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A role for the Fibroblast Growth Factor Receptor in cell fate decisions in the developing vertebrate retina

S. McFarlane[1,*], M. E. Zuber[2] and C. E. Holt[2]

[1]Department of Cell Biology and Anatomy, Neuroscience Research Group, HMRB Room 171, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada, T2N 4N1
[2]Department of Anatomy, University of Cambridge, Downing Street, Cambridge, CB2 3DY, UK
*Author for correspondence (e-mail: smcfarla@acs.ucalgary.ca)

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SUMMARY

The mature vertebrate retina contains seven major cell types that develop from an apparently homogenous population of precursor cells. Clonal analyses have suggested that environmental influences play a major role in specifying retinal cell identity. Fibroblast growth factor-2 is present in the developing retina and regulates the survival, proliferation and differentiation of developing retinal cells in culture. Here we have tested whether fibroblast growth factor receptor signaling biases retinal cell fate decisions in vivo. Fibroblast growth factor receptors were inhibited in retinal precursors in *Xenopus* embryos by expressing a dominant negative form of the receptor, XFD. Dorsal animal blastomeres that give rise to the retina were injected with cDNA expression constructs for XFD and a control non-functional mutant receptor, D48, and the cell fates of transgene-expressing cells in the mature retina determined. Fibroblast growth factor receptor blockade results in almost a 50% loss of photoreceptors and amacrine cells, and a concurrent 3.5-fold increase in Müller glia, suggesting a shift towards a Müller cell fate in the absence of a fibroblast growth factor receptor signal. Inhibition of non-fibroblast-growth-factor-mediated receptor signaling with a third mutant receptor, HAVØ, alters cell fate in an opposite manner. These results suggest that it is the balance of fibroblast growth factor and non-fibroblast growth factor ligand signals that influences retinal cell genesis.

Key words: *Xenopus*, Photoreceptor, Retina, Müller glia, Dominant negative, in vivo

INTRODUCTION

The generation of cell diversity in the developing nervous system is a complex process. It is somewhat simpler in the vertebrate retina, where only seven different cell types are generated from a seemingly homogeneous optic cup. The relative simplicity and accessibility of the retina makes it a good model system for identifying the mechanisms and molecules involved in the specification of cell fate during development (Cepko et al., 1996). In several species, clonal analysis has provided strong evidence that the local environment has an instructive role in retinal cell fate decisions (Holt et al., 1988; Turner and Cepko 1987; Turner et al., 1990; Wetts and Fraser, 1988). These analyses demonstrate that multiple retinal cell types can be generated from a single precursor cell, indicating that non-lineage-dependent mechanisms are involved in specifying cell identity.

A retinal cell's fate is determined by the nature of the cues in its environment and by its responsiveness to those signals. Recently, Notch-Delta signaling was implicated as a mechanism to generate retinal cell diversity by regulating when cells become competent to respond to external signals (Dorsky et al., 1995, 1997; Cepko et al., 1996). Notch-Delta signaling may delay the initial responsiveness of retinal precursor cells allowing subsequent populations of cells to be influenced by later specification signals. Much less is known about the identity of the endogenous inductive cues.

Short-range signals, such as growth factors and hormones, appear to be necessary to induce specific retinal cell types (Harris and Messersmith, 1992; Reh, 1992; Altshuler and Cepko, 1992; Lillien, 1995; Fuhrmann et al., 1995; Kelley et al., 1994, 1995). In particular, fibroblast growth factor (FGF) signaling has been implicated in retinal cell fate choice. Members of the FGF family (Gao and Hollyfield, 1995; Consigli et al., 1993; Bugra et al., 1993; de Longh and McAvoy, 1993) and their receptors (Wanaka et al., 1991; Patstone et al., 1993) are expressed in the developing retina of many species. In the South African clawed frog, *Xenopus laevis*, we have shown that FGF-2 and FGFR protein are expressed in the developing eye (McFarlane et al., 1995). Furthermore, others have demonstrated that message for XFGFR-2, XFGFR-4, FGF-2, FGF-3, FGF-9 and a newly cloned non-FGF ligand for the FGFR, FRL-2, are expressed in the eye primordium (Song and Slack, 1994, 1996; Friesel and Brown, 1992; Riou et al., 1996; Shiozaki et al., 1995; Tannahill et al., 1992; Kinoshita et al., 1995).

In vitro, the FGF transduction pathway has been implicated in regulating the proliferation, survival and differentiation of retinal cells (Sievers, 1987; Bahr et al., 1989; Guillemot and Cepko, 1992; Park and Hollenberg, 1989, 1991; Pittack et al., 1991; Mascarelli et al., 1991; Tcheng et al., 1994; Hicks and Courtois, 1992). Evidence for the regulation of retinal cell fate by FGFR ligands in vivo, however, remains limited. Null mutations of FGFR-1 and FGFR-2 in transgenic mice are embryonic lethal (Deng et al., 1994; Yamaguchi et al., 1994), and thus have not provided clues as to a role for FGFR signaling in retinal cell determination. The well-characterized developing *Xenopus* retina is ideal for investigating an in vivo role for FGFRs in cell genesis, since introduction of foreign genes into cells can be spatially and temporally regulated (Holt et al., 1990; Dorsky et al., 1997), and the retina develops rapidly (Holt et al., 1988).

To determine if FGFR signal transduction influences cell fate during retinogenesis, we inhibited FGFR function in *Xenopus* retinal neuroepithelial cells in vivo using a dominant negative form of the receptor. This mutant receptor is a truncated form of the FGFR that lacks the intracellular tyrosine kinase domain and is thought to inhibit endogenous FGFRs via FGF-stimulated formation of non-functional heterodimers with native receptors (Amaya et al., 1991). We recently used the dominant negative to demonstrate a role for FGFR signaling in the formation of the projection between the retina and the optic tectum in live embryos (McFarlane et al., 1996). In this paper, we determined that (1) several members of FGFR family are expressed in the developing *Xenopus* retina at the time cell fate choices are being made, and (2) inhibiting FGFR signal transduction in retinal precursors alters cell fates, as observed by a 57% decrease in the number of photoreceptors and a 3.5-fold increase in Müller glia. These results provide strong evidence that an FGFR ligand(s) acts as an inductive cue to influence retinal cell specification in the developing vertebrate eye.

MATERIALS AND METHODS

Animals

Embryos were attained by in vitro fertilization of eggs obtained from adult female *Xenopus laevis* injected with human chorionic gonadotropin (Sigma). For blastomere injections, embryos were dejellied in 2% cysteine (pH 8.0) and used at the 16-cell stage. Embryos used for in situ hybridization were kept in 10% Holtfreter's solution (Holtfreter, 1943) with the temperature varied between 14°C and 25°C to control their speed of development. Embryos were staged according to Nieuwkoop and Faber (1994).

Constructs

FGFR constructs used here were described previously (Amaya et al., 1991, 1993; McFarlane et al., 1996) and are shown in Fig. 2. Constructs include dominant negative (XFD), non-functional D48 and HAVNOT (HAVØ) versions of the receptor. In some cases, XFD and D48 receptors containing a single C-terminal myc tag were used. All constructs were in a modified CS2-DNA vector. A CS2 cDNA construct encoding green fluorescent protein (GFP) courtesy of D. Turner was also used as a control. Plasmids were purified from *Escherichia coli* using the Qiagen Maxi-prep kit.

Blastomere injections

Dejellied 16-cell-stage embryos were transferred into 6% Ficoll (Sigma) in 100% modified Ringers (MMR) and were injected with one of the FGFR cDNA plasmids using a borosilicate glass needle, pulled on an electrode puller (Sutter Instrument Company). Injections were made using a Picospritzer II (General Valve Corporation) into dorsal animal blastomeres (D1.1) that, according to a blastomere fate map, give rise to 50% of the ipsilateral retina (Huang and Moody, 1993). To minimize blebbing after injection, embryos were transferred to 6% Ficoll and 5% MMR and left at 14°C overnight. Subsequent development occurred at room temperature in 10% Holtfreters until stage 40. Embryos were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C.

Immunocytochemistry

Fixed embryos were washed in 0.1 M phosphate buffer, transferred to 30% sucrose and embedded in OCT (optimal cutting temperature, Baxter) and 12 μm frozen sections cut with a cryostat (Leica). Immunostaining was carried out as described previously (McFarlane et al., 1996). Briefly, sections were incubated in primary antibody overnight, rinsed and then incubated with either a rhodamine- or fluorescein-conjugated secondary antibody for 1 hour. Sections were mounted in an anti-bleaching medium (p-phenylenediamine (Sigma), 85% glycerol), and cells counted and photographed using a Nikon Optiphot-2 microscope equipped with a cooled CCD camera (Spectrasource). CCD images were captured on to a computer, imported into NIH Image and processed using Adobe Photoshop software. The retina was assayed for changes in the proportions of transgene-expressing cell types, which were easily identified on the basis of morphology and laminar position (Dorsky et al., 1995, 1997). Cells were counted in every second section in the central third of the retina, where laminar morphology is most distinct. Only retinas with at least 15 cells expressing the transgene were analyzed. The mean percentages for the different cell types for XFD-, D48- and HAVØ-expressing retina were compared using a Kruskal-Wallis Nonparametric ANOVA. A Dunn's Multiple Comparisons test was used to determine statistical significance (*P* value) between the different experimental groups.

Antibodies

Primary antibodies used in this study were as follows: polyclonal rabbit anti-*Xenopus* FGFR (provided by T. Musci; Amaya et al., 1993) at a dilution of 1:40 to recognize XFD, HAVØ and D48 transfected cells; a protein G-purified monoclonal mouse anti-myc (9E10) (Evan et al., 1985; Santa Cruz) at a dilution of 1:1500 to visualize c-myc-tagged versions of the D48 and XFD transgenic proteins. We showed previously that XFD and XFD-myc transfected retinal cells can be identified by this immunostaining method and that similar results are obtained with the two constructs (McFarlane et al., 1996). For confirmation of retinal cell identity, in some cases, we performed double labeling with anti-FGFR (or anti-myc) and antibodies against cell-specific markers: PRs; 1:200 anti-calbindin (Sigma) to identify cones (Dorsky et al., 1997) and 1:1 anti-rhodopsin to identify rods (Adamus et al., 1991). Müller glia: anti-R5 (Drager et al., 1984). RGCs: anti-neurofilament (1:2 mouse monoclonal RMO270, from V. Lee). RITC- or FITC-coupled goat anti-mouse or anti-rabbit (Jackson Laboratories) at a dilution of 1:500 was used as the secondary antibody.

BrdU labeling

Labeling was carried out as described previously (McFarlane et al., 1995; Dorsky et al., 1995). At stage 33/34 or stage 40, the gut of each embryo was injected with 5 mg/ml bromodeoxyuridine (BrdU; Sigma) diluted in water and phenol red (to visualize injections). 3 hours after injection, embryos were fixed overnight in 4% paraformaldehyde, and processed as 12 μm cryostat sections. In some experiments, embryos injected at stage 33/34 were left to develop until stage 40 before fixing. Sections were treated briefly (15 minutes) with 2 M HCl and rinsed well before double labeling. Sections were first

labeled with anti-FGFR followed by a RITC-coupled secondary (1:500), and then with mouse monoclonal anti-BrdU (Sigma) at a dilution of 1:1000 followed by a FITC-coupled secondary antibody (1:500).

Quantitation of cell survival

Embryos injected with XFD or D48 cDNA at the 16-cell stage were allowed to develop until stage 35/36 and then fixed in 4% paraformaldehyde overnight at 4°C. Transgene-expressing cells were labeled with anti-FGFR in 12 μm transverse retinal sections. Following the immunocytochemical procedure, apoptotic cells were labeled using the TUNEL method using terminal transferase to incorporate digoxigenin (DIG)-labeled nucleotides into the DNA of dying cells (Apotag kit, Oncor). The labeling was carried out as the manufacturer's instructions. Apoptotic cells were counted in every second section through the central retina, which corresponds to the region where identified cell counts were performed.

In situ reactions

Antisense and sense DIG-labeled RNA probes were generated by in vitro transcription of linearized plasmid constructs containing FGFR-1 (pBSSKII(+).XFGFR1-EC), FGFR-2 (pBSSKII(+).XFGFR2-EC) and FGFR-4 (pBSSKII(+).XFGFR4-EC) using either T7 or T3 RNA polymerase (Boehringer-Mannheim, Indianapolis, IN) according to manufacturer's instructions. Antisense probes: FGFR-1, FGFR-2, and FGFR-4 plasmids were linearized with *Xho*I, *Xba*I and *Not*I (Boehringer-Mannheim) and transcribed with T3, T7 and T7, respectively. Sense (control) probes: FGFR-1, FGFR-2, and FGFR-4 plasmids were linearized with *Xba*I, *Xho*I and *Xho*I and transcribed with T7, T3 and T3, respectively. Following synthesis, cRNA was ethanol precipitated and resuspended in hybridization buffer.

Embryos of appropriate developmental stages (Nieuwkoop and Faber, 1994) were fixed overnight at 4°C in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$ and 3.7% formaldehyde) following removal of jelly coat and vitelline membrane (when necessary). In situ hybridizations were performed on 12 μm cryostat sections as previously described (Dorsky et al., 1997) substituting MAB (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and MAB/2% BMBR (Boehringer-Mannheim Blocking Reagent) for PBT (PBS, 0.2% BSA and 0.2% TX-100) and PBT/20% goat serum, respectively. After hybridizations and subsequent washes, the DIG-labeled probes were detected using alkaline phosphatase (AP)-conjugated anti-DIG antibodies (Boehringer-Mannheim) and AP substrates BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium) (Sigma, St. Louis, MO). Photographs of stained tissue were processed using Adobe Photoshop software.

RESULTS

To test if FGFR signaling is involved in retinal cell fate decisions, we first determined whether FGFRs are expressed in the retina during cell specification. In order to participate in cell fate choice FGFRs would have to be expressed during a 2-day period, from stage 22 when the retina consists of proliferating neuroepithelial cells to stage 37/38 at the termination of the major wave of retinal cell birth. At stage 24-25, approximately 24 hours after fertilization, the first retinal cells are born. Retinal ganglion cells (RGCs), are generated first, followed shortly by the appearance of horizontal cells and cone photoreceptors (Holt et al., 1988; Belecky-Adams et al., 1996; La Vail et al., 1991). Cell commitment may partly occur during cell division as RGCs begin to differentiate within minutes of completing mitosis (Waid and McLoon, 1995). Development of the *Xenopus* retina is rapid, as all the retinal cell types are generated within 36 hours of the first RGCs being born (stage 37/38). The cells are distributed into three layers; the outer nuclear (ONL) or photoreceptor layer, the inner nuclear layer (INL; Müller glia, amacrine, bipolar and horizontal cells) and the RGC layer. Three FGFR subfamilies have been cloned in *Xenopus* and include FGFR-1, FGFR-2 and FGFR-4 (Friesel and Dawid, 1991; Friesel and Brown, 1992; Riou et al, 1996; Shiozaki et al., 1995). In situ hybridization, using digoxigenin-labeled antisense riboprobes, was used to determine the spatial and temporal expression patterns of the FGFRs during retinal development.

Messenger RNA for all three FGFRs is detected throughout the early proliferating eye primordium (stage 22-25). By stage 28, when the first RGCs are initiating axons, the expression patterns for the three receptors diverge (Fig. 1). FGFR-1 mRNA continues to be expressed throughout the neural retina during retinogenesis. By stage 37/38, however, FGFR-1 expression declines in the maturer central retina yet remains high in the peripheral retina, including the proliferative ciliary marginal zone (CMZ). In contrast, beginning at stage 28, FGFR-2 message levels decrease in the neural retina and signal is found predominantly in the developing lens and in mesenchymal cells surrounding the eye (Fig. 1C). A similar expression pattern is seen in fully differentiated eyes (stage 41). FGFR-4 message continues to be expressed throughout the stage 28 retina, but is expressed most highly in cells adjacent to the pigment epithelium (PE) (Fig. 1D). At later stages (stage 37/38 to 41) FGFR-4 message is found in photoreceptors, in a subset of cells in the INL, and in the CMZ (data not shown). These data suggest that all three FGFRs are expressed by the proliferating neuroepithelial cells in the early developing eye

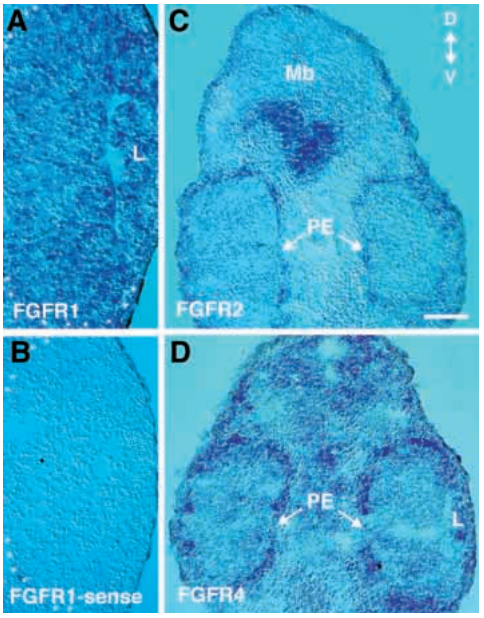

Fig. 1. Several different FGFRs are expressed in the early developing retina. Localization of FGFR mRNA in 12 μm sections through stage 28 *Xenopus* retina by in situ hybridization. (A) Anti-sense FGFR-1. (B) Sense FGFR-1. (C) Anti-sense FGFR-2. Arrows show where pigment epithelium (PE) is developing. (D) Anti-sense FGFR-4. PE, pigment epithelium; L, lens; Mb, midbrain; D, dorsal; V, ventral. Bar in B is 100 μm.

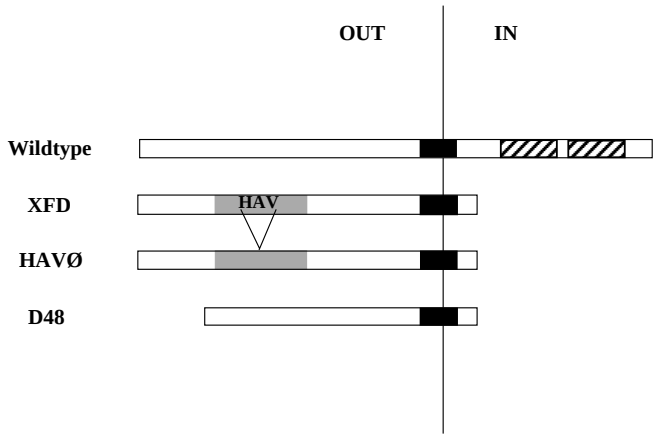

Fig. 2. FGFR cDNA expression constructs. Schematic representation of cDNA plasmids (Amaya et al., 1991, 1993) injected into dorsal animal blastomeres. In the wild-type receptor, the black box is transmembrane region and hatched boxes represent a split tyrosine kinase domain. The tyrosine kinase domain is missing in all three mutant FGFR constructs. The HAVØ mutant is missing an additional three amino acids, Histidine, Alanine and Valine (HAV), in its extracellular domain. Whereas, D48, the non-functional control construct, has an extracellular deletion of 48 amino acids, including the HAV sequence.

primordium (stage 24-28) and could potentially transduce either proliferative or determination signals.

Inhibiting FGFR function in retinal precursors reduces the number that go on to become photoreceptors

To test whether the FGFRs expressed by proliferating retinal precursor cells transduce an endogenous signal that influences cell specification in the developing retina we chose to inhibit the activity of FGFRs in these cells using XFD, a dominant negative form of the FGFR (Amaya et al., 1991) (Fig. 2). The XFD mutant is thought to inhibit the function of all FGFR members (Ueno et al., 1992), and was used previously to examine the role of FGFRs in axon extension and guidance in the developing *Xenopus* visual system (McFarlane et al., 1996). We found that XFD specifically inhibits FGF signaling in *Xenopus* retinal cells, but not signaling through a non-FGF receptor tyrosine kinase, trk B. To inhibit FGFR signaling in retinal precursors, the XFD cDNA construct was injected into embryos at the 16-cell stage, targeting the dorsal animal blastomeres which produce over half of the ipsilateral retina (Huang and Moody, 1993). Previously, we used a transfection approach in neurula stage embryos to express the dominant negative receptor in developing retinal cells (McFarlane et al., 1996). In this study, blastomere injections were performed because of the concern that transfected cells might not express blocking levels of the dominant negative until after determination signals have been received, and because a much larger fraction of the retina expresses the transgene. By targeting dorsal animal blastomeres, we avoided previously documented effects of the dominant negative FGFR on gastrulation (Amaya et al., 1991). D48, a non-functional version of the receptor was used as a control (Amaya et al., 1993; McFarlane et al., 1996) (Fig. 2). This construct was previously shown to have no effect on RGC axon extension or guidance (McFarlane et al., 1996).

At stage 40, when the mature organization of the retina is established, injected embryos were fixed and cells expressing the FGFR transgenes were visualized by immunostaining with either a polyclonal antibody against the *Xenopus* FGFR (Amaya et al., 1993), or a monoclonal myc antibody when myc-tagged transgenes were used (9E10; Evan et al., 1985). Transgene-expressing cells were easily identified on the basis of morphology and laminar position (Dorsky et al., 1995, 1997), and then counted in the central third of the retina, where proper lamination is present. In some experiments, cellular classification was confirmed by immunostaining for cell-specific markers (see Materials and Methods). Retinas were assayed for possible changes in the distribution of transgene-expressing cells amongst the three layers and seven different retinal cell types (Figs 3, 4). When precursor cells express the FGFR control construct, D48, they show a similar distribution amongst the three retinal cell layers as was previously observed for blastomere injections of fluorescent dextran (Huang and Moody, 1993), or a control cDNA construct encoding green fluorescent protein (GFP) (Dorsky et al., 1997). In contrast, when FGFR function was inhibited in retinal precursors approximately 50% fewer transgene-expressing cells were present in the ONL.

To determine which cell fate choices FGFR signaling may influence, we assayed the effects of inhibiting FGFR function on the production of all retinal cell types. Retinal cells are generated in a conserved temporal order with RGC, horizontal and cone cells born first, followed by amacrine, rod, bipolar

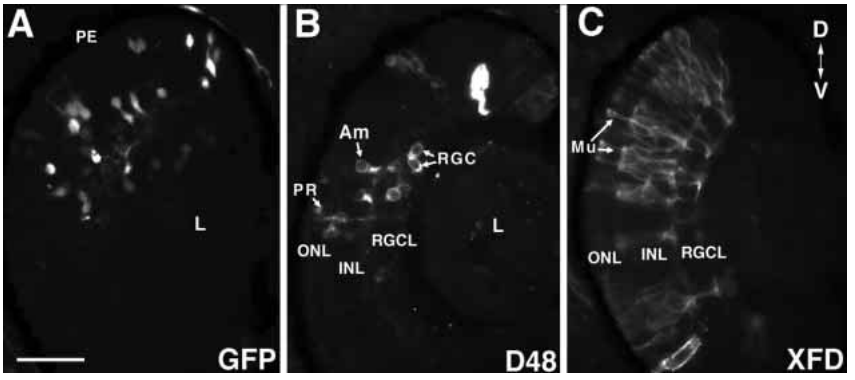

Fig. 3. FGFRs may transduce an environmental cue involved in photoreceptor determination. (A-C) Cross sections through stage 40 *Xenopus* retina. Plasmids containing GFP (A), D48 (B) and XFD (C) cDNA were injected into dorsal animal blastomeres at the 16-cell stage (Huang and Moody, 1993). To visualize FGFR transgene-expressing cells, sections were immunostained with an antibody against FGFR. In contrast to control injected embryos, many XFD-expressing retinal cells are Müller glia and few are photoreceptors. PE, pigment epithelium; L, lens; PR, photoreceptor; Mu, Müller glia; Am, amacrine cell; RGC, retinal ganglion cell; ONL, outer nuclear layer; INL, inner nuclear layer; RGCL, RGC layer; D, dorsal; V, ventral. Bar in A is 75 μm.

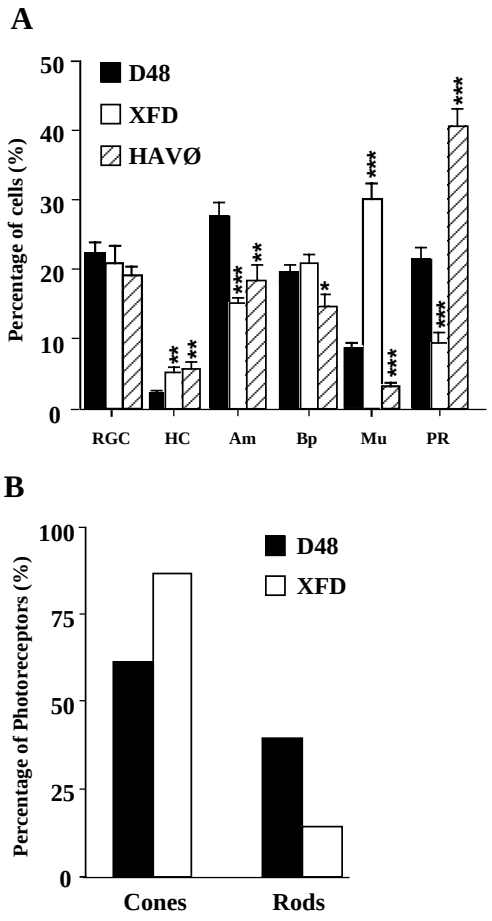

Fig. 4. Inhibiting FGFRs in retinal precursors enhances the development of Müller glia and inhibits rod production. (A) Graph of the distribution of control, D48, and dominant negative, XFD, transgene-expressing cells in stage 40 retina. Also included are the data from retina expressing HAVØ (see Fig. 6). Cells were identified on the basis of laminar position and morphology. Error bars are s.e.m. XFD, n=31 embryos (1187 cells); D48, n=30 embryos (1181 cells); HAVØ, n=20 embryos (1468 cells). Data is from 4-6 separate experiments. *, P<0.05; **, P<0.01; ***, P<0.001; unpaired nonparametric ANOVA. (B) Graph of the percentage of D48- and XFD-expressing (n=42 and n=34, respectively) photoreceptors that are rods and cones, as determined by anti-rhodopsin and anti-calbindin staining, respectively.

and Müller glial cells (Holt et al., 1988; Belecky-Adams et al., 1996; La Vail et al., 1991). In the rapidly developing *Xenopus* retina, there is considerable overlap in the genesis of the various retinal cell types, nevertheless, RGCs are always the first to be born and Müller glia the last. When FGFR signaling is inhibited in retinal precursor cells, the cell fates they normally assume are differentially affected. For instance, no effect was seen in the percentage of XFD-expressing cells in the RGC layer, suggesting that FGFR function may not be required for the generation of RGCs. In contrast, many more XFD-expressing cells become Müller glia cells and fewer become photoreceptors, as compared to D48-expressing retina (Fig. 3B,C). Fig. 4A shows the distribution of control D48 and dominant negative-expressing cells into all retinal cell types. As mentioned above, 57% fewer retinal precursors expressing the XFD transgene become photoreceptors as compared to retina expressing the D48 control construct. Similarly, 47% fewer amacrine cells express the XFD protein as compared to D48. To determine whether the reduction in photoreceptors is selective for either cone or rod photoreceptors, in a separate set of experiments XFD-expressing retina were double labeled with either anti-calbindin or anti-rhodopsin to identify cones and rods, respectively (Dorsky et al., 1997; Adamus et al., 1991) (Fig. 4B). Almost 40% of D48-expressing photoreceptors are rods, similar to what is observed with dorsal animal blastomere injections of a cDNA plasmid encoding GFP (Dorsky et al., 1997). In contrast, fewer than 10% of XFD-expressing photoreceptors were labeled with anti-rhodopsin. These results indicate that there is a large reduction in the number of retinal precursors that become rods when FGFRs are inhibited.

The decrease in rods observed with FGFR inhibition in retinal precursors was associated with a large increase in the number of glia produced. Over three times as many Müller glia are observed in XFD-expressing retina when compared to the control, D48 construct. To ensure that cells identified as Müller glia by morphological means are indeed Müller cells, and not undifferentiated neuroepithelial cells, we stained stage 41 retina of embryos injected with the XFD construct with an antibody that labels Müller glial processes (R5; Drager et al., 1984). Fig. 5A,B shows that XFD-expressing cells identified as Müller glia are double-labeled with R5. In addition, we labeled proliferating cells in stage 40 XFD-expressing retina with BrdU and showed that cells identified as Müller glia are not BrdU positive, and thus are non-proliferative cells (Fig. 5C).

Changes in the representation of Müller glia and rod photoreceptors in XFD-expressing retina could result from a change in cell fate choice, a change in the timing of Müller or rod cell differentiation, or from a selective change in the survival of the two cell types. Early differentiation of Müller glia cells would remove precursors that could have become rod cells, resulting in an increase in Müller cells and a decrease in rod photoreceptors. To address this possibility, we looked for Müller glia in earlier stage 33/34 retina. At this stage, Müller cells have yet to differentiate (Dorsky et al., 1997) and over 60% of photoreceptors are postmitotic (Holt et al., 1988). BrdU labeling of stage 33/34 retina verified that the vast majority of cells (87%, n=343) with processes that spanned the retina and cell bodies in the INL were BrdU positive and thus most likely undifferentiated, proliferating neuroepithelial cells. Moreover, when these BrdU-labeled retina were left to develop until stage 40, almost all XFD-expressing Müller glia were BrdU positive (91%, n=93) indicating that they were born after stage 33/34. These data support the suggestion that the absence of an FGFR signal does not cause Müller glia to differentiate early.

To address whether precursors that fail to receive an FGF signal die, cell death was examined in retina expressing XFD. No significant difference was observed in the average number of apoptotic cells/12 μm retinal section in eyes not expressing a transgene (4.5±0.8, n=9 eyes) or expressing either XFD (4.4±0.7, n=12), or D48 (3.7±0.5, n=14). These results indicate that inhibiting FGFRs in retinal precursors is not increasing cell death in developing retina. Taken together, these data suggest that inhibiting FGFRs in precursors lessens the probability that they become photoreceptors or amacrine cells and instead increases the likelihood they become Müller glia.

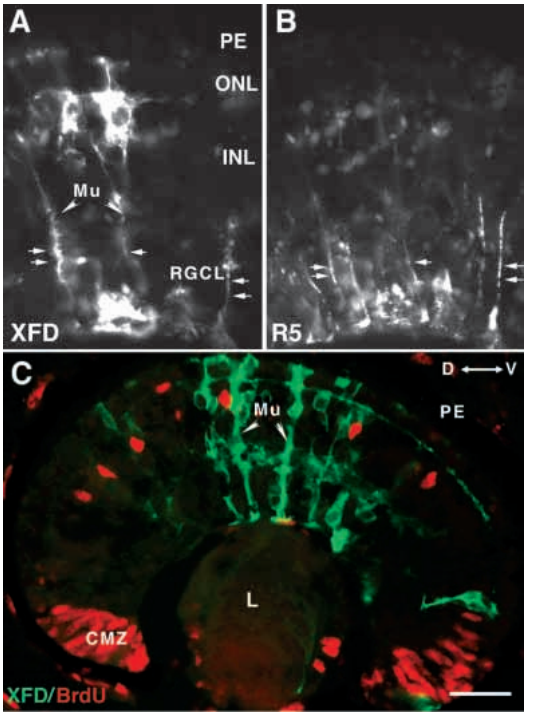

Fig. 5. Inhibiting FGFRs may promote the Müller glia cell fate. (A,B) Cross section through a stage 40 retina double labeled with an antibody against FGFR to visualize XFD-expressing cells (A) and with R5, an antibody against Müller (Mu) glia (B). (C) Cross section through a stage 40 retina of a BrdU-injected embryo double labeled with anti-FGFR (green) and an antibody against BrdU (red) to identify proliferating cells. Double-labeled cells are yellow. XFD-positive cells are not BrdU positive unless they are in the ciliary marginal zone (CMZ). PE, pigment epithelium; D, dorsal; V, ventral; L, lens. Bar in C is 20 μm for A and B, and 50 μm for C.

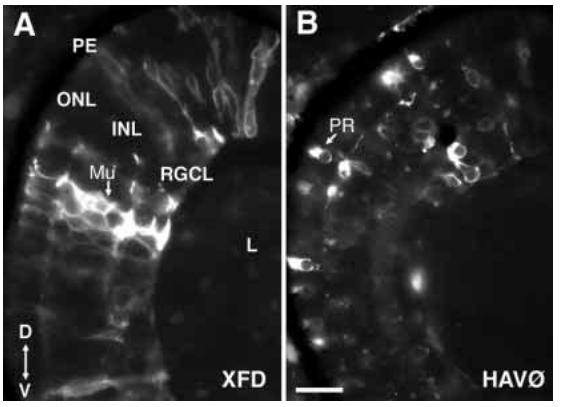

Fig. 6. A non-FGF ligand for the FGFR may participate in cell fate decisions in the developing retina. (A,B) Cross sections through stage 40 retina labeled with anti-FGFR to visualize transgene-expressing cells. Embryos were injected at the 16-cell stage with a plasmid containing a cDNA encoding either the dominant negative, XFD (A), or a mutant FGFR, HAVØ (B), that can inhibit the signaling of at least one non-FGF ligand through the FGFR. L, lens; PR, photoreceptor; Mu, Müller glia; D, dorsal; V, ventral. Bar in B is 20 μm.

An FGF-independent signal may influence retinal cell fate choice

Recently, it has been suggested that cell adhesion molecules (CAMs) and novel non-FGF peptide ligands such as fibroblast growth factor receptor ligands (FRLs) and fibroblast growth factor homologous factors (FHFs) also signal through the FGFR (Williams et al., 1994; Kinoshita et al., 1995; Smallwood et al., 1996). To examine whether a non-FGF signal might be influencing retinal cell genesis, we took advantage of a mutant FGFR construct, HAVNOT (HAVØ) (Fig. 2). In *Xenopus*, HAVØ does not affect FGF signaling, but blocks FGFR activation by at least one of the novel non-FGF ligands, presumably by a dominant negative mechanism (Kinoshita et al., 1995). In animal cap assays, HAVØ has no effect on FGF signaling, yet inhibits FRL-1-activated mesoderm induction (Amaya et al., 1993; Kinoshita et al., 1995). In contrast, XFD inhibits both ligands (Kinoshita et al., 1995). In a previous study, we found that HAVØ and XFD had distinct effects on *Xenopus* RGC axon extension and target recognition (McFarlane et al., 1996). HAVØ inhibited RGC axon initiation but did not affect axon extension or target recognition, both of which XFD profoundly impaired. These results suggested that HAVØ, while unable to inhibit FGF signaling, is able to block the activity of some as yet unidentified ligand for the FGFR in retinal cells, perhaps an FRL-like molecule (Kinoshita et al., 1995). In this study, we found that HAVØ also affects cell specification in the retina in a fashion opposite to the effect of XFD (Fig. 6). Almost twice as many HAVØ-expressing retinal precursors became photoreceptors when compared to D48-expressing controls. This is four times as many XFD-expressing photoreceptors (Fig. 4A). As a result, there are fewer amacrine (33.3% decrease) and Müller glia (67% decrease) cells expressing HAVØ. These results indicate that, in the developing *Xenopus* retina, there may also be an FGF-independent mechanism influencing cell fate.

DISCUSSION

In this paper we provide evidence supporting a role for FGFRs in transducing a signal in the environment of developing retinal precursors that biases them towards specific cell fates. Message for three different members of the FGFR family, FGFR-1, FGFR-2 and FGFR-4, are expressed in the early developing *Xenopus* retina. Inhibiting the function of these receptors in vivo in developing retinal cells using a dominant negative form of the FGFR (Amaya et al., 1991) apparently causes precursors that would normally have received a signal to become a rod photoreceptor or amacrine cell to adopt a later developmental fate, that of a Müller glia.

Clonal analysis in the developing retina of chick, rat and *Xenopus* indicates that precursor cells are able to produce all of the retinal cell types (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). Based on these data, and results demonstrating effects of growth factors and hormones on retinal cell specification in culture (reviewed by Cepko et al., 1996), it has been suggested that the environment plays a major role in determining cell fate in the developing retina. The fact that expression of the FGFR dominant negative in retinal neuroepithelial cells alters the ratios of cell types in the maturing eye might indicate that

FGFRs transduce a signal that influences cell specification. If blocking precursor FGFRs affects cell survival, proliferation or the timing of cell generation, however, a change in cell ratios would also be predicted. The observed decrease in photoreceptor and amacrine cells could be explained by a dependence of these two cell types on an FGFR ligand as a survival factor. Several points argue against this possibility. Supposing photoreceptors and amacrine cells are born but die before the retina are analyzed, then there should be evidence of increased cell death in earlier stage retina. Yet, we saw no evidence of increased apoptotic cell death in stage 35/36 retina, when over 80% of the complement of photoreceptor and amacrine cells are postmitotic (Holt et al., 1988). Moreover, in transgenic mice expressing the dominant negative FGFR under the control of an opsin promoter, abnormal photoreceptor cell death occurred gradually over a period of months, not 2 days (Campochiaro et al., 1996). Finally, selective death of photoreceptor and amacrine cells would result in a greater contribution to the total retinal cell population by all other cell types. Instead, we only observed an increase in Müller glia.

FGFR inhibition could alternatively be causing an increase in cell proliferation. If prolonging proliferation delayed differentiation (Bouvier and Mytilineou, 1995), cells would adopt later cell fates, explaining both the increase in Müller glia and decrease in photoreceptors. Since FGFs are potent mitotic agents for retinal cells in culture (Lillien and Cepko, 1992), it seems unlikely that blocking FGFRs would enhance proliferation. Consistent with this argument, we saw no obvious increase in mitotically active, BrdU-positive, cells in XFD-expressing stage 33/34 or stage 40 retina. Finally, based on this hypothesis, we would have expected to see a decrease in the first cells born. RGC levels, however, appear unaffected by expression of the dominant negative. A differential effect of FGFR inhibition on the timing of generation of rod cells and Müller glia where rods are born later and/or glia early, also seems unable to explain the data. XFD-expressing Müller glia are not observed in younger stage 33/34 retina when rods are normally being born (Holt et al., 1988) and there is no evidence for a population of XFD-expressing, BrdU-positive neuroepithelial cells in stage 40 retina that could later become rods. Thus, it seems likely that the effect of inhibiting FGFRs in retinal precursors is due to a direct influence on cell identity.

FGFR signaling does not participate in the determination of all retinal cell types. No changes were observed in the proportion of bipolar cells and RGCs when FGFR signaling was inhibited with the dominant negative. We have shown previously that inhibiting FGFR function in developing retina by in vivo transfection of XFD has no effect on specification of RGCs (McFarlane et al., 1996). These data contradict several reports showing an increase in RGCs in FGF-2-treated cultures, as assayed by RA4 or neurofilament immunostaining (Guillemot and Cepko, 1992; Pittack et al., 1997), and a recent report in which anti-FGF-2 antibody treatment of chick eye bud cultures resulted in a decrease in neurofilament staining (Pittack et al., 1997). One might expect that blocking endogenous FGFR activity in vivo would produce different results from overstimulation of the receptors in culture. The difference in blocking ligand or receptor activity on RGC specification is more puzzling. It may reflect an effect on the timing of RGC genesis, in that in rabbit retinal explants an FGF-2 antibody can delay, but does not prevent, the appearance of RGCs (Zhao and Barnstable, 1996). A delay in cell birth in the *Xenopus* retina, which develops rapidly in 36 hours, may not be apparent. Alternatively, it could suggest that different signals are used to induce RGCs in *Xenopus* and chick, and that *Xenopus* ganglion cell production is not influenced by a signal transduced by FGFRs.

Photoreceptor specification, and in particular rod development, appears affected by a signal acting through the FGFR. Our data suggest that photoreceptor and Müller glia cell fates are linked, as inhibition of FGFR signal transduction is associated with a decrease in photoreceptors and an increase in Müller glia. An alternate explanation, whereby an FGFR signal independently affects the timing of generation of two separate lineages is not supported by our data. If the two lineages were separate, in order to get an increase in Müller glia and a decrease in rod cells the absence of an FGFR signal would have to both speed Müller cell differentiation and delay rod cell genesis past stage 40. Yet, we found that Müller glia do not differentiate early, and that almost all XFD-expressing cells at stage 40 are postmitotic and have a differentiated morphology. Moreover, a relationship between these two cell types has previously been suggested, based on the observation that viral-overexpression of the epidermal growth factor receptor in the developing rat retina results in a reduction of rods and an enhancement of Müller cell development (Lillien, 1995). These results support a model where retinal progenitors progress through different stages of competence for the production of particular cell types (Cepko et al., 1996). Our data suggest that when precursors fail to receive the 'rod' signal they become Müller cells, which are the predominant late developing cells (Reichenbach et al., 1991; Dorsky et al., 1995). This progression to a Müller cell fate could occur by default, or could result from the undifferentiated precursor being competent to respond to the next determination cue, which pushes it to adopt a glial identity.

What are the candidate signals that the precursor FGFRs could transduce? Certain hints come from both in situ hybridization expression patterns of different ligands and culture data. Several FGFs are expressed in the developing *Xenopus* eye primordium including FGF-2 (Song and Slack, 1994), FGF-3 (Tannahill et al., 1992) and FGF-9 (Song and Slack, 1996). Of these, only the effects of FGF-2 on retinal cell genesis in vivo and in culture have been investigated. FGF-2 influences the specification of several different retinal cell types including RGCs (Guillemot and Cepko, 1992; Pittack et al., 1997), Müller glia (Tcheng et al., 1994) and photoreceptors (Hicks and Courtois, 1992; Tcheng et al., 1994). In *Xenopus*, pigment epithelium treated with FGF-2 transdifferentiates, generating all retinal cell types including both neural retina and glia (Sakaguchi et al., 1997). This effect is specific to FGF-2 and supports the possibility that FGF-2 may act as an inductive cue in the developing *Xenopus* retina. Interestingly, FGF-2 enhances the number of rods in both rat and teleost retinal cultures (Hicks and Courtois, 1992; Mack and Fernald, 1993). These data are in agreement with the decrease in rods that we observe when this ligand's receptor is inhibited in retinal precursors by expression of the dominant negative.

An intriguing possibility is that FGFRs may alternatively, or in addition, transduce a non-FGF signal that influences cell fate choice. Recent evidence suggest that FGFR is somewhat of a misnomer, as the molecule can also serve as a receptor for cell

adhesion molecules (Byers et al., 1992; Williams et al., 1994; reviewed by Doherty et al., 1996), FRL-1 and FRL-2 (Kinoshita et al., 1995) and FHFs (Smallwood et al., 1996). At least one member of each group is expressed in the developing retina (Kinoshita et al., 1995; Smallwood et al., 1996; Riehl et al., 1996; Kintner and Melton, 1987). Our data provide some support for the possibility that a non-FGF cue may affect retinal cell specification. The HAVØ mutant FGFR receptor modifies retinal cell genesis; many more photoreceptors are produced, at the expense of Müller glia. Since it has been demonstrated in the *Xenopus* animal cap assay that HAVØ is unable to block FGF signaling (Amaya et al., 1993), but can block FRL-1 signaling (Kinoshita et al., 1995), it is possible that HAVØ inhibits a non-FGF ligand that normally pushes cells towards a non-photoreceptor fate. In the absence of this cue(s), precursors will go on to assume a photoreceptor cell fate. Whereas, precursors expressing XFD, which are unable to respond to both FGF and non-FGF ligands, would be relegated to adopting the default glial cell fate. It will be interesting to determine, once they become available, whether non-FGF ligands such as the FRLs and FHF-1 promote a non-photoreceptor cell fate.

Thus, an as yet unidentified non-FGF ligand appears to influence retinal cell specification. The fact that HAVØ and XFD have different effects on retinal cell genesis suggests that an FGF cue also biases cell fate choices. Whether FGFs and non-FGF ligands compete for the same FGFRs on cells is unclear, but it raises the intriguing possibility that a single family of receptors receives cues that are both inhibitory and stimulatory for the development of a particular cell type and that it is the balance of these signals, in conjunction with other environmental cues, that determines what fate a cell adopts.

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