Erratum

A balance of FGF, BMP and WNT signalling positions the future placode territory in the head

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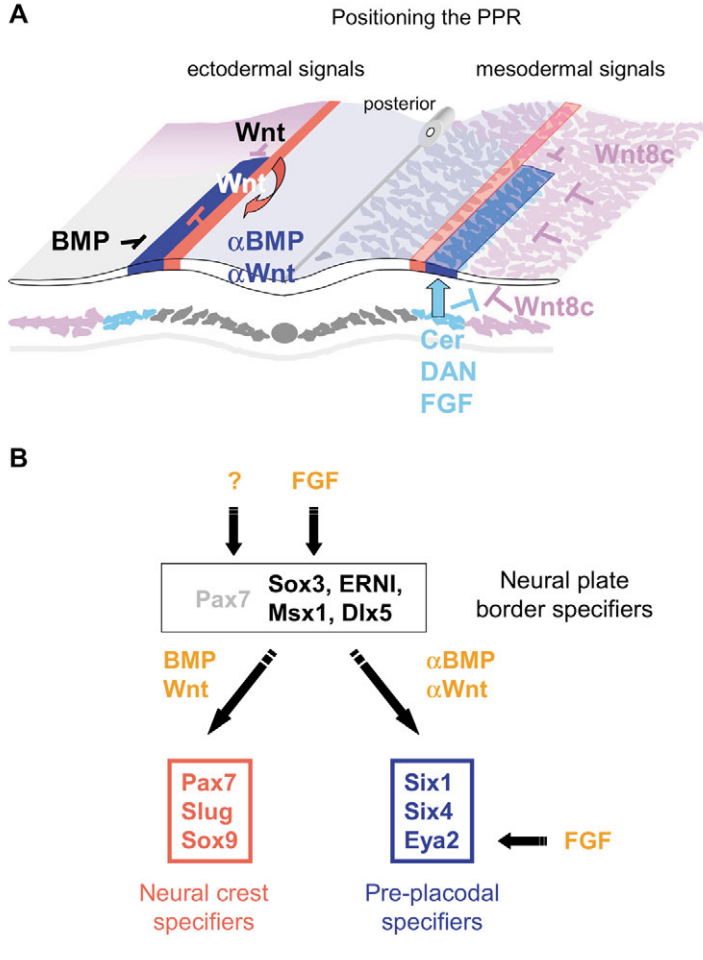
Some of the detail of Fig. 7 was lost on conversion of the electronic file for publication purposes.

The correct version of the figure is printed below.

We apologise to the authors and readers for this mistake.



A balance of FGF, BMP and WNT signalling positions the future placode territory in the head

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Accepted 6 July 2005

Development 132, 4051-4062
Published by The Company of Biologists 2005
doi:10.1242/dev.01964

Summary

The sensory nervous system in the vertebrate head arises from two different cell populations: neural crest and placodal cells. By contrast, in the trunk it originates from neural crest only. How do placode precursors become restricted exclusively to the head and how do multipotent ectodermal cells make the decision to become placodes or neural crest? At neural plate stages, future placode cells are confined to a narrow band in the head ectoderm, the pre-placodal region (PPR). Here, we identify the head mesoderm as the source of PPR inducing signals, reinforced by factors from the neural plate. We show that several independent signals are needed: attenuation of BMP and WNT is required for PPR formation. Together with activation of the FGF pathway, BMP and WNT antagonists can induce the PPR in naïve ectoderm. We also show that WNT signalling plays a crucial role in restricting placode formation to the head. Finally, we demonstrate that the decision of multipotent cells to become placode or neural crest precursors is mediated by WNT proteins: activation of the WNT pathway promotes the generation of neural crest at the expense of placodes. This mechanism explains how the placode territory becomes confined to the head, and how neural crest and placode fates diversify.

Key words: Chick, Quail, Placode, Lens, Inner ear, Olfactory epithelium, Cranial ganglia

Introduction

The sensory nervous system of the vertebrate head has dual origin, arising from neural crest and placode cells. All cranial sensory ganglia receive contributions from both cell populations, whereas crucial components of the special sense organs – the eye, ear, nasal epithelium and lateral line – are largely derived from sensory placodes (D'Amico-Martel and Noden, 1983; Couly and Le Douarin, 1985; Couly and Le Douarin, 1988; Noden, 1993) (for a review, see Baker and Bronner-Fraser, 2001). Placodes are transient ectodermal thickenings that develop next to the anterior neural tube (Knouff, 1935) and generate a large variety of different cell types from simple lens fibres to sensory neurons and hair cells. Because of this, they have often been treated as completely separate entities, unrelated to each other. However, embryological and molecular evidence suggests that placode precursors arise from a unique domain of the ectoderm next to the neural plate, the pre-placodal region (PPR), and that specification of all placodes may be initiated by a single mechanism (see below for details) (for reviews, see Torres and Giraldez, 1998; Baker and Bronner-Fraser, 2001; Streit, 2004).

Patterning events during gastrula and early neurula stages lead to the subdivision of the ectoderm into at least four distinct domains: neural plate, neural crest, PPR and future epidermis. Although in some species the PPR can be identified as a continuous 'primitive placodal thickening' encircling the anterior neural plate (Platt, 1896; Knouff, 1935; van Oostrom and Verwoerd, 1972; Verwoerd and van Oostrom, 1979; Miyake et al., 1997), in other organisms the subdivision first becomes apparent through the expression of molecular markers specific for each of the four tissues. A number of markers have been identified recently that are uniquely expressed in the ectoderm surrounding the anterior neural plate (for reviews, see Baker and Bronner-Fraser, 2001; Streit, 2004), reflecting the position of sensory placode precursors as determined by fate maps (Kozlowski et al., 1997; Whitlock and Westerfield, 2000; Streit, 2002; Bhattacharyya et al., 2004). Among these are members of the Six, Eya and Dach families (Mishima and Tomarev, 1998; Esteve and Bovolenta, 1999; Sahly et al., 1999; Pandur and Moody, 2000; Streit, 2002; McLarren et al., 2003; Schlosser and Ahrens, 2004), which are known to act as a molecular complex to regulate transcription of downstream target genes (Chen et al., 1997; Ohto et al., 1999; Ikeda et al., 2002; Li et al., 2003; Silver et al., 2003). Importantly, this complex controls various aspects of sensory organ development from the fly to vertebrates (for reviews, see Kawakami et al., 2000; Wawersik and Maas, 2000; Hanson, 2001; Donner and Maas, 2004), and in *Xenopus*, Six1 has recently been shown to promote generic placodal fate in the early embryo (Brugmann et al., 2004). Thus, the PPR is unique with respect to morphology, cell fate and gene expression and the expression of the Six/Eya/Dach cassette may be regarded as crucial step in its induction.

In addition, special properties can be attributed to the PPR that distinguish it from non-placodal ectoderm. First, unlike the rest of the ectoderm, cells within the PPR are competent to form any placode until fairly late developmental stages (Jacobson, 1963b; Baker et al., 1999; Groves and Bronner-

Fraser, 2000). Second, while a number of signalling molecules and transcription factors have been described that, when misexpressed, result in the formation of ectopic placodes, they can only do so close to the location of endogenous placodes (i.e. within the PPR) or when associated with ectopic neural tissue (Oliver et al., 1996; Altmann et al., 1997; Begbie et al., 1999; Chow et al., 1999; Koster et al., 2000; Ladher et al., 2000; Vendrell et al., 2000; Lagutin et al., 2001; Nissen et al., 2003; Shimada et al., 2003; Solomon et al., 2003). These observations suggest that by the time Six, Eya and Dach gene expression overlaps at the border of the neural plate, PPR cells have received signals that bias them towards a placode fate and/or confer generic placode character without imposing specific placode identity.

Although some of the molecular events that control specification of individual placodes, such as lens and inner ear, have been investigated extensively (for reviews, see Baker and Bronner-Fraser, 2001; Chow and Lang, 2001; Brown et al., 2003; Riley and Phillips, 2003), we know very little about the signalling mechanisms that establish the PPR and are therefore responsible for initiating the development of all sensory placodes. Two recent studies in *Xenopus* provided evidence that a balance of BMP and its antagonists is in part responsible for positioning the PPR (Brugmann et al., 2004; Glavic et al., 2004). However, modulation of BMP only affects cells close to the neural plate border and cannot induce a PPR in future epidermis away from its normal location. Thus, the signals that confer PPR properties to naive cells away form the endogenous domain are still elusive. Two other important questions have so far remained unanswered. First, unlike neural crest cells, placodes are exclusively found in the head – what are the molecular mechanisms responsible for this restriction? Second, in the head ectoderm, future neural crest and placode cells arise in close proximity and their precursors are initially intermingled – how do multipotent ectodermal cells decide between these fates?

Here, we use *Six1*, *Six4*, *Eya2* and *Dach1* as molecular markers to define the temporal and spatial aspects of PPR induction in relation to neural and neural crest induction. We then identify the head mesoderm as both necessary and sufficient to induce the PPR, while the neural plate seems to play a reinforcing role. Using gain- and loss-of-function experiments, we demonstrate that activation of the FGF pathway, together with WNT and BMP antagonists, imparts PPR character to naive, ectodermal cells. In addition, we show that WNT signalling is required to restrict PPR development to ensure that placode derivatives only form in the head ectoderm next to the neural tube. Finally, we demonstrate that WNT signalling mediates the decision between neural crest and placode fates: activation of the canonical WNT pathway promotes the formation of neural crest cells at the expense of placodes. Together, our data provide the first molecular model for how cranial neural crest and placode induction is integrated during ectodermal patterning and how the induction of sensory placodes is initiated.

Materials and methods

Embryo culture and grafting

Fertile hens' (Winter Farm, Hertfordshire UK) and quails' eggs (Potter Farm, Cambridgeshire, UK) were incubated at 38°C for 16-27 hours to harvest embryos between primitive streak (stage 3$^+$/4) and head fold stages (stage 6/7). Host embryos were maintained in New Culture (Stern and Ireland, 1981) and grafts placed into the inner third of the area opaca. Quail nodes were excised from primitive streak stage embryos, while the neural plate, head and trunk mesoderm were dissected from stage 5-7 embryos using fine needles and 0.05 mg/ml dispase. FGF8 and BSA coated heparin beads were prepared as described before (Streit et al., 2000); Ag1X2 beads were coated with 100 μM SU5402 in PBS (diluted from a 10 mM stock in DMSO) as previously described (Streit et al., 2000), whereas control beads were incubated in DMSO at appropriate concentration. For whole-embryo treatment with FGF inhibitor, embryos were pre-incubated in 50 μM SU5402 in Pannett-Compton saline for 1 hour at room temperature in the dark, rinsed briefly in saline before being set up for New culture. Control embryos were incubated in appropriate dilutions of DMSO.

Electroporation

For targeted misexpression of secreted and intracellular factors, primitive streak stage embryos were electroporated as described before (McLarren et al., 2003). Crescent, N-frizzled8, activated β-catenin and SMAD6 were cloned into pCAβ-IRES-GFP (McLarren et al., 2003; Linker and Stern, 2004), while WNT8C was cloned into pCAβ and co-electroporated with control GFP vector.

Whole-mount in situ hybridisation and immunocytochemistry

In situ hybridisation and immunostaining using the quail-specific antibody QCPN (Developmental Studies Hybridoma Bank; Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; Department of Biological Sciences, University of Iowa, Iowa City 52242 under contract N01-HD-2-3144 from NICHD) and anti-GFP antibodies (Molecular Probes, USA) was performed as described (Streit et al., 1998; McLarren et al., 2003). After processing specimens were vibratome sectioned (25-35 μm).

Results

Signals from the head mesoderm and the neural plate converge to induce the pre-placodal region

To begin to unravel the cellular and molecular interactions that position the placode territory in relation to other ectodermal derivatives, we sought to identify the source of signals that induce the PPR in naïve ectoderm. The organiser (Hensen's node in amniotes) is a potent source of neural-inducing signals, but also regulates the expression of genes expressed outside the neural plate (Pera et al., 1999; Streit and Stern, 1999). To test whether node-derived signals can induce PPR markers directly, we grafted a quail node (stage 3$^+$/4) into the extra-embryonic area of a chick host of the same stage, a territory competent to generate both central and peripheral nervous system, including crest and placode cells (Woodside, 1937; Gallera and Ivanov, 1964; Gallera, 1971). The early neural marker *Sox3* is rapidly induced in a patch of cells overlying the graft (5/5 in 5 hours; Fig. 1A) (see Streit and Stern, 1999; Streit et al., 2000). However, the non-neural ectoderm marker *Dlx5* (3/6; Fig. 1C) and the PPR markers *Six4* (15/19; Fig. 1B), *Six1* and *Eya2* (not shown; *Six1*: 3/5; *Eya2*: 4/6) are induced only in a ring-like structure at the edge of the ectopic neural plate, and only after prolonged exposure to node signals (16 hours). It has previously been shown that the definitive neural marker *Sox2* requires 9 hours of exposure to organiser signals (Streit and Stern, 1999). Thus, neural induction precedes the generation of

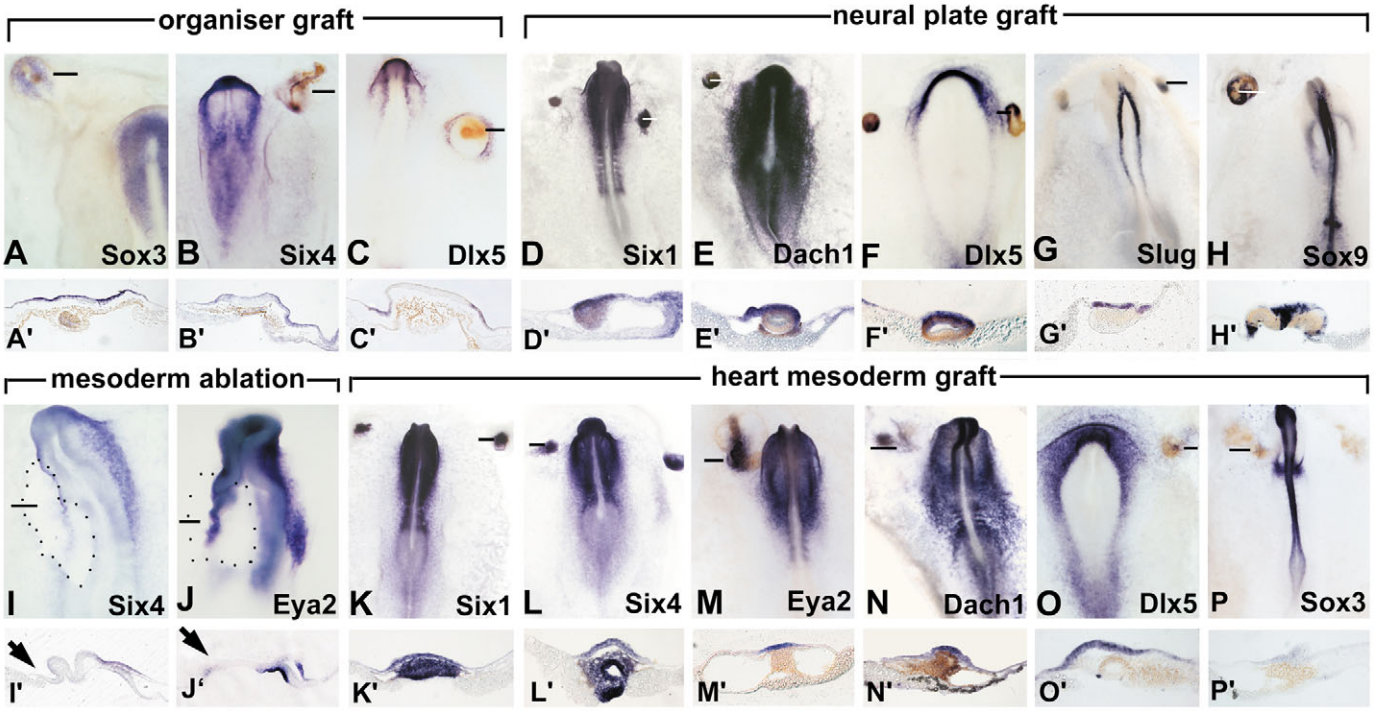

Fig. 1. The pre-placodal region is induced by converging signals from the heart mesoderm and the neural plate. In the extra-embryonic region, Hensen's node (A-C,A′-C′; brown) rapidly induces the pre-neural marker *Sox3* (A,A′; blue), while the pre-placodal marker *Six4* (B,B′; blue) and the epidermal marker *Dlx5* (C,C′; blue) are induced only at the edge of the neural plate after 14-16 hours. Stage 5/6 neural plate grafts (D-H,D′-H′; brown) induce the expression of the pre-placodal markers *Six1* (D,D′; blue) and *Dach1* (E,E′; blue), as well as *Dlx5* (F,F′) and the crest markers *Slug* (G,G′; blue) and *Sox9* (H,H′; blue). Head mesoderm ablation (I,J; I′,J′; broken line in I,J) results in the loss of *Six4* (I,I′, arrow) and *Eya2* (J,J′, arrow). Grafts of head mesoderm (K-P,K′-P′; brown) induce the expression of *Six1* (K,K′; blue), *Six4* (L,L′; blue), *Eya2* (M,M′; blue), *Dach1* (N,N′; blue), *Dlx5* (O,O′; blue) and low levels of *Sox3* (P,P′; blue). Horizontal lines in A-P indicate the level of the sections shown in A′-P′, respectively. Brown staining in A-H,A′-H′,K-P and K′-P′ represents QCPN immunoreactivity labelling quail tissue.

the pre-placodal region, suggesting that the neural plate itself may be responsible for its induction.

To test this possibility, we grafted quail neural plates (stage 5-6) into the extra-embryonic ectoderm of chick hosts (stage 3^{+}/4). Neural crest (*Slug*, 8/10, Fig. 1G,G′; *Pax7*, 5/6; *Sox9*, 3/6, Fig. 1H,H′) and non-neural ectoderm markers (*Dlx5*; 11/22, Fig. 1F,F′) are induced in both host and donor tissue, as is *Dach1* (3/3; Fig. 1E,E′). *Six1* expression is only rarely observed in the host epiblast (2/12), but is strongly upregulated in the neural plate itself (10/12; Fig. 1D,D′). By contrast, *Eya2* (1/13) and *Six4* (0/46) are almost never induced in the host and expression in the graft is found only in a few cases. Thus, while the interaction between neural and non-neural tissue is sufficient to induce neural crest derivatives from both tissues (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995) (this study), reciprocal signalling between them can only induce a subset of pre-placodal markers, suggesting that additional factors are required.

The head mesoderm comes to underlie the PPR at the time when *Six1*, *Six4* and *Eya2* start to be expressed at neurula stages (Rosenquist, 1970; Redkar et al., 2001; Hochgreb et

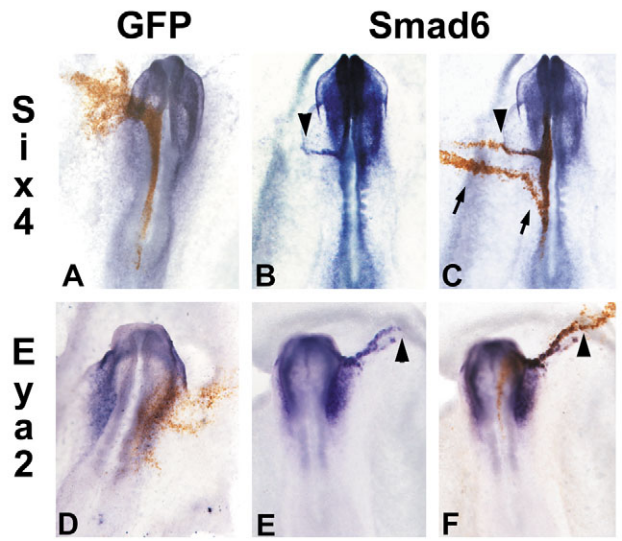

Fig. 2. Inhibition of BMP signalling expands the expression of the pre-placodal genes *Six4* and *Eya2*. Primitive streak stage embryos were electroporated with control GFP containing vector (A,D) or with SMAD6-IRES-GFP vector (B,C,E,F). Expression of *Six4* (A-C; blue) and *Eya2* (D-F, blue) was assessed by in situ hybridisation and the electroporated cells were visualised by staining with GFP antibody (brown in A,C,D,F). While *Six4* and *Eya2* expression is normal in control embryos (A,D), expression of both genes is expanded when BMP signalling is inhibited (B,C,E,F). *Six4* expression is not observed in the trunk region (arrows in C) and ectopic expression of both *Six4* (B,C, arrowheads) and *Eya2* (E,F, arrowheads) is limited laterally.

al., 2003), and is therefore a good candidate to mediate their induction. To test this, we ablated the head mesoderm (including the prospective heart, but excluding paraxial mesoderm) unilaterally before the onset of PPR gene expression. *Six4* (7/8) and *Eya2* (3/6) expression is lost from the overlying ectoderm (Fig. 1I,J), while neural (*Sox3*; *n*=5), neural crest (*Pax7*; *n*=5) and non-neural ectoderm markers (*Dlx5*; *n*=8) are unaffected. Moreover, grafts of stage 5-6 quail head mesoderm, but not trunk paraxial or lateral mesoderm (*n*=36), induce *Six1* (11/21), *Six4* (21/25), *Eya2* (14/42) and *Dach1* (5/6) in chick extra-embryonic ectoderm at stage 4 (Fig. 1K-N,K′-N′). Likewise, they induce *Dlx5* (Fig. 1O) and low levels of *Sox3* (Fig. 1P), which are also characteristic of the pre-placodal territory. By contrast, neural crest (*Pax7*, *n*=6; *Slug*, *n*=2), definitive neural (*Sox2*, *n*=7) or markers specific for mature placodes (*Pax2*, *n*=12; *Pax6*, *n*=6) are never induced. Induction of *Eya2* by head mesoderm only occurs in 33% cases; however, when mesoderm is combined with neural plate, the frequency of induction is doubled (66%).

Together, these experiments identify the mesoderm underlying the PPR as a novel signalling centre in the vertebrate head that directs cell fate decisions in the overlying ectoderm: signals derived from this mesoderm are necessary for specification of sensory placode precursors and also sufficient to induce PPR markers in very remote cells that have never been exposed to neural-inducing factors or to signals from the neural plate. Furthermore, our results show that the neural plate cooperates with mesoderm-derived signals. By contrast, juxtaposition of neural plate and epidermis is sufficient to induce neural crest cells and their derivatives (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995), suggesting that different signalling mechanisms mediate the decision of multipotent ectodermal progenitor cells to form neural crest and placode precursors.

Inhibition of the BMP pathway expands the placode territory into lateral head ectoderm, but is not sufficient to induce it ectopically

What are the signals that position placode precursors to a narrow band in the head ectoderm and prevent sensory organ formation in the trunk? The mesoderm underlying the PPR is a source of many different signalling molecules including FGFs (Shamim and Mason, 1999; Ohuchi et al., 2000), the BMP antagonist DAN (Ogita et al., 2001), and the BMP and WNT antagonist Cerberus (Rodriguez Esteban et al., 1999; Chapman et al., 2002). It has been suggested that a gradient of BMP activity patterns the ectoderm at gastrula stages such that high levels of BMP activity generate epidermis, intermediate levels neural crest and placodes, whereas low or no BMP activity is required for the formation of the neural plate (Sasai and De Robertis, 1997; Wilson et al., 1997; Nguyen et al., 1998; Aybar and Mayor, 2002; Tribulo et al., 2003; Brugmann et al., 2004; Glavic et al., 2004). To modulate BMP signalling in the PPR, we electroporated SMAD6, a cell-autonomous BMP inhibitor, in a line spanning the PPR and the adjacent lateral ectoderm. Although control electroporated embryos show normal expression of all genes tested (*n*=20; Fig. 2A,D), SMAD6 expands the expression of *Six4* (10/12) and *Eya2* (6/9), but not *Six1* (0/10), into the non-placodal ectoderm (Fig. 2B,C,E,F). However, ectopic *Six4* and *Eya2* expression is never observed in the extra-embryonic epiblast or when SMAD6 is misexpressed in the trunk. Thus, inhibition of BMP signalling can expand the PPR but is not sufficient to induce PPR markers in an ectopic location. Moreover, the exclusion of pre-placodal markers from the trunk and lateral head ectoderm suggests that additional inhibitory signals may restrict them to a domain surrounding the anterior neural plate.

WNT signalling restricts the placode territory to the head ectoderm next to the neural plate

WNT proteins are good candidates for such inhibitory signals. The mesoderm lateral and

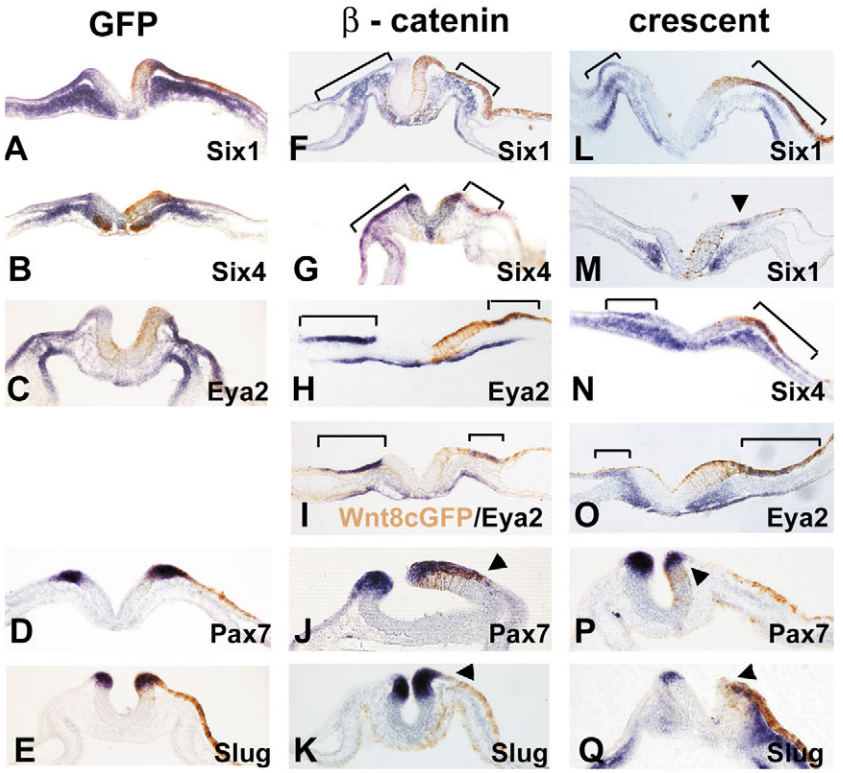

Fig. 3. WNT signalling antagonises the formation of the pre-placodal region (PPR). Primitive streak stage embryos were electroporated with GFP (A-E), β-catenin (F-H,J,K), WNT8C (I) or Crescent (L-Q), and analysed for PPR and neural crest marker expression. Sections through control embryos (A-E) do not show any change in the expression of *Six1* (A), *Six4* (B), *Eya2* (C), *Pax7* (D) and *Slug* (E). Misexpression of β-catenin (F-H,J,K; brown) or WNT8C (I, brown) narrows (brackets in F-I) *Six1* (F), *Six4* (G) and *Eya2* (H,I) expression. By contrast, *Pax7* (J) and *Slug* (K) expression expands into the placodal territory (arrowheads). Inhibition of WNT by Crescent (L-Q, brown) expands *Six1* (L,M), *Six4* (N) and *Eya2* (O) expression into lateral (brackets in L,N,O) and trunk ectoderm (M, arrowhead), while *Pax7* (P, arrowhead) and *Slug* (Q, arrowhead) expression is reduced.

posterior to the PPR expresses high levels of *Wnt8c* (Fig. 4A,A′,A′′), while *Wnt6* is found in trunk ectoderm (Garcia-Castro et al., 2002; Schubert et al., 2002). Electroporation of the soluble WNT antagonist Crescent, but not GFP alone (Fig. 3A-E; *n*=15), into the ectoderm lateral and posterior to the PPR causes an expansion of *Six1* (12/20), *Six4* (7/12) and *Eya2* (12/22) in both directions (Fig. 3L-O). However, misexpression of Crescent in more distant epiblast away from the endogenous PPR has no effect (*n*=33). As attenuation of WNT signalling can also influence mesoderm specification and is required for the formation of heart mesoderm (Marvin et al., 2001), we tested whether the expansion of PPR markers might be due to an alteration of mesoderm patterning. We observed no change in the expression of *Nkx2.5*, a marker for heart mesoderm that normally underlies the PPR (Fig. 4B,C; *n*=10). Thus, as with inhibition of BMP signalling, attenuation of the WNT pathway can expand the PPR, but cannot induce it de novo. However, unlike attenuation of BMP, WNT inhibition can also expand *Six4* and *Eya2* expression into trunk ectoderm.

In a complementary approach, we addressed the issue of whether attenuation of WNT signalling is required to generate sensory placode precursors. To activate the canonical WNT pathway, we misexpressed activated β-catenin or WNT8C and GFP alone (control) in the pre-placodal region. Control embryos occasionally show reduced expression of *Six1* (2/10), *Six4* (5/20) and *Eya2* (3/15). However, when the WNT pathway is activated the expression of all three markers is lost in the electroporated cells (Fig. 3F-I; *Six1*, 6/10; *Six4*, 11/15; *Eya2*, 10/12 show loss). Thus, whereas inhibition of WNT signalling expands the pre-placodal territory, the activation of the canonical pathway suppresses it. We therefore suggest that WNT signals from the lateral and posterior mesoderm and from the trunk ectoderm are critical to restrict sensory placode formation to a narrow band of the head ectoderm and thus ensure that placode derivatives are never found in the trunk.

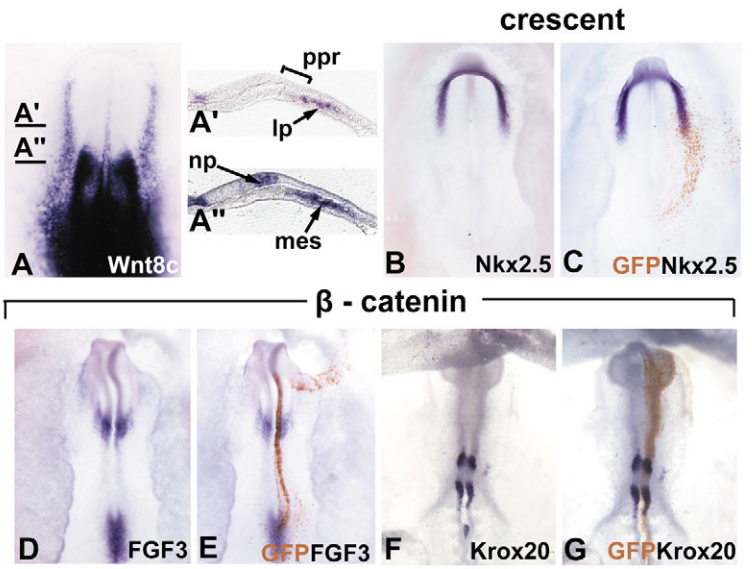

Fig. 4. Modulation of the WNT pathway does not change mesoderm or neural plate patterning. At head-fold stages, *Wnt8c* is expressed in the cranial lateral plate mesoderm (A,A′; lp) next to the pre-placodal region (ppr; bracket), in the trunk mesoderm (A,A′′; mes) and the neural plate posterior to the pre-placodal region (A,A′′; np). Lines in A indicate the positions of the sections shown in A′ and A′′, respectively. (B,C) Misexpression of Crescent (brown in C) does not result in expansion of the heart mesoderm marker *Nkx2.5*. (D-G) Misexpression of activated β-catenin (brown in E and G) does not alter the expression of the regional neural markers *Fgf3* (D,E) and *Krox20* (F,G).

Activation of the WNT pathway promotes neural crest formation at the expense of placodes

In the head ectoderm, neural crest and placode precursors arise in close proximity, but segregate over time (Streit, 2002). Subdivision of the cranial ectoderm into four distinct domains – neural plate, neural crest, PPR and future epidermis – is first apparent around neurula stages (stage 5), when the neural plate has already acquired columnar morphology and expresses definitive neural markers like *Sox2* (Fig. 5F,F′). The ectoderm immediately adjacent to it expresses the early neural crest marker *Pax7*, except in its most anterior aspect (Fig. 5E,E′), and placode precursors can be identified by their co-expression of *Six1*, *Six4*, *Eya2* and *Dach1*. Whereas *Dach1* is found in the entire ectoderm, including the neural plate (Fig. 5D,D′), *Six1*, *Six4* and *Eya2* transcripts are confined to a horseshoe-shaped domain surrounding the anterior neural plate (Fig. 5A-C,A′-C′), which medially overlaps with part of the *Pax7* domain, but never with *Sox2*. Expression of placode and crest markers separates at the 3- to 4-somite stage (Fig. 5G-L,G′-L′): *Pax7* becomes restricted to the neural folds (Fig. 5K,K′), where *Slug* is now also detected (Fig. 5L,L′). These changes of expression patterns reflect the segregation of neural crest and placode fates as the neural folds form (Streit, 2002).

What are molecular mechanisms that control the decision of cells to follow either fate? Our results suggest that attenuation of WNT signalling is required for the induction of the pre-placodal territory. By contrast, neural crest formation depends on activation of the canonical WNT pathway (LaBonne and Bronner-Fraser, 1998; Garcia-Castro et al., 2002; Monsoro-Burq et al., 2003) and both *Wnt1* and *Wnt6* begin to be expressed in the neural folds at the same time as *Slug* and *Sox9*. We find that in the presence of activated β-catenin the neural crest markers *Pax7* (7/8) and *Slug* (6/7) are expanded into the pre-placodal territory (Fig. 3J,K), leading to the simultaneous downregulation of *Six1*, *Six4* and *Eya2* (Fig. 3F-I). However, activation of the canonical WNT pathway lateral to the endogenous neural crest domain never leads to ectopic neural crest cell formation. It is possible that activation of the canonical WNT pathway in the neural folds changes the regional character of the neural plate and thus indirectly leads to the loss of placodal gene expression. However, the expression of *Fgf3* (*n*=5), as a marker for rhombomeres 5-6, and of *Krox20* (rhombomeres 3 and 5; *n*=5) is normal in embryos where the canonical WNT pathway is activated (Fig. 4D-G), indicating that the effect observed is not due to changes in the regional identity of the neural plate.

These results suggest that WNT activity regulates the cell fate choice between crest and placode precursors. This is confirmed by misexpression of the WNT antagonists Crescent or N-frizzled 8, which results in the loss of *Pax7* (5/7; Fig. 3P) and *Slug* (5/6; Fig. 3Q). However, pre-placodal specific

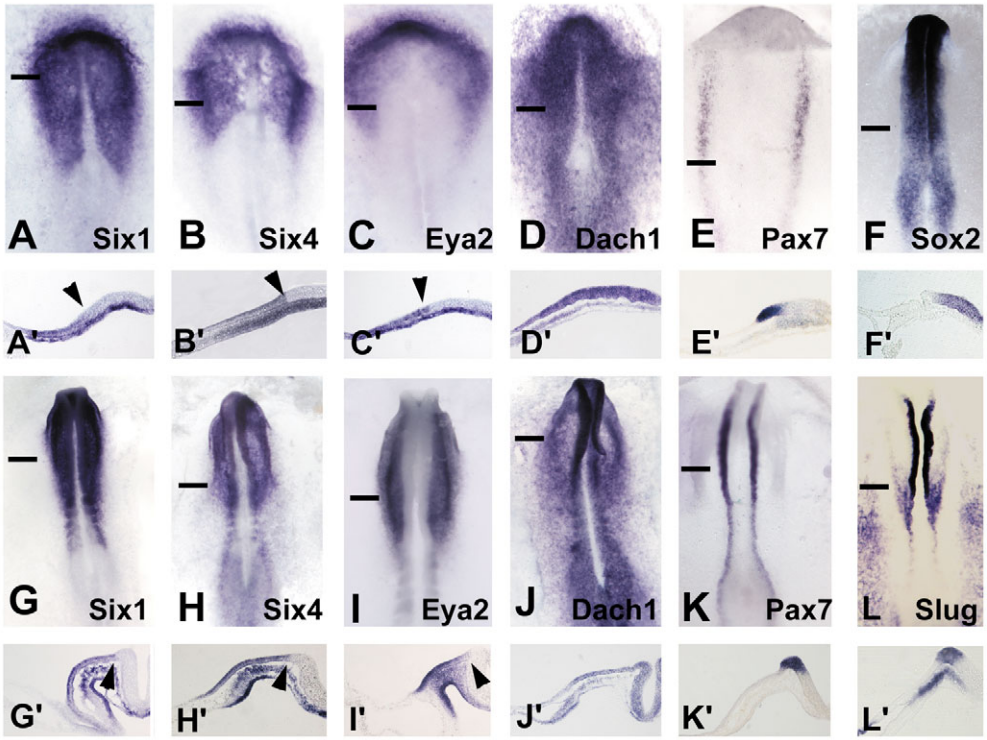

Fig. 5. Segregation of pre-placodal region (PPR) and neural crest markers between head-fold (A-F′) and early somite (G-J′) stages. At stage 5-6 (A-F′), *Six1* (A,A′), *Six4* (B,B′) and *Eya2* (C,C′) are expressed in the ectoderm encircling the anterior neural plate, while *Dach1* (D,D′) is found in the entire ectoderm. *Pax7* (E,E′) is expressed in a band of cells along the neural plate and partially overlaps with *Six1*, *Six4* and *Eya2* expression. PPR markers never colocalise with the neural plate marker *Sox2* (F,F′). By stage 8, *Six1* (G,G′), *Six4* (H,H′) and *Eya2* (I,I′) continue to surround the neural plate, but there is no overlap with *Pax7* (K,K′) or *Slug* (L,L′), which are confined to the neural folds. *Dach1* remains expressed in most of the ectoderm (J,J′). Arrowheads in A′-C′,G-I′ label the medial limit of PPR gene expression; lines in A-L indicate the plane of the sections shown in A′-L′, respectively.

transcripts never expanded into the crest territory. This may be due to the fact that the neural folds express high levels of *Bmp4* (Liem et al., 1995; Streit and Stern, 1999), while formation of the pre-placodal territory requires BMP inhibition (this study). Together these results suggest that segregation of crest and placodal precursors at the border of the neural plate is mediated by WNT signalling: activation of the WNT pathway expands the crest territory at the expense of the pre-placodal region.

FGF signalling cooperates with BMP and WNT antagonists to induce the pre-placode territory

Our results so far show that attenuation of BMP and WNT signalling leads to an expansion of the pre-placodal domain. However, in neither case do we observe ectopic expression of pre-placodal markers, which are separate from the endogenous domain. We therefore tested whether simultaneous inhibition of both pathways could mimic the pre-placode inducing activity of head mesoderm. We misexpressed SMAD6 and Crescent at primitive streak stages to target the ectoderm next to the PPR or the extra-embryonic region and assayed the expression of *Six4* and *Eya2* at early somite stages. The expression of both genes in the presence of both antagonists expands further than in the presence of either inhibitor alone (Fig. 6A-D; *Six4*, 6/6; Eya2, 5/5). However, induced expression of these markers is always continuous with the endogenous pre-placodal region: inhibition of BMP and WNT signalling cannot mimic the PPR inducing activity of head mesoderm. Thus, additional signals are required to recapitulate induction of the PPR in competent ectoderm.

We have previously shown that FGF signalling induces *Msx1*, *Sox3* (Fig. 6E; 4/4) and *Erni* (Streit and Stern, 1999; Streit et al., 2000). While the expression of *Sox3* and *Erni* is widespread before gastrulation, all three genes are later co-expressed with PPR genes at the border of the neural plate. We now find that FGF8-coated, but not control, heparin beads grafted into the extra-embryonic region can also induce the expression of the non-neural ectoderm marker *Dlx5* (Fig. 6F; 12/16) and the PPR marker *Eya2* (Fig. 6H; 10/16) in the absence of the mesodermal markers *Brachyury* (n=5) and *Tbx6l* (n=6). However, FGF8 cannot elicit expression of *Six1* (n=16) or *Six4* (Fig. 6G; n=48). Thus, although FGF signalling promotes the expression of some genes characteristic of the neural plate border, it is not sufficient to induce the complete set of PPR genes.

However, it is possible that FGFs are required upstream and/or in parallel with WNT and/or BMP inhibition to induce a complete ectopic PPR. We therefore co-electroporated SMAD6 or Crescent into the extra-embryonic region of primitive streak stage embryos and grafted FGF8 coated heparin beads next to the electroporated site. Protein expression from electroporated constructs is normally first observed after about 4 hours; therefore, cells are initially exposed to FGF alone, while BMP and WNT antagonists are produced with a delay of a few hours. After overnight culture, neither SMAD6/FGF8 nor Crescent/FGF8 misexpression results in ectopic induction of *Six4* in an isolated patch of cells away from the endogenous PPR (not shown). However, when all three factors are combined, *Six4* is induced in cells next to the FGF8 bead where SMAD6 and Crescent are co-expressed (Fig. 6I,J; 12/15). If the FGF8 bead is removed after 4-5 hours and replaced with a bead coated with the FGF inhibitor SU5402 (Mohammadi et al., 1997), *Six4* induction is still observed (Fig. 5K,L; 7/8), indicating that sustained FGF signalling is not required for *Six4* induction. These observations demonstrate that FGF signalling together with inhibition of both BMP and WNT pathways is sufficient to induce the pre-placodal territory in naïve ectoderm.

To investigate whether FGF signalling is indeed required for

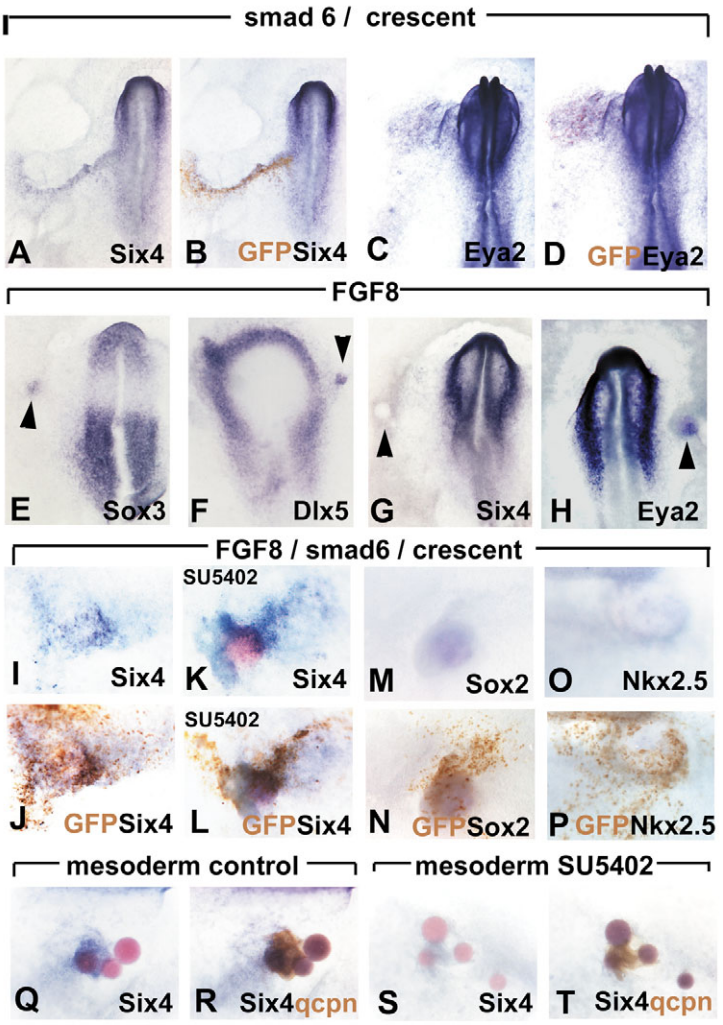

Fig. 6. Cooperation of FGF and BMP and WNT antagonists to induce the pre-placodal region. Misexpression of SMAD6 and Crescent (A-D, brown in B,D) leads to an expansion of *Six4* (A,B; blue) and *Eya2* (C,D; blue) expression into the extra-embryonic epiblast. (E-G) FGF8 induces ectopic *Sox3* (E, arrowhead), *Dlx5* (F, arrowhead) and *Eya2* (H, arrowhead), but not *Six4* (G, arrowhead). Misexpression of SMAD6, Crescent and FGF8 (I-P) results in ectopic induction of *Six4* (I,J; blue) in the absence of the neural marker *Sox2* (M,N) and of the heart mesoderm marker *Nkx2.5* (O,P). When the FGF8 bead is replaced with an SU5402 bead after 5 hours (K,L), induction of *Six4* expression is still observed. Brown staining in J,L,N,P represents immunostaining with anti-GFP antibody to visualise electroporated cells. (Q-T) Head mesoderm (brown in R,T) was grafted into the extra-embryonic region together with control (Q,R) or SU5402-coated beads (S,T). *Six4* expression (blue) is induced in controls (Q,R), but not when FGF signalling is inhibited (S,T). Quail grafts are labelled using QCPN antibody (brown in R,T).

normal expression of PPR markers, head process stage embryos were incubated in the FGF signalling inhibitor SU5402 (50 μM) for 1 hour and then cultured in the presence of the inhibitor until they had reached head fold to early somite stages. No *Six4* expression was observed in the PPR, while control embryos pre-incubated in the appropriate amount of DMSO (solvent for SU5402) showed normal levels of expression (not shown). We have shown above that head mesoderm is able to induce PPR markers in cells that normally never contribute to placodes. Is FGF activity in this mesoderm necessary for their induction? Head mesoderm was grafted into the extra-embryonic region together with either control or SU5402-coated beads, and the expression of *Six4* was assessed after overnight culture. Whereas in controls *Six4* is induced in the overlying epiblast (Fig. 6Q,R; 9/10), its induction is considerably reduced when FGF signalling is inhibited (Fig. 6S,T; 2/10).

Together, these results show that FGF signals from the mesoderm are required for PPR induction and act in concert with BMP and WNT antagonists. In addition, they suggest that FGF signalling may act upstream of BMP and WNT antagonists to initiate PPR induction.

Discussion

The head mesoderm induces sensory placode precursors

Numerous studies have revealed that the induction of sensory placodes at precise positions along the developing neural tube is a multi-step process that requires inputs from more than one tissue and the sequential or parallel action of several signalling pathways (for reviews, see Baker and Bronner-Fraser, 2001; Streit, 2004). In vertebrates, several signals and transcription factors have been identified that control the formation of individual placodes and which, when overexpressed, induce ectopic placodes expressing appropriate markers. However, these molecules can only do so immediately adjacent to endogenous placodes – within the PPR – or when neural tissue is induced as well (Oliver et al., 1996; Altmann et al., 1997; Begbie et al., 1999; Chow et al., 1999; Koster et al., 2000; Ladher et al., 2000; Vendrell et al., 2000; Lagutin et al., 2001; Nissen et al., 2003; Solomon et al., 2003). These observations indicate that the PPR is not only defined by cells of specific fates and by expressing a defined set of molecular markers, but also by its unique properties to respond to placode inducing agents. Therefore, to unravel the signalling events that generate all sensory placodes it is essential to understand the mechanisms that specify ectodermal cells as PPR.

Our results demonstrate that the head mesoderm provides crucial signals that are necessary and sufficient to confer generic, pre-placode marker expression to naïve ectodermal cells in the absence of characteristics of individual placodes. Previous studies have implicated signals from the mesendoderm in the induction of individual placodes, namely the olfactory, lens and otic primordia (Zwilling, 1940; Raven and Kloos, 1945; Yntema, 1950; Yntema, 1955; Jacobson, 1963a; Orts-Llorca and Jimenez-Collado, 1971; Henry and Grainger, 1990; Gallagher et al., 1996). However, owing to the lack of molecular markers, species differences and different timing of the experiments, its precise role remained ill defined. Although recent studies clearly demonstrate a role for mesoderm underlying the otic placode in its induction (Ladher et al., 2000; Wright and Mansour, 2003), its importance in imparting generic placode character to the overlying ectoderm has so far not been recognised. Although our experiments do not specifically test different subpopulations of head mesoderm for their PPR-inducing properties, the heart precursors are

among the inducing cells. Fate map and cell movement studies in amniotes, amphibians and teleosts demonstrate that in the cranial region the heart mesoderm comes to underlie the PPR precisely at the time when *Six* and *Eya* gene expression becomes confined to the overlying ectoderm (Rosenquist, 1970; Kimmel and Warga, 1988; Keller and Tibbetts, 1989; Sater and Jacobson, 1989; Sater and Jacobson, 1990; Warga and Kimmel, 1990; Parameswaran and Tam, 1995; Tam et al., 1997; Redkar et al., 2001; Hochgreb et al., 2003). In addition, like PPR markers in the ectoderm, the early heart-specific transcript *Nkx2.5* is expressed in a horseshoe-shaped domain in the mesoderm. By contrast, input from the neural plate seems to play a minor role in PPR induction. As in *Xenopus* (Glavic et al., 2004; Ahrens and Schlosser, 2005), we find that the neural plate induces *Six1* expression, but it fails to elicit the expression of other markers characteristic for the PPR. Thus, the formation of the common primordium that generates all sensory placodes in the vertebrate head is likely to be initiated through signals that emanate from the head mesoderm, including future heart tissue.

We propose that mesoderm-derived signals are essential to activate placode specific gene expression downstream of the Six/Eya/Dach cascade. While the complex of Six and Dach acts as a transcriptional repressor, the recruitment of Eya into this complex brings in transcriptional co-activators and initiates transcription of downstream targets. At gastrula stages, low levels of *Six1* and *Six4* expression are detected in the neural plate (Esteve and Bovolenta, 1999) (A.L. and A.S., unpublished), where *Dach1* is strongly expressed, thus preventing the activation of target genes. At the time when the definitive neural marker *Sox2* becomes strongly upregulated in the neural plate (stage 4^{+}), *Six1* and *Six4* expression is lost from this domain and confined to the PPR (see Fig. 5). At the same time, the onset of *Eya2* is observed in the PPR, allowing the activation of downstream genes. Thus, the crucial step in the initiation of placode induction may be regulated by the appearance of Eya2 under the influence of mesodermal signals.

Making the difference between cranial neural crest and placode precursors

Like placodes, neural crest cells contribute to the cranial sensory nervous system. Both arise at the border of the neural plate and their precursors are initially mixed. Over time, however, these two cell fates segregate (Streit, 2002). What are the cellular and molecular mechanisms that control this cell fate decision? Unlike the PPR, interaction between the neural plate and future epidermis is sufficient to induce neural crest cells, including some of their derivatives (Moury and Jacobson, 1990; Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Mayor et al., 1997). In addition, signals from gastrula stage dorsolateral mesoderm have been implicated (Raven and Kloos, 1945; Mancilla and Mayor, 1996; Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq et al., 2003). We find that at neurula stages neither cranial (PPR-inducing) nor trunk mesoderm is able to induce neural crest markers in competent ectoderm, whereas trunk paraxial mesoderm from slightly later stages can (A.S., unpublished). Thus, the signals that induce definitive PPR and neural crest character are secreted by different tissues at different times in development.

Multiple signalling pathways have been implicated in the induction of neural crest cells (for reviews, see Aybar et al., 2002; Knecht and Bronner-Fraser, 2002). At gastrula stages, the neural crest domain is thought to be positioned in the ectoderm by intermediate levels of BMP activity, while subsequently FGF, WNT and RA are required to generate definitive neural crest (Mayor et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Garcia-Castro et al., 2002; Villanueva et al., 2002; Monsoro-Burq et al., 2003). One view holds that FGFs, WNT proteins and RA act as posteriorising agents that impart posterior, i.e. neural crest, properties onto cells with ‘anterior neural plate border’ character (for reviews, see Aybar et al., 2002; Knecht and Bronner-Fraser, 2002). However, cranial neural crest cells are generated immediately adjacent to sensory placodes and are therefore unlikely to be subjected to posteriorising signals. Furthermore, we show that in the head, WNT signalling plays an important role in mediolateral patterning of the ectoderm without affecting the position of regional neural markers (*Fgf3* and *Krox20*): activation of the canonical WNT pathway suppresses the formation of sensory placodes while expanding the crest territory. We propose that cells at the border of the anterior neural plate initially have the potential to become both crest and placode, reflected by the fact that their precursors are interspersed (Streit, 2002). As the neural folds form, *Wnt* expression is initiated in the folds concomitant with the onset of *Slug*. Cells that receive WNT signals develop along the neural crest cell lineage, while those that are protected from WNT become placodes (Fig. 7).

Interestingly, even in the absence of WNT activity, placode specific gene expression never expands into the neural folds, suggesting that they contain additional inhibitory signals. Indeed, the neural folds express high levels of *Bmp4* and *Bmp7* transcripts (Liem et al., 1995; Streit and Stern, 1999) and show elevated levels of BMP activity, as determined by the presence of phosphorylated SMAD1 (Faure et al., 2002; Linker and Stern, 2004). By contrast, BMP activity in the placode territory is low (Faure et al., 2002). In agreement with these observations, the generation of neural crest cells requires some level of BMP activity (Liem et al., 1995; Sasai and De Robertis, 1997; Wilson et al., 1997; Nguyen et al., 1998; Aybar and Mayor, 2002; Tribulo et al., 2003; Glavic et al., 2004), while placode precursors require its attenuation (this study).

Thus, the decision of multipotent ectodermal cells to give rise to crest or placode cells is controlled by modulation of local signals that emanate from surrounding tissues. Signals from the neural folds – WNT and BMPs – trigger neural crest development, while signals from the head mesoderm (for which the BMP antagonist DAN and the WNT/BMP antagonist Cerberus are good candidates) protect placode precursors from their inhibitory influence.

Temporal and spatial integration of signalling events to position the placode territory

Several studies in amphibians and fish suggest that in the ectoderm different fates are allocated through a gradient of BMP activity (Sasai and De Robertis, 1997; Wilson et al., 1997; Nguyen et al., 1998; Aybar and Mayor, 2002; Tribulo et al., 2003; Brugmann et al., 2004; Glavic et al., 2004). However, the formation of the otic and olfactory placodes is differentially affected in zebrafish mutants that show different residual levels of BMP activity (Nguyen et al., 1998), making it unlikely that

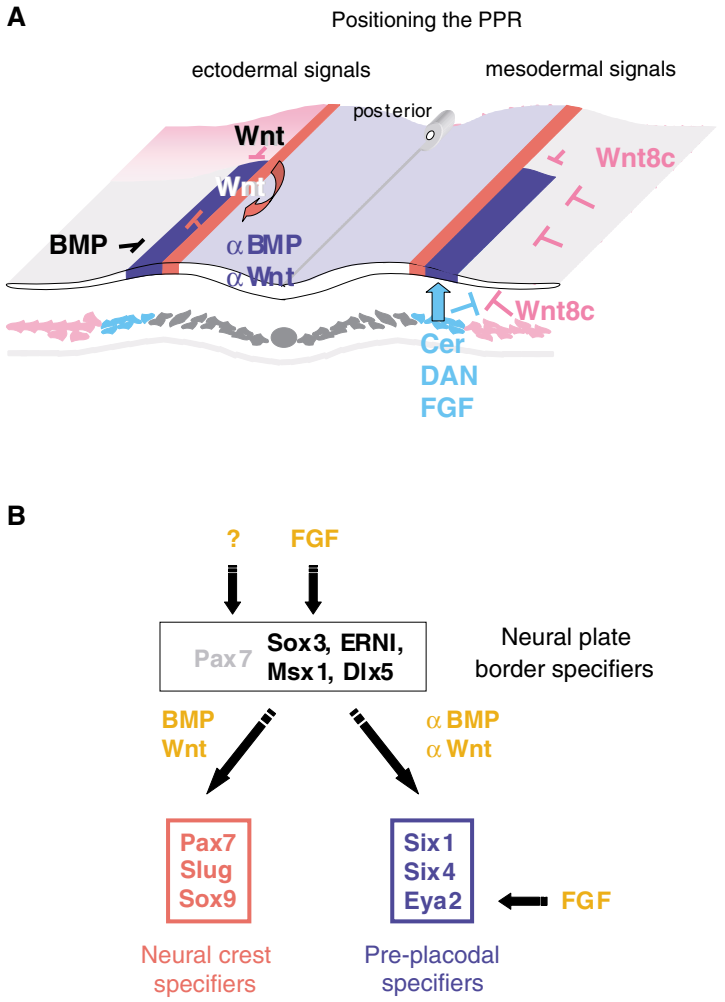

Fig. 7. Model of the signalling interactions that position the pre-placodal region in the cranial ectoderm. (A) The section through an embryo viewed from anterior to posterior represents signalling within the ectoderm on the left and mesoderm-derived signals on the right. The pre-placodal region (dark blue) lies next to the neural crest (red) at the border of the anterior neural plate and is induced by FGF together with BMP and WNT antagonists derived from the underlying mesoderm (light blue). WNT signals from the lateral and posterior mesoderm (pink, right) cooperate with WNT from the trunk ectoderm (pink, left) to limit the PPR. BMP signals from the lateral ectoderm (grey) also prevent expansion of the PPR into more lateral regions. WNT proteins in the neural folds promote neural crest formation (red, left), but inhibit PPR gene expression. The neural plate expresses BMP and WNT inhibitors, which may account for its limited PPR inducing ability. (B) Model for neural crest and placode specification at the border of the neural plate; the terminology follows a recent molecular network for neural crest cell specification proposed by Meulemans and Bronner-Fraser (Meulemans and Bronner-Fraser, 2004), which classifies three categories of molecules: secreted inducers (orange), neural plate border specifiers (black) and neural crest specifiers (red). We propose that the Six/Eya/Dach network may act as pre-placode specifier (blue). Pax7 was classified as a neural plate border specifier (Meulemans and Bronner-Fraser, 2004) and is, like *Msx1*, expressed early; however, so far it is not clear which upstream signalling pathways induce *Pax7*. Our experiments reveal that at neural fold stages its expression is controlled by WNT. FGF signalling plays a dual role: it promotes border specifiers and later pre-placode specifiers, while levels of WNT and BMP activity control neural crest and placode fates in the border.

a BMP gradient alone determines the position where placodes develop. Our findings reveal that although BMP antagonism plays a role in the formation of the pre-placodal territory, its induction by the head mesoderm and neural plate requires the temporal and spatial integration of at least three signalling pathways and is tightly coordinated with the generation of other cell types in the cranial ectoderm (Fig. 7).

We find that FGF signalling cooperates with WNT and BMP antagonists to impart generic placode character to uncommitted ectoderm. In the chick, activation of the FGF pathway in naïve ectoderm leads to rapid expression of pre-neural markers such as *Sox3* and *Erni*, both of which are later co-expressed at the border of the neural plate (Streit and Stern, 1999; Streit et al., 2000) (see Fig. 7B). However, activation of the FGF pathway is not sufficient to specify cells that arise from this border: neural crest (Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2003) and placode precursors (this study) (Ahrens and Schlosser, 2005). The observation that continued FGF signalling is not required for pre-placodal *Six4* expression, but can directly induce *Eya2*, suggests that FGFs may play a dual role. Early FGF signalling may confer 'border character' (Streit and Stern, 1999) to ectodermal cells to make them responsive to PPR and crest inducing signals (Fig. 7B). Our finding that ectopic PPR induction only occurs in the presence of active FGF signalling supports this notion. Later, FGFs from the head mesoderm, probably FGF4 (Shamim and Mason, 1999), initiate the expression of *Eya2* in the placode territory as a crucial step to activate downstream target genes.

Simultaneously, the head mesoderm provides both BMP and WNT antagonists, most likely DAN (Ogita et al., 2001) and Cerberus (Rodriguez Esteban et al., 1999; Chapman et al., 2002), to counteract the inhibitory effect of both factors on the generation of placode precursors (Fig. 7). Our results show that attenuation of either the BMP or WNT pathway leads to an expansion of the PPR into the adjacent ectoderm. However, while the expansion in response to BMP inhibition is limited to the head ectoderm, WNT antagonism also results in the expression of PPR specific genes in the trunk. This is in agreement with recent findings in *Xenopus* reporting that simultaneous overexpression of BMP and WNT antagonist expands *Six1* expression posteriorly along the induced secondary axis (Brugmann et al., 2004). In the chick, *Wnt8c* is expressed in trunk mesoderm and the mesoderm lateral to the heart primordium (Hume and Dodd, 1993) (this study), whereas *Wnt6* is found in trunk ectoderm (Garcia-Castro et al., 2002; Schubert et al., 2002). We propose that WNT activity from surrounding tissues is essential to restrict the placode territory to the head ectoderm next to the neural plate and thus ensure that sensory placodes are confined to the head. To allow

placode formation, WNT antagonists in cooperation with FGF and anti-BMPs from the head mesoderm protect placode precursors from this inhibitory influence.

We thank Drs Paola Bovolenta, Oliver Guillermo, Robert A. Kosher, Ivor Mason, Tom Schultheiss and Stanislav Tomarev for cDNA plasmids. Our special thanks go to Dr Claudia Linker and Dr Federica Bertocchini for the SMAD6 and WNT8C expression constructs, respectively. Thanks to Gerhard Schlosser for sharing unpublished information. We are grateful to Claudio D. Stern and Andy Groves for helpful discussions and comments on the manuscript. This work was funded by the BBSRC, Defeating Deafness, Guy's and St Thomas Charitable Foundation, and a studentship from the Dental Institute.

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