Neural crest determination by co-activation of *Pax3* and *Zic1* genes in *Xenopus* ectoderm

Takahiko Sato[1,2], Noriaki Sasai[1] and Yoshiki Sasai[1,2,*]

[1]Organogenesis and Neurogenesis Group, Center for Developmental Biology, RIKEN, Kobe 650-0047, Japan
[2]Department of Medical Embryology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
*Author for correspondence (e-mail: sasaicdb@mub.biglobe.ne.jp)

Development

Summary

A number of regulatory genes have been implicated in neural crest development. However, the molecular mechanism of how neural crest determination is initiated in the exact ectodermal location still remains elusive. Here, we show that the cooperative function of *Pax3* and *Zic1* determines the neural crest fate in the amphibian ectoderm. *Pax3* and *Zic1* are expressed in an overlapping manner in the presumptive neural crest area of the *Xenopus* gastrula, even prior to the onset of the expression of the early bona fide neural crest marker genes *Foxd3* and *Slug*. Misexpression of both *Pax3* and *Zic1* together efficiently induces ectopic neural crest differentiation in the ventral ectoderm, whereas overexpression of either one of them only expands the expression of neural crest markers within the dorsolateral ectoderm. The induction of neural crest differentiation by *Pax3* and *Zic1* requires Wnt signaling. Loss-of-function studies in vivo and in the animal cap show that co-presence of Pax3 and Zic1 is essential for the initiation of neural crest differentiation. Thus, co-activation of *Pax3* and *Zic1*, in concert with Wnt, plays a decisive role for early neural crest determination in the correct place of the *Xenopus* ectoderm.

Key words: Neural Crest, *Pax3*, *Zic1*, Wnt, Fate determination

Introduction

The neural crest is a unique population of ectoderm derivatives that possess a remarkable ability to migrate and differentiate into a large variety of peripheral cells (Anderson, 1997; Le Douarin and Kalcheim, 1999). The neural crest arises from the boundary between the neural plate and epidermis, along the anterior-posterior (AP) axis posterior to the forebrain level (Le Douarin and Kalcheim, 1999). As the neural crest is a vertebrate trait and is absent in the preceding protochordates (Wada, 2001), its origin and the mechanism of fate determination are attractive paradigms in both embryology and evolutional biology.

A number of regulatory molecules in neural crest development have been identified and appear to participate in the 'multiple-step' fate determination of this lineage (Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002; Villianueva et al., 2002; Meulemans and Bronner-Fraser, 2004). However, the mechanism of how the initial determination of the neural crest fate occurs at the restricted area of the ectoderm remains to be understood. It has been suggested that graded BMP signals along the dorsal-ventral (DV) axis play a role in the DV specification of the ectoderm (the neural plate, the neural crest and the epidermis) (Sasai and De Robertis, 1997; Dale and Jones, 1999; Mayor and Aybar, 2001). Wnt factors, which are expressed in the dorsal neural tube, are also implicated in the promotion of neural crest development (Wolda et al., 1993; Saint-Jeannet et al., 1997; Garcia-Castro et al., 2002). Nevertheless, it is poorly understood how these factors define the exact boundaries of the neural plate, the neural crest and the epidermis in the dorsolateral ectoderm. In addition, it is unclear whether these factors promote the initial determination of the neural crest or the maintenance/consolidation of differentiation.

Along the anteroposterior (AP) axis, the anterior limit of the neural crest corresponds to the anterior midbrain level. The forebrain level is devoid of typical neural crest tissues and is surrounded by specialized ectodermal tissues, such as the preplacode regions. In *Xenopus*, the cephalic neural crest anlage appears relatively early during embryogenesis, and neural crest-specific markers are detected even at the mid-gastrula stage. Moreover, in the frog research, neural crest determination can also be analyzed by using isolated ectodermal explants (animal cap assay), demonstrating that *Xenopus* is a suitable system to study the early phase of ectodermal specification into the neural crest fate.

The transcription factors Foxd3 and Slug are early bona fide markers of the presumptive neural crest region in *Xenopus*, and play essential roles in the specification of the neural crest fate in frog (LaBonne and Bronner-Fraser, 1998; LaBonne and Bronner-Fraser, 2000; Sasai et al., 2001). In this study, we have investigated upstream regulations of neural crest differentiation, particularly by focusing on the roles of the transcription factors Pax3 (Bang et al., 1999) and Zic1 (Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 1998). Mouse genetic studies have indicated that Pax3 (Goulding et al., 1991) is an essential regulator of neural crest development (Gruss and Walther, 1992). The *Pax3* mutant (*splotch*) mouse exhibits defects in neural crest derivatives, such as pigment cells, peripheral ganglia and cardiac neural crest-derived structures (Epstein et al., 1991; Tassabehji et al., 1992; Conway et al.,

1997). The human Waadernburg syndrome, which is caused by a mutation in *Pax3*, also involves pigmentation defects (Tassabehji et al., 1992). However, knowledge about the molecular function of Pax3 during early neural crest development is still limited. In particular, the role of Pax3 in the initial step of neural crest determination is largely unknown. Zic1 has been implicated in the regulation of neural induction and neural crest development (Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 1998). However, because *Zic1* is expressed in wider areas than the presumptive neural crest (such as the anterior neural fold) (Mizuseki et al., 1998) (and see below), the expression of *Zic1* alone cannot explain the spatially restricted pattern of neural crest development.

In this study, we examine the hypothesis that co-activation of *Pax3* and *Zic1* genes is the decisive event for the initiation of neural crest differentiation in the *Xenopus* ectoderm.

Materials and methods

Isolation of *Xenopus Pax3* and plasmid construction

The *Xenopus Pax3A* cDNA (containing a full coding region; accession number AY757358) was isolated from a *Xenopus* neurula library (Matsui et al., 2000) by screening with a short RT-PCR fragment of *Pax3* (Kuo et al., 1998) as a probe. *Pax3B* (another pseudoallele; AY757359) was isolated by RT-PCR by using the EST sequences (TC73183). *Pax3*, *Foxd3*, *Wnt3a* and *Bmp4* cDNAs were subcloned into a pCS2 vector (Sasai et al., 2001), and *Zic1* (Mizuseki et al., 1998) (BJ96772) and *Msx1* (X58773,TC155963) cDNAs were inserted in a *pCMV TnT* vector (Promega). For overexpression studies, we used *Pax3A* and *Zic1A*. For the glucocorticoid receptor (GR)-fusion construction, the ligand-binding domain amplified by PCR from the Sox2-GR plasmid (Kishi et al., 2000) was fused into the C terminus of Pax3 or Zic1 by PCR. These were then subcloned into *pCS2* (*Pax3-GR*) and *pCMV-TnT* (*Zic1-GR*).

MO experiments

The morpholino antisense oligonucleotides (MOs) were designed as follows (a lowercase letter indicates a mismatch):

Pax3A-MO, 5′-TTCCCTTGCCAAGTATTAAATCCAA-3′;

5 mis-pairs *Pax3A-MO*, 5′-TTgCCaTGCCAAcTATTAAtTCgAA-3′;

Pax3B-MO, 5′-TTCCCTTACAAAGAACTAAATCCAA-3′;

5 mis-pairs *Pax3B-MO*, 5′-TTgCCaTACAAAcAACTAAtTCgAA-3′;

Zic1-MO, 5′-AAGTCTTCCAACAATGGGCAGCGAA-3′;

5 mis-pairs *Zic1-MO*, 5′-AAGTgTTaCAACAATcGGgAGgGAA-3′;

Foxd3-MO, 5′-CACTGCCGCTGCCTGACAGGGTCAT-3′;

5 mis-pairs *Foxd3-MO*, 5′-CAgTGaCGCTGCgTGACAGcGTgAT-3′;

Msx1-MO, 5′-CATACAGAGAGATCCGAGCTGAGAA-3′;

5 mis-pairs *Msx1-MO*, 5′-CATACAcAGAcATCgGAcCTcAGAA-3′.

In this study, '*Pax3-MO*' is the 1:1 mixture of *Pax3A-MO* and *Pax3B-MO*. The *MOs* do not contain sequences complimentary to the RNAs used in the rescue experiments. *β-catenin-MO* was purchased from Gene Tools.

Embryonic manipulation and in situ hybridization

The developmental stage of the *Xenopus* embryos was determined according to the normal table of Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For microinjection studies, synthetic mRNA (produced using mMESSAGE mMACHINE; Ambion) and MOs were injected into two adjacent left animal blastomeres or two ventral blastomeres of eight-cell-stage embryos. The injected embryos were fixed with MEMFA (Sive et al., 2000) at the neurula stages. For animal cap assays, synthetic mRNAs were injected into all animal blastomeres of eight-cell embryos, and animal cap explants were prepared at stage 9 and cultured in $1\times$ Low calcium and magnesium Ringer's solution (LCMR) with 0.1% BSA until stage 15. Whole-mount in situ hybridization analyses were performed as previously described (Sasai et al., 2001). For double in situ hybridization, signals with a biotin-labeled probe were visualized by using BCIP red (magenta; BIOSYNTH AG) and without NBT, and signals with a digoxigenin (DIG)-labeled probe were visualized with BM purple (indigo; Roche).

Dissociated animal caps and RT-PCR analysis

For dissociated animal cap assays, animal cap explants were prepared from injected embryos at stage 9, subsequently dissociated in the calcium- and magnesium-free medium (Sive et al., 2000) supplemented with 0.1% BSA, and cultured on four-well plates (Nalge Nunc International) until stage 15. The dissociated animal cap cells were treated with 100 ng/ml of recombinant mouse Wnt3a protein (R&D Systems) during the indicated period. To exclude the possibility of dissociation-induced artifacts, *Foxd3* induction by *Pax3* injection and Wnt3a protein treatment was also confirmed in undissociated animal caps from which the impermeable outer layers were stripped. Total RNA was extracted by using the RNeasy Micro kit (QIAGEN) and RT-PCR was performed as previously described (Mizuseki et al., 1998; Sasai et al., 2001). The PCR primers used in this study for the first time were nrp (forward 5′-TCACGACATG-AGCTGGACTC-3′, reverse 5′-CACAAACCCGAATCCTCTGT-3′) and Pax3 3′UTR (forward 5′-TTTACCCGTTACTCATGGATAG-TGT-3′, reverse 5′-AATGTCACATAAAATCCAAAAAGGA-3′),

Western blot

Total proteins of injected or treated animal caps were extracted by dissolving in extraction buffer [10 mM Tris-HCl (pH 7.4), 1% NP40, and protease inhibitor cocktail (SIGMA)]. The cell extract (50 µg) was subjected to SDS-polyacrylamide gel electrophoresis. Western blot analysis was performed by using the anti-FLAG M2 antibody (SIGMA), the anti-HA antibody (Roche), and ECL western blotting detection reagents (Amersham).

Results

Overlapping of *Pax3* and *Zic1* expression in the presumptive neural crest region

Prior to the onset of *Foxd3* expression during late gastrulation, both *Pax3* and *Zic1* are expressed during the early and mid-gastrula stages in the dorsolateral ectoderm (see Fig. S1A-F in the supplementary material), where the neural crest arises. The expression patterns of *Pax3* and *Zic1* at the lateral ectoderm are overlapping but distinguishable (Fig. 1A,B and Fig. S1D,E in the supplementary material). The anterior borders of the *Pax3* and *Foxd3* expression domains are located at the midbrain level (Fig. 1A,C,D, and data not shown), whereas *Zic1* expression extends to the anterior-most end of the neural ridge (Fig. 1B,E). Posterior to the anterior limit of *Pax3*, *Foxd3* expression is located within the $Zic1^+$ region (Fig. 1E), which mostly overlaps with the $Pax3^+$ area (Fig. 1D,F; arrowheads). Taken together, the $Foxd3^+$ primordial neural crest (at least, its major part) arises in the dorsolateral head ectoderm expressing *Pax3* and *Zic1*.

A gradient of BMP activity (especially, at the intermediate level) has been implicated in neural crest determination along the DV axis (Marchant et al., 1998; Nguyen et al., 1998; Schmid et al., 2000). We therefore investigated the effects of
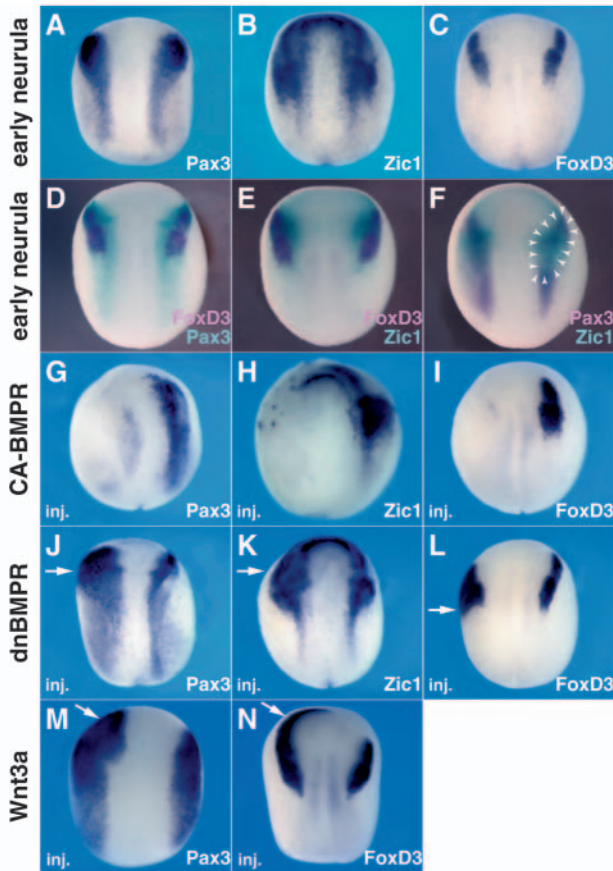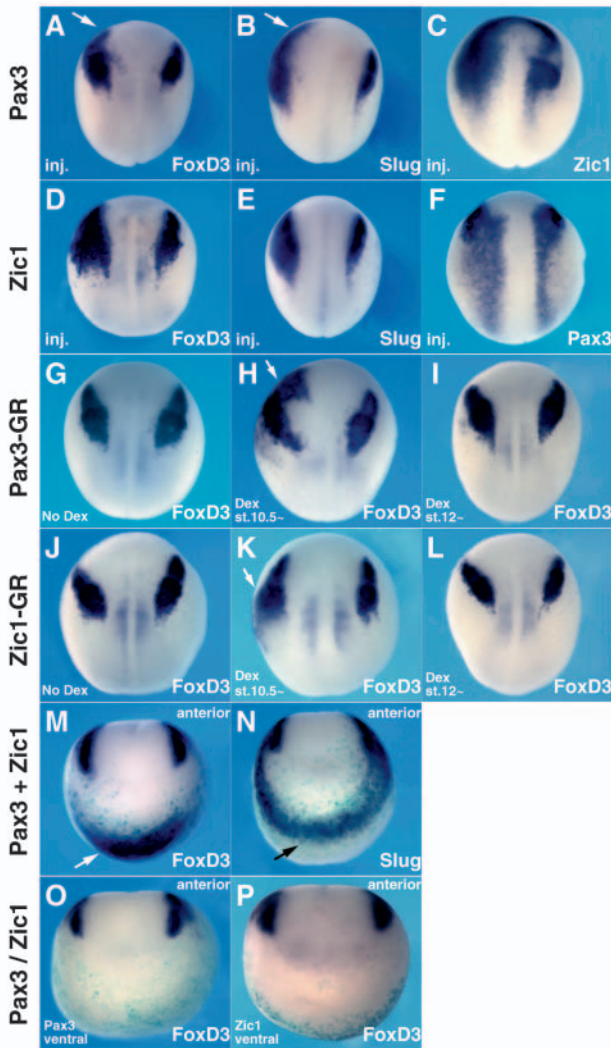
Fig. 2. Overexpression of *Pax3* and *Zic1* induces ectopic expression of neural crest markers. Synthetic mRNA was injected into two left animal blastomeres (A-L) or into the ventral animal blastomeres (M-P) at the eight-cell stage. Embryos were harvested at stage 15 and subjected to in situ hybridization with *Foxd3* (A,D,G-M,O,P), *Slug* (B,E,N), *Zic1* (C) and *Pax3* (F) probes. *Pax3* mRNA injection (50 pg/cell; A-C), *Zic1* mRNA injection (50 pg/cell; D-F), *Pax3-GR* mRNA injection (100 pg/cell; G-I), *Zic1-GR* mRNA injection (100 pg/cell; J-L), and injection of both *Pax3* and *Zic1* mRNAs (50 pg/cell each; E-H) with *lacZ* mRNA (200 pg/cell). Dexamethasone (Dex; 10 µM) was added to culture solution at stage 10.5 (H,K) or stage 12 (I,L). Arrows in A,B,H,K,M and N indicate ectopic expression of each marker gene.

dorsolateral region of the ectoderm, as shown above, but rarely in the ventral region (<5%; not shown). Similarly, when injected separately, *Pax3* and *Zic1* moderately expanded the expression of each other in the dorsolateral region (51%, *n*=55 and 66%, *n*=64, respectively; Fig. 2C,F), but not in the ventral ectoderm.

These findings prompted us to test whether co-expression of *Pax3* and *Zic1* induced ectopic neural crest differentiation in the ventral ectoderm by injecting RNAs into either the ventral animal blastomeres of the eight-cell stage (Fig. 2M-P), or the

ventral-most animal blastomeres of 16-cell stage embryo (see Fig. S1H in the supplementary material). Single injection of *Pax3* did not induce substantial induction of either *Foxd3* or *Zic1* in the ventral ectoderm (Fig. 2O and Fig. S1G; data not shown). Similarly, *Zic1* injection alone did not induce either *Foxd3* or *Pax3* on the ventral side (Fig. 2P and Fig. S1H; data not shown). When injected together, *Pax3* and *Zic1* caused ectopic expression of *Foxd3* and *Slug* ventrally at a distance from their orthotopic sites of expression (71%, *n*=42 and 60%, *n*=55, respectively; Fig. 2M,N, arrow, and Fig. S1H). In this condition, little ectopic expression of the neural marker *nrp1* was induced ventrally (*n*=27), whereas the epidermal marker *Keratin* was suppressed (52%, *n*=25; not shown). The combined mRNA injection did not induce ectopic expression of the preplacodal marker *Six1* (Brugmann et al., 2004) (not shown), or the dorsal neural tube marker *308a* (Tsuda et al., 2002) (not shown).

These findings demonstrate that co-activation of *Pax3* and *Zic1* is sufficient to induce ectopic neural crest differentiation in vivo, even in the ventral ectoderm, at the cost of epidermal differentiation.

Both *Pax3* and *Zic1* are required for neural crest determination in the embryo

We next investigated the roles of *Pax3* and *Zic1* in normal development of the neural crest by performing loss-of-function studies using morpholino antisense oligonucleotides (MOs), which inhibit the translation of *Pax3* and *Zic1*, respectively (see Fig. S2 in the supplementary material). Injection of *Pax3-MO* suppressed the expression of *Foxd3* and *Slug* (suppression in 75%, *n*=60 and 72%, *n*=54, respectively; Fig. 3A,B), whereas injection of the five-base-mispaired control MO did not (*n*=28 and *n*=29, respectively; not shown). Suppression of *Foxd3* was reversed by co-injecting wild-type *Pax3* mRNA (no suppression in 64%, *n*=35; Fig. 3C). Interestingly, *Zic1* expression was not suppressed in the *Pax3-MO*-injected embryo (*n*=38; Fig. 3D; rather it was upregulated, probably because of some feed-back mechanisms), indicating that loss of the Pax3 function inhibits neural crest differentiation even in the presence of *Zic1* expression. The suppression of *Foxd3* by *Pax3-MO* was not reversed by overexpression of *Zic1* (*n*=42; Fig. 3E) or *Msx1* (*n*=23; Fig. 3F).

Similar results were obtained with *Zic1-MO*. Injection of *Zic1-MO* (but not of the five-base-mispaired control MO) caused suppression of *Foxd3* and *Slug* (76%, *n*=37 and 69%, *n*=42, respectively; Fig. 3G,H). The suppression was rescued by co-injection of wild-type *Zic1* (no suppression in 69%, *n*=32; Fig. 3I), but not by co-injection of *Pax3* (*n*=39; Fig. 3K) or *Msx1* (*n*=28; Fig. 3L). *Zic1-MO* injection did not appear to suppress *Pax3* expression (Fig. 3J). Conversely, attenuation of the Foxd3 function by *Foxd3-MO*, which inhibited *Slug* expression (*n*=24; Fig. 3M), did not suppress *Pax3* or *Zic1* expression (*n*=36 and *n*=38, respectively; Fig. 3N,O), consistent with the idea that *Pax3* and *Zic1* work upstream of *Foxd3*, not downstream. Similarly, attenuation of Msx1 by *Msx1-MO* injection inhibited *Foxd3* expression (68%, *n*=31; Fig. 3P) without suppressing *Pax3* or *Zic1* (*n*=28 and *n*=25, respectively; Fig. 3Q,R). Collectively, these observations demonstrate that co-presence of the Pax3 and Zic1 functions is essential for the upstream regulation of neural crest formation in the embryo.

We next examined whether Pax3 and Zic1 were required for the upregulation of *Foxd3* expression caused by attenuation of BMP signaling (see Fig. 1L). Injection of *Pax3-MO* and/or *Zic1-MO* reversed the expansion of *Foxd3* expression caused by *dnBMPR* (Fig. 3S-U, and data not shown), suggesting that

Fig. 3. Both Pax3 and Zic1 are required for neural crest determination in vivo. *Pax3-MO* (20 ng/cell; A-F), *Zic1-MO* (20 ng/cell; G-L), *Foxd3-MO* (20 ng/cell; M-O), *Msx1-MO* (50 ng/cell; P-R) or *dnBMPR* (200 pg/cell; S-U) were injected unilaterally into the animal blastomeres of eight-cell-stage embryos. *Foxd3* expression suppressed by *Pax3-MO* (A) was rescued by *Pax3* mRNA (C), but not by *Zic1* nor *Msx1* (E,F). *Zic1-MO*-induced suppression of *Foxd3* (G) was reversed by *Zic1* mRNA (I), but not by *Pax3* nor *Msx1* (K,L). *Msx1-MO* suppresses the expression of *Foxd3* but not of *Pax3* and *Zic1* (P-R). *Foxd3* expression, upregulated by *dnBMPR* (S), was suppressed by *Pax3-MO* and *Zic1-MO* (T,U). Whole-mount in situ hybridization with *Foxd3* (A,C,E-G,I,K,L,P,S-U), *Slug* (B,H,M), *Pax3* (J,N,Q) and *Zic1* (D,O,R) probes.

attenuated BMP signaling requires both Pax3 and Zic1 for its enhancing effect on neural crest differentiation in vivo.

Requirement of Pax3 and Zic1 for neural crest differentiation in the animal cap explant

To further understand the mechanism of Pax3 and Zic1 functions in neural crest differentiation, we next studied ectodermal explants. Unlike the observation of the in vivo study (Fig. 2A), overexpression of *Pax3* alone caused little induction of *Foxd3* in the animal cap explants (*n*=37; Fig. 4A), suggesting that animal caps lack some signals that are necessary for *Pax3* to induce *Foxd3*. We therefore tested co-injection of *Wnt3a*, which has been shown to promote neural crest differentiation (LaBonne and Bronner-Fraser, 1998). Co-injection of *Pax3* and *Wnt3a* (but not *Wnt3a* alone, Fig. 4B inset) induced strong expression of *Foxd3* (67%, *n*=42; Fig. 4B), and also of *Zic1* (75%, *n*=24; Fig. 4C), in the animal caps, whereas neither *Six1* nor *308a* were induced (data not shown). The strong *Foxd3* induction was inhibited by co-injecting *Zic1-MO* (*n*=32, Fig. 4D).

Zic1 (50 pg/cell) alone induced *Foxd3* expression in the animal caps but only weakly (weak staining in 22%, *n*=37; Fig. 4E). By contrast, *Zic1* and *Wnt3a* induced strong expression of *Foxd3* (60%, *n*=38; Fig. 4F) and *Pax3* (69%, *n*=35; Fig. 4G). The strong induction by *Zic1* and *Wnt3a* was suppressed by co-injecting *Pax3-MO* (*n*=32; Fig. 4H). These findings show that both Pax3 and Zic1 activities are required for neural crest differentiation in Wnt-treated animal cap cells.

We next investigated whether the induction of neural crest differentiation by *Pax3* and *Zic1* was affected by enhanced BMP signaling. *Foxd3* induction by *Pax3* and *Wnt3a* was strongly inhibited by co-expression of *Bmp4* (4%, *n*=25; Fig. 4B,C,I,J; the level of *Bmp4* was sufficient to suppress neural induction by *Chordin*; Fig. 4O,P). Similarly, the induction of *Foxd3* and *Pax3* by *Zic1* and *Wnt3a* was reversed by *Bmp4* (expansion in 6%, *n*=15, and 17%, *n*=18, respectively; Fig. 4F,G,K,L). By contrast, *Foxd3* induction by the combination of *Pax3*, *Zic1* and *Wnt3a* (86%, *n*=28; Fig. 4M) was not remarkably affected by the presence of BMP4 signals (67%, *n*=27; Fig. 4N). These findings indicate that co-presence of *Pax3* and *Zic1* initiates neural crest differentiation in Wnt3a-treated animal cap ectoderm regardless of BMP signaling. These observations are consistent with the in vivo observation that injection of both *Pax3* and *Zic1* (but not each alone) induced *Foxd3* on the ventral side, where BMP signals are high (Fainsod et al., 1994).

Neural crest development involves multiple determination steps and is influenced by complex tissue interactions, such as the one between the neural plate and epidermis (Knecht and Bronner-Fraser, 2002). Therefore, one question as to the mode of action for Pax3 and Zic1 is whether they work together in the precursors of the neural crest or cooperate in a non-cell-autonomous manner by functioning in different kinds of cells. To understand the cell-autonomous nature of the differentiation control, we examined *Pax3*-induced neural crest differentiation by using dissociated animal cap cells. We excised the animal caps at stage 9 and dissociated them into single cells in calcium- and magnesium-free Ringer solution (Fig. 4Q). The dissociated cells were cultured in the presence of Wnt3a protein (added at the time equivalent to embryonic stage 9-13; harvested when siblings reached stage 15). *Foxd3* expression was induced in the dissociated *Pax3*-injected animal caps when
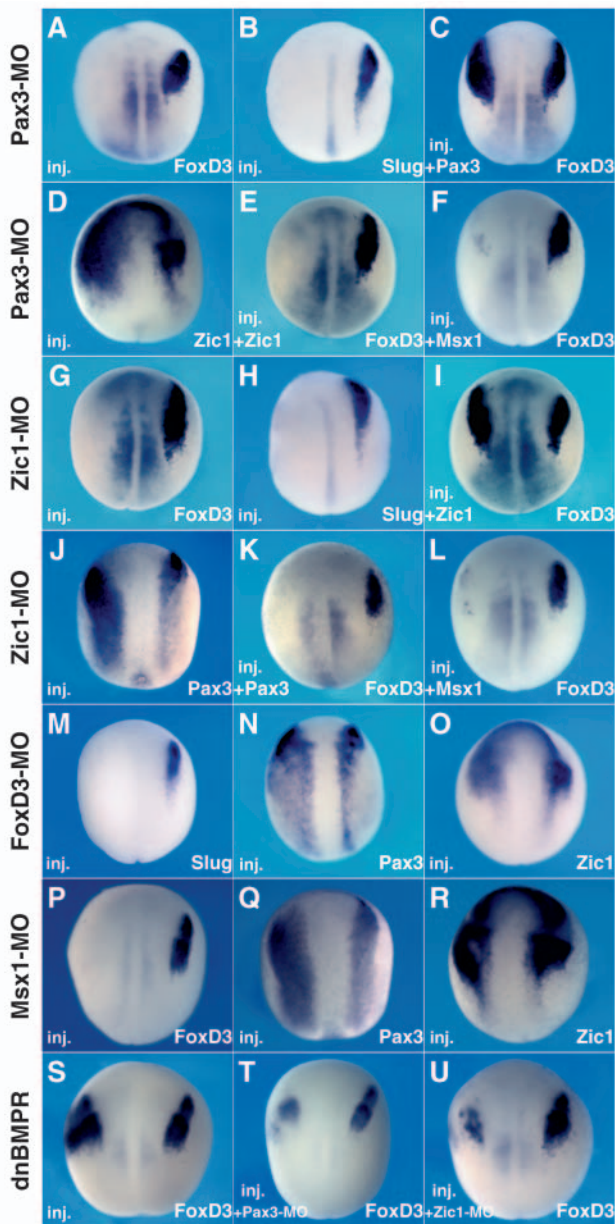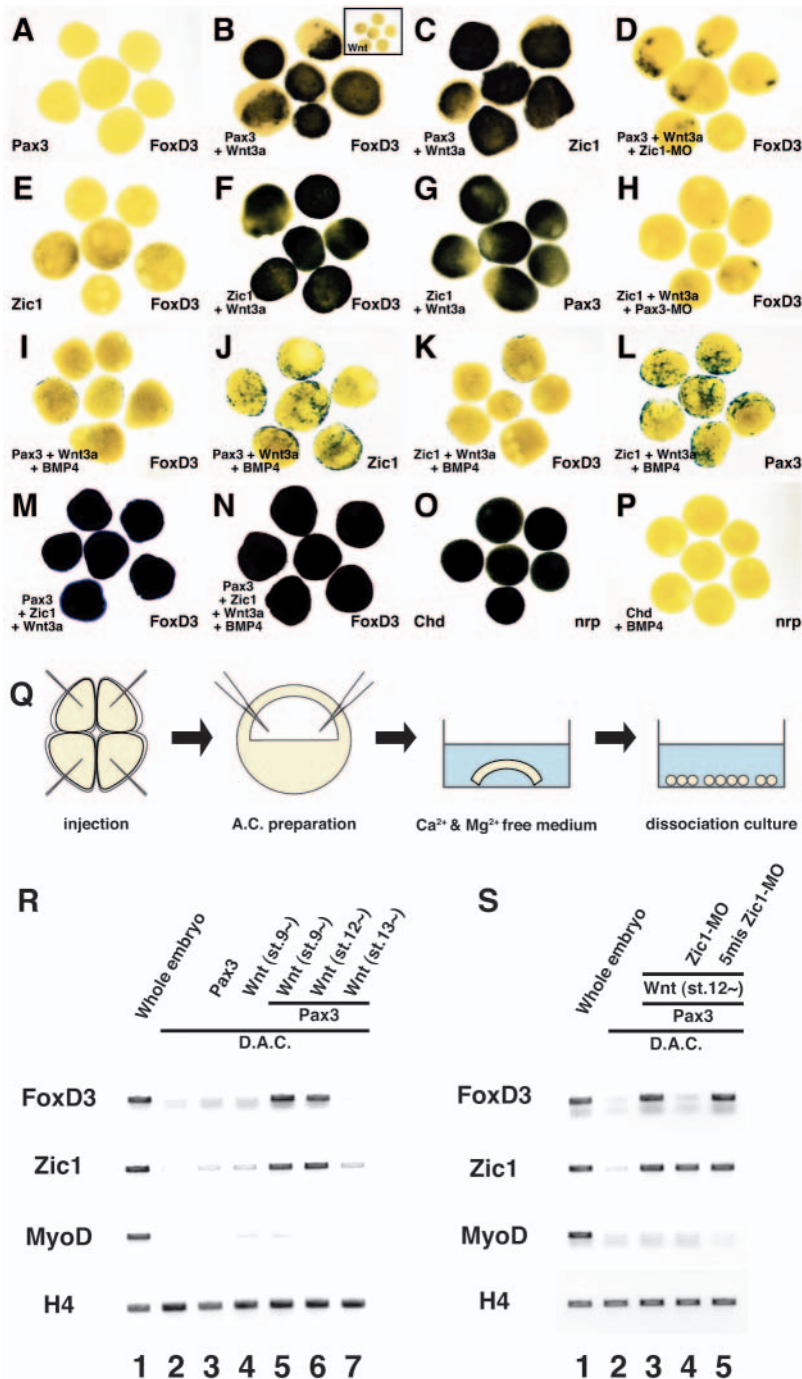
Fig. 4. *Pax3* and *Zic1* promote neural crest differentiation in Wnt-treated animal cap ectoderm. All animal blastomeres of eight-cell-stage embryos were injected with: *Pax3* (50 pg/cell; A,R,S); *Pax3* and *Wnt3a* (50 pg of each/cell; B,C); *Zic1* (50 pg/cell; E); *Zic1* and *Wnt3a* (50 pg of each/cell; F,G); *Pax3* (50 pg/cell), *Wnt3a* (50 pg/cell) and *Zic1-MO* (10 ng/cell; D); *Zic1* (50 pg/cell), *Wnt3a* (50 pg/cell) and *Pax3-MO* (10 ng/cell; H); *Wnt3a* (50 pg/cell; inset of B); *Pax3*, *Wnt3a* and *Bmp4* (50 pg of each /cell; I,J); *Zic1*, *Wnt3a* and *Bmp4* (50 pg of each/cell; K,L); *Pax3*, *Zic1* and *Wnt3a* (50 pg of each/cell; M); *Pax3*, *Zic1*, *Wnt3a* and *Bmp4* (50 pg of each/cell; N); *Chd* (50 pg/cell; O); *Chd* and *Bmp4* (50 pg of each/cell; P); or *Pax3* (50 pg/cell) and *Zic1-MO* (10 ng/cell; S). *Bmp4* injection was performed by using plasmid DNA (*pCS2-Bmp4*). Animal cap explants were prepared at stage 9 and harvested at stage 15 for in situ hybridization with *Foxd3* (A,B,D-F,H,I,K,M,N), *Zic1* (C,J), *Pax3* (G,L) or *nrp* (O,P) probes. (Q) Scheme of the procedure of the animal cap dissociation experiments. (R,S) Animal cap cells were dissociated at stage 9 and harvested for RT-PCR when the siblings reached stage 15. Wnt3a proteins were added at the stage indicated above.

cap cells, in which non-autonomous regulation is unlikely to occur, support the idea that these two genes act together in a cell-autonomous manner.

Roles of Pax3, Zic1 and Wnt signals in neural crest determination in vivo

Pax3 and Zic1 are essential for the neural crest specification of the ectoderm, both in the embryo and in the animal cap. In gain-of-function experiments, Pax3 and Zic1 require the co-presence of exogenous Wnt signals to evoke neural crest induction in the animal cap explant (Fig. 4), but not in the embryo (Fig. 2). Therefore, we next tested whether endogenous Wnt signals were essential for *Pax3* and *Zic1* to initiate neural crest differentiation in vivo. As shown in Fig. 2, misexpression of both *Pax3* and *Zic1* induces ectopic formation of neural crest cells in the ventral ectoderm (Fig. 5A,B; red, *lacZ* tracer). This induction was significantly suppressed when Wnt signaling was blocked by co-injection of *β-catenin-MO* (no significant induction observed, see Fig. 5C,D, $n=32$ and $n=22$, respectively) or *dnTCF3* mRNA (no significant induction observed, see Fig. S3C,D in the supplementary material, $n=63$ and $n=56$, respectively). This suppression was reversed by additional co-injection of wild-type *β-catenin* (ectopic *Foxd3* and *Slug* induction in 36%, $n=28$ and 33%, $n=27$, respectively; Fig. 5E,F) or *TCF3* (ectopic *Foxd3* and *Slug* induction in 36%, $n=28$ and 33%, $n=27$, respectively; Fig. S3E,F in the supplementary material) mRNA. These findings indicate that neural crest induction by *Pax3* and *Zic1* in the embryo is also dependent on Wnt signaling.

Discussion

Our working model, deduced from the present study, for the control of neural crest determination is as follows (Fig. 6). Both

the Wnt treatment started at stage 9 and 12, but not at stage 13 (Fig. 4R, lanes 5-7).

Pax3 injection and Wnt3a treatment induced strong *Zic1* expression in dissociated animal caps (Fig. 4R, lanes 5, 6). We then investigated whether Zic1 was required for neural crest differentiation induced by *Pax3* and Wnt3a in the dissociated animal caps. Co-injection of *Zic1-MO* inhibited *Foxd3* expression induced by *Pax3* and Wnt3a (Fig. 4S, lane 4), showing that Zic1 is essential for *Pax3* to induce neural crest differentiation in dissociated animal cap cells under these conditions. Conversely, induction of *Foxd3* and *Pax3* by *Zic1* and Wnt3a was also observed in dissociated animal cap cells (data not shown). These findings with the 'dissociated' animal
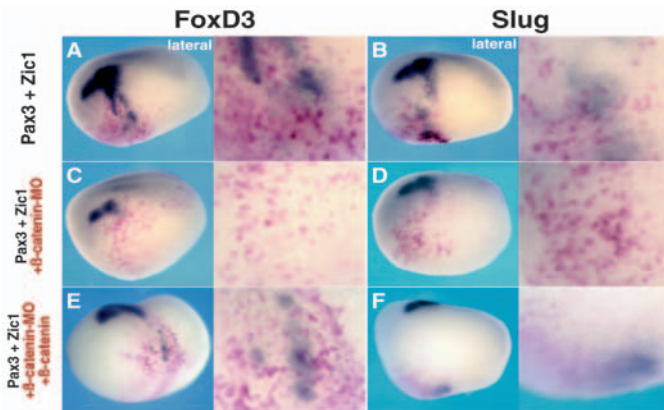
Fig. 5. Co-activation of *Pax3* and *Zic1* in concert with Wnt signaling is essential for neural crest determination of the ectoderm in vivo. (A-F) Synthetic mRNA was injected into a ventral animal blastomere at the eight-cell stage. Embryos were harvested at stage 15 for in situ hybridization with *Foxd3* (A,C,E) or *Slug* (B,D,F) probes. A higher magnification view is shown in the right half of each panel. *Pax3* and *Zic1* mRNA injection with *lacZ* mRNA (A-F; β-gal activity was visualized by incubating with Red-Gal), *β-catenin-MO* (Gene Tools; 10 ng/cell; C-F), and *β-catenin* (50 pg of DNA/cell; E,F).

Fig. 6. Working model for neural crest determination by combined functions of Pax3, Zic1 and Wnt.

Pax3 and Zic1 are independently required for neural crest differentiation, as depicted by *Foxd3* and *Slug* expression (Fig. 3A,B,G,H), whereas the transcription of either *Pax3* or *Zic1* does not require the activity of the other (Fig. 3D,J). Co-activation of *Pax3* and *Zic1* induces *Foxd3* and *Slug* in the ectoderm by working together with Wnt signals both in vivo and in vitro (Figs 4, 5). Taken together, these findings indicate that the co-activation of *Pax3* and *Zic1* is an essential upstream event that actively regulates the initiation of neural crest development in concert with Wnt signaling.

It is intriguing to understand how the roles of Pax3 and Zic1 shown in this study are interpreted with respect to the contexts of the models proposed previously, such as the 'two-signal' model (LaBonne and Bronner-Fraser, 1998) and the double gradient model (Villanueva et al., 2002). The early DV gradient of BMP4 activity (moderately-low BMP signaling in particular) appears to lie upstream of early expression of *Pax3* and *Zic1* (Fig. 1G-L). In addition, the absence of strong BMP signals is necessary for the mutual induction between *Pax3* and *Zic1* in the ectoderm (Fig. 4J,L). Interestingly, neural crest differentiation becomes insensitive to strong BMP signals in the presence of *Pax3*, *Zic1* and Wnt signals together (Fig. 4N). Furthermore, the upregulation of *Foxd3* expression by *dnBMPR* is reversed by co-injection of *Pax3-MO* or *Zic1-MO* (Fig. 3S-U). These findings suggest that the induction of *Pax3* and *Zic1* plays a major role in the interpretation of the BMP4 gradient for the control of neural crest development.

In future investigation, the molecular dissection of the regulatory regions of the *Pax3* and *Zic1* genes would be very intriguing with regard to the 'read-out' of the BMP activity gradient, and should be an attractive topic for promoter analyses using the transgenic frog technique. In addition, whether the co-expression of Pax3 and Zic1 directly attenuates BMP intracellular signaling should be examined to further clarify the mechanism of interactions.

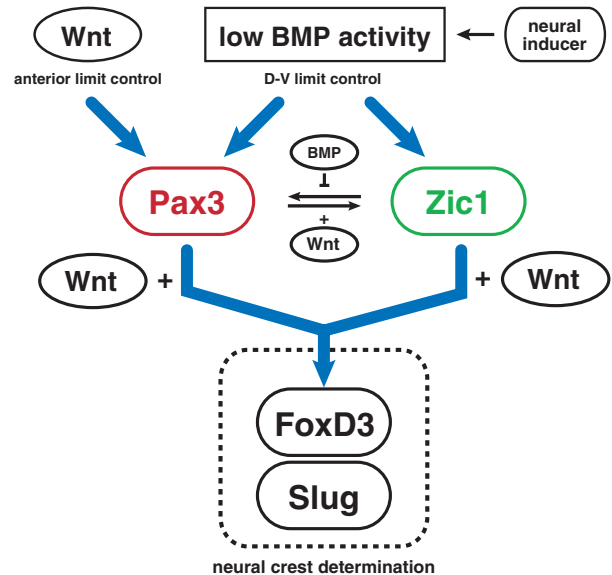The present study indicates two related but distinct roles of

Wnt signals for the initiation of neural crest differentiation. Wnt signaling is likely to play a role in the control of the anterior limit of *Pax3* expression at early inductive phase (Fig. 1M,N) (Bang et al., 1999). In addition, Wnt signaling has a cooperative function with Pax3 and Zic1 factors for *Foxd3/Slug* induction, and for mutual induction between *Pax3* and *Zic1* (Fig. 4). The earlier Wnt function may be relevant to the 'posteriorizing signals' in the double gradient model (Villanueva et al., 2002). The later cooperative effect of Wnt could be interpreted in line with the 'lateralizing signals' of the two-signal model (LaBonne and Bronner-Fraser, 1998), which are suggested to enhance and reinforce neural crest differentiation in weakly neuralized ectoderm. However, the exact relationship between these functions needs to be clarified in future investigation. Also, which particular Wnt factors act at each regulatory step in *Xenopus* neural crest differentiation [such as Wnt6 and Wnt8 suggested for chick and zebrafish neural crest induction (Garcia-Castro et al., 2002; Lewis et al., 2004)] is an important question to be studied in future.

Recently, the role of FGF8 in neural crest differentiation has been suggested with regard to paraxial mesoderm-derived inductive signals (Monsoro-Burq et al., 2003). Our preliminary study has indicated that *Fgf8* also induces *Pax3* and *Zic1* in vivo, and in the animal cap (see Fig. S4 in the supplementary material). *Fgf8*-induced *Foxd3* expression in Chd-treated animal caps requires both Pax3 and Zic1, whereas the induction of *Pax3* and *Zic1* themselves are not affected by *Zic1-MO* and *Pax3-MO*, respectively (see Fig. S4D-J). These findings suggest that FGF8 is another inductive signal candidate for *Pax3* and *Zic1* expression.

In careful comparison, the *Foxd3*-expressing area appears to be slightly narrower than the *Pax3+/Zic1+* region (which includes the lateral-most part of the neural plate; Fig. 1D-F, data not shown). One interpretation for this could be that *Foxd3* expression is inhibited by certain neural plate-specific factors on the medial side. Another possibility is that Wnt signaling, which is required for Pax3 and Zic1 to induce *Foxd3*, is finely

regulated by unknown local mechanisms. In addition, more precise spatial regulation may be controlled by the interaction of *Pax3* and *Zic1* with other transcription factors implicated in neural crest development (e.g. *Msx*, *Sox*, *Dlx*, *Myc* and *Ap2* genes) (Gammill and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2004).

Regarding the possible interaction with *Msx1* (Suzuki et al., 1997b; Tribulo et al., 2003), *Msx1-MO* suppresses *Foxd3* expression without inhibiting *Pax3* and *Zic1* expression in the neural crest region (Fig. 3P-R). Conversely, the attenuation of the Pax3 and Zic1 functions with MOs does not inhibit *Msx1* expression (data not shown), suggesting that *Msx1*-mediated BMP signaling does not function upstream of *Pax3* and *Zic1*, but rather acts in an independent manner at certain steps of neural crest differentiation. Consistently, unlike *Pax3* and *Zic1*, *Msx1* does not induce *Foxd3* expression in the animal cap even in the presence of *Wnt3a* (data not shown). The understanding of the exact pathway network connecting Msx1 and Pax3/Zic1/Wnt in neural crest differentiation requires further careful consideration (Monsoro-Burq et al., 2005).

Do other Zic family members also participate in the initial step of neural crest differentiation? In *Xenopus*, at least three members (*Zic2*, *Zic3* and *Zic5*) are expressed in overlapping patterns with *Zic1* (Nakata et al., 1998; Nakata et al., 2000). Although these family members show moderately high homology to Zic1 (54-57% identity of amino acid residues), the present study using the specific MO has shown that Zic1 is indispensable for *Xenopus* neural crest development. Interestingly, *Zic1-MO* injection does not suppress the expression of *Zic2*, *Zic3* and *Zic5* in the neural crest regions (see Fig. S5 in the supplementary material; instead, some moderate upregulation was seen as shown by arrow), suggesting that these family genes are not simply downstream of *Zic1*. It remains to be determined in future whether the neural crest phenotype of *Zic1* knockdown reflects the quantitative change of total Zic-related activity in the presumptive neural crest, or the qualitative differences of the role of Zic1 from the others. In mice, the gene disruption of mouse *Zic2* (but not mouse *Zic1*) causes defects in neural crest development (Aruga et al., 1998; Nagai et al., 2000). The exact roles of the Zic family members may be unambiguously studied by using reverse genetics analyses, such as compound mutant mice.

Finally, a biochemical analysis of the cooperative function of Pax3 and Zic1 would be an intriguing and challenging topic for future study. Do they bind directly and cooperatively to the regulatory regions of target genes such as *Foxd3*? In our preliminary experiments, we have so far failed to detect co-immunoprecipitation of Pax3 and Zic1 proteins from the lysate of 293 cells overexpressing the two genes. Target DNA-dependent interactions of Pax3 and Zic1 proteins remain to be investigated and, for detailed study, must await the identification of their responsive elements in the regulatory regions of the *Foxd3* and *Slug* genes.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/10/2355/DC1

References

Anderson, D. J. (1997). Cellular and molecular biology of neural crest cell lineage determination. *Trends Genet.* **13**, 276-280.

Aruga, J., Minowa, O., Yaginuma, H., Kuno, J., Nagai, T., Noda, T. and Mikoshiba, K. (1998). Mouse *Zic1* is involved in cerebellar development. *J. Neurosci.* **18**, 284-293.

Aybar, M. J. and Mayor, R. (2002). Early induction of neural crest cells: lessons learned from frog, fish and chick. *Curr. Opin. Genet. Dev.* **12**, 452-458.

Bang, A. G., Papalopulu, N., Goulding, M. D. and Kintner, C. (1999). Expression of *Pax-3* in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. *Dev. Biol.* **212**, 366-380.

Brugmann, S. A., Pandur, P. D., Kenyon, K. L., Pignoni, F. and Moody, S. A. (2004). Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. *Development* **131**, 5871-5881.

Conway, S. J., Henderson, D. J. and Copp, A. J. (1997). *Pax3* is required for cardiac neural crest migration in the mouse: evidence from the *splotch* (Sp^{2H}) mutant. *Development* **124**, 505-514.

Dale, L. and Jones, C. M. (1999). BMP signaling in early *Xenopus* development. *BioEssays* **21**, 751-760.

Epstein, D. J., Vekemans, M. and Gros, P. (1991). *splotch* (Sp^{2H}), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of *Pax-3*. *Cell* **67**, 767-774.

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *EMBO J.* **13**, 5015-5025.

Gammill, L. S. and Bronner-Fraser, M. (2003). Neural crest specification: migrating into genomics. *Nat. Rev. Neurosci.* **4**, 795-805.

Garcia-Castro, M. I., Marcelle, C. and Bronner-Fraser, M. (2002). Ectodermal Wnt function as a neural crest inducer. *Science* **297**, 848-851.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselium, J. R. and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10**, 1135-1147.

Gruss, P. and Walther, C. (1992). Pax in development. *Cell* **69**, 719-722.

Kishi, M., Mizuseki, K., Sasai, N., Yamazaki, H., Shiota, K., Nakanishi, S. and Sasai, Y. (2000). Requirement of *Sox2*-mediated signaling for differentiation of early *Xenopus* neuroectoderm. *Development* **127**, 791-800.

Knecht, A. K. and Bronner-Fraser, M. (2002). Induction of the neural crest: a multigene process. *Nat. Rev. Genet.* **3**, 453-461.

Kuo, J. S., Patel, M., Gamse, J., Merzdorf, C., Liu, X., Apekin, V. and Sive, H. (1998). opl: a zinc finger protein that regulates neural determination and patterning in *Xenopus*. *Development* **125**, 2867-2882.

LaBonne, C. and Bronner-Fraser, M. (1998). Neural crest induction in *Xenopus*: evidence for a two-signal model. *Development* **125**, 2403-2414.

LaBonne, C. and Bronner-Fraser, M. (2000). Snail-related transcriptional repressors are required in *Xenopus* for both the induction of the neural crest and its subsequent migration. *Dev. Biol.* **221**, 195-205.

Le Douarin, N. M. and Kalcheim, C. (1999). *The Neural Crest*. Cambridge: Cambridge University Press.

Lewis, J. L., Bonner, J., Modrell, M., Ragland, J. W., Moon, R. T., Dorsky, R. I. and Raible, D. W. (2004). Reiterated Wnt signaling during zebrafish neural crest development. *Development* **131**, 1299-1308.

Marchant, L., Linker, C., Ruiz, P., Guerrero, N. and Mayor, R. (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev. Biol.* **198**, 319-329.

Matsui, M., Mizuseki, K., Nakatani, J., Nakanishi, S. and Sasai, Y. (2000). *Xenopus* Kielin: a dorsalizing factor containing multiple chordin-type repeats secreted from the embryonic midline. *Proc. Natl. Acad. Sci. USA* **97**, 5291-5296.

Mayor, R. and Aybar, M. J. (2001). Induction and development of neural crest in *Xenopus laevis*. *Cell Tissue Res.* **305**, 203-209.

Meulemans, D. and Bronner-Fraser, M. (2004). Gene-regulatory interactions in neural crest evolution and development. *Dev. Cell* **7**, 291-299.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S. and Sasai, Y. (1998). *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**, 579-587.

Molenaar, M., ven de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86**, 391-399.

Monsoro-Burq, A. H., Fletcher, R. B. and Harland, R. M. (2003). Neural crest induction by paraxial mesoderm in *Xenopus* embryos requires FGF signals. *Development* **130**, 3111-3124.

Monsoro-Burq, A. H., Wang, E. and Harland, R. (2005). *Msx1* and *Pax3* cooperate to mediate FGF8 and WNT signals during *Xenopus* neural crest induction. *Dev. Cell* **8**, 167-178.

Nagai, T., Aruga, J., Minowa, O., Sugimoto, T., Ohno, Y., Noda, T. and Mikoshiba, K. (2000). Zic2 regulates the kinetics of neurulation. *Proc. Natl. Acad. Sci. USA* **97**, 1618-1623.

Nakata, K., Nagai, T., Aruga, J. and Mikoshiba, K. (1998). *Xenopus* Zic family and its role in neural and neural crest development. *Mech. Dev.* **75**, 43-51.

Nakata, K., Koyabu, Y., Aruga, J. and Mikoshiba, K. (2000). A novel member of the *Xenopus Zic* family, *Zic5*, mediates neural crest development. *Mech. Dev.* **99**, 83-91.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M. and Mullins, M. C. (1998). Ventral and lateral regions of the Zebrafish gastula, including the neural crest progenitors, are established by a *bmp2b/swirl* pathway of genes. *Dev. Biol.* **199**, 93-110.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of* Xenopus laevis *(Daudin).* Amsterdam: North Holland Publishing Company.

Saint-Jeannet, J. P., He, X., Varmus, H. E. and David, I. B. (1997). Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc. Natl. Acad. Sci. USA* **94**, 13713-13718.

Sasai, N., Mizuseki, K. and Sasai, Y. (2001). Requirement of *FoxD3*-class signaling for neural crest determination in *Xenopus*. *Development* **128**, 2525-2536.

Sasai, Y. and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5-20.

Schmid, B., Furthauer, M., Connors, S. A., Trout, J., Thisse, B., Thisse, C. and Mullins, M. C. (2000). Equivalent genetic roles for *bmp7/snailhouse* and *bmp2b/swirl* in dorsoventral pattern formation. *Development* **127**, 957-967.

Sive, H. L., Grainger, R. M. and Harland, R. M. (2000). *Early Development of* Xenopus laevis: *A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press.

Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255-10259.

Suzuki, A., Kaneko, E., Ueno, N. and Hemmati-Brivanlou, A. (1997a). Regulation of epidermal induction by BMP2 and BMP4 signaling. *Dev. Biol.* **189**, 112-122.

Suzuki, A., Ueno, N. and Hemmati-Brivanlou, A. (1997b). *Xenopus* msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* **124**, 3037-3044.

Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P. and Strachan, T. (1992). Waardenburg's syndrome patients have mutations in the human homologue of the *Pax-3* paired box gene. *Nature* **355**, 635-636.

Tribulo, C., Ayber, M. J., Nguyen, V. H., Mullins, M. C. and Mayor, R. (2003). Regulation of Msx genes by a Bmp gradient is essential for neural crest specification. *Development* **130**, 6441-6452.

Tsuda, H., Sasai, N., Matsuo-Takasaki, M., Sakuragi, M., Murakami, Y. and Sasai, Y. (2002). Dorsalization of the neural tube by *Xenopus* Tiarin, a novel patterning factor secreted by the flanking nonneural head ectoderm. *Neuron* **33**, 515-528.

Villanueva, S., Glavic, A., Ruiz, P. and Mayor, R. (2002). Posteriorization by FGF, Wnt, and retinoic acid is required for neural crest induction. *Dev. Biol.* **241**, 289-301.

Wada, H. (2001). Origin and evolution of the neural crest: a hypothetical reconstruction of its evolutionary history. *Dev. Growth Differ.* **43**, 509-520.

Wolda, S. L., Moody, C. J. and Moon, R. T. (1993). Overlapping expression of Xwnt-3A and Xwnt-1 in neural tissue of *Xenopus laevis* embryos. *Dev. Biol.* **155**, 46-57.