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

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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 evolutionary 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; Villanueva 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 (Bengtsson 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; 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.
1997). The human Waardenburg 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 ATY57358) was isolated from a Xenopus neurula library (Matsu et al., 2000) by screening with a short RT-PCR fragment of Pax3 (Kuo et al., 1998) as a probe. Pax3B (another pseudoallele; ATY57359) 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 we used inserted in a pCS2 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′-TTGCTTCTGAAGCTATTACATTACCA-3′; 5 mis-pairs with Pax3A-MO, 5′-TTGCTTCTGAAGCTATTACATTACCA-3′;
- Pax3B-MO, 5′-TTGCTTCTGAAGCTATTACATTACCA-3′; 5 mis-pairs with Pax3B-MO, 5′-TTGCTTCTGAAGCTATTACATTACCA-3′;
- Zic1-MO, 5′-AGGTCTTCTGTAAATGCAAGCATATGACAC-3′; 5 mis-pairs with Zic1-MO, 5′-AGGTCTTCTGTAAATGCAAGCATATGACAC-3′;
- Foxd3-MO, 5′-CAGTCGTCTGCTGTTGGGATATG-3′; 5 mis-pairs with Foxd3-MO, 5′-CAGTCGTCTGCTGTTGGGATATG-3′;
- Mx1-MO, 5′-TACAGACAGGATCTGCTGTGCAGG-3′; 5 mis-pairs with Mx1-MO, 5′-TACAGACAGGATCTGCTGTGCAGG-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× 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 undisassociated 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 npr (forward 5′-TCACGACTAGCTGACTC-3′, reverse 5′-CACAAAACCGAATCCTCGT-3′) and Pax3 3′UTR (forward 5′-TTTACCCGTACTACTGAATAGTG-3′, reverse 5′-AATGTACATATAATCATTAGA-3′).

Western blot
Total proteins of injected or treated animal caps were extracted 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
BMP signaling on the expression of Pax3 and Zic1 in the ectoderm. When BMP signaling was augmented in a cell-autonomous manner by injecting constitutively active (CA)-BMPR (Suzuki et al., 1997a) mRNA into two left animal blastomeres of eight-cell embryos, the neural crest marker Foxd3 and the neural plate marker Sox2 were suppressed, whereas the epidermal marker Keratin was dorsally induced (Fig. 11 and data not shown). In this condition, Pax3 and Zic1 were significantly suppressed (83%, n=24 and 62%, n=26, respectively; Fig. 1G,H). Conversely, attenuation of BMP signaling by overexpressing dnBMPR (Suzuki et al., 1994) expanded the expression domains of Pax3, Zic1 and Foxd3 laterally (Pax3, 72%, n=29; Zic1, 58%, n=26; Foxd3, 70%, n=27; Fig. 11J,K,L), probably due to the ventral shift (and expansion) of the ‘intermediate BMP activity’ zone in the ectoderm. These observations suggest that Pax3 and Zic1 are transcriptionally controlled by the BMP gradient along the DV axis in a manner similar to the control of the neural crest formation.

Next, we investigated possible signals that controlled the anterior limit of Pax3 and Foxd3 expression. Because a previous study had indicated that Wnt signaling plays a positive regulatory role in Pax3 expression (Bang et al., 1999), we examined Wnt signaling by comparing its effects on the expression of Pax3 and Foxd3. Unilateral injection of the Wnt3a-expressing plasmid caused significant ‘rostral’ expansion of Pax3 and Foxd3 (67%, n=39 and 84%, n=38, respectively; Fig. 1M,N), whereas both genes were suppressed in embryos injected with dominant-negative Tcf3 (dnTcf3, which blocks canonical Wnt signals) (Molemaar et al., 1996) mRNA (Pax3, 77%, n=26; Foxd3, 67%, n=27; not shown). These findings support the idea that Wnt signaling plays a crucial role in the spatial regulation of the Pax3 and Foxd3 expression domains along the AP axis.

Collectively, expression of the early neural crest markers is closely associated with the overlapping expression of Pax3 and Zic1, both in normal embryos and in embryos with modified DV and AP patterns.

Overexpression of Pax3 and Zic1 induces neural crest differentiation in the embryonic ectoderm

To understand the causal relationship among Pax3, Zic1 and neural crest differentiation, we next performed gain-of-function studies by mRNA injection into two unilateral animal blastomeres of eight-cell embryos. Overexpression of either Pax3 and Zic1 alone expanded Foxd3 (65%, n=48 for Pax3; 74%, n=65 for Zic1) and Slug (63%, n=51 for Pax3; 79%, n=66 for Zic1) expression in the dorsolateral ectoderm (Fig. 2G,L). In particular, Pax3 injection frequently induced Foxd3 and Slug expression in the anterior neural ridge region (48%, n=48 and 41%, n=51, respectively; arrows in Fig. 2A,B), where neither the neural crest markers nor Pax3 is normally expressed (note that Zic1 is normally expressed there, as shown in Fig. 1B).

We next examined the time window of the ability of Pax3 and Zic1 to induce Foxd3 by using inducible GR (glucocorticoid receptor)-fusion constructs (Pax3-GR and Zic1-GR; Fig. 2G-L). No effects were seen in the injected embryos without Dex (dexamethasone) administration (n=20 and n=24, respectively; Fig. 2G,J). When Dex was added at stage 9 (data not shown) or stage 10.5, expansion of Foxd3 expression was observed in mid-neurula embryos injected with Pax3-GR and Zic1-GR (86%, n=28 and 68%, n=22, respectively; Fig. 2H,K). By contrast, addition of Dex at stage 12 caused little effect on Foxd3 expression (Fig. 2L; n=25 and n=22, respectively), although western blot analysis showed the presence of the protein products of Pax3-GR and Zic1-GR at substantial levels throughout these stages (see Fig. S1G in the supplementary material; in this case, HA-tagged Pax3-GR and Zic1-GR, which showed indistinguishable activity, were used). These findings suggest that the time window of the Pax3 and Zic1 actions on Fox3 induction is confined to stages prior to late gastrulation, when the expression of Foxd3 (also Slug) first appears in the presumptive neural crest (Fig. S1 in the supplementary material).

Misexpression of either Pax3 or Zic1 alone frequently caused ectopic expansion of Foxd3 and Slug within the
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, 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 Mxsl (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-MO (n=39; Fig. 3K) or Mxsl (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 Mxsl by Mxsl-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.

**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 (J,L). Arrows in A,B,H,K,M and N indicate ectopic expression of each marker gene.
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 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) 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. 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.](image-url)
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 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
Pax3 and Zic1 are independently required for neural crest differentiation, as depicted by 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).

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 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.

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; Tribul 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). Requirement of Sox2-mediated signaling for differentiation of early Xenopus neuroectoderm. Development 127, 791-800.


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