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CDX4 and retinoic acid interact to position the hindbrain–spinal cord transition

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ABSTRACT

The sub-division of the posterior-most territory of the neural plate results in the formation of two distinct neural structures, the hindbrain and the spinal cord. Although many of the molecular signals regulating the development of these individual structures have been elucidated, the mechanisms involved in delineating the boundary between the hindbrain and spinal cord remain elusive. Two molecules, retinoic acid (RA) and the Cdx4 transcription factor have been previously implicated as important regulators of hindbrain and spinal cord development, respectively. Here, we provide evidence that suggests multiple regulatory interactions occur between RA signaling and the Cdx4 transcription factor to establish the anterior–posterior (AP) position of the transition between the hindbrain and spinal cord. Using chemical inhibitors to alter RA concentrations and morpholinos to knock-down Cdx4 function in zebrafish, we show that Cdx4 acts to prevent RA degradation in the presumptive spinal cord domain by suppressing expression of the RA degradation enzyme, Cyp26a1. In the hindbrain, RA signaling modulates its own concentration by activating the expression of *cyp26a1* and inhibiting the expansion of *cdx4*. Therefore, interactions between Cyp26a1 and Cdx4 modulate RA levels along the AP axis to segregate the posterior neural plate into the hindbrain and spinal cord territories.

1. Introduction

Following the initial induction of neural tissue, molecular cues act to partition the neural tube along the anterior–posterior (AP) axis to delineate four discrete territories of the nascent nervous system (Stern, 2001; Melton et al., 2004), the forebrain, midbrain, hindbrain and spinal cord. In contrast to the forebrain and midbrain, which are segregated by junctions that also serve as organizing centers (Martinez-Barbera et al., 2001; Rhinn and Brand, 2001), no morphological boundaries have been detected that separate the hindbrain territory from the spinal cord. However, the transition zone between the hindbrain and spinal cord territories can be distinguished by the position of tissue specific neurons and the expression of *Hox* genes, with the group 1–4 *Hox* genes expressed within the native hindbrain region and the group 5–13 *Hox* genes expressed throughout regions of the spinal cord (Gaunt et al., 1989; Gruss and Kessel, 1991; Hunt et al., 1991; Nolte et al., 2006; Prince et al., 1998a, 1998b).

Due to their collinear and nested expression in the hindbrain and spinal cord and their established importance in conferring identity to various tissues, the regulatory mechanisms governing regionalization of the hindbrain and spinal cord have been largely attributed to the function of Hox genes (Reviewed in Krumlauf et al., 1993; Schilling and Knight, 2001). However, alteration in hindbrain and spinal cord size or shifts of the hindbrain–spinal cord transition has so far not been reported in functional studies of Hox genes (Carpenter et al., 1993; Chisaka and Capecchi, 1991). Consequently, these findings suggest that additional mechanisms regulate the subdivision of the neural tissue into the domains of the hindbrain and spinal cord.

In contrast, perturbation of Retinoic Acid (RA) signaling, a crucial regulator of *Hox* gene expression in the hindbrain affects both hindbrain and spinal cord development. This is observed in embryos exposed to excess RA where the rostral expansion of posterior hindbrain *Hox* gene expression is also accompanied by a rostral expansion of spinal cord motor neurons (Emoto et al., 2005; Hernandez et al., 2007; Marshall et al., 1992; Wood et al., 1994) suggesting that the underlying phenotype of elevating RA signaling could be a rostral shift of the hindbrain–spinal cord (Hb–Sc) transition. Alternatively, these results could also indicate that increased RA levels may induce a transformation of cell fate from

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hindbrain to spinal cord. Previous findings employing RA reporters have suggested that, in the neural tube, RA signaling is present in the spinal cord at a higher level than in the hindbrain (Maden et al., 1998; Shimozono et al., 2013; Wagner et al., 1992). Since cells of both the posterior hindbrain and spinal cord are located adjacent to the somitic mesoderm source of RA, the concentration difference between these two regions has been partly attributed to the function of Cyp26, a family of RA degradation enzymes expressed exclusively in the hindbrain (Gu et al., 2005; Hernandez et al., 2007; White et al., 2007). For example, Cyp26-deficient embryos have a smaller hindbrain territory and a rostrally expanded spinal cord (Emoto et al., 2005; Hernandez et al., 2007), a similar phenotype found in embryos treated with excess RA (Durston et al., 1989; Marshall et al., 1992).

In addition to RA signaling, perturbation of CDX/Caudal, a family of transcription factors in vertebrates having at least one family member expressed in the spinal cord (Bel-Vialar et al., 2002; Charité et al., 1998; Isaacs et al., 1998; Shimizu et al., 2006; Skromne et al., 2007), also leads to AP positional shifts of the Hb-Sc transition. For example, *cdx*4 zebrafish mutants and morphants have caudally expanded hindbrains and reduced spinal cords (Shimizu et al., 2006; Skromne et al., 2007), congruent with a caudal-ward shift of the Hb–Sc transition. In contrast, loss of Cdx1a (Skromne et al., 2007) does not result in the mis-alignment of the Hb–Sc transition. Therefore, this Hb–Sc transition shift observed in Cdx4 deficient embryos suggests that Cdx4 may also be involved in delineating the sizes of the hindbrain versus spinal cord territories; however the regulatory role of Cdx4 and how it interacts with RA signaling in directing this process remains to be established.

Using various chemical inhibitors and genetic manipulations to alter RA signaling and Cdx4 function, we show that coordinated modulation of RA signaling at the Hb–Sc transition is required for proper AP localization of the Hb–Sc transition. Through gene expression analysis, we show that this is achieved through reciprocal regulation between Cdx4, which inhibits the expression of the RA degradation enzyme, *cyp26a1*, in the spinal cord, and RA, which suppresses *cdx*4 expression in the hindbrain. Further comparison between the arrangement of *hox* gene expressions and motor neuron positions in our epistasis experiments indicates that RA signaling and Cdx4 act to set the position of the Hb–Sc transition and do so independently from their roles in regulating *hox* gene expression.

2. Materials and methods

2.1. Fish husbandry, microinjections and pharmacological treatments

Zebrafish (*Danio rerio*) were raised and handled using standard protocols. Embryos were collected from *AB stock fish, *Tg(isl1:gfp)* (Higashijima et al., 2000) or gir^{rw716} (Cyp26a1 mutant, Emoto et al., 2005), grown at 28 °C in embryo media and staged following Kimmel (2005). Microinjections of *cdx*4 morpholinos were performed at the 1 cell stage as previously described using standard injection protocol (20 ng/μl; Skromne et al., 2007). Uninjected and Cdx4 morphants were incubated in the dark with 10 μM DEAB (inhibitor of RA synthesis; Aldrich) starting at 5.3 h post-fertilization (hpf) or 10 μM R115866 (Hernandez et al., 2007; Cyp26 inhibitor; JanssenPharmaceutica) starting at 4.3 hpf. Control embryos were incubated with the vehicle, 0.1% DMSO in embryo media.

2.2. Whole mount in situ hybridization and antibody staining

Gene expression was detected by standard *in-situ* protocol using NBT/BCIP and Fast Red as the enzyme substrate. Antisense RNA labeled with DIG or FITC were generated for *cdx*4 (Skromne et al., 2007); *cyp26a1*, *cyp26b1*, *cyp26c1* (Hernandez et al., 2007); *hoxa2b*, *hoxb1a*, *hoxb3a*, *hoxb4a*, *hoxd4a*, *hoxb8a*, (Prince et al., 1998a, 1998b); *krx*20 (Oxtoby and Jowett, 1993); *myoD* (Weinberg et al., 1996); *radical fringe* (Cheng et al., 2004); *mafb* (Moens et al. 1998). Antibody staining was performed following previously described method (Skromne et al., 2007). Mouse anti-myosin heavy chain (A4.1025, Developmental Studies, Hybridoma Bank, IA, USA); mouse anti-neurofilament 169 K (RMO44, Zymed, CA, USA); mouse zn5 antibody (Trevarrow et al.,1990); polycolonal rabbit anti-GFP (Invitrogen); mouse anti-acetylated tubulin (Sigma-Aldrich); were used at a dilution of 1:100, 1:50, 1:1000, 1:500 and 1:500 respectively. Anti-mouse Alexa-488 and Alexa-546 secondary antibodies were used at a concentration of 1:2000 (Skromne et al., 2007).

2.3. Imaging and embryo processing

Embryos processed for *in-situ* hybridization and antibody staining were deyolked and flat mounted on slides. Fluorescent images were taken using a Zeiss LSM 710 confocal microscope and images were processed using ImageJ. Additional images were collected on a Zeiss compound microscope using a Nikon-5000 camera. Images were compiled using Adobe Photoshop CS5.1.

3. Results

3.1. Cdx4 and RA have opposite activities in the specification and patterning of hindbrain and spinal cord territories

Although previous papers have reported shifts in *hox* gene expression in Cdx4 and RA deficient embryos, no findings so far have measured and carefully compared the shifts in patterning gene expression to changes in the size of the hindbrain and spinal cord territory. To begin to understand how Cdx4 and RA regulate AP specification and patterning of the hindbrain and spinal cord territories, we first analyzed the spatial distribution of hindbrain and spinal cord *hox* gene transcripts and motor neuron populations relative to somite landmarks in embryos deficient for Cdx4 or RA signaling.

As previously reported (Davidson and Zon, 2006; Shimizu et al., 2006; Skromne et al., 2007; Hayward et al., 2015), loss of Cdx4 had no effect on the expression of anterior *hox* genes (*hoxa2b,* and *hoxb3a*; Fig. 1A, B, E, F), but the expression of a posterior hindbrain gene (*hoxb4a,* Fig. 1C, G) is caudally expanded by 2-somite lengths into what is normally the spinal cord domain. Concurrently, loss of Cdx4 results in caudally shifted spinal cord *hoxb8a* expression (Fig. 1D, H), suggesting a caudal-ward expansion of the hindbrain with a concurrent caudal reduction of the spinal cord. These early changes in *hox* expression were mirrored by late changes in motor neuron architecture. For example, loss of Cdx4 caused a 2-somite length caudal expansion in the distribution of *isl*1-positive hindbrain vagal motor neurons (Fig. 2C), and an equal 2-somite length caudal shift in the position of Zn5-positive spinal motor neurons (Fig. 2D). Thus, Cdx4 is necessary for the anterior placement of the Hb–Sc transition.

Next we examined the function of RA on the Hb–Sc transition by exposing embryos to the RA synthesis inhibitor DEAB starting at the beginning of gastrulation (5.3 hpf). Consistent with previous reports (Begemann et al., 2001; Grandel et al., 2002), RA deficient embryos had a slight caudal expansion of anterior hindbrain gene