Mouse Zic5 deficiency results in neural tube defects and hypoplasia of cephalic neural crest derivatives

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Abstract

Zic family genes encode zinc finger proteins, which are homologues of the Drosophila pair-rule gene odd-paired. In the present study, we characterized the fifth member of the mouse Zic family gene, mouse Zic5. Zic5 is located near Zic2, which is responsible for human brain malformation syndrome (holoprosencephaly, or HPE). In embryonic stages, Zic5 was expressed in dorsal part of neural tissues and limbs. Expression of Zic5 overlapped with those of other Zic genes, most closely with Zic2, but was not identical. Targeted disruption of Zic5 resulted in insufficient neural tube closure at the rostral end, similar to that seen in Zic2 mutant mice. In addition, the Zic5-deficient mice exhibited malformation of neural-crest-derived facial bones, especially the mandible, which had not been observed in other Zic family mutants. During the embryonic stages, there were delays in the development of the first branchial arch and extension of the trigeminal and facial nerves. Neural crest marker staining revealed fewer neural crest cells in the dorsal cephalic region of the mutant embryos without significant changes in their migration. When mouse Zic5 was overexpressed in Xenopus embryos, expression of a neural crest marker was enhanced. These findings suggested that Zic5 is involved in the generation of neural crest tissue in the dorsal cephalic region of the mutant embryos without significant changes in their migration. When mouse Zic5 was overexpressed in Xenopus embryos, expression of a neural crest marker was enhanced. These findings suggested that Zic5 is involved in the generation of neural crest tissue in mouse development. ZIC5 is also located close to ZIC2 in humans, and deletions of 13q32, where ZIC2 is located, lead to congenital brain and digit malformations known as the “13q32 deletion syndrome”. Based on both their similar expression pattern in mouse embryos and the malformations observed in Zic5-deficient mutant mice, human ZIC5 might be involved in the deletion syndrome.

Keywords: Zic family; Zic5; Transcription factor; Gene targeting; Neural tube defect; Neural crest

Introduction

Zic genes encode zinc finger transcription factors that mediate diverse events in vertebrate development (Aruga et al., 1998; Nagai et al., 2000; Nakata et al., 1997); reviewed in Aruga, 2004). The Zic family was originally identified as a group of genes expressed in adult mouse cerebellum (Aruga et al., 1994), and its members share a conserved zinc finger domain similar to the Drosophila pair-rule gene odd-paired (opa), which is required for the timely activation of a segment polarity gene, wingless, in the parasegment of the embryo (Benedyk et al., 1994). The zinc finger domains of Zic proteins, consisting of five tandemly repeated C2H2-type zinc finger motifs, are also similar to those of Gli-Ci zinc finger proteins (Hui et al., 1995; Ruppert et al., 1988). Multiple Zic genes have been identified both in mouse and Xenopus, and they are expressed in overlapping, but distinct patterns (Nagai et al., 1997; Nakata et al., 2000).

Four Zic genes have been reported in mice to date (Aruga et al., 1994, 1996a,b). In developing animals, Zic gene expressions can be detected at gastrulation in the ectoderm and mesoderm. During organogenesis, their expression is restricted to the dorsal neural tube, somites, and limb buds

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(Nagai et al., 1997). At the adult stage, all mouse Zic genes are strongly expressed in the cerebellum. Despite the overlap in expression among the Zics, mutants for each gene show distinct phenotypes in neural development. Disruption of the mouse Zic1 gene results in dysgenesis of the cerebellum and a behavioral abnormality (Aruga et al., 1998). Reduced expression of another member of the Zic family, Zic2, leads to holoprosencephaly (HPE), exencephaly, spina bifida, and skeletal abnormalities (Nagai et al., 2000). Similarly, mutations in human ZIC2 lead to a congenital brain anomaly, known as HPE (Brown et al., 1998). Mutations in Zic3 result in skeletal abnormalities, neural tube defects (NTDs), and abdominal and thoracic situs disturbance (Carrel et al., 2000; Franke et al., 2003; Klootwijk et al., 2000; Purandare et al., 2002). In humans, ZIC3 mutations are responsible for the derangements of the left–right body axis, in some cases in combination with a lumbosacral NTD (Gebbia et al., 1997). All these findings suggest that Zic family genes exert an essential role in neural development.

Recently, Furushima et al. (2000) described the cloning of a mouse cDNA encoding a novel zinc finger protein called Opr (opa related). Opr has a zinc finger motif very similar to that of Zic proteins, suggesting that opr is a novel member of the Zic gene family. Opr expression has been found in the anterior neural plate, dorsal part of the neural tube, and limbs, and seems to overlap with the expression of other Zic family genes. However, functional aspects of Opr as well as Zic4 have not been described.

On the other hand, gain-of-function studies in Xenopus suggest that they have a conserved role in the initial phase of neural and neural crest development. Four Zic genes have been reported in Xenopus (XZic1, XZic2, XZic3, and XZic5), and all four are expressed in the prospective neural plate and the neural plate border region (Nakata et al., 1997, 1998, 2000). In Xenopus, expression of the Zic genes are very early response to neural inducing signals, and ectopic expression of these Zic genes leads to neural plate and neural crest formation (Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 1997, 1998, 2000). However, in mice, the role of Zic family genes in neural crest development is not definitive because neural-crest-derived tissues form normally in Zic1, Zic2, and Zic3 mutant mice. The only finding concerning defective neural crest derivatives is the poor dorsal root ganglion formation in the hypomorphic Zic2 mutants (Nagai et al., 2000), which is difficult to interpret in the presence of severe neural and skeletal abnormalities.

In our attempts to isolate and characterize additional Zic-related genes, we had identified the fifth member of Zic family (Zic5), which was identical to Opr. To investigate the role of Zic5/Opr in mouse development, we disrupted the gene by homologous recombination in embryonic stem (ES) cells. Mutant embryos showed defects in the anterior neural tube and malformations of neural-crest-derived facial bones, including the mandible. Development of the first branchial arch and extension of the trigeminal and facial nerves were also impaired in mutant embryos. Overexpression experiments in Xenopus embryos demonstrated that mouse Zic5 has neural-crest-tissue-inducing activity. Our results suggest that Zic5 plays crucial roles in neural and neural crest development.

**Materials and methods**

**Animals**

Animal experiments were conducted essentially as described previously (Aruga et al., 1998). Animals were mated overnight, and the females were examined for a vaginal plug in the following morning. Noon on the day a vaginal plug was detected was recorded as day E0.5. The mice were maintained by the Research Resource Center, RIKEN Brain Science Institute. All animal experiments were carried out according to the guidelines for animal experimentation in RIKEN. The developmental stage at E8.5–9.5 was precisely matched between wild-type and mutant embryos by counting the number of the somite.

**Isolation of mouse Zic5 cDNA clones**

To search for additional members of the Zic gene family, we screened a mouse 129/SV genomic library (Stratagene) with low stringency, using the zinc finger domain of mouse Zic1 as a probe (Aruga et al., 1996a). Full-length Zic5 cDNA was obtained by screening the lambda gt11 mouse cerebellum cDNA library (Furuichi et al., 1989) with the genomic DNA fragment as a probe.

**In situ hybridization**

In situ hybridizations were performed using single-strand digoxigenin (DIG)-UTP (Boehringer Mannheim)-labeled RNA probes, essentially as described by Wilkinson (1992). Briefly, embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). For whole-mount analysis, specimens were gradually dehydrated in a graded methanol/PBS series up to 100% methanol and stored at −20°C. For sections, embryos were immersed in a 30% sucrose solution and then embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co.). Sections of 8–15 μm thickness were prepared using cryostat. Hybridization was carried out overnight at 60°C in a hybridization buffer (50% formamide, 5× SSPE, 5% SDS, 1 mg/ml yeast transfer RNA) and denatured RNA probes. DIG-labeled molecules were detected by using NBT/BCIP (Boehringer Mannheim) as a substrate for the anti-DIG-antibody-coupled alkaline phosphatase. The probe for Zic5 was prepared from the 3′ untranslated region of the cDNA by using sense primer (5′-AATACATCAACCAGGACCC-3′) and antisense primer (5′-TGGACAGGATGTACCCTAAC-3′). The Zic1, Zic2, and Xenopus Slug (Xshl) probes have been described previously (Nagai et al., 1997; Nakata et al.,...
The significance was determined using a somite stage embryos derived from six litters. Statistical analysis was performed as described elsewhere (Aruga et al., 1998). Serial sections were prepared, and the most comparable sets of sections were selected for the analysis. All the images were digitized with a Fuji HC-2500 3CCD camera, and areas were measured using NIH Image program (developed at the National Institutes of Health, Bethesda, MD; available on the Internet at http://rsb.info.nih.gov/nih-image/). The morphometric analysis in this study was performed on at least eight sets of 8–14 somite stage embryos derived from six litters. Statistical significance was determined using a t test. P values < 0.05 were considered significant.

Immunohistochemistry

For immunohistochemical staining, cryosections were incubated for 12 h at 4°C with antibody-binding solution consisting of primary antibody, 2% normal goat serum, and 0.1% TritonX-100 in PBS. The bound primary antibody was detected by Cy3-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG (Jackson Immunoresearch). To detect Zic5 protein, polyclonal antibody was raised against its carboxy-terminal region (amino acid numbers 585–622 in the accession no. NP_075363), which is not conserved at all in Zic1, Zic2, Zic3, and Zic4 proteins. The anti-Zic5 antibody (ZC5a) specifically bound Zic5 and showed no cross-reactivity with Zic1, Zic2, Zic3, or Zic4 in immunoblotting and immunocytochemistry analyses using Zic1-, Zic2-, Zic3-, and Zic4-expressing cultured cell lysates (T.I., Akira Ishiguro, and J.A., unpublished data). Immunoblot analysis for Zic2 protein was performed as described (Nagai et al., 2000) with anti-Zic2 antibody (Chemicon). Anti-actin antibody was purchased from Santa Cruz. Whole-mount immunohistochemistry for neurofilament was performed as described (Davis et al., 1991) with some modifications. Embryos were fixed in methanol/DMSO (dimethylsulfoxide) (4:1) overnight at 4°C, bleached in 5% H2O2/PBST (PBS containing 0.5% Triton X-100) for 6 h at room temperature, and blocked in PBSMT (PBST containing 2% skim milk) for 1 h. 2H3 antibody (Developmental Studies Hybridoma Bank; DSHB) against a 165-kDa neurofilament protein was used at a 1/10 dilution in PBSMT. After 2–3 days incubation with first antibodies, the embryos were washed in PBSMT four times for 1 h each and incubated for 1–2 days with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch) at 1/400 dilution. The embryos were then rinsed with PBST and incubated for 30 min with 100 μg/ml diaminobenzidine/PBST, and an equal volume of 0.03% H2O2/PBST was added. The color reaction was stopped by several washes in PBST. The embryos were then cleared in BABB, benzyl alcohol/benzyl benzoate (1:2).

RNA extraction and reverse transcription-PCR (RT-PCR)

RNAs were isolated from embryos with TRIzol reagent (GibcoBRL). After DNaseI treatment, reverse transcription was performed with Superscript II reverse transcriptase (GibcoBRL). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected to monitor RNA recovery. For

Fig. 1. Targeted disruption of the Zic5 gene. (A) The wild-type Zic5 gene consists of two exons. The first exon contains initiator methionine and three of five C82H-type zinc finger motifs. The homologous regions for the targeting vector are indicated by the crossbars. Note that the Zic2 gene is located approximately 10 kb upstream of the Zic5 gene. The fragment hybridizing with the 5′ and 3′ probes after EcoRV digestion is shown in pink. The targeting vector contains 5.5- and 1.2-kb regions homologous to the Zic5 gene and neomycin-resistance gene, driven by the PGK promoter (NEO), flanked with loxP sequences. The diphtheria toxin A fragment gene driven by the MC1 promoter (DT) was inserted in the 3′ end of the Zic5 gene to eliminate nonspecific integration. The sizes of NEO and DT cassettes are not proportional in the scheme. In properly mutated allele, the protein coding region of the Zic5 exon 1 has been replaced by the neomycin-resistance gene expression unit (neo mutant allele). The location of the 5′, 3′, and neo probes used for Southern blotting are shown. The fragments of the +neo mutant allele hybridizing with the probes after EcoRV digestion are shown in blue and yellow, respectively. The neomycin-resistance gene has been excised by Cre recombinase. The fragment of the Cre-recombinant allele (Δneo mutant allele) hybridizing with the probes after EcoRV digestion is shown in green. BII, BglII; E, EcoRI; Ev, EcoRV; S, Sall; X, Xhol. The expected size of the hybridizing fragments with the 5′, 3′, and neo probes after EcoRV digestion is shown. (B) Confirmation of homologous recombination of the mutant alleles by Southern blotting. The wild-type (wt, +neo mutant, and Δneo mutant DNA fragments detected by the 5′ (upper), 3′ (middle), and neo probes (lower) are as shown in A. (C–E) Immunohistochemistry of the heterozygote (C) and Δneo mutant (D) hindbrain sections with the anti-Zic5 antibody. A heterozygote section (E) in which only secondary antibody was applied was used as a control. No significant immunoreactivity is detected in the Zic5 Δ/− mutant, the same as the control section. (F) Expression of the other Zic genes, Zic1, Zic2, and Zic3, was unaffected by the Zic5 mutation. RT-PCR analysis was performed on cdnas prepared from wild-type and Δneo mutant E9.0 whole embryos. (G) Expression of Zic2 was not affected by the Zic5 mutation. RT-PCR analysis was performed on cdnas prepared from head region of wild-type and Δneo mutant at E8.5, E9.0, and E10.0. The results were confirmed by real-time quantitative RT-PCR analysis. Zic2 transcript levels in Zic5 Δ/− were 96.4 ± 6.3% [E8.5 (−/−, n = 3; +/+; n = 5)], 94.4 ± 8.1% [E9.0 (−/−, n = 4; +/+; n = 4)], and 112 ± 10.2% [E10.0 (−/−, n = 4; +/+; n = 5)] of the wild type. (H) Immunoblot using the anti-Zic2 antibody. Whole embryo lysates from Zic5 Δ/− and Zic5 Δ/+ at E10.5 and E11.5 were analyzed. The density of the bands representing Zic2 protein (55.5 kDa) was comparable between Zic5 Δ/− and Zic5 Δ/+. Zic2 protein produced in HEK293 cell line served as a control (upper panel). To ensure that the same amounts of proteins were loaded, the blot was stripped and reprobed with anti-actin antibody (lower panel). The positions of the molecular weight markers are shown on the right. Zic2/293, Zic2 protein produced in HEK293 cell line served as control; mock transfection with a control vector (pEF-BOS). (I, J) Spatial expression of Zic2 was unaffected by the Zic5 mutation. Zic5 heterozygous (I) and homozygous Δneo mutant embryos (J) at E9.25 were subjected to whole-mount in situ hybridization with a Zic2 probe. (K) Growth retardation in the Zic5 Δ/− mice. The Δneo Zic5 Δ/− mutant (right) is significantly smaller than the heterozygous littermate (left) and exhibits a gait abnormality (3 weeks of age).
quantification of the transcripts, RT-PCR was performed after they were in the log-linear phase of the amplification curve at the indicated cycles (Aruga et al., 2002a). The PCR cycles, annealing temperature, and primer sequences were: G3PDH, 22 cycles, 68°C, 5'-CCGGTGCTGAGTATGTC-GTGGAGTCTAC-3' and 5'-CTTTCCAGAAGGGGC-CATCCCCACGTCTTC-3'; Zic1, 27 cycles, 66°C, 5'-TG AACAATGGCCTGACATCAC-3' and 5'-TTGTCAGCTG-CATGTGCTTC-3'; Zic2, 28 cycles, 66°C, 5'-CAGCTTAA-GCAATCCAGAAAAGCTGCAAC-3' and 5'-ACAGCCTCGGAACTCACACTGGAAAGG-3'; Zic3, 30 cycles, 66°C, 5'-GCTTCTTCTGGGCTTCAATG-3' and 5'-CAGACGTGGTTGTTCTGCTC-3'. Quantitative real-time RT-PCR was used for quantification of Zic 2 and Wnt3a expression using gene-specific double fluorescently labeled probes in a 7700 ABI PRISM Sequence Detector System (Perkin Elmer-Applied Biosystems, Foster City, CA). PCR primers and TaqMan fluorogenic probes were designed using the Primer Express v5.0 software program (Applied Biosystems). 6-Carboxy fluorescein (FAM) was used as 5' fluorescent reporter while tetramethylrhodamine (TAMRA) was added to the 3' end as quencher. The primer sequences were: Zic2, 5'-GCTCATCTGCAAGTG-GATCGA-3' and 5'-CTCTGGATGTGTCGTAAGAG-3'; Wnt3a, 5'-AGGGCGGGGTGTTGAA-3' and 5'-TCCAGAACAGGGC-AAGAT-3'. The fluorogenic probe used in detection were: Zic2, 5'-FAM-CTAAGCAATCC- GCAATCCAGAAAAGCTGCAAC-TAMRA-3', and Wnt3a, 5'-FAM-TGACACCGCTGACACAGCC-TAMRA-3'. Rodent GAPDH Control reagent (Applied Biosystems) was used according to the manufacturers’ instructions. Amplification was performed by initial polymerase activation for 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 s, annealing and elongation at 60°C for 1 min. For real-time detection PCR, input cDNA was analyzed in duplicate or triplicate per primer pair per sample and the corresponding threshold cycle (Ct) values were measured. The relative amount of each cDNA was normalized to the level of GAPDH in each samples. The starting amount of cDNA in each sample was calculated by preparing a standard curve using known dilutions of cDNA standards. The standard curve was generated from the linear relationship existing between the Ct value and the logarithm of the starting quantity. Expression of each target in Zic5-/- embryos was determined relative to the signal observed in the wild-type samples according to standard procedure (ABI Prism 7700 manual).

Construction of targeting vector

Using full-length mouse Zic5 cDNA as a probe, a mouse genomic library of the 129/SV strain (Stratagene) was screened again to obtain the entire region of mouse Zic5 gene. The genomic organization of the mouse Zic5 locus was determined by restriction enzyme mapping, Southern blotting, and nucleotide sequencing. To construct the targeting vector, a 5.5-kb SalI/BglII fragment of the Zic5 gene was subcloned upstream of a PGK-neo expression cassette (Rudnicki et al., 1992) flanked by a loxp sequence, and the 1.5-kb XhoI–XhoI Zic5 fragment was subcloned downstream of the cassette. The combined fragment was inserted into the DT-A (diphtheria toxin A-fragment) expression vector (Yagi et al., 1993), generating the targeting vector pTVZ5-DT. The resulting targeting vector was linearized at the SalI site of the 5' homologous fragment before electroporation.

Generation of mutant mice

ES cells (E14 cells) were electroporated with 20 µg of linearized targeting vector DNA and selected for G418 resistance (175 µg/ml) as described elsewhere (Gomi et al., 1995). Correct homologous recombination was verified by Southern blot analysis after EcoRV digestion using a 0.8-kb EcoRV/BglII 5' probe, a 1.2-kb BamHI/EcoRV 3' probe, or a 0.6-kb PstI PGK-neo probe (neo-probe) (Figs. 1A, B). Recombinants were obtained at a frequency of 6 per 516 G418-resistant clones. Three independently targeted ES clones were injected into C57BL/6J blastocysts, and each ES clone yielded chimeric mice capable of transmitting the disrupted allele through the germline. To excise the PGK-neo cassette, germline transmitted chimeras were first mated with Cre females, which expressed Cre in their zygotes (Sakai and Miyazaki, 1997). The correct excision of the PGK-neo cassette was confirmed by Southern blotting. The mice carrying the mutated Zic5 allele were backcrossed to C57BL/6J for three to four generations (B6N3 or B6N4) before analysis. Genotypes were determined by Southern hybridization or PCR analysis of DNAs from tail or yolk sac. PCR was performed at 94°C for 30 s, 66°C for 30 s, and 72°C for 1 min with 35 cycles, with Z5WF, 5'-AACCCCTGCCCCCTCCGTTACCCCTGG-3', Z5WA, 5'-TGACACCGCTGACACAGCC-TAMRA-3', and Z5CreA, 5'-ACAGAAGCCACTTCTCCACGCGCCAAAC-3', to detect the mutated allele (Z5WF and Z5CreA) and wild-type (Z5WF and Z5WA) Zic5 allele.

The frequencies of the phenotypes described in the present study (i.e. NTDs and mandibular abnormalities) were comparable in the three mouse lines (B6N3, lacking neo cassettes) derived from three independent ES clones (data not shown). Thus, the phenotypes are considered to correctly reflect the effects of the inserted mutation.

Histological and skeletal analysis

Specimens were either fixed in Bouin’s solution or 4% PFA. After fixation, embryos were dehydrated through graded alcohols, embedded in paraffin, sectioned at a thickness of 8–10 µm, and stained with hematoxylin and eosin. Skeletal analysis of fetuses and newborn animals was performed after alcian blue/alizarin red staining (Hogan et al., 1994).
Misexpression in Xenopus embryos

*Xenopus laevis* were purchased from Hamamatsu Seibutsu Kyozai (Shizuoka, Japan). Embryos were obtained by artificial fertilization, and the jelly coats were removed by immersing them in 1% sodium mercaptoacetate (pH 9.0) for a few minutes. Embryos were cultured in 0.1 × Steinberg’s solution and staged according to Nieuwkoop and Faber (1967). The cDNA encoding the entire ORF of *Xenopus Zic5* (Nakata et al., 2000) and mouse Zic5 was subcloned into EcoRI–XbaI site of myc-tagged expression vector, pMT-CS2+ (Turner and Weintraub, 1994). The myc-epitope tag was used to check the construction by immunoblotting. mRNA for injection was synthesized by in vitro transcription. *Xenopus Zic*5 mRNA (MT-XZic5) (500 pg) and mouse Zic5 mRNA (MT-mZic5) (500 pg) were injected into the dorsal right blastomere of two-cell stage embryos in 3% Ficoll in 1 × MMR. Injected embryos were cultured until stage 19 to detect *Xslu* (Mayer et al., 1995) in 1 × Steinberg’s solution.

**Results**

Identification and expression profile of *Zic5*

An additional *Zic*-related gene was identified by low stringency hybridization on a genomic library and excluding clones containing the *Zic1*, *Zic2*, *Zic3*, and *Zic4* genes. The nucleotide sequence of the cDNA, which we named mouse *Zic5*, revealed an open reading frame for a protein with the zinc finger motifs. The genomic organization of the *Zic5* gene is comparable to that of other *Zic* genes and *opa* (Fig. 1A, wild-type allele). The *Zic5* gene contains one intron in its open reading frame, the same as *Zic4*, whereas the *Zic1*, *Zic2*, *Zic3*, and *opa* genes contain two introns. The position of the intron in the *Zic5* gene coincides with that of the first intron in the *Zic1*, *Zic2*, *Zic3*, *Zic4*, and *opa* genes, suggesting that *Zic5* and other *Zic* genes are derived from a common ancestral gene. Partial sequencing of the genomic clones revealed the *Zic2* gene to be located approximately 10 kb upstream of the *Zic5* gene in a reverse orientation (Fig. 1A). In the mouse genome, *Zic5* is thought to be located in telomeric end of chromosome 14, where *Zic2* was mapped previously (Aruga et al., 1996a).

The *Zic5* transcript was detected in mouse ES cells (data not shown), indicating that expression begins before implantation. At the egg cylinder stage (E7.0), expression was detected in the anterior half of the ectoderm, and weak expression was also observed in the posterior ectoderm and mesoderm (Figs. 2A, D). One day later, following formation of the neural plate, expression had become enhanced within the neural plate, but was soon restricted to the head fold (Fig. 2B). The neural fold and its derivatives continued to express *Zic5* (Figs. 2C, E–H). At E11.5, *Zic5* was expressed in specific regions of several developing organs, including dorsal areas of the brain, dorsal spinal cord, and distal regions of the developing limb (Fig. 2H). *Zic5* expression overlapped with those of other *Zic* genes, most closely with *Zic2*, but was not identical. In particular, *Zic5* expression in the neural tube was more dorsally restricted than that of *Zic1* and *Zic2*, and expression in somite and sclerotome was weaker than that of other *Zic* genes (Figs. 2K–M).

In the course of the study, Furushima et al. (2000) reported the structure and expression profile of a novel zinc finger protein, *Opr*, which was demonstrated to be identical to *Zic5*. In the embryonic stages, *Opr* was expressed in the dorsal areas of the developing central nervous system and limb buds, and this expression pattern is essentially the same as observed in the present study. In this study, we use “*Zic5*” since the name has been used for the human, mouse, zebrafish, and *Xenopus* orthologues of this gene in current databases (accession no. NM_0331302, NM_022987, NM_205727, AB034983).

Targeted disruption of *Zic5* and generation of mutant mice

To analyze the role of the *Zic5* gene, a targeting vector was constructed to delete 2.3 kb of the genomic sequence that includes an initiator methionine and three of the five zinc finger motifs (Fig. 1A). DNAs from G418-resistant ES colonies were analyzed by Southern blotting (Fig. 1B). To avoid possible interference of the PGK promoter with *Zic2* expression, we flanked the PGK-neo cassette with two loxP sites to permit removal of the PGK-neo cassette by Cre recombinase, yielding a mutant allele called the *Δneo* mutant allele. Cre-mediated removal of the PGK-neo cassette was achieved by crossing the heterozygous animals with CAG-Cre transgenic partners (Fig. 1B). Ablation of the *Zic5* protein was confirmed with anti-Zic5 antibody, which specifically bound *Zic5* (Figs. 1C–E). Immunohistochemistry of the dorsal hindbrain derived from E13.5 *Zic5*+/− (Fig. 1C) and *Zic5*−/− (Fig. 1D) showed no significant immunoreactivity, a finding comparable to the immunoreactivity of the second antibody (Fig. 1E) in the *Zic5*+/− mutant.

To investigate the possibility that the targeted mutation affects the expression of other *Zic* genes, we first performed quantitative RT-PCR analyses of RNAs extracted from different stages and regions of the embryos (E8.5–10.0) (Figs. 1F, G). The levels of the transcripts of *Zic1*, *Zic2*, and *Zic3* detected in *Zic5*−/− E9.0 whole embryos were the same as their levels in *Zic5*+/− embryos (Fig. 1F). The amounts of the *Zic2* transcripts in the head region of the several stages of embryos (E8.5–10.0) showed no significant difference between the *Zic5* mutant and wild type as confirmed by both conventional and real-time quantitative RT-PCR (Fig. 1G and legend). Consistent with these observations, the amount of *Zic2* proteins in the mutant was almost comparable to that in wild type as revealed by immunoblot analysis using anti-Zic2 antibody (Fig. 1H). We next performed whole-mount in situ hybridization for *Zic2* in *Zic5*−/− embryos (Figs. 1I, J). *Zic2* is characteristically expressed in the dorsal areas of the brain,
spinal cord, and somites (Nagai et al., 1997) (Fig. 1I). The spatial expression pattern showed no differences between Zic5\textsuperscript{-/-} (Fig. 1J) and Zic5\textsuperscript{+/-} (Fig. 1I). These results suggest that the mutation introduced to the Zic5 allele did not affect the expression of Zic2 at the level of detection, and thus that the phenotypes of the Zic5 mice described in the present study likely reflect the function of Zic5 itself.

Based on their external appearance and fertility, mice heterozygous for the Zic5 allele appeared to be normal. Examination of the embryonic genotypes at various developmental stages (days 8.0–18.5) revealed that each genotype was present in the expected Mendelian ratio (Table 1). However, the percentages of homozygotes at birth (P0–P3) and thereafter were lower than expected, suggesting that...
postnatal lethality had occurred. After birth, the Zic5−/− mice were significantly smaller than their littermates (Fig. 1K), and their average body weight at 3 weeks of age was 32% of their wild-type littermates [Zic5+/+, 17.7 ± 3.1 g (n = 11); Zic5−/−, 5.7 ± 0.9 g (n = 16); P < 0.001, t test]. Most of the Zic5−/− mutants died within 2 months, and attempts to mate homozygous males with homozygous females were rarely successful. The Zic5−/− mice manifested behavioral abnormalities 1 week after birth, frequently exhibiting abnormal gait and posture. Some of the behavioral abnormalities may be attributable to infrequent hydrocephalus (4/16 Zic5−/− at 3 weeks of age). The other postnatal Zic5−/− mice did not show any clear evidence of morphological abnormalities in the brain or other organs. Examination of Zic5+/− embryos and neonates revealed the anomalies described below in addition to the abnormalities observed after birth.

### Neural tube closure is impaired in Zic5-deficient mice

When examined at E13–18, approximately 15% of the Zic5−/− embryos exhibited exencephaly in forebrain,

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of litters</th>
<th>Zic5+/+ (%)</th>
<th>Zic5+/− (%)</th>
<th>Zic5−/− (%)</th>
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<tr>
<td>E10.5–11.5</td>
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<td>18 (21.7)</td>
<td>42 (50.1)</td>
<td>23 (27.7)</td>
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<td>51 (53.7)</td>
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<tr>
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<td>43 (53.8)</td>
<td>14 (17.5)</td>
</tr>
<tr>
<td>Weaninga</td>
<td>18</td>
<td>42 (29.2)</td>
<td>80 (55.6)</td>
<td>22 (15.2)b</td>
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<tr>
<td>Total</td>
<td>69</td>
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a Typically 1–2 weeks of age.
b Most of the −/− were runted and died before 2 months old.

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Fig. 3. Neural tube closure defects in Zic5−/− mutant embryos. (A) Lateral view of the Zic5+/− (left) and Zic5−/− (right) embryos at E10.5. Defective closure of the neural tube in the Zic5−/− embryo is indicated by the white arrow. The defects are generally centered in the midbrain–hindbrain and can extend rostrally and caudally. (B, C) Dorsal view of the Zic5+/− (B) and Zic5−/− (C) embryos at E11.5. Open neural tube is indicated by arrowheads. hb, hindbrain; ov, otic vesicle. (D) Appearance of the exencephalic phenotypes in Zic5−/− mutant embryos at E17.5. ex, exencephaly. (E, F) Sagittal histological sections of Zic5+/− (E) and Zic5−/− (F) embryos at E11.5. Sections were stained with hematoxylin and eosin. Asterisk, medial hinge point; arrowheads, dorsolateral hinge points. (G, H) The brains of the Zic5+/− (G) and exencephalic Zic5−/− (H) embryos at E17.5 are shown in sagittal sections. Note the disorganized cerebral cortex, anteriorly displaced mesencephalon, and absence of cranium in the exencephalic brain. c, cerebral cortex; cr, cranium; me, mesencephalon; sp, spinal cord. (I–L) Lateral (I, J) and dorsal view (K, L) of E9.0 Zic5+/+ (I, K) and Zic5−/− (J, L) littermate embryos stained with Wnt3a probe by whole-mount in situ hybridization. Lines in I indicate the rostrocaudal extension of Wnt3a expression in the dorsal neural tube. Arrows in K and L indicate the regions of open neural tube. Reduction of Wnt3a expression is indicated by the dashed line in J, and by the arrowheads in L.
midbrain, and hindbrain regions (12/79 Zic5+/− embryos, Fig. 3D). While the percentage of Zic5 mutant embryos with NTD was low, that was not an unusual finding because mouse embryos subjected to inactivation of a critical gene via homologous recombination rarely show NTDs with complete penetrance (Juriloff and Harris, 2000). Exencephaly typically reflects a defect in closure of the anterior neural tube, which normally occurs between E8.5 and E9.5 (Copp et al., 1990; Fleming and Copp, 2000). In Zic5+/− embryos, the cranial neural tube (forebrain, midbrain, and hindbrain) closed at E9, whereas in the exencephaly embryo, it remained open at E10.5 (Figs. 3A, C). Transverse sections of the hindbrain region of the E11.5 NTD embryos showed the incomplete neural tube closure, but the medial and dorsolateral hinge points were essentially conserved (Figs. 3E, F). Later, at E17.5, disorganized cerebral cortex, anteriorly displaced diencephalon, and disorganized basal ganglia and hippocampus were observed in the sagittal section (Figs. 3G, H). In the Zic5−/− embryos, we found no other cranial neural tube abnormalities such as holoprosencephaly, or impaired caudal neural closure, which are the most frequent abnormalities in the Zic2 mutant (Nagai et al., 2000). Investigation of the expression patterns of Shh, Pax3, Pax7, Msx2, and Zic2 in the closed neural tube of the mutant embryos at E9.5–11.5 revealed that loss of Zic5 had not affected the organization of the doroventral properties of the neural tube (data not shown).

Previous analyses of NTDs in Zic2 mutant embryos revealed that reduced expression of Zic2 results in neurulation delay and impaired differentiation of the dorsal neural tube concomitant with the lag in expression of Wnt3a (Nagai et al., 2000). We therefore examined the expression of Wnt3a in Zic5 mutant embryos by whole-mount in situ hybridization. At E9.0, Wnt3a transcript was detected in both the dorsal primitive streak region and the dorsal central nervous system (CNS) (Takada et al., 1994). In the wild-type embryos, the expression extended rostrally from the diencephalon to the cervical levels (Figs. 3I, K). Wnt3a expression in Zic5−/− CNS was lower than in wild-type CNS at E9.0, whereas the expression in the primitive streak region was comparable (6/14 Zic5−/− embryos, Figs. 3J, L). The amount of Wnt3a transcript in head region of Zic5−/− at this stage was reduced to 74.1 ± 8.3% that of wild type, as determined by real-time RT-PCR analyzes (Zic5+/+, n = 6; Zic5−/−, n = 4).

However, when the development proceeded at E10.5, expression of Wnt3a both in the CNS and the primitive streak region was detected at essentially the same levels as observed in the wild-type littermates (data not shown), indicating that there is a delay in Wnt3a expression similar to the delay in Zic2 mutants. This delay was associated with a delay in the neurulation process in the Zic5−/− CNS (dashed line in Fig. 3J). These observations suggest that Zic5 controls the progression of neurulation and the timing of anterior neural tube closure.

Zic5 homozygous mutants have facial bone abnormalities

The facial skeletal system was also affected in the Zic5−/− mutant as well as their neural tissues. Examination at birth revealed abnormalities in craniofacial bones, especially in the mandible (8/19 Zic5−/− neonates, Fig. 4). The cranial vault of newborn Zic5−/− mice was slightly smaller than in wild type or Zic5+/− mice and seemed to reflect the sum of minor changes in size and shape in individual calvarial elements (Figs. 4C, D). The mandible was reduced in size, and the two halves of the mandible were abnormally fused in the middle. The angular processes of the affected mandibles were small, and 4 out of 19 Zic5−/− neonates had a solitary incisor abnormally located in the midline (Figs. 4J–L). The premaxillary bones were slightly reduced in size [Figs. 4D, F; Zic5+/−, 2.09 ± 0.16 mm (n = 12); Zic5−/−, 1.72 ± 0.20 mm (n = 17); P < 0.05, t test]. These facial bone phenotypes were observed irrespective of the presence of NTD. In addition to the facial bones, small numbers of Zic5−/− mice showed phenotypic alternations in the sternum (3/46, E16-P0 Zic5−/− embryos) and vertebral arches (4/46), which are frequently observed in Zic1−/− mice (Aruga et al., 1999), as well as in tail structure (4/46), which are typically found in Zic3 mutants (Carrel et al., 2000; Klootwijk et al., 2000; Purandare et al., 2002) (T.I. and J.A., unpublished observations), suggesting that Zic5 plays a role in skeletal development in cooperation with other Zic genes in these tissues. However, involvement of Zic5 in mandibular bone growth and/or fusion is less likely because Zic5 is not significantly expressed in the mandible and first branchial arch (Fig. 2F, H, J, and data not shown).

Development of cephalic neural crest-derived tissue is impaired in the Zic5 mutant

Normal development of cranial structures, including the mandible, which is derived from the first branchial arch, requires an essential contribution by the neural crest cells (Le Douarin, 1982). The impaired facial skeletal elements in the Zic5 mutant raised the possibility that the loss of Zic5 function affects neural crest properties and led us to hypothesize that the failure of mandible outgrowth in Zic5−/− might be due to a lack of neural crest migration into the first branchial arch primordium. To investigate the possibility of a neural crest defect in Zic5 mutants, we first assayed the established neural crest markers AP2, Sox10, and Cadherin6 (Britsch et al., 2001; Inoue et al., 1997; Mitchell et al., 1991).

The merging of streams of neural crest cells destined for the branchial arches examined by these markers was largely unchanged in mutant and wild-type embryos at E9.0–9.5 (Figs. 5A, B), suggesting that differentiation process of the neural crest cells was essentially unaffected in Zic5−/− embryos. The proximodistal properties of the branchial arches and the contributions of neural crest cells in the first branchial arch seemed comparable based on the expression...
Fig. 4. Craniofacial abnormalities in Zic5-deficient mice. (A, B) Lateral profile of wild type (A) and Zic5<sup>−/−</sup> (B) newborn mice. The mandible is shortened and hypoplastic in the mutant (white arrow in B). (C–F) Skeletal stain analyses of the heads of wild type (C, E) and Zic5<sup>−/−</sup> (D, F) newborn mice. Notice the truncation of the mandible and flattened face involving the shortened premaxillary bones in the mutant (arrows in D and F). (G–L) Rostral view (G, J), frontal view (H, K), and lateral view (I, L) of the dissected mandible of wild type (G–I) and Zic5<sup>−/−</sup> (J–L) neonates. In addition to reduced size, the angular process of the mutant is hypoplastic (asterisks in J–L). Mandible is frequently fused, and a single incisor tooth is abnormally located in the midline (arrows in J–L). a, angular process; c, coronoid process; co, condylar process; fb, frontal bone; i, lower incisor tooth; ipb, interparietal bone; ma, mandible; mx, premaxillary bone; nb, nasal bone; pb, parietal bone.
Fig. 5. (A–D) Analyses of cranial neural crest cell migration in Zic5 mutants. Wild type (A, C) and mutant embryos (B, D) were hybridized with AP2 (A, B) and Sox10 (C, D) antisense RNA probes. Whole-mount in situ hybridization of E9.0 (A, B) and 9.5 (C, D) embryos. The expression patterns of the genes are largely unchanged in the Zic5 mutant embryos. The first branchial arch of the Zic5/C0/C0 embryos is frequently smaller and less constricted (arrow in D) than those of the wild-type littermates, and the Sox10-positive cranial ganglia are often smaller (arrowhead in D). r, rhombomere; ba1, first branchial arch.

(E–H) Histological sections of E9.5 wild type (E, F) and Zic5 mutant (G, H) embryos showing the distribution of Twist-positive neural crest cells (G, H) and the proximodistal properties of the first branchial arch revealed by Msx2 expression (F, H). m→l, mediolateral axis; p→d, proximodistal axis; i, first branchial arch; ii, second branchial arch. (I–J) Neural crest properties in the midbrain–hindbrain region of wild-type (I, J) and Zic5 mutant (K, L) at E9.0. Transverse sections were hybridized with Sox10 (I, K) and Cadherin6 (J, L) antisense RNA probes. Note the slightly reduced numbers of neural crest cells in the mutant embryos (asterisks in K and L). (M, N) Morphometric analyses of the neural crest cells. Areas (M) and cell density (N) of the Sox10- and Cadherin6-stained areas. The embryos were derived from E8.5 to 9.0 (8–14 somite stage) littermates. Error bars indicate SD. White bar, wild type; black bar, Zic5/C0/C0. *P < 0.05.
patterns of Msx2 and Twist (Figs. 5E–H). However, the first branchial arches of a subset of Zic5−/− embryos were smaller and less constricted in appearance than those of the wild-type littermates (arrow in Fig. 5D). Histological analysis suggested a reduction in the number of Sox10- and Cadherin6-expressing area in the lower midbrain/upper hindbrain of the mutant embryos at E8.5–9.0 (Figs. 5I–L). Although the reduction seemed slight, morphometric analysis confirmed that the reduction was significant in the stained areas for Sox10 and Cadherin6 (Fig. 5M), whereas the mean cell densities of the stained areas for Sox10 and Cadherin6 were not changed significantly (Fig. 5N). These results indicate that the cell numbers of the neural crest cells were reduced in the early phase of neural crest development. Consistent with the observations, the Sox10-positive cranial ganglia were often faint or small at later stages (E9.5) (7/15 Zic5−/−, arrowhead in Fig. 5D). Cell death frequency was not changed in these regions (data not shown). These results led us to speculate that neural crest cell generation is decreased in Zic5−/−. Cephalic neural crest cells are also known to contribute to the development of the peripheral nervous system. To confirm the idea, we used anti-neurofilament antibody to compare axonal projections at E10.5 and E11.5 (Fig. 6). At E10.5, the trigeminal ganglion of Zic5−/− was slightly smaller and had fewer axonal projections into the surrounding mesenchyme (9/14 Zic5−/−, Fig. 6B). When examined at E11.5, the reductions in axonal projections from the trigeminal and facial ganglion were more evident in the Zic5−/− embryos. The trigeminal ganglion of the Zic5−/− was smaller (9/14 Zic5−/−), and the projection into the mandibular and maxillary components of the first arch were decreased in number and shorter than in Zic5+/− (6/12), although their patterning and orientations seemed normal (Fig. 6D). The glossopharyngeal ganglion was also smaller (4/12 Zic5−/−, asterisk in Fig. 6D). In contrast to the alterations in the cranial ganglia, neurofilament and Sox10 staining in caudal regions of the mutant embryos showed no significant change in dorsal root ganglion and sympathetic ganglia (data not shown). These findings suggested that cephalic neural crest derivatives are selectively affected, and there is a reduction in number of cranial peripheral nervous system-constituting cells without a qualitative change in axonal projection. The impaired neural crest derivatives in the Zic5−/− embryos are therefore thought to reflect decreased generation of neural crest cells.

Overexpression of mouse Zic5 induces neural crest tissues in Xenopus embryos

The above results suggest that Zic5 may be involved in neural crest development in mice. We previously observed that overexpression of Xenopus Zic5, as well as of Xenopus

![Image](attachment:image1.png)

Fig. 6. Neurons in wild type (A, C) and homozygous mutant embryo (B, D) were visualized at E10.5 (A, B) and E11.5 (C, D). The embryos were stained with anti-neurofilament antibody 2H3. Lateral views of the embryos. (A, B) The trigeminal ganglia are slightly smaller in the E10.5 Zic5−/− embryos, and they have fewer axonal projections into the surrounding mesenchyme (9/14 Zic5−/−, Fig. 6B). When examined at E11.5, the reductions in axonal projections from the trigeminal and facial ganglion were more evident in the Zic5−/− embryos. The trigeminal ganglion of the Zic5−/− was smaller (9/14 Zic5−/−), and the projection into the mandibular and maxillary components of the first arch were decreased in number and shorter than in Zic5+/− (6/12), although their patterning and orientations seemed normal (Fig. 6D). The glossopharyngeal ganglion was also smaller (4/12 Zic5−/−, asterisk in Fig. 6D). In contrast to the alterations in the cranial ganglia, neurofilament and Sox10 staining in caudal regions of the mutant embryos showed no significant change in dorsal root ganglion and sympathetic ganglia (data not shown). These findings suggested that cephalic neural crest derivatives are selectively affected, and there is a reduction in number of cranial peripheral nervous system-constituting cells without a qualitative change in axonal projection. The impaired neural crest derivatives in the Zic5−/− embryos are therefore thought to reflect decreased generation of neural crest cells.

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**Fig. 7.** Ectopic expression of mouse Zic5 induces neural crest tissues in Xenopus embryos. (A) A normal stage-19 embryo was subjected to whole-mount in situ hybridization with the Xenopus Slug (Xslug) RNA probe. Expression of Xslug was symmetric along the midline. (B, C) Five hundred picograms of MT-Xenopus Zic5 (B) or MT-mouse Zic5 (C) mRNA was injected into the right blastomere of two-cell stage embryos. Whole-mount in situ hybridization was performed with the neural crest marker Xslug at stage 19. Xslug expressing regions showed lateral expansion on the injected side.

Zic1, Zic2, and Zic3, induced ectopic pigment cells and neural crest markers in Xenopus embryos (Nakata et al., 1997, 1998, 2000). To examine the potential role of Zic5 in neural crest induction, we next misexpressed mouse Zic5 mRNA in Xenopus embryos (Fig. 7). As previously observed, injection of Xenopus Zic5 mRNA increased expression of the neural crest marker Xslug in the injected side [Fig. 7B, 78/109 of MT-XZic5 (500 pg)-injected embryos]. Injection of the same amount of mouse Zic5 mRNA also increased Xslug expression in the injected side [Fig. 7C, 79/109].

**Fig. 8.** Expression of Zic genes during neurulation and neural tube defects (NTDs) in Zic mutants. (A–C) Expression of Zic2 (A), Zic3 (B), and Zic5 (C) at the neurulation stage. Locations of significant expression are indicated by arrows. (D) Schema depicting the expression of Zic genes along the anterior–posterior (AP) axis of the embryos. Expression of Zic2, Zic3, and Zic5 along the AP axis of the neural tube is shown as color density in the vertical bars. The Zic genes are commonly expressed in the neural fold and dorsal part of the closed neural tube. A: anterior, P: posterior. (E) Location of NTDs in Zic2, Zic3, and Zic5 mutant embryos at E13.5. Holoprosencephaly in the Zic2 mutant and facial defects in the Zic5 mutant are also indicated. fb, forebrain; nf, neural fold; sc, spinal cord; sm, somite.
61/93 of MT-mZic5 (500 pg)-injected embryos. These results suggest that mouse Zic5 has neural-crest-inducing activity similar to that of Xenopus Zic5.

Discussion

Neural tube defects in Zic5<sup>−/−</sup> mice

Targeted disruption of the Zic5 gene demonstrated that Zic5 is crucial for the anterior neural tube closure. Recent studies on neurulation suggest the presence of factors both intrinsic and extrinsic to the neuroepithelium that are essential for neural tube closure (Harris and Juriloff, 1999). For example, targeted mutations in the gene for Twist and Cart1 demonstrate a critical role for surrounding head mesenchyme in neurulation (Chen and Behringer, 1995; Zhao et al., 1996). In contrast, NTDs caused by targeted mutations in the Jnk, p300, Ski, and Hes1 genes are due to intrinsic defects in the proliferation, survival, and differentiation of neuroepithelial cells (Berk et al., 1997; Ishibashi et al., 1995; Kuan et al., 1999; Yao et al., 1998). The NTDs in Zic5<sup>−/−</sup> may be caused by a defect intrinsic to the neural plate, since Zic5 is expressed strongly in the head fold region of the E8.5 neural plate (Figs. 2B, E) but barely expressed in the head mesenchyme (Figs. 2I, J). The reduction of Wnt3a expression in the E9.0 Zic5 mutant can be interpreted that Zic5 is required for timely differentiation of the dorsal neural tube, which may be essential to normal progression of neurulation.

The phenotypes of other Zic gene-deficient mice provide clues to consider the role of Zic5 in neural tube development because recent studies have shown functional similarities among mouse Zic proteins (Koyabu et al., 2001; Mizugishi et al., 2001). Reduction of cerebellar granule cell proliferation involving premature neuronal differentiation has been observed in Zic1 mutants and Zic1/Zic2 combined mutants (Aruga et al., 1998, 2002a). Recent analyses of the dorsal spinal cord of both Zic1-deficient mice and Zic1-overexpressing mice have demonstrated that Zic1 controls the expansion of neuronal precursors by inhibiting neuronal differentiation (Aruga et al., 2002b). All these findings support the idea that Zic5 may also control neuronal differentiation and cell proliferation in the neural folds. Although cell proliferation was not apparently changed in the dorsal neural tube of the Zic5 mutant (data not shown), it is conceivable that a slight reduction under the detected level might occur, considering mild phenotypes. Further examination of its differentiation controlling ability is necessary.

Interestingly, loss of Zic2 or Zic3 also give rise to NTDs that differentially occur along the anterior–posterior (AP) axis of the neural tube. We note a significant correlation between the NTD types and the expression profiles of Zic2, Zic3, and Zic5 during neurulation (Fig. 8). Although Zic2 and Zic5 are strongly expressed in prospective forebrain to hindbrain (Figs. 8A, C), Zic3 expression is strongest in the prospective midbrain–hindbrain junction, and significantly less in the trunk region (Fig. 8B). The difference in their expression may underlie the preferential occurrence of NTD in the hindbrain of the Zic3 mutant and in the forebrain to hindbrain of the Zic2 and Zic5 mutants (Figs. 8D, E). The impaired closure of the caudal neural tube in the Zic2 mutant seems to reflect the significant expression of Zic2 in the posterior neural fold. In addition, the enhanced expression of Zic2 in the paraxial mesoderm, where Zic5 was not significantly expressed, may be related to the differential occurrence of the caudal NTD. Zic genes may have functional redundancy in neurulation and differentially contribute to the neural tube closure along the AP axis. Because most of the Zic5-expression regions are overlapped with that of other Zic genes, lower penetrance of the phenotypes in the Zic5 mutant could be the result of partial compensation of the Zic5 activities by the other Zics. Rescue experiments and examination of compound Zic family gene mutants should lead to better understanding.

Zic5 is involved in cephalic neural crest development

We showed the decrement of the cephalic neural crest tissues in Zic5-deficient mice. In the course of neural crest development, Zic5 is expressed in the neural fold of the hindbrain region and in cephalic neural crest cells at onset of migration at E8.5–9.0 (Figs. 2C, E, F, G), but not significantly expressed in the first branchial arch and head mesenchyme (Figs. 2I, J). Since the Zic5 expression is restricted to dorsal neural fold and dorsal neural tube, Zic5 could affect the proliferation of neural crest precursors, as well as the differentiation and early migration processes. However, together with the observation that overexpression of mouse Zic5 in Xenopus embryos results in an enhanced expression of a neural crest marker, our results favor a model in which localized Zic5 signaling regulates neural crest production and/or proliferation in the dorsal neural tube rather than its involvement in migration of the cell population or differentiation of the ganglionic precursors.

Recently, involvement of Zic2 in neural crest production was suggested in Kumba (Ku) mutant mice (Elms et al., 2003), which contained an ENU-induced point missense mutation in the fourth zinc finger domain of Zic2. In Zic2<sup>ku/ku</sup>, insufficient amount of neural crest is generated, and neural crest production is delayed, leading to a depletion in the number of neural crest cells at all axial levels (the cranial region including first and second branchial arches and the trunk). Combining our results, it is likely that both Zic2 and Zic5 are essential for the neural crest production.

Limited impairments of cephalic neural crest in Zic5 mutant may partly be explained by functional compensation by Zic2 at the trunk level. However, we need additional explanation since both genes have very similar rostrocaudal expression profile in neural tube (Fig. 8). Nakata et al. (2000) showed that Xenopus Zic5 induces neural crest tissues without marked induction of anterior neural tissues.
whereas other Xenopus Zic genes induces both components, suggesting a functional difference between Xenopus Zic5 protein and other Xenopus Zic proteins. Since the mouse Zic5 protein is similar to Xenopus Zic5, being distant from other Zic proteins in phylogenetic tree analysis (Furushima et al., 2000), it is possible that there is a functional difference in neural crest production between mouse Zic5 and other mouse Zic proteins.

Recent studies of the signals involved in neural crest induction have suggested some molecules are critically involved in neural crest development (Gammill and Bronner-Fraser, 2003; LaBonne and Bronner-Fraser, 1999). In mice, Wnt1 and Wnt3a are expressed in the dorsal neural tube, where Zic5 is expressed, shortly after its closure (Holleyday et al., 1995; Parr et al., 1993). Combined mutation of these genes results in a diminution of dorsal neural precursors and some neural crest derivatives (Ikeya et al., 1997). The involvement of Wnt-mediated signals has also been shown in amphibians and avians (Chang and Hemmati-Brivanlou, 1998; Garcia-Castro et al., 2002; Saint-Jeannet et al., 1997; Tan et al., 2001). Delayed expression of Wnt3a (Figs. 3J, L) in the dorsal neural tube of Zic5 and Zic2 (Nagai et al., 2000) mutant embryos at E9.0 suggests the possibility that these Zic genes utilize Wnt signaling to exert their roles in neural crest development. In addition, Notch signaling should be noted as another mechanism controlling neural crest development (Cornell and Eisen, 2000; De Bellard et al., 2002; Endo et al., 2002) since Zic1 can activate the Notch signaling pathway (Aruga et al., 2002b). Further clarification of the relationship between Zic family genes and these signals would be beneficial for understanding the molecular mechanism of neural crest development.

Possible relation to human NTD syndrome

The human ZIC2 gene has been found to be responsible for human HPE (Brown et al., 1998, 2001, 2002; Orioli et al., 2001). Deletions that include 13q32, where ZIC2 is located, lead to congenital malformations, including brain anomalies, such as HPE or exencephaly, and digital anomalies, known as “13q32 deletion syndrome” (Brown et al., 1993, 1995). Nagai et al. (2000) demonstrated that reduced expression of mouse Zic2 leads to HPE, exencephaly, spina bifida, and skeletal abnormalities. The phenotypes of the human disease are similar to those found in the Zic2 mutant mice, except for the absence of spina bifida. Taken together, these findings established that ZIC2 mutations cause HPE and that ZIC2 hemizygosity is at least part of the basis of the severe malformations seen in patients with the syndrome (Ming and Muenke, 2002). Based on human genomic databases, we found putative human ZIC5 in human chromosome 13q32 in the vicinity of the ZIC2 gene, approximately 11 kb apart to centromere. This implies that most of the 13q32 deletions, which include the ZIC2 gene, include the adjacent ZIC5 gene as well. The close location of the ZIC5 gene to the ZIC2 gene on the chromosome, their similar expression pattern in mouse embryos, and the brain malformation in the Zic5⁻/⁻ mice lead to the hypothesis that ZIC5 also contributes to 13q32 deletion syndrome. Analysis of the Zic2/Zic5 double mutant mice should provide further insights into their involvement in the deletion syndrome.

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