of retinogenesis suggests that intrinsic and extrinsic factors together determine the choice of cell fate (Cepko, 1999; Harris, 1997; Livesey and Cepko, 2001). In this model, it is hypothesized that retinal progenitors pass through successive and intrinsically distinct states of competence to respond to devel-

opmentally varying environmental cues for the ordered generation of different cell types.

Recent advances by molecular and cellular approaches have begun to identify the intrinsic and extrinsic factors that are involved in the determination and differentiation of these cell types. A number of extrinsic factors have been found to affect the retinogenic potential of progenitors, among them FGFs, EGFs, CNTF, Shh, taurine, retinoic acid, thyroid hormone, and Notch/Delta signaling molecules (Altshuler et al., 1993; Austin et al., 1995; Ezzeddine et al., 1997; Furukawa et al., 2000; Guillemot and Cepko, 1992; Kelley et al., 1994, 1995; Lillien, 1995; Young and Cepko, 2004; Zhang and Yang, 2001). Various transcription factors, on the other hand, have been shown to serve as major intrinsic retinogenic factors, among them the bHLH (basic-helix-loop-helix) factors Mash1, Math3, Math5, NeuroD1, Ngn2, Hes1, and Hesr2 (Brown et al., 2001; Furukawa et al., 2000; Inoue et al., 2002; Morrow et al., 1999; Satow et al., 2001; Tomita et al., 1996, 2000; Wang et al., 2001; Yan et al., 2001) and homeoproteins Pax6, Rax, Chx10, and Prox1 (Burmeister et al., 1996; Dyer et al., 2003; Furukawa et al., 2000; Marquardt et al., 2001; Mathers et al., 1997).

The amacrine and horizontal cells are two classes of important interneurons that modulate and integrate visual signals in the retinal circuitry. Both classes are born early from multipotent progenitor cells, starting from embryonic day 11 (E11) during mouse retinogenesis (Young, 1985). The molecular basis of the determination and differentiation of these two cell types remains poorly understood at present. It has been shown by gene targeting that Pax6 is required by retinal progenitors to acquire multipotency for the generation of all but one cell type (Marquardt et al., 2001). Pax6 appears to control the competence states of progenitors by regulating the expression of bHLH genes, which encode transcription factors known to control competence and fate commitment of progenitors (Marquardt et al., 2001). However, Pax6 appears to be dispensable for the generation of amacrine neurons, raising the possibility that another intrinsic regulator(s) exists that confers retinal progenitors with the potential for amacrine cell genesis. Gene targeting and overexpression studies have demonstrated that Math3 and NeuroD1 play a redundant role in fate determination of amacrine cells, since these neurons are normally formed in single mutants null for either gene but are completely absent in compound mutants deficient for both (Inoue et al., 2002; Morrow et al., 1999). Loss of Pax6 function does not alter NeuroD1 expression in progenitors (Marquardt et al., 2001), thereby allowing the differentiation of amacrine cells to occur. However, Pax6 and another homeobox gene, Barhl2, are expressed by differentiating and mature amacrine cells and have been implicated as playing a role in the specification and/or differentiation of glycinergic amacrine cells (Marquardt et al., 2001; Mo et al., 2004). Much less is currently known about the molecular basis underlying horizontal cell development, although a recent report implicates Prox1 as a crucial intrinsic factor that

*Correspondence: xiang@cabm.rutgers.edu
[1]These authors contributed equally to this work.

controls fate commitment of this cell type (Dyer et al., 2003).

The forkhead/winged helix transcription factors constitute a large family of important proteins characterized by a 110 amino acid DNA binding domain that can fold into a variant of the helix-turn-helix motif that consists of three α helices flanked by two large loops (wings). They are involved in a wide variety of biological processes as key regulators in development and metabolism (Carlsson and Mahlapuu, 2002; Gajiwala and Burley, 2000). *Foxn4* is a recently identified member of this gene family that displays a prominent expression pattern in retinal progenitor cells (Gouge et al., 2001). In this study, we have investigated the detailed spatial and temporal expression pattern of the Foxn4 protein as well as its biological function during mouse retinogenesis. Our data indicate that Foxn4 is expressed by a subset of lineage-biased retinal progenitors and that it controls the genesis of amacrine and horizontal cells by regulating the expression of retinogenic factors that are involved in their fate commitment.

Results

Expression Pattern of Foxn4 during Retinogenesis
To determine the spatial and temporal expression pattern of Foxn4 during retinogenesis, we raised a specific anti-Foxn4 polyclonal antibody for immunofluorescent analysis of developing mouse retinas. While no Foxn4 expression is observed in the developing eye at E10.5, it is detectable at E11.5 in the central region of the retina (Figures 1A and 1B). At E13.5, Foxn4 expression spreads from the center to the entire retina, with most progenitor cells in the outer neuroblastic layer abundantly expressing Foxn4 (Figure 1C). However, Foxn4 expression is absent from the inner neuroblastic layer of the retina and the lens vesicle (Figures 1B and 1C). At E15.5–E17.5, Foxn4 continues to be expressed strongly in a large subset of progenitor cells within the outer neuroblastic layer (Figures 1D and 1E). At postnatal day 1 (P1), however, Foxn4 expression begins to be downregulated (Figure 1F). By P4, only a small number of cells can be seen to express Foxn4 in the outer neuroblastic layer (Figure 1G). In the P6 retina, the central area completely loses Foxn4 expression, while only occasional cells are positive for Foxn4 in the intermediate and peripheral regions (Figures 1H–1J). Thus, both the onset and downregulation of Foxn4 expression follow a central to peripheral gradient of retinogenesis. Foxn4 is not expressed in late postnatal and adult retinas (Figure 1K).

The outer neuroblastic layer of the developing retina consists of a mixture of dividing progenitor cells and some newly generated postmitotic neurons/glial cells. To determine whether Foxn4 was expressed only by mitotic progenitors, we pulse labeled S phase cells in the E17.5 retina by BrdU and performed double immunostaining using anti-Foxn4 and anti-BrdU antibodies. We found that all Foxn4$^+$ cells were colocalized with proliferative cells labeled by the anti-BrdU antibody, even though not all BrdU$^+$ cells were stained by the anti-Foxn4 antibody (Figures 1L–1N), indicating that Foxn4 is expressed solely by a subset of mitotic progenitors. Consistent with this observation, in E14.5 retinas, double

immunostaining revealed that Foxn4-immunoreactive cells overlapped with progenitors immunoreactive for Pax6 or syntaxin in the outer neuroblastic layer but not with the Pax6$^+$ or syntaxin$^+$ postmitotic cells in the inner neuroblastic layer (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/43/6/795/DC1). In fact, during the entire period of retinogenesis, there is a complete absence of Foxn4 expression from the inner retinal layers, where only postmitotic cells reside (Figures 1C–1K). The expression of Foxn4 in the syntaxin$^+$ progenitor cells, which represent a subset of progenitors with a lineage biased toward amacrine and horizontal cells (Alexiades and Cepko, 1997), suggests that Foxn4 may be involved in the genesis of these two cell types.

Retinal Dysplasia in *Foxn4*$^{-/-}$ Mice
To study in vivo the role of *Foxn4* during retinal development, we generated a targeted *Foxn4* allele in mice via homologous recombination in embryonic stem cells. In this targeted allele, seven of the nine coding exons, including the forkhead/winged helix DNA binding domain, were replaced by *IRES-lacZ* and *PGK-Neo* cassettes (Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/43/6/795/DC1). The failure to detect an in situ hybridization signal for *Foxn4* in embryonic retinas of homozygous mutants confirmed that the mutation is a null allele (Figures 4A and 4B). Phenotypically, all heterozygotes behaved in a manner similar to that of their wild-type littermates; however, most homozygous mutants displayed early postnatal lethality, while surviving null mutants exhibited noticeable body size reduction starting at P8.

To determine the fate of progenitors that would normally express *Foxn4* in the *Foxn4*$^{-/-}$ retina, we followed the generation and destination of *lacZ*$^+$ cells. In heterozygotes, *lacZ* expression commenced at E11.5 and peaked around E14.5 (Figures 2A and 2C), while it became downregulated at P0 and was absent by P8 (Figures 2E and 2G). β-galactosidase activity was confined to the outer neuroblastic layer as well as the nerve fiber layer, consistent with labeling of progenitor cells with a bipolar morphology (Figures 2C and 2E). Thus, the knockin *lacZ* reporter faithfully recapitulated the spatial and temporal expression pattern of the endogenous *Foxn4* gene. In the *Foxn4*$^{-/-}$ retina, β-galactosidase activity was similarly seen in the outer neuroblastic layer at E11.5 and E14.5, albeit much stronger (Figures 2B and 2D), indicating that retinal progenitors that would normally express *Foxn4* were produced in the null retina. However, there was no downregulation of *lacZ* expression by P0 in the null retina, and even by P8 many cells in the outer nuclear layer (ONL) could still be seen to prominently express *lacZ* (Figures 2E–2H). Thus, in the *Foxn4*$^{-/-}$ retina, there appeared to be a significant temporal delay in the downregulation of *lacZ* expression in progenitor cells.

By examining retinal sections stained with hematoxylin-eosin (HE), we investigated whether loss of *Foxn4* function would affect the gross morphology and structure of mutant retinas. While the wild-type and null retinas were similar in thickness and structure at P0 (data not shown), we found that at P8 and P20, *Foxn4*$^{-/-}$ retinas were obviously reduced in thickness compared
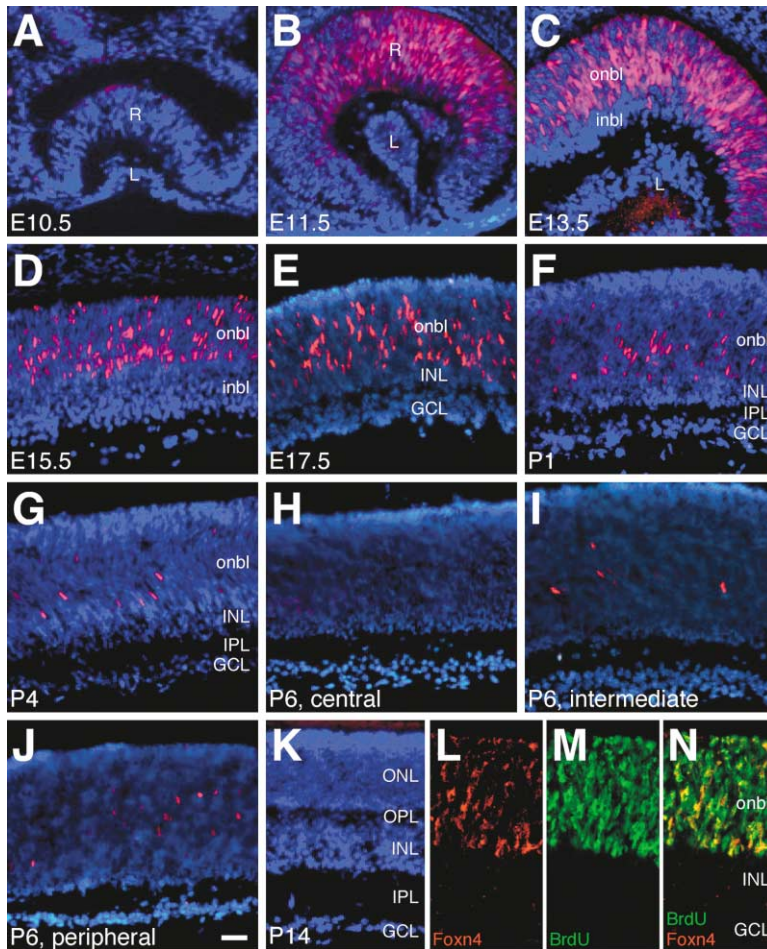
Figure 1. Expression Pattern of the Foxn4 Protein during Mouse Retinogenesis

(A–K) Retinal sections from the indicated developmental stages were immunostained with an anti-Foxn4 antibody (red) and weakly counterstained with DAPI (blue). The expression of Foxn4 commences at E11.5 in the central retina, peaks around E13.5 in the outer neuroblastic layer, and continues to be strong at E15.5–E17.5. However, it begins to be downregulated significantly at P0 and eventually disappears around P6–P7. At P6, Foxn4 expression is completely downregulated from the central retina, while only occasional cells express Foxn4 in the intermediate and peripheral regions (H–J). (L–N) Retinal sections from BrdU-labeled E17.5 embryos were double immunostained with anti-Foxn4 (red) and anti-BrdU (green) antibodies. All Foxn4-immunoreactive nuclei colocalize with the S phase nuclei immunoreactive for BrdU (N). Abbreviations for this and other figures are as follows: GCL, ganglion cell layer; inbl, inner neuroblastic layer; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; onbl, outer neuroblastic layer; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; R, retina; S, photoreceptor segment. Scale bar equals 25 μm in (A)–(K) and 20.5 μm in (L)–(N).

with control *Foxn4*$^{+/+}$ and *Foxn4*$^{+/-}$ retinas (Figures 2I–2L). In *Foxn4*$^{-/-}$ retinas, the inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) were much thinner, and only a small number of scattered cells were present within the GCL (Figures 2I–2L). There were also clusters of cells that aberrantly protruded from the INL into the IPL (Figure 2J). Additionally, the outer plexiform layer (OPL), albeit present at P8 in the null retina, was invisible at P20 (Figures 2J and 2L). The ONL, however, showed no decrease in thickness; on the contrary, it was overtly thicker than that of the control retina at P20 (Figures 2I–2L), indicating that the absence of *Foxn4* causes anomalous development of inner retinal cell types. We noted that there were also many clusters of ONL cells that abnormally protruded into the segments of photoreceptor cells in late postnatal null retinas (Figure 2L), suggesting a possible improper migration and/or differentiation of *lacZ*$^{+}$ progenitor cells still present at P8 in *Foxn4*$^{-/-}$ retinas (Figure 2H).

Defect in the Genesis of Amacrine and Horizontal Cells in *Foxn4*$^{-/-}$ Retinas

Utilizing a variety of cell type-specific markers, we investigated the genesis and differentiation of different cell types in developing *Foxn4*$^{-/-}$ retinas. First, consistent with the significant thinning of the INL, IPL, and GCL, there was a dramatic reduction of amacrine cells immu-

noreactive for syntaxin, γ-aminobutyric acid (GABA), GABA transporter 1 (GAT-1), glycine transporter 1 (GLYT1), calretinin, Pax6, Barhl2, or calbindin in P8 and P20 *Foxn4*$^{-/-}$ retinas (Figures 3A–3N and Supplemental Figure S3I at http://www.neuron.org/cgi/content/full/43/6/795/DC1). For instance, the number of cells immunoreactive for GABA, GAT-1, and GLYT1 in P8 null retinas was reduced by 94.4%, 83.4%, and 74%, respectively (Supplemental Figure S3I). Second, as shown by four horizontal cell markers, including Pax6, Barhl2, calbindin, and Lim1, we found that horizontal cells were completely absent from P8 and P20 *Foxn4*$^{-/-}$ retinas (Figures 3I–3P and Supplemental Figure S3I). The absence of horizontal cells in the null retina was consistent with a lack of the OPL at P20 (Figures 2K and 2L). Third, there was no or only modest difference between wild-type and null retinas in the number of cone cells immunoreactive for the α isoform of protein kinase C (PKCα) (Figures 3S and 3T), bipolar cells immunoreactive for PKCα or Chx10 (Figures 3S and 3T and Supplemental Figure S2I), ganglion cells immunoreactive for Brn3a (Figures 3U and 3V and Supplemental Figure S3I), or Müller cells immunoreactive for glutamine synthase (GS) (Figures 3W and 3X and Supplemental Figure S3I). Fourth, consistent with an increase in the thickness of the ONL (Figures 2K and 2L), recoverin immunoreactivity, a marker for both rod and cone photoreceptors, was sig-
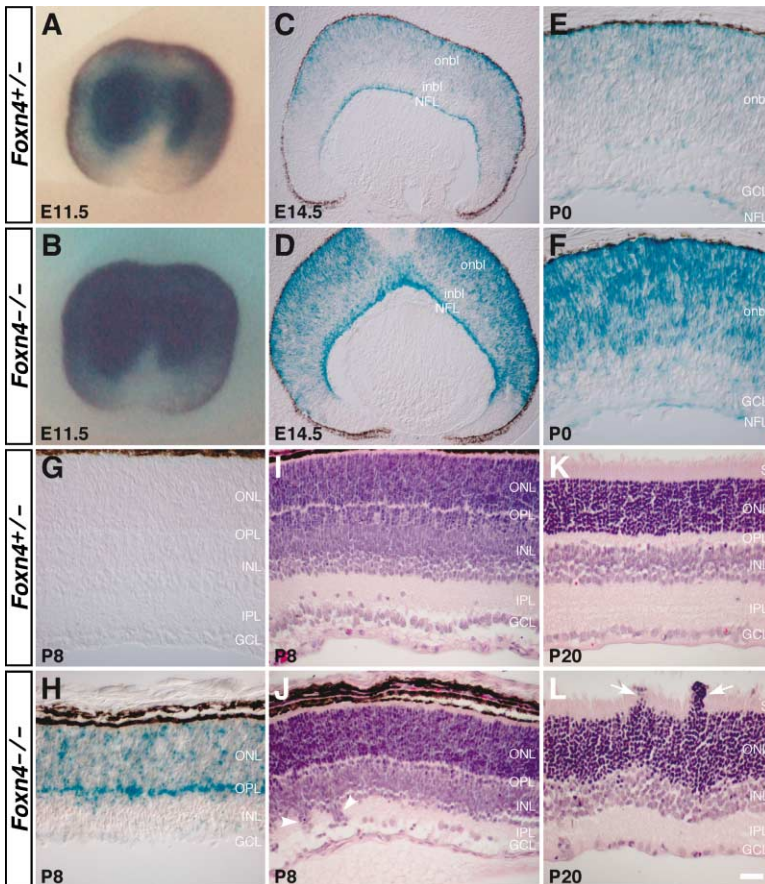
Figure 2. Gross Abnormalities of the *Foxn4*$^{-/-}$ Retina

(A–H) β-galactosidase activity was visualized in whole-mount eyes (A and B) and retinal sections (C–H) at the indicated developmental stages. *lacZ* expression is initiated in the retina at E11.5 in both *Foxn4*$^{+/-}$ and *Foxn4*$^{-/-}$ retinas. By P0, it is downregulated in *Foxn4*$^{+/-}$ retinas but remains high in *Foxn4*$^{-/-}$ retinas. At P8, *lacZ* expression is still found in the ONL of *Foxn4*$^{-/-}$ retinas but is completely absent from *Foxn4*$^{+/-}$ retinas. (I–L) Laminar structures were visualized in P8 and P20 retinal sections by hematoxylin-eosin staining. Compared to the control, there is a significant decrease in the thickness of the INL, IPL, and GCL of *Foxn4*$^{-/-}$ retinas. The OPL is also missing from the mutant retina by P20. In addition, in *Foxn4*$^{-/-}$ retinas, there are anomalous clusters of cells that protruded from the INL into the IPL (indicated by arrowheads) or from the ONL into the photoreceptor segments (indicated by arrows). Scale bar equals 50 μm in (C) and (D) and 25 μm in (E)–(L).

nificantly increased in the *Foxn4*$^{-/-}$ retina (Figures 3Q and 3R). Thus, Foxn4 appears to be dispensable for the genesis of photoreceptor, bipolar, ganglion, and Müller cells. Notably, most Brn3a$^+$ ganglion cells were located within the inner margin of the INL in *Foxn4*$^{-/-}$ retinas (compare Figures 3U and 3V). This dislocation of ganglion cells together with the marked reduction of amacrine cells may explain the near absence of a GCL in the null retina (Figures 2J and 2L).

To test the possibility that normal numbers of amacrine and horizontal cells might be initially generated in the null retina but quickly degenerate later in development, we used markers to detect these two cell types in E17.5 and P0 retinas. No immunoreactivity for syntaxin, Lim1, calbindin, or Pax6 was detected in the outer neuroblastic layer of *Foxn4*$^{-/-}$ retinas at E17.5 and P0 (Supplemental Figures S3A–S3H at http://www.neuron.org/cgi/content/full/43/6/795/DC1), indicating that horizontal cells are not generated during early retinogenesis in null mice. Similarly, the number of cells immunoreactive for syntaxin and calbindin was greatly reduced in the INL and GCL of *Foxn4*$^{-/-}$ retinas (Supplemental Figures S3A, S3B, S3E, and S3F). At P0, Pax6-immunoreactive cells in the INL were missing from *Foxn4*$^{-/-}$ retinas but were normally present in the GCL (Supplemental Figures S3G and S3H), suggesting that the great majority of amacrine cells fail to be born in *Foxn4*$^{-/-}$ retinas, but ganglion cells are normally produced. To further confirm this notion, we labeled newborn cells by BrdU at E14.5

followed by analyses of their cell types at P4. In wild-type retinas, the major cell types born at E14.5 (heavily labeled) were photoreceptors in the ONL, amacrine cells in the INL, and ganglion and displaced amacrine cells in the GCL (Figure 5C). In null retinas, however, amacrine cells were essentially absent, whereas photoreceptor and ganglion cells appeared to be properly produced (Figure 5D), thereby demonstrating that most amacrine cells fail to be born in the mutant.

Downregulation of Math3, NeuroD1, and Prox1 Expression in *Foxn4*$^{-/-}$ Retinas

To determine the molecular basis of the defect in amacrine and horizontal cells in *Foxn4*$^{-/-}$ retinas, we investigated whether loss of *Foxn4* function would affect the expression of retinogenic genes required for the generation of these two cell types. The bHLH factors Math3 and NeuroD1 are redundantly required for the determination of amacrine cells (Inoue et al., 2002). As revealed by RNA in situ hybridization, the expression of both genes was significantly downregulated in progenitors of E14.5 *Foxn4*$^{-/-}$ retinas (Figures 4C–4F). Similarly, the Prox1 homeodomain factor has been shown to play a pivotal role in the commitment of a horizontal cell fate (Dyer et al., 2003). Compared to wild-type controls, the strong *Prox1* expression in a subset of progenitor cells was absent from *Foxn4*$^{-/-}$ retinas, even though the weak retinal expression and the strong lens expression were not altered (Figures 4G and 4H). Other bHLH and homeo-
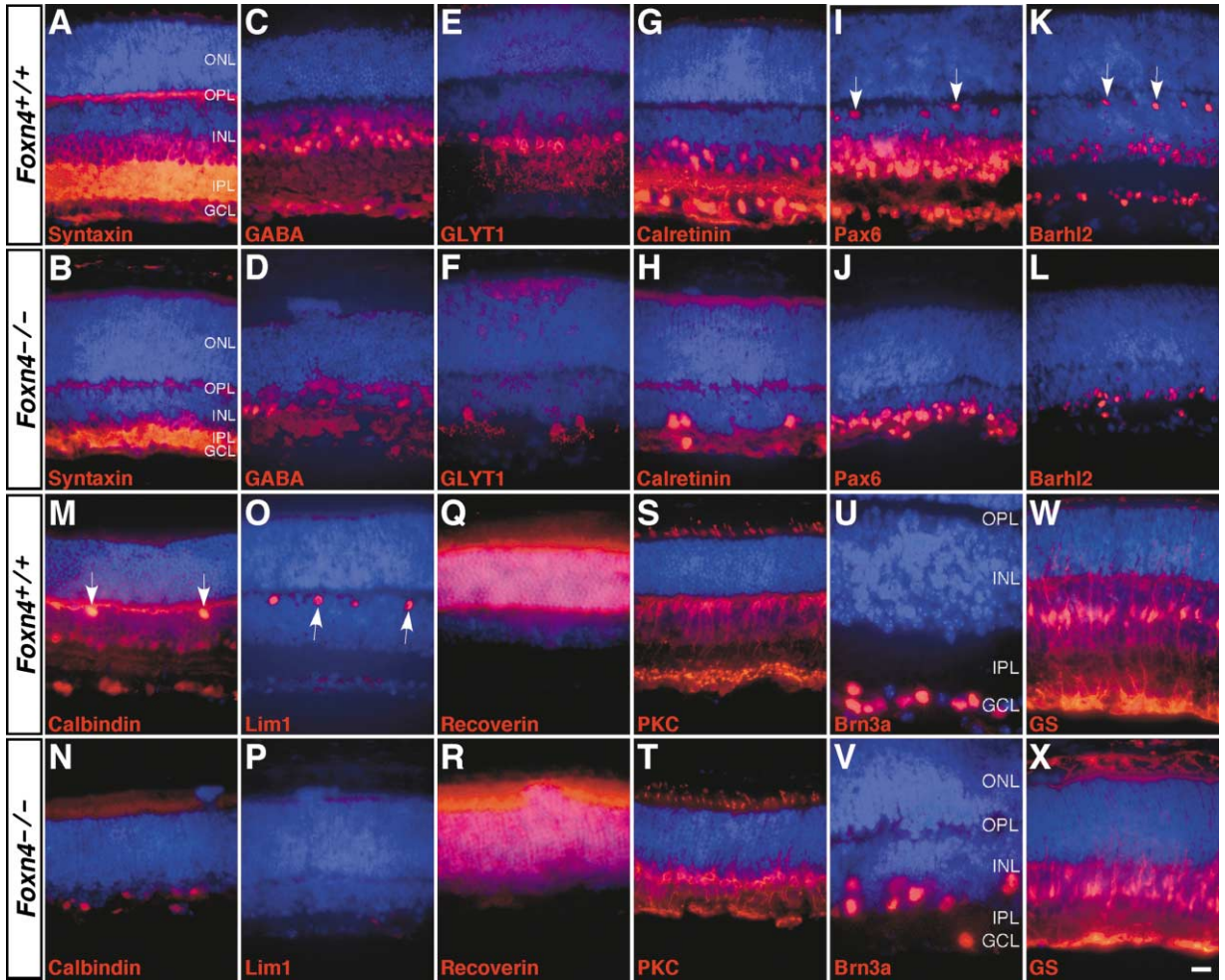
Figure 3. Effect of Targeted *Foxn4* Deletion on the Formation of Different Retinal Cell Types

Sections from P8 (A–L, O, P, and U–X) or P20 (M, N, and Q–T) *Foxn4*$^{+/+}$ and *Foxn4*$^{-/-}$ retinas were immunostained with antibodies against syntaxin (A and B), GABA (C and D), glycine transporter 1 (GLYT1) (E and F), calretinin (G and H), Pax6 (I and J), Barhl2 (K and L), calbindin D-28k (M and N), Lim1 (O and P), recoverin (Q and R), protein kinase C (PKCα) (S and T), Brn3a (U and V), or glutamine synthase (GS) (W and X). All sections were weakly counterstained with DAPI. Loss of *Foxn4* function results in a dramatic decrease in the number of amacrine cells immunoreactive for syntaxin (A and B), GABA (C and D), GLYT1 (E and F), calretinin (G and H), Pax6 (I and J), Barhl2 (K and L), or calbindin (M and N) in *Foxn4*$^{-/-}$ retinas. In addition, it causes a complete absence of horizontal cells immunoreactive for Pax6, Barhl2, calbindin, or Lim1 (I–P). However, there is no or only mild difference between *Foxn4*$^{+/+}$ and *Foxn4*$^{-/-}$ retinas in the number of cone cells or rod bipolar cells immunoreactive for PKCα (S and T), ganglion cells immunoreactive for Brn3a (U and V), or Müller cells immunoreactive for GS (W and X), although recoverin immunoreactivity was significantly increased (Q and R). Arrows point to representative horizontal cells. Scale bar equals 16.7 μm in (A)–(T), (W), and (X) and 10 μm in (U) and (V).

domain retinogenic factors, including Math5, Ngn2, Chx10, and Pax6, however, did not exhibit any alteration in their expression in progenitors of the mutant retina (Figures 4K–4P and data not shown). Thus, Foxn4 appears to control the genesis of amacrine and horizontal cells through the activation of retinogenic genes that are required for their fate specification. Interestingly, consistent with the increase of recoverin immunoreactivity (Figure 3Q and 3R), the expression of *Crx*, a homeobox gene that is required for photoreceptor differentiation (Chen et al., 1997; Furukawa et al., 1997, 1999), was significantly upregulated in *Foxn4*$^{-/-}$ retinas (Figures 4I and 4J), suggesting that the progenitors that would normally differentiate into amacrine and horizontal cells adopted a photoreceptor fate in the mutant.

Diminished Progenitor Proliferation and Elevated Cell Death in *Foxn4*$^{-/-}$ Retinas

Given the expression of *Foxn4* in actively proliferating retinal progenitor cells, we examined whether loss of *Foxn4* function would influence their proliferation. At E15.5 and E17.5, BrdU-labeled cells were reduced by approximately 25% in the *Foxn4*$^{-/-}$ retina compared to the wild-type (Figures 5A, 5B, and 5I), indicating that *Foxn4* is required for proper proliferation of a subset of retinal progenitors.

The gradual dysplasia of postnatal mutant retinas prompted us to investigate whether they gradually degenerate by apoptotic cell death as a secondary defect. As determined by TUNEL labeling, while no significant difference was observed in the number of labeled cells
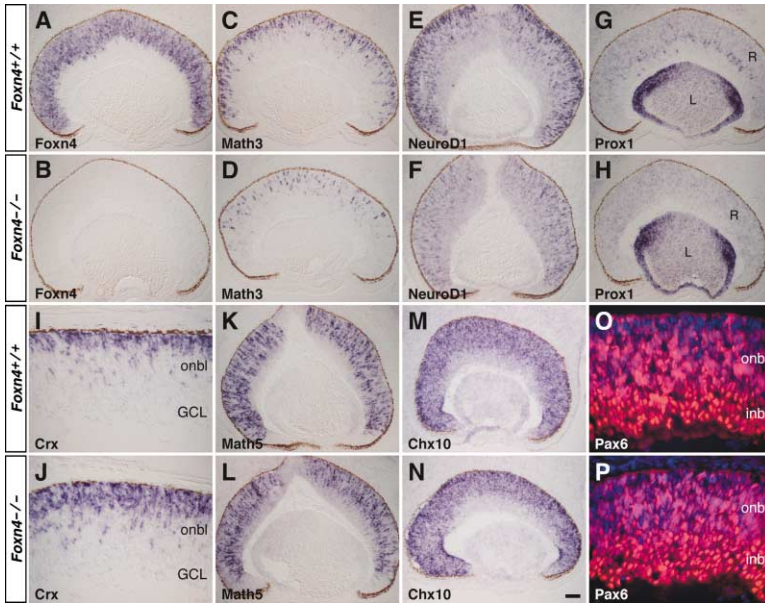
Figure 4. Reduction of *Math3*, *NeuroD1*, and *Prox1* Expression in *Foxn4$^{-/-}$* Retinas

(A–N) RNA in situ hybridization analysis shows an absence of *Foxn4* expression (A and B) as well as a significant reduction of *Math3*, *NeuroD1*, and *Prox1* expression (C–H) in E14.5 *Foxn4$^{-/-}$* retinas. In contrast, there is a significant increase of *Crx* expression in P0 *Foxn4$^{-/-}$* retinas (I and J). Similar levels of *Math5* and *Chx10* expression are seen between E14.5 *Foxn4$^{+/+}$* and *Foxn4$^{-/-}$* retinas (K–N). (O and P) Similar levels of Pax6 immunoreactivity (red) are present between E14.5 *Foxn4$^{+/+}$* and *Foxn4$^{-/-}$* retinas. Scale bar equals 50 μm in (A)–(H) and (K)–(N), 25 μm in (I) and (J), and 16.7 μm in (O) and (P).

between control and mutant retinas at embryonic stages, the null retina displayed a significant increase of cell death at postnatal stages (Figures 5E–5H and 5J). In *Foxn4$^{-/-}$* retinas, cells undergoing apoptosis were seen in all retinal layers, and many were in ectopias of the ONL (Figures 5F and 5H), suggesting that these ectopic cells may be improperly differentiated and eventually degenerate by apoptosis.

Foxn4 Acts as a Potent Factor to Promote an Amacrine Cell Fate

Given its necessity, we tested whether Foxn4 was also sufficient to promote the fates of amacrine and horizontal cells from retinal progenitors by a gain-of-function approach. Overexpression of Foxn4 in retinal progenitors was achieved by a replication-incompetent murine retroviral vector derived from LZRSpBMN-Z that carries a GFP reporter gene (Kim et al., 2002; Kinsella and Nolan, 1996). Similar results were obtained in analyses conducted with retinas infected in vivo at P0 or with retinal explants infected in vitro at P0 or E17.5. While most GFP$^+$ cells became photoreceptors located within the ONL in control retinas, the large majority of GFP$^+$ cells moved into the INL and had larger cell bodies in retinas infected with Foxn4-GFP viruses (Figures 6B and 6C). To more accurately assess cell distribution, we quantified the number of GFP$^+$ cells located in different retinal layers. In retinas infected with Foxn4-GFP viruses, we found that the percentage of GFP$^+$ cells located within the INL increased from 20% in the control to about 81%, and those within the GCL increased from 0.2% to 4% (Figure 6D). However, the percentage of GFP$^+$ cells distributed within the ONL dropped sharply from 80% in the control to only 16% in retinas infected with Foxn4-GFP viruses (Figure 6D). Thus, forced Foxn4 expression dramatically increases the formation of cells located in the inner retinal layers at the expense of photoreceptor cells.

Several cell types, including bipolar, horizontal, amacrine, Müller, and ganglion cells, reside in the INL and

GCL of the retina. To determine which cell types were generated by retinal progenitors infected with Foxn4-GFP viruses, we first investigated whether more amacrine cells were formed in these retinas. The number of amacrine cells was determined by dissociating retinal cells followed by immunocytochemistry for syntaxin. Compared to the control retina, dissociated GFP$^+$ cells from retinas infected with Foxn4-GFP viruses were often larger (Figures 6E–6H), consistent with the observation in retinal sections (Figures 6B and 6C). In addition, the large majority of these cells were immunoreactive for syntaxin but not for rhodopsin (Figures 6F and 6H), while the opposite was true for the control retina (Figures 6E and 6G). Quantitatively, 73% of all GFP$^+$ cells in retinas infected with Foxn4-GFP viruses were immunoreactive for syntaxin, an 11-fold increase from less than 7% in the control (Figure 6I). By contrast, the percentage of rod cells immunoreactive for rhodopsin dropped from 70% in the control to only 3% in retinas infected with Foxn4-GFP viruses (Figure 6I). Therefore, forced Foxn4 expression in retinal progenitors greatly facilitates an amacrine cell fate while dramatically inhibiting the rod fate.

To examine the effect of forced Foxn4 expression on different retinal cell types, we utilized various cell type-specific markers to analyze virus-infected retinas. First, overexpressed Foxn4 increased several-fold the number of GFP$^+$ amacrine cells that were immunoreactive for calbindin, GAT-1, GLYT1, or Pax6 (Figures 7A–7H and 8). Notably, unlike Barhl2, which only increases glycinergic amacrine cells (Mo et al., 2004), Foxn4 promoted the formation of both GABAergic (GAT-1$^+$) and glycinergic (GLYT1$^+$) neurons. Secondly, consistent with a potent suppression of rod cells (Figure 6), overexpressed Foxn4 decreased the number of GFP$^+$ cells immunoreactive for recoverin by more than 10-fold (Figures 7I, 7J, and 8). Overexpression of Foxn4 caused a similar marked reduction in the number of GFP$^+$ cells that became PKCα-immunoreactive bipolar cells or GS-immunoreactive Müller cells (Figures 7K–7N and 8). Thirdly, overexpressed Foxn4 exerted no effect on the
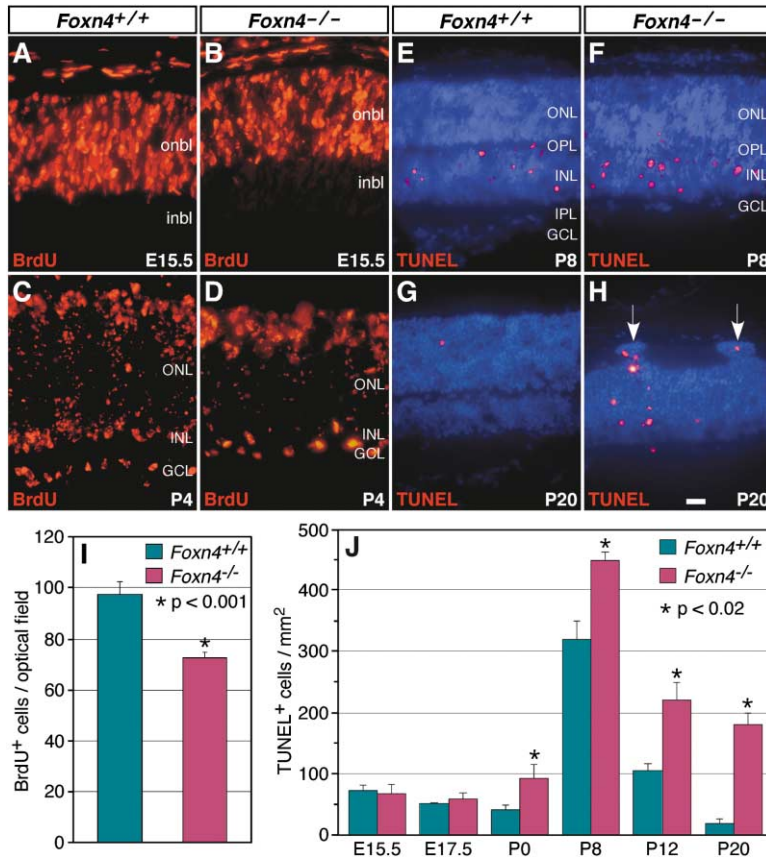
Figure 5. Decreased Progenitor Proliferation and Amacrine Cell Genesis as Well as Elevated Cell Death in the *Foxn4*^{−/−} Retina

(A and B) Dividing retinal progenitor cells were labeled by BrdU at E15.5 and visualized by immunostaining with an anti-BrdU antibody. There are fewer labeled cells in the null retina. (C and D) Cells born at E14.5 were labeled by BrdU and then visualized by BrdU immunohistochemistry at P4. Labeled amacrine cells present in the INL of the control retina are essentially missing from the *Foxn4*^{−/−} retina, whereas normal numbers of labeled photoreceptor and ganglion cells are present in the outer and inner edges of the *Foxn4*^{−/−} retina, respectively. (E–H) Cells undergoing apoptosis (red) were TUNEL labeled in wild-type and null retinas at P8 and P20. A significant increase of apoptotic cells is seen in the *Foxn4*^{−/−} retina at both stages. (I) Quantitation of dividing cells in E15.5 *Foxn4*^{+/+} and *Foxn4*^{−/−} retinas. Each histogram represents the mean $\pm$ SD for four retinas. (J) Quantitation of apoptotic cell death in *Foxn4*^{+/+} and *Foxn4*^{−/−} retinas during development. Each histogram represents the mean $\pm$ SD for three or four retinas. Scale bar equals 16.7 μm in (A)–(H).

number of GFP^{+} horizontal cells immunoreactive for calbindin or GFP^{+} ganglion cells immunoreactive for Brn3a (Figures 7A, 7B, 7O, 7P, and 8). Finally, we observed some unidentified cells located at the edge between the ONL and OPL in retinas infected with Foxn4-GFP viruses (Figures 6C, 7P, and 7R). They usually extended a thick process spanning the entire thickness of the ONL and expressed syntaxin but did not express any mature amacrine or other markers, suggesting that they might have been attempting to differentiate into amacrine cells but were dislocated. Thus, Foxn4 can strongly bias P0 retinal progenitors toward an amacrine cell fate while greatly suppressing the fates of photoreceptor, bipolar, and Müller cells.

Since forced Foxn4 expression could lead to a drastic change in the composition of retinal cell types, we investigated whether the laminar structure of the retina could be affected by this alteration. P0 retinal explants were repeatedly infected with control-GFP or Foxn4-GFP viruses to achieve superinfection of most retinoblasts. As a result, numerous cells within the ONL were found to be positive for GFP in the control retina (Figure 7Q), consistent with the fact that the great majority of cells generated postnatally are rod cells, but there was no alteration in the laminar organization (Figures 7S and 7U). In retinas infected with Foxn4-GFP viruses, however, numerous GFP^{+} cells were distributed in the entire thickness of the retina, concentrating in particular within the inner three-quarters of the retina (Figure 7R). DAPI and HE staining revealed that the layered structure was totally disrupted in retinas infected with Foxn4-GFP vi-

ruses (Figures 7T and 7V). The photoreceptor segments, OPL, INL, IPL, and GCL were no longer recognizable, although a thinned ONL appeared to remain (Figures 7T and 7V).

Discussion

Foxn4 Controls the Genesis of Amacrine Cells by Regulating *Math3* and *NeuroD1* Expression in Retinal Progenitors

Several lines of evidence that are described in this report suggest that Foxn4 is involved in competence acquisition and fate commitment of amacrine cells. First, during mouse retinogenesis, amacrine cells are born over a time course spanning from E11 to P4, with most of them generated between E12 and P0 (Young, 1985). We have shown that Foxn4 is expressed only by a subset of dividing retinal progenitors and that its temporal pattern of expression closely correlates with the birthdates of amacrine cells. Second, our gene targeting experiments have demonstrated that loss of *Foxn4* function results in a marked decrease in the genesis of amacrine cells. Finally, overexpression of Foxn4 potently promotes amacrine cell formation, indicating that Foxn4 is not only necessary but also sufficient for the genesis of amacrine cells by retinal progenitors.

The presence of abundant *lacZ*^{+} cells in early *Foxn4*^{−/−} retinas implies that the progenitors that would normally express Foxn4 are produced in the null retina; however, they appear not to be competent for the genesis of amacrine neurons, since these are greatly de-
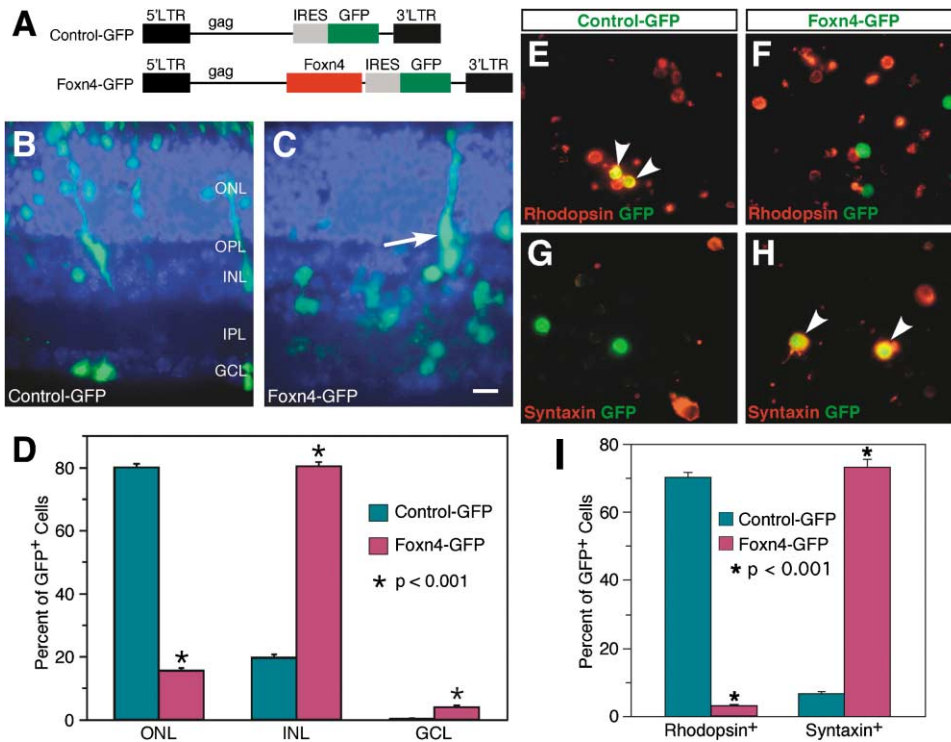
Figure 6. Overexpressed Foxn4 Potently Promotes the Formation of Amacrine Cells

(A) Schematic of control-GFP and Foxn4-GFP retroviral constructs. In Foxn4-GFP, a bicistronic transcript containing an internal ribosomal entry site (IRES) is produced from the viral LTR promoter, which allows efficient expression of both Foxn4 and GFP. (B and C) Virus-transduced GFP$^+$ cells were visualized in retinal sections that were weakly counterstained with DAPI. Forced Foxn4 expression caused a dramatic increase of cells situated within inner retinal layers. The arrow in (C) points to a cell extending a thick process into the ONL. (D) Percentages of virus-transduced cells located in different cellular layers of the retina (means $\pm$ SD). More than 500 GFP$^+$ cells in four retinas were scored for either control-GFP or Foxn4-GFP virus. (E–H) Dissociated retinal cells infected with control-GFP (E and G) or Fonx4-GFP (F and H) viruses were immunostained with antibodies against rhodopsin (E and F) or syntaxin (G and H). Forced Foxn4 expression led to a marked increase of syntaxin$^+$ amacrine cells but a dramatic decrease of rhodopsin$^+$ rod cells. (I) Quantitation of virus-transduced retinal cells that became immunoreactive for rhodopsin or syntaxin (means $\pm$ SD). Scale bar equals 10 μm in (B) and (C).

creased in the mutant. Given the overlap between Foxn4$^+$ and Pax6$^+$ progenitor cells, it appears that the subset of progenitors competent for amacrine cell genesis are positive for both Foxn4 and Pax6 expression (Pax6$^+$Foxn4$^+$) (Figure 9). The subsequent activation of Math3 and NeuroD1, two bHLH factors that are redundantly required for the specification of amacrine cells (Inoue et al., 2002; Morrow et al., 1999), may then function to select an amacrine cell fate from the competent Pax6$^+$Foxn4$^+$ progenitors (Figure 9). Our analyses have also revealed that Foxn4 positively regulates the expression of Math3 and NeuroD1 but is not required for the expression of Pax6 or Pax6-regulated bHLH factors. For instance, the absence of *Foxn4* causes no changes in the expression levels of *Math5*, which is involved in acquisition of ganglion cell competence (Brown et al., 2001; Liu et al., 2001; Wang et al., 2001; Yang et al., 2003).

The presence of residual amacrine cells in *Foxn4*$^{-/-}$ retinas suggests that a minor number of amacrine neurons can be derived from Pax6$^+$Foxn4$^-$ progenitors (Figure 9), thereby indicating a slight functional redundancy between Foxn4 and another retinogenic factor in the control of amacrine cell genesis. Since forced Pax6 expression can weakly promote an amacrine cell fate in combination with Math3 or NeuroD1, although Pax6 alone

lacks such an activity (Inoue et al., 2002), one likely candidate for this putative redundant factor is Pax6 itself. Consistent with the notion that Foxn4 plays a dominant role in fate commitment of amacrine cells, overexpressed Foxn4 alone, unlike Pax6, has a potent ability to bias retinal progenitors toward an amacrine cell fate.

Foxn4 Controls the Genesis of Horizontal Cells by Regulating *Prox1* Expression in Retinal Progenitors

We have shown that Foxn4 is required for the genesis of horizontal cells; this requirement is similar to that of Pax6 (Marquardt et al., 2001). In early *Foxn4*$^{-/-}$ retinas, we have observed a complete failure in the generation of horizontal cells as measured by cell-type specific markers, including syntaxin, calbindin, Lim1, Pax6, and Barhl2. Thus, both Pax6 and Foxn4 are nonredundantly required by progenitors to acquire the state of competence for the genesis of horizontal cells. By inference, only double-positive progenitor cells (Pax6$^+$Foxn4$^+$) may be able to generate horizontal cells, whereas single-positive progenitor cells (Pax6$^+$Foxn4$^-$ and Pax6$^-$Foxn4$^+$) may be incompetent (Figure 9). Further lineage-tracing studies are needed to test the validity of this speculation.
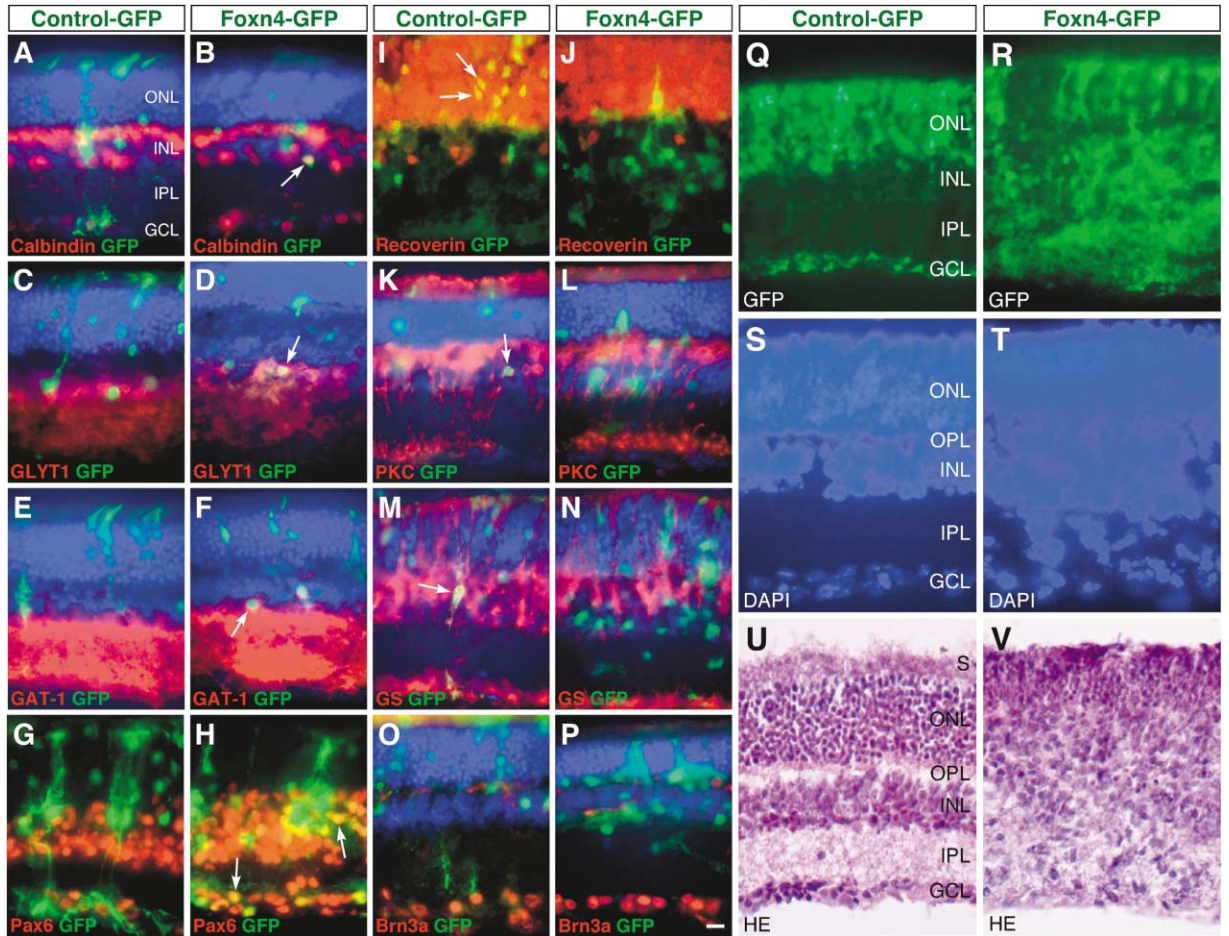
Prox1 has been shown to potently promote a hori-

Figure 7. Effect of Overexpressed Foxn4 on the Formation of Different Retinal Cell Types

(A–P) Sections from retinas infected with control-GFP or Foxn4-GFP viruses were double immunostained with an anti-GFP antibody and antibodies against calbindin, GLYT1, GAT-1, Pax6, recoverin, PKCα, glutamine synthase (GS), or Brn3a. Sections in (A)–(F) and (K)–(P) were weakly counterstained with DAPI. Forced Foxn4 expression resulted in a great increase in the number of amacrine cells immunoreactive for calbindin, GLYT1, GAT-1, or Pax6 (A–H), whereas it dramatically suppressed the formation of photoreceptor cells immunoreactive for recoverin, rod bipolar cells immunoreactive for PKCα, and Müller cells immunoreactive for GS (I–N). It had no effect on ganglion cells immunoreactive for Brn3a (O and P). Arrows point to representative colocalized cells. (Q–V) Sections of retinal explants superinfected with control-GFP or Foxn4-GFP viruses were visualized by GFP fluorescence (Q and R), DAPI staining (S and T), or hematoxylin-eosin staining (U and V). Superinfection with Foxn4-GFP viruses resulted in a complete disorganization of the layered retinal structure (S–V). Scale bar equals 12.4 μm in (Q) and (R), 10.8 μm in (U) and (V), 10 μm in (A)–(P), and 7.4 μm in (S) and (T).

zontal cell fate when overexpressed in P0 retinal progenitors (Dyer et al., 2003). When Foxn4 or Pax6 were similarly overexpressed in P0 or E17.5 retinal progenitors, however, no effect on horizontal cells could be observed (Inoue et al., 2002), indicating that neither Foxn4 nor Pax6 is sufficient to promote a horizontal cell fate from retinal progenitors, although loss-of-function experiments have demonstrated that they are both necessary (Marquardt et al., 2001). Thus, unlike its role in the genesis of amacrine cells, Foxn4 appears to be required only for competence acquirement during the genesis of horizontal cells but is insufficient for their fate determination. Since Prox1 is believed to be the major intrinsic factor that is both necessary and sufficient for the promotion of horizontal cells from competent progenitors (Dyer et al., 2003), we tested whether Foxn4 controls competence acquirement by regulating Prox1 expression. As expected, our analyses have indeed revealed

that Foxn4 positively regulates the expression of Prox1 but not the expression of other tested retinogenic homeodomain factors, including Pax6 and Chx10. Chx10 plays a key role in the specification of bipolar cells (Burmeister et al., 1996), and our data show that loss of *Foxn4* function does not lead to any alteration in *Chx10* expression within retinal progenitors (Figure 4).

Loss of *Foxn4* Function Causes A Switch in Cell Fate

In early *Foxn4*$^{-/-}$ retinas, β-galactosidase staining revealed that the progenitors that would normally express Foxn4 were initially produced. The delay in the downregulation of *lacZ* expression as well as the increase in the thickness of the ONL in the mutant retina, however, suggests that these progenitors would most likely change their fates and largely differentiate as rod photoreceptor cells in the absence of *Foxn4*. Indeed, com-
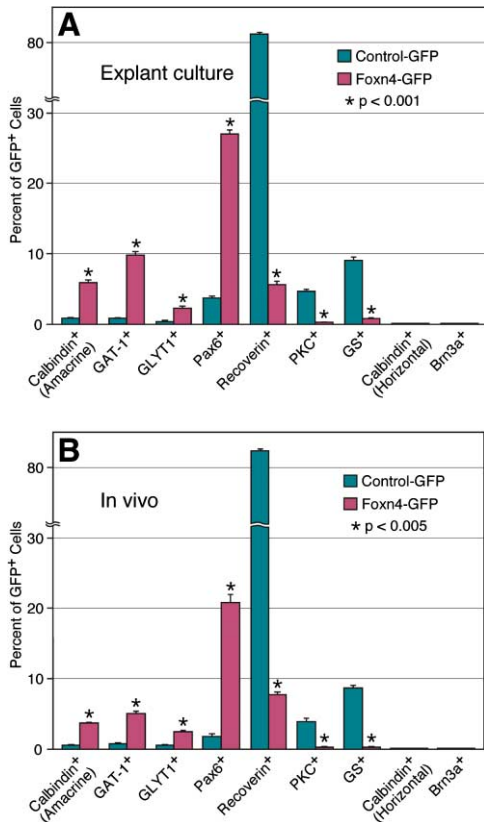
Figure 8. Quantitation of Virus-Transduced Retinal Cells that Became Immunoreactive for a Series of Cell Type-Specific Markers

P0 retinal explants infected in vitro (A) or in vivo (B). Each histogram represents the mean ± SD for three or four retinas. More than 500 GFP+ cells were scored in each retina. GAT-1, GABA transporter 1; GLYT1, glycine transporter 1; GS, glutamine synthase.

pared to the control retina, we found that *Crx* expression was significantly upregulated and that more cells in the ONL expressed recoverin and rhodopsin in the mutant (Figures 3 and 4 and data not shown), thereby indicating that the subset of Pax6+Foxn4+ progenitors, whose fates may be biased toward amacrine and horizontal cells (Figure 9), would assume a rod fate without Foxn4.

Notably, as indicated by the formation of aberrant ONL ectopias, some progenitor cells in the mutant retina may fail to differentiate properly into rods despite an alteration in their fates (Figure 2).

The observed increase of photoreceptors in *Foxn4*−/− mice differs from the fact that in *Math3*; *NeuroD1* double mutants, the loss of amacrine cells is accompanied by an increase of many ganglion cells and some Müller cells and a significant decrease of photoreceptors (Inoue et al., 2002). Although it remains to be determined what exactly causes this difference, it can be explained by the differential expression patterns of *Foxn4*, *Math3*, and *NeuroD1*. For instance, the decrease of photoreceptors in the double mutant is caused by the deficiency of *NeuroD1*, which is required for terminal differentiation and survival of photoreceptors (Morrow et al., 1999; Pennesi et al., 2003). In *Foxn4*−/− retinas, *NeuroD1* expression may be downregulated only in dividing progenitors due to the restriction of *Foxn4* expression in mitotically active cells. Therefore, *NeuroD1* may be normally expressed in differentiating and mature photoreceptors and be able to maintain the overproduced photoreceptors that result from the upregulation of *Crx* expression in *Foxn4*−/− retinas. Similarly, the absence of *Foxn4* would be unlikely to cause a downregulation of *Math3*/*NeuroD1* expression in postmitotic progenitors, where *Math5* expression is also found and thought to be repressed by Math3/NeuroD1 (Inoue et al., 2002; Yang et al., 2003). Thus, unlike the upregulation of *Math5* expression that would lead to more ganglion cells in *Math3*; *NeuroD1* double mutants (Inoue et al., 2002), there is no change of *Math5* expression in *Foxn4*−/− retinas (Figure 4).

During retinogenesis, it is believed that retinogenic factors that are involved in cell fate determination promote the exit of progenitors from the cell cycle and then direct them to commit to the fates available to progenitors at the given temporal stage. The cell fate switch phenotypes seen in *Foxn4* and several other mutants roughly conform to this hypothesis. For instance, *Prox1*−/− retinas lack horizontal cells but contain more rods and Müller cells (Dyer et al., 2003); *Math5*−/− retinas lack ganglion cells but contain more cones and starburst amacrine cells (Brown et al., 2001; Wang et al., 2001); and similarly a fish *ath5* mutant lacks ganglion cells but
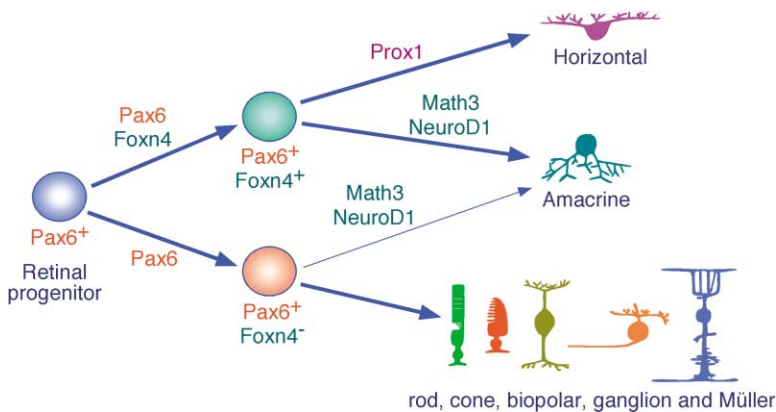

Figure 9. Schematic Illustration of the Proposed Mechanism by which Foxn4 Controls the Genesis of Amacrine and Horizontal Cells by Retinal Progenitors

Retinal progenitors with the activation of both Foxn4 and Pax6 (Pax6+Foxn4+) are competent for the genesis of all horizontal cells and the great majority of amacrine cells. Only a small number of amacrine cells are generated from the Pax6+Foxn4− progenitors. Foxn4 confers progenitors with the competence for the genesis of amacrine and horizontal cells by activating the expression of Math3, NeuroD1, and Prox1, three retinogenic factors involved in the specification of these two cell types. The rod, cone, bipolar, ganglion, and Müller cells are likely to be largely derived from the Pax6+Foxn4− progenitors via activation of other retinogenic factors, which are not shown here for simplicity.

contain more amacrine, bipolar, and Müller cells (Kay et al., 2001). However, more than one mechanism appears to be used by progenitors to control cell fate commitment. First, although Prox1 and Ath5 are found to drive cell cycle exit (Dyer et al., 2003; Kay et al., 2001), our work suggests that Foxn4 may have a minor role in promoting cell proliferation or may be required for both cell cycle exit and progression/reentry. Similarly, Pax6 is required for proper proliferation of progenitors (Marquardt et al., 2001). Second, as discussed above, Foxn4 and other retinogenic factors may both activate and repress a battery of target genes that indirectly influence multiple fate decisions. This may explain why in *Foxn4*$^{-/-}$ retinas there are not greater numbers of ganglion and cone cells, which are alternative fates available at the time of amacrine and horizontal cell genesis, or more rods and other amacrine subtypes in *Math5* mutants.

Foxn4 and Lineage-Biased Retinal Progenitors

Utilizing antibody-coupled fluorescent latex microspheres to label retinal progenitors, Alexiades and Cepko found that VC1.1$^+$/syntaxin$^+$ progenitors represent a subset of progenitor cells whose progeny is significantly biased toward amacrine and horizontal cells (Alexiades and Cepko, 1997). Our work suggests that the Pax6$^+$Foxn4$^+$ progenitors most likely correspond to these VC1.1$^+$ progenitors (Figure 9). First, the VC1.1 epitope or syntaxin has been shown to be expressed by 35%–40% of proliferative progenitors in early embryonic rat retinas. The percentage peaks at 70%–80% around E14–E15, then decreases in late embryonic stages, eventually disappearing by P7 from dividing progenitors (Alexiades and Cepko, 1997). Foxn4 exhibits a similar expression pattern that closely parallels the transient expression pattern of VC1.1/syntaxin in progenitor cells. Second, we have shown that all Foxn4$^+$ progenitors appear to overlap those expressing syntaxin. Finally, our data suggest that the Pax6$^+$Foxn4$^+$ progenitors may give rise to all horizontal cells and the great majority of amacrine cells (Figure 9).

The apparent fate change of Pax6$^+$Foxn4$^+$ progenitors in the absence of *Foxn4* helps us to understand certain properties of retinal progenitor cells. First, from late embryonic to early postnatal stages, VC1.1$^+$ progenitors are found to give rise to increasing numbers of rod cells and a small number of bipolar cells (Alexiades and Cepko, 1997). One possible explanation for this change in fate bias might be due to the downregulation of Foxn4 expression in the VC1.1$^+$ progenitors during late embryonic and early postnatal stages, leading to an increasing preference for a rod cell fate. Second, retinoic acid has been shown to promote the formation of rod photoreceptors by retinal progenitors while concomitantly inhibiting the differentiation of amacrine cells (Kelley et al., 1994). Conceivably, retinoic acid may exhibit this bifunctional activity by acting preferentially on the putative lineage-biased Pax6$^+$Foxn4$^+$ progenitors rather than on the Pax6$^+$Foxn4$^-$ progenitors.

Taken together, our gene targeting and overexpression analyses have demonstrated that Foxn4 is both necessary and sufficient for fate determination of amacrine cells, as well as nonredundantly required for acqui-

sition of the horizontal cell competence. Foxn4 appears to control the genesis of these two cell types by activating the expression of Math3, NeuroD1, and Prox1, three retinogenic factors that are involved in the specification of amacrine or horizontal cells. Thus, our work identifies an essential intrinsic regulator, Foxn4, which together with Pax6 and other factors confers retinal progenitors with the full potential for the genesis of all seven classes of retinal cells. Conceivably, a similar combinatorial mechanism may be utilized by other neural progenitor/stem cells to establish multipotency.

Experimental Procedures

Gene Targeting

λ phage clones covering the entire *Foxn4* coding region were obtained by screening a 129Sv/J mouse genomic library (Stratagene) using a *Foxn4* cDNA as probe. To construct the targeting vector, a 5 kb 3′ arm fragment was assembled by standard cloning techniques with EcoRI/BamHI sites added at the ends and then inserted along with a KpnI/EcoRI adaptor into the KpnI/BamHI sites of pPNTloxP (Partanen et al., 1998; Tybulewicz et al., 1991). Similarly, a 5.5 kb 5′ arm fragment was assembled by standard cloning techniques and then inserted into the NotI/XbaI sites of the modified pSDKlacZpA (Shalaby et al., 1995) containing an internal ribosomal entry site (IRES) sequence. The fusion of 5′ arm-IRES-lacZpA was next excised by NotI and SalI and subsequently cloned into the NotI/XhoI sites of pPNTloxP containing the 3′ arm. The construct was linearized and electroporated into the CMT1 ES cells (Specialty Media, Phillipsburg, NJ), which were cultured and screened following the manufacturer's protocol. Targeted ES cell clones were identified by the presence of both a 5 kb wild-type band and an 8.5 kb recombinant band on BamHI-digested Southern blot using a 3′ diagnostic probe. These clones were injected into blastocysts to derive chimeric mice, which were bred with C57BL/6J mice to produce heterozygotes.

In Situ Hybridization, X-Gal Staining, BrdU Labeling, and TUNEL Assay

RNA in situ hybridization was carried out as described using digoxigenin-labeled antisense riboprobes (Sciavolino et al., 1997). The following probes were used: *Foxn4* was a PCR-amplified partial coding sequence (665–1554 bp of the open reading frame); *Math3, Math5, Ngn2,* and *NeuroD1* were full-length coding sequences amplified by PCR from mouse genomic DNA; *Prox1* was a PCR-amplified sequence of the fifth exon; *Chx10* and *Crx* were previously described cDNAs (Chen et al., 1997; Liu et al., 1994). X-gal staining was also performed as described (Li et al., 2004).

BrdU labeling of dividing retinal progenitor cells was carried out according to a previous description (Xiang, 1998). The number of BrdU$^+$ cells was scored on retinal sections of the intermediate region in a high-power (600×) optical field using a reticule mounted on the microscope. Four fields were counted for each retina, and four independent retinas were scored for each type. For birthdating analysis, newborn retinal cells were pulse labeled by BrdU at E14.5 and visualized by immunostaining with an anti-BrdU antibody at P4.

TUNEL assay was performed using the In Situ Cell Death Detection Kit, TMR Red (Roche Diagnostics) following the manufacturer's protocol except that all sections were counterstained with 0.02 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Three or four independent retinas from six developmental stages (E15.5, E17.5, P0, P8, P12, P20) were collected and analyzed. For each retina, fluorescent-positive cells were counted from three to five sections under the microscope, and images of entire sections were captured with a microscope-mounted digital camera. The area of each section was measured using the NIH Image software, and the density of apoptotic cells was calculated. All data were tested for significance using two-sample Student's t test with unequal variances.

Plasmid Construction and Virus Preparation and Infection

To obtain a *Foxn4* cDNA, mRNA was extracted from E17.5 C57BL/6J mouse retinas using the Oligotex Direct mRNA Micro Kit (Qiagen)

followed by cDNA synthesis using the Marathon cDNA Amplification Kit (Clontech). The full-length *Foxn4* open reading frame was then amplified by PCR using the retinal cDNA as template, and multiple clones were sequenced with the same result. The deduced protein sequence (521 amino acids) is identical to that in the mouse genome database but differs from a published sequence in 22 amino acids (Gouge et al., 2001). To construct the Foxn4-GFP plasmid, the *Foxn4* cDNA fragment was inserted into the BamHI/EcoRI sites of the control-GFP vector (Mo et al., 2004). Virus preparation, in vivo and in vitro infection of mouse retinas, and retinal explant culture were all performed as described previously (Mo et al., 2004). For viral superinfection, P0 retinal explants were infected with fresh viruses every 6 hr for a total of five times at the beginning the culture.

Generation of a Polyclonal Anti-Foxn4 Antibody and Immunohistochemistry

DNA fragment corresponding to amino acids 50–194 of the mouse Foxn4 protein was amplified by PCR and inserted into the pGEMEX (Promega) and pMAL-c$_2$X (New England Biolabs) vectors to express fusion proteins with the bacteriophage T7 gene 10 protein and bacterial maltose binding protein, respectively. Antibody production and affinity purification were performed as described previously (Xiang et al., 1993, 1995).

The preparation of retinal sections, immunofluorescent labeling of retinal sections and dissociated cells, and quantification of immunoreactive cells were carried out as described (Liu et al., 2000a; Mo et al., 2004). The following primary antibodies were used: anti-Foxn4 (rabbit polyclonal; this work); anti-syntaxin (mouse monoclonal, Sigma); anti-PKCα (mouse monoclonal, Amersham); anti-glutamine synthase (mouse monoclonal, Chemicon); anti-Pax6 (mouse monoclonal, Developmental Studies Hybridoma Bank; rabbit polyclonal, Chemicon); anti-Brn3a (mouse monoclonal, Chemicon); anti-calretinin (mouse monoclonal, Chemicon); anti-BrdU (mouse monoclonal, BD Biosciences); anti-GFP (mouse monoclonal, Chemicon; rabbit polyclonal, MBL International Corp.); anti-calbindin-D28K (rabbit polyclonal, Swant); anti-GABA (rabbit polyclonal, Sigma); anti-GAT-1 (rabbit polyclonal, Chemicon); anti-recoverin (rabbit polyclonal; Dizhoor et al., 1991); anti-rhodopsin (mouse monoclonal 1D4; Hodges et al., 1988); anti-Barhl2 (rabbit polyclonal; Mo et al., 2004); anti-Lim1 (rabbit polyclonal; Liu et al., 2000b); anti-GLYT1 (goat polyclonal, Chemicon); and anti-Chx10 (sheep polyclonal, Exalpha). The following secondary antibodies were used: rhodamine-conjugated donkey anti-goat IgG; rhodamine-conjugated donkey anti-sheep IgG; rhodamine-conjugated donkey anti-mouse IgG; rhodamine-conjugated donkey anti-rabbit IgG; Cy2-conjugated donkey anti-mouse IgG; and Cy2-conjugated donkey anti-rabbit IgG (all from Jackson Immunoresearch Laboratories).

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