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Role of BMP signaling and the homeoprotein iroquois in the specification of the cranial placodal field

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Abstract

Different types of placodes originate at the anterior border of the neural plate but it is still an unresolved question whether individual placodes arise as distinct ectodermal specializations in situ or whether all or a subset of the placodes originate from a common preplacodal field. We have analyzed the expression and function of the homeoprotein *Iro1* in *Xenopus* and zebrafish embryos, and we have compared its expression with several preplacodal and placodal markers. Our results indicate that the *iro1* genes are expressed in the preplacodal region, being one of the earliest markers for this area.

We show that an interaction between the neural plate and the epidermis is able to induce the expression of several preplacodal markers, including *Xiro1*, by a similar mechanism to that previously shown for neural crest induction. In addition, we analyzed the role of BMP in the specification of the preplacodal field by studying the expression of the preplacodal markers *Six1*, *Xiro1*, and several specific placodal markers. We experimentally modified the level of BMP activity by three different methods. First, we implanted beads soaked with noggin in early neurula stage *Xenopus* embryos; second, we injected the mRNA that encodes a dominant negative of the BMP receptor into *Xenopus* and zebrafish embryos; and third, we grafted cells expressing chordin into zebrafish embryos. The results obtained using all three methods show that a reduction in the level of BMP activity leads to an expansion of the preplacodal and placodal region similar to what has been described for neural crest regions.

By using conditional constructs of *Xiro1*, we performed gain and loss of function experiments. We show that *Xiro1* play an important role in the specification of both the preplacodal field as well as individual placodes. We have also used inducible dominant negative and activator constructs of Notch signaling components to analyze the role of these factors on placodal development. Our results indicate that the a precise level of BMP activity is required to induce the neural plate border, including placodes and neural crest cells, that in this border the *iro1* gene is activated, and that this activation is required for the specification of the placodes.

Keywords: Preplacodal field; Placodes; Neural crest; Placode induction; *Iro1*; *Six1*; Notch; Delta; BMP gradient; Neurogenesis; *Xenopus*; Zebrafish

Introduction

Cranial placodes are discrete regions of thickened epithelium that form in characteristic positions in the head of vertebrate embryos. They contribute to the formation of nose, eyes, ears, lateral line, and cranial sensory ganglia (reviewed in Baker and Bronner-Fraser, 2001). Like the neural crest, which forms the rest of the peripheral nervous system (reviewed in LaBonne and Bronner-Fraser, 1999), cranial placodes form at the border region of the neural plate and the epidermis. Individual placodes are often described as completely separate entities, unrelated by lineage. However, there are substantial morphological and molecular data from several different species to support the existence of a general preplacodal domain within the cranial neural plate border at the gastrula and neurula stages (Baker and Bronner-Fraser, 2001). In amphibians, placodes form as a thickening of the inner layer of the ectoderm (Northcutt and Brandle, 1995; Schlosser and Northcutt, 2000). The homeobox gene *Six1* is expressed in *Xenopus* at the late neural fold stage

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(stage 18; Nieuwkoop and Faber, 1967) in a continuous band surrounding the anterior neural plate (Ghanbari et al., 2001; Pandur and Moody, 2000), and is apparently one of the earliest molecular markers for the preplacodal domain.

Given the existence of a preplacodal ectoderm field at the border of the anterior neural plate and that the neural crest is also specified at this border, although at a more posterior location, it seems reasonable to propose that some of the mechanisms that specify the preplacodal ectoderm could be similar to those that specify the neural crest region. Progress has been made in recent years concerning the cellular and molecular mechanisms that induce neural crest in chick, *Xenopus* and zebrafish; this evidence has been extensively reviewed (Aybar and Mayor, 2002; Garcia-Castro and Bronner-Fraser, 1999; LaBonne and Bronner-Fraser, 1999; Mayor and Aybar, 2001; Mayor et al., 1999). It has been shown that an interaction between the neural plate and the epidermis is sufficient to induce neural crest (Dickinson et al., 1995; Mancilla and Mayor, 1996; Selleck and Bronner-Fraser, 1995). Several molecules like BMPs, Wnts, FGF, and retinoic acid have been implicated in this induction (Garcia-Castro et al., 2002; Monsoro-Burq et al., 2003; Villanueva et al., 2002). It has also been proposed that in *Xenopus* and zebrafish, a precise level of BMP, intermediate to the levels required to specify neural plate and epidermis, is required to specify neural crest at the border of the neural plate (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998; Tribulo et al., 2003). The participation of BMP in neural crest induction has also been shown in chick (Liem et al., 1995). The Notch/Delta cell signaling pathway has been proposed to be involved in neural crest specification in chick, zebrafish, and *Xenopus* (Cornell and Eisen, 2000, 2002; Endo et al., 2002; Glavic et al., 2004). Moreover, in the chick, this system influences the induction of the neural crest by up-regulating BMP transcription (Endo et al., 2002) while, in *Xenopus* embryos, Notch/Delta signaling seems to regulate neural crest development by inhibition of BMP4 transcription (Glavic et al., 2004).

In summary, neural crest induction is a multistep process, involving signals from the epidermis, the neural plate, and the mesoderm. In this work, several of the cellular and molecular factors that are involved in the early induction of the neural crest were tested to analyze whether they also participate in the specification of the preplacodal field.

The *iro* genes belong to the TALE class of homeobox-encoding proteins (Bürglin, 1997). Since their discovery as prepattern factors required for proneural and provein gene activation (Gómez-Skarmeta and Modolell, 1996; Leyns et al., 1996), they have been shown to participate in many developmental processes (reviewed in Cavodeassi et al., 2001). In *Drosophila*, the *iro* genes are required for the formation of the dorsal eye, head, and mesothorax (Cavodeassi et al., 2000; Diez del Corral et al., 1999). In *Xenopus laevis*, they participate in the specification of the Spemann Organizer (Glavic et al., 2001), the neuroectoderm (Gómez-Skarmeta et al., 2001), the midbrain–hindbrain boundary (Glavic et al., 2002), and the neural crest (Glavic et al., 2004). During late development, the *iro* genes participate in patterning the *Drosophila* imaginal discs and the vertebrate neuroectoderm and heart (Bao et al., 1999; Bellefroid et al., 1998; Bruneau et al., 2001; Cavodeassi et al., 1999; Christoffels et al., 2000; Gómez-Skarmeta and Modolell, 1996; Gómez-Skarmeta et al., 1998; Kehl et al., 1998; Leyns et al., 1996).

Since the identification of the first *iro* gene in vertebrates, it was proposed that one of the expression domains corresponded to the placodal region (Cheng et al., 2001; Gómez-Skarmeta et al., 1998); however, this observation has not been functionally tested. In this work, we show that *iro* gene expression at the border of the neural plate in *Xenopus* and zebrafish corresponds to part of the preplacodal domain, being the earliest marker known for that region. We also show that the expression of *iro* persists in some placodes once they are segregated. By making use of dominant negative and activated forms of the *iro* gene, we show that it plays an important role in the development of the preplacodal field as well as on specific placodes. In addition, we show that an interaction between the neural plate and the epidermis is enough to induce the preplacodal domain and that BMPs are also involved in this induction in *Xenopus* and zebrafish embryos. Finally, we analyze the role that Notch signaling has on placodal specification.

Materials and methods

Xenopus and zebrafish embryos and micromanipulation

Xenopus embryos were obtained as described previously (Gómez-Skarmeta et al., 1998) and staged according to Nieuwkoop and Faber (1967). Dissections and conjugates were performed as described by Mancilla and Mayor (1996). Zebrafish embryos were raised and maintained under standard conditions (Westerfield, 1995).

Xenopus Chordin mRNA was made by in vitro transcription using linearized XChSp6 plasmid (kind gift of J. Larrain) and transcribing with Sp6 polymerase. One cell stage zebrafish embryos were injected through the chorion with approximately 500 pg RNA and fluorescein-dextran (FLDx, Molecular Probes) and they were grown at 28°C until the blastula stage. At this stage, embryos were enzymatically dechorionated with pronase and were disrupted by pipetting in Ca^{2+}-free Holtfreter's medium. Cells were washed in this solution by centrifuging three times at 1000 × *g* for 1 min. Cells were loaded into a pulled Pasteur pipette and about 50 cells were transplanted into host embryos at 70% epiboly. Transplanted embryos were grown at 28°C, fixed at tailbud stage (10 hpf), and processed for in situ hybridization and antifluorescein antibody labeling.

Morpholino injections in zebrafish

To inhibit mesoderm formation, the following combination of two different *spadetail* (*spt*) and *notail* (*ntl*) morpholinos (MOs) was injected in one-cell stage zebrafish embryos. The *spt* MO was a kind gift of Sharon Amacher and Bruce Draper. The mix of MOs was a kind gift of Kate Lewis. The mix used here had final concentrations of *ntl* MO, 1 mg/ml; *spt* MO#2, 0.075 mg/ml; *spt* MO#1, 0.75 mg/ml. The *ntl* MO sequence has been previously published (Nasevicius and Ekker, 2000) and the *spt* MO sequences are:

spt MO #1, 5-AGCCTGCATTATTTAGCCTTCTCTA-3; and
spt MO #2, 5'-GATGTCCTCTAAAAGAAAATGTCAG-3.

In vitro RNA synthesis and microinjection of mRNAs

The *Xiro1*, Notch, Delta, *Su(H),* and BMPR dominant negative constructs have all been described previously (Aybar et al., 2003; Glavic et al., 2001; Marchant et al., 1998; McLaughlin et al., 2000). Briefly, two *Xiro1* contructs were used here: an inducible dominant negative, called *HDGR*, which is composed of the *Xiro1* homeodomian (*HD*) bound to the glucocorticoid binding domain (*GR*); and the inducible *Xiro1*, named *HDEnGR*, in which the *Xiro1* homeodomain was fused to the *Drosophila Engrailed* repressor element (*EnR*) and to the glucocorticoid binding domain (*GR*). All cDNAs were linearized and transcribed, as described by Harland and Weintraub (1985) with the GTP cap analog (New England Biolabs). SP6, T3, or T7 RNA polymerases were used. After DNAse treatment, RNA was extracted using phenol–chloroform, column purified, and precipitated with ethanol. For injections and lineage tracing, mRNAs were resuspended in DEPC–water and coinjected with FLDx (Molecular Probes) using 8–12 nl needles in two-cell stage embryos as described in Aybar et al. (2003). Dexamethasone treatment was performed as described by Kolm and Sive (1995). Dexamethasone was included in the culture medium at stage 12–12.5 and maintained until the embryos were fixed. Wild-type one-cell stage zebrafish embryos were microinjected with 100 pg of the *Xenopus* dominant negative BMP receptor; the RNA was prepared as described above and was dissolved in nuclease-free water with 10% phenol red.

Whole-mount in situ hybridization

For *Xenopus* embryos, antisense RNA probes for *Xiro1* (Gómez-Skarmeta et al., 1998), *Xslug* (Mayor et al., 1995), *Pax2* (Heller and Brandli, 1997), *Fgf8* (Christen and Slack, 1997), N-tubulin (Good et al., 1989), *Sox2* (R. Grainger, personal communication), *Delta1* (Chitnis et al., 1995), *neurogenin1* (Ma et al., 1996), *Six1* (Ghanbari et al., 2001) were synthesized from cDNAs using digoxigenin or fluorescein (Boehringer Mannheim) as a label. Specimens were prepared, hybridized, and stained using the method of Harland (1991). NBT/BCIP or BCIP alone were used as substrate for alkaline phosphatase. Zebrafish embryos were fixed overnight in 4% paraformaldehyde. Antisense RNA probes for zebrafish gene *iro1* (Wang et al., 2001) and *Foxd3* (Odenthal and Nusslein-Volhard, 1998) were synthesized from cDNAs using digoxigenin (Boehringer Mannheim) as a label. Hybridization was done as previously described (Jowett and Lettice, 1994).

Noggin treatment

Acrylic beads (Sigma) were incubated overnight with 100 μg/ml of noggin protein (a kind gift from R. Harland). The beads where grafted into embryos at the appropriate stage and the expression of several markers was later analyzed by in situ hybridization. PBS soaked beads were used as controls.

Results

iro1 is expressed in the preplacodal field and its derivatives in Xenopus and zebrafish

It has been suggested that the expression of *iro1*, which is outside of the neural plate, corresponds to the placodal field but this has not been demonstrated by comparing its expression with specific preplacodal or placodal markers (Cheng et al., 2001; Gómez-Skarmeta et al., 1998). We decided to perform a detailed analysis of this domain of *iro1* expression in *Xenopus* and zebrafish embryos. We compared in *Xenopus* the expression of *Xiro1* and the preplacodal marker *Six1* at different stages (Figs. 1A–H,J,K). Our results show that *Xiro1* is expressed in a region adjacent to the neural plate from stage 15 onward (arrowhead in Fig. 1A), when no expression of *Six1* can be detected (Fig. 1D). At stage 17, when the first expression of *Six1* is observed (Figs. 1E,F), a strong expression of *Xiro1* was observed as a continuous band surrounding the anterior region of the neural plate (Figs. 1B,C), in addition to the expression observed in the neural plate (star in Fig. 1). At stage 19, there is a down-regulation of *Xiro1* in the most anterior region (Figs. 1G,H, arrow), while the expression of *Six1* is still observed in all the anterior neural plate border (Figs. 1J,K). A similar overlap in the expression of *ziro1* and the preplacodal marker *Six4.1* was observed in zebrafish embryos, with the absence of *ziro1* in the most anterior part of the neural plate (Figs. 1I,L). It should be noticed at the early neurula stages, from stage 15 to 18, there is a continuous expression of *Xiro1* from the neural plate to the preplacodal regions, including the prospective neural crest cells as it has been recently shown (Glavic et al., 2004). However, at more advanced stages of development, a down-regulation of

Xiro1 is observed in the neural crest region (arrow in Fig. 1M), which can be confirmed in sections and by its comparison with the expression of the neural crest marker *Slug* (Figs. 1N,O). In addition, when the expression of the neural crest marker *Slug* and the placodal marker *Six1* are compared at advanced stages, a clear gap of expression is observed (arrow in Figs. 1P,R). Thus, the neural crest that are at the border of the neural plate marked by *Sox2* (Fig. 1S) are not immediately adjacent to the preplacodal field at the late neurula stages. In summary, the *iro1* gene is expressed at the early neurula stage as a continuous band that includes part of the most anterior prospective neural crest and the preplacodal field in the neural folds (Fig. 1T). As development progresses, *iro1* is switched off from the most anterior domain of the neural plate border and from the prospective neural crest regions; at the same time, the neural crest and the placodal markers become segregated, generating a gap in the region between these two domains of expression (Fig. 1U). This observation indicates that the initial broader expression of *iro1* is followed by a restriction to the preplacodal domain. It should be mentioned that the absence of an early fate map of the preplacodal region in *Xenopus* embryos does not allow us to unequivocally assign the region of *iro1* expression as preplacodal cells at early stages, specially as the expression of the gene is very dynamic. However, at late neurula stages, there is a clear overlap in the expression of *iro1* and the preplacodal marker *Six1* and *Six4.1* and, furthermore, there is a clear gap in the expression of these two markers and the neural crest marker *Slug*. Thus, to use *iro1* as a preplacodal marker care should be taken to use it only at late neurula stages (after stage 19).

To test if the expression of *Xiro1* in the preplacodal field in *Xenopus* is transient, we performed in situ hybridization of *Xiro1* at later stages and compared its expression with markers for specific placodes. Embryos were analyzed at stages 20 and 33. At stage 20, the expression of *Xiro1* and *Six1* overlaps around the eye as can bee seen in simple and double in situ hybridizations (Figs. 2A,C,E). At stage 33 and in later stages (Figs. 2B,D,F), the expression of *Xiro1* overlaps with the expression of specific placodal markers, such as otic placodes *Six1* and *Pax2* (Chitnis et al., 1995; Heller and Brandli, 1997; Kamachi et al., 1998; Mizuseki et al., 1998; Penzel et al., 1997). It should be mentioned that the neural tube expression of *Xiro1* makes more difficult its analysis at these advanced stages, particularly in double in situ hybridization.

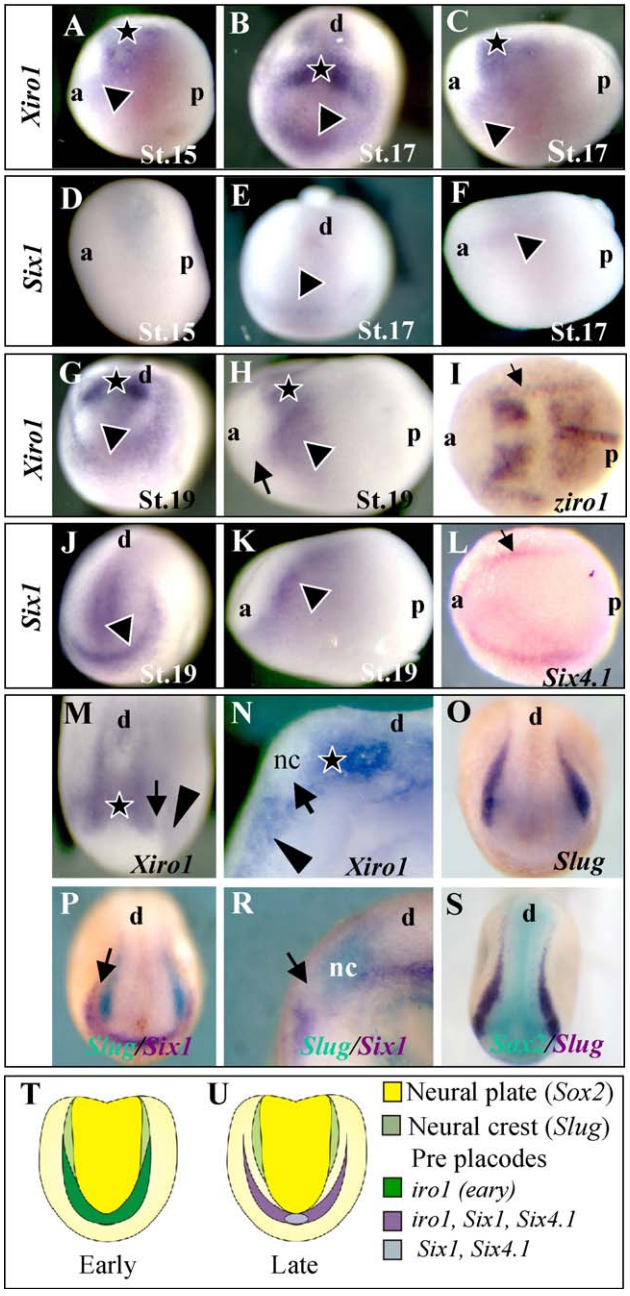

Fig. 1. The *iro* genes are expressed in the placodal field of *Xenopus* and zebrafish embryos. In situ hybridization of *iro1*, preplacodal, placodal, and neural crest markers was performed at different stages of *Xenopus* (A–H, J, K, M–S) and zebrafish embryos (I, L). a: anterior; p: posterior; d: dorsal; star: neural plate expression of *iro1*; arrowhead: preplacodal expression of *iro1*. (A) *Xiro1* expression in a stage 15 embryo. Lateral view. (B, C) *Xiro1* expression in a stage 17 embryo, in an anterior (B) and lateral (C) view. (D) *Six1* in situ hybridization of a stage 15 embryo. Lateral view. (E, F) *Six1* expression in a stage 17 embryo, in an anterior (E) and lateral (F) view. (G, H) *Xiro1* expression in a stage 19 embryo, in an anterior (G) and lateral (H) view. (I) *ziro1* expression in a tail bud stage zebrafish embryo (10.5 h postfertilization, hpf). Dorsal view, arrow indicates the preplacodal domain of expression. (J, K) *Xiro1* expression in a stage 19 embryo, in an anterior (J) and lateral (K) view. (L) *Six4.1* expression in a tail bud stage zebrafish embryo (10.5 h postfertilization, hpf). Dorsal view, arrow indicates the preplacodal domain of expression. (M) *Xiro1* expression in a stage 21 embryo, dorsal view, posterior to the top. Arrow indicates the gap of expression between the neural (star) and placodal (arrowhead) expression of *Xiro1*. (N) section of embryo shown in M. (O) *Slug* expression in a stage 20 embryo. Dorsoanterior view. (P) Double in situ hybridization of a stage 20 embryo for *Six1* (purple) and *Slug* (green). Anterior view. (R) Section of embryo shown in P. Note the gap in the expression of both genes indicated by the arrowhead. (S) Double in situ hybridization of a stage 20 embryo for *Slug* (purple) and *Sox2* (green). Anterior view. (T, U) Summary of neural plate, neural crest, and preplacodal markers, for early (T) and late (U) neurula stages.

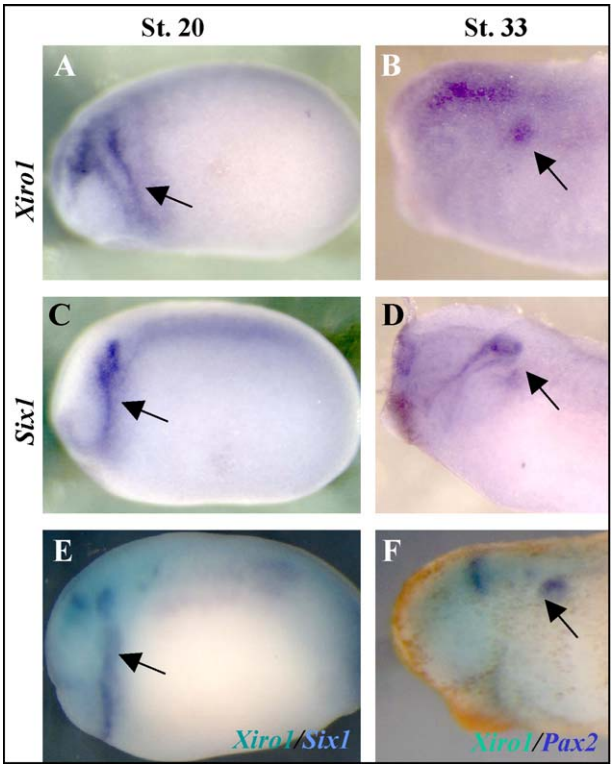

Fig. 2. Expression of *Xiro1* at later stages. Anterior to the left, dorsal to the top: arrow, indicates placodal expression. (A, B) *Xiro1* expression at stage 20 (A) and 33 (B). (C, D) *Six1* expression at stage 20 (C) and 33 (D). (E) Double in situ hybridization for *Xiro1* (green) and *Six1* (purple) at stage 20. (F) Double in situ hybridization for *Xiro1* (green) and *Pax2* (purple) at stage 33. Arrow in F indicates the otic placode.

The preplacodal markers Six1 and Xiro1 can be induced by an interaction between neural plate and epidermis

It has been previously shown that an interaction between neural plate and epidermis induces the neural crest, which, like the placodes, are at the border of the neural plate (Dickinson et al., 1995; Mancilla and Mayor, 1996; Selleck et al., 1995). We decided to test whether a similar interaction was enough to induce the known preplacodal marker *six1* and the *iro1* gene. Embryos were injected at the one-cell stage with the lineage tracer FLDx. At the early neurula stage (13/14, Nieuwkoop and Faber, 1967), a piece of anterior neural plate was grafted into the ventral epidermis of a normal embryo at the same stage. The grafted embryo was cultured until stage 22/23, and the expression of *Six1* and *Xiro1* was analyzed by in situ hybridization (Fig. 3A). We also analyzed the expression of the neural crest marker *Slug* as a control for the induction (Mancilla and Mayor, 1996). As expected, *Slug* was induced at the border of the graft (not shown; Mancilla and Mayor, 1996), and interestingly, *Six1* was also induced in the epidermis surrounding the graft as can be seen in whole mount and in sections (Figs. 3B,D,F,G), as well as *Xiro1* (Figs. 3C,E,H). As control, the explants were cultured in isolation but no *Xiro1* or *Six1* expression was detected (inset in Figs. 3B,C). The expression of the preplacodal markers was always observed in the epidermal region, and in some few cases a gap between the neural graft and the induced expression of *Six1* was observed (12%, $n = 27$; Fig. 3G, bracket). The proportion of grafts where the expression of the three genes was induced was very similar (above 40%, n above 25 for each gene). These results indicate that an interaction between neural plate and epidermis can induce not only neural crest cells but also cells expressing preplacodal marker, and that the preplacodal cells are induced in the epidermal side.

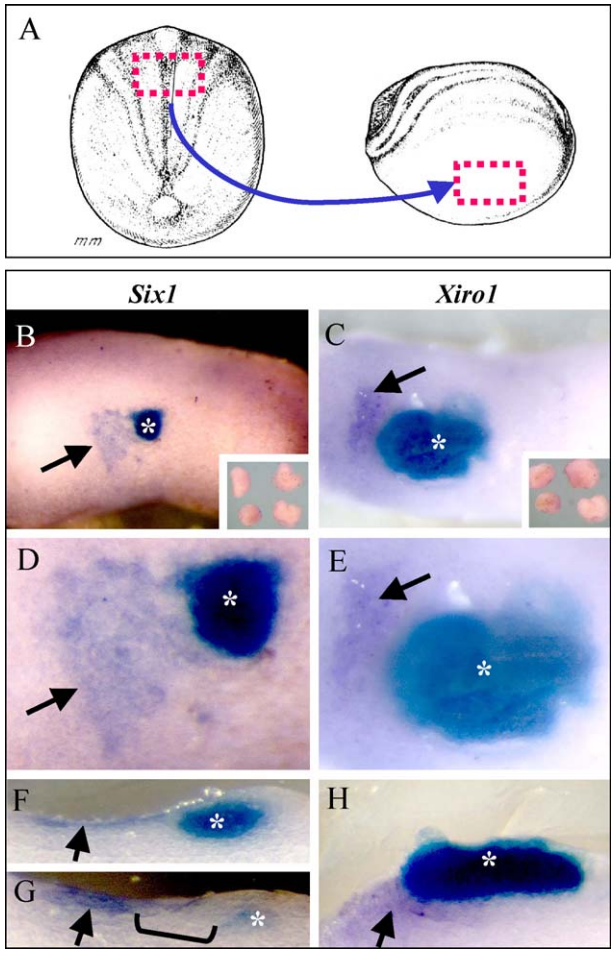

Fig. 3. The preplacodal markers *Six1* and *Xiro1* are induced by an interaction between neural plate and epidermis. (A) A piece of anterior neural plate was dissected from a stage 13 embryo, previously labeled with FLDx at the one-cell stage, and grafted into the ventral epidermis of a normal stage 13 embryo. The grafted embryo was cultured until stage 25, when the expression of the preplacodal markers *Six1* (B, D, F, G) or *Xiro1* (C, E, H) was analyzed. Inset: control neural plate cultured in isolation, where no expression is seen. Arrow: induction of the marker; star: graft. Anterior to the left. (B) Induction of *Six1* in the epidermis adjacent to the graft. (C) Induction of *Xiro1* in the epidermis adjacent to the graft. (D) Higher magnification of the graft shown in B. (E) Higher magnification of the graft shown in C. (F–H) Section of different grafts, showing that the induced cells (arrow) are sometimes not adjacent to the graft (bracket in G). Forty-eight percent of the grafts showed *Six1* induction, $n = 29$; 45% of the grafts showed *Xiro1* induction, $n = 28$.

BMPs are involved in the induction of the preplacodal and placodal markers in zebrafish and Xenopus

BMPs have been involved in the induction of neural crest in chick, *Xenopus*, and zebrafish embryos (Liem et al., 1995; Marchant et al., 1998; Nguyen et al., 1998). In *Xenopus* and zebrafish, a BMP gradient model has been proposed (Aybar and Mayor, 2002), where a specific level of BMP, intermediate to that required to induce neural plate and epidermis, specifies neural crest. As the neural crest is adjacent to the placodes, and both tissues are induced by an interaction between neural plate and epidermis, we decided to test whether decreasing BMP activity in *Xenopus* and zebrafish embryos produced an expansion of the placodal field in a similar way to what has been described for neural crest. Three different methods were used to decrease BMP activity in zebrafish and *Xenopus* embryos: (i) zebrafish and *Xenopus* embryos were injected with a dominant negative of BMP receptor (ΔBMPR); (ii) chordin-expressing cells were grafted into zebrafish blastula embryos; and (iii) beads soaked with noggin were implanted near the preplacodal field in *Xenopus* embryos.

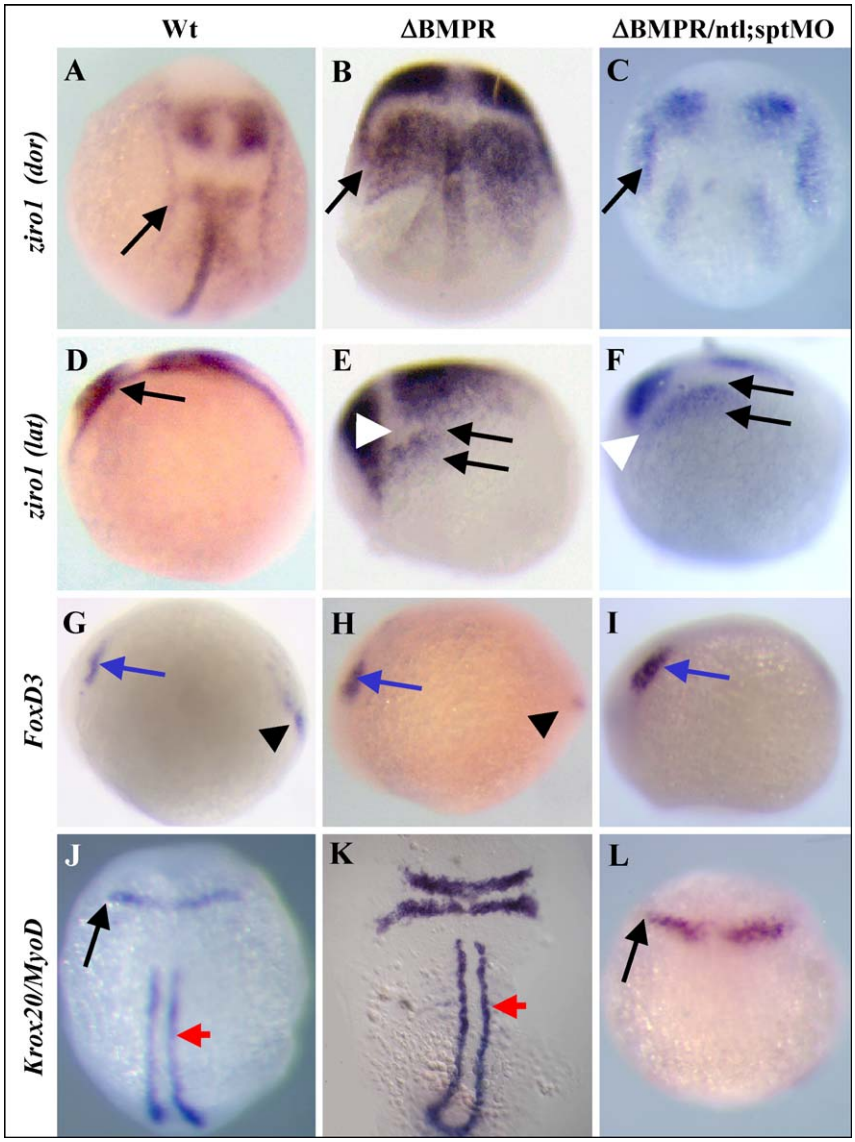

Fig. 4. Specification of the preplacodal field by BMP in zebrafish embryos. Ten hours postfertilization embryos analyzed by whole-mount in situ hybridization for the expression *ziro1* (A–F), *FoxD3* (G–I), and *Krox20/MyoD* (J, L). A–C and J–L are shown in dorsal view with anterior to the top, while D–I are shown in lateral view with anterior to the left. The placodal domains of *ziro1* expression is indicated with arrow. Blue arrow: neural crest; white arrowhead: absence of *ziro1* expression in the neural crest; black and red arrowhead: mesoderm. (A, D, G, J) Wild-type embryos. (B, E, H, K) Embryos injected at the one-cell stage with 100 pg of ΔBMPR mRNA: note the expansion in the midbrain, hindbrain, and placodal field of *ziro1* expression, and the moderate expansion of the neural crest and mesoderm domains. (C, F, I, L) Embryos injected at the one-cell stage with 100 pg of ΔBMPR mRNA and a mixture of *ntl* and *spt* morpholinos to inhibit mesoderm formation. Note the expansion of the placodal domain of ziro1 (C, F), the moderate expansion of *FoxD3* and *Krox20* (I, L), and the absence of *MyoD* expression (L). Note that the embryo in K is slightly older than the embryos in J and L, this is why two bands of *Krox20* expression are seen in K, but only one in J and L.

Zebrafish embryos were injected with 100 pg of ΔBMPR mRNA and the expression of several markers was analyzed. Control embryos showed expression of *ziro1* as a thin line of cells (arrow in Figs. 4A,D); however, after ΔBMPR injection, that region was dramatically expanded (arrow in Figs. 4B,E). Other domains of *ziro1* expression in the neural plate were also expanded, and also there was a moderate expansion of the neural crest region, as can be recognized by the gap between the neural plate and placodal region of *ziro1* expression (white arrowhead in Fig. 4E) and by the slight expansion of *FoxD3* expression (Figs. 4G,H). We should mention that when the levels of BMP activity are even lower, as in the *snh* mutants, a wider expansion of the neural crest was observed as has been previously published (Nguyen et al., 1998). As in all these injected embryos, not only the ectoderm but also the mesodermal patterning is affected; a possible explanation of these results is that the expansion of the preplacodal markers could be a secondary consequence of a primary expansion of mesoderm, which in turn induces the neural markers in the ectoderm. To test whether the expansion of the mesoderm played any role in the expansion of the preplacodal marker, two experiments were performed. First, we proceeded to inhibit the formation of dorsal mesoderm in some of the injected embryos and the preplacodal marker was analyzed. It has previously described that *Krox-20* is characteristically expanded when the level of BMP activity is diminished (Nguyen et al., 1998). We analyzed the expression of *Krox20* as a neural marker and *MyoD* as a mesodermal marker. The expression of both genes can be clearly distinguished in wild type (Fig. 4J) and in embryos injected with ΔBMPR (Fig. 4K), showing the injected embryo a moderate expansion of both markers. To inhibit mesoderm formation, we proceeded to coinject a mix of *ntl* (Nasevicius and Ekker, 2000) and *spt* morpholinos together with the ΔBMPR mRNA. This injection lead to a total inhibition in the expression of *MyoD* (100% of inhibition, $n = 100$), but no effect in the expression of *Krox20* was observed (Fig. 4L). When the expression of *ziro1* was analyzed, no effect in the expansion of this gene in the preplacodal domain was observed (arrow in Figs. 4C,F), indicating that the expansion of this ectodermal domain does not depend on the mesoderm. Note that other domains of *ziro1* expression, such as the mesoderm and some regions of the neural plate, are affected by the morpholino treatment (Figs. 4C,F), as well as the mesodermal expression of *FoxD3* (arrowhead in Figs. 4G–I).

A second experiment aimed to rule out the role of the mesoderm in preplacodal expansion was carried out (Fig.

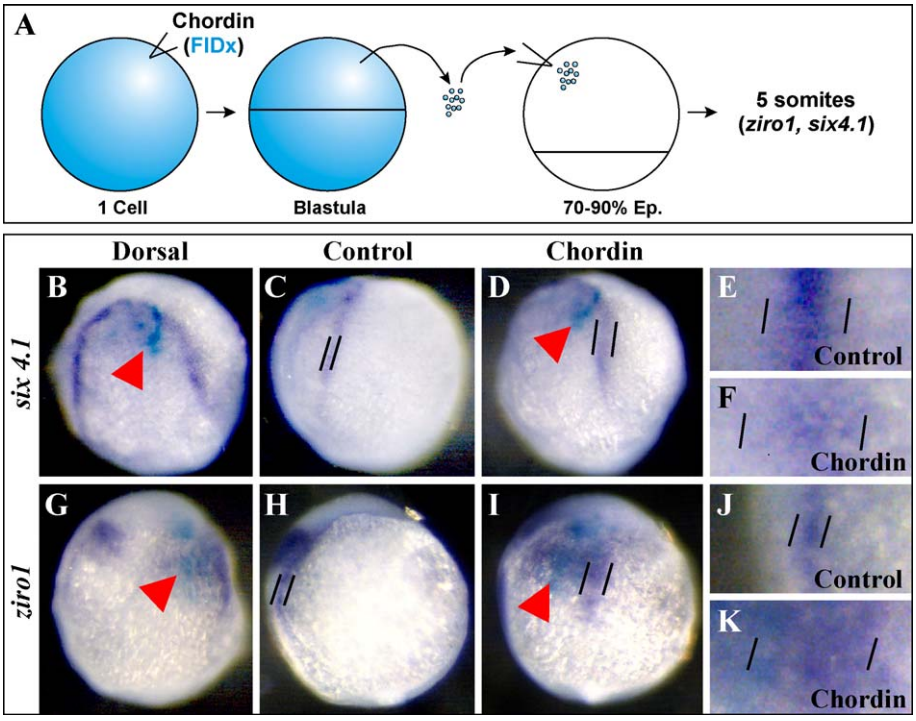

Fig. 5. Induction of preplacodal markers by chordin expressing cells (A). Five hundred picograms of Chordin mRNA and 7 ng/μl of FLDx were injected at the one-cell stage and the embryo cultured until the blastula stage, when ectodermal cells were dissociated and grafted into the lateral ectoderm of a gastrula stage embryo (70–90% epiboly). The grafted embryos were cultured until the one- to two-somite stage, and the expression of preplacodal markers was analyzed by in situ hybridization. The grafted cells were visualized by alkaline phosphatase-mediated FITC inmunostaining and recognized by the blue color of the FLDx staining; they are indicated by a red arrowhead. (B–F) Analysis of *Six4.1*. (B) Dorsal view. Control (C) and grafted (D) sides of embryo shown in B. (E, F) Higher magnification of embryos shown in C and D. Note that the preplacodal marker is expanded a few cell diameters on the side that received the graft, but the border of expression is not as sharp as on the control side (E, F). (G–K) Analysis of *ziro1*. (G) Dorsal view. Control (H) and grafted (I) sides of the embryo shown in G. (J, K) Higher magnification of embryos shown in H and I. Note that the preplacodal marker is expanded a few cell diameters on the side that received the graft (E, F).

5A). Zebrafish embryos were injected with 500 pg of chordin mRNA together with the lineage marker FLDx. At the blastula stage, cells from the injected embryos were dissociated and 20–50 cells were grafted into the lateral region of a gastrula embryo (between 70% and 90% epiboly). These embryos were cultured until the one- to two-somite stage and the expression of the preplacodal markers *Six 4.1* and *ziro1* was analyzed. A moderate but clear expansion of both markers was observed in the preplacodal region adjacent to the grafted cells (Figs. 5B–K). The normal expression of *Six4.1* and *ziro1* that usually does not include more than two or three cells was expanded to more than six cells in the region of the graft (Figs. 5E–K). We were not able to see ectopic induction of preplacodal markers when the grafted cells were in a more ventral position. We never observed induction or expansion of the mesodermal marker *MyoD*. Taken together, these results indicate that a decrease in the level of BMP activity in the ectoderm leads to an expansion of the preplacodal cells that express *Six1.4* and *ziro1*.

Equivalent experiments were also performed in *Xenopus* embryos. Injection of 125 pg of ΔBMPR mRNA in one blastomere of a two-cell stage embryo (Fig. 6A) or grafting of noggin beads in *Xenopus* embryos at the early neurula stage (12) (Fig. 6B) led to a similar expansion of the preplacodal domain of *Xiro1* (Figs. 6C,F). We also analyzed the effect of expansion of the placodal field on the expression of specific placodal markers. Two examples are shown:

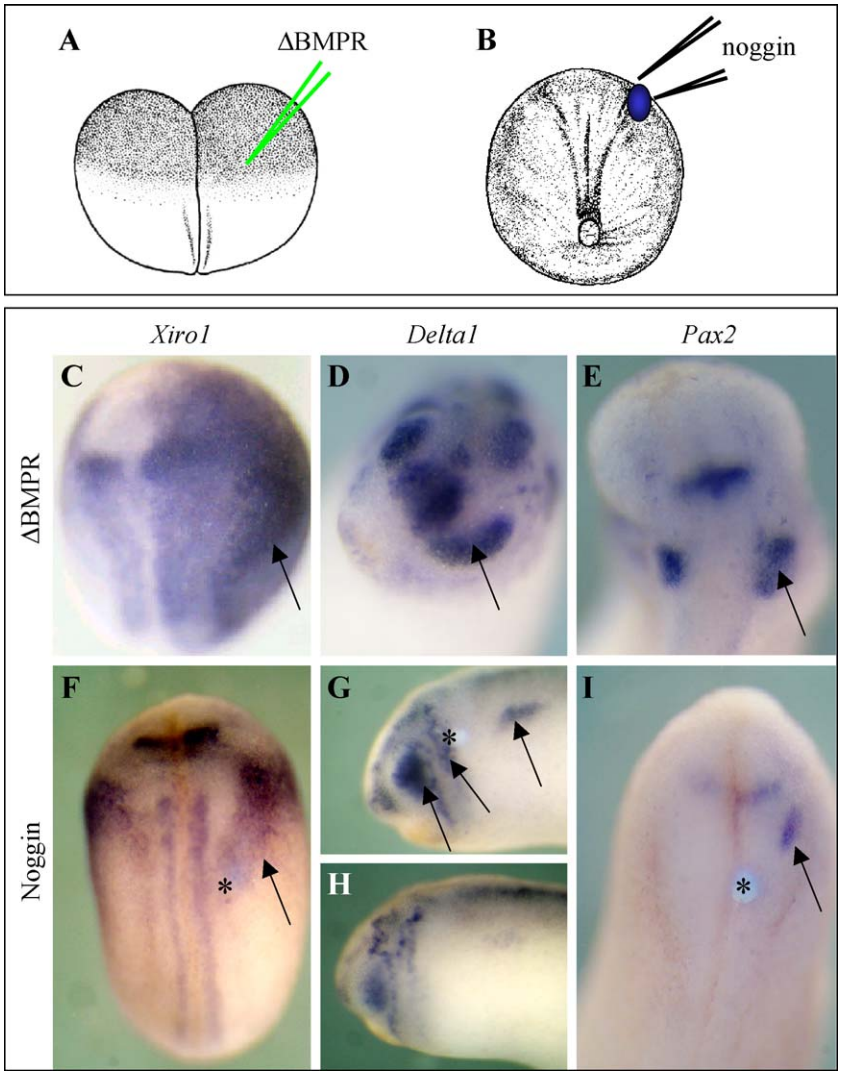

Fig. 6. Specification of the preplacodal field and placodes by BMP in *Xenopus* embryos. The level of BMP activity was changed by injection of the mRNA that codes for the dominant negative of the BMP receptor (ΔBMPR) in *Xenopus* (A, C–E) or by implanting beads soaked with noggin in early neurula *Xenopus* embryos (B, F–I). The expression of the preplacodal (*Xiro1*) and specific placodal markers (*Delta1*, *Pax2*) was analyzed under these conditions. (C–E) An expansion in the preplacodal marker *Xiro1* (C) or the placodal markers *Delta1* (D) and *Pax2* (E) was observed when 125 pg of ΔBMPR mRNA were injected in one blastomere of a two-cell stage embryo. Arrows: expansion of the markers in the injected side. (F–I) Implantation of noggin soaked beads (asterisks) leads to an expansion of the preplacodal marker *Xiro1* (F) and the placodal markers *Delta1* (G, grafted side; H, control side) and *Pax2* (I). Arrow indicates the expansion of the markers in the experimental side.

The expression of *Delta1* and *Pax2*, which are expanded in both treatments (Figs. 6D,E,G–I). Thus, a reduction of BMP activity leads to an expansion of the placodal field in zebrafish and *Xenopus* embryos.

Role of iro1 and Notch signaling on placodal development

We have shown here that *iro1* is expressed in the preplacodal field and that the treatments that affect *iro1* expression also affect the development of the placodes. We decided to analyze the role of *iro1* on the development of the preplacodal field and on specific placodes. To overcome the early effects of *Xiro1* on mesoderm development, we used inducible fusion constructs that have been previously described (Glavic et al., 2001, 2002, 2004; Gómez-Skarmeta et al., 2001). It has been shown that *Xiro1* acts as a transcriptional repressor (Glavic et al., 2001; Gómez-Skarmeta et al., 2001). Thus, activated forms of *Xiro1* (*HDEnGR*) or its dominant negatives (*HDGR*) were injected in one blastomere of a two-cell stage embryo. It should be mentioned that the uninjected side of the same embryo is compared to the experimental condition as a control in each experiment. When mRNA encoding either *Xiro1* (not shown) or its inducible repressor fusion (*HDEnGR*) were injected and then activated at stage 12, the expression of the preplacodal marker *Six1* and the placodal markers *Pax2* and *Sox2* were augmented (Figs. 7A,C,E,G,I,L). Conversely, both activation at stage 12 of the inducible dominant negative fusion (*HDGR*) or the inducible activator fusion (*HDE1AGR*, not shown) inhibited *Six1*, *Pax2*, and *Xsox2* expression (Figs. 7B,D,F,H,J,M). The expression of the preplacodal marker *Six1* is almost completely abolished in the injected side (Figs. 7B,D). *Pax2* is expressed in the otic placode, and this is the region that is most affected by both treatments (Figs. 7F,H), while *Sox2* expression is mainly affected in the lens and otic placodes (Fig. 7J,M). As controls for the specificity of the *Xiro1* dominant negative construct (*HDEnGR*), we performed a rescue experiment. Embryos were injected in one blastomere of a 2-cell stage embryo with *HDGR*. As previously described, a clear inhibition of *Six1* was observed in the injected side (Fig. 7N). However, when the same group of embryos was coinjected with a mixture of *HDEnGR* and *HDGR* mRNA, a complete rescue in the expression of *Six1* was observed and in some cases a stronger expression was visible in the injected side (Fig. 7O). Finally, to rule out the possibility that the inhibition in the expression of placodal markers was a consequence of an expansion of neural plate, as it has been described for inhibition of the neural crest markers by an early overexpression of *Xiro1* (Gómez-Skarmeta et al., 1998), the expression of *Sox2* was analyzed. No effect on the expression of neural plate marker *Sox2* was observed

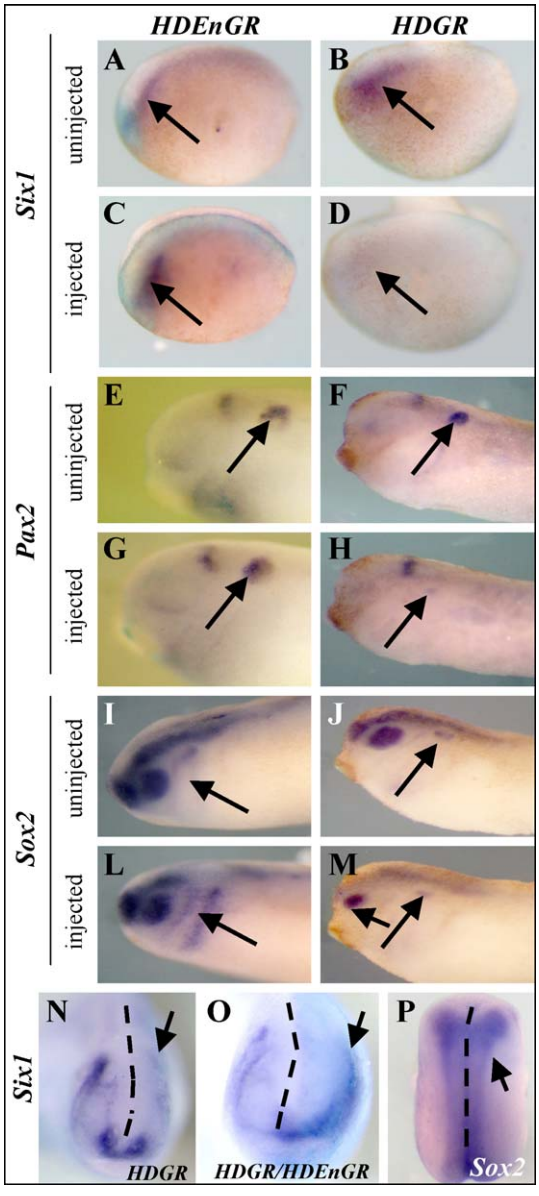

Fig. 7. *Xiro1* is required for preplacodal and placodal development. Two-cell stage embryos were injected in one blastomere with 1 ng of the inducible forms of a repressor form of *Xiro1* (*HDEnGR*) (A, C, E, G, I, L), or with a dominant negative form of *Xiro1* (*HDGR*) (B, D, F, H, J, M). The embryos were treated with dexomethasone at stage 12 and the expression of *Six1*, *Pax2*, and *Sox2* was analyzed by in situ hybridization. The injected side was visualized by alkaline phosphatase-mediated FITC inmunostaining. The uninjected (A, B, E, F, I, J) and injected (C, D, G, H, L, M) side of the same embryos are shown. (A, C, E, G, I, L). The injection of *HDEnGR* leads to a moderate enlargement of *Six1* expression (A, C, arrow) and to a moderate expansion of the otic placodal marker *Pax2* (E, G; arrow) and the epibranchial placode marker *Sox2* (I, L; arrow). (B, D, F, H, J, M) The injection of *HDGR* leads to a complete inhibition of the preplacodal marker *Six1* (B, D, arrow) and to an inhibition of the otic placodal marker *Pax2* (F, H; arrow) and the lens and epibranchial placode marker *Sox2* (J, M; arrows). (N, O) Rescue experiment. Embryos were injected with *HDGR* as described, showing the characteristic inhibition of *Six1* in the injected side (arrow); however, this effect was rescued by coinjection of *HDGR* and *HDEnGR*, as is seen in the injected side (arrow). (P) *Sox2* expression in stage 16 embryo injected with *HDGR*. No effect in the neural plate can be detected. Each experiment was performed at least twice with a minimum of 42 embryos. The percentage effect for each experiment was approximately 65%. For the rescue experiment, the percentage of effect was less than 10%.

when the *Xiro1* gene was activated after stage 12 (Fig. 7P). This indicates that *Xiro1* is able to affect neural plate development at earlier stages than placodes or neural crest cells (this work and Glavic et al., 2004). Taken together, these results indicate that *iro1* is involved in the specification of the placodal field (*Six1*) as its inhibition affect the expression of preplacodal as well as placodal markers.

As our results indicate that BMP signals and the *iro1* gene seem to be involved in the early development of the preplacodal field, which is similar to what has been described for the neural crest, we decided to explore if the Notch signal was also involved in placode development. It has been recently shown that Notch signaling is important in controlling neural crest specification (Glavic et al., 2004). Several molecular tools have been developed to modify the activity of the Notch signaling pathway at different levels (Chitnis et al., 1995; Coffman et al., 1993; McLaughlin et al., 2000). Using these constructs, we have analyzed the participation of Notch signaling in placodal specification. Ligand activation of Notch by Delta results in the proteolytic cleavage of its transmembrane domain, releasing the cytoplasmic region (*NICD*, Struhl and Adachi, 2000). *NICD* translocates to the nucleus where it interacts with the transcriptional repressor *Suppressor of hairless* (*Su(H)*), forming a transcriptional activator complex (Artavanis-Tsakonas et al., 1999). We injected the mRNA coding for *NICD* and a dominant negative of *Su(H)*, *S(H)DBM* dominant negative constructs, *NICDGR* and *Su(H)DBMGR*, and the constructs were activated after stage 12. Activation of Notch signaling produced by injection of *NICDGR* mRNA and dexamethasone treatment at stage 12 did not produce any effect on *Six1* (not shown), *Pax2* or *Sox2* expression in the placodes (Figs. 8A,B,E,F). Injection of *Su(H)DBMGR* mRNA in one blastomere of a two-cell stage embryo and induction with dexamethasone at the late gastrula stage (stage 12) produced no effect on *Pax2* and *Sox2* expression in the placodal regions (Figs. 8C,D,G,H). A small effect on *Sox2* expression in the optic vesicle was observed (Fig. 8H). In conclusion, our results suggest that Notch signaling is not involved in specification of any of the placodes analyzed.

Discussion

The preplacodal field

Not much is known about how the different types of placodes originate at the anterior border of the neural plate, because most studies on placodal development have focused on late embryonic stages. It is still an unresolved question whether individual placodes arise as distinct ectodermal specializations in situ (Northcutt and Brandle, 1995; Schlosser and Northcutt, 2000) or whether all or a subset

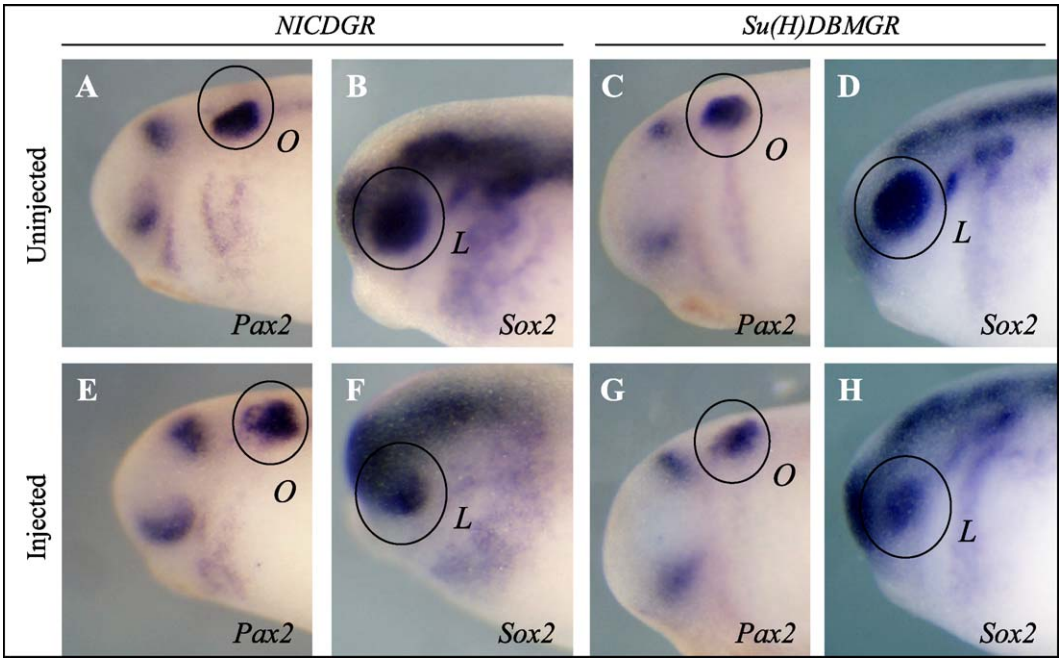

Fig. 8. Notch signaling on placode development. Two-cell stage embryos were injected in one blastomere with 0.7 ng of *NICDGR* (A, B, E, F) or 0.25 ng of *Su(H)DBMGR* (C, D, G, H). Injected embryos were cultured from stage 12 in medium containing dexamethasone and the expression *Pax2* (A, E, C, G) and *Sox2* (B, F, D, H) was analyzed by in situ hybridization. The injected side was recognized by alkaline phosphatase-mediated FITC immunostaining. The uninjected (A–D) and injected (E–H) sides of the same embryos are shown. The expression of markers in several placodes is indicated. O. otic; L: lens. (A, C, E, G). The injection of *NICDGR* does not produce any obvious effect in the expression of the otic placodal marker *Pax2* (A, E) or the epibranchial placode marker *Sox2* (B, F). The injection of *Su(H)DBMGR* does not have any effect on the expression of the otic placodal marker *Pax2* (C, G) and the epibranchial placode marker *Sox2* (D, H). Each experiment was done at least twice with a minimum of 45 embryos.

of the placodes originate from a common placodal primordium, placodal anlagen, preplacodal, or placodal field (Baker and Bronner-Fraser, 2001; Knouff, 1935; Kozlowski et al., 1997; Miyake et al., 1997). Placodes are often described as completely separate entities. However, several placodes may arise from discrete ectodermal thickenings, especially in fish and amphibians. There are substantial morphological and molecular data from several different species to support the existence of a general preplacodal domain within the cranial neural plate border at the gastrula and neurula stages. At the midgastrula stage in zebrafish, fate maps indicate that all the placodal precursors are present in an overlapping territory at the border of the anterior neural plate (Kozlowski et al., 1997). In amphibians, such as the Urodele *Necturus* and the frog *Rana*, placodes seems to originate from a single thickening of the inner (sensory) layer of the ectoderm (Northcutt and Brandle, 1995; Schlosser and Northcutt, 2000). The primitive placodal thickening is broad in the head but abruptly narrows at the head/trunk interface. A preplacodal thickening incorporating all future placodes has not been observed in the frog *Xenopus* or the Urodele *Ambystoma*, although several placodes are initially part of discrete multiplacodal areas (Northcutt and Brandle, 1995; Schlosser and Northcutt, 2000). Although there is no morphological evidence for a common placodal primordium in frogs, it cannot be ruled out that a common placodal cell state is induced before placodal specialization develops. In support of this idea is the expression of several genes in different species in the region that corresponds to the prospective placodal filed. In zebrafish, the homeobox genes *dlx3*, *dlx7*, the homeodomain transcription factor *Six4.1*, and the transcription cofactor *eye1* are expressed at late gastrula stages in a strip corresponding to cells of the future neural plate border and, at later stages, some of those genes are restricted to specific placodes (Akimenko et al., 1994; Ellies et al., 1997; Kobayashi et al., 2000; Sahaly et al., 1999). In *Xenopus*, the homeobox gene *Six1* is expressed in the lateral neural folds at neurula stage (stage 17/18) in a band surrounding the anterior neural plate (Pandur and Moody, 2000). In the chick, *Six4* is expressed in neurula stage embryos (stage 6) in a horseshoe-shaped crescent surrounding the developing anterior neural plate, corresponding precisely to the placodal fate map (Esteve et al., 1999). In summary, morphological evidence in some organisms and molecular evidence in others, support the notion of a preplacodal field from which many, or all, placodes are derived.

Some of the earliest markers for this placodal field described for zebrafish, chick, and *Xenopus* are the *Six* genes. In *Xenopus*, as described above, the *Six1* gene is initially detected at the midneurula stage (17/18) in the placodal field (Ghanbari et al., 2001; Pandur and Moody, 2000). In this work, we show that the *Xiro1* gene is expressed in *Xenopus* in the placodal field at an even earlier stage (15); this observation makes *iro1* the earliest marker for the placodal field known so far. However, we should mention that to employ *Xiro1* expression as a preplacodal marker, later stages of development should be used, as these earlier stages show a continuous expression between the neural plate and the placodes. In addition, *iro1* is expressed in *Xenopus* and zebrafish in many of the placodal derivatives. These observations also support the idea of a preplacodal field, which can be identified at an early stage by the expression of *iro1*.

Induction of the preplacodal field

Given the existence of a preplacodal field at the border of the neural plate and that one of the earliest markers for this region is the expression of the *iro1* gene, we decided to analyze how the expression of this gene and the preplacodal marker *Six1* is induced at the anterior border of the neural plate. As the neural plate border give rise to neural crest and to the placodal field, we argued that perhaps similar mechanisms could be involved in the induction of these two tissues. It is known that the neural crest is induced by an interaction between neural plate and epidermis (Dickinson et al., 1995; Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). As the placodes are found in the anterior neural fold, an expected source of inductive signal is the interaction between anterior neural plate and epidermis. By making grafts of anterior neural plate into lateral epidermis, we found that the interaction between these two tissues is able to induce *Xiro1* and *Six1* at the border of the graft. Our results suggest that the interaction between neural plate and epidermis is able to correctly specify the border of the neural plate including preplacodal cells. A similar observation was found for the induction of the trigeminal placode in chick, where the perturbation of the correct neural tube–ectoderm interaction inhibited the formation of this placode (Stark et al., 1997). The most likely explanation for this observation is that the neural plate and epidermis produce inductive signals that specify a neural plate border region, perhaps including cells that originally lie on either side of the border, giving rise to neural crest and placodes. This idea is supported by the observation that cells fated to become epidermis, neural crest, and neural plate have a common precursor at these earlier stages (Selleck and Bronner-Fraser, 1995).

Enormous progress has been made in recent years concerning the identification of the molecules involved in neural crest induction (reviewed in Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002). Wnts and Wnt receptors have been involved in the induction of the neural crest (reviewed in Wu et al., 2003; Yanfeng et al., 2003). It has been proposed in chick that Wnt6B could correspond to the epidermal signal that is able to transform neural plate cells into neural crest (Garcia-Castro et al., 2002). The participation of Wnt signals in neural crest induction in *Xenopus* has also been explored (LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997; Villanueva et al., 2002). However, it has been proposed that the role of Wnt signals, together

with FGF and retinoic acid, is the transformation of the anterior neural fold into neural crest cells by the process of "posteriorization". Thus, it will be interesting to test whether Wnt signaling is also involved in the induction of the placodal field, perhaps in the form of a signal that arises from the epidermis.

Other molecules implicated in neural crest induction are the BMPs. In the chick, BMP4 is the principal molecule implicated in neural crest formation. Thus, treatments that block BMP activity inhibit neural crest development, while increasing BMP activity, or applying it ectopically, expands the neural crest population (Liem et al., 1995; Selleck et al., 1998; Streit and Stern, 1999). However, data from *Xenopus* and zebrafish support the notion that early induction of neural crest cells depends on a gradient of BMP (reviewed in Aybar and Mayor, 2002; Chitnis, 1999). In these species, neural crest cells are specified at the border between the neural plate and the epidermis, in a zone where intermediate concentrations of BMPs are established; that is, where the BMP4 concentration is lower than that required to induce epidermis and higher than that which induces neural tissue (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Morgan and Sargent, 1997; Nguyen et al., 1998; Villanueva et al., 2002; Wilson et al., 1997). We show in this work that, by changing the level of BMP activity in *Xenopus* and zebrafish, the preplacodal markers *Six1* and *iro1* are displaced. A moderate reduction of BMP, achieved by implanting noggin-beads, grafting chording expressing cells or by injection of low levels of ΔBMPR into *Xenopus* and zebrafish embryos, leads to an expansion of *Six4.1*, *iro1*, and several placodal markers. Interestingly, the enlargement of the placodal domain is larger that the expansion of the neural crest; this is easily explained by a change in the BMP gradient where higher levels (epidermal) are more strongly affected than lower levels. These results agree with the gradient model proposed for neural crest specification. We propose that the gradient that specifies the neural crest at a precise level of BMP also specifies the preplacodal field. Interestingly, different members of the *Dlx* family of genes, which are expressed in different positions adjacent to the neural plate border, are induced by different levels of BMP activity, being the most ventral genes induced by higher levels of BMP activity (Luo et al., 2001). Some of these *Dlx* genes, like *dlx5*, are expressed in the placodal field. Taken together, these results support the notion that a gradient of BMP specifies the preplacodal region and the neural crest at the neural plate border. Fate maps studies in chick suggest that the preplacodal region and the neural crest territories overlap extensively (Streit, 2002). We show in *Xenopus* by comparing the expression of *Six1* and *Slug* that there is no overlap between these two territories at the mid/late neurula stages. However, when the expression of *Xiro1* is analyzed at earlier stages, a continuous band of expression covers neural crest and preplacodal domains. This observation suggests that at the early neurula stages, neural crest and placodes have overlapping territories, but at late neurula stages, these territories become not only segregated but also a gap is generated between them.

Once the preplacodal field is induced, additional signals are required to specify the identity of each of the placodes in a similar way as neural crest derivatives are specified at later stages. Signals from the ectoderm, mesoderm, and neural tissue have all been implicated in placode induction; the precise combination, however, is often entirely different for each placode. Molecules identified in the induction of hypophyseal, lens, otic, and epibranchial placodes are BMPs, FGFs, and Wnts (see review by Baker and Bronner-Fraser, 2001).

iro genes in early specification of placodes

Iroquois genes have been found in species from nematodes to humans and share two main features: a conserved homeodomain of the TALE superclass and a characteristic domain called the Iro box (Bürglin, 1997; Cavodeassi et al., 2001; Gómez-Skarmeta and Modolell, 2002). These genes participate in several developmental processes including sensory organ development, compartment boundary formation in *Drosophila*, dorsal mesoderm formation, neural plate induction, dorsoventral patterning of the neural tube, and midbrain–hindbrain development (Bellefroid et al., 1998; Bosse et al., 1997; Briscoe et al., 2000; Cohen et al., 2000; Diez del Corral et al., 1999; Glavic et al., 2001, 2002; Gómez-Skarmeta and Modolell, 1996; Gómez-Skarmeta et al., 1998, 2001; Itoh et al., 2002; Kudoh and Dawid, 2001; Leyns et al., 1996; Papayannopoulos et al., 1998).

We have recently shown that the *iro1* gene is involved in neural crest specification (Glavic et al., 2004), and we show here that the *iro1* gene also participates in the early specification of the preplacode field. Inhibition of *iro1* activity by dominant negatives induced at the late gastrula stage produces a specific inhibition in the expression of preplacodal markers as well as in the expression of specific placode markers, without affecting neural plate markers. As *iro1* is expressed in the placodal region by the early neurula stage and the inhibition of its activity around this stage produces the described effect on specific placodes, we propose an early role of *iro1* on preplacodal field specification. However, as the *iro1* gene is also expressed at later stages of placodal development, *iro1* likely also plays a later role, but additional experiments will be required to test this. The inhibition of the neurogenic genes (not shown) that label the neuronal precursors after *iro1* inhibition is probably due to the absence of the entire placodes. Thus, our results support a general role of *iro1* in placodal specification rather than in controlling placodal neurogenesis.

Notch signaling has been implicated in many developmental processes (reviewed in Artavanis-Tsakonas et al., 1999) including neural crest induction (Endo et al., 2002; Glavic et al., 2004). The *Delta1* ligand has a dynamic pattern of expression in *Xenopus* embryos, and at the early neurula stage, it is expressed at the anterior neural plate

border (Glavic et al., 2004), while *Notch* is expressed in the entire neuroectoderm overlapping with neural crest markers (Coffman et al., 1990; 1993). Furthermore, *Hairy2A*, a downstream target of the Notch signaling pathway (Dawson et al., 1995; Wettstein et al., 1997), is also found at the neural plate border. Thus, the elements required to activate Notch signaling are present at the right place to be involved in placodal development. We activated and inhibited Notch signaling at the late gastrula stage and analyzed the effect on several placodal markers, but no effect was observed. We could not alter Notch signaling at earlier stages as it is known that this produces general effects on neural development (Coffman et al., 1993) and, in consequence, any effect on placodal development observed under such conditions could be a consequence of affecting the neural plate. In conclusion, we did not find any evidence that Notch signaling was involved in early placodal specification.

In conclusion, an initial interaction between neural plate and epidermis specifies the neural plate border that includes neural crest and placodes. We propose that increasing levels of BMP subdivide the ectoderm into neural plate–neural crest/placodes–epidermis, respectively. These different levels of BMP activity could be originated by interactions between BMPs produced by the ectoderm and BMP-binding molecules secreted from the dorsal mesoderm or from the neural plate, as it is known that the anterior neural plate also expresses noggin (Knecht and Harland, 1997). The specific activity of BMP required to specify the preplacodal field is able to induce the expression of the homeoprotein *Iro1*, which plays an early role on preplacodal specification.

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