Zic2 and Zic3 synergistically control neurulation and segmentation of paraxial mesoderm in mouse embryo

Takashi Inouea, Maya Otta, Katsuhiko Mikoshibab, Jun Arugaa,⁎

a Laboratory for Comparative Neurogenesis, RIKEN Brain Science Institute, Wako-shi, Saitama 351-0198, Japan
b Laboratory of Developmental Neurobiology, RIKEN Brain Science Institute, Wako-shi, Saitama 351-0198, Japan

Received for publication 10 August 2006; revised 4 April 2007; accepted 5 April 2007
Available online 12 April 2007

Abstract

Zic family zinc-finger proteins play various roles in animal development. In mice, five Zic genes (Zic1–5) have been reported. Despite the partly overlapping expression profiles of these genes, mouse mutants for each Zic show distinct phenotypes. To uncover possible redundant roles, we characterized Zic2/Zic3 compound mutant mice. Zic2 and Zic3 are both expressed in presomitic mesoderm, forming and newly generated somites with differential spatiotemporal accentuation. Mice heterozygous for the hypomorphic Zic2 allele together with null Zic3 allele generally showed severe malformations of the axial skeleton, including asymmetric or rostro-caudally bridged vertebrae, and reduction of the number of caudal vertebral bones, that are not obvious in single mutants. These defects were preceded by perturbed somitic marker expression, and reduced paraxial mesoderm progenitors in the primitive streak. These results suggest that Zic2 and Zic3 cooperatively control the segmentation of paraxial mesoderm at multiple stages. In addition to the segmentation abnormality, the compound mutant also showed neural tube defects that ran the entire rostro-caudal extent (craniorachischisis), suggesting that neurulation is another developmental process where Zic2 and Zic3 have redundant functions.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Zic; Compound mutation; Neurulation; Somitogenesis; Gastrulation

Introduction

Zinc finger proteins belonging to the Zic family play critical roles in animal development (reviewed in Herman and El-Hodiri, 2002; Aruga, 2004; Grinberg and Millen, 2005). The proteins contain a phylogenetically conserved zinc finger domain that is composed of tandem repeats of five C2H2 motifs. In mammals, there are five Zic-related genes — Zic1, Zic2, Zic3, Zic4, and Zic5 — all of which have been characterized at least in part as to their expression profiles, molecular functions, and loss-of-function phenotypes (references in previously listed reviews). However, the relationships among the five mammalian Zic proteins in the developmental context are poorly understood. In a previous study, Zic1 and Zic2 were shown to control cerebellar development synergistically (Aruga et al., 2002a), but the other combinations of mammalian Zic genes have not been addressed yet.

Among the five mammalian Zic genes, Zic2 and Zic3 have critical roles in embryogenesis. In human, ZIC2 causes holoprosencephaly (Brown et al., 1998), which is characterized by impaired development of the medial part of the forebrain. Zic2 hypomorphic mutant mice show not only holoprosencephaly but also neural tube defects (NTDs) in both the rostral and caudal ends of the neural tube, abnormalities in retinal axon projection and axial and appendage skeletal patterning, and suppressed neural crest formation (Nagai et al., 2000; Elms et al., 2003; Herrera et al., 2003). In comparison, human ZIC3 has been identified as a causal gene of X-linked heterotaxy (situs ambiguous) syndrome (Gebbia et al., 1997). Mouse Zic3 mutants show abnormalities related to left–right axis determination and a variety of developmental abnormalities, such as malposition of the primitive streak, NTDs, axial skeletal patterning defects, and hypoplastic cerebellum (Carrel et al.,...
2000; Klootwijk et al., 2000; Purandare et al., 2002; Aruga et al., 2004; Ware et al., 2006). The developmental abnormalities caused by deficiencies of Zic2 or Zic3 seem to partly overlap as regards the manifestation of NTDs and skeletal defects such as tail deformity. However, the rostro-caudal positions of NTDs and the types of tail deformity differ between Zic2 and Zic3 mutants, raising questions concerning their functional relationship in each developmental process.

Accumulating evidence provides a clear picture of the expression profiles of the two genes in mouse (Aruga et al., 1996; Nagai et al., 1997, 2000; Aruga et al., 2002a; Purandare et al., 2002; Brown et al., 2003; Elms et al., 2003, 2004; Inoue et al., 2004). Summarization of this literature suggests that Zic2 and Zic3 generally are expressed in partly overlapping, but distinct, manners. Although some comparative studies clearly revealed this point (Nagai et al., 1997; Elms et al., 2004; Inoue et al., 2004), some ambiguities remain. There also are similarities between Zic2 and Zic3 in terms of their molecular functions. They bind the same DNA target sequences, act as transcriptional activators in reporter gene assays (Mizugishi et al., 2001), and have common interacting proteins (Koyabu et al., 2001; Mizugishi et al., 2004). These similarities suggest that some of the roles of Zic2 and Zic3 in mammalian development are redundant.

While re-examining the expression profiles of Zic2 and Zic3 in mouse embryos, we found that the two genes are uniquely expressed during somitogenesis. Somites, which are transient epithelial spheres of paraxial mesoderm cells, give rise to metameric structures, such as vertebrae and ribs. Somites are synchronously generated from the anterior end of the unsegmented mesenchymal precursor tissue, called the pre-somatic mesoderm (PSM), in an anterior to posterior direction in a rhythmic fashion at regular spatiotemporal intervals. Somite formation periodically removes an unsegmented paraxial mesoderm from the PSM, and new mesodermal cells are continuously added on PSM posterior regions that include the primitive streak during gastrulation and the tailbud at later stages (Dubrulle and Pourquie, 2004). Before morphologic segmentation, a segmental prepattern characterized by segmental gene expression is established in the anterior PSM (Saga and Takeda, 2001; Pourquie, 2003; Rida et al., 2004). For instance, expression of mouse Mesp2, a bHLH transcription factor, is initially expressed in a segment-wide domain in the anterior PSM (Saga et al., 1997; Saga and Takeda, 2001). This initial segmental expression domain is defined by a molecular oscillator, called the “segmentation clock,” which includes the Notch signaling pathway and hairy/enhancer of split-related transcription factors (Holley and Takeda, 2002; Maroto and Pourquie, 2001) and is related to the gradient of FGF and Wnt signaling in the posterior PSM (Galceran et al., 2004; Hofman et al., 2004; Kawamura et al., 2005). Although the involvement of Zic genes in somitogenesis is unknown, the expression pattern tempted us to examine their relevance to above processes.

To clarify the presumed redundant roles, we generated Zic2/Zic3 mutant mice and analyzed their embryonic defects. Comparison of the phenotypes of the compound mutants with those of the single mutants indicated that Zic2 and Zic3 have common roles, both in segmentation of paraxial mesoderm and in neurulation.

Materials and methods

Animals

C57BL/6d and BALB/c mice were purchased from Nihon SLC (Shizuoka, Japan). Mice heterozygous for the Zic2 “knock-down” mutation (Zic2mm/hrm, http://www.informatics.jax.org) (Nagai et al., 2000) were backcrossed to C57BL/6d and BALB/c 12 or 13 times. These backcrossed mice then were used in subsequent matings. Zic2 mutant mice were genotyped and maintained as described (Nagai et al., 2000; Aruga et al., 2002a). The spontaneous mutation in the Bent tail (Bn) (Garber, 1952) mouse was revealed to be a deletion of a region of the X chromosome including Zic3 (Zic33 knock-out) (Carrel et al., 2000; Kootwijk et al., 2000). Bn mice were originally purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and have been kept in a mixed C57BL/6d × BALB/c background. The phenotypes of Bn mice are very similar to that of Zic3-deficient mice generated by targeted mutation (Purandare et al., 2002; Aruga et al., 2004). Ware et al. (2006) described a variation in the gastrulation phenotypes of the Zic3 knockout mice and classified them into four types in their study. We observed similar variations in the gastrulation phenotypes of Zic3 mutants and confirmed that embryos representing each of the four types exist in the Bn/Y (Zic33b/-) or Bn/Bn (Zic33b/-) E6.5–9.0 embryos. When we examined at E6.5–9.5, 32.5% (n = 84) of the Bn/Y embryos showed no obvious morphologic abnormalities [type IV in Ware et al. (2006)]. Zic2 k/d × Zic3 Bn/Y males were generated by mating Zic3 Bn/+ females with Zic2 k/d males. Crosses of viable Zic2 k/d × Zic3 Bn/+ females by Zic2 k/d × Zic3 Bn/+ males generated double homozygous males (Zic2 k/d × Zic3 Bn/Y). Animals were maintained by the Research Resource Center, RIKEN Brain Science Institute. All animal experiments were carried out in accordance with the guidelines for animal experiments in RIKEN. All efforts were made to minimize the number of animals used. Noon of the day on which vaginal plugs were first observed in the morning was defined as embryonic day 0.5 (E0.5). Staging criteria described for E6.5–7.5 embryos (Downs and Davies, 1993) were used to determine the stage of gastrulation of embryos. The genotype of the Zic2 hypomorphic allele was determined (Nagai et al., 2000). Genotyping of the Zic3 and Zic33 alleles was performed as described (Kootwijk et al., 2000; Franke et al., 2003).

In situ hybridization, immunofluorescence, and histology

In situ hybridization (ISH) was performed essentially as described (Nagai et al., 1997). RNA probes for Zic2 and Zic3 were described previously (Nagai et al., 1997). RNA probes for Fgf8, Foxa2, Mesp2, Max1, Otx2, Paraxis, Pax1, Brachyury(T), and Uncx4.1 were generated by RT–PCR; their sequences will be provided upon request. RNA probes for Bmp3a and Pax3 were described by Takada et al. (1994) and Goulding et al. (1991), respectively. For E6.0–7.5 embryos, genotypes were identified after ISH by digestion of the embryos in a protease K-containing lysis buffer and subsequent PCR analysis. For

Fig. 1. Localization of mouse Zic2 and Zic3 mRNA and protein in early-stage mouse embryos. In all pictures, anterior is to the right, and all intact embryos are shown in lateral view except in panels G and P (dorsal view). The distributions of Zic2 (A–I) and Zic3 (J–R) mRNA are shown at E7.0 (A, J), E7.5 (B, C, K, L), E8.0 (D, E, M, N), E8.5 (F, G, O, P), E8.75 (H, Q), and E9.0 (I, R). Embryos were examined by ISH. Zic proteins were detected by immunofluorescence staining using anti-panzic antibody (S–V) in E7.0 (S) and E7.5 (T) embryos in sagittal sections and in E8.0 (U) and E8.5 (V) embryos in transverse sections. The positions of the transverse sections are shown in panels B and F. Arrowheads in panels D–I and panels M, O–R indicate the expression of Zic2 and Zic3 in the anterior PSM and newly generated somites. al, allantois; ba, branchial arch; ch, chorion; fb, forebrain; c, eye; cc, embryonic ectoderm; ex, extraembryonic ectoderm; hb, hindbrain; hf, head fold; mes, embryonic mesoderm; nf, neural fold; ps, primitive streak; psm, presomatic mesoderm; r, rhombomere, s, somite; sc, spinal cord.
histological examination, specimens were fixed in either Bouin’s solution or 4% paraformaldehyde, 0.1 M sodium phosphate (pH 7.4). After fixation, embryos were dehydrated through graded alcohols, embedded in paraffin, sectioned at a thickness of 8 to 10 μm, and stained with hematoxylin and eosin. Immunohistochemistry was performed as described previously (Aruga et al., 2002a). Embryos were excised, fixed in 4% paraformaldehyde in PBS at 4 °C overnight, cryoprotected in 30% sucrose in PBS overnight, and embedded in OCT compound; 6- to 10-μm cryosections were cut and examined.

Fig. 2. Expression of Zic2 and Zic3 in PSM and somites at E10.5. (A–E) WMISH for Zic2 (A–C), Zic3 (D), and Zic1 (E) mRNA. Lateral view of whole embryos. Expression of Zic2 and Zic3 in the anterior PSM and newly generated somites is indicated by arrowheads in panels A–D. The expression pattern of Zic2 is variable in the anterior PSM and newly generated somites. Expression of Zic2 in the tail dorsal neural tube is indicated by asterisks in panel A. (F–N) Expression analysis of Mesp2, Zic2, and Zic3 in the tail at E10.5. Expression of Mesp2 (F–H), Zic2 (I–K), and Zic3 (L–N) mRNA was compared by ISH of three sets of adjacent sections. (F, I, L) and (G, J, M) are sets of adjacent longitudinal sections through tail tip, respectively. (H, K, N) are transverse sections through S-1 region. Somites are numbered so that the newest somite is S1, the forming somite is S0, and the next somite to be formed is S-1, which is positive for Mesp2 expression. fl, forelimb; gt, gut; hl, hindlimb; nc, notochord; nt, neural tube; pnm, presomatic mesoderm; sc spinal cord; sm, somite. Solid and dotted lines indicate areas of enhanced and weak expression, respectively. (O–R) Distribution of Zic and Mesp2 proteins in the PSM and newly formed somites at E10.5. Immunopositive signals seem to mostly represent Zic2 protein at these stages. Zic (O, P) and Mesp2 (Q, R) proteins were detected by immunofluorescence labeling of two pairs of adjacent sections, (O, Q) and (P, R). Solid and dotted lines indicate regions of enhanced and weak expression, respectively. (S) Schematic drawing of expression domains of Zic2 and Zic3 mRNA, and Zic2 protein.
immunohistochemically. Fixed sections were washed three times with PBS and preincubated in PBS containing 5% normal donkey serum and 0.1% Triton X-100 for 30 min and then incubated in 1% normal donkey serum and 0.1% Triton X-100 with the primary antibodies. Anti-pan-Zic antibodies were generated by immunizing rabbits with glutathione S-transferase-Zic2 carboxy terminal region (amino acid number, 416–530 in NP_033600) and subsequent affinity purification at a company (MBL, Nagoya, Japan). The antibody primarily recognizes Zic2 protein, but also cross-reacts weakly to Zic1 and Zic3 in an immunoblot analysis (data not shown). The other primary antibodies used were: rabbit anti-Mesp2 (provided by Yumiko Saga) (Morigoto et al., 2005), mouse anti-165-kDa neurofilament (2H3, Developmental Studies Hybridoma Bank; DSHB), goat anti-Notch1 (Santa Cruz), rabbit anti-NICD (Notch intracellular domain) (Cell Signaling Technology), and rabbit anti-phosphohistone H3 (Upstate). For immunofluorescence detection of NICD and Mesp2, frozen sections were immersed in 0.01 M citrate buffer (pH 6.0), or Target Retrieval Solution (Dako), and autoclaved at 105°C for 15 min to enable antigen retrieval (Tokunaga et al., 2004; Morimoto et al., 2005). To detect these antibodies, Cy3-(Jackson ImmunoResearch Laboratory), Alexa488-, and Alexa594-labeled secondary antibodies (Molecular Probes) were used. Fluorescently labeled Solution (Dako), and autoclaved at 105°C for 15 min to enable antigen retrieval (Tokunaga et al., 2004; Morimoto et al., 2005). To detect these antibodies, Cy3-(Jackson ImmunoResearch Laboratory), Alexa488-, and Alexa594-labeled secondary antibodies (Molecular Probes) were used. Fluorescently labeled sections were immersed in 0.01 M citrate buffer (pH 6.0), or Target Retrieval Solution (Dako), and autoclaved at 105°C for 15 min to enable antigen retrieval (Tokunaga et al., 2004; Morimoto et al., 2005). Skeletal analysis of fetuses and newborn animals was performed after 2H3 anti-neurofilament antibody was performed as described (Inoue et al., 2004). Whole-mount immunohistochemical staining AxioCam color CCD camera. All images were analyzed with Adobe Photoshop CS software (Adobe Systems). Cell proliferation analyses

Wild-type (n=6), Zic3 Bn/Y (n=5), and Zic2 kd/+ Zic3 Bn/Y (n=4) embryos were fixed at E7.75 and 8.5, and embedded in OCT compound. Tissue sections (7 μm) were subjected for immunofluorescence staining with anti-phosphohistone H3 antibody and nuclear staining with DAPI. The labeled cells were counted in four comparable sections per each embryo. Mitotic indices (percentage of cells undergoing mitosis) were calculated as percentages of the phosphohistone H3-positive nuclei among the DAPI-stained nuclei. At least, 16 sections from each genotype at each stage were evaluated to determine the mean percentages and the standard deviations. Statistical significance was assessed by t-test.

Results and discussion

Expression of Zic2 and Zic3 in early-stage embryos

We first performed a series of ISH to compare the expression profiles of Zic2 and Zic3 (Fig. 1). At E7.0 (mid to late streak stage), Zic2 was expressed broadly in both the extra-embryonic and embryonic components of the egg cylinder (Fig. 1A). As development proceeded, Zic2 expression increased, first in the anterior ectoderm and mesoderm of the headfold (Figs. 1B, C) and later in dorsal neural folds, somites (Figs. 1D–I), and segmenting trunk, with enhancement in a few caudal segments (arrowheads in Figs. 1D–I) of somite-stage embryos (E8.0–9.0). In contrast, Zic3 transcripts were located more posteriorly in both ectoderm and mesoderm at the mid to late primitive streak stage (E7.0; Fig. 1J) and early neural fold stages (E7.5–8.0; Figs. 1K–N), and in the hindbrain regions (rhombomere [r] 1, 2, and 4) at later stages (E8.5–9.0; Figs. 1O–R). Similarly to Zic2, Zic3 was expressed in the dorsal neural tube, somites, and the segmenting trunk, with enhancement in the caudal segments (arrowheads in Figs. 1M, O–R) in somite-stage embryos.

To characterize the distribution of Zic proteins at these stages, we conducted immunofluorescence staining (Figs. 1S–V). As judged by anti-Zic antibody, which originally raised against Zic2, but weakly cross reacts to Zic1 and Zic3, Zic proteins were located in the cell nuclei of the ectodermal and mesodermal cells of the primitive streak, headfold, and neural fold of the developing embryos. The distribution of the proteins corresponded to the region in which either Zic2 or Zic3 mRNA was detected, suggesting that there is no strong spatial difference in translation efficiency in these tissues. Therefore, in streak- to somite-stage embryos, expression overlapped throughout most of the neuroectoderm, with differing accentuation rostro-caudally (Zic2, stronger in the anterior; Zic3, stronger in the hindbrain and spinal cord-forming region), in the dorsal neural tube, and in some parts of the paraxial mesoderm.

Because expression of Zic2 and Zic3 occurred consistently in somites and the segmenting trunk of embryos from E8.0 through E11.5 (Fig. 1 and data not shown), we performed detailed examination of Zic2 and Zic3 expression in the tail (Fig. 2), in which the segmentation process has been well studied. As comparative markers, we used Mesp2 mRNA and Mesp2 protein, whose differential expression represents various stages of somitogenesis (reviewed in Aulehla and Herrmann, 2004; Dubrulle and Pourquie, 2004; Saga and Takeda, 2001). We also examined expression of Zic1 for comparison.

We detected Zic2 expression in the somites, anterior PSM (arrowheads in Figs. 2A–C), and caudal end of the neural tube (asterisks in Fig. 2A) in E10.5 tails (Figs. 2A–C, 1–J). Expression of Zic2 in the PSM varied among the embryos examined. In 63% of E10.5 embryos (12 of 19), Zic2 expression in the PSM and somite was detected as two distinct stripes (arrowheads in Figs. 2A, B). Comparison of the expression region of Zic2 with that of Mesp2, which is expressed in the anterior portion of the prospective somite (S-1) (Saga et al., 1997; Takahashi et al., 2000) in adjacent section, revealed two stripes of Zic2 mRNA corresponding to S0 (forming somite) and S1 (most newly formed somite) (Figs. 2F, I), and also expressed anterior part of S1 weakly. Elsewhere, however, the expression of Zic2 in S1 was weaker than in S0 (Fig. 2B, 2 of 19 embryos examined) or was detected as a single broad stripe (Fig. 2C, 5 of 19 embryos examined) in which the posterior half of the broad stripe corresponded to S-1 (demarcated by asterisks in Fig. 2). These variations may be better interpreted as changes in Zic2 expression that are synchronous with the segmentation process, i.e., Zic2 expression is initiated in S-1, is enhanced in the forming S0 somite, and is decreased at S1 and segmented somites (Fig. 2S).

In contrast to Zic2, Zic3 expression in the tail was present as a single stripe throughout E8.0 to E11.5 (Fig. 1, and data not shown). At E10.5, Zic3 expression in the tail occurred broadly throughout the PSM and somites but not in the neural tube, and appeared as a single stripe in the anterior PSM with a gradient to the posterior end (Figs. 2D, L, M). Comparison of the expression of Zic3 with that of Mesp2 revealed that the stripe of Zic3 coincided with that of Mesp2 (S-1; Figs. 2F, G, L, M, 17 of 20 embryos). In segmented somites, Zic3 expression was...
distributed evenly rostro-caudally (Figs. 2D, L, M). Zic2 was not expressed in the PSM from E8.0 to E11.5 (Fig. 2E, and data not shown) but is abundantly expressed in more rostral somites and their derivatives (Nagai et al., 1997; Aruga et al., 1999). Antibody staining for Zic (Figs. 2O, P) and Mesp2 (Figs. 2Q, R) proteins also revealed that expression of Zic proteins is weak in anterior S-1 and strong in S0 and S1, and occasionally in S2. Examination of the Zic2 mRNA and Zic protein in neighboring sections revealed that Zic2 mRNA localization pattern was found to be mostly consistent with the immunopositive signals (Supplemental Fig. 1A, C). However, we observed bands of Zic protein in S-1, S0, and S2 while the Zic2 mRNA was strong in S-1, S0 and S1, but diminished in S2. This result suggests that the Zic2 mRNA disappears earlier than the protein. Similar difference was observed between Mesp2 mRNA and protein in the neighboring sections (Supplemental Fig. 1D, E). Mesp2 mRNA was predominantly detected in S-1 while Mesp2 protein was both in S-1 and S0 as reported by Morimoto et al. (2005). Collectively, the results of the expression pattern analysis raised a possibility that Zic2 and Zic3 act together in the neuroectoderm, primitive streak, somites, and anterior PSM.

Zic2 and Zic3 cooperatively control neurulation

To characterize the embryonic defects of Zic2/Zic3 compound mutants, two types of mating were carried out. One was a Zic2 kd/+ × Zic3 Bn/+ intercross to generate a heterozygous Zic2 hypomorphic allele together with nullizygous Zic3 (Zic2 kd/kd;Zic3 Bn/Y) (Table 1), and the other was a Zic2 kd/+;Zic3 +/+ × Zic3 Bn/+ intercross to generate the homozygous Zic2 hypomorphic allele together with nullizygous Zic3 (Zic2 kd/kd;Zic3 Bn/Y) (Table 2). E6.5–18.5 embryos were harvested and genotyped by PCR.

We first characterized Zic2 kd/+;Zic3 Bn/Y embryos. By the end of embryonic development, most of the Zic2 kd/+;Zic3 Bn/Y embryos had been resorbed (Table 1, and data not shown). However, several attempts to collect surviving Zic2 kd/+;Zic3 Bn/Y embryos until E16–17.5 were successful (n = 6). We found that 4 of the 6 Zic2 kd/+;Zic3 Bn/Y embryos at these stages showed marked NTD from the midbrain to the caudalmost region (craniorachischisis) (Figs. 3C, D), whereas NTD restricted to caudal or midbrain–hindbrain regions occurred in Zic2 kd/kd (Fig. 3A) or Zic3 Bn/Y (Fig. 3B) embryos, respectively, as described previously (Klootwijk et al., 2000; Nagai et al., 2000). The neurulation abnormalities could be traced back to E8.5–9.5 (Fig. 3E). At E9.5, Zic3 Bn/Y embryos showed growth retardation, slight rotation defects, and NTD in the hindbrain region. The frequency of hindbrain NTD was 29% (7 of 24) in E9.5–18.5. In contrast, 42% (5 of 12) Zic2 kd/+;Zic3 Bn/Y embryos at E9.0–10.5 showed craniorachischisis extending from the midbrain to the caudalmost region (Figs. 3E–G), and 6 of the 12 embryos showed more severe defects that precluded evaluation of neurulation (Figs. 7J and 9, C, F). Therefore, the severity of NTD was strongly enhanced in Zic2 kd/+;Zic3 Bn/Y in comparison with that in the single mutants. Besides NTD, Zic2 kd/+;Zic3 Bn/Y embryos also showed shortened rostro-caudal body length and dilated heart morphology (5 of 12 embryos at E9.0–10.5), compared with those in wild-type and Zic3 Bn/Y littermates. As a consequence, these results suggest that Zic2 and Zic3 commonly regulate neural
tube closure of the cervical to thoracic region. Together with previous results (Nagai et al., 2000; Klootwijk et al., 2000), it became clear that mouse Zic2 and Zic3 are essential for the neural tube closure at any rostro-caudal levels. It is possible that Zic5 also coordinately controls the neurulation with Zic2 and Zic3, considering its overlapping expression and the NTD phenotype in Zic5 mutant (Inoue et al., 2004).

Skeletal patterning defects in Zic2/Zic3 compound mutant mice

We next examined the skeletal phenotypes of surviving Zic2 kd/+ Zic3 Bn/Y embryos at E16–17.5 and compared them with those of wild-type, Zic2 kd/kd, and Zic3 Bn/Y embryos (Fig. 4). Zic2 kd/+ Zic3 Bn/Y embryos were easily identified because they showed shortened body length and abnormally bent and short tails (Fig. 4D) compared with those of wild-type (Fig. 4A), Zic2 kd/kd (Fig. 4B) and Zic3 Bn/Y (Fig. 4C) embryos. These defects were observed in all (6 of 6) Zic2 kd/+ Zic3 Bn/Y embryos at this stage; they were also apparent in preskeletal cartilage at E14.5 (4 of 4 embryos, data not shown). Quantitative analysis revealed that the shortening of the body was due to a decrease in the number of vertebrae (Fig. 4E). The reduction was strong in the tail region but weak in the cervical, thoracic, and lumbar regions. The size of the vertebral bodies was comparable, except in the tail compartment (Fig. 5). Whereas the most affected embryos were Zic2 kd/+ Zic3 Bn/Y, Zic2 kd/kd and Zic3 Bn/Y embryos also showed significant decreases in the number of vertebrae in the tail region (Figs. 4B, C, E); Zic2 kd/kd embryos also had curly tails, whereas those of Zic3 Bn/Y embryos were kinked.

Detailed examination of the defects in the axial skeleton revealed that the disorganization extended along the entire rostro-caudal axis in Zic mutants at E16.5–17.5 (Fig. 5; Table 3). Defects were localized dorsally in the vertebral arch and ventrally in the vertebral bodies, ribs, and sterni, indicating that almost all axial skeletal bones were more or less affected. In Zic2 kd/kd embryos, vertebral arches were frequently fused to the adjacent ones in the cervical and thoracic regions (Fig. 5B). The vertebral arches were absent in the region of the caudal NTD (spina bifida), and the arch rudiments were fused with rostro-caudally adjacent ones throughout the NTD region (Fig. 5F). In addition, vertebral bodies were occasionally asymmetrically malformed (hemivertebrae, open arrowhead in Fig. 5B) and rostro-caudally fused (open arrowheads in Fig. 5F) in these regions. In the tail region, the dorsal parts of the vertebral bodies were fused together (11 of 12 Zic2 kd/kd, Fig. 5N) to form a "curly tail".

Skeletal abnormality in Zic3 Bn/Y embryos was milder than that of Zic2 kd/kd. Rostro-caudal fusion of the vertebral arches occurred only in the cervical and thoracic regions (Fig. 5C; Table 3, and data not shown). In the tails of Zic3 Bn/Y embryos, the vertebral bodies were asymmetrically aligned along both the dorsoventral and left–right axes, but they were rarely fused to each other (Figs. 5K, O; Table 3). We speculated that these differences may underlie the differences in tail phenotypes between Zic2 kd/kd and Zic3 Bn/Y embryos [i.e. Zic2 kd/kd curly tail (Nagai et al., 2000); Zic3 Bn/Y kinky tail (Carrel et al., 2000; Klootwijk et al., 2000)]. Another Zic2-hypomorphic mutant, Zic2 ku/ku, and Zic3 knockouts also show curly and kinky tail phenotypes, respectively (Purandare et al., 2002;
Elms et al., 2003), confirming that these tail phenotypes are inherent to the loss of Zic2 and Zic3 functions.

As expected, Zic2 kd/+ Zic3 Bn/Y embryos showed more severe skeletal defects than did Zic2 kd/kd and Zic3 Bn/Y (Figs. 5D, H, L, P; Table 3). The vertebral arch rudiments were fused throughout the cervical to lumbar regions along the NTD (Figs. 5D, H), and asymmetrical malformation and rostro-caudal fusion of the vertebral bodies occurred frequently at all rostro-caudal levels (open arrowheads in Figs. 5D, H, and arrowheads in Figs. 5L, P). Vertebrae were fused strongly to each other, particularly in the tail region (Figs. 5L, P). The deformation was also observed in the rib and sternebrae, in which rostro-caudal position of the proximal ribs were asymmetric (Fig. 5T). These synergistic effects of Zic2 kd and Zic3 Bn mutations on skeletal deformation may reflect the redundant functions of Zic2 and Zic3.

Skeletal abnormalities are also found in Zic1-deficient mice (Aruga et al., 1999). In Zic1−/− embryos, the defects in the vertebral arch can be traced back to a disturbance in metameric cartilaginous condensation of the dorsal sclerotome cells. In Zic1-deficiency, early rostro-caudal specification of the somites and differentiation of the somites into sclerotome, myotome, and dermomyotome occur properly. Therefore, dorsal skeletal defects, such as deformities of vertebral arches like those in Zic2 kd/kd, Zic3 Bn/Y, and Zic2 kd/+ Zic3 Bn/Y embryos, could occur by a similar mechanism, considering the similar expression of Zic1 in the dorsal spinal cord (Nagai et al., 1997; Aruga et al., 1999). However, the axial truncation and frequent asymmetrical and rostro-caudal malformation of the axial skeleton in Zic2, Zic3, and Zic2/Zic3 mutants need alternative explanations, because these defects rarely are found in Zic1−/− embryos (Aruga et al., 1999). Because Zic2 and Zic3 are expressed strongly in undifferentiated PSM and S-1 and S0 somites, where Zic1 is not expressed (Fig. 2E), we hypothesized that not only sclerotome differentiation but also the initial generation or patterning of the somites could be disturbed in Zic2, Zic3, and Zic2/Zic3 compound mutants.

Somitogenesis is disorganized in Zic2 kd/kd and Zic3 Bn/Y embryos

To test whether Zic2 and Zic3 play roles in somite segmentation or patterning, we next analyzed somitogenesis in Zic2 kd/kd and Zic3 Bn/Y embryos (Fig. 6). First, we examined Zic2 kd/kd embryos. When examined at E9.5–12.5, Zic2 kd/kd were distinguishable from their littermates by caudal spina bifida and dorsally curved tails after E11.5 (Figs. 6A, C, S). In Zic2 kd/kd embryos, stripes of Uncx4.1, which is a marker for the posterior compartment of each somite, were frequently irregular along the rostro-caudal axis at E11.5 (6 of 6 embryos, arrowheads in Fig. 6C). The disturbance involved
abnormal intrusion of Uncx4.1-positive cells into the anterior compartment, especially in dorsal somites (asterisks in Figs. 6F, G, I and Supplemental Fig. 2C). Histological examination revealed that the Uncx4.1 stripes were partially close to each other in the thoracic region (Fig. 6F), and were continuous in the dorsal region in the curved parts of the tail region (Figs. 6G, I and Supplemental Fig. 2C). In a neighboring section, a somite anterior compartment marker, Tbx18 (Kraus et al., 2001; Bussen et al., 2004), was not expressed in the region where ectopic Uncx4.1 expression was observed (Supplemental Fig. 2D), suggesting the transformation of the anterior compartment into the posterior one or an intrusion of the posterior compartment into the anterior. Consistent with these observations, immunohistochemical detection of neurofilament (Dodd et al., 1988) at E10.5 revealed either lost or irregular arrangement of ganglia and spinal nerve axons in Zic2 kd/kd (Fig. 6K, and data not shown). The segmental pattern disturbance was also indicated by molecular markers for somite derivatives, such as Pax1 (sclerotome, Deutsch et al., 1991), Pax3 (dermomyotome, Goulding et al., 1994), and Paraxis (sclerotome and dermomyotome, Burgess et al., 1995) (Supplemental Fig. 2H–J). However, the results also suggested that the differentiation into these components was not severely impaired.

The somitic defects of Zic3 Bn/Y seemed milder than those of Zic2 kd/kd. Uncx4.1 signals in Zic3 Bn/Y were weak, and both Uncx4.1-positive and -negative stripes were thinner in some embryos (6 of 14 Zic3 Bn/Y or Zic3 Bn/Bn embryos; Figs. 6L, N, O). The irregular stripe pattern seen in Zic2 kd/kd embryos was rare but was significantly detected in Zic3 Bn/Y (1 of 6 Zic3 Bn/Y embryos; asterisk in Fig. 6Q).

Recently, it was suggested that somite boundary is generated by suppression of Notch activity by Mesp2 (Morimoto et al., 2004).
Table 3

Distribution and frequency of skeletal malformations in Zic2 kd/kd, Zic3 Bn/Y, and Zic2 Kd/+ Zic3 Bn/Y mutant at E16.5–17.5

<table>
<thead>
<tr>
<th>Genotype combination</th>
<th>Notch1 expression</th>
<th>Fusion of vertebrae bodies</th>
<th>Asymmetric vertebrae bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zic2 kd/kd (n=11)</td>
<td></td>
<td>0.91</td>
<td>0.91</td>
</tr>
<tr>
<td>Zic3 Bn/Y (n=12)</td>
<td></td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>Zic2 Kd/+ Zic3 Bn/Y</td>
<td></td>
<td>0.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Zic3 Bn/Y (n=6)</td>
<td></td>
<td>0.33</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Table 3: Distribution and frequency of skeletal malformations in Zic2 kd/kd, Zic3 Bn/Y, and Zic2 Kd/+ Zic3 Bn/Y mutant at E16.5–17.5.

We therefore examined the distributions of Notch1 and activated Notch1 proteins in the Zic2 kd/kd PSM at E10.5–12.5 (Figs. 6W, X–AA). There were no clear differences between Zic2 kd/kd mutants (n=18) and their wild-type littermates (n=22) in either Notch1 (Fig. 6W) or activated Notch1 (NICD, in Figs. 6X–AA) immunofluorescence staining. Examination of other markers for anterior PSM, EphA4 (Nieto et al., 1992; Nakajima et al., 2006) and Mesp2 also revealed no clear differences between the mutants and wild-type (Supplemental Fig. 3A–B, D–E; and data not shown). In addition, in the neighboring section, correct stripe of Uncox4.1 expression in the newly formed somites indicated that anterior–posterior somite polarity was normally specified in Zic2 kd/kd mutant (Supplemental Fig. 3F).

Besides the segmentation anomaly, Zic2 kd/kd mutants had slightly enlarged tailbuds when examined at E11.5–12.5 (asterisks in Fig. 6T). Histological sections through this region revealed disorganized cellular condensation in a continuous tissue from caudal neural tube (arrowheads in Fig. 6V). Together with the strong Zic2 expression in the caudal end of the neural tube, this phenotype may reflect a role of Zic2 in tail neurogenesis.

Segmentation and mesodermal patterning defects in the Zic2/Zic3 mutants

The phenotypes of the Zic2 and Zic3 single-mutant somites described above indicate that Zic2 and Zic3 have a role in somitogenesis. To reveal their possible synergy, we next examined the somite defects of Zic2 kd/+ Zic3 Bn/Y embryos. When examined at E8.5 (Fig. 7C) and E9.5 (Figs. 7F, G, J), Zic2 kd/+ Zic3 Bn/Y embryos showed a deficit in posterior development (Figs. 3E–G and 7C, F, G). Body lengths were shortened, and Uncox4.1 transcripts were weakly and irregularly distributed in Zic2 kd/+ Zic3 Bn/Y embryos (Figs. 7F–G). The Uncox4.1-expressing somites were reduced in number, and the stripes of Uncox4.1 expression were weaker and thinner in Zic2 kd/+ Zic3 Bn/Y embryos than in Zic3 Bn/Y embryos, suggesting lack of expansion of the somitic mesoderm. In addition, we noted that several Zic2 kd/+ Zic3 Bn/Y showed bilateral somite defects at E8.5–9.5, which was revealed by asymmetric Uncox4.1 expression (inset in Fig. 7F). Expression of a mesodermal marker, Brachyury(T), was abnormally weak in the Zic2 kd/+ Zic3 Bn/Y PSM (Figs. 7C, F, G). Mox1 is expressed in all cell types of somites, with enhancement in the caudal half, and in the anterior PSM in wild-type embryos at E9.5 (Fig. 7H; Candia et al., 1992). However, in Zic3 Bn/Y embryos, the Mox1 stripe was slightly weaker but regular (Fig. 7I and data not shown). By contrast, Mox1 signals appeared irregular, and without clear segmentation, in Zic2 kd/+ Zic3 Bn/Y embryos (arrowheads in Fig. 7J).

The analyses of Zic2 kd/+ Zic3 Bn/Y also indicated that PSM was present in the combined mutant, but the size was smaller than those of wild-type and Zic3-single mutant (Figs. 7C, F, G, J, 8G, and 9C, F). To determine if the cell proliferation is affected in the combined mutant, we examined the mitotic frequencies in the primitive streak in wild-type, Zic3 Bn/Y, and Zic2 kd/+ Zic3 Bn/Y embryos at E7.75 and E8.5, using a mitotic marker phospho-histone H3 (phospho-H3) (Fig. 8). As a result, primitive streak of Zic2 kd/+ Zic3 Bn/Y contained fewer phospho-H3-positive cells in the mesodermal layers through these stages (Figs. 8C, F), and the mitotic indices were significantly lower than that of wild-type and Zic3 Bn/Y at both stages (Figs. 8H, I). The mitotic indices of the overlying ectoderm were also reduced, but were not significantly different among the three genotypes (Figs. 8H, I). As another possible explanation for the hypoplasticity of the PSM, change in the cell death frequency was possible. However, we did not see significant differences of the cell death frequency among the three genotypes (not shown). Collectively, these results suggested that Zic2 and Zic3 have a redundant function in promoting the cell proliferation of PSM progenitor cells.

In relation to the reduced proliferation of the early mesodermal cells, Zic2 kd/+ Zic3 Bn/Y embryos showed altered expression of some marker genes. Wnt3a (Takada et al., 1994) and Fgf8 (Dubrulle et al., 2001) expression was generally reduced in their sizes (Figs. 9C, F, respectively). Not only area but also staining intensity was diminished for Wnt3a signals [43% of Zic2 kd/+ Zic3 Bn/Y mutants (n=9), arrowheads in Fig. 9F]. These results indicate that differentiation of mesodermal tissue may also be impaired in Zic2 kd/+ Zic3 Bn/Y mutants. The finding is important because Brachyury, Wnt3a, and Fgf8 play crucial roles in mesodermal development.

Finally, we sought to analyze somite-related abnormalities of Zic2 kd/kd Zic3 Bn/Y embryos. However, the Zic2 kd/kd Zic3 Bn/Y embryos showed early embryonic lethality, and we failed to collect any embryos later than E8.5 (Table 2). The three Zic2 kd/kd Zic3 Bn/Y embryos that we obtained at E8.5 showed poor development of somitic structures (Fig. 9G). These data also support the idea that at least one allele of Zic2 or Zic3 is...
Fig. 6. Somite structures are disrupted in Zic2 and Zic3 mutant mice. (A) Zic2 kd/kd (left) and wild-type (right) embryos at E12.5. White line indicates open neural tube (spina bifida). Dashed line indicates the dorsally curled tail in Zic2 kd/kd in comparison to wild-type tail region (black line). (B, C, R, S) WMISH with somite posterior compartment marker, Uncx4.1 RNA probes at E11.5. Zic2 kd/+ (B, R) and Zic2 kd/kd (C, S). In the Zic2 kd/kd embryo (C), Uncx4.1 expression was partly diffused (arrowheads in panel C), suggesting the disturbance of the anterior–posterior polarity of somite. sb, spina bifida. (D–I) ISH with Uncx4.1 RNA probes at E11.5 (D–G) and E12.5 (H–I) in wild-type (D–E, H) and Zic2 kd/kd (F–G, I) embryos in longitudinal sections through thoracic (D, F) and tail regions (E, G, H, I). Fusion of the Uncx4.1-expressing region is indicated by asterisks. Curly-tail regions of Zic2 kd/kd frequently show dorsally restricted bridging of Uncx4.1 stripes (G, I). ap, anterior compartment of the somite; d, dorsal; pc, posterior compartment of the somite; sb, spina bifida; v, ventral. (J–K) Immunostaining with anti-neurofilament antibody in wild-type (J) and in Zic2 kd/kd (K) embryos at E10.5. Arrowheads indicate misrouting of spinal nerve projections from their path. Partial loss of the metameric pattern of the somites are indicated by lines and an asterisk. drg, dorsal root ganglia; scg, sensory chain ganglia; sn, sensory nerve. (L–O) WMISH with Uncx4.1 RNA probes in E9.75 wild-type (L–M), Zic3 Bn/Y (L, N), and Zic3 Bn/Bn (L, O) embryos. Restricted and faint staining of Uncx4.1 is indicated by dashed lines. The difference between (N) and (O) represents a variance generally found in the Zic3-null mouse. Zic3 Bn/Y and Zic3 Bn/Bn were indistinguishable with regard to all embryonic defects. (P–Q) ISH with Uncx4.1 RNA probes in wild-type (P), and Zic3 Bn/Y (Q) in longitudinal sections through lumbar region at E11.5. Fusion of the Uncx4.1-expressing region is indicated by asterisk. T) Lateral views of the tails of wild-type, Zic2 kd/+ and Zic2 kd/kd mutants at E11.5. Morphologic differences in the tail tips are indicated by asterisks. (U–V) Longitudinal sections through the caudal end of the neural tube in the tail of Zic2 kd/+ (U) and Zic2 kd/kd (V). Abnormal cell cluster extended from the caudal end of the neural tube (white arrowheads). (W) Distribution of Notch1 protein in wild-type, Zic2 kd/+ and Zic2 kd/kd embryos in longitudinal sections of PSM at E11.5. Notch1 protein (green) is detected in S-1/S0/S1 compartment of PSM. (X–AA) Detection of the activated form of Notch1 protein (NICD) in wild-type (X, Y) and Zic2 kd/kd (Z, AA) embryos in longitudinal sections of PSM at E11.5. (X, Z) and (Y, AA) represent the representative two patterns of NICD distribution. Thick red lines indicate areas of enhanced expression of NICD. nt, neural tube; PSM, presomitic mesoderm.
required for somitogenesis. In E7.5 Zic2 kd/kd Zic3 Bn/Y embryos, Brachyury staining revealed either a bent path or decreased intensity (Fig. 9H and data not shown), and signals for Foxa2 (a marker for anterior axial mesoderm; Sasaki and Hogan, 1996; Ang and Rossant, 1994) were weak and detected only in the distal tips of the embryos (Fig. 9I). In addition, anterior neuroectoderm demarcated by Otx2 (Simeone et al., 1993; Kimura et al., 2000) was reduced in E8.5 Zic2 kd/+ Zic3 Bn/Y embryos (Fig. 9F). These findings indicate that a broad range of embryonic patterning was impaired in Zic2/Zic3 compound mutants. However, these defects were essentially similar to those observed in Zic3 knockout mice (Ware et al., 2006). Except for their roles in segmentation, we find it difficult to conclude unambiguously that Zic2 and Zic3 cooperate in embryonic patterning.

Roles of Zic2 and Zic3 in somitogenesis and mesodermal development

In this study, we analyzed the expression patterns of Zic2 and Zic3 during gastrulation to somite stages and somitic defects in Zic2 single, Zic3 single, and Zic2/Zic3 compound mutant mice. By analyzing combined mutants, we provide evidence that Zic2 and Zic3 exert functional redundancy in mesodermal patterning and somitogenesis. Following observations may be essential to consider the involvement of Zic2 and
Zic3 in somite development. (1) Zic2 and Zic3 are expressed in the mesoderm and primitive streak region during gastrulation. In somite stages, Zic2 expression is enhanced in the anterior parts of PSM (in both S0 and S-1) and newly generated somites (S1), whereas Zic3 is expressed more evenly from S-1 to the tail tip and in segmented somites; (2) The skeletal pattern reveals the segmentation defects, and somite markers occur unevenly along the anterior–posterior in Zic2 single, Zic3 single, and Zic2/Zic3 compound mutants. Subdivision in each somite is more severely impaired in Zic2 kd/+ Zic3 Bn/Y and Zic2 kd/kd Zic3 Bn/Y compound mutant embryos than in single mutants; (3) Not clear in the single mutant, caudal structures (primitive streak during gastrulation and PSM in later stage) are severely impaired in the Zic2/Zic3 compound mutant. (4) Early mesodermal patterning through gastrulation is also defective in Zic2/Zic3 mutants.

In light of these observations, we propose two major roles of Zic2 and Zic3 in mesodermal segmentation. The first one is that they maintain somite compartment rather than its specification in the anterior PSM. We first postulated that Zic2 and Zic3 could specify somite compartment (i.e. anterior–posterior) at the anterior PSM. However, there were no cases of continuous disappearance of somite regional markers’ expression or the disturbance in the anterior PSM markers (Mesp2, EphA4, and NICD). In addition, somite polarity of newly formed somites was unchanged in Zic2 kd/kd embryos. These results suggest...
that determination of somitic polarity and its border may not directly depend on \(Zic2\) and \(Zic3\). Instead, the primary role for \(Zic2\) and \(Zic3\) may be in the maintenance of the somitic integrity by controlling separation of cells and the cell number for formed somite compartments during somite development. Accordingly, segmental disturbance and regional defects of
axial skeletons were also observed in Zic1 mutant (Aruga et al., 1999), which is expressed later than Zic2 and Zic3 in the somitogenesis (Fig. 2E).

The other possible role is that Zic2 and Zic3 are required for generation of paraxial mesodermal progenitors from primitive streak and tailbud. It is known that the PSM is constantly supplied caudally with new cells by progenitors located in the primitive streak and tailbud during somitogenesis, and maintenance of PSM size is dependent on these tissues (Tam, 1986; Yamaguchi et al., 1999). We have shown that loss of Zic2, Zic3 or both Zic2 and Zic3 lead to a reduction in the body length (total number of generated somites), the size of primitive streak and the PSM. Thus, a reduction of streak- or tailbud-derived progenitors can be an explanation for the axial truncation of in Zic2, Zic3, and Zic2/3 mutants. Taken together with the reduced PSM progenitor cell proliferation in the Zic2 kd/+ Zic3 Bn/Y mutant, Zic2 and Zic3 may function in the primitive streak and tailbud to control the proliferation of paraxial mesoderm progenitors. The role of Zic2 and Zic3 in the regulation of cell proliferation seems to be analogous to the role of Zics in other developmental contexts. For example, in cerebella of Zic1 single and Zic1/Zic2 combined mutants, cell proliferation of granule neuron progenitor was reduced (Aruga et al., 1998, 2002a), and in spinal cord Zic1 controls the expansion of neuronal precursors by inhibiting neuronal differentiation (Aruga et al., 2002b). The apparent similarity in the cell proliferation regulation might be related to the functional similarities (i.e. DNA binding property and transcriptional regulation ability) among mouse Zic proteins (Koyabu et al., 2001; Mizugishi et al., 2001).

Our results indicate that Zic2 and Zic3 synergistically control multiple steps of mesodermal segmentation in mammals. The abnormality in Zic1 mutants was most pronounced in the dorsal components of sclerotome-derived structures such as the vertebral arch. Therefore, the subgroup of vertebrate Zic genes comprising Zic1, Zic2, and Zic3 has a common role in the establishment of metamers. It is interesting that these three genes strongly preserve some evolutionary conserved domains that are present in the bilaterian ancestor but that have been lost by unsegmented urochordates (Aruga et al., 2006). The redundant and unique functions of Zic1, Zic2, and Zic3 may have contributed to the establishment of segmented mesoderm in vertebrates during the course of evolution.

Acknowledgments

We thank Yumiko Saga (National Institute of Genetics) for anti-Mesp2 antibody, Andy McMahon (Harvard University) and Shinji Takada (National Institute for Basic Biology) for Wnt3a probe, Peter Gruss (Max-Planck Institute) for Pax3 probe, Akinori Tokunaga and Hideyuki Okano (Keio University) for helpful advice, and Yayoi Nozaki and RRC RIKEN BSI for technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.04.003.

References


