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Enforced expression of the transcription factor HOXD3 under the control of the *Wnt1* regulatory element modulates cell adhesion properties in the developing mouse neural tube

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Abstract

HOX genes expressed in a specific spatial and temporal manner play a crucial role in determining the body plan during the early development of vertebrates. In adult tissues, many *HOX* genes participate in normal hematopoiesis and carcinogenesis. We previously found that overexpression of the homeobox gene *HOXD3* alters expression levels of cell adhesion molecules in human cancer cell lines. Here, we have investigated whether *HOXD3* expression is related to the cell adhesion processes during mouse development focusing on dorsal midline cells or roof-plate cells of the neural tube and neural crest cells. We created transgenic mouse embryos, in which *HOXD3* is expressed in the dorsal midline under the control of the *Wnt1* regulatory element, and analyzed these embryos at embryonic day 10.5–13.5. In *HOXD3*-expressing transgenic embryos, although neural crest-derived structures in the trunk region appeared to be normal, striking abnormalities were found in the neural tube. In transgenic embryos expressing the *lacZ* gene under the control of the *Wnt1* regulatory element, expression of *lacZ* was restricted to roof-plate cells within the neural tube. By contrast, in *HOXD3*-expressing transgenic embryos, expression of *HOXD3* was not only located in the dorsal neural tube, but also had spread inside the ventricular zone in more ventral regions of the neural tube. These findings show that the *HOXD3* transgene is expressed more broadly than the *Wnt1* gene is normally expressed. Expression of both *Wnt1* and *Msx1*, marker genes in the roof plate, was further extended ventrally in *HOXD3*-expressing embryos than in normal embryos, suggesting that expression of the *HOXD3* transgene expands the roof plate ventrally within the neural tube. In the ventricular zone of *HOXD3*-expressing embryos at embryonic day 10.5, we observed an increase in the number of mitotic cells and failure of interkinetic nuclear migration of progenitor cells. Furthermore, in *HOXD3*-expressing embryos at embryonic day 12.5, the ventricular zone, in which progenitor cells became more loosely connected to each other, was composed of a large number of cells that did not express N-cadherin. Our results indicate that expression of *HOXD3* is closely associated with modulation of cell-adhesive properties during embryonic development.
Key words: *HOXD3*; mouse; N-cadherin; neural tube; *Wnt1* regulatory element.

Introduction

HOX genes encode DNA-binding proteins that function as transcriptional regulators. In mammals, such as humans and mice, 39 *HOX* genes are organized in four separate clusters called *HOX A*, *B*, *C* and *D*. Individual members of *HOX* genes in the four clusters are classified into 13 paralogous groups, according to the similarity of their nucleotide sequences and the position of the genes within the clusters (Scott, 1992). As for members that belong to the different para-logous groups, there is a 'colinear' relationship between an anteroposterior pattern of embryonic expression domains, a so-called *HOX*-code, and ordering of the genes within each complex (Duboule & Dolle, 1989). A number of mutational analyses show that the *HOX*-code in embryonic segments along the anteroposterior

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axis plays a pivotal role in determining the regional specificity of cells. For example, the *Hoxb1* gene, located at the most 3′ side within the cluster, is responsible for the generation of branchiomotor neurons that innervate the muscles of facial expression (Arenkiel et al. 2004), whereas the *Hoxd13*, located at the most 5′ side within the cluster, plays a crucial role in the formation of the sacrum and the penile bone (Dolle et al. 1993), and normal morphogenesis of the internal anal sphincter (Kondo et al. 1996).

Hoxd3 is a third paralogous member of the *Hox* gene family, and functions in the formation of somitic mesoderm- and neural crest-derived structures during mouse development (Condie & Capecchi, 1993; Manley & Capecchi, 1998). On the other hand, using human cell lines such as erythroleukemia HEL and lung cancer A549 cells, we found that overexpression of the *HOXD3* gene results in an increase of cell-extracellular matrix adhesiveness and cell motility accompanied by increasing expression levels of β3 integrin (Taniguchi et al. 1995; Hamada et al. 2001). It has been demonstrated that, in cultured human endothelial cells, *HOXD3* expression mediates conversion of endothelium from the resting to the angiogenic state, and consequently causes increased expression of β3 integrin (Boudreau et al. 1997). In these cells, HOXD3 protein binds directly to the promoter region of the *β3 integrin* gene (Boudreau & Varner, 2004). In addition, we have shown that, in HEL cells, deletion of *HOXD3* expression gives rise to simultaneous suppression of β3 integrin expression and activation of *cadherin 4* expression; overexpression of *HOXD3* causes the reverse (Taniguchi et al. 2003). These *in vitro* studies have revealed that expression of *HOXD3* is deeply implicated with the regulation of cell adhesion processes.

During development of the vertebrate embryo, neural crest cells delaminate from the neural plate or neural tube by changing their shape and properties from those of neuroepithelial cells to those of mesenchymal cells. This epithelial-to-mesenchymal transformation is accompanied by a loss of cell-to-cell adhesion molecules such as N-cadherin, and a gain of cell-to-extracellular matrix adhesion molecules such as β1 and β3 integrins on the crest cells during their migratory phase (Hatta et al. 1987; Akitaya & Bronner-Fraser, 1992; Monier-Gavelle & Duband, 1997). In the present study, we have investigated the role of the *HOXD3* gene in the change of cell adhesiveness focusing on dorsal midline or roof-plate cells of the neural tube and neural crest cells during mouse development. It is possible that a transgene is expressed specifically in the dorsal neural tube and the early migratory neural crest population under the control of the *Wnt1* promoter and enhancer (Echelard et al. 1994). We have employed this system, and created transgenic mouse embryos in which *HOXD3* is expressed in dorsal midline cells and early migrating neural crest cells. In the transgenic embryos, we have found that, although neural crest-derived structures appear to be normal, remarkable abnormalities take place in the neural tube. Our results show that *HOXD3* expression driven by the *Wnt1* regulatory element brings about expansion of roof-plate cells in a dorsal-to-ventral direction within the neural tube and reduces expression levels of N-cadherin in many neural progenitor cells, raising the possibility that HOXD3 plays a role in the regulation of cell-adhesive properties during embryonic development.

Materials and methods

Construction of the expression vectors

Two constructs were prepared to induce *lacZ* and *HOXD3* expression under the control of the *Wnt1* enhancer, respectively. The construct to induce *lacZ* expression was generated according to the following processes. By digesting the plasmid DNA named pCAGGS-lacZ with *Hin*dIII, a 3.5-kb fragment, containing a 3-kb *lacZ* DNA and a 0.5-kb rabbit β-globin DNA with the polyadenylation signal, was isolated. This fragment was inserted into the *Wnt1* misexpression construct, pWexp2 (Echelard et al. 1994), which was linearized by *Eco*RV. This expression vector was designated as Wexp-lacZ (Fig. 1A). The construct to induce *HOXD3* expression was prepared in the following procedures. An oligonucleotide polylinker, containing an *Hpa*I restriction endonuclease cleavage site, was inserted into the expression vector, pMAMneo-HOXD3 (+) (Taniguchi et al. 1995), linearized with *Spe*I. By digesting this construct with *Hpa*I, a 4.1-kb fragment, containing a 3.4-kb human *HOXD3* genomic DNA with the full coding region and intron and a 0.7-kb SV40 DNA with the polyadenylation signal, was isolated. This fragment was inserted into pWexp2, in which the translational start site of the *Wnt1* gene is disrupted through inserting a multiple cloning site. This expression vector was called Wexp-HOXD3 (Fig. 1B). For microinjection, the Wexp-lacZ and Wexp-HOXD3 vector DNAs were digested with *Sal*I and *Aat*II, respectively. A 14.3-kb *Sal*I-*Sal*I fragment containing the *lacZ* gene and a 14.9-kb *Aat*II-*Aat*II fragment containing the *HOXD3* gene were separated on agarose gels, isolated by electroelution, and purified by phenol/chloroform extraction.

Generation of transgenic mouse embryos

Transgenic mouse embryos were generated according to the method of Hogan (Hogan et al. 1986). Briefly, 6-week-old Crj: BDF1 female mice were superovulated by intraperitoneal injection of 5 IU gonadotropin from pregnant mare's serum 46 h prior to injection of 5 IU of human chorionic gonadotropin. Female mice were then mated with C57BL/6NCrj male mice. Fertilized eggs were flushed from the oviducts with M2 medium and then freed of cumulus cells by hyaluronidase treatment. DNA fragments (2 ng mL^{-1}) were microinjected into the pronuclei of the zygotes. The injected zygotes were transferred to pseudopregnant recipient Crj: CD-1 female mice. For two experiments, pregnant females were injected intraperitoneally with 2 mg bromodeoxyuridine (BrdU; Sigma, St. Louis, MO, USA) per 30 g body weight one hour prior to killing. Founder (F_0) embryos were collected at 10.5, 11.5, 12.5 and 13.5 days post coitum (dpc).

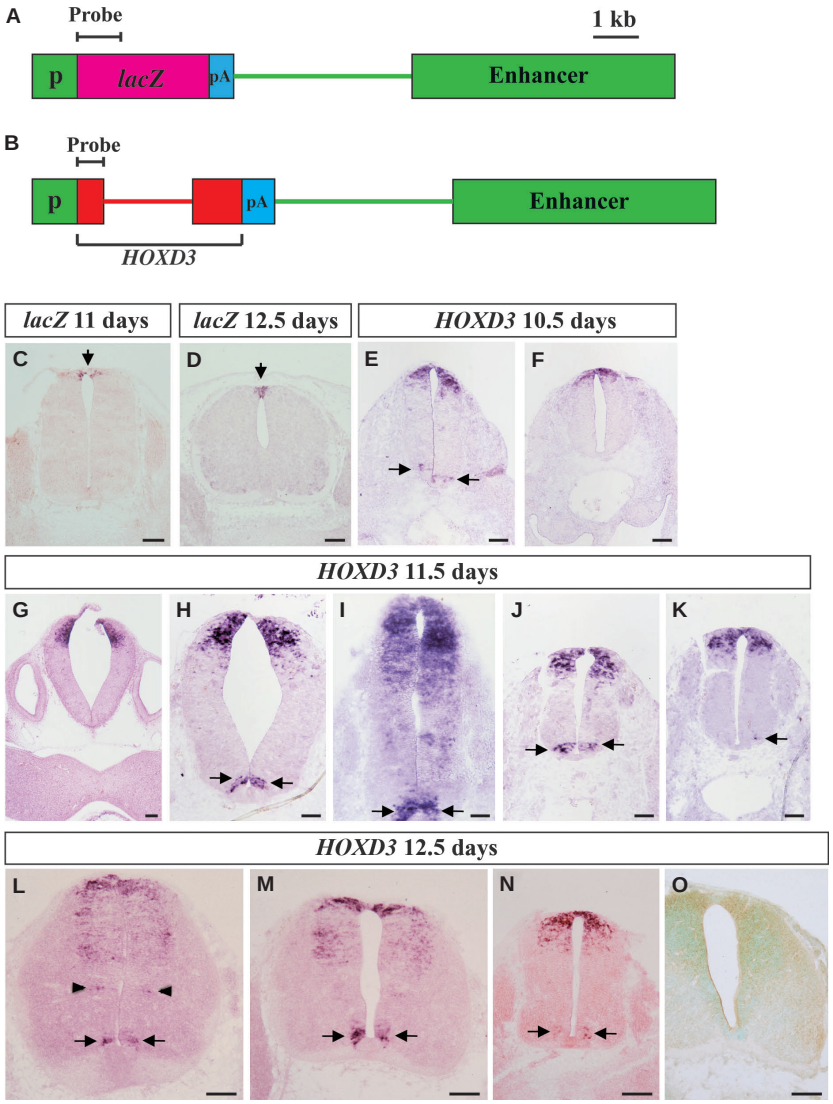

Fig. 1 Expression of the *lacZ* and *HOXD3* genes in transgenic embryos. (A,B) Structure of the Wexp-lacZ and Wexp-HOXD3 transgenes. Green boxes 'p' and 'enhancer' indicate the promoter and enhancer of the *Wnt1* gene, respectively. A purple box shows the *lacZ* gene. Two red boxes indicate the exons of the *HOXD3* gene, and a red bar shows the intron of the *HOXD3* gene. Blue boxes 'pA' in (A) and (B) represent the rabbit *β*-globin and SV40 polyadenylation signals, respectively. (C,D) Expression of the *lacZ* gene in transverse sections of the neural tube at the thoracic level of Wexp-lacZ transgenic embryos. The transcript of *lacZ* was detected by *in situ* hybridization with the *lacZ* RNA probe. Expression of *lacZ*, indicated by arrows, is seen restrictedly in the roof plate. (E–N) Expression of the *HOXD3* mRNA in transverse sections of Wexp-HOXD3 transgenic embryos. *HOXD3* expression was detected with the *HOXD3* RNA probe at the thoracic (E) and lumbar (F) levels in 10.5-days transgenic embryos. *HOXD3* expression was detected in 11.5-days transgenic embryos at the levels of the auditory vesicles (G), posterior hindbrain (H), cervical region (I), lumbar region (J) and hindlimb (K). In 12.5-days transgenic embryos, *HOXD3* expression was detected at the cervical (L), thoracic (M) and hindlimb (N) levels. (O) Expression of the HOXD3 protein in transverse sections of Wexp-HOXD3 transgenic embryos. Transverse sections at the thoracic level in 12.5-days transgenic embryos were treated with anti-HOXD3 antibody and methyl green. HOXD3-positive cells were stained brown. Expression of *HOXD3* is seen from the roof plate to more ventral regions of the neural tube. Small groups of *HOXD3*-expressing cells immediately adjacent to the floor plate are indicated by arrows. Scale bar: 100 μm.

Genotype determination

Genomic DNA isolated from the extraembryonic yolk sac was analyzed by Southern blot or polymerase chain reaction (PCR) to determine the genotype of F_0 embryos. For Southern blot analysis, *Eco*RI-digested DNA separated on agarose gels was probed with a 520-bp fragment that covers the nucleotide region encoding an N-terminal part of the HOXD3 protein (Taniguchi et al. 1995). In a PCR assay, pairs of primers from the *Wnt1* promoter region (5′-AACTATAAGAGGCCTATAA-GAGGCGGTGCC-3′) and the *lacZ* coding region (5′-GGCGAT-TAAGTTGGGTAACGCCAGGGTTTT-3′), or the *HOXD3* coding region (5′-CTCTTTCATCCAGGGGAAGATCTGCTTGCT-3′) were used to determine whether an embryo was transgenic. By PCR

analysis, the transgenes were amplified, yielding a 0.6-kb product for *lacZ* and a 0.7-kb product for *HOXD3*.

Histological analysis

Embryos were fixed overnight at 4 °C in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS). After the whole embryos were photographed with a stereomicroscope, they were dehydrated in a series of graded ethanols and embedded in paraffin, according to standard procedures. The embryos were serially sectioned at 3–6 μm in the transverse plane. Representative sections were stained with hematoxylin and eosin, and photographed with a photomicroscope. Alternatively, the fixed embryos were dehydrated, suspended in 30% sucrose in PBS, and frozen in OCT compound (MilesCo Scientific, Princeton, MN, USA) using dry ice-ethanol. Twenty-micrometer-thick cryostat sections were then made, mounted on slides and dried. For *in situ* hybridization and immunohistochemistry, the paraffin sections were stored at 4 °C and the frozen sections were stored at −80 °C until used.

In situ hybridization

Whole-mount *in situ* hybridization was performed essentially as described by Xu & Wilkinson (1998). Embryos were fixed overnight at 4 °C in 4% paraformaldehyde in PBS, then dehydrated in ethanol and stored at −20 °C until used. *In situ* hybridization on paraffin sections was performed according to the protocol of M. Kessel's laboratory (Gottingen), except that hybridization was carried out overnight at 70 °C. *In situ* hybridization on frozen sections was carried out in accordance with the manufacturer's manual (Nippon Gene, Tokyo, Japan). Using a DIG RNA labeling kit (Roche, Basel, Switzerland), digoxigenin-labeled RNA probes were transcribed by T7 RNA polymerase from the promoter of pBluescript II (Stratagene, La Jolla, CA, USA) or pT7Blue (Novagen, Darmstadt, Germany), into which various genes were cloned. A 1.1-kb *Eco*RI-*Ban*III fragment of the *lacZ* gene was inserted into pBluescript II. This construct was used as a template for the *lacZ* RNA probe. To synthesize the *HOXD3* RNA probe, a 525-bp *Eco*RI-*Pst*I fragment covering the nucleotide sequence of the first exon in the *HOXD3* gene (Taniguchi et al. 1992) was cloned into pBluescript II. To amplify a 415-bp fragment containing the *Wnt1* gene, a pair of primers from the *Wnt1* 5′ untranslated region (5′-UTR) (5′-AGACTTATTAGAGCCAGCCTGGGAA-3′) and the *Wnt1* second exon (5′-AATTGCCATTTGCACTCTCGCACAG-3′) were used in an reverse transcriptase (RT)-PCR assay. The resultant fragment was inserted into pT7Blue. This construct was linearized with *Nco*I whose restriction site is located in the nucleotide sequence containing the *Wnt1* initiation codon ATG, and the *Wnt1* RNA probe was synthesized with the linearized construct. Thus, the 126-bp *Wnt1* 5′-UTR is eliminated from the *Wnt1* RNA probe. The *Sox10* probe was transcribed from a cloned 890-bp fragment of the mouse 3′-UTR, which was a gift from Ralf Kist. The remaining probes were synthesized with the pT7Blue vector into which RT-PCR products from 10.5-day-old mouse embryo RNA were cloned. Primer pairs used for PCR amplification were as follows: *Hoxd3*, forward primer (5′-GAAGGCTGCATACTACGAGA-3′) and reverse primer (5′-CGGGTGGGCTCTTGTCCTCA-3′); *Msx1*, forward primer (5′-AGAAGCAGTACCTGTCTATTGCCGA-3′) and reverse primer (5′-AATCTCTTGGCCTCTGCATCCTTAG-3′); *Math1*, forward primer (5′-ATCAGACCTTGCAGAAGAGACTAGG-3′) and reverse primer (5′-TTGAAGGACGGGATAACGTTGCGCA-3′); *Pax3*, forward primer (5′-CTGAACCTGATTTACCGCTGAAGAG-3′) and reverse primer (5′-GTGGGCAGACTGTCCACATTCTTCA-3′); *Jagged1*, forward primer (5′-GAGACTCCTTCACCTGTGTCTGCAA-3′) and reverse primer (5′-TTCGCTGCAAATGTGTTCGGTGGTA-3′); *Isl1*, forward primer (5′-GCGGCAATCAAATTCACGACCAGTA-3′) and reverse primer (5′-TGAAACCACACTCGGATGACTCTGG-3′); *Cadherin 6*, forward primer (5′-ATGGCTGATGTTGGCACATTTGTGG-3′) and reverse primer (5′-TCACTGTGATGTTGTGCCACAGCAG-3′); *Noelin 1*, forward primer (5′-CATAAGCAGCATCTAGCCAGAGAGT-3′) and reverse primer (5′-CGGATGACATGGAAGAGGGTGACAT-3′).

Immunohistochemistry

Deparaffinized sections were washed in 0.1 M Tris–HCl (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20 (TNT), blocked with 5% skim milk in TNT, and incubated with a rabbit anti-human N-cadherin antibody (Calbiochem, Darmstadt, Germany) overnight at 4 °C. Frozen sections were washed in TNT, blocked with 5% sheep serum in TNT, and incubated with a rabbit anti-human HOXD3 antibody (Abcam, Cambridge, UK). When using anti-human HuC/D (Molecular Probes, Eugene, OR, USA) and anti-BrdU (Becton Dickinson, Franklin Lakes, NJ, USA) monoclonal antibodies, samples were treated with 0.1% pepsin (Wako, Osaka, Japan) in 0.1 N HCl for 20 min at room temperature and 2 N HCl for 20 min at room temperature before blocking. Sections were then incubated with biotinylated secondary antibodies for 1 h at room temperature, washed and further incubated with the Vectastatin ABC reagent (Vector Laboratories, Burlingame, CA, USA) in PBS according to the manufacturer's instructions. Sections were washed in PBS and stained with 3,3′-diaminobenzidine.

Laser microdissection (LMD) and dot blot analysis

For LMD, we used 20-μm-thick frozen sections that were obtained from 4% paraformaldehyde-fixed embryos at 12.5 days. A region including the entire ventricular zone from three serial sections was cut and collected by microscopic laser-based dissection using a laser microdissection system (PALM MicroBeam IV, Carl Zeiss, Oberkochen, Germany). Proteins from the dissected samples were extracted by the method of peptide extraction (Takada et al. 2010) with a slight modification. The samples were homogenized in 80 μL of 8 M urea solution with an ultrasonic homogenizer. After homogenization, 90 μL of 50 mM ammonium bicarbonate buffer (pH 8.0) was added, followed by the addition of 10 μL of 1 mM dithiothreitol solution. The samples were incubated at 37 °C for 60 min. Next, 10 μL of 100 mM iodoacetamide solution was added, and the samples were incubated at 37 °C for 30 min. Moreover, 10 μL of trypsin solution (5 $\mu g\ mL^{-1}$) (Promega, Madison, WI, USA, Mass Spectrometry Grade) was added, and proteins in the samples were digested by overnight incubation at 37 °C. Fifty microliters of the digestion products was spotted onto transfer membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated overnight at 4 °C with a rabbit anti-human N-cadherin antibody (Calbiochem) at 1 : 1000 dilution and a monoclonal anti-β-actin antibody (Sigma) at 1 : 5000 dilution. Then, the membranes were incubated for 1 h at 37 °C with

horseradish peroxidase-conjugated secondary antibodies. The chemiluminescence reaction was performed with ECLplus (GE Healthcare, Buckinghamshire, UK). Immunoreactive signals were captured by ATTO Cool Saver (ATTO, Tokyo, Japan) and analyzed by ATTO CS analyzer software (Atto). Before dot blotting was performed, we had confirmed that the antigenic peptides used to make anti-N-cadherin and anti-β-actin antibodies were isolated by trypsin from N-cadherin and β-actin proteins using ProteinProspector MS-Digest available at prospector2.ucsf.edu/.

Results

Expression of the *lacZ* and *HOXD3* genes exogenously introduced in histological sections of transgenic embryos

In order to induce *HOXD3* expression in dorsal midline cells of the neural tube during embryonic development, we attempted to generate transgenic mice in which the *HOXD3* gene was expressed under the control of the *Wnt1* regulatory element. We expected that *HOXD3* expression gave rise to aberrant migration of neural crest cells and malformation of the neural tube leading to embryonic lethality, so we concentrated on producing and analyzing F_0 transgenic embryos. As a result, we found that *HOXD3* exogenously introduced was more broadly expressed than endogenous *Wnt1*. Thus, to know whether this broad expression is dependent on *HOXD3* expression itself or not, we generated transgenic embryos in which expression of the *lacZ* gene was driven by the *Wnt1* regulatory element, and compared the location of *HOXD3* expression in *HOXD3*-expressing transgenic embryos with that of *lacZ* expression in *lacZ*-expressing transgenic embryos.

In an experiment where the expression vector Wexp-lacZ was microinjected, a total of 101 F_0 embryos at 11 and 12.5 dpc were collected. Seven of all collected embryos carried the Wexp-lacZ DNA. To determine whether *lacZ* was expressed in the embryos, *in situ* hybridization was performed on the paraffin or frozen transverse sections with the *lacZ* antisense probe shown in Fig. 1A. Four of seven transgenic embryos expressed the *lacZ* gene. The remaining three transgenic embryos did not express the *lacZ* gene at all. In all embryos expressing the *lacZ* gene, expression of *lacZ* was restricted to roof-plate cells within the neural tube (shown by arrows in Fig. 1C,D). From these results, we could confirm that the transgene was expressed correctly and restrictedly in roof-plate cells by the *Wnt1* regulatory element we used.

We carried out three experiments, in which the expression vector Wexp-HOXD3 was microinjected, and collected F_0 embryos at 10.5, 11.5, 12.5 and 13.5 days. Of 149 collected embryos, 27 embryos carried the Wexp-HOXD3 DNA (Table 1). To determine whether *HOXD3* was expressed in the transgenic embryos, *in situ* hybridization was performed on the paraffin or frozen transverse sections at the levels from the caudal myelencephalon to the spinal cord with the human *HOXD3* antisense probe shown in Fig. 1B. Of 25 transgenic embryos in which we could examine expression of the *HOXD3* transgene, 13 embryos expressed the *HOXD3* gene and the remaining 12 embryos showed no *HOXD3*-positive signals (Table 1). In all *HOXD3*-expressing transgenic embryos, expression of *HOXD3* was not only located in the roof plate, but also had spread in more ventral regions of the neural tube (Table 1). In the transgenic embryos at 10.5 days, *HOXD3*-expressing cells were localized in the roof plate and its vicinity (Fig. 1E,F). At 11.5 days, *HOXD3* expression was not confined in the roof plate, and was located in the greater part of the dorsal neural tube at the hindbrain level to the hindlimb level (Fig. 1G–K). The broadest expression of *HOXD3* in the neural tube was seen at the cervical level. A large number of *HOXD3*-expressing cells had spread over the dorsal half of the ventricular zone and a small number of the cells

Table 1 Summary of Wexp-HOXD3 transgenic experiments.

Embryonic stage (dpc)	Number of collected embryos	Number of transgenic embryos	Number of embryos expressing *HOXD3* from the roof plate to more ventral regions	Number of embryos expressing *HOXD3* restrictedly in the roof plate	Number of embryos showing no *HOXD3* expression
Experiment I					
10.5	24	7*	3	0	2
12.5	19	2	1	0	1
Experiment II					
10.5	41	4	2	0	2
12.5	23	4	2	0	2
Experiment III					
11.5	26	7	4	0	3
13.5	16	3	1	0	2

*Two embryos were not prepared for *in situ* hybridization.

scattered into more ventral regions (Fig. 1I). No *HOXD3*-positive signals were found in the intermediate and marginal zones. At 12.5 days, *HOXD3*-expressing cells were distributed in the dorsal half region and the central small parts (shown by arrowheads in Fig. 1L) of the ventricular zone at the cervical level, located in the dorsal half part of the ventricular zone at the thoracic level, and localized in the roof plate and its vicinity at the hindlimb level (Fig. 1L-N). In addition to the expression of *HOXD3*, a small population of *HOXD3*-expressing cells was found immediately adjacent to the floor plate (shown by arrows in Fig. 1E,H–N). This expression extended from the caudal myelencephalon to more posterior regions of the spinal cord at 10.5–13.5 days. Except the neural tube, the heart alone expressed *HOXD3*. *HOXD3*-positive cells were scattered in the walls of the atrium and ventricle of the heart in *HOXD3*-expressing embryos, whereas no *lacZ*-positive signals were observed in the heart in *lacZ*-expressing embryos (see Supporting Information Fig. S1a,b). By means of an antibody to the HOXD3 protein, we detected HOXD3-positive cells from the dorsal neural tube to more ventral regions inside the ventricular zone in *HOXD3*-expressing transgenic embryos (Fig. 1O). On the other hand, HOXD3-positive cells were not detected in the neural tube of normal embryos (not shown). These results from the experiments of microinjection of *lacZ* and *HOXD3* suggest that the HOXD3 protein, which is produced from the *HOXD3* transgene, is expressed in broader regions than *Wnt1* is normally expressed.

In the neural tube of normal embryos, endogenous *Hoxd3* expression was detected at background levels by the human *HOXD3* probe (Fig. 2A,B). In the neural tube of *HOXD3*-expressing transgenic embryos, a profile of *HOXD3* expression recognized by the human *HOXD3* probe was similar to that detected by the mouse *Hoxd3* probe (see

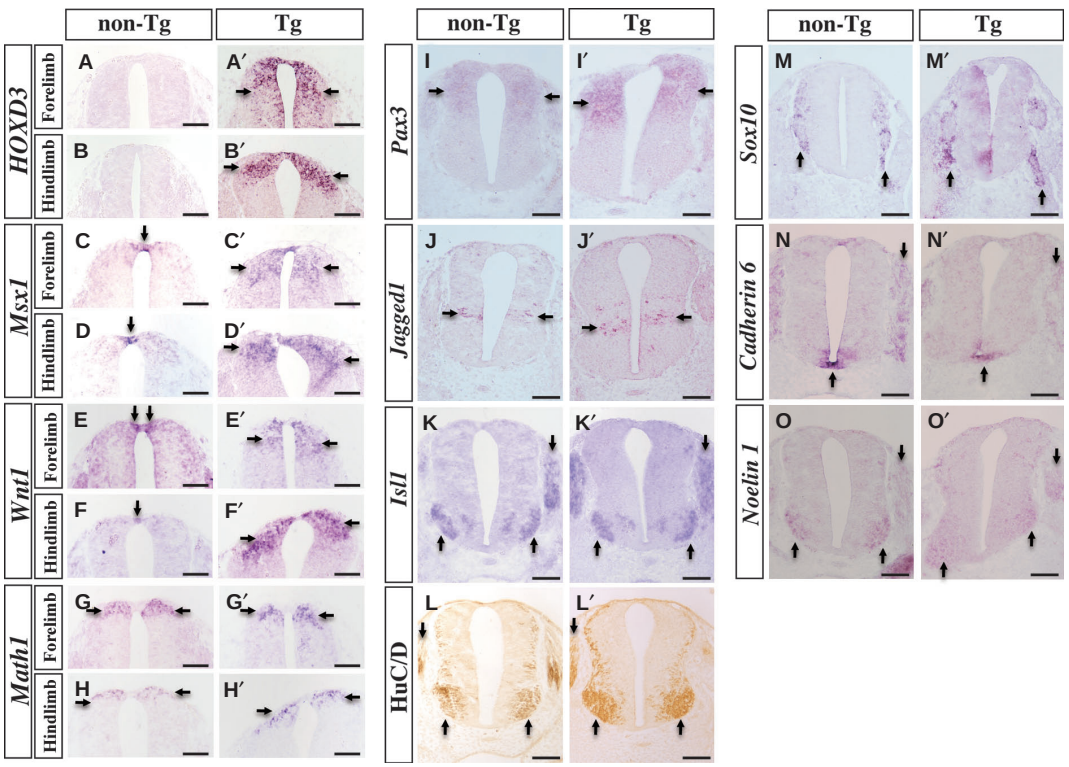

Fig. 2 Expression of marker genes for the dorsal neural tube, neural progenitors, differentiated neurons and neural crest cells. Expression of *HOXD3* and marker genes in transverse sections of *HOXD3*-expressing transgenic embryos (Tg) at 11.5 days was compared with that in transverse sections of non-transgenic embryos (non-Tg) at 11.5 days using *in situ* hybridization or immunohistochemistry. Expression of *HOXD3* and marker genes is indicated by arrows. (A,A′,B,B′) Expression of endogenous *Hoxd3* at the forelimb (A) and hindlimb (B) levels in non-Tg embryos and expression of the *HOXD3* transgene at the forelimb (A′) and hindlimb (B′) levels in Tg embryos. (C,C′,D,D′) Expression of *Msx1* at the forelimb (C) and hindlimb (D) levels in non-Tg embryos and at the forelimb (C′) and hindlimb (D′) levels in Tg embryos. (E,E′,F,F′) Expression of *Wnt1* at the forelimb (E) and hindlimb (F) levels in non-Tg embryos and at the forelimb (E′) and hindlimb (F′) levels in Tg embryos. (G,G′,H,H′) Expression of *Math1* at the forelimb (G) and hindlimb (H) levels in non-Tg embryos and at the forelimb (G′) and hindlimb (H′) levels in Tg embryos. Expression of both *Wnt1* and *Msx1* in Tg embryos is extended further ventrally than that in non-Tg embryos, whereas expression of *Math1* in Tg embryos is similar to that in non-Tg embryos. (I–O,I′–O′) Expression of *Pax3* (I), *Jagged1* (J), *Isl1* (K), HuC/D (L), *Sox10* (M), *Cadherin 6* (N) and *Noelin 1* (O) in non-Tg embryos, and expression of *Pax3* (I′), *Jagged1* (J′), *Isl1* (K′), HuC/D (L′), *Sox10* (M′), *Cadherin 6* (N′) and *Noelin 1* (O′) in Tg embryos at the hindlimb level. Expression patterns of markers for neural progenitors, differentiated neurons and neural crest cells in non-Tg embryos are very similar to those in Tg embryos. Scale bar: 100 μm.

Supporting Information Fig. S2a,b). These results indicate that, in addition to endogenous *Hoxd3* expression from the caudal myelencephalon to the spinal cord (Tan et al. 1996), the *HOXD3* gene exogenously introduced is excessively expressed in the neural tube of the transgenic embryos. In our study, we primarily examined the effect of overexpression of the *HOXD3* gene in the neural tube.

Expression of marker genes for the dorsal neural tube, neural progenitors, neurons and neural crest cells

To assess whether the expression of the *HOXD3* transgene influences expression of marker genes for the dorsal neural tube, we compared expression of *HOXD3*, *Msx1*, *Wnt1* and *Math1* in 11.5-days *HOXD3*-expressing embryos with that in 11.5-days normal embryos. Endogenous *Hoxd3* expression in normal embryos was detected at background levels (Fig. 2A,B), whereas expression of the *HOXD3* transgene was detected from the roof plate to more ventral regions of the neural tube (Fig. 2A′,B′). The expression of *Msx1* gene has been reported as becoming restricted to cells in and immediately adjacent to the roof plate between E9.5 and E11 (Hill et al. 1989; Robert et al. 1989). This expression pattern was identical to that of normal embryos (Fig. 2C,D). However, in *HOXD3*-expressing embryos, *Msx1* expression extended further down the roof plate into more ventral regions (Fig. 2C′,D′). Similarly, *Wnt1* expression in *HOXD3*-expressing embryos (Fig. 2E′,F′) was expanded more ventrally in the neural tube than that in normal embryos (Fig. 2E,F). Expression of *Math1* flanking the roof plate in normal embryos (Fig. 2G,H; Akazawa et al. 1995) was in accord with that in *HOXD3*-expressing embryos (Fig. 2G′,H′). These results suggest that expression of the *HOXD3* transgene gives rise to expansion of roof-plate cells in a dorsal-to-ventral direction within the neural tube.

To examine effects of the roof-plate expansion on the expression domains of marker genes for neural progenitors and differentiated neurons, we compared expression of *Pax3*, *Jagged1*, *Isl1* and HuC/D in 11.5-days normal embryos with that in 11.5-days *HOXD3*-expressing embryos. *Pax3* is expressed in progenitor cells in the dorsal half part of the neural tube (Goulding et al. 1991), whereas *Jagged1* is expressed in progenitor cells of the central part of the neural tube (Lindsell et al. 1995). There was little difference in both the expression domains of *Pax3* and *Jagged1* between normal and *HOXD3*-expressing embryos (Fig. 2I,I′,J,J′). *Isl1* encoding a transcription factor and HuC/D, an RNA-binding protein, are neuron-specific markers (Szabo et al. 1991; Pfaff et al. 1996). The expression domains of *Isl1* and HuC/D in normal embryos were identical with those in *HOXD3*-expressing embryos (Fig. 2K,K′,L,L′). It is possible that the expansion of roof-plate cells may not affect the identity of neural progenitor cells and the differentiation of neurons from progenitor cells within the neural tube.

To elucidate whether neural crest marker genes are downstream targets of the HOXD3 protein, we compared expression of *Sox10*, *Cadherin 6* and *Noelin 1*, which is maintained during neural crest cell migration (Inoue et al. 1997; Kuhlbrodt et al. 1998; Moreno & Bronner-Fraser, 2002), between 11.5-days normal and *HOXD3*-expressing embryos. Consequently, it was confirmed that expression of *HOXD3* in the neural tube was not related to that of *Sox10*, *Cadherin 6* and *Noelin 1* (Fig. 2M–O,M′–O′).

Morphological abnormalities of the neural tube in transgenic embryos expressing *HOXD3*

Comparing histological and immunohistochemical sections of normal embryos with those of *HOXD3*-expressing embryos, we investigated whether the expansion of roof-plate cells causes morphological abnormalities within the spinal cord. As a result, in spite of partial expression of *HOXD3* in the spinal cord, unusual features were found to appear throughout the neural tube along a dorsoventral axis.

In *HOXD3*-expressing embryos at 10.5 days, apparent disarrangement and overgrowth of progenitor cells in the ventricular zone were observed. In contrast to proliferating progenitor cells stretching from the luminal surface to the outside surface in normal embryos (Fig. 3A,E,G), progenitor cells in *HOXD3*-expressing embryos seemed to be crowded into the outer layer without stretching (Fig. 3B,F,H). So, to confirm whether the roof-plate expansion brings about an increase in progenitor population, we measured the number of M-phase nuclei on the luminal surface of the spinal cord at the hindlimb level. We found that the number of mitotic nuclei in *HOXD3*-expressing embryos was about 1.5-fold higher than in normal embryos (Fig. 3I–K). Furthermore, we labeled non-transgenic and transgenic embryos with BrdU, and examined BrdU incorporation into S-phase nuclei of neural tube cells by means of immunohistochemistry using an anti-BrdU antibody. In normal embryos, S-phase nuclei in progenitor cells were localized in the outside surface of the neural tube (Fig. 3L), and extended constantly in a luminal-to-basal direction (Fig. 3N). By contrast, in *HOXD3*-expressing embryos, S-phase nuclei were distributed over the entire ventricular zone (Fig. 3M). Some nuclei were rounded, and some were stretched in irregular directions (Fig. 3O). These results suggest that the *HOXD3* expression gives rise to not only overgrowth of progenitor cells, but also failure in the movement of nuclei from the luminal surface of the neural tube to the outside surface within these cells during the progression of the cell cycle. However, there was little apparent difference in the morphology of neural crest-derived structures, such as the dorsal root ganglia (Fig. 3A,B) and sympathetic ganglia (Fig. 3C,D).

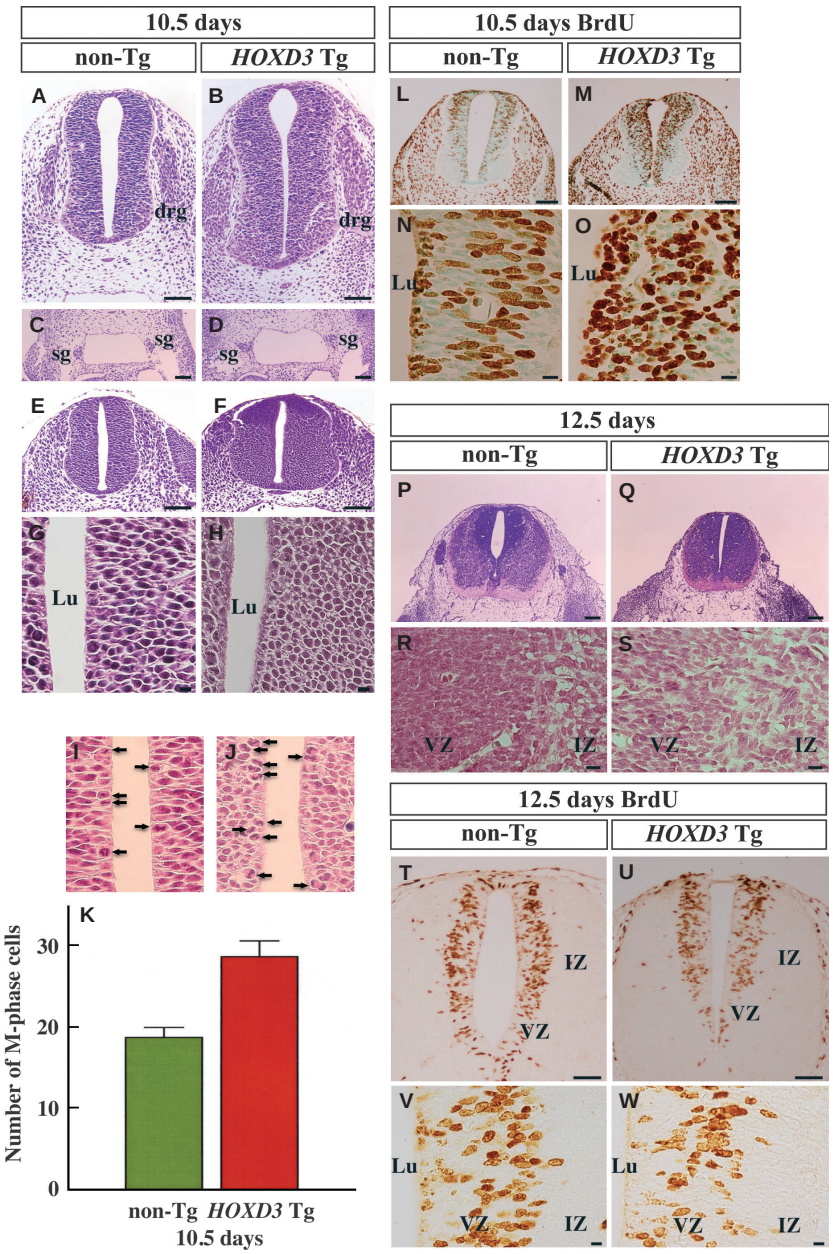

Fig. 3 Morphology of the neural tube and neural crest-derived structures. (A–H) Transverse sections of non-transgenic embryos (non-Tg: A,C,E,G) at 10.5 days and *HOXD3*-expressing transgenic embryos (*HOXD3* Tg: B,D,F,H) at 10.5 days were stained with hematoxylin and eosin. (A–D) Pictures from sections at the thoracic level; (E,F) pictures from sections at the hindlimb level. (G,H) Higher-magnification views of the neural tube in (E,F), respectively. (I–K) Comparison of the number of M-phase cells in the neural tube. The number of M-phase cells on the luminal surface of the neural tube at the hindlimb level was counted and averaged from five transverse paraffin sections of a non-transgenic embryo (non-Tg) and a *HOXD3*-expressing transgenic embryo (*HOXD3* Tg) at 10.5 days. Examples of M-phase cells in which chromosomes could be apparently observed are shown by arrows in I (non-Tg) and J (*HOXD3* Tg). Error bars on a graph indicate the range of standard deviations in the result from four independent non-Tg and *HOXD3* Tg embryos. (L–O) Transverse sections of 10.5-days non-Tg embryos (L,N) and 10.5-days *HOXD3* Tg embryos (M,O) at the hindlimb level were treated with anti-BrdU antibody and methyl green. (N,O) Higher-magnification views of the neural tube in (L,M), respectively. (P–S) Transverse sections of 12.5-days non-Tg embryos (P,R) and 12.5-days *HOXD3* Tg embryos (Q,S) at the cervical level were stained with hematoxylin and eosin. (R,S) Higher-magnification views of the boundary between the ventricular and intermediate zones with thinner sections (3 μm) in non-Tg embryos and *HOXD3* Tg embryos, respectively. (T–W) Transverse sections of 12.5-days non-Tg embryos (T,V) and 12.5-days *HOXD3* Tg embryos (U,W) at the cervical level were treated with anti-BrdU antibody. (V,W) Enlarged versions of (T,U), respectively. Failure of interkinetic nuclear migration of progenitor cells, which is seen in 10.5-days *HOXD3* Tg embryos, is not observed in 12.5-days *HOXD3* Tg embryos. drg, dorsal root ganglia; IZ, intermediate zone; Lu, lumen of the neural tube; sg, sympathetic ganglia; VZ, ventricular zone. Scale bars: 100 μm (A–F,L,M,P,Q,T,U); 10 μm (G,H,N,O,R,S,V,W).

At 12.5 days, in contrast to the distinct boundary between the ventricular and intermediate zones in normal embryos (Fig. 3P), the boundary seemed to be obscure in *HOXD3*-expressing embryos (Fig. 3Q). High-magnification images of the boundary with thinner histological sections revealed that progenitor cells in the ventricular zone were more loosely connected to each other in *HOXD3*-expressing embryos than in normal embryos (Fig. 3R,S). This result suggests that *HOXD3* expression causes a decrease in cell-to-cell adhesion among progenitor cells in the ventricular zone. In both normal and *HOXD3*-expressing embryos at 12.5 days, S-phase nuclei labeled by BrdU in progenitor cells were primarily located in the outside surface of the ventricular zone (Fig. 3T–W). Failure in the movement of nuclei within progenitor cells, which was seen in *HOXD3*-expressing embryos at 10.5 days, was removed at 12.5 days.

Alterations of N-cadherin protein expression in the neural tube of *HOXD3*-expressing embryos

Using histological sections, we observed that cell-to-cell adhesion among progenitor cells in the ventricular zone was reduced in *HOXD3*-expressing embryos at 12.5 days. Therefore, we compared expression levels of N-cadherin protein in the neural tube of normal embryos with those

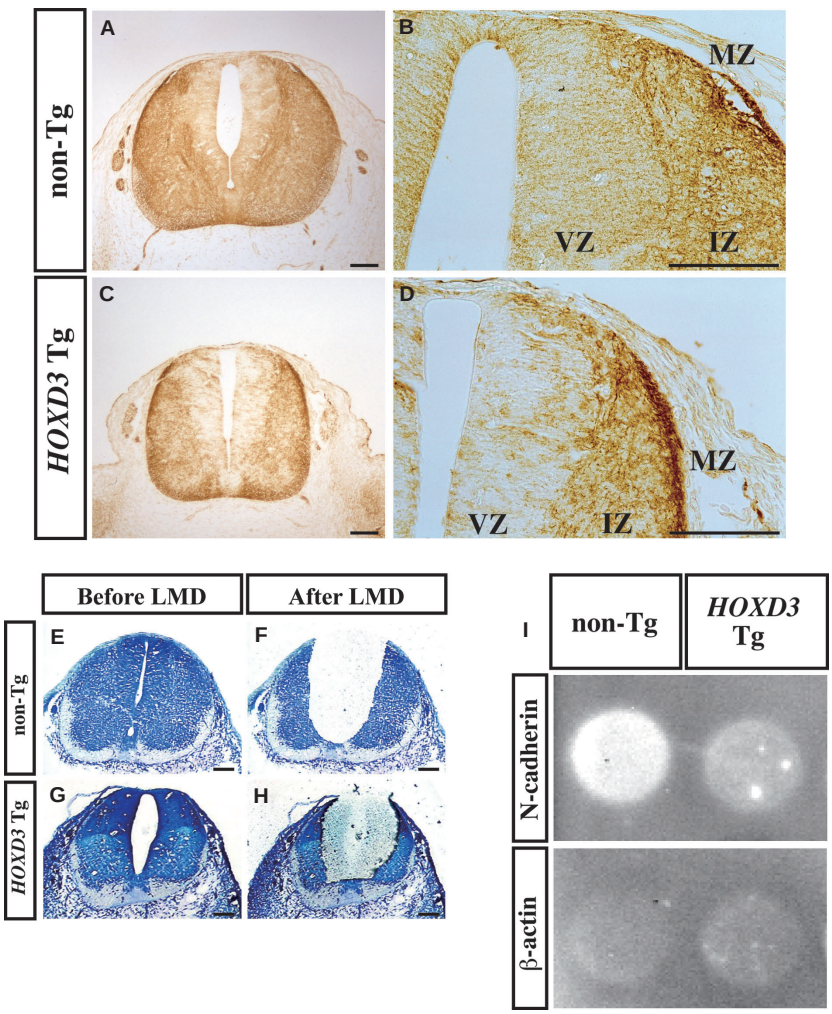

Fig. 4 Expression of N-cadherin protein in the neural tube. (A–D) Transverse paraffin sections at the cervical level in non-transgenic embryos (non-Tg: A,B) and *HOXD3*-expressing transgenic embryos (*HOXD3* Tg: C,D) at 12.5 days were treated with anti-human N-cadherin antibody. (B,D) Enlarged versions of the dorsal neural tube in (A,C), respectively. N-cadherin is strongly expressed in the ventricular zone of non-Tg embryos, whereas the ventricular zone in *HOXD3* Tg embryos is composed of a large number of progenitor cells that do not express N-cadherin. IZ, intermediate zone; MZ, marginal zone; VZ, ventricular zone. (E–H) From transverse frozen sections at the cervical level in non-Tg and *HOXD3* Tg embryos, a region including the entire ventricular zone was dissected with a laser microdissection (LMD) system. Toluidine blue-stained sections before LMD (E,G) and after LMD (F,H) are shown. (I) Peptides extracted from the dissected samples were immunoreacted with anti-N-cadherin and anti-β-actin antibodies using dot blot analysis. The signal intensity of N-cadherin normalized to β-actin in non-Tg embryos is 2.5-fold higher than that in *HOXD3* Tg embryos. Scale bar: 100 μm.

of *HOXD3*-expressing embryos by means of immunohistochemistry. In 12.5-days normal embryos, progenitor cells radially arranged in the ventricular zone strongly expressed N-cadherin protein (Fig. 4A,B). On the other hand, in the ventricular zone of *HOXD3*-expressing embryos, there were a large number of progenitor cells that did not express N-cadherin protein (Fig. 4C,D). N-cadherin-negative progenitor cells were distributed throughout the ventricular zone, which was more extensive than the area where *HOXD3*-expressing cells were localized (compare Fig. 4C with Fig. 1L). To further confirm the reduction in expression levels of N-cadherin in *HOXD3*-expressing embryos, we dissected a region including the entire ventricular zone from frozen sections of 12.5-days paraformaldehyde-fixed embryos (Fig. 4E–H). Then, peptides extracted from the dissected samples were immunoreacted with N-cadherin and β-actin antibodies by dot blot analysis. The analysis showed a 60% reduction in the signal intensity of N-cadherin normalized to β-actin in *HOXD3*-expressing embryos relative to that in normal embryos (Fig. 4I). In 11.5-days *HOXD3*-expressing embryos, the ventricular zone was composed of both N-cadherin-positive and N-cadherin-negative progenitor cell populations in a mosaic manner (see Supporting Information Fig. S3e,f). These observations suggest that *HOXD3*-expressing cells have a negative effect on N-cadherin expression more broadly than in the area where these cells are located.

In addition to the above difference, a variation of N-cadherin expression was found. In *HOXD3*-expressing embryos, more excessive N-cadherin protein accumulated in the marginal zone of the spinal cord than in the intermediate zone (Fig. 4A,C; see Supporting Information Fig. S3e,f). It is likely that N-cadherin protein clustered around the axonal termination of the neurons that arose from N-cadherin non-expressing progenitor cells in the neural tube.

Discussion

To elucidate a role for HOXD3 in the regulation of cell adhesion process during embryonic development, we analyzed transgenic mouse embryos in which the human *HOXD3* gene is expressed in the dorsal midline under the control of the *Wnt1* regulatory element. Our analysis shows that expression of *HOXD3* causes expansion of dorsal midline cells within the neural tube and has a negative effect on N-cadherin expression in a number of neural progenitor cells. These findings suggest that *HOXD3* expression plays a role in the regulation of cell adhesive properties.

The reasons why we used the human gene instead of the mouse gene are as follows. First, we presumed that the expression of human genes, exogenously introduced in individual cells of mouse embryos, would be more clearly detected. Actually, histochemistry by *in situ* hybridization showed that, in paraffin sections of mouse embryos, the human probe recognizes *HOXD3* expression with a lower background than the mouse probe. Thus, introduction of the human *HOXD3* gene would facilitate the detection of *HOXD3*-positive cells within the neural tube of mouse embryos. Second, because the amino acid sequence of the HOXD3 protein is 95% identical with that of the mouse Hoxd3 protein (Taniguchi et al. 1992; Tan et al. 1996), it was expected that there would be little difference in function between the two proteins. In fact, as for *Hoxd11*, *Hoxd12* and *Hoxd13*, the functional equivalence between the mouse and human proteins is verified by the experiment that the mouse vertebral defect caused by deleting *Hoxd* genes was almost rescued by the introduction of the human DNA including *HOXD* genes (Spitz et al. 2001).

In transgenic embryos that we generated using the expression vector Wexp-lacZ, expression of *lacZ* is localized exclusively in roof-plate cells. Thus, the *Wnt1* regulatory element in the expression vector can correctly drive expression of the transgene in roof-plate cells. On the other hand, in transgenic embryos that we created using the expression vector Wexp-HOXD3, expression of *HOXD3* is not only localized in the roof plate but also has spread in more ventral regions of the ventricular zone in the neural tube. Broader expression of *HOXD3* is seen in all transgenic embryos expressing *HOXD3*, so this variation does not come from insertion site-specific modulation of the transgene expression pattern. Because the expression pattern of the *HOXD3* transgene obtained by using the human *HOXD3* probe is identical with that obtained by using the mouse *Hoxd3* probe, the *HOXD3* transgene is much more abundantly expressed than the endogenous *Hoxd3* gene in the neural tube. Moreover, expression of both *Msx1* and *Wnt1*, roof-plate markers, is further extended ventrally in parallel with *HOXD3* expression. These findings suggest that overexpression of the *HOXD3* gene gives rise to expansion of the roof plate in a dorsal-to-ventral direction within the neural tube. However, because there is little difference in expression patterns of progenitor cell markers, *Pax3* and *Jagged1*, and neuron-specific markers, *Isl1* and HuC/D, between normal and *HOXD3*-expressing embryos, the roof-plate expansion does not seem to affect the identity of neural progenitor cells and the differentiation of neurons.

In the ventricular zone of the neural tube in *HOXD3*-expressing embryos at 10.5 days, there are many more neural progenitor cells. It has been reported that ectopic *Wnt1* expression throughout the neural tube causes a dramatic increase in the number of progenitor cells in the ventricular zone (Dickinson et al. 1994). Thus, it is likely that the expansion of roof-plate cells causes elevated expression of Wnt1 proteins, whose ventral diffusion non-autonomously leads to overgrowth of progenitor cells. Many progenitor cells in *HOXD3*-expressing embryos at 10.5 days do not exhibit interkinetic nuclear migration, in which their nuclei migrate between the luminal surface and the basal part of

the ventricular zone in synchrony with the cell cycle. This implies that the expansion of roof-plate cells interrupts extension of many progenitor cells in a luminal-to-basal direction. It is possible that roof-plate cells expressing *HOXD3* may be propagated in the dorsal neural tube and migrate in a dorsal-to-ventral direction within the neural tube. Therefore, excessive cells in the neural tube of *HOXD3*-expressing embryos might be derived from roof-plate cells as well as neural progenitor cells. However, *HOXD3*-expressing cells spreading inside the neural tube do not express *Sox10*, *Cadherin 6* and *Noelin 1*, which are markers for migrating neural crest cells. At present, we do not have definite evidence that *HOXD3*-expressing cells migrate within the neural tube.

In *HOXD3*-expressing embryos at 12.5 days, loss of N-cadherin expression in a large number of progenitor cells leads to a decrease in cell-to-cell adhesion among these cells. Although expression of *HOXD3* is localized in the dorsal half of the neural tube and the positions immediately adjacent to the floor plate, progenitor cells that do not express N-cadherin are distributed throughout the ventricular zone. This finding indicates that *HOXD3*-expressing cells have a negative effect on N-cadherin expression in more extensive areas than in the area where these cells are localized. The mechanism by which *HOXD3* expression reduces expression levels of N-cadherin protein remains to be resolved. A possibility is that N-cadherin expression is regulated in a non-cell autonomous manner. Signal molecules produced in the expanded roof plate or secreted proteins whose expression is induced by HOXD3 may be involved in a decrease of N-cadherin expression.

In this study, we show that overexpression of *HOXD3*, a human counterpart of the mouse *Hoxd3* gene, under the control of the *Wnt1* enhancer induces expansion of roof-plate cells and reduces expression levels of N-cadherin in a number of neural progenitor cells within the neural tube. *HOXD3*-expressing cells have a negative effect on N-cadherin expression more extensively than in the area where these cells are located. This regulatory system conferred by HOXD3 might be employed in various aspects in the morphogenesis where the *Hoxd3* gene actually functions during embryonic development.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of the transgenes in the heart.

Fig. S2. Expression of the *HOXD3* transgene detected by the human *HOXD3* and the mouse *Hoxd3* probe.

Fig. S3. Morphology of the neural tube and expression of N-cadherin in non-transgenic and *HOXD3*-transgenic embryos at 11.5 days.

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