Requirement of FoxD3-class signaling for neural crest determination in Xenopus

Noriaki Sasai1,2, Kenji Mizuseki1 and Yoshiki Sasai1,3,*

1Department of Medical Embryology and Neurobiology, and 2Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Sakyo, Kyoto 606-8507, Japan
3Organogenesis and Neurogenesis Group, Center for Developmental Biology, RIKEN, Kobe 650-0047, Japan

*Author for correspondence (e-mail: sasai@phy.med.kyoto-u.ac.jp)

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SUMMARY

Fox factors (winged-helix transcription factors) play important roles in early embryonic patterning. We show here that FoxD3 (Forkhead 6) regulates neural crest determination in Xenopus embryos. Expression of FoxD3 in the presumptive neural crest region starts at the late gastrula stage in a manner similar to that of Slug, and overlaps with that of Zic-r1. When overexpressed in the embryo and in ectodermal explants, FoxD3 induces expression of neural crest markers. Attenuation of FoxD3-related signaling by a dominant-negative FoxD3 construct (FoxD3delN) inhibits neural crest differentiation in vivo without suppressing the CNS marker Sox2. Interestingly, these loss-of-function phenotypes are reversed by coinjecting Slug. In animal cap explants, neural crest differentiation induced by Slug and Wnt3a is also inhibited by FoxD3delN but not by a dominant-negative form of XBF2. Loss-of-function studies using dominant-negative forms of FoxD3 and Slug indicate that Slug induction by Zic factors requires FoxD3-related signaling, and that FoxD3 and Slug have different requirements in inducing downstream neural crest markers. These data demonstrate that FoxD3 (or its closely related factor) is an essential upstream regulator of neural crest determination.

Key words: FoxD3, Slug, Neural crest, Dominant-negative mutant, Xenopus

INTRODUCTION

Neural crest cells originate from the ectoderm at the junction of the prospective neural plate and the prospective epidermis (Le Douarin and Kalcheim, 1999; Mayor et al., 1999). These unique cells are characterized by their extensive migration and ability to generate a large spectrum of cell types (Selleck et al., 1993; Bronner-Fraser, 1994; LaBonne and Bronner-Fraser, 1999). The derivatives of the neural crest include neurons and Schwann cells in the peripheral nervous system, adrenal medulla cells, pigment cells, facial cartilage cells and smooth muscle cells.

In Xenopus, the earliest gene markers of prospective neural crest are two genes encoding Zinc-finger transcription factors related to Drosophila snail, Xenopus Snail and Slug, which start to be expressed by the late gastrula stage (Essex et al., 1993; Mayor et al., 1995; reviewed in Mayor et al., 1999). Although Slug is suggested to function in specification and migration of neural crest cells (Nieto et al., 1994; Carl et al., 1999; LaBonne and Bronner-Fraser, 2000), relatively little is understood about the molecular mechanisms underlying neural crest determination in the early ectoderm.

Molecular embryological studies have indicated that several genes may be involved in neural crest determination. The neural inducers Noggin (Lamb et al., 1993) and Chordin (Chd; Sasai et al., 1995) do not induce neural crest cells when overexpressed alone in the Xenopus animal cap assay. In contrast, when Wnt or FGF acts in concert with Noggin or Chd, Slug is efficiently induced in the animal cap ectoderm (McGrew et al., 1995; Mayor et al., 1995; Sasai et al., 1996; Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998). However, it remains to be elucidated how Wnt or FGF signals cooperate with anti-BMP signaling. Overexpression of transcription factors such as Zic and SoxD induce neural crest cells in the animal cap (Nakata et al., 1997; Nakata et al., 1998; Mizuseki et al., 1998a; Mizuseki et al., 1998b). Also, Sox2 overexpression together with FGF treatment induces neural crest markers and melanophores (Mizuseki et al., 1998a). However, as these transcription factors induce gene markers of both the central nervous system (CNS) and the neural crest, it is not clear whether their role in neural crest formation is direct or indirect.

Formation of the neural crest can be induced also by juxtaposing presumptive neural plate and epidermal tissues both in vitro and in vivo (Moury and Jacobson, 1990; Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Liem et al., 1995; Mancilla and Mayor, 1996). This raises the possibility that the neural crest formation is dependent on interactions between neural and non-neural tissues. However, zebrafish studies have suggested that a BMP gradient, which
patterns tissues along the dorsoventral axis during gastrulation, plays a crucial role in the formation and positioning of the neural crest (Nguyen et al., 1998; Barth et al., 1999). A Xenopus study (Marchant et al., 1998) also supports this mechanism.

To further understand the molecular regulation of neural crest formation, we investigated the role of the neural crest-specific winged-helix transcription factor FoxD3 (formerly Forkhead 6) in Xenopus. FoxD3 expression starts in the presumptive neural crest at the late gastrula stage, as early as that of Slug. Gain-of-function and loss-of function studies showed that FoxD3 acts as an essential upstream regulator of neural crest determination in complex regulatory pathways together with Slug and Zic factors.

MATERIALS AND METHODS

Isolation of XFD6/FoxD3

To search for genes activated by neural inducers plus FGF, a differential screen was performed as follows. 50 ng of sog (fly Chd; Holley et al., 1995) mRNA (tester) or water (driver) were injected into 4 animal blastomeres of 8-cell Xenopus embryos. Animal caps were dissected at stage 10.25 and cultured in 1× LCMR supplemented with 0.2% BSA (Mizuseki et al., 1998a). 50 µg/ml human recombinant bFGF (Promega) was added to the culture medium of sog-injected caps. Animal caps were harvested for RNA isolation at stage 13. Testor-specific and control probes were prepared by subtracting (driver from tester) and (driver from driver), respectively. cDNA subtraction was performed using a PCR-select subtraction kit (Clontech), 437 positive clones that gave tester-specific hybridization signals were obtained from screening 2×106 pfu of a stage 13 Xenopus neural plate library. One clone (99A; GenBank accession no. AB014611) expressed in the neural crest regions turned out to be a homologue of zebrafish Forkhead 6. In addition, Zic-r1 (Mizuseki et al., 1998a), Zic2 (Brewster et al., 1998), Sox2 (Mizuseki et al., 1998a), Six3 (Zhou et al., 2000), and XFD127XLFLIP/FoxD5c (Söltner et al., 1999; Fetka et al., 2000) were also isolated. These genes were induced by Chd+FGF in the animal cap as expected from the cloning strategy.

Embryonic manipulation and in situ hybridization

Staging of embryos was done according to the normal table of Nieuwkoop and Faber. Synthetic RNA was injected using a fine glass capillary and a pneumonic pressure injector (Narishige) in Barth’s solution, and then embryos were transferred into 0.1× Barth’s solution (Gurdon, 1976) until further manipulation or harvesting. RNA was injected into all four animal blastomeres of the 8-cell embryo unless stated otherwise. All the injection experiments were carried out at least twice and gave reproducible results. The total amount of injected mRNA was kept constant by adding neutral GFP mRNA when necessary.

For animal cap assays, animal caps were excised at stage 10.5 and cultured in 1× LCMR supplemented with 0.2% BSA until the required stage. Regarding FoxD3 injection, we noticed that FoxD3-induced mesodermal differentiation in the animal caps was dependent on the dose and the stage of animal cap preparation. FoxD3-injected animal caps (100 pg/cap) contained a small amount of MyoD-positive tissues when caps were prepared from stage 9 embryos, while caps from stage 10.5 embryos did not express any of the mesodermal markers tested (Fig. 3G and not shown). It has also been noted that when a several-times higher dose of FoxD3 mRNA is injected, more efficient mesodermal induction is observed in blastula animal caps (Dan Kessler, personal communication) but not in gastrula caps (not shown). Therefore, in this study we selected conditions that do not induce mesodermal differentiation.

Whole-mount in situ hybridization was performed as described previously (Chitnis et al., 1995) with minor modifications (Sive et al., 1998). For double in situ hybridization, one DIG-labelled probe was stained with BCIP (light blue; Promega) and the other fluorescein-labelled probe was stained with BM purple (indigo; Boehringer Mannheim).

Plasmid construction

The entire coding region of 99A was subcloned into pCS2 vector at the EcoRI and XhoI sites (pCS2-FoxD3). To generate the FoxD3delN construct, the carboxyl-terminal part of FoxD3 (amino acid residues 195-371) was amplified by PCR and subcloned into pCS2-NLS. XBF2delN (amino acid residues 169-345) was constructed in a similar way and used as a specificity control. To generate a FoxD3-VP16 construct, the DNA-binding domain of FoxD3 (amino acid residues 85-194) was fused to the VP16 activation domain (Sadowski et al., 1988) and subcloned into pCS2 vector at the StuI site. pCS2-FoxD3delN-GR was constructed by fusing the human glucocorticoid receptor ligand-binding domain to the FoxD3 carboxyl-terminal domain (Kolm and Sive, 1995). A FoxA4 (amino acid residues 110-219)-VP16 construct was similarly generated, and used as a control for specificity. FoxA4, formerly called XFKH1/HNF3β/pintallavis/ XFD1 (Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992; Knöchel et al., 1992), shares 60% amino acid identity with FoxD3 in the DNA-binding domain. FoxA4-VP16 is active and mimics wild-type HNF3β (Pani et al., 1992) in inducing target genes. For instance, FoxA4-VP16 induced the floor-plate marker Kielin in the animal cap when cojected with Chd (Matsui et al., 2000, and data not shown). To generate a FoxD3-EnR construct, the DNA-binding domain of FoxD3 was fused to the Drosophila engrailed repressor domain (EnR; Conlon et al., 1996), and was subcloned into pCS2 vector at the ClaI site. The entire coding regions of Slug and Wnt3a were amplified by RT-PCR from stage17 embryo cDNA and subcloned into pCS2 vector. For mRNA injection, the plasmids were linearized with SacI (FoxD3- EnR) or NotI (the other constructs), and transcribed with SP6 polymerase (mMessage mMACHINE, Ambion).

RT-PCR analysis

RT-PCR was performed as described previously (Kengaku and Okamoto, 1995; Mizuseki et al., 1998a; Kuo et al., 1998; Nakata et al., 1998; Kishi et al., 2000). The other primers used in this study were as follows: Zic-r1 (Mizuseki et al., 1998a) (forward primer; ATGAAATGGTGCCCGGACAAC, reverse primer; CACTCTGATG-TGTGCTACAG; 282 bp, 25 cycles), Ets-l (Meyer et al., 1997) (forward primer; GAGCCCTAAGAAATACGCTGC, reverse primer; CATACAGTTTAAAGAGG; 231 bp, 28 cycles), Ephrin-B2 (Smith et al., 1997) (forward primer; AGGAGATCGAG-GGTGATCTGC, reverse primer; TTTTAGCATTGAAAGGTAGG- ACTTC, 210bp, 28cycles), FoxD3 (forward primer; TCTCTGGGG- CAATCACACTC, reverse primer; GTACATTTGTTGATAAAGG; 278 bp, 28 cycles), and SoxD (Mizuseki et al., 1998b) (forward primer; ACCAGGACTCTATGCTTAC, reverse primer; CTAGGTTCAAGTACGATAGA; 240 bp, 28 cycles).

RESULTS

Isolation of Xenopus FoxD3

We previously reported a systematic differential hybridization screen for downstream genes of the neural inducer Chd (Mizuseki et al., 1998a). Although the screen identified several early regulators of neural differentiation, including Zic, Sox2 and SoxD, we failed to isolate region-specific neural genes by this strategy (Mizuseki et al., 1998a; Mizuseki et al., 1998b). One possible reason is that Chd induces in the animal cap mainly archencephalic tissues, which remain immature until
late stages (Sasai et al., 1995). In contrast, the combination of Chd and FGF promotes differentiation of more mature cells with various regional markers, such as the floor plate, neural crest and posterior neural markers (Sasai et al., 1996). Therefore, we attempted to isolate genes activated by Chd and FGF in the animal cap ectoderm during the early phase of differentiation.

Chd/FGF-treated animal caps and control caps were prepared at early gastrula stages and harvested at the late gastrula stage equivalent as described in Materials and Methods. A differential screen was performed on 2×10^4 pfu of a *Xenopus* neural plate cDNA library (stage 13) by using RNAs from treated and control animal caps as probes. We identified 437 clones expressed preferentially in Chd/FGF-treated caps. Among them, two clones encoded *Fox* class transcription factors. One is expressed in the CNS midline and turned out to be *FoxD3* (formerly called *XFD-12/XFLIP*; King and Moore, 1994; Solter et al., 1999; Fetka et al., 2000). The other is a *Xenopus* homologue of zebrafish *forkhead 6* (Scheucher et al., 1995; Odenthal and Nüsslein-Volhard, 1998; Kelsh et al., 2000; now renamed *FoxD3;* Kaestner et al., 2000). Because of its intriguing expression in the neural crest, *FoxD3* was chosen for further investigation.

**Spatial and temporal distribution of *Xenopus* *FoxD3***

*FoxD3* expression starts at stage 10.25 on the dorsal surface of the gastrula (posterior neuroectoderm region). Fig. 1A shows *FoxD3* expression at stage 11. *FoxD3* is not expressed in the involuting marginal zone located just above the dorsal lip (indicated by the arrowhead) at this stage. By stages 11.5-12, *FoxD3* expression in the posterior neuroectoderm is gradually fading, while it becomes detectable in the dorsal mesoderm (Fig. 1C and data not shown). By stage 12.5, the presumptive cephalic neural crest regions start to express *FoxD3* (Fig. 1E). The onset and the spatial distribution of *FoxD3* in the neural crest regions are similar to those of *Slug* (Fig. 1F). Double in situ hybridization (Fig. 1I,J) with *Sox2* (light blue; a CNS-specific marker at this stage) shows that the expression patterns of *FoxD3* and *Slug* (indigo) in the ectoderm are indistinguishable in terms of positioning relative to the *Sox2* distribution. *FoxD3* expression (indigo; Fig. 1K) is located in the lateral and posterior part of the *Zic*-r1-positive area (light blue). Histological analyses (Fig. 1L) showed *FoxD3* expression in the ectoderm adjacent to the neural plate (arrowhead and np) and in the paraxial mesoderm (arrow). At late tailbud stages, *FoxD3* is also expressed in the trunk neural crest and in the migrating cephalic neural crest cells (data not shown).

*FoxD3* expression largely overlaps with *Slug* expression, and demarcates the neural crest lineage in the ectoderm as early as the late gastrula stage.

**Regulation of *FoxD3* expression in the animal cap ectoderm***

To understand the molecular basis of *FoxD3* expression, we performed RNA microinjection studies by using animal cap explants. Wnt signals (Wnt3a, Wnt8 and Wnt1) have been implicated in neural crest formation (Mayor et al., 1995; Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998). In mice, Wnt1 and Wnt3a are expressed in the dorsal roof of the neural tube (Wolda et al., 1993; McGrew et al., 1997), and are essential for proper neural crest development and dorsal CNS specification (Ikeya et al., 1997). Overexpression of Chd and Wnt3a efficiently induced *FoxD3* and *Slug* (Fig. 2G,H; 86%, n=44 and 90%, n=41, respectively) whereas little induction was observed after injection of *Chd* or Wnt3a mRNA alone (Fig. 2C-F; n=30 each). Overexpression of *Slug* and Wnt3a, but not *Slug* alone, activated *FoxD3* and *Slug* transcription (Fig. 2I-L).

As shown above, Wnt requires additional signals (e.g., Chd) to initiate neural crest differentiation in the ectodermal explant. In contrast, *Zic-r1* injection by itself was sufficient to induce *FoxD3* and *Slug* transcription (Fig. 2M,N; 97%, n=38 and 94%, n=36, respectively). Since the onset of *Zic* expression occurs before that of *FoxD3* in the neural crest regions (Mizuseki et al., 1995; Odenthal and Nüsslein-Volhard, 1998; Kelsh et al., 2000; now renamed *FoxD3;* Kaestner et al., 2000). Because of its intriguing expression in the neural crest, *FoxD3* was chosen for further investigation.

![Fig. 1. Expression of *FoxD3* in early *Xenopus* embryos and comparison to that of *Slug.* (A-H) Spatial and temporal expression of *FoxD3* (A,C,E,G) and *Slug* (B,D,F,H) analyzed by whole-mount in situ hybridization. (A,B) Early gastrula stage 11 (vegetal view), arrowhead indicates dorsal lip; (C,D) mid-gastrula stage 12; (E,F) late gastrula stage 12.5; and (G,H) mid-neurula stage 16. (I-K) Double-labeled in situ hybridization. (I) *Sox2* (light blue) and *FoxD3* (indigo). (J) *Sox2* (light blue) and *Slug* (indigo). (K) *Zic-r1* (light blue) and *FoxD3* (indigo). (C-K) Dorsal views. (L) Histological analysis of *FoxD3* distribution at mid-neurula stage. np, neural plate.](image-url)
al., 1998a; Nakata et al., 1998), Zic factors appear to be good candidates for activators of FoxD3.

**Induction of neural crest and neural markers by overexpression of FoxD3**

The intriguing expression pattern of FoxD3 in the presumptive neural crest regions led us to test the effects of FoxD3 on neural crest differentiation. We injected FoxD3 mRNA into two left animal blastomeres of 8-cell embryos and analyzed them at the tailbud stage (Fig. 3). FoxD3 overexpression caused ectopic expression of neural crest markers such as Slug (40%, n=58; Fig. 3A), endogenous FoxD3 (40%, n=43; Fig. 3B), Ets-1 (55%, n=88; Fig. 3C) and AP-2 (Saint-Jeannet et al., 1997) (43%, n=61; data not shown). We next tested whether FoxD3 overexpression induced ectopic expression of the CNS marker Sox2. Sox2 was also induced by FoxD3 injection in ectopic positions (60%, n=34) (Fig. 3D). Thus, overexpression of FoxD3 induces neural crest and neural markers in vivo.

To determine whether FoxD3 can induce neural crest cells in the isolated animal cap explant, FoxD3 mRNA was injected into 4 animal blastomeres (25 pg/cell) of 8-cell embryos, and animal caps were excised from stage 10.5 gastrula embryos. In this case, animal caps were prepared from embryos from an albino mother and a pigmented father, so that pigments would be derived only from zygotic synthesis (Mizuseki et al., 1998a). After 2 days incubation in vitro, FoxD3-injected caps contained a significant number of melanophores (Fig. 3F; 65%, n=33), in contrast to control caps (0%, n=25; Fig. 3E; inset, a sibling embryo), showing that FoxD3 induces production of a mature type of neural crest derivative.

We then tested whether FoxD3 can induce early neural crest markers in the animal cap using RT-PCR (Fig. 3G). After 1 day in culture, injection of FoxD3 induced Slug, Twist, endogenous FoxD3, Zic factors (Zic-r1, Zic2; neural crest and dorsal CNS markers at the neurula stage), Ets-1 (a late neural crest marker) and Ephrin-B2 (a late neural crest marker; Smith et al., 1997; data not shown) in the animal cap. These results demonstrate that FoxD3 can initiate neural crest differentiation in the ectodermal explant. FoxD3 did not induce the mesodermal marker M-actin (Fig. 3G) or the neural inducers Noggin or Chd (data not shown) under these conditions (see Materials and Methods).

FoxD3 overexpression also induced the panneural markers Sox2, NCAM, SoxD and Xnngn-r1 (primary neurons), and suppressed the epidermal marker Keratin (Fig. 3G). Expression of regional markers such as Otx2 (forebrain), En2 (midbrain-hindbrain border), Krox20 (hindbrain), Xlhx1 (anterior spinal cord), FoxB9 (posterior spinal cord), Pax3 (dorsal CNS) and Pax6 (forebrain and ventral CNS) was also observed in FoxD3-injected caps (data not shown). These results show that FoxD3 induces neural crest and neural differentiation in the isolated ectodermal explant.

Previous studies have reported several Fox family factors expressed in early Xenopus neuroectoderm such as FoxA4 (HNF3β), XBF1 (a homologue of mouse FoxG1) and XBF2 (a homologue of mouse FoxD1; Kauffman and Knöchel, 1996; Bourguignon and Papalopulu, 1998; Mariani and Harland, 1998; Gómez-Skarmeta et al., 1999). It has been shown by RNA microinjection that XBF1 and XBF2 possess strong neuralizing activities as does FoxD3 (Bourguignon and Papalopulu, 1998; Mariani and Harland, 1998). Taken together with our results, these observations raise two crucial questions in terms of specificity. First, does FoxD3 induce neural crest markers by acting on its own target genes, or by acting on the target genes of related Fox genes such as XBF2 (FoxD3 has the highest homology to XBF2 in the DNA-binding domain among the Fox family factors; 89%)? Second, does FoxD3 induce neural crest differentiation primarily by acting in the neural crest precursors, or secondarily by promoting ectopic formation of CNS tissues (as indicated by ectopic Sox2 induction)? The latter possibility is suggested by the finding that neural plate tissues induce neural crest formation when juxtaposed with epidermal tissues (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996).

To start addressing these questions, we first examined the effect of XBF2 overexpression in the animal cap explant. A previous study showed that XBF2 overexpression in vivo induces ectopic neural differentiation without activating Slug.
transcription (Mariani and Harland, 1998). Consistent with the previous in vivo data, overexpression of XBF2 in the animal cap induces the CNS marker Sox2, but not the neural crest marker Slug (Fig. 3H). This suggests some specificity for the role of FoxD3 in neural crest differentiation. To further understand the specific roles of FoxD3 in neural crest formation, we performed detailed dominant-negative studies.

**A specific dominant-negative mutant, FoxD3delN, suppresses neural crest differentiation but not neural plate development in vivo**

We generated a candidate for a dominant-negative FoxD3 by deleting the amino-terminal domain including the conserved DNA binding domain (FoxD3delN; Fig. 4A); a similar strategy has been successfully used to generate dominant-negative constructs of Sox2 and SoxD (Kishi et al., 2000; Mizuseki et al., 1998b). Coexpression of FoxD3delN in the animal cap suppressed Sox2 and Slug induction by FoxD3 (Fig. 4B lanes 3 and 4) and this suppression was rescued by increasing wild-type FoxD3 (lane 5). In contrast, FoxD3delN did not suppress Sox2 induction by XBF2 (lane 6) even at a high dose (lane 7). In a reverse experiment, a similar specificity was observed with XBF2delN (XBF2 lacking the DNA-binding domain) and FoxD3 (lanes 8-12). These results indicate that FoxD3delN works as a specific dominant-negative FoxD3 construct which antagonizes the activity of FoxD3 but not that of XBF2. This is consistent with the fact that FoxD3 does not share significant homology with other FoxD subfamily factors such as XBF2 in the carboxyl-terminal domain. However, despite the specificity shown above, the possibility that FoxD3delN may also interfere other FoxD3-related factors (including unidentified ones) cannot be excluded. Therefore, in the context of this work, a signaling activity that is disturbed by FoxD3delN is termed an activity of ‘FoxD3-related signaling’.

FoxD3delN was injected into the right animal blastomeres of 8-cell embryos and the effects on differentiation markers were examined at the neural plate stage. The neural crest markers Slug, FoxD3, Twist and Ets-1 were strongly suppressed by FoxD3delN injection (reduced in 87% of 47 embryos, 64% of 33, 82% of 38, 78% of 23, respectively; Fig. 5A,B and data not shown). Suppression of the neural crest markers by FoxD3delN suggests that FoxD3 (or its closely related factor) is essential for neural crest differentiation. The CNS marker Sox2 was not suppressed in the neural plate but rather induced in the regions of injected embryos usually fated to be neural crest (79%, n=53; Fig. 5C). FoxD3delN did not significantly affect the expression of the epidermal marker Keratin or the mesodermal marker MyoD (n=25) (data not shown). The finding that the CNS marker was not suppressed by the dominant-negative FoxD3 supports the idea that FoxD3 plays a role primarily in neural crest differentiation and argues against the alternative possibility of secondary effects due to perturbation of the CNS determination.

FoxD3delN suppressed not only late neural crest markers such as Twist, but also early neural crest-specific transcription factor gene, Slug. This led us to test whether injection of Slug may reverse the phenotypes caused by FoxD3delN (Fig. 5D-O). Injection of Slug alone moderately upregulated expression of the neural crest markers Slug, FoxD3 and Twist (Fig. 5D,G,J). Coinjection of FoxD3delN and Slug reversed the suppressing effects of FoxD3delN on the neural crest markers (Fig. 5E,F, Slug, 81%, n=37; panels H,I, FoxD3, 83%, n=40; panels K,L, Twist, 74%, n=42), and inhibited ectopic expression of Sox2 (panels N,O, 75%, n=40). These data suggest a critical role for Slug in FoxD3 signaling during neural crest determination.

**FoxD3 is required for neural crest differentiation in the animal cap explant**

To further examine the direct involvement of FoxD3 in neural crest determination, we analyzed effects of FoxD3delN by using an animal cap assay in which neural crest forms in the absence of CNS tissues. A previous study showed that overexpression of Slug and Wnt induces neural crest markers without activating the neural plate marker Sox2 (LaBonne and Bronner-Fraser, 1998). Consistent with that report, coinjection
Slug and Wnt3a mRNAs induced the neural crest markers Slug, FoxD3 and Ets-1 without inducing the CNS marker Sox2 or the mesodermal marker M-actin (Fig. 5P, lanes 2 and 3). Slug + Wnt3a did not induce expression of XBF1, XBF2 or FoxD5 in the animal cap (data not shown). Induction of the neural crest markers by Slug and Wnt3a was clearly opposed by coinjection of FoxD3delN (lane 4) but not by the control XBF2delN (lane 5). These results show that the requirement for FoxD3 in neural crest differentiation is independent of the presence of CNS tissues, and strongly support the idea that FoxD3 is involved directly as a key regulatory factor in neural crest differentiation.

Differential requirements for Slug and FoxD3 in neural crest development

The present study (Fig. 5) and previous reports (LaBonne and N. Sasai, K. Mizuseki and Y. Sasai) show that FoxD3 is required for neural crest development both in vivo and in animal caps. (A-C) Injection of FoxD3delN mRNA (50 pg) into two right blastomeres at the 8-cell stage suppressed Slug (A), and endogenous FoxD3 (detected with 3’UTR probe) (B). Sox2 was induced in the expected neural crest region (shown by an arrow in C) at stage 16. (D-O) Co-injection of FoxD3delN and Slug rescues the expression of neural crest markers. Injection of Slug mRNA (100 pg) into two right blastomeres at 8-cell stage moderately expands the expression of endogenous Slug (detected with 3’UTR probe) (D), FoxD3 (G) and Twist (J), but not Sox2 (M). Injection of FoxD3delN suppressed expression of the neural crest markers (E,H,K), while co-injection of FoxD3delN and Slug rescued their expression (F,I,L). Expansion of Sox2 caused by FoxD3delN was suppressed by coinjecting Slug (N,O). (P) Animal caps were prepared from embryos injected with Slug (50 pg) + Wnt3a (50 pg; lane 3), Slug (50 pg) + Wnt3a (50 pg) + FoxD3delN (100 pg; lane 4), and Slug (50 pg) + Wnt3a (50 pg) + XBF2delN (100 pg; lane 5) mRNAs. They were harvested at stage 17 equivalent and analyzed by RT-PCR.
Fig. 6. Differential requirement of FoxD3 and Slug in neural crest development.
(A) A truncated mutant that encodes only the DNA-binding domain of Slug (bottom; SlugBD, labeled dn Slug). (B) Injection of SlugBD mRNA (100 pg) into two right blastomeres at 8-cell stage suppressed endogenous Slug expression (detected with 3'UTR probe) at stage 15 on the injected side. (C,D) Slug expression was suppressed in animal caps by the co-injection of 100 pg of SlugBD mRNA with Slug (50 pg) and Wnt3a (50 pg) mRNAs at stage 17 (C) (animal caps injected with Slug (50 pg) and Wnt3a (50 pg); C inset). This suppression was reversed by increasing wild-type Slug mRNA to 200 pg (D). (E) Effects of SlugBD on neural crest markers in FoxD3-injected animal caps. Control mRNA, FoxD3 mRNA (25 pg), or combination of FoxD3 (25 pg) and SlugBD (100 pg) mRNAs were injected into animal blastomeres in 8-cell stage embryos. Animal caps were prepared at stage 10.5 and harvested at stage 21 for RT-PCR analysis. (F) Animal caps were prepared from embryos injected with Zip-r1 (100 pg; lane 3), Zip-r1 + FoxD3delN (100 pg; lane 4), Zip-r1 + SlugBD (100 pg; lane 5), Chd (50 pg) + Wnt3a (50 pg; lane 6), Chd + Wnt3a + FoxD3delN (lane 7), or Chd + Wnt3a + SlugBD (lane 8) mRNAs. Animal caps were harvested at stage 14 for RT-PCR analysis. (G) Animal caps were prepared at stage 10.5 from embryos injected with control (100 pg; lanes 2 and 6), Slug (50 pg; lanes 3 and 7), FoxD3 (25 pg; lanes 4 and 8), or FoxD3 + Slug (lanes 5 and 9) mRNAs, with (lanes 6-9) or without (lanes 2-5) Chordin (50 pg) mRNA.

Bronner-Fraser, 2000) have demonstrated that two early neural crest-specific transcription factors, FoxD3 and Slug, are required for neural crest development. These two genes are expressed in an overlapping manner (Fig. 1) and are regulated by similar upstream genes (Fig. 2). Therefore, we next examined whether FoxD3 and Slug function in the same pathway or have distinct roles in neural crest determination.

A recent report using dominant-negative constructs has shown that Slug function is required for expression of Slug and Twist and for proper migration of neural crest cells (LaBonne and Bronner-Fraser, 2000). A dominant-negative Slug construct (dn Slug) lacking the amino-terminal domain (SlugBD; Fig. 6A) was generated in accordance with that report, and used to analyze the requirement for Slug in FoxD3 signaling. Consistent with the previous report, SlugBD overexpression in the right half of the embryo inhibited endogenous Slug expression on the ipsilateral side (88%, n=32; Fig. 6B). SlugBD injection also suppressed other neural crest markers such as FoxD3 (81%, n=21), Twist (86%, n=21) and Ets-1 (100%, n=21), but not Sox2 (n=18; not shown). Injection of SlugBD also inhibited Slug induction by Slug and Wnt3a in the animal cap (no Slug induction, n=32; Fig. 6C; inset, Slug and Wnt3a only, Slug induction 95%, n=21). This inhibition was rescued by increasing the amount of wild-type Slug (Slug induction 80%, n=40; Fig. 6D), showing that the dominant-negative effect was specific to Slug (or Snail-related factors; Essex et al., 1993).

Injection of SlugBD completely suppressed the induction of Slug, FoxD3 and Twist by FoxD3 in the animal cap (Fig. 6E, lanes 3,4). In contrast, induction of Ets-1, a late neural crest marker, was not strongly affected by SlugBD (lanes 3,4). These results suggest the following relationship between FoxD3 and Slug functions. First, Slug-related activity is essential for induction or maintenance of Slug and FoxD3 in FoxD3-injected caps. Second, Slug is essential for FoxD3 to induce Twist. Third, FoxD3 can induce Ets-1 without Slug activity in the animal cap, indicating that Twist and Ets-1 are controlled in a different manner by Slug and by FoxD3. In the embryo, however, SlugBD injection does suppress both Twist and Ets-1 as discussed above. One explanation for this discrepancy is...
that the SlugBD-resistant portion of Ets-1 expression is irrelevant to its neural crest expression. This seems unlikely to be the case, as Ets-1 expression in the ectoderm is neural crest-specific during early tailbud stages (Meyer et al., 1997; we have also confirmed it ourselves). Another interpretation, which we think is more likely, is that suppression of Ets-1 by SlugBD in vivo is caused secondarily by downregulation of the upstream regulator FoxD3.

Next we further examined the mutual requirements for FoxD3 and Slug in the animal cap assay (Fig. 6F). First, we overexpressed Zic-r1 with FoxD3delN or SlugBD in animal caps, and harvested the caps for RT-PCR analysis at the early neurula stage. Induction of FoxD3 and Slug by Zic-r1 was completely suppressed by FoxD3delN (Fig. 6F lanes 3,4). In contrast, SlugBD strongly suppressed induction of Slug itself but not of FoxD3 (lane 5). Similar results were obtained in induction experiments using Zic2 and Zic3 (data not shown). These results suggest that induction of Slug by Zic factors is dependent on FoxD3 signaling while induction of FoxD3 does not rely on Slug.

As FoxD3 and Slug are also induced by a combination of the extracellular signals Chd and Wnt (Fig. 2G), we next tested the requirements for FoxD3 and Slug in this induction system. As in the experiment examining induction by Zic (Fig. 6F lane 5), SlugBD did not strongly inhibit FoxD3 induction by Chd+Wnt3a (lane 8). Notably, a clear difference was found between the effects of FoxD3delN on the induction of Slug by Zic and by Chd+Wnt3a (compare lanes 4 and 7). Slug induction by Chd+Wnt3a was not affected by FoxD3delN (lane 7), suggesting that Slug induction by Chd+Wnt3a mainly utilizes signaling pathways other than that involving Zic and FoxD3.

The data above indicate a close relationship between FoxD3 and Slug in early determination steps of neural crest development. This led us to test whether coinjection of FoxD3 and Slug exerts synergistic effects on neural crest determination. First, we coinjected FoxD3 and Slug into embryos and analyzed possible cooperative effects. We did not observe remarkable synergism beyond additive effects in vivo (data not shown). To further test possible cooperativity of FoxD3 and Slug, we performed detailed animal cap experiments. As shown in Fig. 6G, coinjection of FoxD3 and Slug (lanes 5,9) did not show significant effects on neural crest and neural markers as compared to FoxD3 injection alone, regardless of the absence (lanes 2-5) or the presence (lanes 6-9) of the neural inducer Chordin. Thus, at least in these gain-of-function studies, Slug does not seem to be a limiting factor, and endogenous Slug induced by FoxD3 is likely to be sufficient to initiate neural crest differentiation.

**FoxD3 and XBF2, two transcriptional repressors, show distinct effects on BMP4 suppression and Slug induction**

Studies with reporter assays have shown that XBF2 is a transcriptional repressor. A chimeric construct of the XBF2 DNA-binding domain and the En repressor domain mimics the neuralizing activity of XBF2, while the chimeric construct of the XBF2 DNA-binding domain and the VP16 activation domain functions as a dominant-negative XBF2 (Mariani and Harland, 1998). Therefore, we tested whether the same principle was applicable to FoxD3. Injection of the FoxD3-EnR construct caused neural differentiation of animal caps without inducing the mesodermal marker M-actin (Fig. 7A). In contrast, FoxD3-VP16 suppressed the neuralizing activity of wild-type FoxD3 and restored Keratin expression (lane 4). This suppression could be reversed by increasing the amount of wild-type mRNA (lane 5), indicating that the suppression was due to attenuation of FoxD3-related signaling. The dominant-negative effects were not observed with the VP16-fused FoxA4 (XFHK1) construct (lane 6). These results indicate that FoxD3, like XBF2, is a transcriptional repressor.

Finally, we attempted to investigate the mode of action of FoxD3 in neural crest induction. One model for the action of XBF2 has been proposed by Mariani and Harland (Mariani and Harland, 1998). According to this model, XBF2 suppresses BMP4 expression in the ectoderm and promotes neural differentiation. We therefore tested a similar mechanism in neural crest induction by FoxD3 by using animal cap assays. Animal caps injected with control GFP, FoxA4 or FoxD3delN mRNA expressed BMP4 strongly, but not Sox2 or Slug (Fig. 7B-G and data not shown). Injection of FoxD3, XBF2 or dominant-negative BMP receptor mRNA suppressed BMP4 expression and induced Sox2 expression (Fig. 7H,L,K,L,N,O). Suppression of BMP4 by blocking BMP signaling is consistent with previous reports. Interestingly, at the doses that gave similar levels of BMP4 suppression, only FoxD3 injection induced Slug expression in the caps (Fig. 7I). Neither XBF1 nor XBF2 induced Slug at any of doses tested (25-100 pg/cell; Fig. 7M and data not shown). These observations indicate that induction of the neural crest marker by FoxD3 cannot be simply explained by suppression of BMP4 alone and involves distinct additional pathways.

**DISCUSSION**

**FoxD3-related signaling is required for neural crest development**

Fox family genes have been shown to play essential roles in the formation of specific ectodermal regions of vertebrate embryos. For instance, targeted disruption of mouse BF-1 (Fox1) causes a dramatical reduction of the telencephalon (Xuan et al., 1995). An essential role for Fox3 in mouse lens development has recently been reported (Blixt et al., 2000). In these cases, specification of the primordial cells of the telecephalon and lens occurs, but their proliferation is much reduced (Xuan et al., 1995; Blixt, 2000). A recent study in Xenopus suggests that FoxG1 exerts distinct effects on determination and proliferation of CNS tissues depending on the dose (Hardcastle and Papalopulu, 2000).

Our present study demonstrates a role of FoxD3-related function in ‘determination’ of the neural crest. Attenuation of FoxD3-related function by FoxD3delN suppresses not only late neural crest markers but also the early neural crest markers Slug and FoxD3 in vivo (Fig. 5), suggesting that initial specification of the neural crest is disturbed. Furthermore, FoxD3, which is expressed in early neural crest primordia, is sufficient for neural crest differentiation of the ectoderm (Fig. 3).

FoxD3 homologues have also been isolated from zebrafish, chicken and mice, and their neural crest expression is found to be conserved across species (Freyaldenhoven et al., 1997; Odenthal and Nüsslein-Volhard, 1998; Labosky and Kaestner,
Possible dual roles of FoxD3-related signaling in neural and neural crest differentiation

As discussed above, our results on the role of FoxD3-related signaling in neural crest differentiation are consistent in both overexpression and dominant-negative studies. Interestingly, the data on the CNS marker present an apparently puzzling situation since both gain-of-function and loss-of-function phenotypes involve upregulation of Sox2 in the embryo (Figs 3D and 5C). Overexpression of wild-type FoxD3 induces ectopic Sox2 expression in vivo (Fig. 3D). In contrast, suppression of the neural crest markers by FoxD3delN is accompanied by expansion of Sox2 (Fig. 5C). However, there are some qualitative differences between these two cases. In the FoxD3 overexpression study, ectopic Sox2 expression can be induced at any injected region of the ectoderm, including ventral and posterior areas separated from the CNS (Fig. 3D). This is a distinct contrast to the dominant-negative data, in which FoxD3delN-induced Sox2 expression is only seen in the cephalic neural crest region and is always contiguous to the endogenous Sox2 expression in the neural plate (Fig. 5C). Another major difference is that Sox2 induction in the animal cap assay is seen with wild-type FoxD3, but not with FoxD3delN (Fig. 3G and data not shown).

One interpretation of these results is that FoxD3 has dual roles depending on the time of action and the region of ectoderm. During early gastrulation, FoxD3 is expressed in posterior neuroectoderm (Fig. 1A) and the ability of FoxD3 to induce neural differentiation is likely to be relevant to this expression. Suppression of BMP4 expression by FoxD3 (Fig. 7H) explains its neuralizing activity at least in part, as attenuation of BMP signaling by organizer factors has been shown to induce differentiation of the neuroectoderm in early gastrula embryos (Sasai et al., 1995; Piccolo et al., 1996; Zimmerman et al., 1996). Although FoxD3delN does not cause suppression of Sox2 in the neural plate (Fig. 5C), this may be explained by some compensatory mechanisms, as a number of related Fox genes are expressed in the CNS (Kaufmann and Knoechel, 1996; Bourguignon and Papalopulu, 1998; Mariani and Harland, 1998; Gomez-Skarmeta et al., 1999). This idea is supported by our observation that injection of FoxD3-VP16, a less specific dominant-negative FoxD3 that interferes with both FoxD3 and XBF2, causes suppression of both Slug and Sox2 in vivo (data not shown).

During late gastrulation, FoxD3 expression is downregulated in the neural plate primordia and becomes apparent in the neural crest primordia (Fig. 1E). This second phase expression is consistent with the neural crest-inducing activity of FoxD3. FoxD3 is the only Fox family gene so far reported to be expressed abundantly in the neural crest primordia of Xenopus gastrulae. (One paper reported a faint and transient expression of XBF2 in the crest primordia of Xenopus neurulae while another paper reported no XBF2 expression in the same region; Gomez-Skarmeta et al., 1999; Mariani and Harland, 1998) The expansion of Sox2 by FoxD3delN at the cost of neural crest markers can be interpreted as a conversion of ectodermal fate from neural crest into a CNS type. According to this interpretation, in the absence of FoxD3-related signaling, ectodermal cells flanking the neural plate cannot differentiate into the neural crest but rather adopt a default CNS fate.
Consistently, epidermal *Keratin* expression is not significantly affected by *FoxD3delN* injection in vivo (data not shown). A similar observation of neural crest-CNS conversion was reported in a previous loss-of-function study of *Slug* (LaBonne and Bronner-Fraser, 2000). Therefore, it seems that early signaling involving *FoxD3* and *Slug* in the neural crest primordia is essential for the segregation of neural crest and CNS fates in the dorsal ectoderm.

We have attempted to establish the existence of stage-specific ‘dual roles’ of *FoxD3* by using inducible forms of *FoxD3*-fused with GR (Kolm and Sive, 1995). Unfortunately, we have not yet succeeded in generating such constructs because, for unknown reasons, all the GR-fused *FoxD3* constructs we made exhibit high background activity in the absence of the activator ligand Dex. Future studies using transgenic frog techniques may resolve this problem. We also tried to understand the temporal requirement of *FoxD3* signaling by using inducible GR-fused dominant-negative *FoxD3* (*FoxD3delN-GR*). This construct works fine, and neural crest markers are suppressed in the embryo injected with *FoxD3delN-GR* only when Dex is added to culture medium (our unpublished observations). In this case, injected embryos treated with Dex from stage 9 on and from stage 12 on exhibit similar extents of neural crest marker suppression, suggesting that *FoxD3* function from the late gastrula stage is essential for neural crest formation.

A model for the roles of *FoxD3*, *Slug* and *Zic* in neural crest development

To understand the molecular cascade in neural crest differentiation, we have studied transcriptional regulations involving *FoxD3* and *Slug*. Our working model of the relationships of *FoxD3*, *Slug* and *Zic* is as follows. *Zic* factors such as *Zic-r1* are induced widely in dorsal ectoderm (presumptive neural plate and neural crest regions) by neural inducers at the early gastrula stage. By the late gastrula stage, dorsoventral patterning in the dorsal ectoderm becomes evident. *Zic* expression is suppressed in the medial portion of the neuroectoderm, and *FoxD3* and *Slug* are induced in the presumptive neural crest regions. Animal cap studies showed that *Zic* factors induce both *FoxD3* and *Slug* in distinct fashions. *Slug* induction by *Zic* is dependent on *FoxD3* signaling (Fig. 6F lane 4) whereas *FoxD3* induction is largely independent of *Slug* activity (lane 5). In contrast, *Slug* induction by *Chd+Wnt* is not blocked by *FoxD3delN* (lane 8), suggesting that *Chd+Wnt* signaling involves a *FoxD3*-independent pathway. It remains to be clarified whether *FoxD3* induction is totally dependent on *Zic* signaling or not. The answer to this question should be attainable once an appropriate dominant-negative *Zic* becomes available.

Once *FoxD3* and *Slug* are induced, mutual activation loops of *Zic-FoxD3* and *FoxD3-Slug* (with *Wnts*) play essential roles in maintenance of these factors (Figs 2M, 3G, 5E). These ‘self-activating’ circuits are likely to support expression of these three factors in the neural crest regions. RNA injection studies also show that *Zic-r1* induces *Slug* while *Slug+Wnt3a* activates *Zic-r1* in the cap (Fig. 2N and data not shown). However, these inductions are not primary events because both are blocked by *FoxD3delN* (Fig. 6F lane 4 and data not shown).

Although the model above explains a majority of the data from this study, additional genes are thought to be involved. For instance, (1) as both *FoxD3* and *Slug* are shown to be repressors (this study and LaBonne and Bronner-Fraser, 2000), their ability to activate theoretically requires unknown intermediate factors. (2) The neural crest primordia express two closely related *Snail* family factors (*Slug* and *Snail*), which are suggested to play somewhat redundant roles (discussed by LaBonne and Bronner-Fraser, 2000). It remains to be clarified whether these two factors have distinct functions in the initiation and maintenance phases. (3) The in vivo expression of the upstream gene *Zic* is broader than that of the downstream gene *FoxD3* (Fig. 1K), suggesting that yet unidentified factor(s) should provide additional positional information. Also, it is important to understand in the future how FGFs, Wnts and *Pax3* (Bang et al., 1999) signals are integrated in our model. (4) It remains to be elucidated whether the effects of dominant-negative *Slug* (*SlugBD*) on neural crest migration (Nieto, 1994; Carl et al., 1999; LaBonne and Bronner-Fraser, 2000) are dependent on *FoxD3*-related signaling or not.

Conclusion

This study provides the first evidence for the requirement of *FoxD3*-related signaling in neural crest formation. In addition, it has been shown that *FoxD3* overexpression is sufficient to initiate neural crest differentiation in the embryonic ectoderm. *FoxD3* is required for induction of *Slug* by *Zic*, while *Slug* is not needed for *FoxD3* induction. Mutual activation of *Zic*, *FoxD3* and *Slug* may be involved in their own maintenance. In addition to these mutual activation loops, *FoxD3* and *Slug* utilize distinct pathways in activating specific downstream genes, such as *Eis-1* and *Twist*. Collectively, *FoxD3* and *Slug* (or their closely related factors) are essential regulators of early neural crest differentiation, which work in concert and in partially non-overlapping pathways.

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Note added in proof

Soon after our revised manuscript was submitted, a study in the chick appeared that also indicated positive roles of *FoxD3* in neural crest development (Kos et al., 2001).

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