The neural crest is a specialized population of cells that delaminate from the dorsal neural tube, migrate into the periphery and differentiate into multiple cell types, including neurons and glia within the peripheral nervous system, melanocytes, cardiac muscle, endocrine cells and chondrocytes (Le Douarin, 1982). Neural crest cells are generated from uncommitted progenitors in the dorsal neural folds and neural tube, a region that also gives rise to roof plate cells and dorsal interneuron cell types (Anderson, 1989; Lee and Jessell, 1999). Recent studies have shown that signals from the ectoderm and the non-axial mesoderm, such as the BMPs, Wnts and FGFs, induce the expression of dorsalizing genes in the neural plate/tube, initiating a transcriptional cascade that specifies early dorsal cell types (LaBonne and Bronner-Fraser, 1999). Several transcription factors are expressed in the dorsal neural tube and are induced in response to Wnt- and BMP-dependent signaling (Lee and Jessell, 1999). These include Pax3, Pax7, Msx1/2 and Zic1-Zic3, all of which have been implicated in the development of neural crest cells but are also expressed in progenitors that give rise to dorsal interneurons (Epstein et al., 1991; Goulding et al., 1993; Bang et al., 1997; Liem et al., 1997; Houzelstein et al., 1997; Mansouri and Gruss, 1998; Nakata et al., 1998). This argues that other transcription factors may be required for the specification of neural crest cells.

The intrinsic factors that specify and segregate neural crest progenitors from multipotent precursors in the dorsal neural tube are poorly defined. One candidate for neural crest determination is the helix-loop-helix transcription factor Id2, which is able to convert epidermal cells to a neural crest cell fate, when ectopically expressed in cranial regions of the embryo (Martinsen and Bronner-Fraser, 1998). However, Id2 expression is restricted to cranial neural crest, and neural crest defects have not been described in Id2 null mice (Martinsen and Bronner-Fraser, 1998; Yokota et al., 1999). The zinc-finger transcription factor Slug is also transiently expressed in neural crest cells. There is evidence that Slug regulates neural crest delamination and migration in Xenopus and chick embryos (Nieto et al., 1994; LaBonne and Bronner-Fraser, 1998; Yokota et al., 1999). The winged-helix transcription factor Foxd3 (CWH3, Hfh2, Genesis) is expressed in the presumptive neural crest region in both chick and mouse embryos, and may therefore play a role in neural crest determination (Freyaldenhoven et al., 1997; Labosky and Kaestner, 1998; Yamagata and Noda, 1998; Nakata et al., 1998).

The intrinsic factors that specify and segregate neural crest...
Hromas et al., 1999; Kos et al., 2001). The temporal expression of Foxd3 closely matches that described for other early markers of neural crest, including Slug and the RhoB GTPase (Liu and Jessell, 1998; Sela-Donenfeld and Kalcheim, 1999). As with Slug and RhoB, Foxd3 mRNA expression in the dorsal neural tube progenitors appears to be specific to neural crest, as evidenced by the emigration of Foxd3 cells from the neural tube (Yamagata and Noda, 1998; Kos et al., 2001). In view of the early and specific expression of Foxd3 in premigratory neural crest cells, we examined the function of Foxd3 in neural crest development. We show that Foxd3 is downstream of Pax3 and is expressed in migratory neural crest cells that give rise to multiple cell types. To explore its role in neural crest development, Foxd3 was misexpressed in the neural tube of stage 10-11 chick embryos. Ectopic Foxd3 was found to induce the expression of migratory neural crest markers, as well as to stimulate their delamination and migration from the neural tube at all dorsoventral levels. This effect was observed at all anterior-posterior levels of the spinal cord, as well as at hindbrain levels. The normal patterning and differentiation of interneurons within the neural tube was also suppressed by Foxd3, suggesting Foxd3 functions as a genetic switch to promote a neural crest fate while simultaneously repressing interneuron differentiation. Taken together, our findings describe a function for Foxd3 in the early specification and segregation of neural crest cells from multipotential progenitors in the dorsal neural tube.

MATERIALS AND METHODS

Whole-mount in situ hybridization

White Leghorn eggs were incubated in a force-draft, humidified incubator at 38°C. Chick embryos were staged according to Hamburger and Hamilton. Mouse embryos were obtained from our breeding colony, with noon on the day of plug discovery designated as day 0.5. Genotyped Splotch+/− (Pax3+/− – Mouse Genome Informatics) embryos were obtained from breeding Splotch heterozygous mice (Epstein et al., 1991). Whole-mount in situ hybridization was performed according to the protocol of Wilkinson (Wilkinson, 1992) using digoxigenin-labeled antisense RNA probes. The probes used were chick Pax3 (Goulding et al., 1993), chick Foxd3 (Freyaldenhoven et al., 1997) and mouse Foxd3 (Labosky and Kaestner, 1998). For vibratome sections, embryos were embedded in 3.5% agar and 8% sucrose in PBS then sectioned at 100 μm.

Immunohistochemistry

Mouse and chick embryos were fixed 1 hour in 4% paraformaldehyde in phosphate-buffered saline (PBS), cryoprotected in 20% sucrose in PBS, and then embedded in OCT (Tissue-Tec) and sectioned at 20 μm. Immunohistochemistry was performed on frozen sections as previously described (Burrill et al., 1997). The following antibodies were used in this study: rat anti-BrdU (Harlan), polyclonal anti-Btn-3.0 (E. Turner), monoclonal anti-Cad-6B and anti-Cad-7 (M. Takeichi), monoclonal anti-β-catenin (Zymed Lab), monoclonal anti-NAPA73 (E/C8, Developmental Studies Hybridoma Bank, DSHB), polyclonal anti-Foxd3 (see below), monoclonal anti-HNK-1 (Bronner-Fraser, 1986), monoclonal anti-IsI1/2 (40.2.D6, DSHB), monoclonal anti-Lhx1/5 (4F2-10, DSHB), polyclonal anti-Lhx2/9 (K. Lee and T. Jessell), polyclonal anti-Mtf (M. Mohici), monoclonal anti-Myc (9E10, ATCC), polyclonal anti-Myc (S. Pfaff), monoclonal anti-NeuN (Chemicon International), monoclonal anti-P0 (1E8, DSHB), polyclonal anti-Pax2 (Zymed Lab), rat anti-Pax3 (M. Gross), monoclonal anti-Pax6 (H. Fujisawa), monoclonal anti-Pax7 (PAX7, DSHB), polyclonal anti-Phox2a (J. Brunet), monoclonal anti-Slug (62.1E6, DSHB), monoclonal anti-RhoB (56.4H7, DSHB), monoclonal anti-TAG1 (23.4-5, DSHB), and mouse anti-Tuj1 (Chemicon). Species-specific secondary antibodies were conjugated to Cy3, Cy2 or FITC, and used as recommended (Jackson Labs). Antisera to Foxd3 was generated by immunizing rabbits with a fusion protein containing amino acids 109-281 of mouse Foxd3 fused to glutathione S-transferase pGEX (Pharmacia). The antiserum was affinity purified before use.

BrdU labeling

E10.5 mouse embryos were pulsed for 90 minutes in utero with bromodeoxyuridine (50 mg/ml in 0.9% saline injected intraperitoneally). Embryos were harvested and processed for immunohistochemical staining as described above. Sections were double-stained with anti-Foxd3 and anti-IsI1 and then processed for staining with an anti-BrdU monoclonal antibody (Moran-Rivard et al., 2001).

In ovo electroporation

Full-length chick Foxd3 cDNA (Freyaldenhoven et al., 1997) was cloned into the pCS2-MT expression vector (provided by D. Turner) and an IRES-EGFP expression vector containing the chick β-actin promoter (provided by C. Krull). Windowed E2 (stage 10-11) chick embryos were electroporated with pCS2-Foxd3MT (Foxd3Myc), pCS2-MT (Myc), or Foxd3IRES-EGFP DNA at 4 μg/μl, as previously described (Muramatsu et al., 1997). Briefly, plasmids were pipetted into the lumen of the neural tube using a picospriter and electroporated into the embryo using a BTX electroporator (five 50 msecond pulses at 25 V). Eggs were sealed and allowed to develop to a specific stage, at which time they were dissected, fixed and processed for immunohistochemistry. Foxd3-Myc expression was detected with Myc monoclonal and polyclonal antibodies.

Full-length Slug cDNA was amplified from stage 10 chick embryo total RNA, cloned into BS vector (Clonetech) and sequenced. For Slug misexpression experiments, a Myc-tagged version of full-length chick Slug cDNA was cloned into the MiwSV expression vector (Suemori et al., 1990) containing the chick β-actin promoter (SlugMyc) and expressed in the neural tube using the electroporation procedure described above. SlugMyc expression was detected with antibodies that recognize the Myc epitope. For co-transfection experiments of Foxd3Myc and SlugMyc, protein expression was detected using Myc polyclonal and Slug monoclonal antibodies, respectively.

RESULTS

Foxd3 is expressed in premigratory and migratory neural crest cells

To characterize Foxd3 expression at the single cell level, an antisera was generated against amino acids 109-281 of the mouse Foxd3 protein. This antibody recognizes the mouse but not the chick Foxd3 protein. In E9.5 mouse embryos, Foxd3-positive cells were detected at both cervical and trunk levels in a domain that spans the dorsal midline where neural crest progenitors are located. From E9.5 to E10.5, Foxd3-positive cells were also seen emigrating from the dorsal neural tube along its entire length (Figs 1A-C, 2A). Foxd3 expression was then compared with that of Pax3 which is transiently expressed in all migrating neural crest cells (Epstein et al., 1991; M. G., unpublished), to examine whether all migrating neural crest cells express Foxd3. In E9.5 embryos, all Pax3-positive migratory crest cells were seen to express Foxd3 (Fig. 1B,C, arrows). Furthermore, in E12.5 embryos, cells expressing Foxd3 were present in both the ventral and dorsolateral
migration pathways that give rise to neurons/Schwann cells and melanocytes, respectively (Fig. 1). Thus, in addition to marking premigratory neural crest cells, Foxd3 is expressed in all neural crest cells during their early phase of migration from the dorsal neural tube.

Double labeling immunohistochemistry was then used to examine Foxd3 expression in various neural crest lineages. In E12.5 embryos, Foxd3 was detected in a population of cells located just beneath the epidermis that do not express $\beta$-catenin, a marker of epidermal cells (Fig. 1H,I). These cells appear to be neural crest cells that migrate between the skin and dermomyotome and are therefore likely to be melanoblasts. They are distinct from the Foxd3-positive/Pax3-positive cells that encompass the dorsal root ganglia (Kioussi et al., 1995). Interestingly, Foxd3-positive cells were not detected in the dermis of E14.5 embryos when melanocyte precursors are no longer being generated (Keshet et al., 1991), nor did we detect double labeled Foxd3-positive/Mitf-positive cells, suggesting that Foxd3 is downregulated as this lineage matures (data not shown).

Foxd3 expression was then compared with NeuN, which marks the nuclei of postmitotic neurons in both dorsal root ganglia and sympathetic ganglia (Fig. 1D,E). Nevertheless, a subset of NeuN-positive cells in the dorsal root ganglia (DRG) did exhibit low levels of Foxd3 expression (Fig. 1D, arrow). To further examine whether Foxd3 expression is excluded from postmitotic neurons, E10.5 mouse embryos were pulsed with BrdU for 90 minutes. Although cross-sections from these embryos showed Foxd3-positive/BrdU-positive cells within the DRG, none of these cells co-expressed Islet1 a marker of postmitotic sensory neurons (Fig. 1F). However, a number of BrdU-positive/Islet1-positive/Foxd3-negative sensory neurons were observed in embryos pulsed with BrdU at E9.5 and analyzed 24 hours later (data not shown). These findings, together with the observation that Foxd3 is expressed in all early migrating crest cells (Fig. 1B,C) argue that crest-derived neuronal precursors downregulate Foxd3 once they become postmitotic.

In E10.5 embryos, the majority of Foxd3-positive neural crest cells are located at the periphery of the sensory and autonomic ganglia as well as along the TuJ1-labeled axons of the peripheral nerves (Fig. 1D,E,G, arrows) indicating that these Foxd3 cells are undifferentiated Schwann cell precursors. Furthermore, Pax3, which is expressed in early non-myelinating Schwann cells (Kioussi et al., 1995), was seen to be co-expressed with Foxd3 cells in dorsal root ganglia in E12.5 embryos (Fig. 1I, arrowhead). Although Foxd3 expression in cells surrounding the peripheral ganglia and axon
tracts was as late as E14, it was downregulated before the onset of Krox20 and SCIP expression, which defines the transition from premyelinating to promyelinating Schwann cells (Zorick et al., 1996; data not shown). Taken together, these data suggest that Foxd3-positive precursors contribute to multiple neural crest lineages in the mouse, including sensory/sympathetic neurons, glia and melanocytes. Studies in the chick also demonstrate widespread Foxd3 expression in neural crest cells that give rise to chondrocytes, neurons and glia (Yamagata and Noda, 1998; Kos et al., 2001). However, although Foxd3 appears to mark all premigratory crest cells in the chick, it is not expressed in migrating trunk melanoblasts at stage 18 (Kos et al., 2001).

**Foxd3 is genetically downstream of Pax3 in dorsal neural tube progenitors**

To further assess the role of Foxd3 in neural crest, Foxd3 expression was analyzed in Pax3 null mutant (*Splotch*) embryos, which lack all neural crest derivatives caudal to the boundary of the hindbrain and spinal cord (Epstein et al., 1991). In wild-type E10 embryos, neural crest cells that express Foxd3 are generated along the length of the neural tube, from midbrain/hindbrain border to the tail, with expression persisting in dorsal root and cranial ganglia (Fig. 2A-D). In *Splotch* embryos however, Foxd3 is not expressed in caudal regions where dorsal root ganglia and sympathetic ganglia are missing (Fig. 2E, arrowhead; Fig. 2F,H arrows). However, Foxd3 is still expressed at hindbrain levels in neural crest cells that contribute to the V, VII and IX cranial ganglia (Fig. 2E,G, arrows). Pax7, a protein closely related to Pax3 in structure, may compensate for Pax3 in Pax3-null embryos, thereby allowing neural crest cells to form at hindbrain and upper cervical levels. Examination of Pax7 expression in E10 *Splotch* embryos, shows that while Pax7 is expressed in the dorsal midline of the neural tube in the hindbrain and anterior cervical spinal cord (Fig. 2I, arrowhead), it is not expressed in this domain further caudally where Foxd3-positive neural crest cells are missing (Fig. 2J, arrowhead).

Two different models have been proposed to account for the neural crest defects observed in *Splotch* embryos: (1) Neural crest cells are specified, but fail to migrate; or (2) neural crest cells are not specified correctly in caudal regions of *Splotch* embryos. While some migration of neural crest cells is observed when neural tubes from *Splotch* embryos are transplanted to chick embryos (Serbedzija and McMahon, 1997), other studies in chick (Goulding et al., 1993) and *Xenopus* (Bang et al., 1999) indicate Pax3 plays a role in the early specification of dorsal cell types, including neural crest cells. The demonstration that Foxd3, a marker of premigratory crest cells, is no longer expressed in the dorsal neural tube/folds of *Splotch* embryos (Fig. 2B,F and Fig. 2A,E, arrowheads) argues that neural crest progenitors are not

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**Fig. 2.** Foxd3 expression in wild-type and *Splotch* mouse embryos. (A,B,E,F) Whole-mount in situ of Foxd3 in E10 wild-type (A,B) and *Splotch* (E,F) mouse embryos. (A) Foxd3 is expressed in migrating cranial neural crest cells (arrows) and in premigratory crest (arrowhead). (E) Foxd3 is expressed in cranial neural crest (arrow) but is absent in more caudal regions of the embryo (arrowhead). (B) Cross sections through caudal neural tube at the level of the arrowheads shows Foxd3 expression in the dorsal neural tube of wild-type embryos. (E) No Foxd3 expression is observed in the open neural tube of *Splotch* embryos. (C,D,G,H) Foxd3 protein expression (green) in the hindbrain (C,G) and caudal spinal cords (D,H) of wild-type (C,D) and *Splotch* (G,H) embryos. Foxd3-positive crest cells are observed in the head regions of *Splotch* (G) embryos at E9.5. In *Splotch* embryos, Foxd3 is expressed in hindbrain-derived crest (G, arrow) but not in trunk-derived crest (H, arrow). (I) Cross section through the anterior cervical neural tube of an E10 *Splotch* embryo showing Pax7 expression (red) in the dorsal midline (arrowhead). Migrating Foxd3-positive neural crest cells are present at this level (green, arrow). (J) Cross-section through the caudal neural tube of an E10 *Splotch* embryo. Pax7 (red) is not expressed in the dorsal midline (arrowhead) at spinal cord levels where neural crest cells are lacking (arrow). Foxd3 (green) is however expressed in a population of postmitotic ventral interneurons (IJ, asterisk). hb, hindbrain; sc, spinal cord.
correctly specified. Furthermore, these findings suggest that Foxd3 may function in the specification of neural crest cells.

**Foxd3 misexpression induces characteristics of migratory neural crest cells**

To examine whether Foxd3 plays a role in specifying neural crest, Foxd3 was ectopically expressed in the neural tubes of stage 10-11 chick embryos by electroporation. After 48 hours, extensive expression of the migratory neural crest markers, HNK-1 (Bronner-Fraser, 1986) and Cad-7 (Nakagawa and Takeichi, 1998) was observed on the electroporated side of the neural tube (Fig. 3A-C), in neural tube progenitor cells that express Myc-tagged chick Foxd3 (Foxd3Myc). This upregulation of HNK-1 and Cad-7 was observed as early as 24 hours after electroporation (Fig. 3D), suggesting that those cells expressing Foxd3Myc have the molecular characteristics of migratory neural crest cells.

A dramatic change in cell morphology was also seen on the electroporated side of the neural tube. In Foxd3Myc electroporated neural tubes, the basement membrane surrounding the neural tube was disrupted and large numbers of Foxd3Myc+ cells were seen migrating laterally at multiple dorsoventral levels (Fig. 3B,E,H,I). This effect was observed at all anteroposterior levels in the spinal cord as well as in the hindbrain (Fig. 3B,E,F,G-I). The upregulation of neural crest markers that accompanies the delamination and migration of ectopic Foxd3Myc+ cells was observed in all Foxd3Myc-electroporated neural tubes analyzed at 48 hours (n=37/37 embryos) and 96 hours (n=10/10 embryos), as well as in the majority of the neural tubes analyzed at 24 hours (n=7/11 embryos). A Foxd3 expression vector lacking the Myc tag (Foxd3IRES-EGFP), also gave the same full phenotype in chick embryos as Foxd3Myc (Fig. 3I), whereas the control Myc expression vector did not induce HNK-1 expression or changes in cell morphology. Taken together, these results demonstrate that Foxd3 induces the morphological characteristics of migrating neural crest cells when misexpressed in the chick neural tube.

The delamination, lateral migration and the expression of HNK-1 and Cad-7 are all properties of migrating neural crest cells. To determine whether constitutive Foxd3 expression causes ectopic ‘neural crest-like’ cells to differentiate as neurons or glia, Foxd3Myc-electroporated embryos were examined 96 hours after electroporation. By this time Foxd3Myc+ cells had undergone extensive migration and were often seen encircling dorsal root ganglia (Fig. 4A,E, arrows). Foxd3Myc+ cells were also seen encompassing TuJ1-positive sensory neurons and their axons in a manner characteristic of early Schwann cell precursors (Fig. 4D, arrow). Foxd3Myc+ cells were rarely, if ever, detected within the neuronal cores of sympathtic (Fig. 4B) or dorsal root (Fig. 4D,E) ganglia that are marked by Phox2a and Brn3.0, respectively (Fedtsova and Turner, 1995; Morin et al., 1997). Whereas no Brn3.0-positive/Foxd3Myc+ or Phox2a-positive/Foxd3Myc+ cells were detected in ganglia at electroporated levels, electroporated cells expressing the Myc-tag alone were frequently detected within the neuronal cores of ganglia, where they co-expressed NeuN, a marker of differentiated neurons (Fig. 4F, arrow). Our observation that Foxd3Myc-electroporated cells fail to contribute to either sensory or autonomic neuronal lineages suggests that persistent ectopic expression of Foxd3 may disrupt neuronal differentiation. This conclusion is consistent with the observation that Foxd3 is normally downregulated as neural crest cells differentiate into postmitotic neurons (Fig. 1D-F), and suggests downregulation of Foxd3 may be an obligatory step in the generation of postmitotic neurons in the peripheral nervous system. The exclusion of Foxd3Myc+ cells from peripheral neurons suggests that ectopic Foxd3 cells either persist as
undifferentiated neural crest precursors or eventually enter another crest lineage. This was examined by analyzing expression of the melanoblast marker Mitf (Mochii et al., 1998) and P0, a marker of Schwann cell precursors (Bhattacharyya et al., 1991), 96 hours after electroporating Foxd3. Ectopic Foxd3Myc+ cells did not co-express Mitf (data not shown), demonstrating that they do not differentiate as melanoblasts, a result that is consistent with the findings of Kos et al. (Kos et al., 2001). However, P0 was co-expressed in a number of Foxd3Myc+ cells in the neural tube, where P0 is normally not expressed (Fig. 4C). This ectopic P0 expression was observed in all Foxd3Myc-electroporated neural tubes analyzed at 96 hours (n=10/10 embryos). By contrast, ectopic P0 staining was never observed in neural tubes electroporated with a control Myc vector (n=4/4 embryos, data not shown). It therefore appears that some Foxd3Myc+ cells upregulate P0, which is expressed in early Schwann cell precursors. In summary, it appears that Foxd3 may need to be downregulated for neural crest cells to differentiate as neurons and melanocytes, while continued expression may promote an early step in Schwann cell development.

Ectopic Foxd3 suppresses interneuron markers
The observation that Foxd3 is expressed in premigratory neural crest progenitors suggests that Foxd3 may restrict dorsal progenitors to a neural crest cell fate. We therefore expected that cells ectopically expressing Foxd3 would no longer differentiate as interneurons. To determine whether dorsal interneuron specification is suppressed by Foxd3, expression of the LIM-homeodomain proteins Lhx2/9, Isl1/2 and Lhx1/5 was examined in Foxd3Myc-electroporated neural tubes. Lhx2/9, Isl1/2 and Lhx1/5 mark three populations of dorsal interneurons, D1, D2 and D3 interneurons, respectively (Ericson et al., 1992; Xu et al., 1993; Liem et al., 1997). Fewer Lhx2/9-positive D1, Isl1/2-positive D2 and Lhx1/5-positive D3 interneurons were found on the electroporated side of Foxd3Myc-electroporated neural tubes (Fig. 5A-E). Cells expressing Foxd3Myc also did not express Brn3.0 (Fig. 5F) a marker of dorsal interneurons (Fedtsova and Turner, 1995). In addition to repressing the differentiation of dorsal interneurons, misexpression of Foxd3 also suppressed Pax2 interneuron development in the intermediate neural tube (Fig. 5G,H, arrow). Once again, Pax2 was specifically repressed in cells that express Foxd3Myc (Fig. 5H arrow). By contrast, embryos electroporated with a Myc control plasmid showed no loss of Pax2 expression after electroporation, with Pax2-positive/Myc-positive cells being widely distributed throughout the normal Pax2 expression domain (Fig. 5I arrow). The observation that Foxd3 was never co-expressed with Lhx2/9, Isl1/2, Lhx1/5, Brn3.0 and Pax2 on the electroporated side (Fig. 5A,C,D,F,G) argues that Foxd3 functions cell autonomously to repress interneuron differentiation.

The transcription factor Pax6 is expressed in dividing progenitors that give rise to Lhx1/5-positive/Pax2-positive interneurons, but is not expressed in the progenitors of neural crest cells (Burrill et al., 1997). Pax6 expression was examined in Foxd3Myc-electroporated neural tubes to test whether Foxd3 suppresses the early developmental program of interneuron progenitors. In neural tubes electroporated with Foxd3Myc, the number of Pax6-positive progenitors was dramatically reduced (Fig. 5J,K, arrow), whereas those electroporated with a Myc control vector showed no change (Fig. 5L, arrow). Moreover, in Foxd3Myc-electroporated embryos, progenitor cells that expressed Foxd3Myc did not express Pax6, and vice versa (Fig. 5J). Thus, it appears that overexpression of Foxd3 is sufficient to override the early differentiation program of interneuron progenitors in the spinal cord. The loss of interneuron specific markers in Foxd3Myc+ cells at multiple dorsoventral levels of the neural tube, coupled with the retention of these markers in Foxd3-negative cells, indicates that Foxd3 functions in a cell autonomous manner to suppress interneuron specification and differentiation.

Neural crest induction by Foxd3 is independent of Slug
The ability of Foxd3 to induce both the markers and morphogenetic behavior of neural crest cells, together with its ability to suppress interneuron differentiation, suggests that Foxd3 is able to induce many of the initial steps of neural crest development. Premigratory crest cells express Slug, RhoB and Cad-6B (Nieto et al., 1994; Liu and Jessell, 1998; Nakagawa and Takeichi, 1998; Sela-Donenfeld and Kalcheim, 1999).
leading us to examine Slug, RhoB and Cad-6B expression in the neural tubes electroporated with Foxd3. Surprisingly, no ectopic expression of either Slug, RhoB or Cad-6B was observed in Foxd3Myc+ cells, either 24 hours or 48 hours after electroporation (Fig. 6A-C; data not shown).

To test whether Slug induces neural crest development in a manner similar to Foxd3, a Slug expression vector was electroporated into chick neural tubes at stage 10-11 and expression of HNK-1 and RhoB was examined. Ectopic Slug expression failed to upregulate either of these neural crest cell markers (Fig. 6D-E). In addition, there was no dissolution of the basal lamina coupled with lateral migration of Slug-positive cells from the neural tube (Fig. 6D-G). Finally, the expression of Pax2 was not disrupted in Slug-positive cells (Fig. 6F), indicating Slug does not suppress interneuron differentiation. The inability of Slug to induce migratory neural crest and suppress interneuron differentiation in a manner analogous to Foxd3 argues that Slug does not act upstream of Foxd3. Consistent with these findings, Foxd3 expression was not upregulated in the dorsal spinal cord by ectopic expression of Slug (Fig. 6G,H).

Previous studies have suggested that Slug is necessary for the delamination of neural crest cells from the dorsal neural tube (Nieto et al., 1994). While misexpression of Foxd3 leads to delamination of Foxd3-positive cells from the neural tube, emigrating cells are only observed 36 hours after introducing Foxd3 into the neural tube. To test whether Slug acts synergistically with Foxd3 to promote the early emigration of neural crest-like cells from the neural tube, Foxd3 and Slug expression vectors were co-electroporated into stage 11 neural tubes and analyzed 24 hours later. No difference was seen in the morphology of the neural tube in co-electroporated versus Foxd3-only electroporated embryos at 24 hours, nor were cells seen migrating from the neural tube, demonstrating that Slug does not synergize with Foxd3 to promote the migration of neural crest cells (Fig. 6I). These results indicate Slug and Foxd3 function independently in neural crest development. Foxd3 induces markers and morphological changes that are characteristic for migratory neural crest cells (Fig. 6D-H). By contrast, Slug when misexpressed in the chick fails to induce any of the features of neural crest cells (Fig. 6D-H).

The inability of Foxd3 to upregulate Slug, RhoB and Cad-6B led us to examine the onset of their expression in the caudal neural tube with respect to Foxd3. The expression of Foxd3 in the spinal cord is shown in Fig. 5.

**Fig. 5.** Ectopic Foxd3 suppresses interneuron differentiation in the spinal cord. (A-L) Cross-sections of neural tubes 48 hours after electroporation of Foxd3Myc or Myc alone (IL). Ectopic expression of Foxd3 (green) leads to the loss of Isl1/2-positive D2 interneurons (A,B), Lhx2/9-positive D1 interneurons (C) and Lhx1/5-positive D3 interneurons (D,E) in the neural tube. The arrowhead marks the Isl1/2-positive cells (B) and the Lhx2/9-positive cells (C) on the unelectroporated side of the neural tube and the arrow shows the loss of dorsal interneurons on the electroporated side. (F) Foxd3 expression also suppresses expression of Brn3.0 (red) in dorsal interneurons. A domain of Brn3.0-expressing cells is present in the dorsal spinal cord (arrow), but these cells do not co-express Foxd3. Foxd3-positive cells migrating from the dorsal spinal cord (arrowhead). (G,H) Ectopic Foxd3 suppresses Pax2-positive interneuron differentiation in the dorsal and intermediate spinal cord. Fewer Pax2-positive (H) cells are present on the electroporated side of the neural tube (arrow) and these cells are Foxd3-negative (G). (I) Control electroporation with a Myc expression vector, showing no reduction in Pax2-positive interneurons (red), many of which co-express Myc (yellow, arrow). (J,K) Ectopic Foxd3 suppresses Pax6 expression in interneuron precursors. (K) Fewer Pax6-positive precursors are present on the electroporated side of neural tube (arrow). (J) Foxd3-positive cells (green) do not express Pax6. (L) Electroporation of Myc does not reduce the Pax6 expression domain (arrow). fp; floor plate.
stage 10 chick neural tubes was compared with that of Pax3, Slug, Cad-6B and RhoB. At these stages, the caudal limit of gene expression serves as a good indicator for the onset of gene expression. Foxd3 was expressed in the dorsal neural tube at the level of somite I (Fig. 7A), as well as at segmental plate levels where the neural tube is closing (Fig. 7B), but not caudally in the neural folds (Fig. 7C). By contrast, Pax3 was expressed throughout the neural tube and in the dorsal neural folds (Fig. 7D-F), a finding that is consistent with our studies in the mouse showing Pax3 is upstream of Foxd3 expression (arrows), nor did it suppress the expression of endogenous Foxd3 within the ventral regions of the neural tube (arrowheads). (I) Co-electroporation of Foxd3 and Slug expression vectors showed no enhancement of cell migration in Foxd3-positive/Slug-positive cells (yellow), 24 hours after electroporation compared with cells expressing ectopic Foxd3 only (see Fig. 3D). fp, floor plate.

**DISCUSSION**

Progenitors in the dorsal neural folds/neural tube give rise to multiple cell types, including different classes of dorsal commissural interneurons, as well as a migratory stem cell population, the neural crest (Lee and Jessell, 1999). In this report, we show that Foxd3 mediates a key early step in the development of the neural crest. Foxd3 restricts neural progenitor cells to the neural crest cell lineage, while concomitantly suppressing interneuron differentiation. Cells overexpressing Foxd3 acquire a ‘neural crest-like’ phenotype, i.e. they express HNK-1 and Cad-7, delaminate from the neural tube and invade the adjacent mesoderm. At later times, a subset of these Foxd3-positive cells exhibit the characteristics of the early Schwann cells, raising the possibility that prolonged Foxd3 expression biases neural crest cells toward this pathway. In summary, our findings indicate that Foxd3 plays an early determination role in the dorsal neural tube by committing multipotential progenitors to the neural crest lineage.
Specification of neural crest by Foxd3

Wnt signaling and is further upregulated by BMPs (Liem et al., 1995; Liem et al., 1997; Bang et al., 1999).

At later stages of development, Foxd3 is also expressed in a subset of postmitotic interneurons in the neural tube (Fig. 2I,J). We propose that the context in which Foxd3 is expressed, i.e. mitotic precursors versus postmitotic interneurons, is important for neural crest specification. The observation that Foxd3 does not block interneuron differentiation, or induce neural crest markers, when it is specifically misexpressed in postmitotic neurons in the neural tube, is consistent with this hypothesis (M. D., unpublished). Thus, the role Foxd3 plays in restricting dorsal progenitor cells to neural crest cell fate appears to be specific for dividing progenitor cells.

Our analysis of Foxd3 expression in mouse embryos indicated that Foxd3 is expressed in all Pax3-positive neural crest cells that emigrate from the neural tube, including cells that migrate along both the ventral and dorsolateral pathways. Thus, Foxd3 appears to be expressed in the precursors of all neural crest lineages in the mouse and may therefore define a population of neural crest ‘stem’ cells.

Although these findings are similar to those of Kos et al. (Kos et al., 2001) in the chick embryo, they differ in one respect. Kos et al. (Kos et al., 2001) observed Foxd3 in ventrally migrating neural crest but not in late migrating cells that enter

Fig. 7. Comparison of Foxd3 expression with Pax3, Slug, Cad-6B and RhoB. Cross sections of stage 10 chick embryos showing gene expression at three caudal levels. Sections were taken at the level of the most caudal somite, (A,D,G,J,M) the level of the segmental plate, (B,E,H,K,N) and at the mid point of the neural folds (C,F,L,O). (A-F) Foxd3 (A-C) and Pax3 (D-F) expression was visualized by whole-mount in situ hybridization. (G-O) Sections showing Slug (G-I), Cad-6B (J-L) and RhoB (M-O) expression at equivalent levels. The panels that are shown for Slug, Cad-6B and RhoB at each AP level represent directly adjacent sections from the same embryo. These sections are carefully matched to those shown for Pax3 and Foxd3.

Fig. 8. Schematic outlining the genetic regulation of neural crest cell differentiation. The genes that are not induced by Foxd3 in committed neural crest progenitor cells are italicized.
the dorsolateral pathway and give rise to melanocytes. We however, observed Foxd3 expression in cells underlying the epidermis, which are likely to be early melanocyte precursors (Fig. 1). This difference in Foxd3 expression may reflect differences in the timing of melanoblast specification in mouse and chick. In the chick, neural crest cells migrate along the dorsolateral pathway at later stages compared with those that migrate ventrally, and late-migrating crest cells are fate-restricted to develop as melanoblasts (Serbedzija et al., 1989; Henion and Weston, 1997; Reedy et al., 1998). In the mouse, however, neural crest cells enter the dorsolateral migration pathway during all stages of neural crest migration (Serbedzija et al., 1990), indicating that neural crest cells, and in particular melanoblasts, are not specified as they emerge from the neural tube.

The findings of Kos et al. (Kos et al., 2001) show that overexpression of Foxd3 prevents the migration of neural crest cells along the dorsolateral migratory pathway, suggesting Foxd3 suppresses melanogenesis. Although our studies did not directly address the function of Foxd3 in melanogenesis, in our experiments, Foxd3-EGFP+ cells were never observed migrating beneath the epidermis. Our studies are also consistent with Foxd3 expression being downregulated as melanocyte precursors begin to differentiate (Fig. 1), in a manner similar to that observed in other neural crest lineages. This suggests that Foxd3 may need to be repressed for neural crest cells to differentiation once they have been specified and have emigrated from the neural tube. In the chick, only specified melanoblasts enter the dorsolateral migratory pathway (Reedy et al., 1998), and these cells downregulate Foxd3 before they leave the neural tube (Kos et al., 2001). As a result, any Foxd3-mediated block in early melanoblast differentiation would most likely be manifested in fewer cells entering the dorsolateral migratory pathway. Interestingly, many of the cells that ectopically express Foxd3 migrate ventrally and acquire the characteristics of Schwann cell precursors, and this is the last neural crest lineage to undergo terminal differentiation (Zorick and Lemke, 1996).

**Foxd3 induces several properties of early migrating neural crest cells**

The cellular properties and migratory behavior of the ectopic Foxd3-expressing cells demonstrate that Foxd3 activates a number of aspects of the neural crest migration program. Foxd3 promotes expression of Cad-7 and HNK-1, both markers of migrating neural crest cells, as well as the delamination of cells at multiple dorsoventral levels from the neural tube (Fig. 3). The migration of these cells appears to coincide with the late phase of neural crest migration, and the Foxd3 cells predominantly populate sites in the periphery that are occupied by Schwann cell precursors. Moreover, some Foxd3 cells express the early Schwann cell marker P0 (Fig. 4) and the migratory routes taken by these Foxd3-expressing cells appears to reflect a bias by these late migrating neural crest cells for the Schwann cell lineage.

Interestingly, the delamination and migration induced by Foxd3 is independent of RhoB, as RhoB expression was not upregulated after misexpression of Foxd3 (Fig. 6). Previous explant studies have demonstrated a role for RhoB in crest emigration from the neural tube, and have shown that this activity is regulated by BMP4 signaling (Liu and Jessell, 1998; Sela-Donenfeld and Kalcheim, 1999). Blocking RhoB activity, however, did not affect the early specification of crest, nor did it affect the migration of crest cells that have already delaminated, suggesting that RhoB activity is not required for cell migration per se (Liu and Jessell, 1998). Our results suggest that Foxd3 activates a RhoB-independent pathway, which is sufficient to drive cells in the neural tube to delaminate and migrate. Nevertheless, in the absence of RhoB, this delamination step may be inefficient, thereby accounting for the delayed onset of Foxd3-positive cell migration from the neural tube.

In a recent study by Kos and colleagues (Kos et al., 2001), expression of a Foxd3-EGFP fusion protein resulted in the upregulation of HNK-1 expression, and increased numbers of HNK-1-positive cells were observed emigrating from the dorsal edge of the neural tube. However, at other dorsoventral levels, all Foxd3-positive/HNK-1-positive cells remained within the neural tube and did not display the same delamination and migratory phenotype that we observe. In a series of experiments using their expression vector, we observed the same phenotype that they reported (data not shown). Kos et al. (Kos et al., 2001) fused Foxd3 with EGFP protein, raising the possibility that the fusion of EGFP sequences to Foxd3 interferes with the normal function of the Foxd3 protein. By contrast, our studies used two Foxd3 expression vectors, including one that encodes Foxd3 alone. Both expression vectors induced delamination and migration of HNK-1-positive cells from all dorsoventral levels of the neural tube (Fig. 3). It is therefore likely that the delamination and migration of neural crest-like cells that we observe reflects the normal endogenous activity of Foxd3.

The mechanism by which Foxd3 induces the delamination and migration of neural crest cells is not clear. Interactions between cell adhesion molecules, such as the cadherin family of proteins may play a role in neural crest delamination. Interestingly, we observe upregulation of Cad-7 expression in ectopic Foxd3 cells, while Cad-6B expression is unchanged. Neural crest cells specifically express Cad-6B while resident in the neural tube, and as they delaminate Cad-6B expression is downregulated and Cad-7 expression is upregulated (Nakagawa and Takeichi, 1998). It has been suggested that this switch in the expression of Cad-6B and Cad-7, allows neural crest cells to dissociate and emigrate from the neural tube (Nakagawa and Takeichi, 1998). Thus, it is possible that Foxd3 promotes delamination by upregulating Cad-7 expression. However, explant studies have shown that BMP4 can upregulate both Cad-6B and Cad-7 expression in premigratory crest, and increased Cad-7 expression under these circumstances does not affect their emigration (Liu and Jessell, 1998). It is also possible that Foxd3 induces crest delamination and migration indirectly by regulating other genes, which are yet to be identified.

**Foxd3 may function independently of Slug during crest development**

We have found that the widespread induction of ‘neural crest-like’ cells by Foxd3 is not accompanied by an upregulation of Slug, nor does Slug induce Foxd3 (Fig. 6). Thus, both genes are activated independently in premigratory neural crest progenitors. This conclusion is consistent with the observation that Foxd3 and Slug have a similar temporal onset of expression in the dorsal neural tube (Fig. 7) and it seems likely that the expression of both genes in the dorsal neural tube is activated by similar dorsalizing signals. Slug expression in the neural tube...
is induced by BMP4/7 (Liem et al., 1995), while Foxd3 expression is dependent on Pax3, which is in turn upregulated by BMP signaling (Liem et al., 1995; Bang et al., 1997).

While studies in chick and Xenopus embryos have outlined a role for Slug in neural crest specification as well as crest migration (Nieto et al., 1994; LaBonne and Bronner-Fraser, 1998; LaBonne and Bronner-Fraser, 2000), our results show that Slug alone is not sufficient for neural crest induction. Recent studies show that neural crest cells can be specified and induced to migrate in the absence of Slug expression (Selleck et al., 1998; Sela-Donenfeld and Kalcheim, 1999). Furthermore, expression of Slug in the dorsal tube is downregulated in anterior regions of stages 22 chick embryos, even though neural crest cells continue to be produced in these regions at this time (Sela-Donenfeld and Kalcheim, 1999).

We find that Slug, when overexpressed, does not cause an expansion of the neural crest, nor does it synergize with Foxd3 to promote neural crest cell migration from the neural tube. Although our experiments did not identify a role for Slug in neural crest development in the chick, they do argue that Foxd3 and Slug function independently of each other in neural crest development.

In conclusion, the results presented argue that Foxd3 plays a Slug-independent role in segregating neural crest cell progenitors away from multipotent progenitors in the dorsal neural tube. We find that Foxd3 misexpression also leads to the delamination of cells from the neural tube in vivo and the upregulation of migratory neural crest markers. Although the expression of Foxd3 in migrating neural crest cells suggests Foxd3 may regulate the delamination and emigration of neural crest cells from the neural tube, this regulation may be indirect.

In summary, our results demonstrate that Foxd3 can induce delamination of cells from the neural tube in vivo and the neural tube. We find that Foxd3 misexpression also leads to the expression of a neural crest marker. It is possible that Foxd3 may regulate the delamination and emigration of neural crest cells from the neural tube, this regulation may be indirect. However, additional Foxd3-independent pathways are also likely to contribute to the early development of neural crest cells.

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