Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor

Samantha A. Brugmann1, Petra D. Pandur1,*, Kristy L. Kenyon2,†, Francesca Pignoni2 and Sally A. Moody1,‡

1Department of Anatomy and Cell Biology, Institute for Biomedical Sciences, The George Washington University, Washington, DC 20037, USA
2Department of Ophthalmology, MEEI, Harvard Medical School, Boston, MA 02138, USA
*Present address: The Burnham Institute, La Jolla, CA 92037, USA
†Present address: Department of Biology, Hobart and William Smith Colleges, Geneva, NY 14456, USA
‡Author for correspondence (e-mail: anasam@gwumc.edu)

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Summary

Cranial placodes, which give rise to sensory organs in the vertebrate head, are important embryonic structures whose development has not been well studied because of their transient nature and paucity of molecular markers. We have used markers of pre-placodal ectoderm (PPE) (six1, eya1) to determine that gradients of both neural inducers and anteroposterior signals are necessary to induce and appropriately position the PPE. Overexpression of six1 expands the PPE at the expense of neural crest and epidermis, whereas knock-down of Six1 results in reduction of the PPE domain and expansion of the neural plate, neural crest and epidermis. Using expression of activator and repressor constructs of six1 or co-expression of wild-type six1 with activating or repressing co-factors (eya1 and groucho, respectively), we demonstrate that Six1 inhibits neural crest and epidermal genes via transcriptional repression and enhances PPE genes via transcriptional activation. Ectopic expression of neural plate, neural crest and epidermal genes in the PPE demonstrates that these factors mutually influence each other to establish the appropriate boundaries between these ectodermal domains.

Key words: Pre-placodal ectoderm, Neural crest, foxD3, zic2, sox2, sox3, keratin, dlx5, dlx6, Cell fate determination, Patterning, Xenopus

Introduction

It was discovered over a century ago that the cranial sensory organs of vertebrates arise from discrete areas of thickened ectoderm, called placodes, that form in characteristic positions in the head (Knouff, 1935; LeDouarin et al., 1986). They give rise to the olfactory, lens, auditory-vestibular and lateral line organs, and contribute to the cranial sensory ganglia. Placodes derive from a band of specialized pre-placodal ectoderm (PPE) that surrounds the anterior neural plate. The PPE is competent to form many different placodes that express distinct developmental fates as the result of interactions with their surrounding tissues (Schlosser and Northcutt, 2000; Baker and Bronner-Fraser, 2001).

Several studies indicate that during gastrulation, embryonic ectoderm is separated into fields with distinct fates (neural plate, neural crest, PPE, epidermis; Fig. 1A) in response to different concentrations of BMP. Genes expressed by the presumptive epidermis are positively regulated by BMPs (Suzuki et al., 1997; Feledy et al., 1999; Beanan and Sargent, 2000; Luo et al., 2001a; Tribulo et al., 2003), whereas anti-BMP factors secreted from the organizer and the dorsal midline mesoderm promote neural plate formation (Weinstein and Hemmati-Brivanlou, 1999; Wilson and Edlund, 2001). It has been suggested that signals responsible for establishing the neural plate also establish a lateral neurogenic ectoderm (LNE) that surrounds the neural plate and gives rise to the intervening neural crest and PPE (Baker and Bronner-Fraser, 2001). The best-studied derivative of the LNE, the neural crest, appears to be induced by intermediate concentrations of anti-BMP factors (Morgan and Sargent, 1997; Marchant et al., 1998; Mayor et al., 1999; Mayor and Aýbar, 2001; Aýbar et al., 2002). In addition, neural crest induction requires signaling pathways that establish the posterior axis of the neural plate (Wnt, FGF, retinoic acid) (LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998; Mayor and Aýbar, 2001; Villanueva et al., 2002; Monsoro-Burq et al., 2003; Glavic et al., 2004a). Whether similar signaling events are involved in establishing the PPE has not been addressed because of a paucity of molecular markers specific for this ectodermal domain.

The LNE also appears to require interactions between the presumptive neural plate and epidermis to initiate the expression of transcription factors that define this border zone and its derivatives (Streit and Stern, 1999; McLarren et al., 2003). For example, Dlx genes are induced by BMP in a concentration-dependent pattern in the epidermis and play a role in positioning the boundaries of the neural plate and the neural crest (Feledy et al., 1999; Beanan and Sargent, 2000; Luo et al., 2001a; Tribulo et al., 2003; McLarren et al., 2003;
Zic genes are initially expressed throughout the neural plate in response to anti-BMP factors, and as they become restricted to its lateral border they initiate neural crest fates (Nakata et al., 1997; Nakata et al., 1998; Brewster et al., 1998; Kao et al., 1998; Mizuseki et al., 1998). The roles that border genes play to specify the fates of the different ectodermal subdomains remain to be elucidated.

Although placodes have long been recognized as important embryonic structures, their transient nature and the lack of specific molecular markers have made it difficult to study the mechanisms by which they form. Recently, however, markers of the PPE during the initial induction of the placodes have been identified in Xenopus. *six1* is homologous to *Drosophila sine oculis*; it is characterized by a homeobox DNA-binding domain and a protein-protein interaction domain called the Six domain.

It is initially expressed in a band surrounding the anterior neural plate and later in all neurogenic placodes (Pandur and Moody, 2000). *eyal* is homologous to *Drosophila eyes absent* (*eya*); it functions as a co-factor for Six genes of the Six1/2 and Six4/5 subfamilies (Pignoni et al., 1997; Ohno et al., 1999; Ikeda et al., 2002) and is expressed in a pattern very similar to that of *six1* (David et al., 2001). We have used these markers to demonstrate that gradients of both neural inducer and anteroposterior signals are required for proper PPE formation. Moreover, we show that *six1* expression is required for the establishment of the PPE, and it promotes the PPE at the expense of the neural crest and epidermis by both activating and repressing target gene expression. Finally, we demonstrate that several genes expressed in the embryonic ectoderm mutually influence each other to define its distinct subdomains.

**Materials and methods**

**Expression constructs**

The full open-reading frames of *Xenopus six1* and *Drosophila groucho* (*Dgroucho;* LD33829, Berkeley *Drosophila* Genome Project) were cloned into expression vectors (pDH105, pCS2+). To generate a chimeric transactivating *six1* construct, the Six domain plus the homeodomain (SDHD; amino acids 9-183) was cloned into expression vectors (pDH105, pCS2+). Transcripts of *six1* were transcribed in vitro, and ligated upstream of the VP16 activation domain in pCS2VP16 (from M. Whitman). To generate a chimeric repressive *six1* construct, the SDHD region was ligated downstream of the Engrailed repressor (EnR) domain in pCS2EnR (from D. Kessler).

**RNA microinjection**

Transcripts of *six1* (400-600 pg), *six1VP16* (100 pg), *six1EnR* (100 pg), *Dgroucho* (400 pg), *noggin* (5-40 pg) (Smith and Harland, 1992), *chordin* (5-40 pg) (Sasai et al., 1994), *dnWnt8* (500 pg) (Hoppler et al., 1996), *frzb-1* (750 pg) (Wang et al., 1997), *bmp4* (10-50 pg) (Dale et al., 1992), *eyal* (400 pg) (David et al., 2001), *zic2* (100 pg) (Brewster et al., 1998), *foxD3* (25 pg) (Sasai et al., 2001) (from D. Kessler), *dlx5* (200 pg) (Luo et al., 2001a), *dlx6* (200 pg) (Luo et al., 2001a) and *sox2* (200 pg) (Mizuseki et al., 1998) (from T. Grammar) were mixed with back-galatosidase (*β*-gal) mRNA (100-200 pg) and microinjected into identified blastomeres with known ectodermal fates (Fig. 1C) as described (Moody, 2000).

**Yeast two hybrid analysis**

To determine if *Xenopus six1* interacts with *Dgroucho*, the Six domain (amino acids 9-123) was cloned into pGBK7T7, and an N-terminal region of *Dgroucho* (amino acids 1-247) was cloned into pGAD424 (Clontech). The yeast strain AH109 was transformed with both vectors and assayed for reporter gene expression according to the MATCHMAKER kit (Clontech).

**Morpholinos**

Morpholino antisense oligonucleotides (MO) were synthesized against two different potential translational start sites in *six1* (5′-GGAGGCAGCATAGACATGGCTCAG-3′ and 5′-CGCACAGCGAACACATACACCGG-3′ (Gene-Tools). An equimolar mixture of the two *six1-MO*, or a standard control MO (5′-GGAGGCAGCATAGACATGGCTCAG-3′) was microinjected (10-16 ng). A Myc-tagged construct (*six1-myc*) containing the *six1* wild-type 5′UTR was generated to assess morpholino knock-down efficacy by immunofluorescent detection of protein. A rescue construct (*six1- rescue*) was generated by replacing the *six1* wild-type 5′UTR with pCS2+ sequence and changing nine bases in the coding region that would interfere with MO binding without altering amino acid sequence.

**Animal cap explants**

The animal pole was injected with mRNAs for *noggin* (50 pg), *cerberus* (50 pg) (Bouwmeester et al., 1996), *bmp4* (50 pg), *Wnt8* (50 pg) (Hoppler and Moon, 1998) or constitutively activated *fgf1* (*cfgfr1*, 50 pg) (Neilson and Friesel, 1996). Animal cap explants were dissected at stages 8.5-9 and cultured in NAM (Messenger and Warner, 1979), in some cases supplemented with recombinant mouse Noggin protein (R & D Systems). Explants were processed for either RT-PCR or in situ hybridization. For each in situ hybridization experiment, control and an entire series of Noggin-treated explants were processed in parallel so that staining intensities could be compared. The intensity of reactivity in experimental caps was compared with control caps, and then sorted into three groups of staining intensity (none, moderate, high). Frequencies of caps in each group were compared between treatments by Chi-square analyses.

**RT-PCR**

Total RNA from animal cap explants was isolated then subjected to first strand cDNA synthesis using oligo(dT) primers. PCR was performed in the linear range using *six1* primers as described (Pandur and Moody, 2000).

**In situ hybridization**

Full-length antisense RNA probes for *six1* (Pandur and Moody, 2000), *eyal* (David et al., 2001), *sox2* (Penzel et al., 1997) (from R. Grainger), *sox3* (Zygler et al., 1998), *sox1* (from T. Grammar and R. Harland), *foxD3* (Sasai et al., 2001) (from D. Kessler), *epidermal specific keratin* (Jonas et al., 1989), *dlx5* and *dlx6* (Luo et al., 2001a), and *zic2* (Brewster et al., 1998) were transcribed in vitro, and embryos were processed by standard protocols (Sive et al., 2000). The widths of the expression domains of marker genes were measured in whole-mount preparations of *β*-gal mRNA-injected control embryos and of experimental transcript-injected embryos at 40× with an eyepiece micrometer, as described (Kenyon et al., 2001). Measurements were expressed as differences between injected and uninjected sides of the same embryo, and the mean differences between groups were analyzed by *t*-tests.

**Results**

*six1* is induced by neural inducers in a concentration-dependent manner

*six1* expression is initiated during gastrulation and consolidates into a discrete band adjacent to the anterior neural plate that corresponds to the classical description of the PPE (Fig. 1A-B). Consistent with the hypothesis that LNE derivatives are induced by the same anti-BMP factors that establish the neural plate, *noggin* and *cerberus* mRNA-injected animal cap explants express *six1*, whereas neither uninjected nor *bmp4* mRNA-injected explants do (Fig. 2A). It has been proposed that neural crest forms in response to an ‘intermediate’ concentration of...
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neural inducers (Morgan and Sargent, 1997; Marchant et al., 1998). To test whether this is also true for the PPE, uninjected animal cap explants were cultured in a range of Noggin protein concentrations then subjected to in situ hybridization detection of six1 expression. Explants were scored as none, moderate or high-expressing in comparison with control explants processed in parallel but cultured in the absence of Noggin. The largest percentage of explants expressing six1 at high levels was observed in explants cultured in 1-5 ng/ml of Noggin protein, and this expression was drastically reduced as Noggin concentration increased to 25 ng/ml (Fig. 2B). To confirm that this response was specific to Noggin activity as an anti-BMP factor, embryos were first injected with bmp4 mRNA then explants were cultured in the presence of 1 ng/ml Noggin. six1 expression was undetected in 66.3% of Noggin-treated BMP-injected explants (n=83), compared with only 3.2% in Noggin-alone treated explants (n=62) (Fig. 2C). These data indicate that six1 expression is most highly induced at low concentrations of anti-BMP factors.

To determine whether the concentration of Noggin that highly induces PPE genes differs from that for other neurogenic fields, the in situ hybridization assay was repeated using foxD3 (neural crest), sox2 (neural plate) and eya1 (an additional PPE marker) (Fig. 2D). Neither foxD3 nor sox2 was induced at the low levels of Noggin that were sufficient for PPE induction, but they began to be highly expressed at higher concentrations (>25ng/ml). eya1 was highly induced by low levels of Noggin and repressed by high levels, similar to six1. These results support the proposal that genes characteristic of the three early neurogenic fields (neural plate, neural crest, PPE) are most highly induced at different concentrations of anti-BMP factors, corresponding to their respective distances from an endogenous source of these factors at the dorsal midline.

Neural inducers alone are not sufficient to induce six1 in the intact embryo

Consistent with the explant data, increased BMP signaling in the lateral ectoderm of the intact embryo, achieved by injecting doses of bmp4 mRNA that do not disrupt axial patterning into ventrolateral blastomeres that contribute significantly to the LNE (V1.2.1, V1.2.2; Fig. 1C), reduced endogenous six1 expression (Fig. 3A; 56% of cases at 20 pg, 67% at 40 pg). To test whether neural inducer alone is sufficient to induce the PPE in the intact embryo, different concentrations of noggin or chordin mRNA (10 pg, 20 pg and 40 pg) were microinjected into a ventral blastomere (V1.1.2; Fig. 1C) that contributes significantly to the ventral epidermis. These concentrations were used because they induce neural genes in explants but infrequently re-pattern the embryo to produce secondary axes. Two morphologies were observed: a dispersed clone with no obvious patterning defects (Fig. 3B) or an elongated clone associated with a ventrally located putative secondary axis (Fig. 3C,D). Staining with sox2 confirmed that every embryo with an elongated clone

FIG. 1. (A) In the stage 16 Xenopus embryo there are four major ectodermal domains: neural plate, neural crest, pre-placodal ectoderm (PPE) and epidermis. (B) The expression pattern of six1 coincides with the PPE. (C) An idealized depiction of the ectodermal fate of the 32-cell embryo showing four dorsal blastomeres (blue) that contribute significantly to the neural plate, two ventrolateral blastomeres (green) that contribute significantly to the neural crest and PPE, and two ventral blastomeres (yellow) that contribute significantly to the ventral epidermis (from Moody, 1987).

FIG. 2. (A) RT-PCR analysis showing that explants injected with noggin or cerberus mRNAs express six1, whereas control and bmp4-injected explants do not. H4, loading control. (B) Explants were cultured in different concentrations of Noggin, and processed for six1 expression at stage 17. The largest percentage of explants stained at high levels was observed between 1 and 5 ng/ml of Noggin. (C) BMP4 antagonizes the 1 ng/ml Noggin-induction of six1. (D) Explants were cultured as in B, and the percentage of explants with high levels of marker gene expression were plotted.
Posteriorizing signals restrict six1 expression to the head

That six1 expression was always confined to the anterior pole of both the endogenous axis and the induced secondary axis suggests that signals associated with establishing the anteroposterior neural axis (e.g. Wnts, FGFs) (Gould and Grainger, 1997; Gamse and Sive, 2000) influence where the PPE forms. To test this, endogenous Wnt signaling was reduced in the LNE of the intact embryo by co-expressing either a Wnt1 family antagonist (Frzb-1) or a dominant-negative form of Wnt8 (dnWnt8) with Noggin. Co-expression of frzb-1+noggin mRNAs did not induce six1 in the absence of a secondary axis (Fig. 3E; n=18), but when a secondary axis formed six1 expression expanded markedly further posterior in every case (Fig. 3F; n=25). Co-expression of dnWnt8+noggin mRNAs caused six1 expression to encircle the entire embryo (Fig. 3G; 84%, n=76), consistent with reports that blocking Wnt signaling anteriorizes the entire embryo (Glinka et al., 1998; Itoh and Sokol, 1999). By contrast, mRNAs encoding molecules that activate Wnt or FGF pathways (Neilson and Friesel, 1996; Fredieu et al., 1997; Pöpperl et al., 1997) repressed six1 expression, both in whole embryos and in explants cultured in Noggin (Fig. 3H-J). Consistent with reports that posteriorizing factors play a positive role in neural crest induction, foxD3 expression was reduced after frzb-1+noggin mRNA injections (Fig. 3K), and expanded after Wnt8 or cfgfr1 mRNA injections (Fig. 3L,M). These data demonstrate that like neural crest genes, normal six1 expression is modulated by both neural inductive and posteriorizing factors. However, unlike the neural crest, PPE formation is positively influenced by the former and negatively regulated by the latter. Therefore, the relative levels of these factors likely influence whether a LNE cell expresses a neural crest versus placodal fate.

Six1 expression expands placode gene expression at the expense of epidermis and neural crest, and is necessary for PPE formation

At neural plate stages, sox2/sox3 neural plate expression domains are separated from the six1/eya1 placodal domains by

containectotic neural tissue (Fig. 3C), whereas none with a dispersed clone did. Regardless of concentration of injected mRNA, in no case was there ectopic six1 expression associated with a dispersed clone (Fig. 3B), but in every elongated clone, ectopic six1 expression occurred at its anterior tip (Fig. 3D). Thus, in contrast to explants, in which Noggin alone induces six1 expression, in whole embryos the additional presence of an axis is necessary for six1 expression.
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Table 1). These changes in gene expression in the lateral ectoderm did not significantly alter the extent of the neural plate domain, as defined by the size of the expression domains of sox2 or sox3 (Fig. 4K; Table 1). These results demonstrate that elevated sixl expression in the lateral ectoderm promotes PPE genes at the expense of epidermal and neural crest genes, but has minimal direct effect on the neural plate domain.

To test whether Six1 is required for the formation of the PPE, two morpholino antisense oligonucleotides that span sixl translation start sites were injected into ventrolateral blastomeres to locally reduce endogenous sixl translation. The PPE markers sox11 (86.4%) and eyal (95.5%) were significantly reduced (Fig. 4F; Table 1), compared with uninjected controls and to control MO-injected embryos. By contrast, the domains of keratin (95.5%) and foxD3 (80%) were significantly expanded (Fig. 4C,I; Table 1). Lateral reduction of Six1 also significantly enlarged the neural plate domains of sox2 (90.9%) and sox3 (95.5%) (Fig. 4L; Table 1). Protein translated from an injected myc-tagged sixl mRNA was repressed in the presence of sixl-MO (Fig. 4M-O), demonstrating the efficacy of the knock down. Furthermore, the sixl-MO expansion of foxD3 expression reverted to control levels by the co-injection of a sixl-rescue mRNA (Fig. 4P). These results demonstrate that Six1 is necessary for the expression of PPE genes, and that in its absence neighboring ectodermal domains expand.

**sixl affects ectodermal genes via both transcriptional activation and repression**

Six1/2 type factors likely function as both transcriptional activators and repressors depending upon the presence of co-factors (Silver et al., 2003). To determine whether the above effects are via transcriptional activation or repression, both activating (sixlVP16) and repressive (sixlEnR) constructs were made. The keratin expression domain was dramatically repressed by sixlVP16 (Fig. 5A; 96.3%; Table 1). The lateral expression of sixlEnR also caused a significant reduction in keratin expression (Fig. 5C; 100%; Table 1). PPE genes (sox11, 95.5%; eyal, 75%) were significantly expanded by sixlVP16, and significantly reduced (68.8% and 90%, respectively) by sixlEnR (Fig. 5E,G; Table 1). The foxD3 expression domain was significantly expanded by sixlVP16 (90.9%), and significantly reduced by sixlEnR (Fig. 5L; 81.8%; Table 1). A neural plate gene (sox2) was unaffected by either construct expressed in the lateral ectoderm (Table 1). These results predict that: (1) keratin expression is repressed by Six1 both directly and indirectly because the sixl-WT and both activating and repressing constructs reduce its domain; (2) PPE genes are transcriptionally activated by Six1 because the effect of sixlVP16 mimics sixl-WT and the effect of sixlEnR is the reverse; (3) foxD3 is transcriptionally repressed by Six1 because the effect of sixlEnR mimics sixl-WT and the effect of sixlVP16 is the reverse.

These predictions are supported by co-expressing sixl-WT with known co-factors that have either activating or repressive functions. The interaction between Six and Eya proteins to cooperatively regulate transcription has been well documented with both Drosophila and vertebrate proteins (Pignoni et al., 1997; Ohno et al., 1999; Ikeda et al., 2002; Silver et al., 2003). The interaction between vertebrate Six and Eya proteins is thought to be necessary to recruit Eya proteins into the nucleus

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**Fig. 4.** Neural plate stage control expression patterns (A,D,G,J). (B,E,H,K) Overexpression of sixl-WT (B) represses keratin. (E) increases the placodal domain of sox11 (inset, control side). (H) represses foxD3, but (K) has no significant effect on sox2 expression. The asterisks indicate the injected sides. (C,F,I,L) Six1-MO knockdown (C) expands the keratin domain closer to the border of the neural plate [np; compare bars on injected (*) versus control sides]. (F) reduces the placodal domain of sox11. (I) increases the width of foxD3 domain, and (L) expands sox2 (bars indicate distance from midline). Quantitation of changes is presented in Table 1. (M) Injection of sixl-myc results in protein expression detected by Myc antibody (green). (N) No protein (green) is detected when sixl-MO is co-injected. (O) Same section as in N showing presence of lysamine-tagged sixl-MO. (P) Injection of sixl-rescue mRNA restores normal foxD3 domain on sixl-MO injected side (*).
(Ohno et al., 1999). Sequences in the SD/HD region target the complex to DNA and the Eya conserved N-terminal domain causes the complex to activate transcription (Pignoni et al., 1997; Xu et al., 1997; Kawakami et al., 2000). We co-expressed six1-WT and eya1-WT mRNAs in ventrolateral blastomeres and observed that for every marker gene examined results were identical to those obtained with the six1EnR constructs (Fig. 5B,F,J; Table 1). The transcriptional co-repressor Groucho also is unable to bind to DNA directly (Courrey and Jia, 2001) and requires protein-protein interactions to assert its repressive activity. In yeast two-hybrid analyses, the vertebrate homologue Six3 has been shown to physically interact via the SD region with Groucho-related (Grg) proteins in mouse and in zebrafish (Kobayashi et al., 2001; Zhu et al., 2002). Although Six3/Six6 homologs are distinct from other Six related factors as they do not interact with Eya type proteins, the Drosophila Six1/2 type homologue Sine oculis does interact with Groucho in yeast two-hybrid analyses (Giot et al., 2003) (K.L.K. and F.P., unpublished). As there are multiple Grg proteins in vertebrates and no biochemical data are available for those specific to Xenopus, we determined that the Six domain of Xenopus Six1 does interact with Drosophila Groucho using yeast two-hybrid analysis (data not shown). Thus, we used Drosophila Groucho for our functional assays in Xenopus embryos. We co-expressed six1-WT and Dgroucho-WT mRNAs in ventrolateral blastomeres and observed that for every marker gene examined results were identical to those obtained with the six1EnR

![Table 1. Differences in expression domain sizes in response to altered levels of six1](image)

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Tissue marked</th>
<th>Control</th>
<th>six1-WT</th>
<th>six1VP16</th>
<th>six1-WT + eya1-WT</th>
<th>six1EnR</th>
<th>six1-WT + Dgroucho</th>
<th>Six1-MO</th>
<th>Control MO</th>
</tr>
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<tbody>
<tr>
<td>keratin</td>
<td>Epidermis</td>
<td>2.3%</td>
<td>(n=19)</td>
<td>29.8%†</td>
<td>304.7%†</td>
<td>250.9%†</td>
<td>190.8%†</td>
<td>76.5%†</td>
<td>25.6%</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.001, n=38)</td>
<td>(P&lt;0.001, n=27)</td>
<td>(P&lt;0.001, n=21)</td>
<td>(P&lt;0.001, n=23)</td>
<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=33)</td>
</tr>
<tr>
<td>sox11</td>
<td>Placode</td>
<td>0.8%</td>
<td>(n=29)</td>
<td>38.4%*</td>
<td>55.2%*</td>
<td>23.5%†</td>
<td>37.6%†</td>
<td>23.0%†</td>
<td>0.8%†</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.001, n=24)</td>
<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=16)</td>
<td>(P&lt;0.001, n=24)</td>
<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=44)</td>
</tr>
<tr>
<td>eya1</td>
<td>Placode</td>
<td>0.1%</td>
<td>(n=38)</td>
<td>34.4%*</td>
<td>27.4%*</td>
<td>Not determined</td>
<td>-</td>
<td>35.5%†</td>
<td>26.8%</td>
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<td></td>
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<td>(P&lt;0.001, n=20)</td>
<td>(P&lt;0.001, n=16)</td>
<td>(P&lt;0.001, n=20)</td>
<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=22)</td>
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<tr>
<td>foxD3</td>
<td>Neural crest</td>
<td>0.9%</td>
<td>(n=39)</td>
<td>21.2%†</td>
<td>67.4%*</td>
<td>125.8%*</td>
<td>29.0%†</td>
<td>33.6%†</td>
<td>25.4%</td>
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<td>(P&lt;0.001, n=22)</td>
<td>(P&lt;0.001, n=20)</td>
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<td>(P&lt;0.001, n=22)</td>
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<td>(P&lt;0.001, n=22)</td>
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<tr>
<td>sox2</td>
<td>Neural plate</td>
<td>0.1%</td>
<td>(n=37)</td>
<td>1.9%* (NC; n=20)</td>
<td>0.9%* (NC; n=23)</td>
<td>0.01%* (NC; n=16)</td>
<td>9.6%* (P&lt;0.01; n=21)</td>
<td>32.4%*</td>
<td>0.7%†</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>(NC; n=46)</td>
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<td>(P&lt;0.001; n=16)</td>
<td>(P&lt;0.001; n=16)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=22)</td>
</tr>
<tr>
<td>sox3</td>
<td>Neural plate</td>
<td>0.1%</td>
<td>(n=40)</td>
<td>4.9%* (NC; n=24)</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
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<td>Not determined</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(NC; n=24)</td>
<td>(P&lt;0.001; n=20)</td>
<td>(P&lt;0.001; n=16)</td>
<td>(P&lt;0.001; n=16)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=22)</td>
</tr>
<tr>
<td>zic2</td>
<td>Lateral patch</td>
<td>2.1%</td>
<td>(n=27)</td>
<td>25.2%*</td>
<td>254.4%*</td>
<td>69.9%*</td>
<td>11.3%* (NC; n=19)</td>
<td>19.3%*</td>
<td>30.7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.001; n=26)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=21)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=22)</td>
</tr>
<tr>
<td>dlx5</td>
<td>Epidermal border</td>
<td>0.5%</td>
<td>(n=34)</td>
<td>17.9%†</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.001; n=14)</td>
<td>(P&lt;0.001; n=15)</td>
<td>(P&lt;0.001; n=15)</td>
<td>(P&lt;0.001; n=15)</td>
<td>(P&lt;0.001; n=15)</td>
<td>(P&lt;0.001; n=15)</td>
<td>(P&lt;0.001; n=15)</td>
</tr>
<tr>
<td>dlx6</td>
<td>Epidermal border</td>
<td>0.02%</td>
<td>(n=22)</td>
<td>12.3%*</td>
<td>29.6%†</td>
<td>23.5%†</td>
<td>35.8%†</td>
<td>17.2%†</td>
<td>26.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.001; n=27)</td>
<td>(P&lt;0.001; n=15)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=20)</td>
<td>(P&lt;0.001; n=22)</td>
<td>(P&lt;0.001; n=25)</td>
<td>(P&lt;0.001; n=25)</td>
</tr>
</tbody>
</table>

Measurements are expressed as the mean percent difference between uninjected and injected sides of embryos. For control embryos, it is the mean differences between left and right sides. Percent differences from experimental embryos were compared with control embryos using Student’s t-test. NC, no significant change (P<0.01). *An increase in expression domain size. †A decrease in expression domain size.

Fig. 5. Constructs that cause transcriptional activation (six1VP16; six1+eya1) reduce keratin expression (A,B), expand the placodal domain of sox11 (E,F) and expand the foxD3 domain (I,J). Constructs that cause transcriptional repression (six1EnR; six1+groucho) reduce keratin expression (C,D), reduce sox11 placodal expression (G,H) and reduce the foxD3 domain (K,L).
construct (Fig. 5D,H,L; Table 1). These data indicate that Six1 functions in the embryo as both a transcriptional activator and repressor, depending upon which co-factor it is able to interact. In Xenopus, both eya1 and at least three Grg genes are expressed in domains that overlap with endogenous six1 expression (Choudhury et al., 1997; Mollenar et al., 2000; David et al., 2001), but relative levels of protein expression or functional activity are not known.

**Subdomains within the LNE depend upon mutual gene interactions**

It has been proposed that interactions between the border of the neural plate and the non-neural ectoderm are crucial for establishing fates within the LNE (Luo et al., 2001a; McLarren et al., 2003; Woda et al., 2003). We investigated whether expanded six1 expression affects genes whose domains lie at the border between the PPE and neural plate (ziec2) or the border between the PPE and epidermis (dlx5, dlx6). Increased six1-WT expression significantly expanded the patch of ziec2 that is expressed lateral to the neural plate (Fig. 6A,B; 73.1%; Table 1), without affecting the neural plate domain (data not shown). This effect was mimicked by both the six1VP16 construct (100%) and co-injection of six1-WT+eya1-WT mRNAs (85.7%), but not by the sixEnR construct or co-injection of six1-WT+Dgroucho mRNAs (Table 1). These results indicate that Six1 positively regulates the lateral ziec2 domain via transcriptional activation. However, reduction of Six1 protein by six1-MO injections also expanded ziec2 expression (Fig. 6C; 90%; Table 1), indicating that other factors that are antagonized by Six1 probably also positively regulate ziec2 (see below). Increased six1-WT expression had two effects on Dlx expression. First, it caused the lateral stripes of dlx5 and dlx6 expression to be located further laterally (Fig. 6E; 71.4% and 70.4%, respectively; Table 1). Second, the dlx5 (78.6%) and dlx6 (51.9%) stripes either had gaps of full repression or were diffuse and less intense compared with the uninjected, control side (Fig. 6E). The six1VP16 construct (93.3%), co-injection of six1-WT+eya1-WT mRNAs (90.0%), the six1EnR construct (80.0%) and co-expression of six1-WT+Dgroucho mRNAs (65.0%) all caused a phenotype similar to six1-WT for dlx6 (Table 1). Both dlx5 (84.6%; Table 1) and dlx6 (Fig. 6F; 100%; Table 1) were moved more laterally or repressed by reduction of Six1 by MO injection, suggesting that other factors antagonized by Six1 also negatively regulate the dlx5/6 genes (see below).

The LNE comprises several subdomains of a number of transcription factors (Schlosser and Ahrens, 2004; Glavic et al., 2004b), many of which are affected by altering the levels of six1 expression. To understand further the regulatory interplay that subdivides the LNE, we investigated the effects of expanded expression of some of these genes on neighboring expression domains. Expression of sox2 in the lateral ectoderm repressed six1 expression (Fig. 6G; 88%, n=17). Expanded expression of foxD3 repressed six1 expression (Fig. 6H; 92%, n=39), caused ectopic sox2 expression (not shown, 100%, n=16), repressed keratin expression (not shown, 100%, n=21), repressed dlx5 expression (not shown, 73%, n=15) and caused ectopic ziec2 expression (Fig. 6I; 76.9%, n=13). Increased ziec2 expression also repressed six1 expression (Fig. 6J; 68.3%, n=41), and expanded foxD3 expression (Fig. 6K; 77.8%, n=18). Increased expression of dlx5 repressed six1 expression (Fig. 6L; 84.6%, n=78). These data demonstrate a dynamic interplay between the transcription factors that regulate subdomain identity within the LNE and along its boundaries (Fig. 7).

**Discussion**

Three potential mechanisms have been proposed for subdividing the embryonic ectoderm into neural plate, neural crest, PPE and epidermis. First, cells are exposed to different levels of BMP and axial signaling. Second, different domains express transcription factors that strongly promote one fate over another. For example, Sox2 promotes a neural stem cell fate (Graham et al., 2003), FoxD3 promotes a neural crest fate (Dottori et al., 2001; Sasai et al., 2001) and AP-2 promotes an epidermal fate (Luo et al., 2002). Third, interactions between the presumptive neural plate and epidermis may create a border zone environment that is conducive for neural crest and PPE fates (Baker and Bronner-Fraser, 1997; Feledy et al., 1999;
The lateral neurogenic ectoderm (LNE), which comprises neural crest and PPE, is located between the neural plate and the epidermis in an intermediate region of a proposed gradient of BMP activity (Weinstein and Hemmati-Brivanlou, 1999; Wilson and Edlund, 2001). It has been shown that neural crest markers are induced by concentrations of neural inducers lower than those required for neural plate markers (Morgan and Sargent, 1997; Marchant et al., 1998), and we demonstrate that even lower concentrations elicit the highest levels of expression of two placodal markers (\(\text{six}1\), \(\text{eya}1\)). This differential responsiveness to Noggin concentrations in explants supports the idea that an endogenous gradient of neural inducing signals provides a first step in separating the embryonic ectoderm into its different domains (Fig. 7). However, in the intact embryo, in which several signaling pathways intersect to influence the fate of the cell, intermediate BMP levels are not sufficient to induce either neural crest or placode fates. The neural crest, which is absent from the most anterior pole of the neural plate but extends to its posterior tip, requires Wnt and FGF signaling (LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998; Mayor and Aybar, 2001; Villanueva et al., 2002; Monsoro-Burq et al., 2003; Glavic et al., 2004a). These pathways are thought to act both to posteriorize the axis and to directly promote neural crest fate (Monsoro-Burq et al., 2003; Lewis et al., 2004). We demonstrate that these same signals antagonize \(\text{six}1\) expression, and propose that their posterior expression restricts placode formation to the head (Fig. 7). In the intact embryo, the expression of endogenous Wnt and FGF antagonists in anterior regions may additionally contribute to restricting placode formation (Leyns et al., 1997; Glinka et al., 1998; Piccolo et al., 1999; Bradley et al., 2000; Nutt et al., 2001; Zhang et al., 2001; Yamaguchi, 2001; Tsang and Dawid, 2004).

These data indicate that PPE and neural crest formation require the combined activities of neural inducing and anterior-posteriorizing factors. However, different concentrations of BMP antagonists favor one fate over the other, and posteriorizing factors promote neural crest over placodal fate. Thus, it is likely that the LNE is initially competent to give rise to both placodal and crest derivatives, and the acquisition of the specific fate of a LNE cell results from local concentrations of the pertinent signaling pathways. This hypothesis is consistent with: (1) observations that although \(\text{six}1\)/\(\text{eya}1\) are most highly expressed at Noggin concentrations lower than those for \(\text{fox}D3\), there is significant overlap in the two dose-response curves; (2) fate maps showing that otic placode precursors are intermingled with future neural crest precursors (Streit, 2002); and (3) observations that the expression domains of some neural crest and placodal marker genes partially overlap (McLarren et al., 2003; Schlosser and Ahrens, 2004; Glavic et al., 2004b).

**\(\text{six}1\) acts as a placodal fate specifying gene**

Studies in vitro have previously shown that Six proteins can function as transcriptional activators and repressors (Kobayashi et al., 2001; Ohto et al., 1999; Silver et al., 2003; Li et al., 2003). However, only evidence for repression has been documented in vivo, specifically for the related factors \(\text{Optx2/Six}6\) and \(\text{Six}3\) (Zuber et al., 1999; Kobayashi et al., 2001). We find that placodal fate specification by \(\text{Six}1\) requires both activating and repressing activities. Analyses of \(\text{six}1\)-\(\text{WT}\) and \(\text{six}1\)-\(\text{MO}\) injected embryos show that \(\text{Six}1\) positively regulates the expression of PPE factors and negatively
regulates other ectodermal genes (Fig. 4). By using Six1VP16 and Six1EnR constructs, we further demonstrate that these effects result from both activating and repressing activities of Six1 in vivo (Fig. 5). These activities may be mediated, at least in part, by the association of Six1 with the transcriptional activator Eya1 or transcriptional repressors of the groucho family (Fig. 5). Whether endogenous Six1 functions as an activator or repressor is probably determined by the relative levels and activities of the different co-factors within six1-expressing cells.

**Definition of sub-domains within the embryonic ectoderm**

Our studies also predict complex in vivo interactions between six1 and other ectodermal genes. sox2 and sox3 are both induced in presumptive neural ectoderm by neural inductive signaling, and promote stabilization of a neural fate (Mizuseki et al., 1998; Penzel et al., 1998). Later, they are both expressed in neural stem cells (Graham et al., 2003). We show that endogenous six1 expression in the LNE precedes sox2/3 placodal expression, indicating that sox2/3 are not upstream of six1. six1 overexpression in the LNE has no significant effect on sox2/3 neural plate expression, indicating that its effects in this border domain are cell autonomous and not due to intermediate signaling. However, when six1 is reduced in the lateral ectoderm, sox2/3 expression expands laterally. This could be a secondary result of the expansion of foxD3, which in turn expands sox2/3 (Sasai et al., 2001) (Fig. 5) and/or a mutual antagonism between six1 and sox2/3. The latter possibility is supported by the observations that six1 expression in the neural plate dramatically represses sox2/3 (data not shown) and that expression of sox2 in the LNE represses six1. At later stages sox2/3 are expressed in placodal domains that presumably overlap with six1 expression. How these genes interact at this later phase of placode development remains to be determined.

**foxD3** is required for neural crest formation, and both explant and in vivo studies show that it induces neural crest and neural plate marker genes (Sasai et al., 2001). We show that foxD3 and six1 have a mutually antagonistic relationship; the over-expression of one gene causes the repression of the other. Conversely, the reduction of SIX1 causes the foxD3 domain to expand, and the reduction of foxD3 by six1 overexpression causes expansion of other placodal markers. It is not yet known whether the interactions between foxD3 and six1 are direct or indirect; because six1 can repress both foxD3 and sox2 expression domains and foxD3 can expand sox2/3 domains (Sasai et al., 2001) (data herein), the interaction could be via sox2/3 regulation.

The zic1, zic2 and zic3 genes, which are likely to be functionally redundant, are first expressed throughout the entire presumptive neural epithelium and then become restricted to the lateral border of the neural plate and neural crest (Nakata et al., 1997; Nakata et al., 1998; Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998; LaBonne and Bronner-Fraser, 1999). The Zic genes appear to be important for the initial phase of both neural plate and neural crest development, and all three can induce ectopic expression of neural crest markers. In explants, zic1 induces foxD3 and slug, and foxD3 induces zic1 and zic2, leading to the proposal that Zic genes act upstream of foxD3 and slug to initiate neural crest fate, and that Zic gene and foxD3 expression is maintained in the neural crest by mutual interactions (Sasai et al., 2001). We corroborate a mutual positive interaction between zic2 and foxD3 by in vivo expression assays (Fig. 6). However, in the LNE foxD3 and the Zic genes do not have identical expression patterns (Sasai et al., 2001) (herein), indicating that they also may be interacting through intermediary genes. We demonstrate a similarly complex interaction between six1 and zic2. Overexpression of six1 expands the zic2 lateral domain, but reduction of Six1 also expands it. We propose that the former phenotype is caused by activation/maintenance of zic2 by six1, whereas the latter phenotype is most probably caused by the expansion of foxD3, which subsequently expands the zic2 domain.

Members of the Dlx gene family represent some of the earliest genes expressed at the border between the neural plate and epidermis (Feledy et al., 1999; Luo et al., 2001a; Luo et al., 2001b; Beanan and Sargent, 2000). In chick, dlx5 is expressed at the neural/non-neural border, overlapping with eya2 and six4 in the pre-placodal thickening where it is proposed to create a border zone in which lateral neurogenic fates can be expressed (McLarren et al., 2003). In Xenopus there is a low level of dlx5/6 expression along the border of the neural plate, but the most intense stripe is adjacent to six1 expression along the anterior neural ridge, and overlapping with the lateral edge of the crescent of six1 PPE expression (Luo et al., 2001a; Luo et al., 2001b) (herein). In chick, dlx5 overexpression results in a weak upregulation of six4 expression (McLarren et al., 2003), whereas in Xenopus wild-type dlx5/dlx6 both strongly reduce six1 expression (this study), and activator Dlx constructs cause a loss of six1 expression (Woda et al., 2003). It is not clear whether these differences are due to species differences in the precise patterning of the embryonic ectoderm, as has been proposed for neural induction (Aybar and Mayor, 2002), or due to the fact that Six1 and Six4 belong to different subclasses of the Six gene family (Kawakami et al., 2000). Regardless, it is clear that in frog six1 has two effects on dlx5/6 expression. Most prominently, overexpression of six1 in the LNE pushes the dlx5/6 stripe laterally away from the neural plate midline. This phenotype is probably due to the expansion of the PPE (eya1, sox11) and reduction of epidermis (keratin), resulting in the formation of a new border between the expanded LNE and the epidermis. This interpretation is consistent with the effects of six1 overexpression on keratin, and further suggests that the effect is not due to movement of the neural plate border because the sox2/3 domains do not change. Likewise, dlx5/6 negatively regulate six1 expression. A previous study also demonstrated a mutual regulation between Dlx genes and six1 (Woda et al., 2003): inhibition of endogenous Dlx activity relocated the six1 expression domain more laterally, whereas activation relocated it more medially. The second effect of six1 is complete repression of dlx5/6 expression in those cells expressing six1. This may result from Six1 either repressing dlx5/6 gene expression or causing changes in gene expression in the affected cells that secondarily create an environment that is not compatible with dlx5/6 expression. Interestingly, foxD3 overexpression has similar effects on dlx5/6, which could be direct, or, unlike six1, could be due to the overlap of sox2/3. We further observed paradoxically similar dlx5/6 phenotypes.
by activator and repressor sixl construct expression and sixl-MO injections. We predict that these can be explained by sixl effects on foxD3. The injection of sixlVP16, sixl-WT+eya-WT and sixl-MO may indirectly reduce dlx5/6 by expansion of foxD3, whereas sixl-WT alone and sixlEnR constructs may directly reduce dlx5/6. These results support the proposal that dlx5/6 contribute to forming the LNE border zone (McLaren et al., 2003; Woda et al., 2003), and additionally demonstrate that they do so by participating in a complex interplay with several genes expressed in adjacent domains. It will be important to determine the precise molecular interactions between these various gene pathways to fully understand their roles in specifying LNE fates.

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References


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