

## The winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate

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### SUMMARY

The neural crest is a migratory cell population that gives rise to multiple cell types in the vertebrate embryo. The intrinsic determinants that segregate neural crest cells from multipotential dorsal progenitors within the neural tube are poorly defined. In this study, we show that the winged helix transcription factor Foxd3 is expressed in both premigratory and migratory neural crest cells. Foxd3 is genetically downstream of Pax3 and is not expressed in regions of Pax3 mutant mice that lack neural crest, implying that Foxd3 may regulate aspects of the neural crest differentiation program. We show that misexpression of Foxd3 in the chick neural tube promotes a neural crest-

like phenotype and suppresses interneuron differentiation. Cells that ectopically express Foxd3 upregulate HNK1 and Cad7, delaminate and emigrate from the neural tube at multiple dorsoventral levels. Foxd3 does not induce Slug and RhoB, nor is its ability to promote a neural crest-like phenotype enhanced by co-expression of Slug. Together these results suggest Foxd3 can function independently of Slug and RhoB to promote the development of neural crest cells from neural tube progenitors.

Key words: Winged-helix genes, Foxd3, Neural crest specification, Neural tube development, Chick, Mouse

### INTRODUCTION

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 largely unknown. 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; LaBonne and Bronner-Fraser, 2000). However, the role of Slug in neural crest formation is not definitive, as neural crest migration can occur in the absence of Slug expression (Selleck et al., 1998; Sela-Donenfeld and Kalcheim, 1999). Furthermore, although Slug misexpression in *Xenopus* leads to expanded expression of neural crest markers, this expression is limited to the dorsal neural folds, thus arguing that additional dorsally restricted factors specify neural crest cell fate (LaBonne and Bronner-Fraser, 2000).

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;

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 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 *Splootch*<sup>-/-</sup> (*Pax3*<sup>-/-</sup> – Mouse Genome Informatics) embryos were obtained from breeding *Splootch* 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), cyroprotected 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-Brn-3.0 (E. Turner), monoclonal anti-Cad-6B and anti-Cad-7 (M. Takeichi), monoclonal anti-β-catenin (Zymed Lab), monoclonal anti-NAP73 (E/C8, Developmental Studies Hybridoma Bank, DSHB), polyclonal anti-*Foxd3* (see below), monoclonal anti-HNK-1 (Bronner-Fraser, 1986), monoclonal anti-Is11/2 (40.2.D6, DSHB), monoclonal anti-Lhx1/5 (4F2-10, DSHB), polyclonal anti-Lhx2/9 (K. Lee and T. Jessell), polyclonal anti-Mitf (M. Mochii), 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-Islet1 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-*Foxd3*MT (*Foxd3*<sup>Myc</sup>), pCS2-MT (Myc), or *Foxd3*<sup>IRES-EGFP</sup> 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 msec 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 (Clontech) 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 (*Slug*<sup>Myc</sup>) and expressed in the neural tube using the electroporation procedure described above. *Slug*<sup>Myc</sup> expression was detected with antibodies that recognize the Myc epitope. For co-transfection experiments of *Foxd3*<sup>Myc</sup> and *Slug*<sup>Myc</sup>, protein expression was detected using Myc polyclonal and *Slug* monoclonal antibodies, respectively.

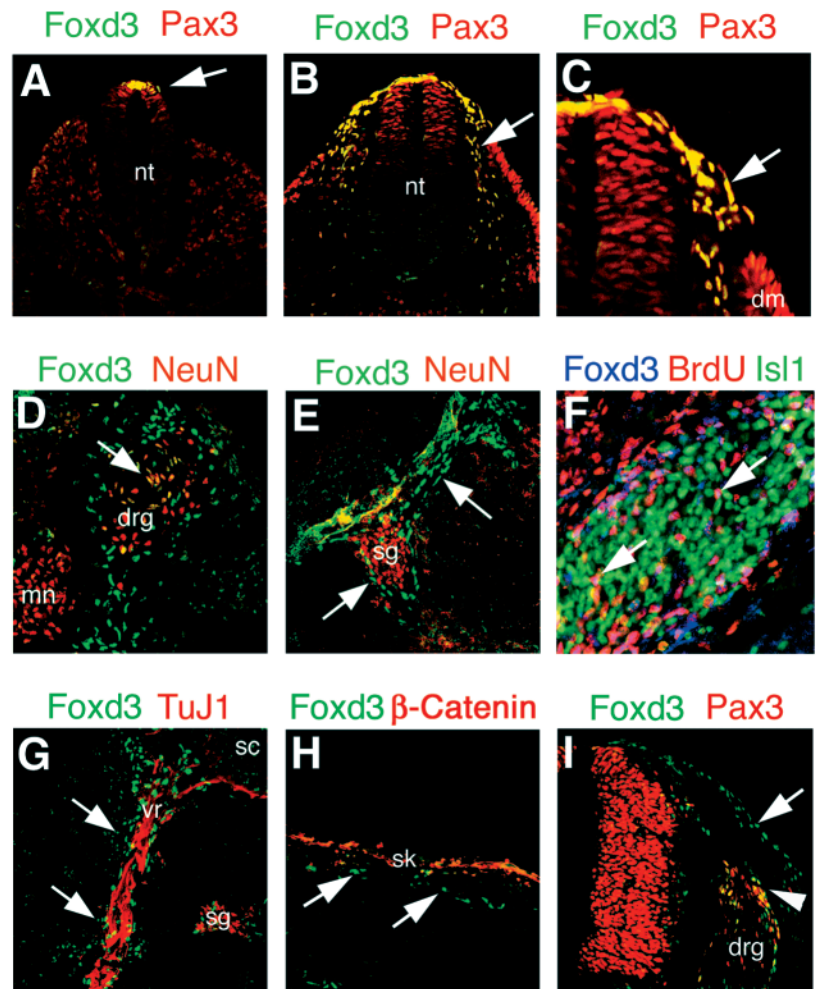
## RESULTS

### *Foxd3* is expressed in premigratory and migratory neural crest cells

To characterize *Foxd3* expression at the single cell level, an antiserum 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

**Fig. 1.** Foxd3 expression in neural crest cells.

(A–C) Immunohistochemical analysis of Foxd3 (green) and Pax3 (red) expression in E9.5 mouse embryos at thoracic (A) and cervical (B,C) levels. Foxd3 is expressed in presumptive premigratory and migrating neural crest cells and in dorsal midline cells. All the Foxd3 cells are also Pax3 positive (yellow). (D–G) Foxd3 expression in E10.5 mouse embryos. (D,E) Differentiating neurons in sensory (drg) and sympathetic ganglia (sg) were detected using an antibody to NeuN (red). (G) Peripheral axons were stained with an antibody to neuron specific-tubulin (TuJ1) (red). Foxd3 (green) is rapidly downregulated in differentiating sensory (D) and autonomic neurons (E) but is maintained in Schwann cell precursors that envelope the ganglia and axons. (F) Cross-sections of embryos pulsed with BrdU for 90 minutes, and triple stained with BrdU (red), Foxd3 (blue) and Islet1 (green). BrdU-positive/Foxd3-positive cells (pink) are found encompassing and within the DRG (arrows); however, none of these cells co-expressed Islet1 (green) a marker of postmitotic sensory neurons. (H,I) Foxd3 (green) is expressed in melanocyte precursors migrating beneath the ectoderm at E12.5 (arrows). (H) The ectodermal layer is stained with  $\beta$ -catenin (red). (I) Foxd3-positive/Pax3-positive cells (yellow) envelope the dorsal root ganglia (arrowhead). Foxd3-positive crest cells are also observed migrating along a dorsolateral pathway beneath the skin (arrow). dm, dermomyotome; drg, dorsal root ganglion; mn, motor neurons; nt, neural tube; sc, spinal cord; sg, sympathetic ganglion; sk, skin; vr, ventral root.



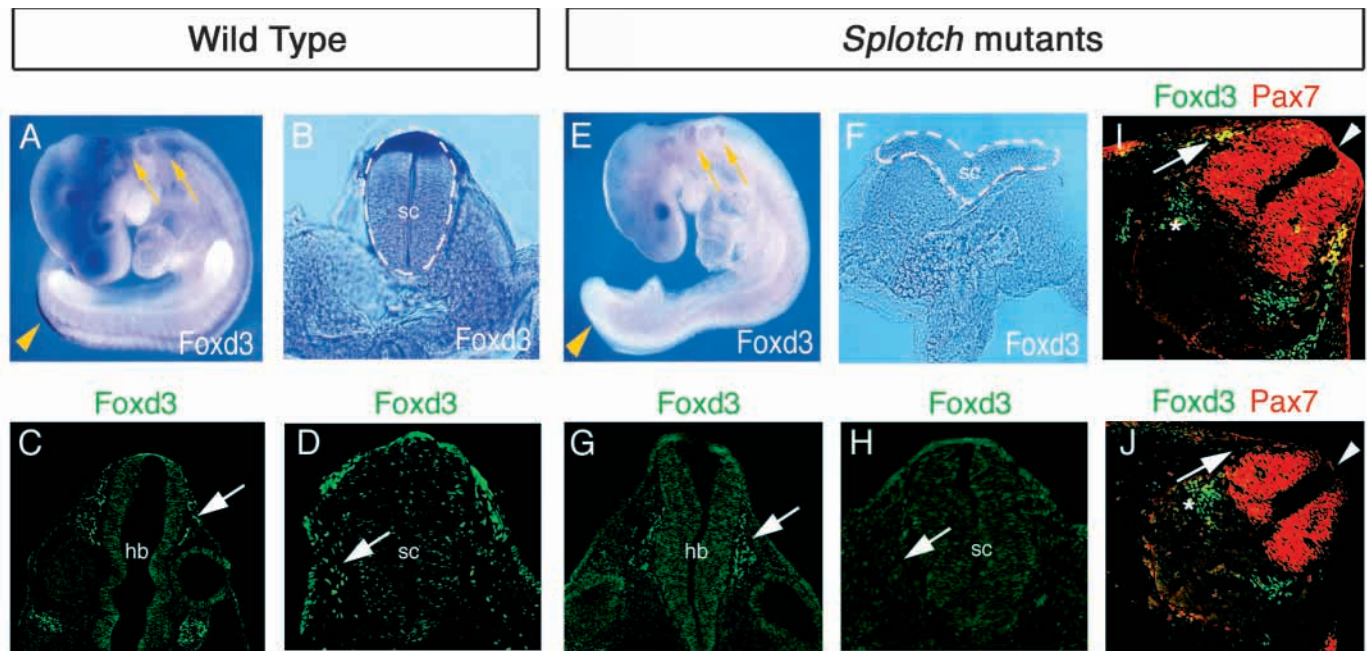
migration pathways that give rise to neurons/Schwann cells and melanocytes, respectively (Fig. 1I). Thus, in addition to marking premigratory neural crest cells, Foxd3 is expressed in all neural crest cells during their early phase of migration from the dorsal neural tube.

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 arrows). 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 (Fig. 1I arrowhead) which are likely to be Schwann cell precursors (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. In E10.5 embryos, the majority of the NeuN-positive cells present in these ganglia did not co-express Foxd3, indicating that Foxd3 is not expressed

in postmitotic sensory and sympathetic neurons (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



**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 (I,J, asterisk). hb, hindbrain; sc, spinal cord.

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 suggests 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 caudal dorsal neural tube/folds of *Splotch* embryos (Fig. 2B,F and Fig. 2A,E, arrowheads) argues that neural crest progenitors are not

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

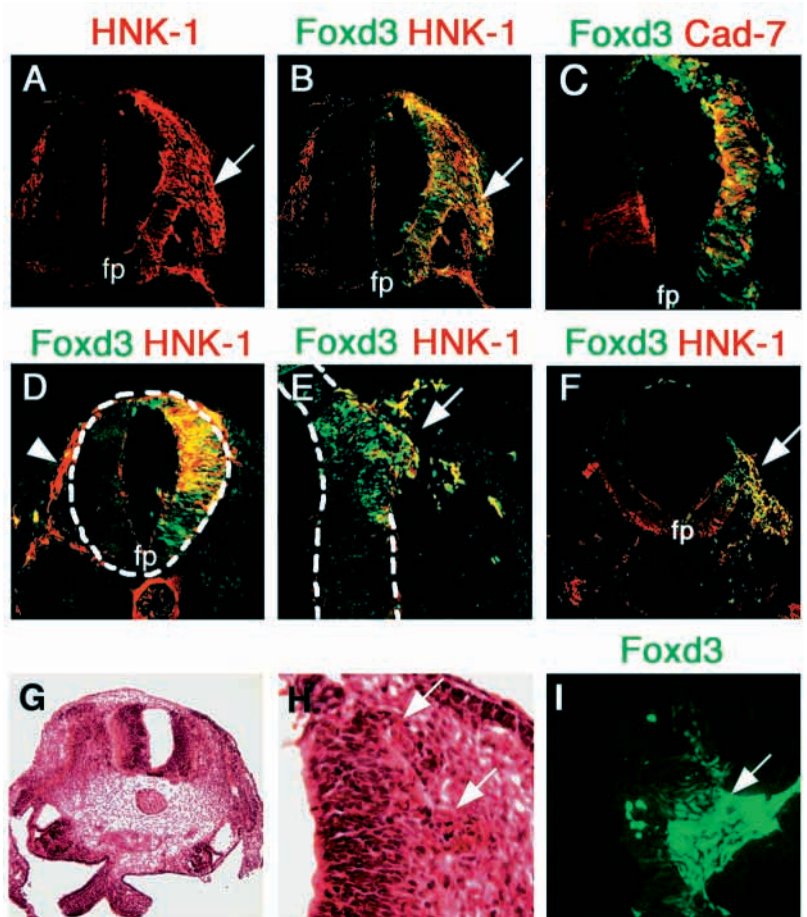
To examine whether Foxd3 plays a role in specifying neural crest, Foxd3 was ectopically expressed in the neural tubes of stage 10-11 chick embryos by electroporation. After 48 hours, extensive expression of the migratory neural crest markers, HNK-1 (Bronner-Fraser, 1986) and Cad-7 (Nakagawa and Takeichi, 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 (Foxd3<sup>Myc</sup>). This upregulation of HNK-1 and Cad-7 was observed as early as 24 hours after electroporation (Fig. 3D), suggesting that those cells expressing Foxd3<sup>Myc</sup> 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 Foxd3<sup>Myc</sup> electroporated neural tubes, the basement membrane surrounding the neural tube was disrupted and large numbers of Foxd3<sup>Myc</sup> 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 Foxd3<sup>Myc</sup> cells was observed in all Foxd3<sup>Myc</sup>-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 (Foxd3<sup>IRES-EGFP</sup>), also gave the same full phenotype in chick embryos as Foxd3<sup>Myc</sup> (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, Foxd3<sup>Myc</sup>-electroporated embryos were examined 96 hours after electroporation. By this time Foxd3<sup>Myc</sup> cells had undergone extensive migration and were often seen encircling dorsal root ganglia (Fig. 4A,E, arrows). Foxd3<sup>Myc</sup> cells were also seen encompassing TuJ1-positive sensory neurons and their axons in a manner characteristic of early Schwann cell precursors (Fig. 4D, arrow). Foxd3<sup>Myc</sup> cells were rarely, if ever, detected within the neuronal cores of sympathetic (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/Foxd3<sup>Myc</sup> or Phox2a-positive/

Foxd3<sup>Myc</sup> 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 Foxd3<sup>Myc</sup>-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 Foxd3<sup>Myc</sup> cells from peripheral neurons suggests that ectopic Foxd3 cells either persist as



**Fig. 3.** Neural crest induction by Foxd3. (A-I) Electroporation of Foxd3<sup>Myc</sup> (A-F) and Foxd3<sup>IRES-EGFP</sup> (G-I) in neural tubes of stage 10 chick embryos. (A-C) Forty-eight hours after Foxd3<sup>Myc</sup> electroporation (stage 22), widespread expression of Foxd3<sup>Myc</sup> leads to ectopic expression of HNK-1 in one half of the trunk neural tube (A,B). Normally, HNK-1 is expressed only on migrating neural crest cells (arrow). At this stage, cells ectopically expressing Foxd3, HNK-1 (B) and Cad-7 (C) are seen migrating away from the neural tube (arrow). (D) Ectopic expression of HNK-1 is observed in the brachial neural tube of stage 18 embryos as early 24 hours after electroporation of Foxd3<sup>Myc</sup>. (E,F) Delamination and migration of ectopic Foxd3<sup>Myc</sup> cells is also observed at hindbrain levels of the neural tube (arrows). (G-I) Bright field images (G,H) of neural tubes electroporated with Foxd3<sup>IRES-EGFP</sup> (I). (H,I) High magnification showing the delamination and emigration of Foxd3<sup>Myc</sup> cells from the intermediate neural tube (arrows). fp, floor plate.

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 *Foxd3*<sup>Myc+</sup> cells did not co-express *Mitf* (data not shown), demonstrating 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 *Foxd3*<sup>Myc+</sup> cells in the neural tube, where P0 is normally not expressed (Fig. 4C). This ectopic P0 expression was observed in all *Foxd3*<sup>Myc</sup>-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 *Foxd3*<sup>Myc+</sup> 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

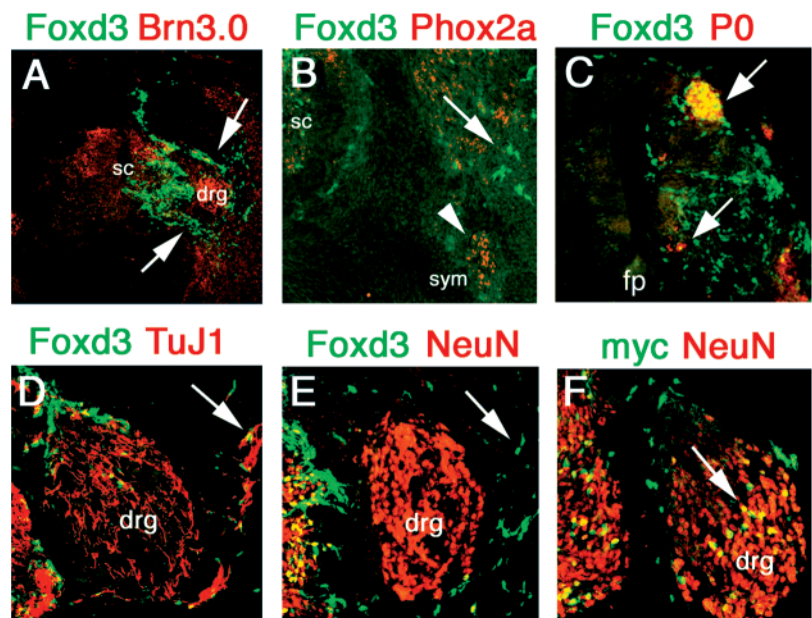
The observation that *Foxd3* is expressed in premigratory neural crest progenitors suggests that *Foxd3* may restrict dorsal progenitors to a neural crest cell fate. We therefore expected that cells ectopically expressing *Foxd3* would no longer differentiate as interneurons. To determine whether dorsal interneuron specification is suppressed by *Foxd3*, expression of the LIM-homeodomain proteins *Lhx2/9*, *Isl1/2* and *Lhx1/5* was examined in *Foxd3*<sup>Myc</sup>-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 *Foxd3*<sup>Myc</sup>-electroporated neural tubes (Fig. 5A–E). Cells expressing *Foxd3*<sup>Myc</sup> 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 *Foxd3*<sup>Myc</sup> (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 *Foxd3*<sup>Myc</sup>-electroporated neural tubes to test whether *Foxd3* suppresses the early developmental program of interneuron progenitors. In neural tubes electroporated with *Foxd3*<sup>Myc</sup>, 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 *Foxd3*<sup>Myc</sup>-electroporated embryos, progenitor cells that expressed *Foxd3*<sup>Myc</sup> 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 *Foxd3*<sup>Myc+</sup> 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),



**Fig. 4.** Effects of *Foxd3*<sup>Myc</sup> electroporation at stage 28. (A,B,D,E) *Foxd3*<sup>Myc</sup>-expressing cells have migrated extensively and can be seen encircling neurons in the dorsal root ganglia (A,D,E, arrows). *Foxd3*<sup>Myc+</sup> cells do not co-express *Brn3.0* (A) or *Phox2a* (B), which mark sensory and sympathetic ganglia, respectively. Ectopic *Foxd3*<sup>Myc+</sup> cells do not express markers of postmitotic neurons, *TuJ1* (D) and *NeuN* (E). (C) Ectopic expression of the Schwann cell marker, P0, in the neural tube induced by *Foxd3* misexpression. *Foxd3*<sup>Myc+</sup>/P0<sup>+</sup> cells are marked by arrows. (F) Electroporation with a control CS2-Myc expression vector. Note that Myc-tag expressing cells enter the dorsal root ganglia and differentiate as sensory neurons (arrow). drg, dorsal root ganglion; fp, floor plate; sc, spinal cord; sym, sympathetic ganglia.

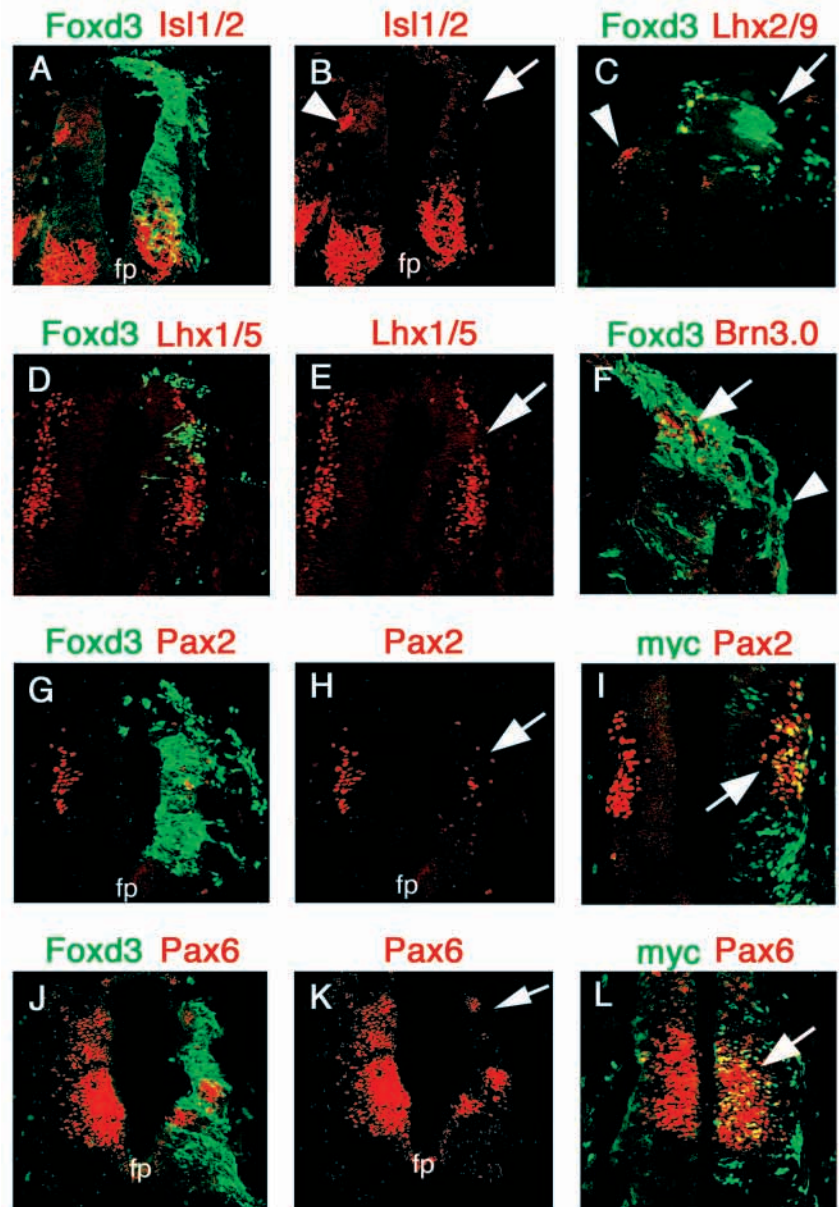
leading us to examine Slug, RhoB and Cad-6B expression in the neural tubes electroporated with Foxd3. Surprisingly, no ectopic expression of either Slug, RhoB or Cad-6B was observed in Foxd3<sup>Myc+</sup> cells, either 24 hours or 48 hours after electroporation (Fig. 6A-C; data not shown).

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).

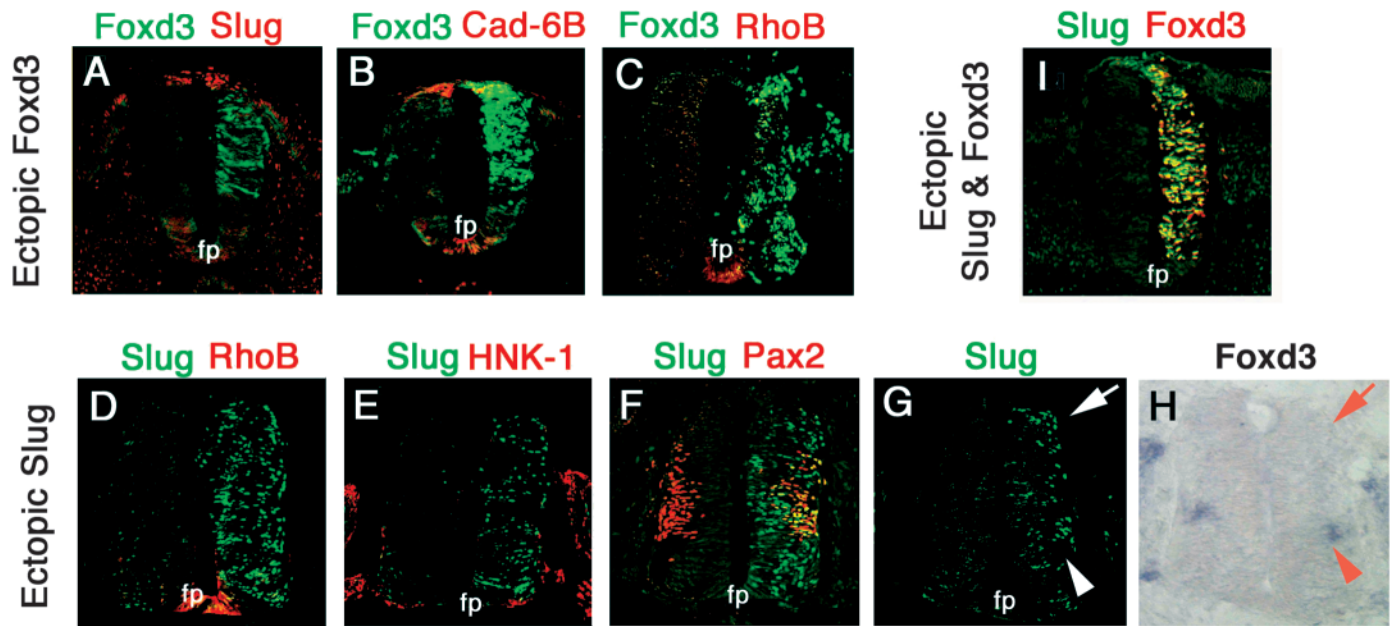
Previous studies have suggested that Slug is necessary for the delamination of neural crest cells from the dorsal neural tube (Nieto et al., 1994). While misexpression of Foxd3 leads to delamination of Foxd3-positive cells from the neural tube, emigrating cells are only observed 36 hours after introducing 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. 3), but is unable to induce RhoB, Slug and Cad-6B (Fig. 6A-C). 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



**Fig. 5.** Ectopic Foxd3 suppresses interneuron differentiation in the spinal cord. (A-L) Cross-sections of neural tubes 48 hours after electroporation of Foxd3<sup>Myc</sup> or Myc alone (I,L). 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.



**Fig. 6.** Comparison of Foxd3 and Slug misexpression in electroporated embryos. (A-C) Ectopic Foxd3 did not upregulate the premigratory crest markers, Slug (A), Cad-6B (B), and RhoB (C) at 24 hours (A,B) or 48 hours (C) after electroporation. (D-H) Ectopic Slug did not induce ectopic expression of RhoB (D) or HNK-1 (E), 48 hours after electroporation. Ectopic Slug-positive cells remained within the neural tube and the interneuron patterning remained normal as shown by Pax2 expression (F). (G) Alternate sections of immunohistochemical (G) and in situ (H) analyses showing ectopic Slug-positive cells (green) and Foxd3 mRNA, respectively. Ectopic expression of Slug did not upregulate 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.

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 (Fig. 2). The expression domains of Slug (Fig. 7G-I) and RhoB (Fig. 7M-O) along the anteroposterior axis of the neural tube were similar to that of Foxd3 (Fig. 7A-C), suggesting a similar temporal onset to their expression in premigratory neural crest. Cad-6B expression, like that of Pax3, was detected at low levels in the neural folds (Fig. 7L). The observation that Cad-6B expression precedes that of Foxd3, and that RhoB/Slug are expressed contemporaneously with Foxd3, is consistent with their expression being upregulated in the dorsal neural folds/tube in a Foxd3-independent manner.

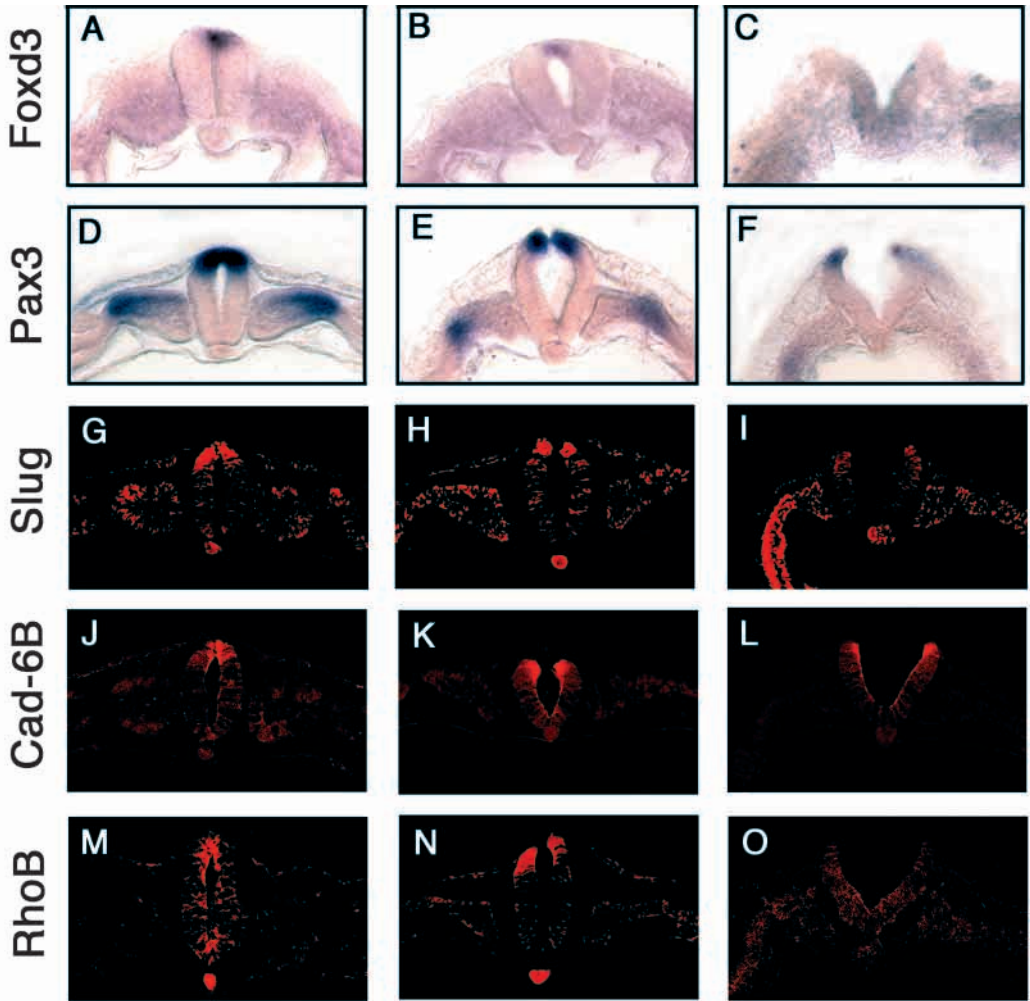
## DISCUSSION

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 progenitors by Foxd3

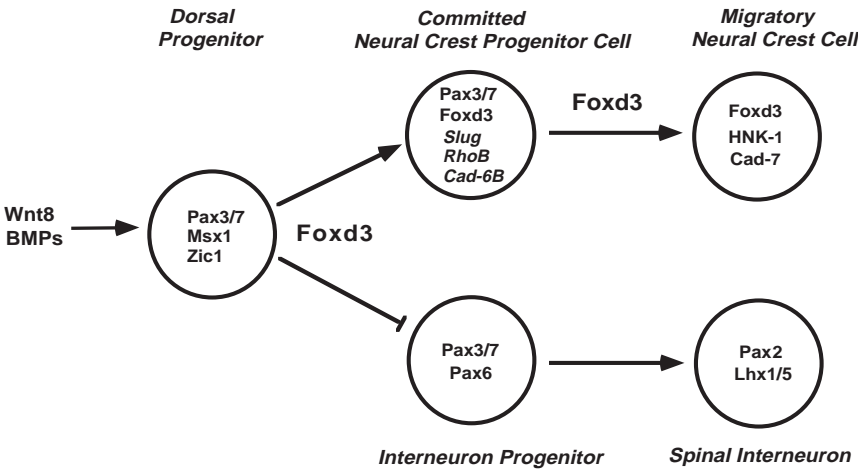
Previous studies have shown that signals emanating from the non-neural ectoderm and paraxial mesoderm, such as the BMPs, Wnts and FGFs, induce the expression of dorsal cell types within the dorsal neural tube (Liem et al., 1995; Liem et al., 1997; LaBonne and Bronner-Fraser, 1999). Our results suggest a model in which Foxd3 functions downstream of these signaling pathways to restrict dorsal progenitors to a neural crest fate, thereby preventing them from differentiating as interneurons. In the model we propose (Fig. 8), expression of Foxd3 is upregulated in prospective neural crest progenitors by crest-inducing signals that are active at the dorsal midline. This induction, which requires the activity of the dorsal patterning genes Pax3 and Pax7, may also depend on other dorsal genes such as the Msx and Zic genes. Although Foxd3 is likely to be upregulated by BMP-dependent signaling from the dorsal midline, our results suggest that this induction may be indirect, as expression of Pax3 in the spinal cord is known to depend on



**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,I,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.

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. 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<sup>Myc+</sup> 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 demonstrates 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 multipotential 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 many of the properties of early migrating neural crest cells, either directly or indirectly. However, additional Foxd3-independent pathways are also likely to contribute to the early development of neural crest cells.

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