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Lens Specification Is the Ground State of All Sensory Placodes, from which FGF Promotes Olfactory Identity

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Summary

The sense organs of the vertebrate head comprise structures as varied as the eye, inner ear, and olfactory epithelium. In the early embryo, these assorted structures share a common developmental origin within the preplacodal region and acquire specific characteristics only later. Here we demonstrate a fundamental similarity in placodal precursors: in the chick all are specified as lens prior to acquiring features of specific sensory or neurogenic placodes. Lens specification becomes progressively restricted in the head ectoderm, initially by FGF and subsequently by signals derived from migrating neural crest cells. We show that FGF8 from the anterior neural ridge is both necessary and sufficient to promote olfactory fate in adjacent ectoderm. Our results reveal that placode precursors share a common ground state as lens and progressive restriction allows the full range of placodal derivatives to form.

Introduction

Since Spemann's original experiments (Spemann, 1901), lens formation has remained an important model for studying inductive interactions during embryogenesis. He suggested that the optic vesicle, the future retina, is the source of lens-inducing signals sufficient to generate lenses in competent ectoderm. This idea has since been revised to propose a multistep model for lens induction. In amphibians, key events in lens formation occur around late gastrula/early neurula stages, before the optic vesicle contacts the presumptive lens ectoderm. Molecular interactions at these preplacode stages are ill defined, whereas the molecular control of lens placode formation (which occurs after optic vesicle contact) is relatively well understood (Grainger, 1996; Ogino and Yasuda, 2000; Lang, 2004). Ultimately, formation of a functional eye depends on the mutual interaction between the differentiating lens vesicle and the optic cup (Coulombre and Coulombre, 1963; Yamamoto and Jeffery, 2000; Lang, 2004).

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One of the key factors in early lens formation is the transcription factor Pax6, whose ability to induce ectopic eyes is conserved throughout the animal kingdom (Halder et al., 1995; Chow et al., 1999; Gehring and Ikeo, 1999; Donner and Maas, 2004). *Pax6* is expressed prior to lens placode formation in the presumptive lens ectoderm (PLE) and is required cell autonomously in the PLE for its transition to a morphological placode (Walther and Gruss, 1991; Grindley et al., 1995; Ashery-Padan et al., 2000). Downstream of Pax6, different transcription factors of the Sox, Maf, Fox, and Six families cooperate with Pax6 and with each other to activate the lens program, initially manifested by the expression of lens-specific crystallins (Kamachi et al., 1995, 1998, 2001; Ogino and Yasuda, 1998; Muta et al., 2002; Zhu et al., 2002; Shimada et al., 2003). BMP signaling in the optic vesicle and in the PLE is necessary for gene expression specific for the lens placode (Furuta and Hogan, 1998; Wawersik et al., 1999; Faber et al., 2002), while FGF signaling is required in the placode (Faber et al., 2001). Thus, our current molecular understanding of lens induction is most complete for the transition from preplacode to placode stages, while the events that initiate this process (upstream of preplacodal Pax6) are unknown.

At early neurula stages, cells fated to become lens are part of a unique domain in the cranial ectoderm which contains the precursors for all sensory placodes and thus has been termed the "preplacodal region" (Kozlowski et al., 1997; Streit, 2002, 2004; Bhattacharyya et al., 2004; Bailey and Streit, 2006; Schlosser, 2006). This region expresses a unique set of genes belonging to the Six, Eya, and Dach families (Bailey and Streit, 2006; Schlosser, 2006), which have been implicated in its own formation and in the control of various aspects of sensory organ development (Xu et al., 1999; Li et al., 2003; Zheng et al., 2003; Kawakami et al., 2000; Hanson, 2001). Within the preplacodal region, precursors for different placodes are initially interspersed, but then separate to form individual placodes at discrete positions along the neural tube (Kozlowski et al., 1997; Streit, 2002; Bhattacharyya et al., 2004). In the adult, placode derivatives contribute to the cranial sensory nervous system giving rise to structures as diverse as the lens, the inner ear, the olfactory epithelium, the adenohypophysis, and the sensory ganglia, and generate a large variety of different cell types (Bailey and Streit, 2006; Schlosser, 2006). Among these, the adenohypophysis and the lens are the only nonneurogenic placodes, the latter being the simplest placode, which produces an anterior epithelial sheet and a posterior mass of lens fibers.

Despite their eventual cellular and functional diversity, it has been proposed that all placodes initially share a common developmental program (Jacobson, 1966; Torres and Giraldez, 1998; Streit, 2004; Bailey and Streit, 2006; Schlosser, 2006). In this model, an early step in placode specification entails the induction of the preplacodal region, which would represent a unique state through which cells have to pass before becoming mature placodes. So far, the strongest support for this model comes from a recent study of otic induction

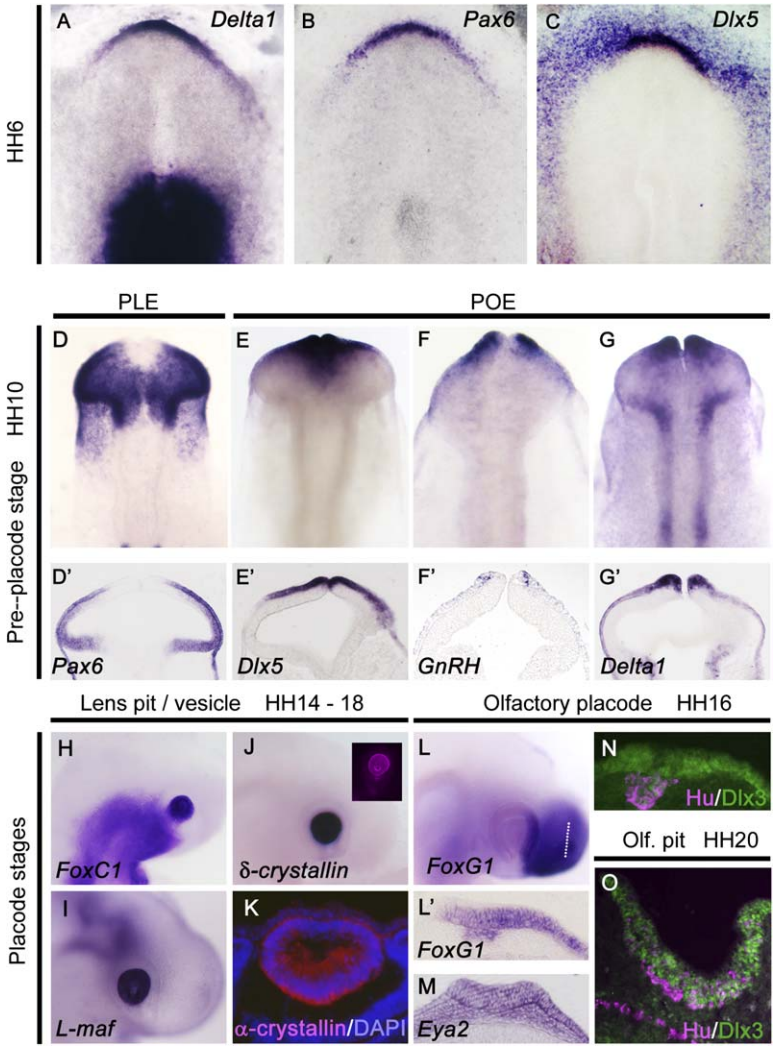

Figure 1. Molecular Markers of Preplacode and Placode Stages

(A–C) At HH6, *Delta1* (A) and *Pax6* (B) are expressed outside the anterior neural plate, while *Dlx5* (C) surrounds the entire neural plate.

(D–G) At HH10, the presumptive lens ectoderm (PLE) occupies the region overlying the optic vesicles, while the presumptive olfactory ectoderm (POE) surrounds the anterior neuropore. The PLE is marked by *Pax6* (D and D′), while the POE is labeled by *Dlx5* (E and E′), *GnRH* (F and F′), and *Delta1* (G and G′).

(D′ and E′) Transverse sections through embryos shown in (D)–(G).

(H–K) The lens placode (H) and vesicle (I–K) express *FoxC1* (H), *L-maf* (I), *δ-crystallin* transcript (J) and protein ([J], inset), and α-crystallin (K).

(L–O) At HH16, the olfactory placode expresses *FoxG1* (L and L′), *Eya2* (M), and Dlx3 protein (N); a subset of migrating cells is Hu$^+$ (N). The line in (L) indicates the plane of section in (L′) and (M)–(O).

(O) At HH20, the olfactory pit expresses Dlx3, and a subset of cells also expresses Hu.

showing that the acquisition of preplacodal-like character is required for cells to respond to an otic-inducing signal (FGF; Martin and Groves, 2006). Although the terms "placode bias" or "generic placode character" have been used to describe the preplacodal state, the exact properties of cells in the placode territory remain ill defined.

Surprisingly, we find that the entire preplacodal region is initially specified as lens tissue. This implies that "lens" is a default state of the preplacodal territory, which must therefore be repressed in the nonlens domains. FGF from the anterior neural ridge initiates lens suppression and simultaneously imparts olfactory fates to neighboring cells. However, FGF alone is not sufficient to restrict lens formation to its correct position in the embryo; we show that neural crest cells inhibit lens specification in vitro, while their ablation in vivo results in ectopic lens formation.

Results

All Sensory Placodes Are Initially Specified as Lens

To characterize the regional identity and developmental state of different parts of the preplacodal region over time, we first analyzed the expression of molecular markers specific for individual presumptive and mature placodes. At head fold stages (HH6), the chick preplacodal region expresses *Six1*, *Six4*, and *Eya2* (Streit, 2004; Schlosser, 2006). Within this domain, *Pax6*, *Delta1*, and *Dlx5* overlap in the anterior placode territory (Figures 1A–1C; Bhattacharyya et al., 2004); however, by the ten-somite stage, *Pax6* (Figures 1D and 1D′) is concentrated in the presumptive lens ectoderm, while *Dlx5* and *Delta1* (Figures 1E, 1E′, 1G, and 1G′) are strongly expressed in the presumptive olfactory region (POE), which also expresses *gonadotropin releasing hormone* (*GnRH*) transiently (Figures 1F and F′). The otic region is *Pax2*$^+$ and *Sox10*$^+$ (Cheng et al., 2000; Groves and Bronner-Fraser, 2000), while the trigeminal territory expresses Pax3 (see Figure S1 in the Supplemental Data available with this article online). At stage HH16, all placodes have acquired their typical morphology; the olfactory placode expresses *FoxG1* (Figures 1L and 1L′), *Eya2* (Figure 1M), and *FGF8* (Karabagli et al., 2002b), is strongly Dlx3$^+$, and has begun to generate Hu$^+$ neurons, a subpopulation of which is Dlx3$^+$ (Figure 1N; stage HH20: Figure 1O). The lens expresses high levels of *L-maf* (Figure 1I), *FoxC1* (Figure 1H), *δ-crystallin*

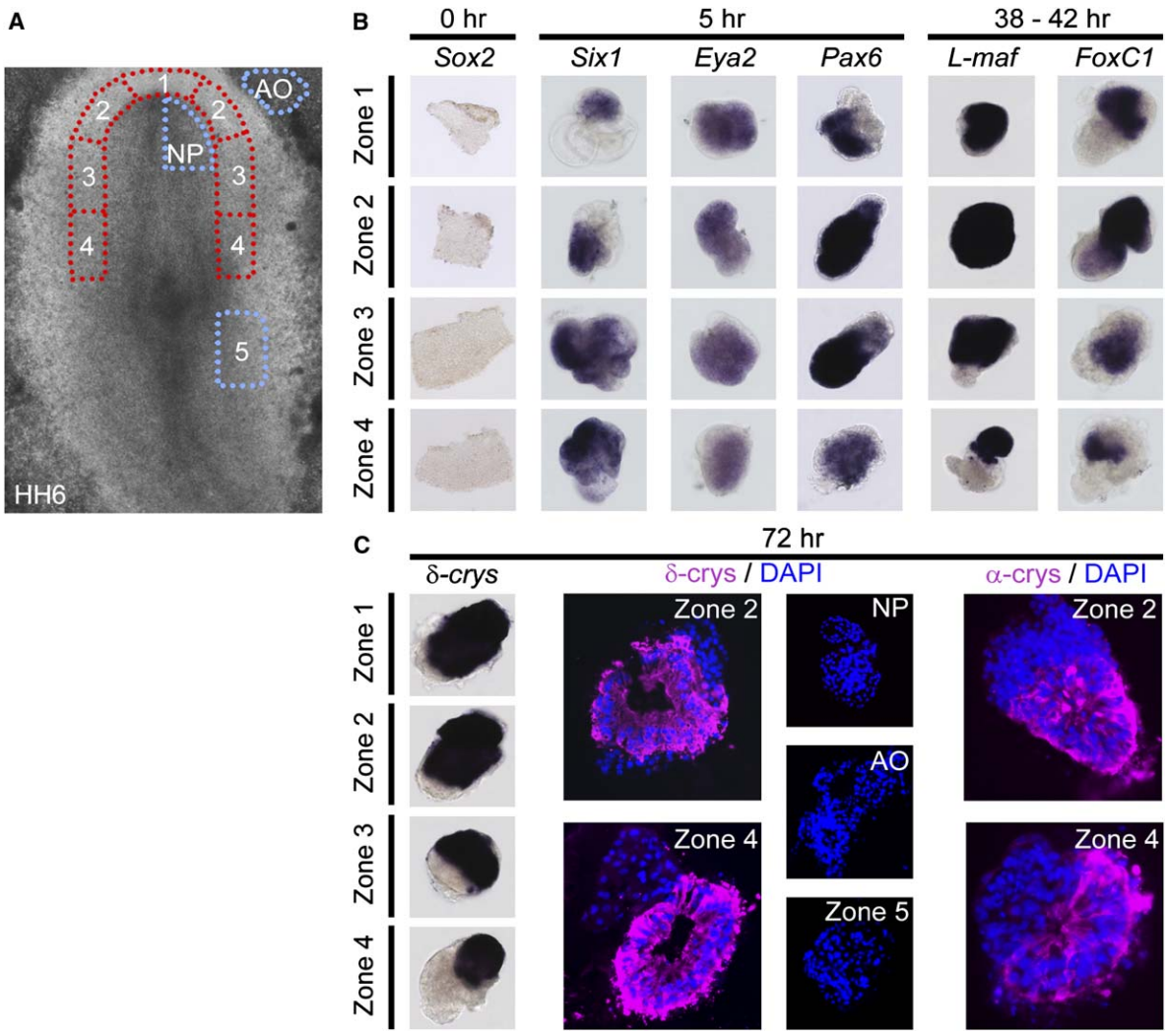

Figure 2. The Preplacodal Region at HH6 Is Specified as Lens

(A) Ectoderm was isolated from different regions of HH6 embryos. The preplacodal region (red) was divided into four zones (1–4). Nonpreplacodal ectoderm is highlighted in blue: the neural plate (NP), trunk ectoderm (zone 5), and area opaca (AO).

(B) Explants from zones 1–4 do not express *Sox2* at the time of dissection (column 1). After 5 hr, they express *Six1*, *Eya2*, and *Pax6* (columns 2–4). After 38–42 hr, they express the lens placode markers *L-maf* (column 5) and *FoxC1* (column 6).

(C) After 72 hr, zones 1–4 express *δ-crystallin* transcript (column 1), forming lens vesicle-like structures with elongated δ- and α-crystallin$^+$ cells surrounding a hollow lumen. Examples from zones 2 and 4 are shown. δ- and α-crystallin$^+$ cells were never found in zone 5, neural plate and area opaca explants.

(Figure 1J), and α-crystallin (Figure 1K) but lacks expression of any member of the Dlx family (Bhattacharyya et al., 2004), while the otic placode continues to be *Pax2*/*Sox10*$^+$ and the adenohypophysis expresses Lhx3 (Figure S1; Zhadanov et al., 1995).

To determine the properties of preplacodal cells, we first sought to determine their state of specification, defined as their autonomous tendency to develop according to a particular fate in the absence of extrinsic signals (Slack, 1991). The preplacodal region from head fold stage embryos was subdivided along the anterior-posterior axis into four regions, each containing a mixture of precursors for different placodes (zone 1: adenohypophyseal, olfactory; zone 2: olfactory, lens; zone 3: few lens, trigeminal, epibranchial, otic; zone 4: epibranchial, otic; Streit, 2004; Bailey and Streit, 2006). As controls, we also dissected anterior neural plate, posterior lateral ectoderm (zone 5), and extraembryonic ectoderm (Figure 2A). After short-term culture, zones 1–4 strongly express the preplacodal markers *Six1* (26/26) and *Eya2* (26/27) (Figure 2B), while zone 5 and extraembryonic ectoderm expresses neither (Table S1). Weak expression of *Eya2* is observed in the neural plate (9/9), while *Six1* is never expressed (0/7; Table S1). At this stage, future otic, trigeminal, olfactory, and adenohypophyseal cells are not yet specified and require additional signals to differentiate according to their normal fate (Table S1; Barabanov and Fedtsova, 1982; Baker et al., 1999; Groves and Bronner-Fraser, 2000). Surprisingly however, we find that all four preplacodal zones generate a large number of δ-crystallin$^+$ cells, often organized into lens-like bodies or lentoids, while the anterior neural plate, trunk, and extraembryonic ectoderm do not (Figure 2C; Table S1).

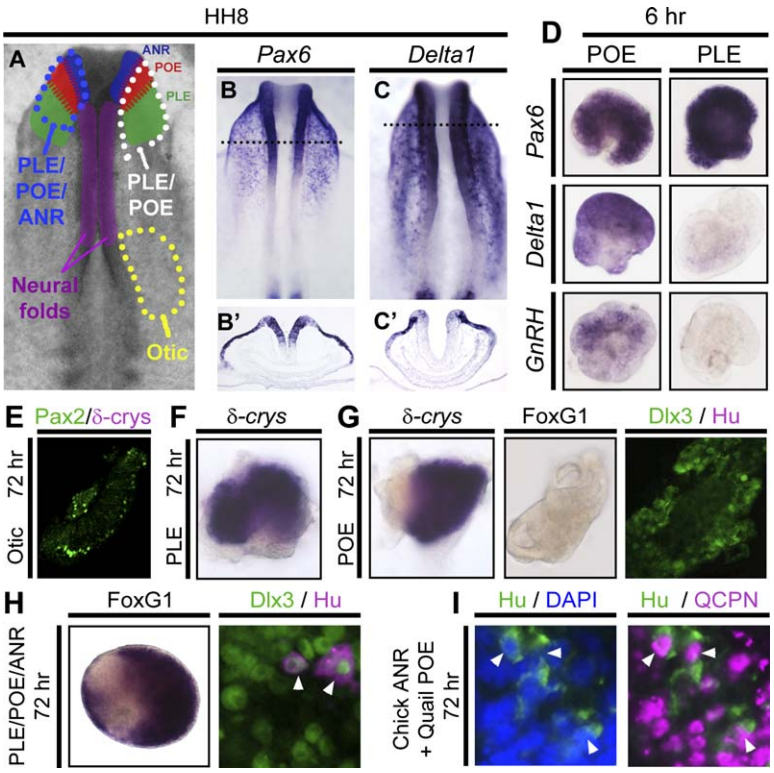

Figure 3. Lens and Olfactory Placode Specification at HH8

(A) Schematic illustration of explanted ectoderm from HH8 embryos. The PLE (green) overlaps slightly (green/red hatching) with the POE (red), which in turn partially overlaps (blue/red hatching) with the anterior neural ridge (ANR, dark blue). The neural folds (purple) give rise to the cranial neural crest. Explanted tissue is demarcated with dotted lines: PLE/POE (white), PLE/POE/ANR (blue), and presumptive otic placode (yellow).

(B and C) At stage HH8, *Pax6* remains expressed in the presumptive lens and olfactory region (B and B′), while *Delta1* begins to be restricted to the future olfactory cells (C and C′).

(D) PLE and POE explants are still specified to express *Pax6* after 6 hr. Only POE is specified to express *Delta1* and *GnRH*, while future lens cells are not.

(E) The otic region has lost lens specification, indicated by the absence of δ-crystallin and the presence of Pax2.

(F and G) In contrast, the PLE (F) and POE (G) continue to be lens specified, expressing *δ-crystallin*; the olfactory placode markers *FoxG1* (F) and Hu (G) are not detected in the POE. Dlx3 is expressed in some cells.

(H) Inclusion of the ANR (PLE/POE/ANR) leads to *FoxG1* expression. A subset of cells (arrowheads) coexpresses nuclear Dlx3 (green) and cytoplasmic Hu (magenta).

(I) Chick ANR induces Hu^+ cells (green) and quail tissue POE (magenta, arrowheads).

The results suggest that, although lens precursors lie at the zone 2/3 boundary and are absent from zones 1 and 4, all four regions are initially specified as lens. To investigate this in more detail, we examined zones 1–5 neural plate and extraembryonic ectoderm explants for the expression of molecular markers that define intermediate stages of lens placode formation. Virtually all zone 1–4 explants, irrespective of their origin, express the PLE marker *Pax6* after 5–6 hr, the transcription factors *L-maf*, *Foxc1*, and *Sox2* after 36–42 hr, and *Foxc1*, *δ-crystallin*, and *α-crystallin* after 72 hr in vitro, confirming their lens character (Figures 2B and 2C; Table S1). Thus, even cells from regions that normally never contribute to the lens, or express any lens marker, will form a lens when isolated from the embryo. These findings indicate that lens specification may represent a ground state for all sensory placodes and that to form other neurogenic placodes, lens character must be suppressed.

Restriction of Lens Specification at Early Somite Stages

To investigate when lens restriction begins, we assessed the specification of placode ectoderm at early somite stages (Figure 3A). At HH stage 8, the presumptive otic ectoderm has largely lost lens specification (3/10 with minimal δ-crystallin$^+$ cells), concomitant with the acquisition of otic properties (Figure 3E; Groves and Bronner-Fraser, 2000). In contrast, the anterior preplacodal region continues to be specified as lens. After 3 days of culture, ventral ectoderm fated as adenohypophysis expresses high levels of δ-crystallin (7/10, not shown; Barabanov and Fedtsova, 1982), as do PLE/POE (31/34), POE (4/4; Figure 3G), and PLE explants (4/4; Figure 3F). In contrast, olfactory placode markers are never expressed in cultured POE (0/9 *FoxG1*; 0/3 *Eya2*; 0/14 Hu^+ neurons; Figures 3G and 4B) or PLE (0/4 *FoxG1*; not shown).

After short-term culture, however, PLE/POE explants express the early olfactory markers *GnRH* (20/23; Figure 4A) and *Delta1* (6/8), as well as the PLE marker *Pax6* (30/30; Figure 4A), in distinct domains. When future olfactory and lens ectoderm are separated, *Pax6* is found in both tissues (POE: 6/6; PLE: 5/5; Figure 3D), while presumptive olfactory markers *GnRH* and *Delta1* are largely restricted to the POE only (POE: 6/7 $GnRH^+$, 5/7 $Delta1^+$; PLE: 0/11 $GnRH^+$, 0/8 $Delta1^+$; Figure 3D).

Thus, lens repression occurs sequentially and is first evident in the future otic territory. Although cells in the anterior preplacodal region are initially specified as early olfactory precursors ($GnRH^+/Delta1^+$), this does not translate into long-term establishment of mature olfactory character, suggesting that additional signals are required to confer stable olfactory fates.

FGF Signaling Suppresses Lens Potential of the Preplacodal Ectoderm, Promotes Olfactory Fate, and Is Required for Olfactory Placode Formation

At early somite stages, the POE is flanked medially by the anterior neural ridge (Figure 3A), which itself contains some olfactory progenitors (Bhattacharyya et al., 2004). The anterior neural ridge is an important signaling center that patterns the forebrain (Houart et al., 1998; Eagleson and Dempewolf, 2002; Gunhaga et al., 2003),

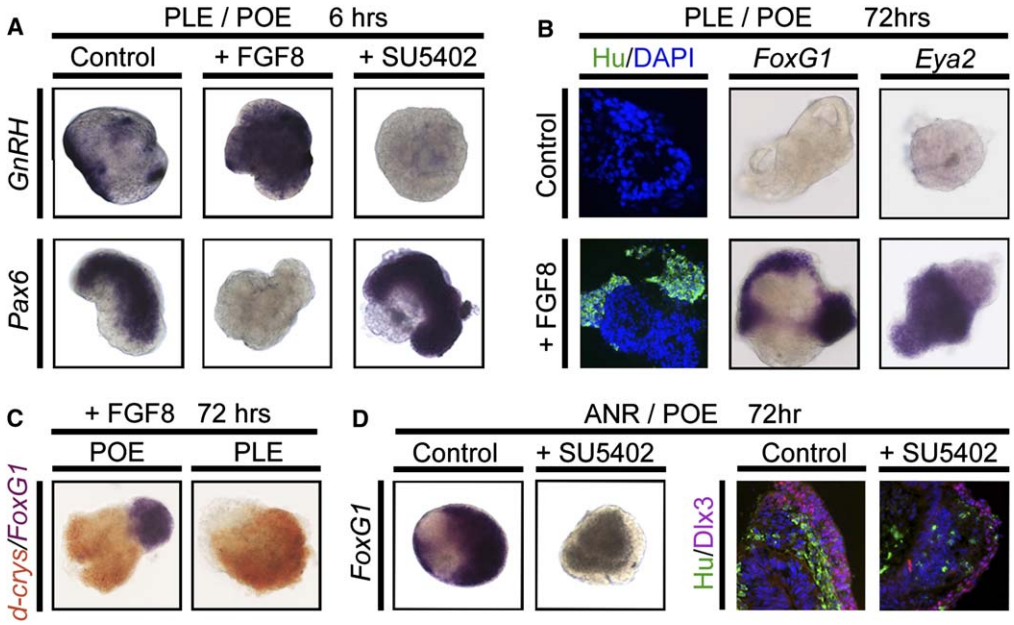

Figure 4. FGF8 Promotes Olfactory Placode Development

(A) FGF signaling is necessary and sufficient for the expression of the POE marker *GnRH*. After 6 hr, control PLE/POE explants show a small *GnRH*$^+$- and a large *Pax6*$^+$-expressing domain (column 1). In the presence of FGF8, explants are almost entirely *GnRH*$^+$, and *Pax6* is lost (column 2). Inhibition of FGF signaling by SU5402 results in a loss of *GnRH* expression and has no effect on *Pax6* (column 3).

(B) FGF8 is sufficient to induce olfactory placode markers. PLE/POE explants cultured in the presence of FGF8 for 72 hr (lower row) are positive for Hu, *FoxG1*, and *Eya2*, while control explants never express olfactory markers (upper row).

(C) POE, but not PLE, explants respond to FGF8 treatment by expressing *FoxG1* (blue). Note: even in the presence of FGF8, a large region of the POE continues to express δ-crystallin (red).

(D) FGF signaling is necessary for olfactory placode development. ANR/POE explants express *FoxG1*, Hu, and Dlx3 after 72 hr (control), but their expression is strongly reduced in the presence of SU5402. Dose-dependent reduction of neurons is shown in Table S2.

and thus its absence may be the reason for the lack of olfactory character in PLE/POE explants. Indeed, when they are cultured together with the anterior neural ridge, they strongly express *FoxG1* (4/4) and generate Hu$^+$ neurons, some of which are Dlx3$^+$ (3/5; Figure 3H).

We next asked whether the anterior neural ridge is the source of these neurons, or induces them in the adjacent ectoderm. To test this, we cultured chick anterior neural ridge together with quail POE; after 3 days, quail neurons (QCPN$^+$ Hu$^+$) are observed in most explants (9/13), demonstrating that the anterior neural ridge can induce neuronal cells in the olfactory ectoderm (Figure 3I).

The anterior neural ridge expresses a number of signaling molecules including FGFs (Rubenstein et al., 1998; Eagleson and Dempewolf, 2002). *FGF8* expression is first observed in this tissue when lens and olfactory precursors begin to segregate (Bhattacharyya et al., 2004) and is subsequently upregulated in the POE (Figures 5A, 5C, and 5C′), making FGF8 a good candidate to promote olfactory placode formation. In addition, we show the presence of phosphorylated ERK1/2 in the anterior neural folds and the adjacent olfactory territory (Figures 5B, 5B′, 5D, and 5D′). ERK1/2 activity increases in the POE, while the lens region is devoid of any pERK1/2, supporting the possibility that olfactory, but not lens precursors, receive FGF signals.

To test whether FGF signaling alone can activate early olfactory-specific genes and simultaneously suppress lens character, FGF8- or BSA (control)-coated beads were transplanted underneath the PLE of stage HH8 embryos. After 5 hr, *Pax6* expression is lost surrounding the FGF8, but not the control beads (7/7; Figures 5E and 5E′). In contrast, *Dlx5* (3/4) and *Delta1* (6/8) expression is expanded and *GnRH* (9/10) expression is induced in the vicinity of the FGF8 source (Figures 5F–5H and 5F′–5H′).

To confirm these results in vitro, PLE/POE explants were cultured in the presence or absence of FGF8 for 6 hr and analyzed for expression of *Pax6* and *GnRH*. We find that all control explants strongly express *Pax6* (11/11), but lose expression in the presence of FGF8 (3/13 with weak expression; Figure 4A). In contrast, while *GnRH* is only expressed in a fraction of cells in the controls (6/6), it is strongly upregulated and expressed in virtually all cells in FGF8-treated explants (8/8; Figure 4A). To test whether FGF signaling is sufficient to generate a mature olfactory placode from nonspecified PLE/POE ectoderm, explants were cultured in the presence of FGF8 for 72 hr. *FoxG1* (12/14), *Fgf8* (3/4; not shown), and *Eya2* (9/10) were strongly induced and Hu$^+$ neurons (13/16) were generated in nearly all explants (Figure 4B). Similar results were observed when HH6 explants from the preplacodal region (zones 1–3) were treated with FGF8, as *FoxG1* expression was induced (not shown; Table S1). When POE and PLE from HH8 embryos were separately exposed to FGF8, only future olfactory cells responded by switching on *FoxG1* (8/9) while PLE remained largely *FoxG1* negative (1/8), suggesting that although FGF signaling suppresses *Pax6* expression in the PLE, this is not sufficient to divert future lens cells toward olfactory fates (Figure 4C).

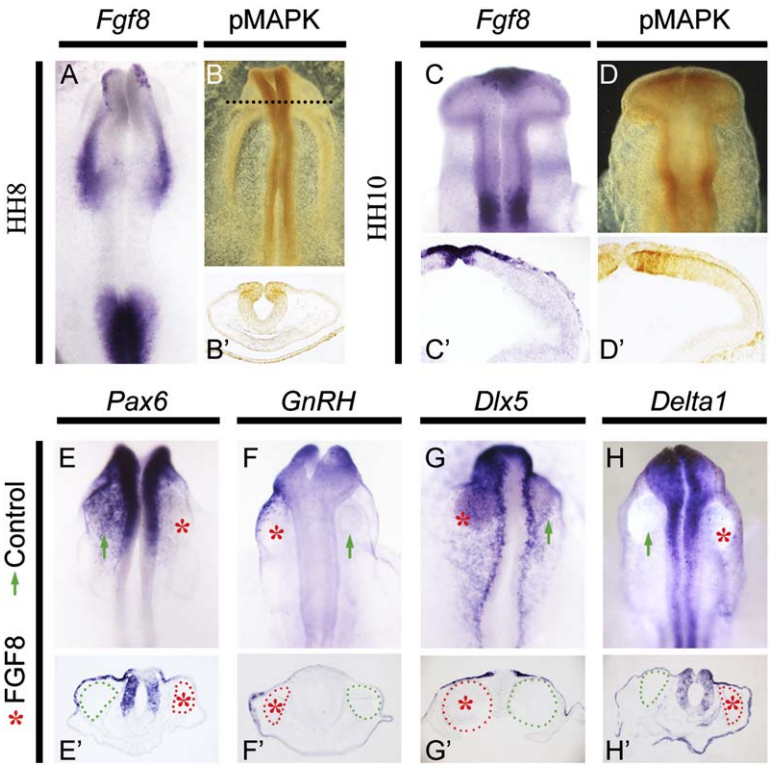

Figure 5. FGF8 Promotes POE Character at the Expense of PLE

(A–D) At HH8, *Fgf8* is expressed in the anterior neural ridge (A); pERK1/2 is observed in the same tissue and the adjacent POE (B and B′); the dotted line in (B) indicates the level of the section shown in (B′). By HH10, *Fgf8* is strongly expressed in the POE (C and C′), and pERK1/2 is detected in the same region (D and D′). (C′) and (D′) show horizontal sections through the embryos in (C) and (D), respectively.

(E–H) FGF8b-coated (red asterisk) and BSA-coated (green arrow) control beads were grafted beneath the PLE at HH8; after 5 hr, FGF8 downregulates *Pax6* (E and E′) and induces *GnRH* (F and F′). FGF8b also causes an expansion of *Dlx5* (G and G′) and *Delta1* (H and H′). (E′)–(H′) show cross-sections through the grafted beads in the embryos shown in (E)–(H), respectively.

Because FGF signaling has been implicated in otic (Riley and Phillips, 2003; Barald and Kelley, 2004) and adenohypophyseal (De Moerlooze et al., 2000; Ohuchi et al., 2000; Herzog et al., 2004) placode formation, we tested whether FGF treatment of PLE/POE explants promotes the expression of markers for other placodes. We find that trigeminal (Pax3; 0/20) and adenohypophyseal (Lhx3; 0/28) markers are never induced (not shown). In contrast, Pax2 is expressed in both control (3/14) and FGF8-treated explants (8/12), while in a few cases a small population of cells expresses the otic/trigeminal marker *Sox10* in the presence of FGF8 (2/11). Although Pax2 is generally considered to be an otic marker, we detect low levels of Pax2 in the olfactory placode (Figure S1; see also Ohyama and Groves, 2004). Together, these experiments demonstrate that FGF8 is sufficient to generate olfactory fates from preplacodal cells that are not specified as olfactory placode.

To investigate whether FGF signaling is necessary for the generation of olfactory cells, we took advantage of the FGF signaling inhibitor SU5402. When PLE/POE explants from early somite stages are cultured in the presence of 10 μM SU5402 for 6 hr, *GnRH* expression is lost or strongly reduced in 86% of explants (n = 28; Figure 4A). In contrast, *Pax6* expression is not affected (n = 14; Figure 4A). While POE cultured together with the anterior neural ridge strongly expresses *FoxG1* and generates Hu^+ neurons after 72 hr (Figure 4D), *FoxG1* expression is lost (n = 7) and the number of Hu^+ cells is reduced in the presence of SU5402 in a dose-dependent manner (Figure 4D; Table S2). Thus, FGF signaling is required for the specification of the presumptive olfactory territory, as well as for the formation of a mature olfactory placode.

Signals from Neural Crest Cells Restrict Lens Fates

The above results show that FGF signaling from the anterior neural ridge, possibly FGF8, promotes olfactory fates from cells in the preplacodal region. This raises the question of whether the anterior neural ridge and/or activation of the FGF pathway are sufficient to suppress lens character. When PLE/POE explants are grown in combination with the anterior neural ridge, δ-crystallin expression is still observed after long-term culture (not shown; n = 3). Likewise, PLE/POE (n = 24), PLE (n = 9), or POE (n = 8; Figure 4C) explants cultured in the continuous presence of FGF8 for 72 hr, or PLE/POE exposed to a 5 hr pulse of FGF8 at the beginning of the culture period (n = 6; not shown), continue to express high levels of δ-crystallin, indicating that FGF signaling alone is not sufficient to repress lens character.

Previous experiments suggested that at relatively late stages of development, nonlens head ectoderm retains lens specification (Barabanov and Fedtsova, 1982; Sullivan et al., 2004), indicating that active repression of lens formation is required to restrict the lens to its normal position next to the optic vesicle. To investigate which tissues mediate this process, we first cultured the PLE together with its underlying mesoderm and endoderm and found that *Pax6* (16 hr: 8/8; 24 hr: 10/10; Figure 6C) and *δ-crystallin* specification is retained (72 hr: 11/11; Figure 6C). However, when the neural folds contiguous with the PLE are also included, *Pax6* expression is reduced by 16 hr (7/7 with a small *Pax6* domain) and virtually absent after 24 hr (6/11 *Pax6* negative; 5/11 weak expression in few cells; Figure 6C). Likewise, *δ-crystallin* expression is lost or strongly reduced (n = 11; Figure 6C), suggesting that neural crest cells (derived from the folds) may inhibit lens specification.

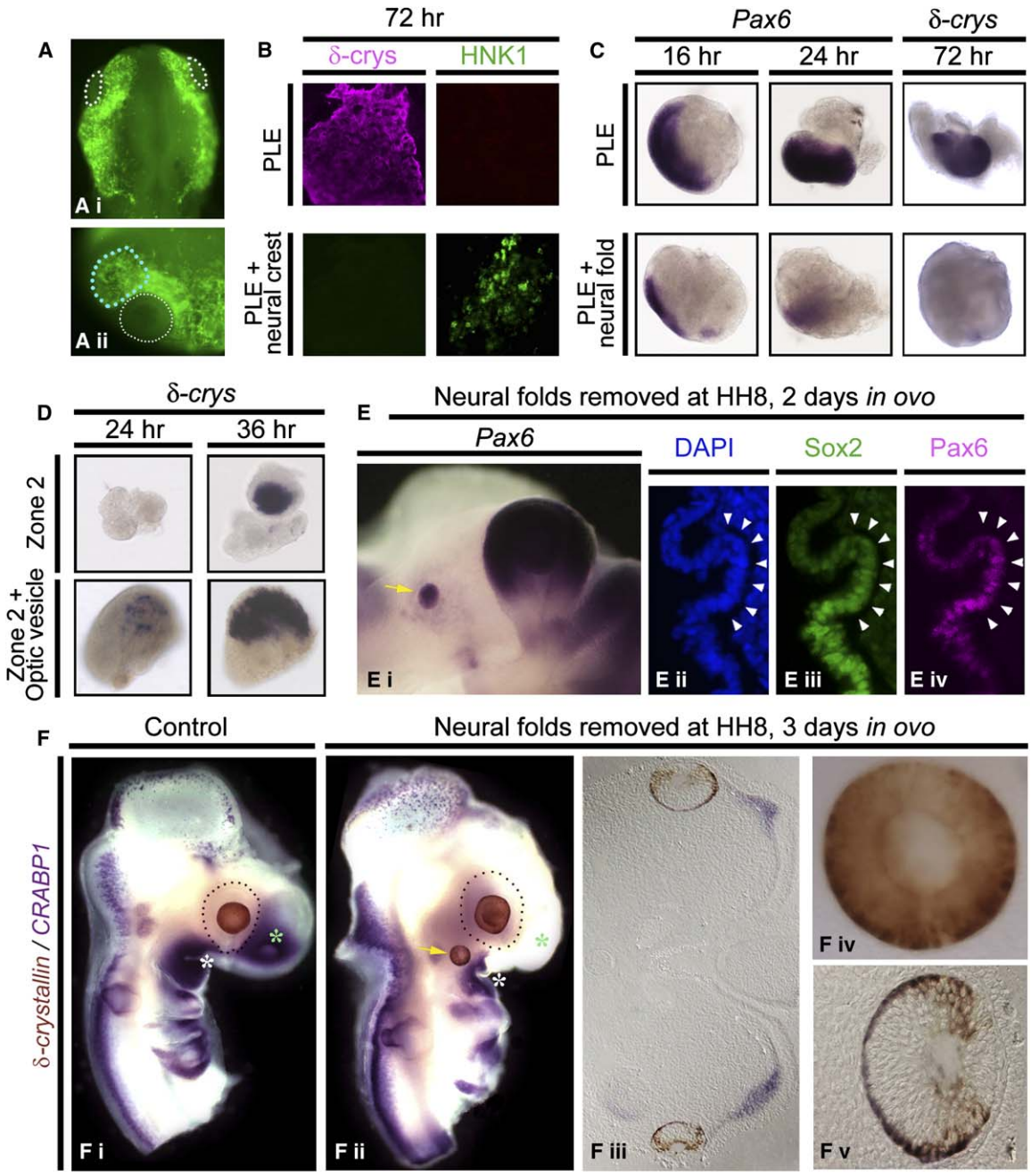

Figure 6. Neural Crest Cells Inhibit Lens Development, and Ectopic Lenses Form in Their Absence

(A) Dorsal (i) and dorsolateral (ii) views of an HH12 embryo stained for HNK1 labeling migrating neural crest cells (blue outline). White outline: lens.

(B) When cultured together with neural crest cells (HNK1$^+$; green), lens specification is inhibited in the PLE, indicated by the absence of δ-crystallin. Control PLE explants are δ-crystallin$^+$ (magenta).

(C) PLE cultured with underlying mesendoderm continues to express *Pax6* and *δ-crystallin*. When the adjacent neural folds, which generate neural crest cells, are included, *Pax6* expression is strongly downregulated after 16 and 24 hr, and *δ-crystallin* is lost completely.

(D) The optic vesicle enhances lens formation from preplacodal ectoderm. When zone 2 ectoderm (Figure 2) is cultured alone, *δ-crystallin* expression begins after 36 hr; however, when cocultured with the optic vesicle, expression begins in a few cells after 24 hr and is strongly expressed after 36 hr.

(E and F) Ablation of neural folds results in ectopic lens formation.

(E) Neural fold (Figure 3A) removal at HH8 leads to *Pax6* maintenance in the ectoderm posterior to the endogenous lens. After 2 days, Pax6 ([i], yellow arrow; [iv]) and Sox2 (iii) expression are found in ectopic lens pits.

(F) In controls (i), the lens (*δ-crystallin*$^+$; brown) is associated with the optic cup (black dotted outline). *CRABP1* (purple) marks neural crest cells of the frontonasal mass (green asterisk) and the branchial arches (white asterisk). Neural fold ablation (ii) leads to formation of ectopic lenses posterior to the endogenous lens. Absence of *CRABP1* indicates successful crest ablation (asterisks). A cross-section (plane indicated by the arrow in [ii]) shows that lenses are induced bilaterally (iii); their vesicle-like structure is apparent (iv), and cross-sections (v) reveal the absence of an optic cup.

This hypothesis is consistent with the distribution of migrating neural crest cells: they are in contact with head ectoderm posterior to the lens and migrate anteriorly across the optic stalk to form the frontonasal mesenchyme (Figure 6A). However, they are never in contact with future lens territory. To test this directly, we cocultured PLE with migratory neural crest cells (Figure 6B) or premigratory neural crest cells from cranial and trunk levels (Figure 3A). While control PLE explants show strong δ-crystallin expression after 3 days in vitro (14/14), no or reduced expression was observed in the presence of HNK1$^+$ neural crest cells from all three sources (migratory NC, 3/7 δ-crystallin$^+$; Figure 6B; neural folds, 1/12; trunk neural folds, 0/3). These experiments demonstrate that neural crest cells are an important source of lens inhibitory signals and ensure that lens formation is restricted to its normal position during embryogenesis.

These results predict that extra lenses should form in the absence of neural crest cells. Classical studies in amphibians (von Woellwarth, 1961) are consistent with this: partial ablation of the neural plate including future neural crest leads to the formation of ectopic lenses. We therefore removed the dorsal neural folds from fore- and midbrain levels at HH8 before the start of neural crest cell emigration. Successful neural crest cell ablation was determined by the lack of *CRABP1* expression in a subset of embryos (Figures 6Fi and 6Fii). Consistent with neural crest being a source of lens repression, we observe ectopic lenses: *Pax6* is expressed in a lens pit-like structure posterior to the endogenous lens 2 days after neural crest cell removal (3/6; Figure 6Ei). These ectopic lenses coexpress Sox2 and Pax6 (4/5; Figures 6Eii–6Eiv), which act together to activate δ-crystallin (Kamachi et al., 2001). While the lens vesicle markers *L-maf* and *δ-crystallin* are not expressed ectopically after 2 days (*L-maf*: 0/3 lentoids; *δ-crystallin*: 0/5 lentoids), they are both present 3 days after neural crest ablation (*L-maf*: 3/4; not shown; *δ-crystallin*: 6/17; Figures 6Fii–6Fv). Thus, although development of ectopic lenses is somewhat delayed compared to the endogenous lens, the molecular events leading to their formation appear to be similar.

Ectopic lenses always form in the absence of an optic vesicle (Figures 6Fii and 6Fv), and this may be one reason for their slow development, as signals from the optic vesicle are known to promote various aspects of lens formation (Furuta and Hogan, 1998; Kamachi et al., 1998; Wawersik et al., 1999). To confirm its lens-promoting role, we combined preplacodal ectoderm from stage HH6 with an optic vesicle from stage HH9. After 36 hr in vitro, the optic vesicle alone never expresses *δ-crystallin* (n = 4; not shown) and does not induce its expression in posterior-lateral ectoderm (zone 5 in Figure 2A; n = 5; not shown). *δ-crystallin* expression in zone 2 preplacodal explants is first observed after 36 hr in culture (24 hrs: 0/3; 36 hr: 3/3; Figure 6D); however, in the presence of the optic vesicle, it is already detected in 2/3 explants after 24 hr and present at high levels in a large domain after 36 hr (4/4; Figure 6D). Together, these results suggest that positive signals from the optic vesicle act in concert with lens-repressing signals from neural crest cells to ensure correct positioning of the lens next to the future retina.

In summary, our results suggest that one of the key processes in placode induction is the restriction of lens character. In the preplacodal region, cells initially possess common properties and are specified as lens. FGF signaling from surrounding tissues initiates lens repression and simultaneously imparts otic and olfactory character. Subsequently, signals emanating from neural crest cells are required for continued repression of lens fates.

Discussion

Lens: Uniting All Sensory Placodes

At neurula stages, precursors for all sensory placodes are localized in a continuous band of head ectoderm, the preplacodal region, which is distinct from future neural, neural crest, and epidermal territories, and expresses a unique set of genes belonging to the Six, Eya, and Dach families (Streit, 2004; Bailey and Streit, 2006; Schlosser, 2006). It has previously been shown that different signaling pathways converge to confer preplacodal character to the ectoderm and to position the preplacodal region to the head ectoderm surrounding the neural plate (Brugmann et al., 2004; Glavic et al., 2004; Ahrens and Schlosser, 2005; Litsiou et al., 2005). In chick, the head mesoderm underlying the placode territory is sufficient and required for its induction and expresses FGFs as well as BMP and Wnt inhibitors (Litsiou et al., 2005). These factors act to protect preplacodal cells from antagonistic influences emanating from surrounding tissues. These include a high level of Wnt from mesoderm lateral and posterior to the preplacodal region and from the neural folds flanking it medially, as well as BMP activity from the nonneural ectoderm and the neural folds. FGFs and modulation of the BMP pathway have also been implicated in the formation of the preplacodal region in *Xenopus* (Brugmann et al., 2004; Glavic et al., 2004; Ahrens and Schlosser, 2005).

There has been an ongoing debate about whether or not the induction of the preplacodal region is a crucial and common step in the sequence of events leading to the formation of individual placodes and, if so, which developmental state preplacodal cells represent in this sequence (Bailey and Streit, 2006). One view holds that the inductions of placodes with individual identity are independent events mediated by distinct tissues and localized signals (Graham and Begbie, 2000; Begbie and Graham, 2001). In contrast, another model suggests that an initial step involves the induction of a common state (termed generic placode state or placode bias), from which precursors for different placodes later diversify (Jacobson, 1963; Torres and Giraldez, 1998; Streit, 2004; Bailey and Streit, 2006). The cellular and structural diversity of placode derivatives has been used to argue against the second model, as has the finding that some placodes (trigeminal and epibranchial; Stark et al., 1997; Baker et al., 1999; Begbie et al., 1999) appear to be induced in a single step by distinct tissues and presumably different signals (Graham and Begbie, 2000; Begbie and Graham, 2001). However, in both cases, the test tissue used to assay for placode induction lies within the preplacodal region, and it cannot be excluded that it had already received initial appropriate inducing signals. More recently, the functional importance of the

preplacodal region has been demonstrated in a study on otic induction which shows that only cells that have acquired preplacodal properties are able to respond to otic-inducing signals (Martin and Groves, 2006). Here we provide, to our knowledge, the first evidence that sensory placodes indeed share common properties before they diverge. Even preplacodal cells that are not fated to become lens and normally never express lens-specific genes go through the sequence of gene expression characteristic of lens tissue and form lentoids when isolated from the embryo.

This observation is supported by previous findings that argued for lens specification in some nonlens territories of the head. In particular, presumptive adenohypophyseal and lens ectoderm seem to represent an "equivalence" domain, as isolated future adenohypophysis forms lens tissue (at least in the presence of serum and embryo extract) (Barabanov and Fedtsova, 1982; Sullivan et al., 2004). Additionally, in the absence of Hedgehog signaling, ectopic midline lenses have been reported in fish and chick (Ede and Kelly, 1964; Kondoh et al., 2000; Varga et al., 2001; Sbrogna et al., 2003; Dutta et al., 2005). Elsewhere in the head, lens specification is also observed in the ectoderm just posterior to the PLE (Barabanov and Fedtsova, 1982; Sullivan et al., 2004). Together with the existing literature, our observations strongly suggest that lens is a "default" state of all sensory placodes, and that development of other placodes not only requires positive inducing signals but also lens repression.

A Two-Step Model for Lens Restriction: Initiation of Lens Suppression by FGFs and Continued Repression by Neural Crest Cells

Here we provide evidence that FGF8 transiently represses lens specification, as indicated by the loss of *Pax6* expression in the PLE. By contrast, FGF signaling has been implicated in the induction of nonlens sensory placodes. In amniotes and fish, FGF signaling is required for otic induction, while FGF misexpression induces an ectopic otic placode in the preplacodal region (Riley and Phillips, 2003; Barald and Kelley, 2004). This pathway is also involved in specification of the adenohypophysis. In fish, FGF3 from the ventral diencephalon is required for the expression of early adenohypophysis markers (Herzog et al., 2004), while in mouse the loss of FGF10 (Ohuchi et al., 2000) or FGFR2IIIb (De Moerlooze et al., 2000) leads to early defects in the adenohypophysis. Our results reveal that FGF8 is also required and sufficient to induce mature olfactory character in preplacodal cells. Tissues adjacent to the PLE appear to lack FGF expression (Karabagli et al., 2002a) and thus presumptive lens markers are maintained. Together, these observations suggest that activation of the FGF pathway is a key event that initiates lens repression in the preplacodal ectoderm.

Although FGF signaling is important for olfactory development, our results also show that FGF alone is not sufficient to prevent lens formation in long-term cultures. Rather, other factors are required to restrict lens to the appropriate position in the embryo. Sullivan and colleagues previously showed that head mesenchyme can repress lens specification; however, the identity of the cells responsible—neural crest cells or paraxial mesoderm—remained unclear (Sullivan et al., 2004). Our results unequivocally identify neural crest cells as the endogenous source of lens-repressing signals. While crest-free head mesoderm does not repress lens formation, neural crest cells effectively abolish lens-specific gene expression and the formation of lentoids. We also show that, remarkably, ectopic lenses form when neural crest cells are ablated. This finding confirms classical studies in amphibian embryos where removal of parts of the anterior neural plate also led to the formation of extra lenses (von Woellwarth, 1961).

It is worth noting that ectopic lenses are never observed in, for example, the olfactory territory, but only in a particular position posterior to the endogenous lens. This ectoderm maintains *Pax6* and *Sox2* at low levels until after the lens placode has formed (Kamachi et al., 1998), which may account for its latent lens-forming ability. Because these proteins regulate each other's expression during lens placode formation (Furuta and Hogan, 1998; Wawersik et al., 1999; Kamachi et al., 2001), it is possible that either or both genes are targets for the neural crest-derived inhibitory signal(s). Although the mechanisms of this inhibition are unknown, our data support a role of the neural crest in keeping *Pax6* expression at low levels, thereby preventing the formation of extraocular lenses.

Regarding the identity of these signals, it is interesting that loss of β-catenin function in the extraocular ectoderm leads to the formation of small ectopic lentoids, raising the possibility that Wnts may have lens-repressive function (Smith et al., 2005). However, preliminary data from our laboratory indicate that Wnt activity alone is not sufficient to repress lens specification in preplacodal ectoderm (A.P.B. and A.S., unpublished observation). In the optic vesicle, *Pax6* appears to be regulated by TGF-β family members (Fuhrmann et al., 2000), which thus may be good candidates to cooperate with Wnts in lens repression.

Together, our findings suggest a two-step model for the restriction of lens specification in the preplacodal region (Figure 7). Before neural crest migration, activation of the FGF pathway in nonlens preplacodal ectoderm initiates the repression of lens, leading to the inhibition of the early lens marker *Pax6* and to the acquisition of otic and olfactory character. Subsequent signals from neural crest cells that come to underlie the head ectoderm posterior to the lens reinforce its suppression and ensure correct positioning of the lens next to the optic vesicle.

Lens Induction: An Early Start

Since Spemann's original experiments on lens induction (Spemann, 1901), this process has attracted continuous interest but still remains partially unresolved (Lang, 2004). Experiments in *Xenopus* led to the idea that lens induction begins during gastrulation with the acquisition of lens competence, followed by a state of lens "bias," lens induction, and placode formation (Henry and Grainger, 1987, 1990; Zygar et al., 1998). Likewise, in amniotes, lens formation has been subdivided into a preplacode and a placode phase (Li et al., 1994; Lang, 2004), highlighting that processes occurring prior to optic vesicle contact with the PLE and placode formation play an essential role in lens development.

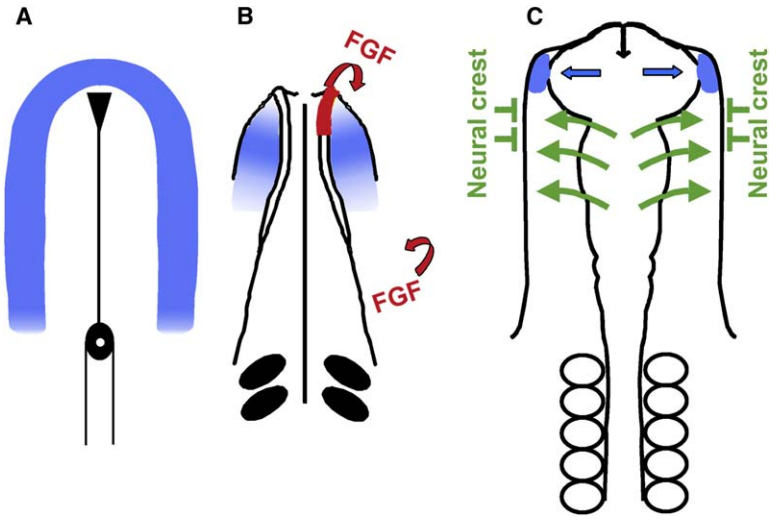

Figure 7. Lens Specification as a Placode State and a Two-Step Model for Its Restriction

(A) At neurula stages, placode precursors share a common ground state that is specified as lens and develop into lentoids when cultured in isolation.

(B) Around early somite stages, activation of the FGF pathway results in promoting otic and olfactory character from lens-specified ectoderm.

(C) After the beginning of their migration, neural crest cells provide additional lens-suppressing signals that restrict lens formation to its normal position together with lens-promoting signals from the optic vesicle.

Our results reveal that lens specification already occurs at neurula stages, long before optic vesicle contact. Thus, this tissue has received all the signals required to initiate the lens program and to develop into lens-like, δ-crystallin-expressing lentoids autonomously. These findings suggest that the initial induction of lens fate may be completed early in development and that subsequent processes restrict, position, and enhance lens formation. We show that FGF signaling initiates lens restriction and that the neural crest is a potent lens repressor. Does the optic vesicle have a primarily mechanical role, impeding the migration of neural crest cells beneath the PLE (McKeehan, 1951; Sullivan et al., 2004)? In rodents, lens specification appears to occur only upon optic vesicle contact (Fisher and Grainger, 2004; Lang, 2004) and, unlike in the chick (Hilfer, 1983), mesenchymal cells are initially found between the vesicle and the PLE (Kaufman, 1979; Furuta and Hogan, 1998). This argues that the optic vesicle is important to eliminate contact between neural crest cells and the lens territory.

However, a number of studies have provided evidence that the optic vesicle does actively promote lens placode formation, via FGF and BMP signaling, and is essential for upregulation of lens-specific genes (Furuta and Hogan, 1998; Kamachi et al., 1998; Wawersik et al., 1999; Faber et al., 2002). In agreement with these findings, our experiments show that the optic vesicle enhances *δ-crystallin* expression in preplacodal explants. It is therefore likely that a balance between promoting and inhibiting signals from the optic vesicle and the neural crest, respectively, ensures correct positioning of the lens next to the future retina.

Multiple Roles for FGF Signaling in Olfactory Development

Here we identify FGF as a signal that initiates the formation of the olfactory placode: activation of the FGF pathway is required for its formation and sufficient to induce it from cells within the preplacodal region. Our results show that FGF8 from the anterior neural ridge acts at early somite stages to induce first the expression of genes specific for the presumptive olfactory region and subsequently late olfactory markers. The initial expression of FGF8 in the anterior neural ridge correlates well with the time when segregation of lens and olfactory precursors is observed and Pax6 expression is lost from future olfactory cells (Bhattacharyya et al., 2004). In the absence of FGF signaling, preplacodal ectoderm is not specified as presumptive olfactory nor does it form a mature olfactory placode. In mouse, FGF8 has been implicated in patterning of both the olfactory placode at placode stages and the subjacent frontonasal mesenchyme (LaMantia et al., 2000; Firnberg and Neubuser, 2002). While FGF inhibition leads to a reduction in medially located N-CAM-positive neurons, FGF8 treatment has the opposite effect. Whether FGF acts directly on placode cells or indirectly via the underlying mesenchyme is unclear, though our results argue in favor of the former hypothesis. Another recent study showed that loss of FGF8 function in the anterior forebrain and facial ectoderm including the olfactory placode leads to abnormal olfactory morphogenesis due to increased apoptosis (Kawauchi et al., 2005). In addition, primary olfactory neural stem cells are lost, leading to the absence of virtually all olfactory placode-derived neurons, suggesting a role for FGF in maintaining this precursor cell population. Thus, FGF signaling plays multiple roles during olfactory placode development and differentiation. An early function is the induction of the presumptive olfactory epithelium (this study), while later it is involved in patterning (LaMantia et al., 2000), cell survival, and stem cell maintenance (Kawauchi et al., 2005).

Conclusions

Here we present evidence supporting the view that all sensory placodes initially share a common developmental program: they are specified as lens. These findings imply that by early neurula stages, preplacodal cells have received sufficient information to initiate the lens program autonomously. Subsequently, lens fate is repressed in precursors for other placodes, and we propose that FGFs play an important role in initiating this process while simultaneously imparting properties characteristic of other placodes. After beginning their migration, neural crest cells provide additional lens-repressing signals, in the absence of which extra lenses

develop. Thus, induction of all cranial placodes is intimately linked with the restriction of lens fates.

Experimental Procedures

Embryo Cultures and Surgical Manipulations

Fertile hens' eggs (Henry Stewart; AA Laboratories) were incubated at 38°C for 24–72 hr to harvest embryos between Hamburger & Hamilton (HH; Hamburger and Hamilton, 1951) stage 6 and 16. Whole embryos were maintained in New Culture (Stern and Ireland, 1981) for 5–6 hr after grafting beads beneath the PLE. Heparin beads were coated with 100 μg/ml FGF8 in PBS containing 0.1% BSA for 1 hr on ice, and then washed and grafted. Neural crest cell ablation was performed in ovo at HH8 before the beginning of their migration using tungsten needles to remove the neural folds and the dorsal half of the neural tube from the posterior forebrain and the midbrain. Embryos were maintained in ovo for another 2–3 days.

Explant Cultures

HH6 and HH8 embryos were harvested in Tyrode's saline. Tissues to be explanted (see Figures 1–3) were dissected using tungsten needles and freed from underlying mesendoderm using 0.05% dispase. Explants were kept on ice before being cultured in collagen gels (Streit et al., 1997) prepared in medium 199 containing N2 supplement. At stage HH8, the POE is fairly small and the boundary between POE and PLE is still ill defined (Bhattacharyya et al., 2004). Although POE explants may contain a small number of lens precursors, for simplicity we refer to these explants as POE.

Explants were kept in vitro for 6–72 hr in the presence or absence of 1 μg/ml FGF8 (R&D) and 2.5 μM, 5 μM, or 10 μM SU5402 (Calbiochem) diluted from a 1 mM stock in DMSO or the appropriate amount of DMSO. Explants were fixed in 4% paraformaldehyde in PBS for 15 min for immunohistochemistry or overnight at 4°C for in situ hybridization.

In Situ Hybridization and Immunohistochemistry

Embryos and explants excised from collagen gels were processed for in situ hybridization as previously described (Streit et al., 1998). The following plasmids were used: Dlx5 (McLarren et al., 2003), Pax6 (Bhattacharyya et al., 2004), Delta1 (a gift from D. Henrique), GnRH (a gift from Dr. Ian Dunn), δ-crystallin (a gift from F. de Pablo), FoxG1 (a gift from P. Vogt), FoxC1 (Buchberger et al., 1998), Fgf8 (a gift from J. Izpisúa Belmonte), CRABP1 (a gift from A. Graham), and Pax2 (a gift from M. Goulding).

Immunohistochemistry was performed on cryosections (Bhattacharyya et al., 2004) using polyclonal antibodies against chick δ- and α-crystallin (gifts from J. Piatigorsky), mouse Pax2 (Zymed), chick Dlx3, human Sox2 (R&D; a gift from C. Stern), and monoclonal antibodies against the neuronal marker HuC/D (Molecular Probes), HNK1 (Developmental Hybridoma Bank, Iowa State University), Pax3 (Developmental Hybridoma Bank), Lhx3 (a gift from J. Briscoe), and pERK1/2 (Sigma). The appropriate Alexafluor 488- and 594-coupled secondary antibodies were purchased from Molecular Probes; nuclei were stained by DAPI (Molecular Probes).

To quantify the number of Hu^+ neurons in each explant, digital images from each section were taken after immunostaining; for each section, the total number of cells was determined by counting nuclei ($DAPI^+$) and the number of Hu^+ cells was determined by counting nonneural ectoderm-derived $Hu/DAPI^+$ cells. An unpaired t test was performed to determine the statistical significance between different conditions.

Supplemental Data

Supplemental Data include one figure and two tables and are available at http://www.developmentalcell.com/cgi/content/full/11/4/505/DC1/.

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