Pea3 Expression Is Regulated by FGF Signaling In Developing Retina

Kathryn Leigh McCabe,1* Chris McGuire,2 and Thomas A. Reh2

FGF signaling has been implicated as an important regulator of retinal development. As a first step in characterizing potential downstream targets of FGF signaling in the retina, we have analyzed expression of Pea3, a member of the Pea3 class of Ets-domain transcription factors, in the developing eye. We find that Pea3 is expressed in the developing retina, and its transcription is regulated by FGF receptor activation. In addition, FGF signaling activates Cath5, a gene necessary for retinal ganglion cell differentiation. These results suggest that FGF signaling via MAPK up-regulates transcription factors that in turn control retinal ganglion cell differentiation. Developmental Dynamics 235:327–335, 2006. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Fibroblast growth factors (FGFs) and their receptors (FGFRs) play a number of roles in eye development, including patterning of the optic vesicle, proliferation and differentiation of progenitor cells, and survival of neurons and photoreceptors (Pittack et al., 1991, 1997; Hicks and Courtois, 1992; Lillien and Cepko, 1992; Guillemot and Cepko, 1992; Lombardo and Slack, 1998; Hyer et al., 1998; Zhao and Barnstable, 1996; Desire et al., 1997; McFarlane et al., 1998; McCabe et al., 1999; Patel and McFarlane, 2000; Fischer et al., 2002; Fischer and Reh, 2002). FGFs also promote neurite outgrowth (Chai and Morris, 1999) and ganglion cell axon targeting (McFarlane et al., 1995, 1996). Several different FGFs, as well as FGFRs, are present in the eye throughout its development (Heuer et al., 1990; de Jongh and McAvoy, 1992, 1993; Bugra et al., 1993; Ohuchi et al., 1994; Tcheng et al., 1994; Wilke et al., 1997). Despite this wealth of evidence showing the importance of FGF signaling during vertebrate eye development, there is little known about the downstream signals resulting from FGFR activation in the retina.

Secreted FGF binds to the extracellular domain of FGFR, a member of the receptor tyrosine kinase family. Ligand binding causes receptor dimerization that, in turn, leads to auto-phosphorylation and activation of the intracellular kinase domain. Intracellular signaling cascades, in particular the Ras-MAPK cascade, relay the signal through a series of sequential phosphorylations of protein kinases. This results in activation of downstream responses including gene transcription. To further identify the molecular mechanisms invoked by FGF in retinal development, we analyzed the effects of activation or inhibition of FGF signaling on expression of the Pea3 subfamily of Ets-domain transcription factors. One of the major pathways through which FGFRs are known to signal is the Ras-MAPK pathway (reviewed by Szebenyi and Fallon, 1999). The nuclear targets for the MAPK family members include at least six subfamilies of Ets proteins (for review, see Wasylyk et al., 1998).

One of these subfamilies of Ets-domain transcription factors is the Pea3 class, which includes Pea3, Erm, and ER81. Members of this subfamily of Ets proteins have been shown to be up-regulated by FGF in several deve...
opining systems. Transcription of ER81 is up-regulated by FGF1 or eFGF and blocked with a dominant negative FGFR mutant in Xenopus animal caps and embryos (Münchberg and Steinbeisser, 1999). In zebrafish embryos, the expression patterns of Pea3 and Erm overlap with the expression patterns of FGF8 and FGF3. Inhibition of FGF signaling with antisense oligonucleotides to FGFs or with the FGF receptor blocker SU5402 leads to a loss of both Erm and Pea3 (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Using overexpression and dominant negative constructs of Pea3, it has been shown that scleraxis, a tendon-specific bHLH transcription factor, is regulated by FGF signaling through Pea3 in the sclerotome (Brent and Tabin, 2004). Similar experiments in nasal mesenchyme and the nasal placodes have shown that FGF is both necessary and sufficient for expression of Pea3 and Erm (Fernberg and Neubuser, 2002). Finally, there is also some evidence for a role for the Pea3 family of transcription factors in eye development. Injection of ER81 mRNA into the dorsal blastomeres of Xenopus embryos causes abnormalities in eye development (Chen et al., 1999). Thus, in all tissues examined to date, all three members of the Pea3 subfamily of transcription factors are regulated in their expression by FGF signaling.

To better characterize a potential role for Pea3, we analyzed its expression during retinal ganglion cell development. Previous work has shown exogenous FGF can stimulate retinal progenitor cells to differentiate into ganglion cells, whereas inhibition of FGF signaling with receptor blockers, antibodies, or dominant negative receptor expression inhibits retinal ganglion cell differentiation in many different species (as previously reviewed above). In this study, we report that Pea3 is expressed in developing retina, and its expression is regulated by FGF signaling. We find that Cath5, a critical gene for ganglion cell development (Brown et al., 2001; Liu et al., 2001; Wang et al., 2001), is also regulated by FGF signaling. These results suggest that Pea3 and Cath5 may be important components in the downstream events triggered by FGF in eye development, and specifically for ganglion cell differentiation.

RESULTS

Pea3 Is Expressed in the Developing Retina

Expression of Pea3 transcription factor was examined by in situ hybridization in embryonic chick retinas at the stages in which we had previously characterized the effects of FGF signaling in retinal ganglion cell differentiation (stages 23–25) (McCabe et al., 1999). At stage 23, Pea3 is expressed throughout the retinal epithelium, with a slightly higher level of expression in a mid-peripheral domain (Fig. 1B, arrow). There is also a very high level of Pea3 expression in the hindbrain (Fig. 1A). We compared the Pea3 expression at this stage with that of the differentiating ganglion cells by labeling the sections with anti-Neurofilament-M (NF-M) antibodies after the in situ hybridization for Pea3 (Fig. 1D,E). We found that while Pea3 expression extends throughout the retinal epithelium, the NF-M expression is confined to a region of central retina (Fig. 1D,E, arrow). By stage 25, the Pea3 expression is substantially reduced in the most peripheral part of the retinal epithelium, and developing ciliary body (Fig. 1C), and the peripheral extent of expression (arrows) corresponds approximately to the extent of retinal ganglion cell differentiation at this stage (data not shown).

To determine whether Pea3 is expressed in the developing ganglion cells, or alternatively in the undifferentiated precursor cells, we analyzed the double-labeled NF-M/Pea3 retinas (Fig. 2). At this stage in the chick retina, NF-M is only expressed in ganglion cells during or after their final mitotic division (McCabe et al., 1999). In our analysis of the Pea3/NF-M co-labeled sections, we found that only the newly generated ganglion cells at the mitotic surface (scleral) were double-labeled (Fig. 2D,E, arrow). Those NF-M immunoreactive ganglion cells that were in either the ganglion cell layer (vitreal surface) or in the neuroblastic layer (presumably migrating to the ganglion cell layer), did not express Pea3. The Pea3-expressing cells in the neuroblastic layer were not labeled with NF-M. These data indicate that postmitotic ganglion cells do not express Pea3, but their precursors do.

FGF1 Up-Regulates mRNA Levels of Pea3

Since Pea3 is expressed in the region of the retina where newly developing retinal ganglion cells are differentiating, we next asked whether its expression was FGF dependent. Whole retinas were cultured with and without 100 ng/ml FGF1 for 24 hr and subsequently analyzed by Northern blot analysis. RNA loading was equalized by using a probe to 18S ribosomal fragment. Pea3 probe hybridizes to a single band (Fig. 3A) that was strongly up-regulated by FGF1 compared to retinas cultured in vehicle alone (Fig. 3A). Densitometry analysis from at least 4 separate experiments for each time point revealed that FGF1 up-regulated Pea3 mRNA over 6-fold compared to control (Fig. 3B). This up-regulation of Pea3 was observed as early as 6 hr after FGF1 treatment and was sustained for up to 24 hr. In contrast, the expression of a related member of this subfamily, ER81, was much less affected by FGF1 (256% control ± 8.7% S.D.).

FGFRs are members of the receptor tyrosine kinase family that have been shown to signal through the mitogen activated protein kinases (MAPK), phospholipase C gamma (PLCγ), and phosphatidylinositol 3 kinase (PI3K) signaling cascades (reviewed by Dally et al., 2005). To test whether the effects of FGF1 on Pea3 expression were mediated through the MAPK pathway, we added the MAPK inhibitor PD98059, along with FGF1, to the whole retina cultures. PD98059 inhibits the MAPK pathway by blocking MEK’s ability to activate MAPK (Dudley et al., 1995; Langlois et al., 1995; Pang et al., 1995; Waters et al., 1995). At 25 μM, PD98059 reduced the effect of FGF1 on Pea3 mRNA levels by more than half (Fig. 3A,B) at all time points. It seems likely that this partial knockdown is due to the relatively low concentration used since the IC_{50} of PD98059 in several systems is 10–25 μM (i.e., Jelovac et al., 2005). Alternatively, these results could indicate
that FGF is signaling through multiple pathways.

One possibility is that the quantitative decrease in Pea3 mRNA levels correlate with a reduction in ganglion cell differentiation. To test this hypothesis, we assayed the effects of decreasing MAPK signaling on retinal differentiation using a peripheral retina explant assay. We have previously reported that NF-M protein is regulated by FGFs in peripheral retinal explants (McCabe et al., 1999). NF-M is an early marker of ganglion cell differentiation. Addition of FGF promoted ganglion cell differentiation in peripheral retina explants, while inhibition of FGF signaling by SU5402 reduced differentiation (McCabe et al., 1999). Accordingly, we assayed whether the MAPK inhibitor PD98059 resulted in a down-regulation of NF-M protein. Retinas were flat-mounted and the dorsal peripheral retina was dissected away. The peripheral retina explant was further divided into two pieces, with one piece cultured in the presence of PD98059 and the other in the equivalent amount of vehicle. Similar to the results of SU5402 (McCabe et al., 1999), 25 μM PD98059 and the other in the equivalent amount of vehicle. Similar to the results of SU5402 (McCabe et al., 1999), 25 μM PD98059 retarded the

Fig. 1. In situ hybridization for Pea3 at different stages of eye development in chick embryos. A,B: Low- and high-magnification images of Pea3 expression at stage 23. Arrow points to a region of somewhat higher expression than we typically observed at this stage. Note expression in lens, as well. C: Expression of Pea3 at stage 25 shows a distinct central to peripheral gradient; the highest levels of expression correspond to regions of ganglion cell differentiation. D,E: Stage 23 section double-labeled for Pea3 (in situ) and NF-M (immunofluorescence), respectively. Arrows point to the region where ganglion cells are newly differentiating. HndBrn, hindbrain.

Fig. 2. Pea3 and NF-M are co-expressed only in newly differentiating ganglion cells. Low (A–C) and high (D–E) magnification micrographs of stage 23 embryo section double-labeled for Pea3 (C,F) and NF-M (A,D). Merged images are shown in B and E. Large arrow in A–C shows the point of onset of ganglion cell differentiation. Small arrow corresponds to a NF-M-positive, Pea3-negative cell in the ganglion cell layer. The box shows the region magnified in D,F. Arrow in D,F points to a double-labeled cell in all panels. We only found examples of double-labeled cells at the ventricular surface. Arrowhead points to an undifferentiated Pea3 positive cell in the neuroblastic layer. RPE, retinal pigmented epithelium.
movement of the front of ganglion cell differentiation by 40% (Fig. 4B,C). Additionally, 10 μM PD98059 resulted in a similar reduction (data not shown). There was no apparent increase in cell death since no difference was noted in the number of necrotic or apoptotic cells as analyzed by Sytox green at 25 μM PD98059 compared to sister-matched vehicle controls (Fig. 4D).

**FGF1 Up-Regulates mRNA Levels of Cath5**

The proneural ath5 gene is a bHLH transcription factor that has been shown in several species to be necessary and sufficient for ganglion cell differentiation (Brown et al., 2001; Hutcheson et al., 2005; Kanekar et al., 1997; Liu et al., 2001; Kay et al., 2005; Ma et al., 2004a,b; Masai et al., 2005; Skowronska-Krawczyk et al., 2004; Wang et al., 2001; Xie et al., 2004). To better understand the potential role of Pea3 in ganglion cell differentiation, we asked whether the chick ath homologue, Cath5 (Liu et al., 2001) is regulated by FGF similarly to Pea3 regulation. To determine whether FGF signaling regulates Cath5 expression, we treated whole retinal explants from embryos of stages 23–24 for 24 hr in the presence or absence of 100 ng/ml FGF. The mRNA from the retinas was subsequently extracted and subjected to Northern blot analysis. Cath5 probe hybridizes to a single band on the Northern blot (Fig. 5A), and is up-regulated approximately 2.5-fold in response to FGF1 (Fig. 5B) after 24 hr.

**DISCUSSION**

We have found that Pea3 is expressed in the developing retina in embryonic chick, and its pattern of expression appears to be mediated by FGF signaling through the MAPK pathway. The period of development when retinal ganglion cells are generated is a well-characterized FGF-dependent developmental event. During this time, Pea3 is initially expressed throughout the epithelium, but by stage 25 Pea3 expression is distinctly higher in central than peripheral retina. The expression of Pea3 is regulated by FGF signaling, increasing
markedly as early as 6 hr of FGF treatment and sustained for up to 24 hr. Conversely, Pea3 is reduced after treatment with the FGF receptor blocker SU5402. These results suggest that endogenous FGF signaling maintains the expression of Pea3 in the developing retina.

The results reveal a correlation between retinal differentiation and up-regulation of Pea3 such that FGF treatment of the retina at stages 23–24 promotes both, whereas FGF inhibition reduces both. This correlation suggests a model in which Pea3 might be a downstream effector of FGF receptor activation that leads to ganglion cell differentiation. Studies in other areas of the nervous system are consistent with this possibility. In motor neuron development, for example, Pea3 and ER81 are specifically expressed in particular motor neuron pools around the time the motor neurons innervate their targets and regulate the specificity of their connections (Lin et al., 1998; Arber et al., 2000). Moreover, Arber and colleagues have shown that Pea3-deficient mice fail to show normal branching patterns of their terminal arbors within the muscles they innervate (Livet et al., 2002; Koo and Pfaff, 2002). In addition, the motor neurons themselves are malpositioned in the spinal cords of these mice, suggesting a function for Pea3 in an earlier stage of differentiation of the motor neurons. In the case of motor neurons, however, GDNF and not FGF is important in triggering the expression of Pea3, since Pea3 expression is markedly reduced in mice deficient in GDNF signaling (Haase et al., 2002). Another member of this subfamily, Erm, has also been shown to be important in the neuronal differentiation from neural crest cells. Transfection of a dominant-negative form of Erm into chick neural crest cells blocked their differentiation into neurons, while not affecting their ability to adopt a glial fate (Paratore et al., 2002).

We have found that Pea3 and, to a lesser extent, ER81, are regulated by FGF in the developing chick retina; Northern blot analysis revealed a sixfold induction of Pea3 in only 6 hr after addition of FGF to the medium of the explanted retinas. In Xenopus, Munchberg and Steinbeisser (1999) found that the homolog to ER81, XER81, is expressed in neural crest cells, eyes, optic vesicles, and pronephros in the embryonic frog. Transcription of XER81 was stimulated by bFGF and eFGF in animal and vegetal cap explants and expression of a dominant negative FGF receptor blocked XER81 transcription in embryos. In zebrafish embryos, the expression patterns of Pea3 and Erm correspond to the expression patterns of FGF8 and FGF3; inhibition of FGF signaling with antisense oligonucleotides to FGFs or with the FGF receptor blocker SU5402 leads to a loss of both Erm and Pea3 (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Similar experiments in nasal mesenchyme and the nasal placodes have also found that FGF is both necessary and sufficient for expression of both Pea3 and Erm (Firnberg and Neubuser, 2002). Liu et al. (2003) have found that Pea3 is regulated by FGFs in the developing mouse lung. Brent and Tabin (2004) have found Pea3 is regulated by FGFs in the sclerome. Hasegawa et al. (2004) found that FGF18 regulates Pea3 expression in the developing neocortex. Thus, in all tissues examined to date, all three members of the Pea3 subfamily of transcription factors are regulated in their expression by FGF signaling.

We find that Pea3 expression is not confined to the domain of the retina in
which ganglion cells develop, but rather initially extends into a region that will become the ciliary epithelium of the ciliary body. Thus, the expression of Pea3 is not sufficient to cause ganglion cell development, and other factors must also regulate this process. DNA binding by Pea3 subfamily members is regulated by cis-acting autoinhibitory domains on either side of the DNA binding domain (Bojovic and Hassell, 2001). Phosphorylation of Erm by PKA adjacent to the DNA-binding domain regulates its ability to bind DNA, such that phosphorylated Erm binds DNA much less effectively than its unphosphorylated form (Baert et al., 2002). If the same type of regulation by phosphorylation occurs with Pea3, it is possible that PKA activity is lower in the central retina where ganglion cells are developing, and Pea3 activity is, therefore, higher in this region. Another potential mechanism for regulation of Pea3 is through interaction with other proteins, either suppressors or co-activators. Pea3 is capable of binding to USF-1 and Id proteins; Id proteins interact with the ETS-domain of Pea3 to cause inhibition of DNA binding, while USF-1 interacts with Pea3 to increase its DNA binding activity (Greenall et al., 2001). Id proteins are expressed in developing retina (Dickmeis et al., 2002; Jen et al., 1997; Zhang et al., 1995). Id-1 and 4 are expressed in the developing mouse retina (Duncan et al., 1992; Ellmeier and Weith, 1995; Jen et al., 1997) and one homolog in the developing chicken retina (Helms et al., 1994). The expression of Pea3 may be regulated by auto-inhibition, the presence of a co-activator or inhibitor, or some combination of the three.

Our data show that FGF signaling through the MAPK signaling cascade up-regulates retinal ganglion cell differentiation as assayed by NF-M expression. We further show that MAPK up-regulates expression of the Ets subfamily member, Pea3, in the retina. In addition, Cath5, a transcription factor known to be necessary and sufficient for ganglion cell differentiation, is also regulated by FGF signaling, perhaps via Pea3. Our data are consistent with a model in which FGF promotes ganglion cell differentiation by upregulation of Pea3 in their precursors. FGF may thus be acting as a competence factor to increase expression of Pea3, which then promotes the ganglion cell fate through the upregulation of Cath5 and NF-M. Further experimental analysis will be required to test this model.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Chicken embryos were staged as described by Hamburger and Hamilton (1951). Embryos were killed by decapitation and the eyes were removed under sterile conditions. The neural retinas were dissected from the lens, vitreous, and extraocular tissue in Hanks Balanced Salt Solution (GIBCO, Gaithersburg, MD) supplemented with 0.6% glucose and 5 mM HEPES. All explants were cultured in 5% FBS (GIBCO) in medium consisting of DMEM-F12 (without glutamate or aspartate; GIBCO) with the following supplements: 0.6% glucose, 0.11% NaHCO3, 5 mM HEPES, 1 U/ml Penicillin, and 1 mg/ml Streptomycin (GIBCO) at 37°C with gentle nutation for 24 hr. PD98059 or SU5402 (Calbiochem, San Diego, CA) was added at time of culture at 25 μM, with controls receiving equal concentration of the carrier, DMSO (Sigma, St. Louis, MO).

Toxicity of PD98059 on the explants was assayed by the addition of Sytox Green (Molecular Probes, Eugene, OR), which binds to DNA in cells when their plasma membrane is compromised either by necrotic or apoptotic cell death. Explants were treated...
with 1 μM Sytox Green at room temperature for 30 min and then rinsed several times before fixation with 4% PFA for 1 hr. Explants were placed in 30% Sucrose/PBS overnight at 4°C and cryosectioned. To assess the number of dying cells, the number of cells at 40× (180 μm) were counted, 8 sections per explant, n = 4 sister matched explants.

For whole retina cultures, retinas were dissected as above with the pigmented epithelium removed from all but the most peripheral retina. Retinas were cultured with nutation for 6, 12, or 24 hr. At the time of culture, SU5402 was added at 25 nM, and either PD98059 or FGF1 (R&D Systems) was added at 12, or 24 hr. At the time of culture, Northern Blots

Fragments of ER81 and Pea3 were cloned as described above. A 170-bp fragment of chicken 18S ribosomal was cloned by RT-PCR from chicken E3.5 retina. Primers were designed to 18S (Accession AF173612). 32P-labeled probes were made by random primer incorporation of radioactive nucleotides (Amersham, Arlington Heights, IL). RNA was resolved by electrophoresis in an agarose-formaldehyde gel and blotted to a nylon membrane as described by the manufacturer (Amersham). Blots were prehybridized for at least 2 hr at 65°C in plaque screen buffer (0.2% polyvinylpyrrolidone, 0.25 M FICOLL, 0.2% BSA, 20 mM Tris (pH 7.5), 1M NaCl, 4 mM Ppi, 1% SDS) or with Express Hybridization solution (Clontech, Palo Alto, CA). All steps were performed with gentle agitation. Hybridization was done at 65°C overnight. Blots were then washed once with 2× SSC, 0.1% SDS for 20 min at 65°C, and twice with 0.2× SSC, 0.1% SDS for 20 min at 65°C. 18S ribosomal probe was hybridized for 1–2 hr and required higher stringency washes: 2× SSC, 0.1% SDS for 20 min at 70°C and twice with 0.1× SSC, 0.1% SDS for 20 min at 70°C. Blots were then exposed to XOMAT X-ray film at −70°C until signal was detected. Blots were stripped with boiling 0.5% SDS under hybridization conditions for lens development. Blots were subsequently re-probed.

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Pea3 IN THE DEVELOPING RETINA 333

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