Development 128, 1467-1479 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV1636

The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos

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Accepted 29 January; published on WWW 22 March 2001

SUMMARY

The winged-helix or forkhead class of transcription factors has been shown to play important roles in cell specification and lineage segregation. We have cloned the chicken homolog of FoxD3, a member of the winged-helix class of transcription factors, and analyzed its expression. Based on its expression in the dorsal neural tube and in all neural crest lineages except the late-emigrating melanoblasts, we predicted that FoxD3 might be important in the segregation of the neural crest lineage from the neural epithelium, and for repressing melanogenesis in early-migrating neural crest cells.

Misexpression of FoxD3 by electroporation in the lateral neural epithelium early in neural crest development produced an expansion of HNK1 immunoreactivity throughout the neural epithelium, although these cells did not undergo an epithelial/mesenchymal transformation. To test whether FoxD3 represses melanogenesis in early migrating neural crest cells, we knocked down expression in cultured neural crest with antisense oligonucleotides and in vivo by treatment with morpholino antisense oligonucleotides. Both experimental approaches resulted in an expansion of the melanoblast lineage, probably at the expense of neuronal and glial lineages. Conversely, persistent expression of FoxD3 in late-migrating neural crest cells using RCAS viruses resulted in the failure of melanoblasts to develop.

We suggest that FoxD3 plays two important roles in neural crest development. First, it is involved in the segregation of the neural crest lineage from the neuroepithelium. Second, it represses melanogenesis, thereby allowing other neural crest derivatives to differentiate during the early stages of neural crest patterning.

Key words: Neural crest, Winged helix transcription factor, FoxD3, Chick, Morpholino antisense oligonucleotides

INTRODUCTION

The neural crest is a population of mesenchymal cells that arises from the neural epithelium, migrates extensively, and differentiates into a wide range of cell types. How the neural crest lineage is segregated from the rest of the neural epithelium, however, remains uncertain. Although an inductive interaction between the epidermal ectoderm and neural folds, probably mediated by members of the bone morphogenetic protein (BMP) family of signaling molecules, is required in order to establish the neural crest lineage (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1996; Baker and Bronner-Fraser, 1997), the molecular players that distinguish a neural crest cell from a neural epithelial cell are unknown. Similarly, although experimental studies have revealed the timing and order in which many neural crest subpopulations are specified (reviewed by Stemple and Anderson, 1993; Wehrle-Haller and Weston, 1997; Le Douarin and Kalcheim, 1999), the molecular basis of cellular identity and the step-wise progression of specification are not understood.

Transcription factors regulate the differentiation of many different cell types during embryogenesis. One family of transcription factors that has been shown to play important roles in cell specification and lineage segregation (Kaufmann and Knochel, 1996) is the winged-helix or forkhead class of transcription factors (recently renamed the Fox proteins for Forkhead box; Kaestner et al., 2000), so named because the DNA-binding domain folds into two distinctive loops. To identify new winged-helix transcription factors expressed in early development, we have screened a chicken library with a probe derived from the mouse Foxa2 sequence (previously called HNF3β). Our investigations of the clone we identified as the chick homolog of FoxD3 (Hromas et al., 1999; Kelsh et al., 2000) suggest that FoxD3 plays two important roles in neural crest development. First, it is involved in the segregation of the neural crest lineage from the neuroepithelium, although its expression is not sufficient for these cells to undergo an epithelial/mesenchymal transformation (EMT). Second, it

represses melanogenesis, thereby allowing other neural crest derivatives to differentiate during the early stages of neural crest patterning.

MATERIALS AND METHODS

Cloning and sequencing of *FoxD3*

A stage-13 chicken cDNA library was screened under conditions of medium stringency with a probe derived from mouse HNF3β. A 1.7 kb cDNA clone was identified and sequenced using the ABI sequencing system at Davis Sequencing (Davis, CA). Because the clone was extremely GC rich, DMSO was added to a final concentration of 10% to the sequencing reaction to reduce the amount of secondary structure in the template. Without DMSO, each reaction generated less than 100bp of readable sequence. With DMSO, each reaction generated 400-600 bp of clean sequence. Both strands of the cDNA clone were sequenced in their entirety. FoxD3 sequences were aligned and formatted using the CLUTALW program at the Biology Workbench website (http://workbench.sdsc.edu/).

Whole-mount in situ hybridization

Probes were made by transcribing *Xho*I-linearized cDNA with T3 RNA polymerase. In situ hybridization was carried out essentially as described previously (Nieto et al., 1996), with slight modifications, as follows. White leghorn chicken embryos (California Golden Eggs, Sacramento) from stages 6-26 (Hamburger and Hamilton, 1951) were collected in cold phosphate-buffered saline (PBS), and extra-embryonic tissues were removed. Embryos were fixed in 4% paraformaldehyde in PBS and dehydrated in a graded series of PBT-MeOH ending with 100% MeOH. Embryos were rehydrated and bleached with 6% hydrogen peroxide and treated with 10 μg/ml proteinase K for 2-15 minutes, depending on the embryonic stages. Hybridization was carried out at 67°C in hybridization buffer for 36 hours. Alkaline phosphatase development was carried out in the presence of 10% PVA (13,000-23,000 MW; Sigma) in CT buffer (100 mM TrisHCl, pH 9.5, 150 mM NaCl, 25 mM $MgCl_2$, 0.5% Tween 20 and 2 mM levamisol) for 3-6 hours at room temperature.

We also confirmed the whole-mount in situ patterns by doing the hybridization directly on frozen sections as described previously (Birren et al., 1992), except we used Fast Red development tablets (Boehringer Mannheim) according to the manufacturer's directions.

Quail neural crest cultures

Fertile Japanese quail eggs (*Coturnix coturnix japonica*) from the Avian Sciences Department (University of California, Davis) were incubated in a humidified 37°C incubator until they reached stage 13-15. Neural tubes at somite level I to X (between the last-formed somite to the tenth from the last-formed somite) were dissected from embryos and separated from surrounding tissues, including ectoderm, somite, and notochord, after a brief digestion in Pancreatin (Gibco). Cleaned neural tubes were transferred to 24-well culture dishes (Nunc) and maintained in Ham's F-12 (Gibco) medium supplemented with 10% fetal bovine serum (FBS; Gibco), 3% 10-day chick embryo extract, and 100 units/ml penicillin/streptomycin (Gibco). In some experiments when we performed immunocytochemistry, the neural tubes were cultured on a small circular coverslip (Fisher) placed in the bottom of each well. Cultures enriched for neuroblasts and glioblasts but deficient in melanoblasts were obtained by removing the neural tube 15 hours after explantation (Henion and Weston, 1997; Reedy et al., 1998a).

Antisense-oligonucleotide treatment of neural crest cultures

Antisense oligonucleotides directed against the transcriptional start site of *FoxD3* (CGGAGCGGGGATGACT) were manufactured by Oligos Etc. (Watsonville, OR). The control sense oligonucleotide was AGTCATCCCCGCTCCG. In our initial experiments, oligonucleotides were added to the cultures in concentrations ranging from 5 to 25 μM. As there appeared to be no difference in cellular response at 5 and 10 μM, we used these concentrations for the experiments described here. After culturing for 15 hours in standard F-12 medium containing 5 or 10 μM oligonucleotides, the neural tubes were carefully scraped from the substratum and the medium replaced with F-12 containing fresh oligonucleotides. For each treatment there were at least three wells containing 2-3 neural tubes each. After an additional 24 hours, the oligonucleotides were removed and the medium replaced with fresh F-12 medium. The cultures were fed every day until day 6, when the cultures were photographed, the cells removed from the substratum with Pancreatin, and counted in a hemacytometer. The data from three experiments are summarized in Table 1. The significance of differences between results was determined using the Tukey method (Pruginin-Bluger et al., 1997) for multiple comparisons with an α of 0.05. Comparisons significant at the 0.05 level are indicated by ***. We used a different preparation of oligonucleotides for each experiment reported in this table.

In two additional experiments, we cultured the neural crest cells on coverslips and then processed them on day 6 for immunocytochemistry. Neural crest cultures were grown on coverslips specially treated for culture (Fisher) and fixed with 4% paraformaldehyde. All antibodies were diluted in a buffer containing 0.5 M NaCl, 0.01 M phosphate buffer pH 7.5, 0.1% sodium azide and 0.2% Triton X-100. Neuronal cells were detected using the neuron-specific monoclonal antibody, 16A11 (Marusich et al., 1994; Molecular Probes). Glial cells were identified using the 7B3 monoclonal antibody (Henion et al., 2000; kind gift from Dr J. A. Weston, University of Oregon). After blocking with 1% BSA, neural crest cells were incubated with either 20 μg/ml 16A11 or 40 μg/ml 7B3 overnight at 4°C in combination with 10 μg/ml Hoechst dye (no. 33258; Sigma) to reveal nuclei. All antibodies were detected with Cy2- or Cy3-conjugated secondary antibodies (Jackson Laboratory; 1:500 dilution). Immunolabeled cells were viewed with a Leitz Dialux 20 fluorescence microscope, and images were captured using an Optronics Magnafire camera.

In ovo electroporation of *FoxD3*-expressing plasmids and morpholino antisense oligonucleotides

An enhanced green fluorescent protein (EGFP)-tagged FoxD3 cDNA construct was prepared by removing the 3′ UTR from our FoxD3 cDNA clone and ligating that 3′ to EGFP in the pEGFP-C2 vector (Clonetech). The orientation and sequence integrity were verified by restriction digest and sequencing.

Stage 12-14 embryos were windowed on their side. Following a subblastodermal injection of India ink, the vitelline membrane was torn and *FoxD3*-EGFP DNA (1 μg/ml diluted in 0.1% Fast Green to visualize the solution) was mouth pipetted into the lumen of the neural tube. The DNA was electroporated into the neural epithelium using Gentronics electrodes (model 512) and a square wave generator (BTX-T820; Gentronics) using 5, 100 msecond, 12 V square pulses. Embryos were allowed to develop for an additional 24 or 48 hours and fixed with ice cold 4% paraformaldehyde.

After fixation, embryos were embedded in Paraplast Plus, sectioned and processed for immunocytochemistry, as described above for cell cultures. HNK1 antibody (prepared from the cell line obtained from ATCC) was used as a marker for neural crest cells. Culture supernatant was diluted 1:100 in PBS containing 1% BSA. Secondary antibodies conjugated with either RITC, Cy2 or Cy3 (Jackson Laboratory; 1:5,000) were used to detect the primary antibody. Neurons and glial cells were identified using 16A11 and 7B3 monoclonal antibodies, as described for cell cultures. Slides were coverslipped using GelMount (Biomedia Corp.) and viewed with a Leitz Dialux 20 microscope.

Fluorescently tagged morpholino antisense oligonucleotides

against *FoxD3* (Gene Tools, LLC; Corvallis, Oregon) were injected into stage 13 embryo neural tubes (500 μM) and then electroporated as describe above. Control embryos were injected with the fluorescent standard control provided by Gene Tools. Embryos were allowed to develop for an additional 24 hours and labeled with HNK1.

Production and injection of *FoxD3*-RCAS

The coding region of the FoxD3 cDNA was cloned into pSLAX-21, excised with *Cla*I, and then ligated into the *Cla*I site of RCASBP(B) (a kind gift from C. Cepko, Harvard Medical School, MA). Both orientations were obtained, as verified by sequencing, and purified by cesium chloride ultracentrifugation. The reverse orientation was used to generate control virus that did not express *FoxD3*. Transfection, harvesting, concentration and titering of the viruses were performed essentially as described elsewhere (Morgan and Fekete, 1996). Briefly, the DNA was transfected by calcium chloride into primary chick embryo fibroblasts, cells were passaged three times, and crude supernatant was harvested after 1 week. The virus-containing supernatant was filtered, concentrated by ultracentrifugation, titered and resuspended at a final concentration of approximately 1-5×10^8/ml.

White leghorn chicken eggs (C/E strain) from Charles River SPAFAS, Inc. (North Franklin, CT) were incubated at 38°C until they reached stages 12-13. Eggs were windowed and a drop of sterile Fast Green dye (1 mg/ml; Sigma) was placed over the embryo to add contrast. Approximately 0.5 ml of virus (with 0.1 mg/ml Fast Green added) was injected into the lumen of the neural tube just posterior to the level of the future wing bud. The injected fluid filled the lumen of the neural tube from the injection site to the head, and embryos in which we observed excessive leakage from the injection site were discarded. Eggs were resealed with tape and placed back in the incubator for 48 hours, or until the embryos reached stages 22-23. Injected embryos were healthy and developed normally to stage 22-23, at which time they were fixed and processed for immunocytochemistry.

Embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature (or overnight at 4°C in 0.4% paraformaldehyde), washed in PBS, dehydrated through PBS/ethanol, and embedded in Paraplast Plus (Fisher). Transverse sections (7 μm) were cut and dried onto Superfrost Plus slides (Fisher). Before staining, the slides were dipped in HistoClear2 (American Diagnostics) to remove the paraffin and rehydrated through a graded alcohol series to PBS. 1% BSA/10% heat-inactivated goat serum in PBS was used to block non-specific antibody binding. Because the RCAS viruses are replication competent, secondary infection of non-neural crest/neuroepithelium was unavoidable. Thus, we used HNK1 (1:10; ATCC) to detect neural crest cells, and the polyclonal antibody p27 (1:1000; SPAFAS) to detect virus-infected cells. The two primary antibodies were added overnight at 4°C. After washing in PBS, the secondary antibodies were added at a final concentration of 1:600 (goat-anti-mouse IgM-Cy3; Chemicon; goat-anti-rabbit Alexa 488; Molecular Probes) for 2 hours at room temperature. Slides were washed in PBS, mounted with GelMount (EM Sciences), and viewed under epifluoresence. The analysis was done blind, and embryos with poor p27 staining (indicative of weak RCAS infection) were discarded. 40-50 serial sections per embryo from three high-expressing embryos were analyzed for each virus.

RESULTS

Cloning and sequencing of *FoxD3*

A stage-13 chicken embryo cDNA library was screened under conditions of medium stringency with a probe derived from mouse HNF3β (Foxa2 – Mouse Genome Informatics; Kaestner et al., 2000). A 1.7kb cDNA clone, which we initially named HNF17, was identified and sequenced in its entirety. Conceptual translation predicts that the HNF17 clone encodes a 396-amino acid winged-helix transcription factor (Fig. 1A). BLAST searching revealed that the amino acid and nucleotide sequences of HNF17 are nearly identical to those of a previously cloned chicken forkhead-like cDNA called CWH-3 (Freyaldenhoven et al., 1997; Fig. 1A). The largest area of difference between CWH-3 and HNF17 is an 18-amino acid region near the N terminus. This area corresponds to a frameshift between HNF17 and CWH-3 caused by three nucleotides in the HNF17 sequence not reported in the CWH-3 sequence (arrows, Fig. 1B).

Other than CWH-3, the most closely related genes to HNF17 in the databases are zebrafish *fkh6* (*foxd3* – Zebrafish Information Network; Odenthal and Nusslein-Volhard, 1998) and mammalian Hfh2 or *Genesis* (Foxd3 – Mouse Genome Informatics; Hromas et al., 1993; Fig. 1C). Given the general acceptance of homology between zebrafish *foxd3* and mouse *Foxd3*, we will hereafter refer to the chicken gene described here as *FoxD3*, according to the newly agreed upon nomenclature for this gene family (Kaestner et al., 2000).

FoxD3 is expressed in premigratory and migratory neural crest cells

FoxD3 expression is almost exclusively correlated with premigratory and migratory neural crest cells. Exceptions include the floorplate and possibly the endocardial cushion cells of the heart. A brief description of *CWH-3* expression was reported previously (Yamagata and Noda, 1998), but our data differ in several important respects, which we detail below.

FoxD3 expression is first detected in the presumptive neural folds at stage 6 (Fig. 2A), but by stage 8, expression has faded from most of the forebrain (Fig. 2D). The anterior prosencephalon is a region of the brain from which neural crest cells do not arise. *FoxD3* is subsequently expressed in an anterior-to-posterior wave along the presumptive neural folds beginning at stage 7 (Fig. 2B), and by stage 8, a few *FoxD3*-expressing cells are observed to be migrating away from the neural folds. At stage 9, expression in the neural folds has spread posteriorly and *FoxD3*-positive cells at the level of the midbrain have begun to migrate away from the neural tube, suggesting that they are neural crest cells.

The HNK1 antibody is routinely used as a marker for the neural crest, but generally this epitope is not expressed until after migration has begun. By stage 10, neural crest cells from the midbrain have spread laterally and they are HNK1 positive (Fig. 2I) coincident with the *FoxD3* expression pattern (Fig. 2G,H). Sections that have been processed for *FoxD3* in situ hybridization and then double-labeled with the HNK1 antibody show that *FoxD3*-positive cells are also HNK1 positive (Fig. 3F), confirming their identity as neural crest cells.

Midbrain and rhombomeres 1 and 2 (r1,r2)

Neural crest cells initiate migration from this region beginning at stage 8 and stop emigrating from the neural tube by stage 11 (Anderson and Meier, 1981; Tosney, 1982; Lumsden et al., 1991). *FoxD3* expression is first seen in the dorsal neural tube in this region at stage 7, and is observed at stage 8 to be in a few scattered cells leaving the neural folds (Fig. 2D). Cessation of *FoxD3* expression in the neural tube at this axial level occurs

A

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cFoxD3 MTLSGGGSDMSGQTALAAEDVDIDVVGEGDDAPGKDGDGEARSPAALPRL
cwh-3  MTLSGGGSDMSGQTALAAEDVDIDVVGEGDDAPGKDGDGEARSPAALP-L

cFoxD3 PLDEAAEPGEPGAGGESGSSGSGSPAPAGPEGGRGGGGGGGGGEEGASGG
cwh-3  PLDEAAEPGEP-ERAARRAAAARQPGPGRPEGGRGGGGGGGGGEEGASGG

cFoxD3 GAAAAAAAGQSKPKSSLVKPPYSYIALITMAILQSPQKKLTLSGICEFIS
cwh-3  GAAAAAAAGQSKPKSSLVKPPYSYIALITMAILQSPQKKLTLSGICEFIS

cFoxD3 NRFPYYREKFPAWQNSIRHNLSLNDCFVKIPREPGNPGKGNYWTLDPQSE
cwh-3  NRFPYYREKFPAWQNSIRHNLSLNDCFVKIPREPGNPGKGNYWTLDPQSE

cFoxD3 DMFDNGSFLRRRKRFKRHQQEHLRDQTALMMQGFGAYGLAGPYGRPYGLP
cwh-3  DMFDNGSFLRRRKRFKRHQQEHLRDQTALMMQGFGAYGLAGPYGRPYGLP

cFoxD3 PGAYPHPAALQYPYIPPVGPMLPPAVPLLPSGELSRKAFNAQLGPSLQLQ
cwh-3  PGAYPHPAALQYPYIPPVGPMLPPACPLLPSGELSRKAFNAQLGPSLQLQ

cFoxD3 LSSLGAAGSIVKSEPSSRPSFSIENIIGGPAASSAPSAQTFLRPPVTVQS
cwh-3  LSSLGAAGSIVKSEPSSRPSFSIENIIGGPAASSAPSAQTFLRPPVTVQS

cFoxD3 GLVAHQPLALARTTAAIAPILSVPTNIIAGQFLQPPAAVQAKWPAQ
cwh-3  GLVAHQPLALARTTAAIAPILSVPTNIIAGQFLQPPAAVQAKWPAQ
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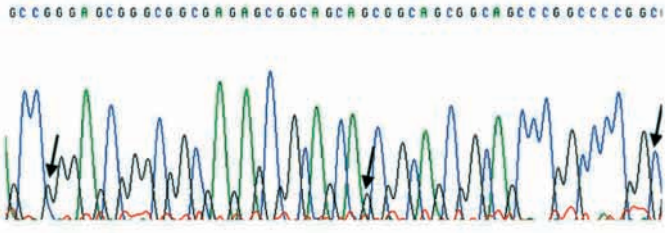
B



C

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mFoxD3 MTLSGSGSASDMSGQTVLTAEDVDIDVVGEGDDGLEEKDSDAGCDSPAGP
hFoxD3 MTLSGGGSASDMSGQTVLTAEDVDIDVVGEGDDGLEEKDSDAGCDSPAGP
zfkd-6 MTLSGGTSASNMSGQTVLTADDVDIDVVGEGD---EGMEQDSDCESQCMQ
cFoxD3 MTLSGGG--SDMSGQTALAAEDVDIDVVGEGDD-APGKDGDGEARSPAAL

mFoxD3 PDLRLDEADEGPPVSAHHGQSQP---QALALPTEAT----GPGNDTGAPE
hFoxD3 PELRLDEADEVPPAAPHHGQPQPPHQQPLTLPKEAAGAGAGPGGDVGAPE
zfkd-6 DRGDEVEEIEVKE-------------RSTS-PCESN----ADGETKG---
cFoxD3 PRLPLDEAAEPGEPGAGGESGSS---GSGS-PAPAG----PEGGRGGG--

mFoxD3 ADGCKG---GEDAVTGGGGPGAGSGATGGLTPNKPKNSLVKPPYSYIALI
hFoxD3 ADGCKGGVGGEEGGASGGGPG-GSGSAGGLAPSKPKNSLVKPPYSYIALI
zfkd-6 -----D---AQESSTG---P----------MQNKPKSSLVKPPYSYIALI
cFoxD3 -GGGGG---GEE-GASGGG----AAAAAAAGQSKPKSSLVKPPYSYIALI

mFoxD3 TMAILQSPQKKLTLSGICEFISNRFPYYREKFPAWQNSIRHNLSLNDCFV
hFoxD3 TMAILQSPQKKLTLSGICEFISNRFPYYREKFPAWQNSIRHNLSLNDCFV
zfkd-6 TMAILQSPQKKLTLSGICEFISNRFPYYREKFPAWQNSIRHNLSLNDCFV
cFoxD3 TMAILQSPQKKLTLSGICEFISNRFPYYREKFPAWQNSIRHNLSLNDCFV

mFoxD3 KIPREPGNPGKGNYWTLDPQSEDMFDNGSFLRRRKRFKRHQQEHLREQTA
hFoxD3 KIPREPGNPGKGNYWTLDPQSEDMFDNGSFLRRRKRFKRHQQEHLREQTA
zfkd-6 KIPREPGNPGKGNYWTLDPQSEDMFDNGSFLRRRKRFKRHQPDILRDQTA
cFoxD3 KIPREPGNPGKGNYWTLDPQSEDMFDNGSFLRRRKRFKRHQQEHLRDQTA

mFoxD3 LMMQSFGAYSLAAAAGAG-----PYGLHPAAAAGAYSHPAAAAAAAGCCG
hFoxD3 LMMQSFGAYSLAAAAGAAGPYGRPYGLHPAAAAGAYSHPAAAAAAAAAAA
zfkd-6 LMMQSFGAYGIGNPYGRH------YGIHP----AAYTHPAALQYP-----
cFoxD3 LMMQGFGAYGLAGPYGRP------YGLPP----GAYPHPAALQYP-----

mFoxD3 APVPVRATTRGSRAAARSAPAALGRAGPQSGRLRLAARSEPAATAQHPGR
hFoxD3 LQYPYALPPVAPVLPPAVPLLPSGELGRKAAAFGSQLGPGLQLQLNSLGA
zfkd-6 YIPPV-----GPMLPPAVPLLPSAELNRKA----FSSQLSPSLQLQLN--
cFoxD3 YIPPV-----GPMLPPAVPLLPSGELSRKA----FNAQLGPSLQLQLS--

mFoxD3 RRGSRGHGGRGGHHVAHQVRAQCAAVVQHREHHRRG-IRAPGGSAGGGGS
hFoxD3 AAAAAGTAGAAGTTASLIKSEPSARPSFSIENIIGGGPAAPGGSAVGAG-
zfkd-6 ---SLSTA-------SIIKSEPSSRPSFSIENIIG---------------
cFoxD3 ---SLGAAG------SIVKSEPSSRPSFSIENIIGG--------------

mFoxD3 GGGAGGGGGSGGGGGAQSFLRPPGTVQSAALMATHQPLSLSRTTATIAPI
hFoxD3 --VAGGTGGSGGGSTAQSFLRPPGTVQSAALMATHQPLSLSRTTATIAPI
zfkd-6 --------VSSSLRAIQTFLRPPVTVQSA-L-LSAQSLSLTRTSAAIAPI
cFoxD3 -------PAASSAPSAQTFLRPPVTVQSG-L-VAHQPLALARTTAAIAPI

mFoxD3 LSVP---LSGQFLQPAASAAAAAAAAVQAKWPAQ
hFoxD3 LSVP---LSGQFLQPAASAAAAAAAAAQAKWPAQ
zfkd-6 LSVPSNIISGQFLPTAS------TAAVS-KWPSQ
cFoxD3 LSVPTNIIAGQFLQPP--------AAVQAKWPAQ
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Fig 1. Comparison of cFoxD3 with a similar chicken sequence (CWH-3) and with FoxD3 homologs in mouse, human and zebrafish. (A) Alignment of the FoxD3 and CWH-3 predicted amino acid sequences. Identical residues are black, similar residues are blue and completely different residues are red. The region of highest discrepancy between the two sequences is boxed. (B) An electropherogram showing the part of the FoxD3 cDNA sequence corresponding to the boxed region in (A). Arrows indicate three nucleotides in the FoxD3 cDNA that were not reported in CWH-3 and cause a short frameshift. (C) Alignment of the predicted FoxD3 amino acid sequences from chicken FoxD3 (cFoxD3), zebrafish foxd3 (zfkd-6), mouse Foxd3 (mFoxd3; formerly Hfh2) and human FOXD3 (hFoxD3). Completely conserved residues are black, identical residues are gray, similar residues are blue and completely different residues are red.

at stage 11+, about the time that neural crest cells stop migrating.

Once migration is underway, this population of the neural crest (also known as the trigeminal crest because of their contribution to the trigeminal ganglion) spreads dorsal to the eye, and into the maxillary and mandibular process (branchial arch 1) (see Lumsden et al., 1991). These neural crest cells give rise to elements of the eye, including the corneal endothelium and stromal fibroblasts, the scleral cartilages and connective components of the eye muscles, and the connective tissue of the face, jaws and tongue (Noden, 1978; Kontges and Lumsden, 1996; Baker et al., 1997). More proximally these crest give rise to neurons and glial cells of the trigeminal ganglion and the glial cells along the trigeminal nerve (cranial nerve V; D'Amico-Martel and Noden, 1983). This pattern of the mesencephalic crest migration is revealed by the HNK1 antibody (Fig. 2I,L,O,R). The pattern of *FoxD3* expression is virtually identical to the HNK1 patterns at this axial level between stages 10-14 (Fig. 2K,N), suggesting that most or all of the derivatives of the neural crest at this axial level express *FoxD3*, at least when they initiate their migration. Expression of both *FoxD3* and HNK1 in this subpopulation of the crest begins to diminish by stage 14 (Fig. 2N,T), especially in branchial arch 1, and to a lesser extent in the facial mesenchyme (e.g. periocular crest, Fig. 2P,Q). Expression of *FoxD3* remains high in the trigeminal ganglion, even after stage 22 (Fig. 2U), in both the neurons and glial cells (Fig. 3I,J).

Preotic crest (r3 and r4)

Several studies (Anderson and Meier, 1981; Graham et al., 1993; Birgbauer et al., 1995) show that a significantly reduced number of neural crest cells originate from r3. Neural crest emigration from r4 begins at stage 8+ and ceases by stage 11+ (Lumsden et al., 1991). Few, if any, cells in the dorsal neural tube at r3 are *FoxD3* positive (Fig. 2E,H,K), whereas the dorsal neural tube at r4 is heavily labeled, consistent with the notion that *FoxD3* is a marker for premigratory neural crest cells. Expression of *FoxD3* in r4 is extinguished by stage13.

Once migration is under way, this population of neural crest (also known as the hyoid crest) migrates dorsolaterally between the ectoderm and paraxial mesoderm into the hyoid arch (branchial arch 2; Fig. 2L,O). These crest give rise to the connective tissue elements of the hyoid arch (e.g. Kontges and Lumsden, 1996). More proximally they differentiate into the glial cells, a few neurons of the vestibular-acoustic ganglion (root ganglia of cranial nerve VII-VIII) and the glial cells of the geniculate ganglion (distal ganglion of cranial nerve VII; D'Amico-Martel and Noden, 1983). The patterns of *FoxD3* expression are virtually identical to HNK1 labeling, suggesting that most, if not all, of the neural crest derivatives at this axial level initially express *FoxD3* (Fig. 2K,N). By stage 13/14,

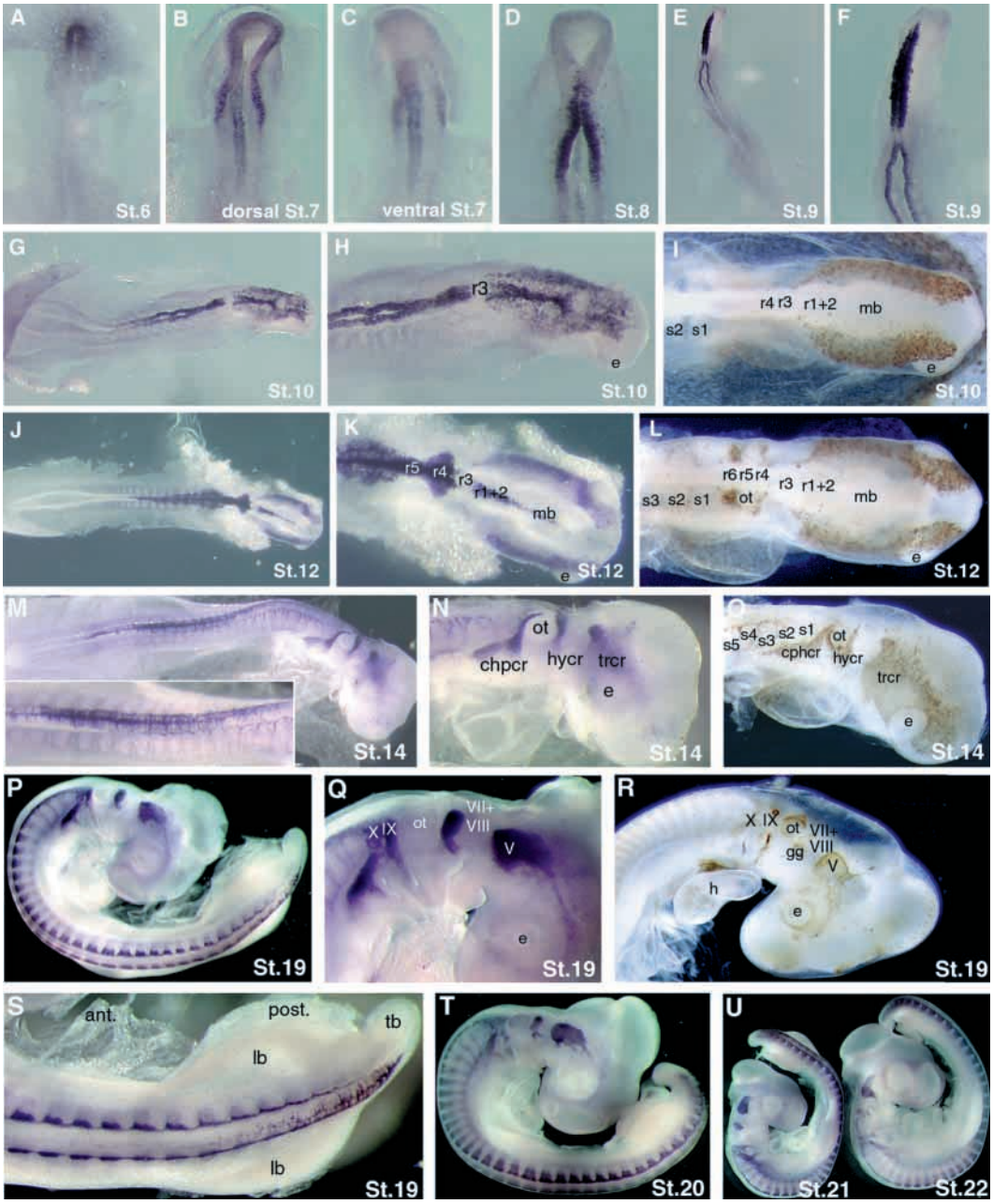

Fig. 2. In situ hybridization patterns of *FoxD3* in stage 6-22 embryos. Stage-matched HNK1-labeled embryos are shown in I, L,O and R. See text for detailed analysis. Note that cells expressing *FoxD3* are the same population of cells that are immunolabeled with HNK1. Expression is downregulated in the neural tube from those regions where melanoblasts are migrating (e.g. M,P,S). e, eye; h, heart; lb, limb bud; mb, midbrain; ot, otic placode; r, rhombomere; s, somite; trcr, trigeminal crest; hyrc, hyoid crest; cphcr, circumpharyngeal crest; V, trigeminal ganglion; VII+VIII, vestibular-acoustic ganglion; IX, glossopharyngeal nerve; X, vagus nerve; gg, geniculate ganglion.

expression of both HNK1 and *FoxD3* fades in arch 2 mesenchyme but remains high in the vestibular-acoustic and geniculate ganglia (Fig. 2N,Q), presumably in the glial cells because they are the predominant neural crest component of these ganglia.

Post-otic crest (r5,r6,r7)

Neural crest cells begin migration at this axial level at stage 9+/10 and cease migration about stage 12 (the exact time has not been reported, to our knowledge). Lineage analysis has demonstrated that, although neural crest cells do originate from r5 (Birgbauer et al., 1995), they do not spread laterally from the neural tube, but instead migrate anteriorly or posteriorly before dispersing laterally at r4 and r6 (Graham et al., 1993; Birgbauer et al., 1995; Shigetani et al., 1995). Extensive *FoxD3* expression in the r5, r6 and r7 neural folds is observed by stage 8+ (Fig. 2D), and persists in a population of cells dispersing lateral to the neural tube at r6 and r7 but not r5 at stage 10 (Fig. 2H; in agreement with Birgbauer et al., 1995). Expression of *FoxD3* in the dorsal neural tube is greatly reduced by stage 13, shortly after neural crest cells cease migration at this axial level, and is eliminated altogether in r5, coincident with the time of extensive cell death observed in that rhombomere (Graham et al., 1993).

Once migration is under way, this population of the neural crest (also known as the circumpharyngeal crest; Kuratani and Kirby, 1991) spreads laterally and posteriorly to populate branchial arches 3 and 4, and to a limited extent branchial arch 6 (Shigetani et al., 1995; Fig. 2O). Distally, these crest give rise to the connective tissue of the arches and elements of the heart.

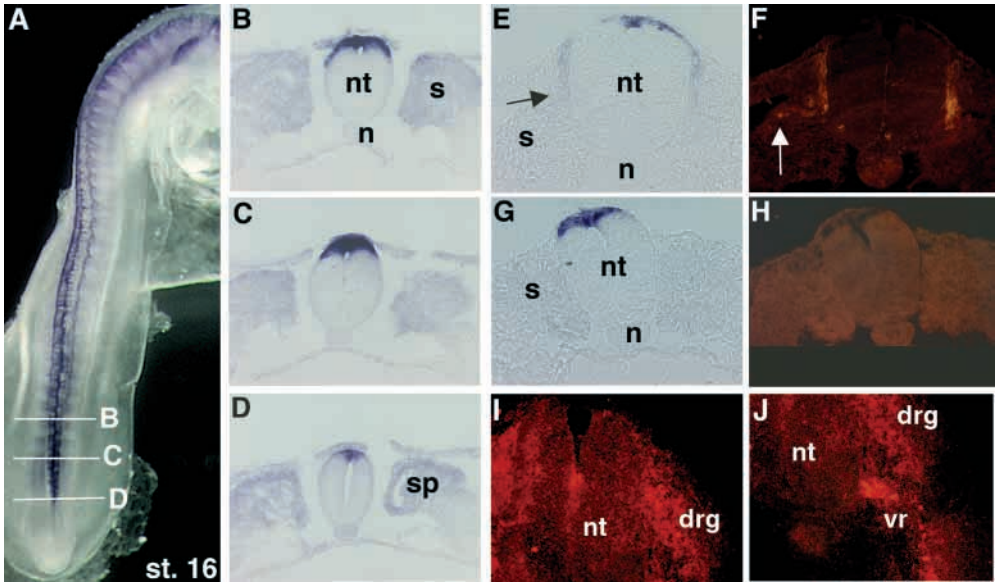

Fig. 3. Expression of *FoxD3* in a stage 16 embryo seen in whole-mount (A) and in sections through the most posterior levels of the trunk (B-D). At the trunk level, *FoxD3* is expressed in the dorsal neural tube and in cells migrating away from the dorsal neural tube (B). Labeling of a section taken through the somite level 8th from the last-formed somite (E) with HNK1 (F) indicates that the *FoxD3*-positive migratory cells are neural crest cells (indicated by arrows). The premigratory neural crest cells are *FoxD3* positive (G), but do not immunolabel with HNK1 (H). In situ hybridization on sections of a stage 22 embryo (I,J) supports the contention that *FoxD3* is expressed largely in glial cells and probably in some neurons. s, somite; sp, segmental plate; nt, neural tube; n, notochord; drg, dorsal root ganglion.

Later migrating crest from this axial level contribute proximally to the neurons of the proximal ganglia (root ganglia) of cranial nerves IX and X, to the glial cells of the distal IX (petrosal) and distal X (nodose) ganglia, and glial cells along cranial nerves IX and X (Fig. 2O,R; D'Amico-Martel and Noden, 1983). Expression patterns of *FoxD3* (Fig. 2H,K,N) at stages 10-14 are similar to the HNK1 patterns, suggesting that most of the subpopulations of the neural crest at this axial level express *FoxD3* during the early stages of their migration. Beginning at stage 14, expression of *FoxD3* in branchial arches 3, 4 and 6 is considerably reduced, and is not evident in the cardiac crest that populates the heart (Fig. 2N,T). Expression remains high in neurons and glial cells of root ganglia IX and X, in the glial cells of the petrosal and nodose ganglia, and along cranial nerves IX (glossopharyngeal) and X (vagus), which presumably is expression in crest-derived glial cells.

Vagal crest (somite level 1-7)

Scanning electron micrographs of the vagal level show that neural crest cells begin to detach at the axial level of somite 1 after stage 10 and appear progressively posteriorly in a wave (Tosney, 1978). It is not clear when emigration ceases at this axial level, as the appropriate DiI-labeling studies have not been published, but evidence exists that some neural crest cells that will give rise to the pigment lineage are still detaching at stage 18 (Kitamura et al., 1992; Reedy et al., 1998b). *FoxD3* expression in the dorsal neural tube at this level begins at stage 9 (Fig. 2E,F), well before emigration begins, is greatly reduced by stage 13, and entirely gone by stage 14 (Fig. 2N). Thus, at this axial level, *FoxD3* expression is lost from the neural tube while neural crest cell detachment is still under way. This is in contrast to *FoxD3* expression at more anterior levels, which persists in the dorsal neural tube throughout the entire time that neural crest cells are detaching.

Neural crest cells at this axial level follow two migratory pathways: dorsolaterally between the ectoderm and somites, and ventrally through the anterior half of each somite. The neural crest cells migrating dorsolaterally do so in two waves (Reedy et al., 1998b). The first wave joins the circumpharyngeal crest (Fig. 2O) and gives rise to the cardiac crest (Kuratani and Kirby, 1991) and possibly the enteric ganglia of the gut (Peters-van der Sanden et al., 1993); this subpopulation is initially *FoxD3* positive (Fig. 2J,K). The second dorsolateral wave, at stage 20, consists of the pigment cell precursors (Reedy et al., 1998b) and these crest are *FoxD3* negative even at the time they detach from the neural tube (Fig. 2N).

The ventrally dispersing neural crest migrate through the anterior half of each somite (Fig. 2O,R) and give rise to the neurons and glial cells of the dorsal root and sympathetic ganglia, and glial cells of the ventral root motor neurons. Both the neurons and glial cells remain *FoxD3* positive until at least stage 22, although expression is reduced compared with levels at the initiation of migration (compare with Fig. 2K,N). There is an intensely *FoxD3*-expressing population of cells near the dorsal root of the sensory ganglion (Fig. 2P,S), which by morphological criteria and their position are probably glial cells (Henion et al., 2000; Wakamatsu et al., 2000).

Trunk neural crest (somite 7-tail)

FoxD3 expression is seen in the premigratory trunk neural crest just shortly before the onset of migration. Neural crest cells begin to emigrate from the neural tube at somite level 8-13 (cervical level of the trunk) at stage 12, and *FoxD3* expression is seen in the dorsal neural tube at stage 10 (i.e. about 10 hours earlier; Fig. 2G). Neural crest cell detachment from the neural tube then progresses in a wave posteriorly. Neural crest cell emigration from the neural tube ceases at approximately stage 18 at the cervical level (Serbedzija et al., 1989). *FoxD3* expression begins disappearing by stage 14, however, well before emigration ceases (Fig. 2M). Thus, just as at the vagal level, early migrating neural crest cells express *FoxD3* but the later migrating cells do not.

At the trunk level, neural crest cells take two migratory paths. The early-migrating crest invade the anterior half of each somite (Rickmann et al., 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987) and give rise to the neurons and glial cells of the sensory and sympathetic ganglia, the glial cells of the ventral root, the adrenal medulla (somite level 18-24 only) and a subpopulation of the enteric crest (sacral level only). Initially these cells are all strongly *FoxD3* positive (Fig. 2M) but expression fades rapidly as the cells enter the somites (Fig. 3E). Nevertheless *FoxD3* expression is still apparent in the ventral trunk crest derivatives even as late as stage 22 (Fig. 2U). A subpopulation of the neural crest at the dorsal root express high levels of *FoxD3* (see Figs 2P,S,T, 3I,J) and are likely to be glial cells based on their position (Henion et al., 2000). From these in situ preparations, we could not ascertain whether the neural crest cells that give rise to the adrenal medulla ever expressed *FoxD3*.

Later migrating trunk neural crest cells take the dorsolateral path, beginning about 24 hours after embarking on the ventral path (Erickson et al., 1992). DiI-labeling studies (Serbedzija et al., 1989; Kitamura et al., 1992) have shown that trunk neural crest cells destined for the dorsolateral path leave the neural tube beginning at stage 18 at the wing bud level, and marker analysis has demonstrated that these late-migrating trunk neural crest cells are already specified as melanoblasts before they enter the dorsolateral path (Kitamura et al., 1992; Reedy et al., 1998a). At the wing bud level, *FoxD3* expression in the dorsal neural tube is lost by stage16, well before presumptive melanoblasts leave the neural tube, and we never detected any *FoxD3*-positive neural crest cells in the dorsolateral path at later stages:

The expression patterns of *FoxD3* in the neural crest suggested two potential functions for this transcription factor.

(1) Because *FoxD3* is expressed in the premigratory neural crest, we predicted that it might be involved in establishing the neural crest lineage and/or controlling the EMT that segregates the neural crest from the neural tube.

(2) Because it is initially expressed in all subpopulations of neural crest cells except melanoblasts, we predicated that FoxD3 might be a repressor of melanogenesis, especially as murine Foxd3 has been shown to be a transcriptional repressor when expressed in embryonic stem cells (Sutton et al., 1996) and delays terminal differentiation when transduced into teratocarcinoma cells (Hromas et al., 1999) or myeloid stem cells (Xu et al., 1998).

Early misexpression of *FoxD3* throughout the neural epithelium results in an expansion of HNK1 expression and an increase in migratory neural crest cells

We used electroporation to misexpress EGFP-tagged *FoxD3* in the lateral and ventral neuroepithelium. Using this technique, protein production is observed in less than 6 hours. We injected expression plasmids carrying *FoxD3*-EGFP into the lumen of stage 12-14 neural tubes and then electroporated the plasmids into the neural epithelium. Production of FoxD3 protein, as evidenced by EGFP fluorescence, was always on one side of the neural tube and the EGFP signal was concentrated in the nucleus, as would be expected for a transcription factor (Fig. 4A,C,E). We noted several dramatic changes in these embryos. First, there was prominent HNK1 immunoreactivity in *FoxD3*-EGFP-positive neural epithelial cells and neural crest cells (Fig. 4B,D,F). Second, we often observed an increase in the number of neural crest cells that were migrating away from the dorsal neural tube (Fig. 4D arrow). Although there was a clear stimulation of HNK1 immunoreactivity in the neural epithelium, and a greatly increased number of HNK1-positive cells emigrating from the dorsal neural tube, there did not appear to be any HNK1-positive cells escaping from the sides of the neural tube, nor was there any evidence of an ectopic EMT. In fact, most of the HNK1-positive/EGFP-positive cells had the typical

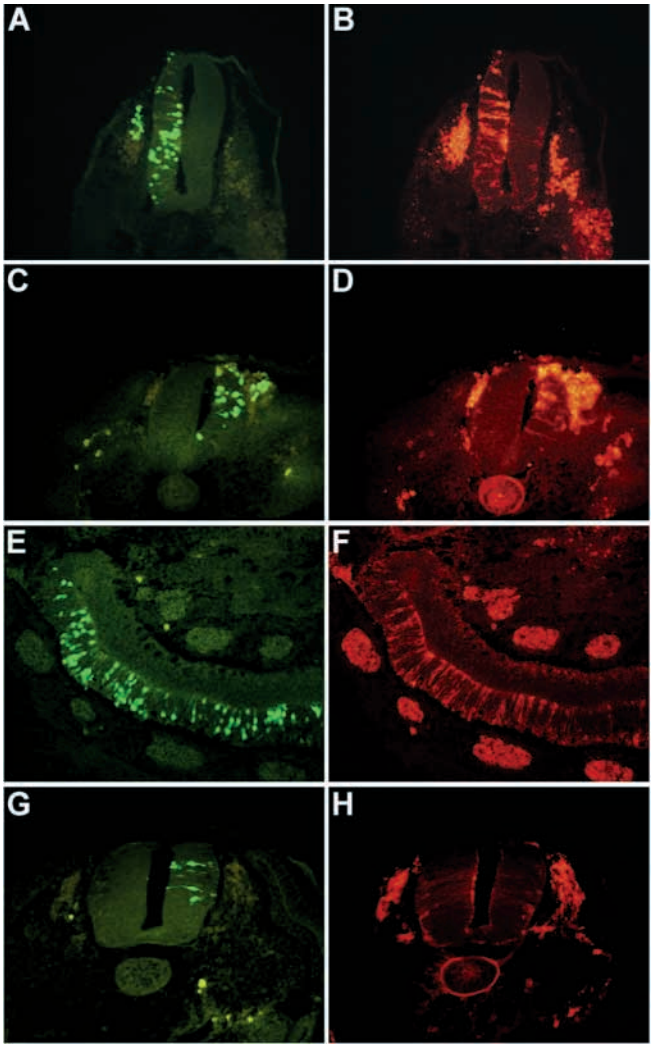

Fig. 4. Sections through three different embryos that were electroporated with the *FoxD3*-EGFP expression vector, fixed after 24 hours and labeled with HNK1 antibody. (A-F) The distribution of EGFP is shown in the left-hand panels (A,C,E) and the HNK1 immunoreactivity of the same section shown in the right-hand panels (B,D,F, respectively). Wherever *FoxD3*-EGFP is misexpressed there is a concomitant expansion of HNK1 immunoreactivity, and also an expansion of neural crest cells emigrating from the dorsal neural tube. Electroporated lateral and ventral neural epithelium still retain a normal epithelial structure, however (especially well seen in the frontal section in F), and do not appear to undergo an EMT. (G,H) In EGFP-control electroporated embryos there is no expansion of HNK1 immunoreactivity.

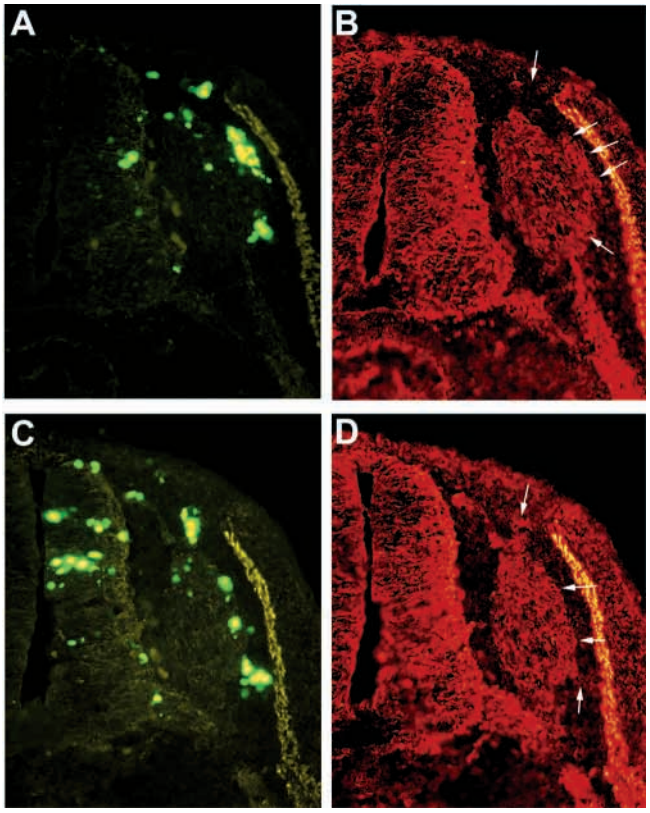

Fig. 5. *FoxD3*-expressing neural crest cells differentiate primarily into glial cells. Sections of *FoxD3*/EGFP-expressing neural crest cells are shown in the left-hand panels and the same sections shown immunolabeled with 7B3 (B,D) on the right. Arrows in B,D indicate the position of some of the electroporated cells. Most of the *FoxD3*-overexpressing cells express the glial marker, but a few do not (e.g. the cells indicated by the most ventral arrow in D). Note that the electroporated cells are often found at the margins of the dorsal root ganglia or under the dermomyotome.

morphology of a pseudostratified epithelium (Fig. 4F). We also cultured *FoxD3*-EGFP-expressing ventral neural tubes on plastic substrata or in collagen gels and still failed to see evidence of an EMT, although these tissues also immunolabeled with HNK1 (data not shown).

Most of the *FoxD3*-EGFP-expressing neural crest cells were found in the ventral pathway and were often clustered around the margins of the sensory ganglion, or under the dermamyotome. Only rarely was an EGFP-positive neural crest cell found in the dorsolateral pathway. To determine the phenotype of the EGFP-positive cells in the ventral pathway, we immunolabeled two embryos (Fig. 5) with either 16A11 (neuronal marker; Marusich et al., 1994) or 7B3 (glial marker; Henion et al., 2000). 25% (n=75) of the EGFP cells in one embryo were 16A11 positive and 79% (n=87) of the cells in the second embryo were 7B3 positive. These data, in addition to the location of the cells in the embryo, suggest that the majority of the EGFP-positive cells were glial cells.

These observations suggest that *FoxD3* misexpression can convert lateral epithelium to a more neural crest-like fate, but that FoxD3 alone is not sufficient for the EMT.

Table 1. Percentage of melanocytes in sense and antisense oligonucleotide-treated cultures

	% Melanocytes (sense treated)	% Melanocytes (antisense treated)
Experiment 1	15	60
	10	27
	6.5	16
Experiment 2	35	47
	37	60
Experiment 3	12	38
	11	22

Two or three neural tubes were plated/well and treated with sense or antisense oligonucleotides. On day 6 of culture, the cells were removed from the wells and counted. For each experiment, three wells were combined for more accurate counting. Thus in experiment 1, a total of nine wells for each treatment were counted, whereas for experiments 2 and 3, a total of six wells/treatment were counted.

Using Tukey grouping, the percentage of melanocytes in antisense-treated cultures was significantly higher than in sense-treated cultures (38.571%, n=7 versus 18.071%, n=7; P<0.05)

Antisense oligonucleotide treatment results in an increase in melanocytes and a reduction of neurons and glial cells in neural crest cultures

We treated 15-hour neural crest outgrowths with antisense oligonucleotides directed against the 5′ end of *FoxD3* for 48 hours and then assessed the number of melanocytes in the cultures on day 6. Normally, most of the neural crest cells that migrate during the first 12-15 hours are neuronal and glial cell precursors, and not melanoblasts (Henion and Weston, 1997; Reedy et al., 1998a). We predicted that a reduction of *FoxD3* expression using antisense oligonucleotides would increase the number of melanocytes in these cultures if FoxD3 acts as a repressor of melanoblast specification. Our results are summarized in Table 1 and Fig. 6. In three different experiments using three different lots of antisense oligonucleotides, we found that 38.5% of the antisense-treated neural crest cells differentiated into melanocytes, compared with only 18% in the sense controls (Fig. 6A,B). These results are statistically significant using the Tukey analysis to compare percentages. There was no significant difference in the total number of cells between the antisense- and sense-treated cultures, suggesting that the oligonucleotides did not affect cell proliferation or survival.

There are three primary neural crest lineages that differentiate from the trunk neural crest: neurons, glial cells and melanocytes. We asked whether there was a concomitant decrease in the number of neurons and glial cells in the antisense-treated cultures by labeling the cultures with immunomarkers for neurons (16A11 antibody; Marusich et al., 1994) and glial cells (7B3 antibody; Henion et al., 2000). Because the cultures were so dense, we could not accurately quantitate the results by counting immunolabeled cultures. However, we noted an obvious decrease in neurons (Fig. 6C,D) and glial cells (Fig. 6E,F) in the antisense-treated cultures. Moreover, the clustering of neurons that is typically seen in neural crest outgrowths was absent.

Morpholino antisense oligonucleotide treatment of embryos

To knock down expression of *FoxD3* in embryos, we electroporated fluorescently tagged morpholino antisense

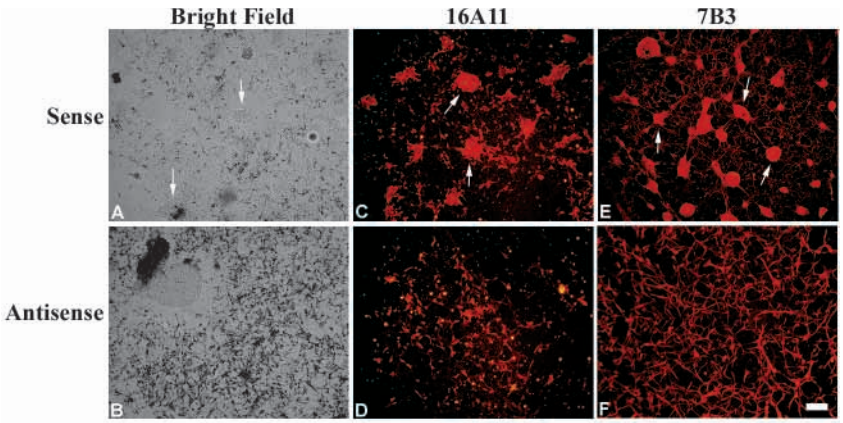

Fig. 6. (A-F) Neural crest cultures treated with sense (A,C,E) and antisense (B,D,F) oligonucleotides. Bright field images reveal a dramatic increase in melanocytes in the antisense cultures (B) compared with the sense-treated control cultures (A). There is a concomitant decrease in the number of neurons with antisense treatment, as revealed with the 16A11 antibody (C,D). Note, in particular, the loss of clusters of neurons (arrows in C), which typically appear in neural crest outgrowths (also indicated by arrows in A). A similar-looking cluster in B is actually a remnant of the neural tube. There appears to be a reduction in glial cells, here immunolabeled with the 7B3 antibody (E,F). Glial cells in culture often associate with neurons (Holton and Weston, 1982), and we also observe glial cells co-localized with the neuronal clusters (arrows).

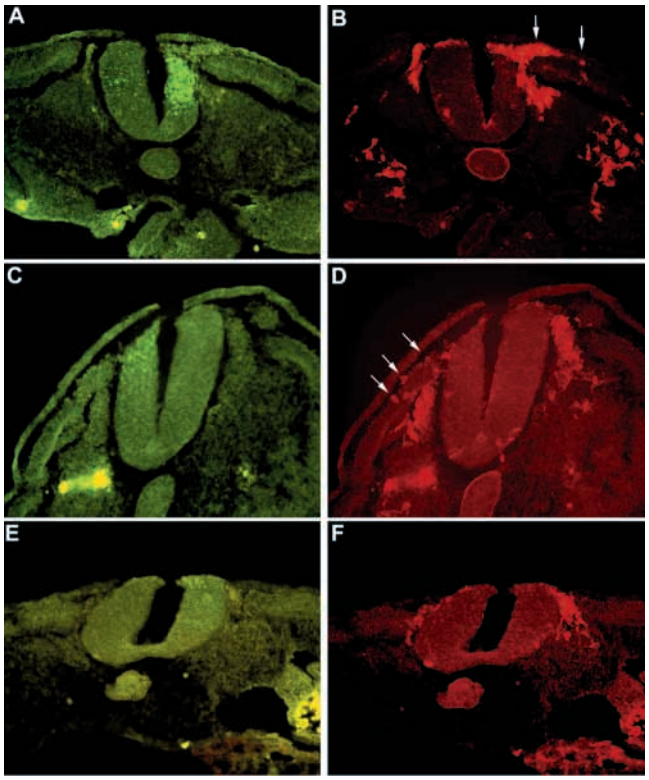

Fig. 7. Electroporation of morpholino antisense oligonucleotides results in a precocious migration of neural crest cells into the dorsolateral path. Fluorescently tagged morpholinos are shown in the left panels and HNK1 labeling of the same sections in the right panels. Electroporation of morpholino antisense oligonucleotides (A-D) produces an early migration of neural crest cells into the dorsolateral space (indicated by arrows), whereas the contralateral control side shows no dorsolateral migration. Fluorescent standard controls (E,F) do not perturb normal neural crest morphogenesis.

oligonucleotides directed against *FoxD3* into stage 13 embryos. A total of five embryos in one experiment developed normally and were analyzed. We observed in all embryos a precocious migration of neural crest cells into the dorsolateral path on the electroporated side (Fig. 7A-D). We presume that these cells were melanoblasts because this is the only neural crest subpopulation that can exploit and survive in the dorsolateral path (Erickson and Goins, 1995; Reedy et al., 1998a; Wakasama et al., 1998). Standard fluorescent controls had no effect on neural crest migration (Fig. 7E,F).

Neural crest cells expressing *FoxD3* using RCAS virus fail to enter the dorsolateral path

Previous studies have shown that melanoblast specification is a prerequisite to dorsolateral migration (Kitamura et al., 1992; Erickson and Goins, 1995; Reedy et al., 1998a; Reedy et al., 1998b). If FoxD3 represses melanogenesis, then late-migrating neural crest cells in which FoxD3 is misexpressed in ovo should fail to enter the dorsolateral path. In order to test this hypothesis we used the avian retrovirus RCASBP(B) to misexpress *FoxD3* in later migrating neural crest cells. Previous studies (Homburger and Fekete, 1996; Perez et al., 1999) have shown that B envelope RCAS viruses are efficient at infecting the neuroepithelium and neural crest cells. For a control, we made an RCAS construct with the *FoxD3* cDNA inserted in the reverse orientation. Both constructs generated infectious virus with an approximate titer of $1\text{-}5\times10^{8}$/ml. Injections were performed just posterior to the wing bud level of stage 12-13 embryos. Protein expression in the RCAS system begins approximately 18-20 hours after injection (Morgan and Fekete, 1996), so injection at stage 12 should result in expression of *FoxD3* beginning at around stage 17-19, which is when melanogenic neural crest cells begin to leave the neural tube at the wing bud level.

Fig. 8 shows the results of this experiment. Embryos injected with *FoxD3*-expressing virus (*FoxD3*-RCAS) had a high percentage of double-labeled cells in the dorsal root ganglion (double-label, yellow; Fig. 8A-C). However, we detected essentially no double-labeled cells in the dorsolateral path in these embryos, even though there were many HNK1-positive/p27-negative neural crest cells in the dorsolateral path (arrows, Fig. 8A-C), indicating that uninfected neural crest cells migrated normally. In contrast, embryos injected with the control virus (control-RCAS) that did not express *FoxD3* had many double-labeled cells in the dorsolateral path (Fig. 8D,E, small arrows). There were also many more p27-positive/HNK1-negative cells in the dorsolateral path of control

infected embryos (green cells in Fig. 8D,E). It is possible that some of these are neural crest cells undergoing melanogenesis, because previous studies (e.g. Erickson et al., 1992) have shown that melanoblasts start losing HNK1 immunoreactivity at stage 22-23. Control-infected cells were also present in the dorsal root ganglion (not shown). Thus, we conclude that misexpression of *FoxD3* in late-migrating neural crest cells prevents their migration into the dorsolateral path, suggesting that FoxD3 inhibits melanoblast specification in vivo.

DISCUSSION

We have cloned the chicken homolog of the *FoxD3* gene, which is nearly identical to the previously reported *CWH-3* gene (Freyaldenhoven et al., 1997). We have examined its expression patterns in detail and based on these patterns hypothesized that FoxD3 has a role in the establishment of the neural crest lineage and represses differentiation of melanocytes. Antisense studies and misexpression using either RCAS viruses or electroporation verify these predictions.

In situ hybridization reveals that *FoxD3* is expressed in the premigratory neural crest but is downregulated in the premigratory melanoblast lineage

We did a detailed analysis of the expression patterns of *FoxD3* and compared these with stage-matched embryos processed for HNK1 immunoreactivity and with previously reported DiI-labeling studies, which have determined the timing of neural crest cell migration and cessation of migration. Although a previous brief report of the expression patterns of *CWH-3* has appeared elsewhere (Yamagata and Noda, 1998), some key points were not made in that study, which we emphasize here.

From our in situ studies, we made several observations that shaped our experimental studies:

(1) *FoxD3* is expressed in the dorsal neural tube just as the neural folds contact each other, it is expressed in the early migratory neural crest cells suggesting that the dorsal neural tube expression is a marker for premigratory neural crest, and at the cranial levels it is downregulated at the time that neural crest cell emigration is known to stop, suggesting that *FoxD3* has a role in the establishment of the neural crest lineage. It was interesting to note that regions of the neural tube from which neural crest cells do not migrate, such as the prosencephalon and r3, never express *FoxD3*, confirming its usefulness as a marker for neural epithelial cells with neural crest potential. Curiously murine *Foxd3* is not expressed in either r3 or r5 (Labosky and Kaestner, 1998), suggesting that in the mouse, there is no neural crest emigration from either of these rhombomeres.

(2) *FoxD3* is expressed in most of the migratory neural crest lineages, even the ectomesenchyme (at least initially), further suggesting that *FoxD3* plays a role in the establishment of the neural crest lineage. Comparison with HNK1-labeled embryos and double labeling of the in situs with HNK1 confirmed which neural crest lineages expressed *FoxD3* (see Results for the detailed analysis). Yamagata and Noda have suggested that *FoxD3* is not expressed in ectomesenchyme (Yamagata and Noda, 1998), but our results show intense expression in all the cranial neural crest and moderate-to-low expression in many of the ectomesenchymal derivatives, such as elements of the eye, even in 5-day old embryos. Similarly, murine ectomesenchyme expresses *Foxd3* (Labosky and Kaestner, 1998). At later stages of migration, expression is gradually reduced in the ectomesenchyme and to a large extent in cranial and trunk neurons, but is still highly expressed in glial cells (see Fig. 3I,J), suggesting that *FoxD3* positively induces glial cell differentiation. Previous studies in mouse did not distinguish

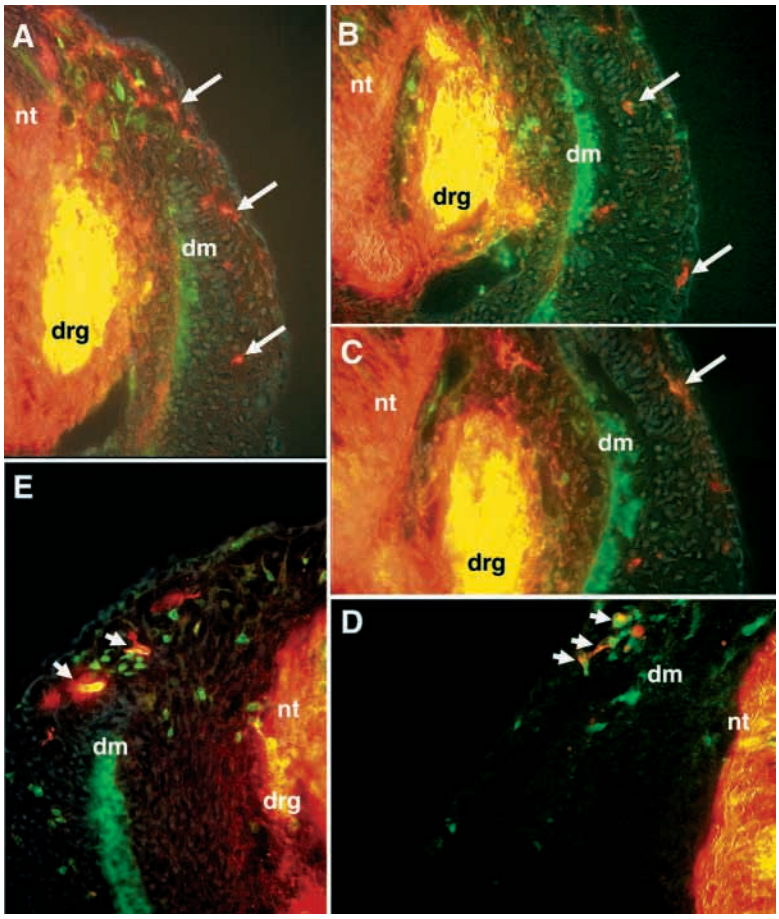

Fig. 8. Embryos infected with control *FoxD3*-expressing (A-C) or control (E,F) RCAS retroviruses. All sections are at the wing-bud level of stage-22 (A,B,E) or stage-23 (C,D) embryos. Neural crest cells are labeled with HNK1 (red), viral-infected cells with p27 (green), and double-labeled cells appear yellow. The weak green label in the dermamyotome is background labeling that we saw with the Alex488-conjugated secondary antibody even in embryos not infected with either virus (not shown). Note that while there are abundant *FoxD3*-expressing neural crest cells in the dorsal root ganglion (drg) in A-C, there are no *FoxD3*-expressing cells in the dorsolateral path, even through there are many uninfected dorsolateral neural crest cells (long arrows) in these embryos. In contrast, control-infected neural crest cells enter the dorsolateral path normally (short arrows, D,E). Sections shown in D and E are through the posterior somite, so there is little or no dorsal root ganglion present, but in other sections (not shown), control-infected neural crest cells were also present in the dorsal root ganglion.

between neuronal and glial cell expression, but in the zebrafish, the *FoxD3* homolog (previously called *fhk6*; Odenthal and Nusslein-Volhard, 1998) is expressed exclusively in glial cells at late stages of migration (Kelsh et al., 2000).

(3) In the vagal and thoracic regions, *FoxD3* is downregulated in the dorsal neural tube just prior to the time that melanoblasts emigrate, and is never expressed in the dorsolaterally migrating neural crest, suggesting that *FoxD3* represses melanogenesis. None of the previous reports of chicken or murine *FoxD3* expression (Labosky and Kaestner, 1998; Yamagata and Noda, 1998; Hromas et al., 1999) reported on the lack of expression in the melanocyte lineage. This expression pattern suggested that *FoxD3* might repress melanogenesis.

FoxD3 expands the neural crest lineage at the expense of the neural epithelium

We believe that *FoxD3* participates in the segregation of the neural crest lineage from the neural epithelium. It is expressed prior to neural crest migration in the region from which the neural crest will migrate, and is not expressed in those regions along the neural tube from which neural crest cells do not emigrate. Overexpression by electroporation in the dorsal neural tube results in an abundance of neural crest cells emigrating from the transfected side of the neural tube. And finally, misexpression in the lateral and ventral neural tube results in an expansion of HNK1 immunoreactivity, which is a widely used marker for the neural crest. Although it might be argued that HNK1 is also expressed at later stages in the neural tube (e.g. Oakley et al., 1994), and therefore *FoxD3* expression is only accelerating its neural tube expression, this seems an unlikely scenario for at least two reasons. First, dorsal expression results in a vastly increased number of migratory neural crest cells, revealing a direct effect on specification of the neural crest lineage. And second, *FoxD3* is predominantly expressed in the neural crest, suggesting that any response from the neural epithelium is in some way related to the establishment of the neural crest lineage and not a CNS lineage.

Although the neural epithelium expresses HNK1, it fails to undergo an EMT. We noted that the HNK1-positive cells all maintain a clear pseudostratified epithelial organization typical of the early neural tube. Even 48 hours after electroporation or viral transfection, there is no evidence of an EMT from the lateral or ventral neural epithelium. Furthermore, culturing of electroporated ventral neural epithelium in collagen gels still fails to induce an EMT. We suggest that *FoxD3* may be an important element in generating the neural crest lineage apart from the rest of the neural epithelium, but a second signaling pathway that induces the expression of the genes for cadherin 6 (Nakagawa and Takeichi, 1998) and RhoB (Liu and Jessell, 1998), among other genes, is required for separation of these cells from the neural tube. Evidence that segregation of the crest lineage and the EMT are independently controlled events also comes from a study by Sela-Donenfeld and Kalcheim, who showed that they could inhibit the EMT by inhibiting BMP signaling (Sela-Donenfeld and Kalcheim, 1999), even though *slug* is still expressed and HNK1-positive cells accumulate in the lumen of the neural tube.

FoxD3 represses melanogenesis

Our results demonstrate that *FoxD3* can repress melanogenesis in the neural crest. We have shown directly that when we treat cultures of neural crest cells with antisense oligonucleotides to *FoxD3*, there is a greatly increased number of neural crest cells that differentiate into melanocytes with a concomitant decrease in the number of neurons and glial cells, as revealed using antibody markers against these latter two lineages. It is still formally possible that in the absence of *FoxD3*, the rate of division of melanoblast precursors is stimulated or that this triggers apoptosis in the neuronal and glial lineages. We did not specifically look at the rate of BrdU incorporation or apoptosis under these conditions, but the fact that we did not see any difference in the absolute number of neural crest cells in the control versus antisense-treated cultures suggests that there was a direct effect on lineage specification rather than a selective proliferative effect on an already established population. An indirect indicator that *FoxD3* represses melanogenesis is that in embryos in which *FoxD3* was misexpressed in late migrating neural crest cells, either by electroporation or retroviruses, there were essentially no *FoxD3*-expressing neural crest cells in the dorsolateral path, whereas uninfected and control- transfected/infected cells were present in the dorsolateral pathway in normal numbers. As previous studies showed that only fate-restricted melanoblasts are able to enter the dorsolateral path (Erickson and Goins, 1995; Wakamatsu et al., 1998; Reedy et al., 1998a), these latter results imply that transfected neural crest cells expressing *FoxD3* are unable to differentiate into melanoblasts. Conversely, when FoxD3 is knocked down using morpholino antisense oligonucleotides, neural crest cells invade the dorsolateral path precociously, showing that melanogenesis is accelerated when *FoxD3* expression is downregulated prematurely.

It is not particularly surprising that *FoxD3* could repress melanogenesis, as many members of this gene family are known to repress differentiation or retain cells in a pluripotent state (summarized by Kaufmann and Knochel, 1996). Moreover the mouse homolog has been shown to repress the differentiation of embryonic stem cells, and is downregulated when stem cells are stimulated to differentiate (Sutton et al., 1996). At least in the latter report, mouse FoxD3 acts as a transcriptional repressor. How FoxD3 might be acting in avian neural crest cells to repress differentiation into melanocytes is not known, but one possibility suggests itself. We have recently shown that members of the BMP family of signaling molecules repress melanogenesis and stimulate neuronal and glial cell differentiation, whereas Wnt signaling has the opposite effect (Jin et al., 2001). One possibility that we are pursuing is that FoxD3 mediates repression of melanogenesis by functioning as a co-factor of Smads in neural crest precursors (Chen et al., 1996). Another alternative is that BMP signaling directly results in the expression of *FoxD3*, which in turn functions as a transcriptional repressor of melanocyte-specific genes, especially MITF. It will be interesting to determine if the promoter of MITF contains a FoxD3 consensus sequence.

Still another possibility is that FoxD3 acts to repress melanogenesis indirectly by directly inducing neural or glial cell differentiation. Our results, as well as other studies (e.g. Henion and Weston, 1997; Jin et al., 2001) indicate that melanocytes are specified at the expense of neural and glial cells. We have no direct evidence to support the idea that FoxD3 positively controls neural or glial cell specification. In

fact, given that *FoxD3* is expressed in every neural crest lineage except the melanocytes, this idea seems unlikely. However, recent studies from zebrafish show that *foxd3* is expressed in premigratory crest but is downregulated at the time of the EMT. Then, at later stages of development, it is once again expressed exclusively in glial derivatives (Kelsh et al., 2000). We have also noted that *FoxD3* is most abundantly expressed in glial cells in older embryos, although it is not exclusively in glial cells. Additionally, when *FoxD3* is misexpressed, many expressing cells accumulate near the sensory ganglion in areas that are generally occupied by glial cells (Henion et al., 2000), and most of the *FoxD3*-expressing cells are 7B3-positive. Thus, it is still formally possible that *FoxD3* acts to repress melanogenesis by stimulating gliogenesis.

We thank Jim Weston for his generous gift of the 7B3 antibody used in this study, Rick Tucker for his financial contributions to the electroporation effort, and Paul Morcos at Gene Tools for his expertise in designing morpholino antisense oligos. We also thank Tina Goins for her help with the preparation of Fig. 2, and Alicia Santiago for contributing the HNK1- labeled whole mount images. This study was supported by NIH grants GM53258 to C. A. E., EY12311 to R. L. J. and postdoctoral fellowship EY06945 to M. V. R.

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Note added in proof

We have recently learned that Mirella Dottori and Martyn Goulding have similarly observed that misexpressed FoxD3 in the lateral neural tube results in the expansion of HNK1 immunoreactivity in the neural epithelium.