Essential role for PDGF signaling in ophthalmic trigeminal placode induction

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Much of the peripheral nervous system of the head is derived from ectodermal thickenings, called placodes, that delaminate or invaginate to form cranial ganglia and sense organs. The trigeminal ganglion, which arises lateral to the midbrain, forms via interactions between the neural tube and adjacent ectoderm. This induction triggers expression of Pax3, ingestion of placode cells and their differentiation into neurons. However, the molecular nature of the underlying signals remains unknown. Here, we investigate the role of PDGF signaling in ophthalmic trigeminal placode induction. By in situ hybridization, PDGF receptor β is expressed in the cranial ectoderm at the time of trigeminal placode formation, with the ligand PDGFD expressed in the midbrain neural folds. Blocking PDGF signaling in vitro results in a dose-dependent abrogation of Pax3 expression in recombinants of quail ectoderm with chick neural tube that recapitulate placode induction. In ovo microinjection of PDGF inhibitor causes a similar loss of neural folds. Blocking PDGF signaling in vitro results in a dose-dependent abrogation of Pax3 expression in recombinants of quail ectoderm with chick neural tube that recapitulate placode induction. In ovo microinjection of PDGF inhibitor causes a similar loss of Pax3 as well as the later placodal marker, CD151, and failure of neuronal differentiation. Conversely, microinjection of exogenous PDGFD increases the number of Pax3+ cells in the trigeminal placode and neurons in the condensing ganglia. Our results provide the first evidence for a signaling pathway involved in ophthalmic (opV) trigeminal placode induction.

KEY WORDS: PDGF, Induction, Neurogenesis, Trigeminal placode, Chicken

INTRODUCTION

Cranial ectodermal placodes are regions of thickened head ectoderm that give rise to many of the defining features of the vertebrate head, including regions of cranial ganglia and sense organs. They differentiate into a variety of cell types, including sensory neurons and receptors, neuroendocrine cells, glia and other support cells. Ectodermal placodes have been formally divided into two categories: ‘neurogenic’ placodes that form trigeminal and epibranchial ganglia or ‘sensory’ placodes that form the ear, nose, lens and hypophysis. The trigeminal ganglion, which innervates much of the head, has neuronal contributions from the placode and neural crest, with the glia being solely derived from neural crest. Most of the cutaneous sensory neurons in the trigeminal ganglia are derived from the ophthalmic and maxillo-mandibular placodes (reviewed by Baker and Bronner-Fraser, 2001). Despite their important role in peripheral nervous system development, the tissue interactions and molecular signaling events leading to placode specification and differentiation remain a mystery.

Growth factors appear to be crucial for specification of particular placode fate. The otic placode, for example, is induced by interactions with hindbrain and/or surrounding tissues (reviewed by Groves, 2005). Similarly, epibranchial placode neurons can be induced by tissue interactions with pharyngeal endoderm (Begbie et al., 1999), though this may represent promotion of neurogenesis rather than initial induction. At least three families of growth factor have been implicated in specification of various placode fates. BMP signaling is required for lens and olfactory placode induction (Sjodal et al., 2007); for epibranchial placodes, BMP7 and FGFs appear to mediate the effects of the pharyngeal endoderm (Begbie et al., 1999) and the mesenchyme, respectively (Nechiporuk et al., 2007; Sun et al., 2007); similarly, FGFs and WNTs are involved in otic placode induction (reviewed by Barald and Kelley, 2004).

The trigeminal placode gives rise to ganglia that provide sensory innervation to much of the face. These cells differentiate early and solely give rise to sensory neurons. Because it generates a single cell fate, the trigeminal placode provides an excellent model for studying the processes underlying placode induction and acquisition of neuronal traits. Unlike some other placodes (e.g. lens and otic) that are morphologically distinct from neighboring ectoderm, trigeminal placode cells cannot be distinguished from surrounding tissue. Cell marking techniques suggest that the ectoderm overlying the presumptive midbrain and rostral hindbrain is fated to contribute to the trigeminal ganglion (Webb and Noden, 1983; Xu et al., 2008). Furthermore, the transcription factor Pax3 and the tetraspanin CD151 serve as molecular markers of the ophthalmic trigeminal placode (Stark et al., 1997; Baker et al., 1999; McCabe et al., 2004). Like otic and epibranchial placodes, the trigeminal placode appears to be induced by tissue interactions. One or several factors derived from the dorsal neural tube (Stark et al., 1997; Baker et al., 1999) can mediate this inductive interaction. However, the molecular nature of the signals involved has yet to be elucidated. The maxillo-mandibular placode is molecularly distinct from the ophthalmic placode and may arise via separate and as yet unknown interactions. For simplicity, we will refer to the ophthalmic trigeminal placode throughout the paper as the opV trigeminal placode.

Platelet derived growth factors (PDGFs) were originally isolated in a search for serum factors that promote proliferation of arterial smooth muscle cells (Ross et al., 1977). Subsequently, they were shown to function in migration, proliferation, survival, deposition of extracellular matrix and tissue remodeling factors in many cell types (reviewed by Hoch and Soriano, 2003). The ligands PDGFA, PDGFB, PDGFC and PDGFD are secreted as disulfide bound homo- or heterodimers. Upon binding, the ligands induce receptor dimerization, phosphorylation and activation of signal transduction cascades. PDGF receptors form both homo- and heterodimers when
activated, each with different affinities towards the four PDGF ligands (reviewed by Reigstad et al., 2005). PDGFRβ homodimers can be activated by ligand homodimers of PDGFB and PDGFD, whereas PDGFRα heterodimers can be activated by PDGFAB, PDGFB, PDGFCC and PDGFRαβ by the ligands PDGFAA, PDGFAB and PDGFCC (reviewed by Hoch and Soriano, 2003).

Here we examine the molecular nature underlying opV trigeminal placode formation, and present evidence that PDGF signaling is required for induction. The PDGF ligand and its receptor PDGFRβ are expressed at the right time and place to play a role in placode development. Furthermore, both in vitro and in vivo inhibition experiments demonstrate that PDGF signaling is required for expression of Pax3. Blocking initial induction reduces later neurogenesis of the ophthalmic lobes of the trigeminal ganglia. Conversely, exogenous PDGFD causes an increase in the number of Pax3+ cells and the overall size of the opV trigeminal placode domain, as well as increasing the number of neurons in the condensing ganglia. These experiments demonstrate that PDGF signaling is essential for ophthalmic trigeminal placode induction.

**MATERIALS AND METHODS**

**Explants**

Fertile chicken (*Gallus gallus domesticus*) and quail eggs (*Coturnix coturnix japonica*) were incubated at 38°C until they reached the proper stage. Ectodermal explants were removed from three- or four-somite stage (ss) (st. 8) embryos and placed in Ringer’s solution on ice. For obtaining neural tubes, a large segment of 12 ss trunk neural tube was dissected with tungsten needles and treated with 1 μg/ml Dispase (Roche; in DMEM, 20 mM Hepes pH 8.0) for 15 minutes on ice, 10 minutes at 38°C, and allowed to recover in F12/N2 medium (Invitrogen) with 0.1% bovine serum albumen (Sigma) for at least 10 minutes on ice. Using glass and tungsten needles, ectoderm was removed from the neural tubes, the dorsum was dissected away from other tissue, and placed in F12/N2 media on ice. Collagen matrix gels were made with commercially available collagen (Collaborative Research) as described (Artinger and Bronner-Fraser, 1993). Tissue was added after the bottom layer of collagen solidified. After positioning ectodermal explants on dorsal neural tubes, a top layer of collagen was added, allowed to set for 10 minutes at 38°C with 5% CO₂, and F12/N2 was added for 18 hours at 38°C with 5% CO₂. Conjugates were cultured with vehicle (DMSO), 10 nM or 100 nM PDGF Receptor Tyrosine Kinase Inhibitor III (PIII) (Calbiochem). For immunohistochemistry, explants were fixed overnight in 4% paraformaldehyde at 4°C. PIII has a half-maximal inhibitory concentration (IC₅₀) of IC₅₀=50-80 nM for PDGFRα and PDGFRβ (Matsuno et al., 2002a; Matsuno et al., 2002b). Pharmacological studies have shown that PIII is selective for PDGFRα and PDGFRβ, and can block other signaling pathways only at very high concentrations, greatly exceeding those used here (Fit3 IC₅₀=230 nM; EGFR, FGFR, Src, PKA and PKC IC₅₀> 30 mM) (Matsuno et al., 2002a; Matsuno et al., 2002b).

**Embryo injections**

Using a fine glass needle, ~1 nl of 1, 2.5, or 5 μM PIPII or 250 ng/μl PDGF-R (R&D Systems) or control (DMSO or BSA, respectively) was injected just under the ectoderm adjacent to the midbrain on the right side of st. 8-10 chick embryos. Embryos were fixed overnight in 4% paraformaldehyde at 4°C for immunohistochemistry and in situ hybridization.

**Immunohistochemistry**

Embryos were cryoprotected, embedded in gelatin and cryosectioned at 10 μm as previously described (Schrist et al., 1995). Gelatin was removed from sections by incubating at 42°C for 10 minutes and rinsed in PBS. Sections were blocked at room temperature for 1 hour using 10% donkey serum, 0.1% Triton and 0.1% BSA in PBS. Primary antibodies in blocking solution were incubated overnight at 4°C at the following concentrations: mouse Pax3 (Developmental hybridoma bank), 1:50; mouse QCPN (Developmental hybridoma bank), 1:10; rabbit 145KDa Neurofilament (Chemicon), 1:500; mouse Hu/C/D (Molecular Probes), 1:250; mouse Phospho-histone H3 (Upstate Biotech), 1:2000. Samples were then rinsed three times in PBS for at least 15 minutes. Secondary antibodies in 0.1% Triton, 0.1% BSA in PBS were incubated for 1 hour at room temperature. Secondary antibodies (goat anti-mouse IgG or IgG2a Alexa 488, goat anti-mouse IgG2a Alexa 568, goat anti-mouse IgG1 or IgG2b Alexa 594, goat anti-rabbit Alexa 594) were used at 1:2000-4000 (Molecular Probes) and washed as above. Sections were counterstained with 1 μg/ml DAPI (Sigma) in PBS for 10 minutes, and rinsed three times in PBS for 5 minutes and coverslipped using Permafluor (Beckman Coulter).

Embryos were immunostained with Pax3, Hu (Wakamatsu and Weston, 1997; Okano and Darnell, 1997) and Neurofilament-M [NF (Shaw and Weber, 1982)], and counter-stained with DAPI. Hu and NF were visualized using the same fluorophore to facilitate identification of neurons.

**Cell death and proliferation**

TUNEL labeling was performed using the In Situ Cell Death kit (Roche) with modifications from the manufacturer’s directions. Samples were subsequently immunostained with the proliferation marker Phospho-histone H3. Cryosectioned slides were twice rinsed in PBS (PBS + 0.5% TritonX-100) for 10 minutes each, permeabilized for 10 minutes with 0.5% TritonX-100, 0.1% sodium citrate in PBS and rinsed twice in PBST. In the dark, the reaction mix was diluted 1:40 with TUNEL buffer. Slides were incubated with 100 μl for 3 hours at 37°C in a humidified chamber, rinsed three times in PBST for 10 minutes, and immunostained for Phospho-histone H3.

**In situ hybridization**

Embryos were fixed overnight in 4% paraformaldehyde (pH 9.5) (Basyuk et al., 2000). Antisense digoxigenin-labeled RNA probes were made according to manufacturer’s directions (Roche). Whole-mount in situ hybridization was performed as described (Kee and Bronner-Fraser, 2001) using NBT/BCIP (Roche) for color detection. Whole-mount pictures were taken using a Zeiss Stemi SVM microscope with an Olympus DP10 digital camera.

**Cell counting**

Explants were sectioned, immunostained and photographed using a Zeiss Axiophot2 Plus. Pax3+/QCPN+ ectodermal cells were counted using Photoshop (Adobe) in the individual color channels using DAPI to verify total cell number. Because of the variation of the size and plane of sectioning explants, as many sections as possible (at least six) were counted per explant to reduce variability. Pax3+/QCPN+ cells were expressed as a percentage of total cell number. All values were normalized to the percentage of Pax3+/QCPN+ cells of total cell number for controls. ANOVA with the Bonferroni multiple comparisons post-hoc test was performed between control and treated groups.

For inhibitor injection, PDGFD injection and dnPDGFRβ electroporation experiments analyzed at early stages, embryos were stained with Pax3 and DAPI. For injection experiments, all Pax3+ cells on the right injected side of the embryo in the ectoderm of the midbrain were counted (at least six sections). Total cell number in the ectoderm was assessed using DAPI. Pax3+ cells were expressed as a percentage of total cell number and normalized to controls. For electroporation experiments, Pax3/GFP+ cells were counted. To evaluate transfection efficiency, all GFP+ cells were expressed as a percentage of total cell number using DAPI. Student’s unpaired t-test was performed between control and treated groups with error bars indicating s.e.m.

Stage 13-15 embryos were stained with Pax3 (green), DAPI (blue), NF (red) and Hu (red), with the latter two visualized with the same fluorophore (Alexa 594). All Pax3/Hu/NF+ cells on the right injected side were counted (at least 10 sections). Average number of cells per section was normalized to controls. ANOVA with the Bonferroni multiple comparisons post-hoc test was performed between control and multiple treated groups. For single treatment experiments, Student’s unpaired t-test was performed.

**Electroporation of dominant-negative PDGFRβ**

The dnPDGFRβ construct was made by cloning the murine PDGFRβ lacking the kinase and autophosphorylation sites (Ueno et al., 1991), which was then subcloned into the pCIG expression construct (Megas and McMahon, 2002) that drives expression through the chicken β-actin
promoter and CMV enhancer. GFP is driven from an IRES sequence downstream of the coding sequence allowing detection of transfected cells. Embryos were electroporated at st. 4-6 to ensure expression of constructs by st. 8. Embryos were cultured ventral side down on filter rings in albumen by the modified New method. DNA (2 μg/ml) was injected between the epiblast and the vitelline membrane. Platinum electrodes were placed vertically over the embryo and electroporated with five pulses of 7 V for 50 msec with 100 msec intervals as previously described (Shiau et al., 2008). Embryos were then cultured in 1 ml of albumen at 38°C for 18 hours until st. 10-11.

RESULTS

Placodes are specified to particular fates via inductive interactions with adjacent tissues. Only recently have some molecular pathways mediating induction been identified. For opV trigeminal placode, one or more unknown factors from the dorsal neural tube (Baker et al., 1999) appear to be responsible for induction. Here, we explore the role of PDGF ligands and receptors during this process.

Identification of PDGF receptors in the forming opV trigeminal placode

As a first step to identify potential signals involved in induction, we performed RT-PCR on presumptive opV trigeminal placode ectoderm to look for transcripts that encode receptors for secreted factors as candidate inducers. To this end, ectoderm adjacent to the presumptive midbrain region of 3-4 somite stage (ss) (st. 8) chicken embryos was dissected and harvested for mRNA. Primers were designed to specifically recognize PDGFRα and PDGFRβ. Both were expressed in ectoderm derived from 3-4 ss (st. 8) embryos (McCabe et al., 2007). In addition, receptors for members of the fibroblast growth factor family, insulin-like growth factors, sonic hedgehog, the transforming growth factor β super family, and WNTs were all expressed in patterns consistent with a role in opV trigeminal placode formation (McCabe et al., 2007).

Because RT-PCR lacks spatial information, we next performed in situ hybridization with specific probes for both PDGFR receptors. Whole-mount in situ revealed that PDGFRβ is expressed in presumptive midbrain-level ectoderm at st. 8, prior to the time of placode induction (Fig. 1E,F); ectodermal expression continues through st. 10 by which time induction has begun (Fig. 1G,H). Interestingly, PDGFRβ is also expressed in the tips of st. 8 neural folds, which may contribute to presumptive placode, but also neural crest and neural tube (Bronner-Fraser and Fraser, 1988). PDGFRβ expression has expanded to include the entire ectoderm by st. 10, when the majority of opV trigeminal placode cells have been induced. PDGFRα is present in the mesenchyme at presumptive midbrain-level at st. 8 (Fig. 1A,B), although ectodermal staining is very faint. At st. 10, PDGFRα is present on migrating neural crest at the level of the midbrain (Fig. 1C,D). As previously shown (Stark et al., 1997; Baker et al., 1999), Pax3 is expressed by future ingressing placodal cells by st. 10 (Fig. 1K,L) and PDGFRβ is expressed both on Pax3+ as well as Pax3– ectodermal cells. Early migrating neural crest cells express only low levels of Pax3 at st. 10 (Fig. 1L).

We next assayed PDGF ligands in the neural tube. Both PDGFA and PDGFDD are expressed in the neural folds, consistent with a possible role for PDGF signaling in opV trigeminal placode induction (Stark et al., 1997). Notably, PDGFα is expressed in the st. 8 neural folds (Fig. 2K,L) and st. 10 neural tube in the midbrain as well as the trunk (Fig. 2M,N,O). Faint expression of PDGFα can be detected in the adjacent ectoderm at st. 8. Importantly, PDGFα is expressed at all axial levels in the neural tube (Fig. 2N,N/O), consistent with the finding that both cranial and trunk neural tube at st. 10-11 are able to induce competent ectoderm to become opV trigeminal placode (Baker et al., 1999). PDGFα signal is also detected migrating neural crest at st. 10 (Fig. 2N,N′), but is absent from ectoderm (Fig. 2N′). PDGFα is expressed in the caudal st. 8 neural folds, just below the presumptive midbrain, making it less likely candidate (Fig. 2A,B). At st.10, PDGFA is strongly expressed in the midbrain and trunk-level ectoderm itself, but not the neural tube (Fig. 2C,D,E). PDGFC, however, is expressed in the presumptive midbrain-level ectoderm at st. 8 (Fig. 2F,G) and maintained at st. 10 (Fig. 2H,I) in a similar pattern to Pax3. In the

Fig. 1. PDGFRα, PDGFRβ and Pax3 mRNA expression at st. 8 and 10. (A) PDGFRα is expressed in the head and somites at st. 8 in whole mount. (B) In section, PDGFRα is detected in the midbrain-level mesenchyme. (C) At st. 10, PDGFRα is in neural crest and somites. (D) Migrating neural crest cells express PDGFRα at the midbrain-level at st. 10. (E,F) PDGFRβ is strongly expressed in ectoderm and neural folds at st. 8. (G,H) PDGFRβ is maintained in ectoderm at st. 10. (I,J) Pax3 is absent from presumptive opV trigeminal placode at st. 8. (K,L) Pax3 marks opV trigeminal placode ectoderm and early migrating neural crest at midbrain level. Broken white lines in whole mounts (A,C,E,G,J,K) indicate level of section (B,D,F,H,I,J,L). ECTO, ectoderm; MES, mesenchyme; NF, neural folds; NC, neural crest.
trunk, PDGFC is also found in the somites and mesoderm (Fig. 2H,J). Owing to the high levels of PDGFRβ expression in the ectoderm, the most likely signaling scenario is that PDGFD through PDGFR ββ; however, PDGFA may be signaling through PDGFRαβ heterodimers at low levels.

**PDGF signaling is necessary for Pax3 induction in vitro**

To recapitulate opV trigeminal placode induction in vitro, we took advantage of the fact that juxtaposition of 3-4 ss (st. 8) presumptive midbrain-level ectoderm with 12 ss (st.11) dorsal neural tube from either cranial or trunk levels induces ectodermal Pax3 expression (Baker et al., 1999; McCabe et al., 2004). As cranial and trunk neural tubes are virtually interchangeable in this assay, we used trunk for ease of dissection. Both placodal ectoderm and dorsal neural tube express Pax3. To identify ectodermal Pax3 expression specifically, we used interspecific recombinants to juxtapose quail ectoderm with chick neural tube, such that ectodermally derived Pax3+ cells also expressed quail specific QCPN (Fig. 3A). After 18 hours, Pax3 is abundantly expressed in the quail ectoderm (Fig. 3C), whereas 3-4 ss (st. 8) ectoderm cultured alone does not express Pax3 (data not shown) (McCabe et al., 2004).

To test the necessity of PDGF ligand/receptor interactions, we blocked all PDGF signaling with an inhibitor, the receptor tyrosine kinase inhibitor III (PIII), that blocks with a half maximal inhibitory concentration (IC50) of 50-80 nM for PDGFRα and PDGFRβ (Matsuno et al., 2002a; Matsuno et al., 2002b). In our experiments, we find that IC50<10nM, which is significantly lower than previously published studies. Pharmacological studies have shown that PIII is selective for PDGFRα and PDGFRβ, and can only block other signaling pathways at very high concentrations, greatly exceeding those used in the present study (Flt3 IC50=230 nM; EGFR, FGFR, Src, PKA and PKC IC50>30 mM) (Matsuno et al., 2002a; Matsuno et al., 2002b). In addition, we have previously shown that EGFR is not present in presumptive opV trigeminal placode (McCabe et al., 2007).

At 18 hours, PIII concentrations of 10 and 100 nM dramatically decreased the number of Pax3+/QCPN+ cells compared with DMSO controls (Fig. 3F,J,N). PIII reduced the number of Pax3+ cells by 58% at 10 nM (n=15 explants) and by 84% at 100 nM (n=11 explants) compared with controls (n=15 explants) (Fig. 3B) with no change in cell viability (Fig. 3E,I,M). The results show that there is a dose-dependent reduction in the numbers of Pax3+ cells after treatment with the PIII inhibitor and suggest a potent role for PDGF signaling in opV trigeminal placode induction in vitro.

**PDGF signaling is necessary for Pax3 and CD151 opV trigeminal placode induction in vivo**

We next examined the effects on opV trigeminal placode formation of blocking PDGF signaling in the developing embryo. To this end, we injected a small volume (~1 nl) of 1, 2.5 and 5 μM of PIII into the mesenchyme in the presumptive midbrain-level at st. 8. As a control, an equivalent concentration of the vehicle DMSO was injected in an identical manner. Because the inhibitor is small and may cross the midline, we compared the injected side of experimental embryos to stage-matched control-injected embryos.
Transverse sections through these embryos revealed a marked reduction of the numbers of Pax3+ cells (Fig. 4B) compared with stage-matched controls (Fig. 4A), with no significant difference in the total number of DAPI+ cells (Fig. 4C,D; Fig. 5D). A 1 μM PIII solution (n=6) resulted in a 71% reduction Pax3+ cells compared with control embryos (n=7) (Fig. 5A). Similarly, 2.5 and 5 μM solutions of PIII resulted in a 67% and 84% reduction (n=8, n=6), respectively. The results demonstrate that PDGF signaling is necessary for opV trigeminal placode induction in vivo.

To assess whether there were changes in cell proliferation or cell death in the presence of the inhibitor, we repeated the assay described in Fig. 5A, and counted the number of dividing cells in ectoderm of 2.5 μM PDGFR inhibitor-injected embryos. Using the mitotic marker Phospho-histone H3, inhibition of PDGFR signaling did not alter the number of dividing cells (Fig. 5A; n=5, P>0.05). Similarly, TUNEL staining showed no difference in numbers of dying cells (Fig. 5C; n=5, P>0.05) and DAPI showed no changes in total cell number in the ectoderm (Fig. 5D; n=5, P>0.05).

Because Pax3 is an early placode marker, we next asked whether PDGF signaling was necessary for expression of a later placode marker. For this purpose, we injected 2.5 μM PIII or vehicle control into the st. 8 cranial mesenchyme adjacent to the midbrain and assessed the effects by in situ hybridization on CD151, a member of the tetraspanin superfamily of proteins that was upregulated in response to opV trigeminal placode induction (McCabe et al., 2004). It is strongly expressed by opV trigeminal placode cells at st. 10 (McCabe et al., 2004) and thus initiates later than the onset of Pax3 expression. Similar to Pax3, we observed a marked reduction in the expression of CD151 on the PIII-injected side relative to the uninjected side of experimental embryos (Fig. 6D-F; n=9), whereas control embryos were unaffected (Fig. 6A,B,C; n=5).

Because pharmacological inhibitors may have specificity problems, we verified the requirement for PDGF signaling in opV trigeminal placode formation using an alternative approach. To this end, we generated a dominant-negative (dn) PDGF receptor β that was truncated and thus bound the ligand but failed to signal. This construct has been shown to inhibit PDGFRβ homodimers, as well as PDGFRαβ heterodimers (Ueno et al., 1991; Ueno et al., 1993). The dnPDGFRβ construct was made as previously described (Ueno et al., 1991) and subcloned into a vector with a chicken specific β-actin promoter [pCIG (Megason and McMahon, 2002)]. Using New Culture to culture whole embryos on paper rings, st. 4-6 embryos were electroporated as described by Shiau et al. (Shiau et al., 2008) with either an empty control pCIG or dnPDGFRβ-pCIG vector and allowed to develop until st. 10-11. Embryos were sectioned and the number of Pax3+/GFP cells were counted in the midbrain. Similar to the PDGFR inhibitor experiments, the dnPDGFRβ construct resulted in a greater than 50% reduction in the number of Pax3+ transfected cells compared with controls (Fig. 7E-G; n=4 embryos each). There was no
significant difference in the transfection efficiency between empty vector pCIG and dnPDGFRB (Fig. 7H; \( P>0.3 \)). These results confirm that PDGF signaling is necessary for opV trigeminal placode induction.

**PDGFR signaling during induction is necessary for subsequent neurogenesis in the ophthalmic lobe of the trigeminal ganglion**

We asked whether inhibiting PDGF signaling blocked neurogenesis, the final step in opV trigeminal induction, by assessing whether neuronal differentiation was attenuated in the presence of the PDGFR inhibitor. To this end, we injected 1 \( \mu \)M PIII, 2.5 \( \mu \)M PIII, or control carrier into the mesenchyme lateral to the midbrain at st. 8 and allowed the embryos to develop to st. 13-14, by which time neurogenesis has commenced. Embryos were immunostained with Pax3, Hu and Neurofilament-M (NF) and counter-stained with DAPI, and cells double-positive for Pax3 and Hu/NF were counted. In order to avoid counting the same neuron multiple times, we used NF (processes, and some cell bodies) and Hu (perinuclear) in the same channel. At this stage, only placode-derived neurons have differentiated in the ophthalmic lobe, whereas neural crest cells differentiate and begin expressing Hu and NF only at st. 18 (d’Amico and Noden, 1980).

The results show that the PDGFR inhibitor PIII blocks neurogenesis in ovo. In control embryos, abundant Pax3/Hu/NF+ cells are present in the condensing ganglion (Fig. 8A-C). By contrast, injection of 2.5 \( \mu \)M PIII causes a marked reduction in the number of Pax3+ neurons (Fig. 8D-F). PIII (1 and 2.5 \( \mu \)M) caused a statistically significant reduction in neurogenesis by \( \sim \)70% of control (control \( n=6; \) 1 \( \mu \)M PIII \( n=3; \) 2.5 \( \mu \)M PIII \( n=5 \)) (Fig. 9A). Therefore, PDGF signaling is necessary for neurogenesis as well as for induction.

Our results are consistent with either a dual role for PDGF signaling, an early role in induction as well as later in neurogenesis, or a single early role with later effects on neurogenesis occurring secondarily as a consequence of those on induction. To test the role of PDGF signaling after specification has begun, embryos were injected with vehicle or 2.5 \( \mu \)M PIII at st. 10, by which time the majority of opV trigeminal placode cells have been specified, and allowed to develop to st. 13/14. To examine effects on neurogenesis, we counted Pax3-, Hu- and NF-expressing cells but found no significant change in the number of Pax3+, Hu/NF+ and Pax3/Hu/NF+ cells within the condensing opV trigeminal ganglion in PIII-treated embryos (Fig. 9B; \( n=6 \) embryos each, \( P>0.05 \)). These results suggest that, once the opV trigeminal placode cells have been specified, they continue to generate neurons in the absence of PDGFR signaling.

**PDGFD increases the number of Pax3 expressing cells and size of the opV trigeminal placode**

Because inhibition of PDGF signaling results in fewer opV trigeminal placode cells, increasing PDGF signaling might be expected to increase the number of placode cells. To test this, we microinjected 250 ng/\( \mu \)l solution of PDGFD into the mesenchyme in the presumptive midbrain-level at st. 8 and assessed the effects of the placode at st. 11. As a control, an equivalent concentration of the vehicle BSA was injected in an identical manner. We found that 250ng/\( \mu \)l of PDGFD resulted in an increase in both the number of...
the Pax3+ cells at midbrain-levels by 32% (Fig. 10K; \( P = 0.0032, n = 8 \) control, \( n = 10 \) PDGF), as well as the overall size of the placode (Fig. 10A,B). The white lines in Fig. 10A-D demarcate the ventral boundaries of the placodes in transverse section. To address whether this occurred by alterations in cell number and/or cell density, we counted the total number of cells in the ectoderm between the midline of the injected side to the last Pax3 cell within the section (see white line, Fig. 10A,B). In the presence of exogenous PDGF, the placode spread laterally into more ventral ectoderm. The number of Pax3+ cells increased by 29% \( (P = 0.0014) \) compared with control, with no change in the density, suggesting that the increase was mediated by an increase in the total number of placode cells, rather than total cell number. When comparing total cell number, we found no significant difference between control and PDGF-injected embryos \( (P > 0.3) \). Interestingly, PDGFAA, PDGFAB, PDGFBB, PDGFCC or PDGFDD alone was not sufficient to induce 3-4 ss (st. 8) presumptive opV trigeminal ectoderm in vitro, nor could beads when placed in permissive epiblast in vivo induce ectopic opV trigeminal placode cells (data not shown), indicating that other factors in addition to PDGF may be necessary for opV trigeminal placode induction.

**PDGF increases the number of neurons in the condensing opV trigeminal ganglia**

To test whether the increase in Pax3+ cells by exogenous PDGFD increased neurogenesis, PDGFD was injected at st. 8 and the embryos were fixed at st. 15 to analyze the numbers of Pax3/Hu/NF+ neurons formed in the condensing opV trigeminal ganglia. We noted a 54% increase in the number of Pax3/Hu/NF+ neurons in the condensing opV trigeminal ganglia \( (P < 0.003, n = 6 \) control and PDGFD embryos) (Fig. 10J,K) versus control injected with BSA (Fig. 10G). Thus, PDGFD injection significantly increases the number of opV trigeminal placode-derived neurons.

**Fig. 6. PIII injection reduces CD151, a marker for opV trigeminal placode cells.** (A,B) Control embryo shows no difference between injected and uninjected sides \( (n=5) \). (C) Magnification of vehicle injected control. (D) A focal injection of 2.5 \( \mu M \) PIII solution causes large reduction in CD151 expression on the right side \( (n=9) \). (E) Section of 2.5 \( \mu M \) PIII embryo shows decrease in CD151 staining on injected side. (F) Magnification of 2.5 \( \mu M \) PIII embryos. Arrowhead indicates side of embryo injected; Inj, injected; ecto, ectoderm.

**Fig. 7. dnPDGFR\( \beta \) blocks Pax3 induction.** (A-C) Control empty pCIG vector co-expresses Pax3 and GFP. (D-F) dnPDGFR\( \beta \)-pCIG vector dramatically reduces the expression of Pax3. (G) Quantification of Pax3/GFP+ shows a greater than 50% reduction with expression of dnPDGFR\( \beta \) \( (*P=0.036) \). (H) Quantification of transfection (GFP/DAPI+ cells) efficiency shows no significant difference between control and dnPDGFR\( \beta \) \( (P>0.35) \). Arrows indicate double-positive Pax3/GFP+ cells.

**Fig. 8. PDGFR signaling is necessary during induction for opV trigeminal ganglion neurogenesis.** (A) A st. 14 control embryo shows Pax3 (green) cells condensing into ophthalmic lobe of the trigeminal ganglion. (B) Hu/NF (red) staining of same section as A shows many of the Pax3+ cells are neurons. (C) Merge of A,B plus DAPI. Arrows indicate double-positive Pax3/GFP+ cells. (D) At st. 14, many fewer Pax3+ have condensed in 2.5 \( \mu M \) PIII embryos (injected at st. 8). (E) Similarly, fewer Hu/NF+ neurons were generated. (F) Merge of D,E plus DAPI. Arrows indicate double-labeled Pax3/Hu/NF cells.
DISCUSSION

PDGF expression and opV trigeminal placode induction

Previous work in the chick has shown that one or more unidentified secreted factors from the dorsal neural tube are necessary for induction of the opV trigeminal placode (Stark et al., 1997; Baker et al., 1999). Here, we show that PDGF has the correct spatiotemporal pattern to fill this role and is necessary for induction of the opV trigeminal placode, as assessed by Pax3 and CD151 expression. This provides the first experimental evidence of a molecular inducer of the ophthalmic trigeminal placode.

Both PDGFD and PDGFRβ are expressed at the correct time and place during chicken opV trigeminal placode development to mediate the induction process. The ligand PDGFD is present in the neural folds and the receptor PDGFRβ in the adjacent ectoderm at st. 8 immediately preceding placode induction (Baker et al., 1999). After this stage, placode cells fail to change fate even when transplanted to another placode-forming region (Baker et al., 1999). By st. 10, the ligand PDGFD is expressed in the neural tube at all axial levels. In vitro data have shown that the inducer for the opV trigeminal placode is present along the entire neuraxis at st. 10-12 (Baker et al., 1999), consistent with the PDGFD expression pattern.

As PDGFC is expressed in the adjacent mesenchyme and PDGFD is present at low levels in the ectoderm, it is possible that there are multiple sources of PDGF ligands not solely arising from the neural folds. Expression studies in the zebrafish and mouse have reported PDGFRα in the opV trigeminal placodes and later in the ganglia (Liu et al., 2002a; Zhang et al., 1998; Andrae et al., 2001), with the ligand PDGFA expressed in the adjacent neural tube (Liu et al., 2002b). This may reflect paralog switches between species. Expression of another ligand, PDGFB, has been reported in olfactory and otic placode in the mouse (Ding et al., 2000). PDGFB does not appear to be expressed in mouse placodes, and no information has yet appeared for PDGFD.

PDGF signaling is necessary for opV trigeminal placode induction

Induction of the opV trigeminal placode upregulates expression of the transcription factor Pax3 concomitant with specification of opV trigeminal placode cells (Baker et al., 1999). Pax3+ cells in the
forming opV trigeminal ganglia can first be detected at st. 9+ and are abundant by st. 10. We show that inhibition of PDGFR receptor function in vitro abrogates Pax3 expression in a dose-dependent manner. In vivo studies further show that blocking PDGFR signaling effects not only Pax3 expression but also a later placodal marker, CD151, as well as the formation of placode-derived neurons. This effect is not due to changes in either cell proliferation or death; rather, these cells continue to express an ectodermal marker, Pax6 (data not shown), suggesting that they may remain in an undifferentiated state. These results show that PDGFR signaling is essential for induction and subsequent differentiation of the opV trigeminal placode.

PDGFs not only function to promote proliferation in many cell types, but also are involved in migration, survival, and deposition of ECM and tissue remodeling factors (reviewed by Hoch and Soriano, 2003). Extensive genetic studies in mouse have revealed many roles for PDGF ligands and their receptors during embryonic development in mouse (reviewed by Betsholtz, 2004). The Patch mutant, which has a large deletion that includes the PDGFRα gene (Morrison-Graham et al., 1992; Orn-Urreger et al., 1992; Schatteman et al., 1992) exhibits disruption of non-neuronal neural crest cells. However, no gross abnormalities in the opV trigeminal placode were noted, possibly because the placodal component is often overlooked or ignored. Indeed, further inspection of the images presented by Morrison-Graham et al. (Morrison-Graham et al., 1992) suggests that the trigeminal ganglia look smaller, thus raising the issue of the role of PDGFRα in the mouse. In the PDGFRα-null, neurofilament expression was analyzed for neural crest derivatives only (Soriano, 1997). Other PDGFRα-null alleles were not analyzed for perturbations of the trigeminal ganglia (Klinghoffer et al., 2002; Hamilton et al., 2003). PDGFRβ mouse nulls die at or shortly before birth with defects in the blood and kidney glomerulus (Soriano, 1994) and have yet to be analyzed for defects in cranial ganglia. Thus, it is not known whether PDGFR signaling is necessary for trigeminal placode induction in the mouse and in the chicken.

**OpV trigeminal placode induction**

Placodal induction probably occurs via two separable steps. The first occurs when ectodermal cells gain general competency to become presumptive placode cells in a ‘pre-placodal domain’ (reviewed by Streit, 2004; Bailey and Streit, 2006; Schlosser, 2006), which expresses a unique combination of Six, Eya and Dach gene family members shortly after neural plate formation (Streit, 2002; McLarren et al., 2003; Bhattacharyya et al., 2004; Kozlowski et al., 2005; Litsiou et al., 2005). Fate-mapping experiments have shown that placodal progenitors cells are interspersed throughout the pre-placodal domain (Kozlowski et al., 1997; Whitlock and Westerfield, 2000; Streit, 2002; Bhattacharyya et al., 2004). Eventually, these cells separate into distinct, identifiable areas along the neural tube (D’Amico-Martel and Noden, 1983; Couly and LeDouarin, 1985; Couly and LeDouarin, 1987; Noden, 1993; Xu et al., 2008). The first step in opV trigeminal placode induction probably occurs during formation of this domain; this is followed by specific induction of the placode towards an opV trigeminal fate in a manner requiring PDGFR signaling (Fig. 11).

Recent studies suggest that all cells within the pre-placodal domain may initially be specified as lens. Subsequently, lens fate is repressed in non-lens placodal regions, followed by induction of alternative placode fates (Bailey et al., 2006). For olfactory placode, FGF from the anterior neural ridge as well as an unidentified inhibitory factor from neural crest cells is required for suppression of lens fate and the subsequent induction of olfactory placode. However, FGF alone is not sufficient to restrict lens fate (Bailey et al., 2006). Similarly for the otic placode, cells must first acquire general competency in the pre-placodal domain, then later are able to respond to FGF signaling to be specified as otic placode (Martin and Groves, 2006). For both otic and olfactory placodes, FGF signaling is not sufficient to induce all markers, indicating that additional factors are required. Sjodal et al. (Sjodal et al., 2007) found that at st. 4, BMP signaling is necessary for both olfactory and lens placodal precursors. This indicates that placode induction is likely to be a multifactorial process. Here, we show that the second step in opV trigeminal placode induction requires PDGFR signaling. Addition of exogenous PDGFD in ovo results in more opV trigeminal placode cells and placode-derived neurons, suggesting that PDGFR ligand is a limiting factor in opV trigeminal placode induction in vivo. However, PDGFs do not appear to be sufficient for opV trigeminal placode induction. PDGFAA, PDGFBB, PDGFCC or PDGFD alone is not sufficient to induce 3-4 ss (st. 8) presumptive opV trigeminal ectoderm in vitro (data not shown). Nor can beads coated with either PDGFAB, BB, CC or DD when placed in permissive epiblast at st. 4-6 generate ectopic opV trigeminal placode cells (data not shown). Therefore, we speculate that factors in addition to PDGFD may be required for opV trigeminal placode induction, similar to olfactory and otic placode induction.

**Placode induction and neurogenesis**

The epibranchial and otic placodes exhibit morphological characteristics long before neurogenesis occurs, making it possible to separate factors involved in induction versus neurogenesis; e.g. the chick epibranchial placode is distinguishable as a thickening at st. 10 (Groves and Bronner-Fraser, 2000; Abu-Elmaged et al., 2001), prior to expression of neuronal markers at st. 16 (Begbie et al., 1999). By contrast, olfactory and opV trigeminal placodes express neuronal markers shortly after induction. The thickened olfactory ectoderm is obvious by st. 14 (Street, 1937), and the neuronal marker, Hu, is observed concomitant with delamination (Fornaro and Geuna, 2001). Unlike olfactory placode, the opV trigeminal is not readily morphologically identifiable; rather small groups of cells begin expressing Pax3 and soon thereafter, express neurofilaments and commence delamination.
It is difficult to separate opV trigeminal placode induction from neurogenesis. In the chick opV trigeminal placode, neuronal specification temporally correlates with Pax3 expression in vitro and in vivo (Baker and Bronner-Fraser, 2000). Accordingly, we find that PDGF signaling is necessary both for Pax3 expression and subsequent neurogenesis. When the PDGF inhibitor is injected in ovo, Pax3 induction was dramatically reduced after 9-12 hours and the number NF/Hu neurons was also significantly reduced after 24 hours. Thus, if opV trigeminal placode cells fail to express Pax3, they do not form neurons. Our data show that specification, as detected by Pax3, cannot be separated from neurogenesis in the opV trigeminal ganglia. If we block PDGF signaling at st. 10 rather than st. 8, we see no significant change in number of neurons. Therefore, absence of PDGF signaling during the inductive period results in the loss of specified opV trigeminal placode cells and consequent loss of opV trigeminal placode-derived neurons.

In contrast to the opV trigeminal placode, epibranchial placodes appear to undergo additional steps between induction and neurogenesis. In explant culture, pharyngeal endoderm or BMP7 can induce neurons from cranial non-neuronal ectoderm (Begbie et al., 1999). Neither pharyngeal endoderm nor BMP7 was able to generate neurons from trunk ectoderm (Begbie et al., 1999), although trunk ectoderm transplanted into the presumptive epibranchial placodes did generate epibranchial neurons (Vogel and Davies, 1993). The differences between these two studies are probably due to timing differences. The ectoderm used by Begbie et al. (Begbie et al., 1999) has the thickened morphology of placodal ectoderm (Groves and Bronner-Fraser, 2000; Abu-Elmagd et al., 2001), and is known to express a presumptive epibranchial marker, Sox3 (Abu-Elmagd et al., 2001). As neuronal markers were used to identify cells as epibranchial, these results suggest that BMP7 is required for neurogenesis and accounts for the effects mediated by pharyngeal endoderm; however, whether a similar situation exists for initial induction is unknown and signals in addition to BMP7 may be required for epibranchial placode formation in chick. The studies of Sun et al. (Sun et al., 2007) argue that FGFs are also involved, and Nechiporuk et al. (Nechiporuk et al., 2007) provide new evidence supporting the hypothesis that mesenchyme is a source of inducing signals. Support for this idea comes from studies in zebrafish mutants that lack endoderm (Nechiporuk et al., 2005) and suggest that at least two inductive signals are at work: one that is endoderm-independent as Fox1 epibranchial placode precursors form in the absence of pharyngeal endoderm; and a second endoderm-dependent process required for neuronal differentiation.

**PDGF transcriptional targets**

Many transcription factors have been implicated in the formation of placodes (reviewed by Schlosser, 2006). Several of these, including GATA and Hairy-related transcription factors, play a role in PDGF-mediated processes in vascular smooth muscle cells, megakaryocytes and hepatocellular carcinomas. For example, GATA2 is upregulated during the PDGF-mediated epithelial to mesenchymal transition during hepatocytic cancer (Gotzmann et al., 2006). In megakaryocyte lines where PDGF promotes proliferation, Chui et al. (Chui et al., 2003) found that PDGF upregulates GATA1 protein over 2.5 times in 2 hours. In addition, PDGF downregulates Hairy-related transcription factors in vascular smooth muscle cells (Wang et al., 2002; Sakata et al., 2004). GATA transcription factors have been found to be present in the pre-placodal region in *Xenopus* embryos (Kelley et al., 1994; Walmsey et al., 1994; Read et al., 1998) and Hairy-related genes in the differentiating trigeminal placode (Turner and Weintraub, 1994; Deblandre et al., 1999; Koyano-Nakagawa et al., 2000; Davis et al., 2001; Tsuji et al., 2003).

In order to find direct transcriptional targets of PDGF, Chen et al. (Chen et al., 2004) used a gene-trap screen to identify genes that are regulated by PDGF. When comparing the outputs of the PDGF transcriptional targets screen (Chen et al., 2004) and a screen looking for genes upregulated by trigeminal placode induction (McCabe et al., 2004), three genes were found in common: calmodulin 2, kinesin family member 4A and 60S ribosomal protein L12. In addition, two families of transcription factors that have been implicated in placodes were found in the enhancer trap screen, Msx1 and FoxI1, both of which are expressed in the pre-placodal domain and differentiating trigeminal placode in *Xenopus* (Lef et al., 1994; Maeda et al., 1997; Suzuki et al., 1997; Feledy et al., 1999; Pohl et al., 2002; Schlosser and Ahrens, 2004), although the function of these genes in PDGF induction of the opV trigeminal placode has yet to be determined. The shared expression of direct PDGF targets and genes downstream of placode induction is particularly intriguing in light of the present data showing that PDGF signaling is required in the induction process.

**Conclusion**

Our results show the PDGF ligands and receptors are present at the right time and place, and are required for ophthalmic trigeminal placode induction and subsequent neurogenesis. Furthermore, exogenous PDGF generates additional opV trigeminal placode cells, indicating that it is a limiting factor. Thus, our combined use of experimental embryology with gain- and loss-of-function analyses helps clarify the molecular nature of tissue interactions underlying ophthalmic trigeminal placode formation.

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