Repression of the hindbrain developmental program by Cdx factors is required for the specification of the vertebrate spinal cord

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The spinal cord is a unique vertebrate feature that originates, together with the hindbrain, from the caudal neural plate. Whereas the hindbrain subdivides into rhombomeres, the spinal cord remains unsegmented. We have identified Cdx transcription factors as key determinants of the spinal cord region in zebrafish. Loss of Cdx1a and Cdx4 functions causes posterior expansion of the hindbrain at the expense of the unsegmented spinal cord. By contrast, cdx4 overexpression in the hindbrain impairs rhombomere segmentation and patterning and induces the expression of spinal cord-specific genes. Using cell transplantation, we demonstrate that Cdx factors function directly within the neural ectoderm to specify spinal cord. Overexpression of 5’ Hox genes fails to rescue hindbrain and spinal cord defects associated with cdx1a/cdx4 loss-of-function, suggesting a Hox-independent mechanism of spinal cord specification. In the absence of Cdx function, the caudal neural plate retains hindbrain characteristics and remains responsive to surrounding signals, particularly retinoic acid, in a manner similar to the native hindbrain. We propose that by preventing the posterior-most region of the neural plate from following a hindbrain developmental program, Cdx factors help determine the size of the prospective hindbrain and spinal cord territories.

KEY WORDS: Cdx, Caudal, Hox, Retinoic acid, Segmentation, Rhombomeres, Hindbrain, Spinal cord, Central nervous system, Chordates, Vertebrates, Evolution

INTRODUCTION

One of the most prominent characteristics that distinguishes the rostral and caudal regions of the vertebrate central nervous system (CNS) is its segmental nature. Both forebrain and hindbrain have been shown to be organized into boundary-restricted, clonally related groups of cells known as neuromeres (Figdor and Stern, 1993; Fraser et al., 1990; Orr, 1887). The forebrain neuromeres (or prosome reres) and the more familiar hindbrain rhombomeres are important for proper neural organization and structural patterning of the adult brain and brain stem (reviewed by Borday et al., 2004; Kiecker and Lumsden, 2005; Puelles and Rubenstein, 2003). In the hindbrain, extensive genetic and molecular data have shown the existence of a regulatory network of transcription and secreted factors that specify the identity and order of rhombomere formation (Waskiewicz et al., 2002) (reviewed by Moens and Prince, 2002). In sharp contrast, the spinal cord remains unsegmented, although an anterior-posterior iterative arrangement of various neuronal populations can be seen to different degrees in Amphioxus, zebrafish and in certain tetrapods (Bone, 1960; Fetcho, 1987; Forehand and Farel, 1982; Myers, 1985; Roberts and Clarke, 1982; Stern et al., 1991). However, these iterated arrangements are not the consequence of cryptic segmentation, as demonstrated by the extensive mixing of cells in clonal analysis experiments (Morin-Kensicki and Eisen, 1997; Stern et al., 1991), but are the result of spinal cord neurons responding to paraxial mesoderm-derived signals (Dwight, 1934; Eisen and Pike, 1991; Ensin et al., 1998; Keynes and Stern, 1984; Lewis and Eisen, 2004). Despite the striking difference in segmentation, the hindbrain and spinal cord share a number of characteristics: both derive from the caudal neural plate (Brown and Storey, 2000; Muhr et al., 1997; Schoenwolf, 1992), are patterned along their anterior-posterior axis by Hox transcription factors (Deschamps and van Nes, 2005; Krumlauf et al., 1993; Lumsden and Krumlauf, 1996) and have a common evolutionary origin (Ghysen, 2003; Hirth et al., 2003). Therefore, we set out to investigate the mechanisms that direct the caudal neural plate to develop as hindbrain or as spinal cord.

Cdx (Caudal) transcription factors have been implicated in the development of trunk and tail structures across all major animal groups by controlling the sequential addition and identity of body segments (Copf et al., 2004). Within vertebrates, the three family members Cdx1, Cdx2 and Cdx4 are expressed in nested domains in the trunk and tail of the embryo (Davidson et al., 2003; Frumkin et al., 1993; Gardner and Wright, 1993; Joly et al., 1992; Lohnes, 2003; Marom et al., 1997; Meyer and Gruss, 1993; Pillemer et al., 1998; Reece-Hoyes et al., 2002). Most of our understanding of Cdx function is restricted to their role in paraxial mesoderm in mouse, where they have been shown to integrate FGF, retinoic acid and Wnt signals into coherent Hox gene expression (reviewed by Deschamps and van Nes, 2005; Lohnes, 2003). This role seems to be conserved in zebrafish and Xenopus (Davidson et al., 2003; Davidson and Zon, 2006; Isaacs et al., 1998; Pownall et al., 1998; Pownall et al., 1996; Shimizu et al., 2006; Shimizu et al., 2005). The function of Cdx genes in CNS development, however, is poorly understood, despite the fact that expression of Cdx genes in the caudal neural plate is highly conserved across vertebrates (Ehrman and Yutzey, 2001; Frumkin et al., 1993; Joly et al., 1992; Marom et al., 1997; Meyer and Gruss, 1993; Nordstrom et al., 2006; Pillemer et al., 1998; Reece-Hoyes et al., 2002).

Using a variety of morphological, cellular and molecular criteria we present evidence that spinal cord specification in zebrafish is dependent on the partially redundant functions of Cdx1a and Cdx4.
In agreement with a previous study (Shimizu et al., 2006), we show that zebrafish embryos lacking full Cdx1a and Cdx4 functions develop an expanded hindbrain. In addition, we show that this expanded hindbrain is organized into segmental units arranged in a mirror-image duplicated pattern of ectopic rhombomeres within the trunc region of the embryo. We also show that Cdx factors can induce the development of spinal cord cell types and posterior Hox gene expression, when misexpressed in rostral regions of the CNS. We propose that Cdx transcription factors normally function to prevent rhombomere formation in the caudal neural plate and that by preventing the posterior-most region of the neural plate from following a segmented developmental program, Cdx transcription factors help determine the size of the prospective hindbrain and spinal cord territories. We hypothesize that this newly proposed function of Cdx transcription factors allowed the development of the dorsal, hollow and unsegmented caudal neural tube that is characteristic of the vertebrate lineage.

MATERIALS AND METHODS

Fish care, microinjection, cell transplantation and pharmacological treatments

Zebrafish (Danio rerio) were raised and handled following standard techniques (Westerfield, 1994). Embryos from wild-type AB stock, kgg21864 (Golling et al., 2002), Tg Isl1:GFP (Hagishijima et al., 2000) and Tg [factin:GFP] (Gillette-Ferguson et al., 2003) were obtained from natural spawning, grown at 28°C and staged as described (Kimmel et al., 1995). Injections were carried out at the one-cell stage. Antisense cdx1a (5 ng) (Shimizu et al., 2005) and cdx4 (20 ng) (Davidson et al., 2003) morpholino oligonucleotides (Gene Tools LLC) were injected alone or in combination. For mRNA overexpression, 25 pg of hoxc6a, hoxb8a or hoxa9a (Prince et al., 1998a), or 40 pg of gap43-RFP (cell membrane marker, gift of E. Amaya, University of Cambridge, Cambridge, UK) capped sense mRNA (SP6 mMessage mMachine Kit, Ambion) was injected using a standard injection protocol (Bruce et al., 2001). Retinoic acid signaling was blocked by incubating embryos in 1 μM BMIS493 in the dark.

Cell transplantation was performed as previously described (Ho and Kane, 1990). Donor embryos were injected at the one-cell stage with 40-kDa lysine-fixable fluorescein (Invitrogen). Donor cells were collected at sphere stage and approximately 20 cells were transplanted 2- to 5-cell diameters away from the margin of a stage-matched unlabeled host.

Transgenic constructs, genotyping and heat shock

To generate a heat-inducible cdx4 expression construct, full-length zebrafish cdx4 was PCR amplified using the following primers: mCdx1-5’-forward, 5’-CGATCCATCGGATCCCGGCGCCACAGCTGTGGATGTAGCTGGTTCTGCAGAAAAGACAGCACATGTATACCCAA-3’ (BamHI and AgeI sites underlined, translation initiation site in bold); and 3’MCsdsexreverse, 5’-TTGTCTAGAAGAGCTTGTACGATAGTTGATGTGCTTAGATGACGATTACCGGCGTGTTGGAGAATTC-3’ (XbaI site underlined). The forward primer changes the 5’ end of the gene to that of mouse Cdx4, rendering it unrecognizable to the zebrafish cdx4 morpholino. BamHI/XbaI-digested PCR product was cloned in-frame into pcDNA3.1− (−)/Myc-His B (Invitrogen). The modified mouse 5′-zebrafish cdx4-myc-his (5′zcdx4) gene was digested with SphI, blunt ended, digested with AgeI and gel purified. Separately, the zebrafish hsp70 promoter (Halloran et al., 2000) and the pBS-ISce-II KS vector (Thermes et al., 2002) were digested with SacI/AgeI and SacI/EcoRV, respectively, and purified. A double ligation was then set up using the purified fragments to generate pshp-psp70:m5′zcdx4-IscE. All constructs were confirmed by sequencing.

Stable transgenic fish were generated by injecting 1 μg pshp-psp70:m5′zcdx4-IscE plasmid and 1 unit of ISce-I meganuclease in 1× ISce-I buffer (New England Biolabs) into fertilized eggs during the first 15 minutes of development (Thermes et al., 2002). Embryos were grown to adulthood, pairwise crossed and their embryos genotyped using the 3pshp70-forward (5′-GATAACTTCTGTTGATAACGTGCC-3′) and BGHrev (5′-TGAAGGGAACACGATCGGAG-3′) primers (ITD). As positive PCR controls, Wnt5a-12 (5′-CATGTTTCACAGGCTGATTGCGACTGA-3′) and Wnt-21 (5′-CTCTCCGCGTGTTGGAGAATTC-3′) primers were included in all reactions. Founder fish whose progeny produced diagnostic bands were out-crossed to wild-type fish. F1 fish were genotyped and individuals carrying the transgene were used as the Tg[pshp70:cdx4] line founders. A standard heat-shock protocol was used to induce transient cdx4 expression (Halloran et al., 2000).

Whole-mount in situ hybridization and immunocytochemistry

Detection of cdx4 (Shimizu et al., 2005); cdx4 (Joly et al., 1992); cyp26a1 (Kudo et al., 2002); epha4a (Xu et al., 1994); hoxb2 (2) (Odenthal and Nusslein-Volhard, 1988); islet1 (Inoue et al., 1994); krox20 (Otxoby and Jowett, 1993); hoxa2, hoxa8a, hoxa9a, hoxa4a, hoxb3a and hoxa4a (Prince et al., 1998a; Prince et al., 1998b); myod (Weinberg et al., 1996); ollg (Park et al., 2002); radical fringe (rfng) (Cheng et al., 2004); radlh2 (also known as aldh1a2 – ZFIN) (Begemann et al., 2001); retinoic acid receptor alpha (RARα; also known as rara – ZFIN) (Hale et al., 2006); and valentino (val) (Moens et al., 1998) expression by in situ hybridization was carried out as previously described (Bruce et al., 2001), using NBT/BCIP or Fast Red as the enzyme substrate.

Antibody labeling was performed as previously described (Svoboda et al., 2001). Monoclonal mouse anti-acetylated Tubulin (Sigma-Aldrich), mouse anti-myosin HC (A4.1025, Developmental Studies Hybridoma Bank, IA, USA), mouse anti-neurofilament 160k (RMO44, Zymed, CA, USA) and polyclonal rabbit anti-GFP conjugated to FITC (Invitrogen) were used at 1:500, 1:100, 1:5000 and 1:1000, respectively. Goat anti-mouse [Alexa Fluor 647 (Invitrogen) and/or FITC (Jackson ImmunoResearch)] or goat anti-rabbit (Alexa Fluor 488, Invitrogen) secondary antibodies were used at 1:2000.

Image processing

Deyolked embryos were manually sectioned using a scalpel. Specimens were photographed with a Nikon D1 digital camera mounted on a Leica MZFL III or Zeiss Axioskop microscope. For confocal microscopy, single optical sections and image stacks were obtained using a Zeiss laser-scanning confocal imaging system (LSM 510). Three-dimensional reconstructions were produced with the Zeiss LSM 510 software and ImageJ 1.32 (NIH). Figure panels were constructed using Photoshop 7.0 (Adobe).

RESULTS

Cdx4 is required for proper anterior-posterior position of the hindbrain-spinal cord transition

In zebrafish, the Cdx genes cdx1a and cdx4 are expressed in the posterior portion of the embryo (see Fig. S1 in the supplementary material) (Davidson et al., 2003; Davidson and Zon, 2006; Joly et al., 1992; Shimizu et al., 2005). We tested the function of Cdx4 in zebrafish CNS development by examining the distribution of hindbrain and spinal neuronal populations in wild-type and cdx4 morpholino-injected embryos (Fig. 1A-J). These embryos, referred to hereafter as cdx4-deficient embryos, are phenotypically indistinguishable from cdx4 loss-of-function mutants kgg21864 and kgg22090 (Davidson et al., 2003; Golling et al., 2002). We used the vagal motor neurons and the T reticular interneurons as landmarks for the posterior hindbrain. These neurons are located in the spinobulbar junction, and they have a characteristic organization in vertebrates that is evolutionarily conserved (Fetcho, 1987; Kimmel et al., 1985; Wake, 1993). We found that in cdx4-deficient embryos, the size of the vagus expanded posteriorly by two somites as compared with wild-type siblings (Fig. 1A,F). Similarly, the RMO44 immunopositive T reticular interneurons also expanded posteriorly in these embryos (Fig. 1B,G). Other hindbrain-specific populations, such as the branchiomotor and reticulospinal neurons of rhombomeres (r) 1-6, appeared unaffected (Fig. 1B,G and data not shown). We also examined three different spinal cord populations in cdx4-deficient embryos: motor neurons and their exit roots,
We injected wild-type and hindbrain-spinal cord transition in zebrafish. To test this hypothesis, background led to an almost complete loss of spinal cord identities: embryos injected with a cell populations described above (Fig. 1K-T). Whereas wild-type morpholino (Shimizu et al., 2005) and examined the distribution of possibility that Deschamps and van Nes, 2005; Lohnes, 2003), raising the patterning of the mouse paraxial mesoderm (reviewed by Cdx transcription factors have been shown to act redundantly in the territory specification of the prospective spinal cord. Cdx1a and Cdx4 act redundantly in the axial location of the transition from hindbrain to spinal cord. Together, these data suggest that Cdx1a and Cdx4 function redundantly in the proper determination of the axial location of the transition from hindbrain to spinal cord.

Cdx1a and Cdx4 act redundantly in the specification of the prospective spinal cord territory
Cdx transcription factors have been shown to act redundantly in the patterning of the mouse paraxial mesoderm (reviewed by Deschamps and van Nes, 2005; Lohnes, 2003), raising the possibility that cdx1a and cdx4 might cooperate in determining the hindbrain-spinal cord transition in zebrafish. To test this hypothesis, we injected wild-type and cdx4-deficient embryos with a cdx1a morpholino (Shimizu et al., 2005) and examined the distribution of cell populations described above (Fig. 1K-T). Whereas wild-type embryos injected with a cdx1a morpholino showed no CNS defects (Fig. 1A-E.K-O), reduction of cdx1a function in a cdx4-deficient background led to an almost complete loss of spinal cord identities: spinal motor neurons and oligodendrocytes were absent, and the number of isl1-positive Rohon-Beard sensory neurons was greatly reduced (Fig. 1R-T). Conversely, there was a posterior expansion of r7 and r8 in these embryos, as shown by the trunk and tail distribution of isl1-GFP-positive branchiomotor neurons (Fig. 1P). Most of these branchiomotor neurons had axonal projections and morphologies characteristic of vagal cells, although separate and distinct neuronal clusters were also observed near the tail of these embryos (Fig. 1P). A posterior expansion of T reticular interneurons was also observed in these embryos (Fig. 1Q). Together, these data suggest that Cdx1a and Cdx4 function redundantly in the specification of spinal cord-specific neuronal identities, and in their absence most of the caudal neural plate takes on an expanded hindbrain fate.

The hindbrain patterning and segmentation program is improperly induced in the CNS of cdx1a/cdx4-deficient embryos
We next examined the expression of Hox patterning genes in the CNS of cdx1a/cdx4-deficient embryos (i.e. in embryos deficient for both genes). In these embryos, the r7 and anterior spinal cord marker hoxd4a was found to be expressed throughout the posterior CNS, whereas expression of the spinal cord markers hoxb6a, hoxb8a and hoxb10a was notably absent (Fig. 2A,B,G,H and data not shown). Within the hindbrain, cdx1a/cdx4-deficient embryos showed no changes in the anterior limit of hoxa2b (r2), hoxb1a (r4), hoxb3a (r5) and hoxd4a expression as compared with wild-type embryos (Fig.
Fig. 2. Loss of Cdx function activates the expression of hindbrain genes in the posterior CNS. Expression of hindbrain markers (purple) in wild-type (A-F, D-F) and cdx1α/cdx4-deficient (G-L, J-L') zebrafish embryos counterstained for the r3 and r5 marker knx20 (red signal). (A, G) Spinal cord hoxb8a expression (bracket in A) is lost in cdx1α/cdx4-deficient embryos (bracket with asterisk in G). (B, H) r7/r8 hoxd4a expression (bracket in B) is expanded caudally in cdx1α/cdx4-deficient embryos (bracket in H) except for the most caudal tip of the CNS (asterisk in H). (C, I) In addition to its normal domain of expression in r4, hoxb1a expression can also be seen in the posterior CNS of cdx1α/cdx4-deficient embryos (bracket in I). (D, J) In the hindbrain, val is expressed in r5 and r6 of wild-type (D) and cdx1α/cdx4-deficient (J) embryos. In the tail region, val is also expressed in the posterior CNS of cdx1α/cdx4-deficient (bracket in J, J') but not wild-type (D') embryos. (E, K) Overlapping expression of epha4a (purple) and knx20 (red) are visualized in r3 and r5 of wild-type (E) and cdx1α/cdx4-deficient (K) embryos. In the tail region, epha4a and knx20 are expressed in the posterior CNS of cdx1α/cdx4-deficient (bracket in K, K') but not wild-type (E') embryos. (F, L) In the hindbrain, radical fringe (rfng) is expressed in seven stripes at the rhombomere boundaries in wild-type (F) and cdx1α/cdx4-deficient embryos (L). In the tail region, rfng is also expressed in the posterior CNS of cdx1α/cdx4-deficient (bracket in L, L') but not wild-type (F) embryos. For each condition, a minimum of 44 embryos from at least three independent experiments were analyzed, with more than 82% of embryos displaying the phenotype shown. Representative 20-somite, stage-matched, whole-mounted embryos are shown in dorsal view, anterior to the left. The position of somite 3, the hindbrain-spinal cord transition in wild-type embryos, is indicated with a white arrowhead. The planes of section are indicated with two short vertical bars. Sections are dorsal to the top, with the neural rod delineated by the dashed line. Scale bars: 100 μm.

2B.C.H.I and data not shown) suggesting that only spinal cord fates fail to be specified in these embryos. Together with our morphological data, these results show that the native hindbrain region in embryos lacking Cdx1a and Cdx4x activities has a normally ordered and nested set of rhombomeric identities and that the increase in hindbrain size seen in these embryos is mostly owing to an expansion of r7 and r8.

We also noticed that in cdx1α/cdx4-deficient embryos, the expanded hoxd4a-positive r7/r8 region did not extend along the entire length of the posterior CNS (Fig. 2H, asterisk). Instead, ectopic expression of more-anterior rhombomere-specific genes such as the r4-specific marker hoxb1a was seen within the posterior CNS (Fig. 2I, bracket). This phenomenon was not restricted to Hox genes. The gene valentino (val; also known as mafb and kr), which is normally expressed in the eyes, hindbrain r5 and r6 and in the tail mesenchyme (Moens et al., 1998), was also found to be expressed in the posterior CNS of these embryos (Fig. 2D, J, J'). Similarly, knx20 (egrbh – ZFIN) expression, normally confined to r3 and r5 (Octoby and Joguet, 1993), was now present in the posterior CNS (Fig. 2G, K, K'). This shows that in cdx1α/cdx4-deficient embryos, several members of the regulatory network controlling rhombomere patterning are ectopically expressed outside of their native hindbrain region.

The regulatory network controlling hindbrain patterning also controls its segmentation into rhombomeres (Moens and Prince, 2002; Waskiewicz et al., 2002). Therefore, we examined the expression of epha4a, radical fringe (rfng) and foxb1.2 (also known as mar) in cdx1α/cdx4-deficient embryos, as these genes have been shown to be involved in rhombomere cell-sorting and boundary formation (Cheng et al., 2004; Cooke et al., 2001; Cooke et al., 2005; Odenthal and Nusslein-Volhard, 1988). In wild-type embryos, epha4a is expressed within odd-numbered rhombomeres as well as in the forebrain, midbrain and tail notochord, whereas expression of rfng and foxb1.2 in the CNS is restricted to the boundaries between rhombomeres (Fig. 2E, F). In cdx1α/cdx4-deficient embryos, these genes were ectopically expressed in small discontinuous domains in the posterior CNS (Fig. 2K-L'), showing the induction of hindbrain segmentation genes beyond their normal domain of expression. Together, these data suggest that in the absence of Cdx1a and Cdx4 activities, the caudal neural plate not only fails to acquire spinal cord characteristics, but it also becomes competent to initiate the molecular program leading to the formation of supernumerary hindbrain segments and boundaries.

Development and mirror-image patterning of supernumerary rhombomeres in the CNS of cdx1α/cdx4-deficient embryos

Despite the fact that several hindbrain- and boundary-specific genes were ectopically expressed in the posterior CNS of cdx1α/cdx4-deficient embryos, we noted the absence of definitively sized additional rhombomeres in this region. We hypothesized that the expansion of the native r7/r8 region in cdx1α/cdx4-deficient embryos could be inhibiting the formation of correctly sized rhombomeres within the transformed caudal neural
Fig. 3. Development of supernumerary rhombomeres and their mirror-image patterning in cdx1a/cdx4-deficient zebrafish embryos with compromised RA signaling. Changes in rfg, foxb1.2 (marl), hox (purple) and krx20 (red) gene expression visualized in wild-type (A-H) and cdx1a/cdx4-deficient (I-P) embryos treated with the retinoic acid (RA) receptor inhibitor BMS493 (BMS) at mid-gastrulation (75% epiboly, 8 hpf). (A,I) hoxc4a expression in r8 is lost in wild-type (bracket with asterisk in A) and cdx1a/cdx4-deficient (I) embryos with compromised RA signaling. (B,J) RA inhibition reduced expression of the r7/8 marker hoxd4a in wild-type (bracket in B) and cdx1a/cdx4-deficient (J) embryos. (C,K) krx20 expression in the hindbrain posterior to r5 is reduced in wild-type embryos (bracket in C) and is limited to a central domain of the CNS in cdx1a/cdx4-deficient embryos (K). (D,L) hoxb3a, which is normally expressed posterior to the r4/r5 boundary (bracket in D), is expressed in a central domain in the CNS of cdx1a/cdx4-deficient embryos that includes the krx20 r5 and ectopic expression domains (L). (E,M) hoxb1a is expressed in r4 of wild-type (E) and cdx1a/cdx4-deficient (M) embryos. The latter also shows an additional hoxb1a-positive domain of expression in the posterior CNS. (F,N) hoxa2b is strongly expressed in r2 and r3 and weakly in r4 in wild-type (F) and cdx1a/cdx4-deficient (N) BMS-treated embryos. (G,O) rfg is expressed in six and nine boundary-like stripes (arrowheads) in wild-type (G) and cdx1a/cdx4-deficient (N) BMS-treated embryos, respectively. (H,P) foxb1.2 is expressed in nine boundary-like stripes (arrowheads) in cdx1a/cdx4-deficient BMS-treated embryos (P), compared with the six stripes seen in their wild-type siblings (H). For each condition, a minimum of 44 embryos from at least three independent experiments was analyzed at the equivalent of the 20-somite stage. More than 82% of embryos displayed the phenotype shown. Representative embryos were dorsal flat-mounted, anterior to the left. Ectopic rhombomere-like krx20 domain of expression is labeled with an arrow. Position of somite 3, the hindbrain-spinal cord transition in wild-type embryos, is indicated with a white arrowhead. Supernumerary rhombomeres are labeled r'. Scale bar: 100 μm.

In order to experimentally reduce the size of the r7/8 region, we took advantage of the observation that retinoic acid (RA) signaling inhibition results in the loss of r6-8 markers in wild-type (Begemann et al., 2004; Maves and Kimmel, 2005) and cdx1a/cdx4-deficient embryos (Shimizu et al., 2006). We treated embryos with the pan-RA receptor inhibitor BMS493 at mid-gastrulation to reduce the size of r7/8 without affecting the development of more-rostral rhombomeres (Begemann et al., 2004; Maves and Kimmel, 2005). As predicted, these treatments led to the loss of the r8 marker hoxc4a and an altered expression limit of the r7/8 marker hoxd4a (Fig. 3A,B,I,J). We then examined the expression of more-anterior Hox genes including hoxa2b (r2/r3), hoxb1a (r4), hoxb3a (r5/r6) and hoxd3a (r6) in comparison with the r3 and r5 marker krx20 (Fig. 3C-F,K-N). Excluding hoxa2b, whose expression domain was confined to r2/3 as in wild-type embryos (Fig. 3N) (Prince et al., 1998b), ectopic expression of these markers was observed in rhombomere-sized domains in the posterior CNS (Fig. 3K-M). These embryos expressed krx20 in three definitive stripes (Fig. 3I-N), contrasting with the more loosely organized expression seen in the posterior of cdx1a/cdx4-deficient embryos without BMS493 treatment (Fig. 2). Within the second and third krx20 stripes, we observed broad hoxb3a expression (r5/r6 marker, Fig. 3I-L) and, nested within it, smaller hoxd3a (r6/7 marker, Fig. 3K) and hoxd4a (r7 marker, Fig. 3J) expression domains. The r4 marker hoxb1a was expressed in two rhombomere-like domains, between the first and second stripe of krx20 expression and posterior to the third krx20-positive domain (Fig. 3M). In addition, we examined the expression of the rhombomere boundary markers rfg and foxb1.2 (Cheng et al., 2004; Odenthal and Nusslein-Volhard, 1988) and found that cdx1a/cdx4/RA-deficient embryos had nine evenly spaced, boundary-like stripes (Fig. 3O,P), instead of the six seen in wild-type embryos (Fig. 2F and data not shown). Together, these results show that upon RA-pathway inhibition, cdx1a/cdx4-deficient embryos can develop three supernumerary rhombomeres in the posterior CNS in addition to the normal seven. These supernumerary rhombomeres express Hox identity genes in a reverse anterior-posterior orientation in what seems a mirror-image duplication of the hindbrain, as follows: r2, r3, r4, r5, r6, r7, r6, r5, r4 (Fig. 3I-L, summarized in Fig. 4).
The formation of supernumerary segments in the posterior CNS region of cdx1a/cdx4/RA-deficient embryos is likely to be owing to the loss of Cdx1a and Cdx4 activity rather than RA signaling, as inhibition of the RA pathway in wild-type embryos results in the loss of posterior rhombomeres (Begemann et al., 2004; Begemann and Meyer, 2001; Begemann et al., 2001). In support of this, close re-examination of hindbrain-specific gene expression in cdx1a/cdx4-deficient embryos not treated with BMS493 showed that these embryos had signs of incipient mirror-image duplication in the CNS, despite having normal expression levels of genes involved in the synthesis, reception and degradation of RA (see Fig. S2 in the supplementary material). For example, the expanded hoxd4a-positive r7/8 territory was flanked by the r5/6 marker val, which in turn was bordered by the r4-marker hoxb1a (Fig. 2H-J, summarized in Fig. 4). Together, these results suggest that the absence of Cdx1a and Cdx4 functions allows the posterior CNS to adopt a segmented pattern of development with the potential to develop supernumerary, hindbrain rhombomere-like fates.

**Cdx function is required in the CNS to prevent hindbrain expansion**

It has been suggested that spinal cord fate may depend on paraxial mesoderm-derived signals (Ensini et al., 1998; Muhr et al., 1999; Muhr et al., 1997; Nordstrom et al., 2002; Nordstrom et al., 2006), raising the question of whether Cdx function is required in the CNS, paraxial mesoderm, or in both tissues, to specify spinal cord. We transplanted cdx1a/cdx4-deficient, fluorescent-labeled cells into the CNS of wild-type host embryos and analyzed the expression of the r5/6 marker val (n=8; Fig. 5A–E), as this gene is ectopically expressed in the posterior CNS of cdx1a/cdx4-deficient but not wild-type embryos (Fig. 2D,J). As shown in Fig. 5, transplanted cdx1a/cdx4-deficient cells were typically found populating the entire length of the CNS by the 20-somite stage. In rostral regions, transplanted cdx1a/cdx4-deficient as well as wild-type host cells located in the native r5/6 territory expressed val (Fig. 5C). In the posterior CNS, only the transplanted cdx1a/cdx4-deficient cells, but not surrounding wild-type cells, expressed val, even when in isolation (n=8; Fig. 5D,E, arrowheads). In reciprocal experiments, transplantation of wild-type cells into the paraxial mesoderm of a cdx1a/cdx4-deficient host failed to prevent val expression within the posterior CNS (n=2; Fig. 5F-H). Taken together, these transplant experiments indicate that Cdx function is required autonomously within neural ectoderm for correct spinal cord fate specification.

We also transplanted wild-type cells into the CNS of cdx1a/cdx4-deficient embryos (n=5; Fig. 5G,J). Wild-type cells were able to contribute to the entire length of the CNS. In rostral regions, wild-type and cdx1a/cdx4-deficient cells were evenly distributed within the tissue, with transplanted cells expressing val only when located in the native r5/6 territory (Fig. 5I). Interestingly, in posterior regions, wild-type cells were found segregating from host cdx1a/cdx4-deficient cells and did not express val (black arrowheads). However, in the posterior CNS, isolated wild-type cells surrounded by cdx1a/cdx4-deficient cells were occasionally seen expressing val (Fig. 5J, white arrowheads). Together, these results suggest that the expression of the hindbrain-specific val gene can be controlled by the level of Cdx function within the CNS.

**cdx4 overexpression in the hindbrain induces spinal cord development**

Our experiments indicate that Cdx activity is required for the caudal neural plate to develop as spinal cord instead of as segmented hindbrain. This hypothesis predicts that cdx4 overexpression in the hindbrain should: (1) interfere with the segmentation of this region; (2) change hindbrain neuronal identities; and (3) induce spinal cord neuronal markers. cdx4 overexpression by mRNA injection at the one-cell stage causes severe gastrulation defects (Davidson et al., 2003) (data not shown). To overcome this limitation, we generated a transgenic fish line, Tg[hsps70:cdx4], carrying a 5’-end modified zebrafish cdx4 gene under the control of the heat-inducible hspt promoter, which enables the rapid and ubiquitous induction of transgene expression at any point during development by incubating the embryos for 1 hour at 37°C (Halloran et al., 2000) (see Fig. S3A,B in the supplementary material and data not shown).

To study the effects of cdx4 overexpression in rhombomere formation, one-cell stage Tg[hsps70:cdx4] embryos were injected with gap43-RFP mRNA, a membrane-tagged red fluorescent protein, to follow the formation of rhombomere boundaries after heat shocking the embryos at the three-somite stage. This labeling method has been used to reveal the rhombomere boundaries before they become morphologically distinct (Moen et al., 1998). In transgenic heat-shocked and control embryos, boundary formation initiated at the six-somite stage (data not shown) and visible boundaries were apparent by the 14-somite stage, although less well defined in embryos carrying the cdx4 transgene (Fig. 6A,B). By the 20-somite stage, however, the characteristic rhombomere bulges seen in wild-type embryos were not present in their transgenic siblings (Fig. 6C,D). At this stage, heat-shocked embryos also showed loss of rfg and foxb1.2 expression at the rhombomere boundaries, and downregulation of the cell adhesion molecule-encoding ephd4a gene in r1, r3 and r5 (Fig. 6E-H). Less severe defects were obtained when the transgene was induced at other developmental stages [from 75% epiboly to the ten-somite stage, 8-14 hours post-fertilization (hpf), data not shown]. These results show that Cdx4 interferes with rhombomere cell sorting and boundary formation.
We then examined the effects of *cdx4* overexpression on hindbrain neuronal populations and patterning. *cdx4* overexpression caused the anterior expansion of r7/8 neuronal populations such as the vagal motor neurons (nX) and the T reticular interneurons (Fig. 6I-L). Furthermore, rostral neuronal populations, including the trigeminal (nV in r2) and facial (nVII in r4) motor neurons as well as the MiD2 (r5), MiD3 (r6) and in some cases Mauthner (r4) reticulospinal neurons, were lost in these embryos (Fig. 6I-L). Furthermore, rostral neuronal populations, including the trigeminal (nV in r2) and facial (nVII in r4) motor neurons as well as the MiD2 (r5), MiD3 (r6) and in some cases Mauthner (r4) reticulospinal neurons, were lost in these embryos (Fig. 6I-L). Since posterior Hox genes are under direct Cdx regulation (Charite et al., 1998) and their activity is required to override anterior Hox function in caudal regions of the embryo (Duboule, 1991), it was important to know whether this hindbrain expansion was due to the posteriorization of hindbrain neuronal populations or whether this was due to the lack of posterior Hox activity or more directly to the loss of Cdx function itself. We overexpressed the posterior Hox genes *hoxa2a, hoxb2a* and *hoxa9a* by injecting 25 pg of the respective mRNAs into *cdx1a/cdx4* deficient embryos (Davidson and Zon, 2006). Although these results suggest that Cdx4 can interfere with normal hindbrain development, they do not address whether Cdx4 is sufficient to initiate the development of spinal cord neuronal populations. We examined the expression of *olig2*, a marker for spinal cord primary motor neurons and oligodendrocytes (Park et al., 2002), after *cdx4* overexpression. We found that this gene was ectopically expressed in the hindbrain of heat-shocked transgenic but not wild-type embryos (Fig. 6Q,R), suggesting that Cdx4 can divert the development of hindbrain cells to a spinal cord fate. Taken together, these results suggest that Cdx factors normally promote spinal cord development by inducing and later patterning neuronal cell types specific to this region, and by interfering with molecular pathways leading to hindbrain patterning and segmentation.

**Hox-independent specification of spinal cord fates by Cdx factors**

Hindbrain expansion in *cdx1a/cdx4* deficient embryos was accompanied by the loss of posterior Hox gene expression (Fig. 2). Since posterior Hox genes are under direct Cdx regulation (Charite et al., 1998) and their activity is required to override anterior Hox function in caudal regions of the embryo (Duboule, 1991), it was important to know whether this hindbrain expansion was due to the lack of posterior Hox activity or more directly to the loss of Cdx function itself. We overexpressed the posterior Hox genes *hoxa6a, hoxb8a* and *hoxa9a* by injecting 25 pg of the respective mRNAs into *cdx1a/cdx4* deficient embryos (Davidson and Zon, 2006). Although we were able to observe the rescue of the red blood cell marker *gata1* in our injected embryos (see Fig. S4 in the supplementary material), we never observed the rescue of the CNS defects seen in the *cdx1a/cdx4* deficient embryos. For example, branchiomotor neurons were still present along the trunk of *cdx1a/cdx4* deficient embryos overexpressing these posterior Hox genes (Fig. 7A-C). This suggests that Cdx factors have a Hox-independent role in spinal cord specification.
We also examined the effect that posterior Hox gene overexpression had on the mirror-image patterning of the CNS of cdx1a/cdx4-deficient embryos (Fig. 7D-I). In the native hindbrain, r3 and r5 krx20 expression was reduced or absent, whereas the r5/r6 val expression domain was mostly unaffected (Fig. 4D-I). We also found, as previously reported (Shimizu et al., 2006), that hindbrain patterning genes can be differentially affected by Hox gene overexpression (Fig. 7D-F, asterisks). This result shows that hindbrain to lose aspects of its segmental character and take on features of the spinal cord. We conclude that Cdx factors specify vertebrate spinal cord cell fates and, by regulating posterior Hox gene expression, additionally influence anterior-posterior patterning.

**DISCUSSION**

Our analysis of cdx1a/cdx4-deficient zebrafish embryos has shown that Cdx function is required within the neural tissue for spinal cord specification and patterning: loss of Cdx function causes the unsegmented spinal cord to become segmented and take on hindbrain features. In reciprocal gain-of-function experiments we find that cdx4 overexpression is sufficient to cause the segmented hindbrain to lose aspects of its segmental character and take on features of the spinal cord. We conclude that Cdx factors specify vertebrate spinal cord cell fates and, by regulating posterior Hox gene expression, additionally influence anterior-posterior patterning.

**Cdx promotes spinal cord development**

In zebrafish, at the beginning of gastrulation, the hindbrain and spinal cord precursor cells are broadly distributed along the margin of the epiblast and are not yet committed to their fate (Woo and Fraser, 1995; Woo and Fraser, 1998). Commitment occurs towards the end of the gastrulation period, when hindbrain and spinal cord cells occupy the anterior and posterior halves of the caudal neural plate, respectively (Woo and Fraser, 1995). This segregation and commitment of prospective hindbrain and spinal cord cells correlates with the restriction of cdx4 transcripts to the posterior third of the neuroectoderm from an initial broad, ventral-to-dorsal gradient of expression at the margin of the epiblast (see Fig. S1 in the supplementary material) (Davidson et al., 2003;
Davidson and Zon, 2006; Shimizu et al., 2005). In addition to the posterior CNS expression domain, \( \text{cdx4} \) and \( \text{cdx1a} \) are expressed in a lateral-posterior domain of the tailbud that contains the spinal cord precursor cells, among other lineages (Kanki and Ho, 1997). This nested expression explains the partially redundant function of \( \text{cdx1a} \) and \( \text{cdx4} \) in spinal cord specification and patterning; only the loss of both genes causes severe tail truncations, absence of spinal cord and, as previously shown, lack of hematopoietic stem cells (Davidson and Zon, 2006). This partially redundant function of \( \text{cdx1a} \) and \( \text{cdx4} \) in the development of the spinal cord is not unlike the situation described in the paraxial mesoderm of mouse (Chawengsaksophak et al., 2004; van den Akker et al., 2002; van Nes et al., 2006).

Notably, the failure of these Cdx-deficient embryos to develop spinal cord does not appear to be caused simply by a tail truncation, but also involves the posterior expansion of the hindbrain territory. The use of isil-GFP transgenic animals as well as various hindbrain-specific markers has shown that whereas the native anterior hindbrain regions appear unaffected, the native posterior hindbrain region, especially the r7/8 region, has greatly expanded its domain to take up the majority of the former spinal cord region in these embryos. Posterior to the expanded r7/8 territory, the remaining CNS expresses ectopic hindbrain-specific markers, including anterior Hox genes, at the expense of spinal cord-specific markers. In addition, our morphological studies have shown that spinal cord-specific characteristics, such as the formation of spinal nerve roots, are lost from the nervous systems of \( \text{cdx1a/cdx4} \)-deficient embryos. Together, our results suggest that Cdx factors are necessary for the specification and development of the spinal cord region.

The loss of Cdx functions in the developing nervous system leads to the formation of a larger than normal hindbrain region in which both expanded and ectopic rhombomeric identities can be found within the former spinal cord territory. We have also observed that in \( \text{cdx1a/cdx4} \)-deficient embryos, the hindbrain boundary markers rfng and foxb1.2 are ectopically expressed within the CNS in the tailbud region, and that this incipient segmentation resolves into recognizable rhombomere-like structures upon partial inhibition of RA signaling. The involvement of Cdx factors in not only spinal cord specification and anterior-posterior patterning, but perhaps also inhibition of segment formation, places these factors at an important regulatory crossroad.

**Cdx repression of hindbrain development**

A formal possibility is that Cdx factors might allow spinal cord specification by repressing hindbrain-specific characteristics within the posterior CNS; such a function would be consistent with the expression pattern of \( \text{cdx4} \) within the spinal cord region of the zebrafish. The creation of a heat-inducible \( \text{cdx4} \) transgenic line has enabled the overexpression of a Cdx factor at the end of the hindbrain determination period. We have shown that \( \text{cdx4} \) overexpression affects correct hindbrain formation. For example, the loss of \( \text{rfng} \) and \( \text{foxb1.2} \) expression within the hindbrain suggests that a relatively late step in the hindbrain segmentation cascade, namely the formation of segmentation boundaries, can be disrupted by \( \text{cdx4} \) overexpression. Therefore, this type of experiment suggests that by interfering with the hindbrain segmentation program, Cdx factors might be able to direct the caudal neural plate cells to a spinal cord fate.

By contrast, the analysis of more-upstream hindbrain segmentation pathway components such as \( \text{krx20} \), \( \text{epha4a} \) and \( \text{hoxb3a} \), gave variable results in our overexpression assays, depending on which region of the hindbrain was analyzed. This variation can be attributed to the heterochrony of the region, as different rhombomeres form at different times during development and express different sets of genes (Moens and Prince, 2002). In these experiments, we confined our analyses to cases in which \( \text{cdx4} \) overexpression was accomplished by heat-shock treatment administered at the three-somite stage, the developmental stage at which \( \text{cdx4} \) overexpression caused the most severe hindbrain abnormalities. At this time, genes involved in hindbrain patterning...
and rhombomeric boundary formation such as *hoxb1a*, *val* and *krx20*, are already expressed in the hindbrain (Moens et al., 1998; Prince et al., 1998b; Waskiewicz et al., 2002). Under these experimental conditions, the hindbrain region expresses what appears to be a mixed hindbrain/spinal cord identity with some aspects of hindbrain fate, such as the formation of vagal motor neurons, now overlapping with aspects of spinal cord fate, such as the ectopic expression of the spinal motor neuron marker *olig2*. It is likely that *cdx4* overexpression at different times of development will give different outcomes, a possibility we are currently testing. Despite this caveat, we have shown that the overexpression of *cdx4* is able to interfere with both the segmentation and specification of individual rhombomeric identities in the zebrafish hindbrain.

**Specification and patterning of the spinal cord territory by Cdx**

Although our work, like that of many others (reviewed by Deschamps and van Nes, 2005; Lohnes, 2003), shows that Cdx genes have roles in the establishment of Hox gene expression limits, we further propose that the initial function of Cdx in establishing the spinal cord field might be independent of a role in Hox gene regulation. We suggest that Cdx factors initially function to establish the prospective spinal cord territory by preventing the posterior-most region of the caudal neural plate from adopting a segmental developmental program (hindbrain fate) and by inducing or promoting the expression of spinal cord-specific gene expression. Consistent with this hypothesis are our data showing that the overexpression of 5′ Hox genes fails to rescue the loss of the spinal cord markers seen in *cdx1a/cdx4*-deficient zebrafish embryos and only causes the posteriorization of the expanded hindbrain. If Cdx functioned solely through the control of Hox gene expression, then the general overexpression of a posterior Hox gene would be predicted to prevent the expansion seen in *cdx1a/cdx4*-deficient embryos. Since this was not the case, we propose that separate hindbrain and spinal cord territories must be established prior to becoming receptive to Hox gene functions. In *cdx1a/cdx4*-deficient embryos, posterior hindbrain identities are still present when 5′ Hox genes are overexpressed. Our conclusion differs from that of Shimizu et al. (Shimizu et al., 2006), who interpreted their overexpression studies as showing that Hox genes could prevent the ectopic expression of posterior hindbrain fates in *cdx1a/cdx4*-deficient embryos. We note that Shimizu et al. (Shimizu et al., 2006) only utilized the r5 marker *krx20*; however, when we additionally evaluated the r5/r6 marker *val* it was clear that ectopic hindbrain fates were still present in the Hox-overexpressing embryos (Fig. 7). Therefore, our data suggest that in vertebrates, Cdx might have homeotic functions independent of those of Hox factors, similar to the function of the *caudal* gene in the *Drosophila* adult (Moreno and Morata, 1999). This homeotic function may act both prior to and independent of any downstream control of Hox genes, similar to the ability of *Drosophila* Caudal to repress *Abd-B* transcription and induce Distal-less, brachycenteron and even skipped gene expression during analia development (Moreno and Morata, 1999). Further work will be required to characterize the Hox-independent function of Cdx during spinal cord development.

Another function of Cdx factors within the nervous system is to allow the hindbrain and spinal cord regions to differentially respond to gradients of FGF, RA and Wnt signals in the embryo. This is illustrated by the striking mirror image expression of ectopic hindbrain patterning genes in *cdx1a/cdx4*-deficient embryos and by the failure of 5′ Hox gene overexpression to prevent this phenotype. As the caudal neural plate fails to be specified as spinal cord in *cdx1a/cdx4*-deficient embryos, it retains hindbrain characteristics and remains responsive to surrounding signals, particularly FGF and RA, in a manner similar to the native hindbrain region. For example, in wild-type embryos, *r4*-derived FGF signals are responsible for inducing and patterning the r5 and r6 regions (Maves et al., 2002). During normal development, the native hindbrain territory is located far removed from the tailbud region, which is another source of FGF signals (Draper et al., 2003; Griffin et al., 1995). However, in *cdx1a/cdx4*-deficient embryos, the expanded hindbrain now comes into close contact with the tailbud. As shown by Shimizu et al. (Shimizu et al., 2006), tailbud-derived FGF signals are able to mimic the FGF-dependent, r5- and r6-inducing activity of r4. This signaling activity, coupled with paraxial mesoderm-derived RA signals, is responsible for the induction of ectopic r4, r5, r6 and r7/8 identities in the trunk region of the embryo (summarized in Fig. 4). Because the gradients of FGF from the tailbud region of the embryo are reversed relative to the FGF gradients found in the native hindbrain region, the pattern of ectopic rhombomeric identities is likewise reversed within the trunk region. Therefore, the mirror-image pattern of rhombomeric identities seen in the trunk region of *cdx1a/cdx4*-deficient embryos could be produced by the normal responses of hindbrain tissues to the same types of signals that they would be exposed to in the hindbrain’s native location. This is further supported by transplantation experiments, in which individual *cdx1a/cdx4*-deficient cells in the spinal cord region of a wild-type host responded to FGF and RA factors as if they were located in the hindbrain. Therefore, we propose that the functions of the *cdx1a* and *cdx4* genes in the nervous system of the zebrafish are to inhibit the hindbrain developmental program by preventing the tissue from inappropriately segmenting and taking on inappropriate anterior-posterior identities.

**Evolutionary implications of Cdx function in spinal cord development**

Based on patterns of expression and functional similarities across species, we propose that the control of hindbrain and spinal cord development by Cdx transcription factors might be common to all vertebrates. Remarkably, the rostral limit of the most anteriorly expressed Cdx gene coincides with the position of the hindbrain/spinal cord transition in zebrafish, *Xenopus*, chick and mouse (Cambronero and Puuelles, 2000; Frumkin et al., 1993; Lohnes, 2003; Marom et al., 1997; Meyer and Gruss, 1993; Pillemer et al., 1998; Reece-Hoyes et al., 2002) (this work); Cdx factors thus define the prospective spinal cord territory in the caudal neural plate and, by exclusion, the region that will give rise to hindbrain. Careful re-examination of Cdx function in chordate neural tube patterning might prove useful in addressing the underlying developmental mechanisms and evolutionary origin of the vertebrate spinal cord.

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