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Spatiotemporally restricted regulation of generic motor neuron programs by miR-196-mediated repression of Hoxb8

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ABSTRACT

Hox transcription factors are key determinants of antero-posterior identity and have been implicated in assigning positionally appropriate neuron subtypes in the neural tube. These roles inherently necessitate stringent control mechanisms that confine Hox protein activities to discrete spatiotemporal domains. Here, we provide evidence that the timing and rostro-caudal extent of Hoxb8 activity in the neural tube is tightly regulated by miR-196, a microRNA species encoded within three Hox gene clusters. In vitro and in vivo sensor-tracer analysis and transcription assays revealed that miR-196 activity restricts the caudal extent of Hoxb8 expression to the thoracic-lumbar intersect via 3' UTR-dependent negative regulation. Spatio-temporally inappropriate Hoxb8 activity, through relief of miR-196-mediated repression or direct misexpression, affected normal progression of motor neuron genesis by affecting generic motor neuron differentiation programs. In addition to uncovering a role for microRNA-dependent restriction of caudal Hox activities, these data thus indicate novel aspects of Hox-dependent neural tube patterning by revealing a requirement of temporal regulation of a generic neuronal specification program.

Introduction

The assembly of functional CNS circuits entails the acquisition of neuronal phenotypes in register with specific positions along the body axis (Edlund and Jessell, 1999; Sur and Rubenstein, 2005). This is particularly well illustrated in the developing spinal cord of the chick where motor neurons (MN) become organized into discrete columnar and pool clusters, in sync with unique axon targeting preferences for select muscle targets along the body axis (Landmesser, 2001; Tanabe and Jessell, 1996). Throughout the rostro-caudal extent of the nascent spinal cord, a gradient of Sonic hedgehog (Shh) protein, secreted from the floor plate, results in the ventro-lateral subdivision of the mitotic progenitor cells into discrete progenitor domains. Certain Shh threshold levels within the ventral neural tube result in the establishment of the MN progenitor (pMN) domain defined by co-expression of Homeodomain (HD) proteins, Nkx6.1 and Pax6, with Nkx2.2 and Irx3 which limit its ventral and dorsal boundaries, respectively (Dessaud et al., 2008; Jessell, 2000; Lee and Pfaff, 2001; Price and Briscoe, 2004). The activity of Nkx6.1/Pax6 within this MN progenitor domain is thought to activate expression of the basic Helix-loop-helix (bHLH) transcription factor Olig2. Olig2 in turn represents a key node in the MN differentiation process, by linking overall neurogenic differentiation programs with the acquisition of a generic MN identity (Marquardt and Pfaff, 2001; Mizuguchi et al., 2001; Novitch et al., 2001; Rowitch et al., 2002). The transient activity of Olig2 within subsets of pMN drives the further progression of MN specification by activating expression of MNR2 and Lim3 LIM-HD MN determinants, as well as neurogenic bHLH proteins, such as Ngn2. It also serves to suppress precocious activation of postmitotic MN markers and gliogenic factors (Lee et al., 2005; Novitch et al., 2001; Tanabe et al., 1998; William et al., 2003). Activation of MNR2/Lim3/Ngn2 just prior to cell cycle exit eventually results in the activation of the LIM-HD proteins Isl1, Isl2 and Hb9, and the consolidation of postmitotic MN identity (Jessell, 2000). Concomitant with postmitotic specification, MNs further diversify into distinct columnar and pool identities in register with their antero-posterior locale. At limb levels, MNs cluster into medial and lateral motor columns (MMC and LMC), sending axons to axial and limb muscles, respectively (Jessell, 2000; Landmesser, 2001).

Hox proteins appear to play a central role in assigning discrete columnar identities with respect to their rostro-caudal position (Dasen et al., 2003, 2005; Kessel, 1994). In higher vertebrates, the 39 Hox genes map to four distinct paralogous clusters, named Hoxa to Hoxd, which exhibit temporal and spatial collinearity (Duboule, 2007; Duboule and Dolle, 1989; Gaunt, 1988; Iimura and Pourquie, 2007; Pearson et al., 2005); genes located more 3' in a cluster are expressed earlier and more rostrally than 5' genes, which are expressed later and more caudally. This collinearity is to a large extent preserved in the spinal cord, resulting in distinctly overlapping expression domains that confer unique combinations of Hox proteins to different rostro-caudally positioned MNs.

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This exceptional spatiotemporal coordination implies tight regulatory mechanisms that confine individual Hox proteins to specific domains. Recently, post-transcriptional regulation through specific noncoding RNAs has been implicated in aiding this process, by repressing transient spatio-temporally inappropriate Hox expression domains (Rinn et al., 2007; Sessa et al., 2007). The repressive effect of microRNAs (miR) is exerted via direct binding to their target sequences in the 3' untranslated region (3'UTR) of an mRNA. The degree/level of repression is further determined by the complementarity of the miR-mRNA sequences, resulting in translational repression or mRNA degradation, for partial or complete complementarities, respectively (Brodersen and Voinnet, 2009; Filipowicz et al., 2008; Nilsen, 2007; Pillai, 2005). miR-196 microRNAs are encoded within the Hoxa, b and c clusters, and were shown to selectively target Hoxa7, b8 and c8, based on bioinformatic and microRNA-reporter analyses (Ohler et al., 2004). Bioinformatic analyses also predicted several additional Hox genes as putative targets of miR-196 (Grimson et al., 2007; Yekta et al., 2008). Viral overexpression of miR-196 in chick embryos effectively prevents induction of Hoxb8 by retinoic acid in the developing forelimb (Hornstein et al., 2005), while antagomiR mediated knockdown of miR-196 in the chick paraxial mesoderm results in a posterior transformation of the last cervical vertebra and vertebral malformations (McGlinn et al., 2009). Furthermore, miR-196 was found to be involved in salamander tail and spinal cord patterning during regenerative processes (Sehm et al., 2009).

Although several Hox genes have been shown to play a role in columnar specification of MNs along the rostro-caudal axis (Dasen and Jessell, 2009; Dasen et al., 2003) and in motor pool identities (Dasen et al., 2005), little is known about their function during basic motor neuron generation. Here we show that in the caudal neural tube, Hoxb8 and miR-196 are expressed in mutually exclusive domains, with miR-196 limiting the caudal-most extent of Hoxb8 expression. This observation suggests a regulatory relationship between Hoxb8 and miR-196 in the neural tube. Through sensor-tracer analysis and translation/transcription assays, we show that in the chick, miR-196 acts via 3'UTR-mediated suppression of Hoxb8 translation. Experimentally disrupting the normal spatiotemporal pattern of Hoxb8, through direct misexpression or miR-196 inhibitor mediated derepression, selectively impairs normal progression of MN determination programs. This study thus provides evidence for spatiotemporal regulation of a generic neuronal specification program through microRNA-mediated confinement of Hox protein activity.

Materials and methods

Northern blot analysis, RNase protection assay, whole mount in situ hybridization

Total RNA was extracted using TRIZOL (Invitrogen). Northern hybridization for the detection of small RNAs was performed as described (Brown, 2001) using LNA (miRCURY™ mmu-miR-196-a detection probe, Exiqon) or DNA probes against miR-196 or U6 small RNAs, respectively (for sequences see Table S1). Northern detection of the chick 3'UTR was performed according to standard protocols using total RNA separated on a formalin denaturing agarose gel and detected using chick Hoxb8-3'UTR antisense RNA probe (Sequence in Table S1). Reverse Transcriptase (RT) semi-quantitative PCR was performed on embryonic total RNA using the primers listed in Table S1, and the Qiagen One-step RT-PCR kit following manufacturer's instructions. Detection of miR-196 by RNase protection assay was performed using the miRVANA™ microRNA detection kit (Ambion) following manufacturer's instructions. For probe sequences refer to Table S1. Chicken embryos were staged as Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1951), and mouse embryos according to days after fertilization plugs (dpc—days post-coitum). Whole mount in situ hybridization was performed using DIG-labeled probes, mapping to nucleotides 302–632 of the chick Hoxb8 mRNA (NM_204911) and nucleotides 1214–1531 of the mouse Hoxb8 mRNA (NM_010461). Whole mount in situ hybridization for the miR-196 was performed as described before (Kloosterman et al., 2006) using miR-196 LNA probe (Exiqon; for sequence see Table S1).

Western blot analysis, immunohistochemistry, BrdU labeling

Western blot analysis was performed on total protein extracts, run on a 10% SDS gel and blotted to nitrocellulose membrane. The following antibodies/dilutions were used: Hoxb8 (mouse, Abnova/1:1000), GFP (mouse, Roche/1:1000), alpha tubulin (mouse, Sigma/1:4000), H2B (rabbit, Upstate/1:2000). Immunostaining was performed as described previously (Sharma et al., 1998). Unless otherwise indicated, transverse (10 μm) or horizontal (100 μm) cryosections were analyzed. The antibodies/dilutions used were as follows: Hoxb8 (mouse, Abnova/1:100), Olig2 (rabbit, Abcam/1:500), dsRED (rabbit, Clontech/1:500), H3P (mouse, CST/1:100), GFP (mouse, Roche/1:500), GFP-Alexafluor488 conjugated (rabbit, Invitrogen/1:1000), p27kip1 (mouse, Abcam/1:500). The Isl1/2 (39.4D5), Isl2(51.4H9), Lim1/2(4F2), Nkx2.2(74.5A5), Nkx6.1 (F55A10), Pax6, Pax7 and MNR2(81.5C10) antibodies were obtained from the developmental studies hybridoma bank, under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences. For BrdU labeling, the embryos were incubated with a 100 μM BrdU (Sigma) solution for 30 min. The immunostaining was performed with GFP-Alexafluor488 conjugated (rabbit, Invitrogen/1:1000) and BrdU (mouse, Roche/1:50) antibodies.

Vector details, cell culture/ transfection methods, microscopy

For all vector maps see Fig. S1. The miR-196 expression vector was generated by cloning a genomic DNA fragment of 675 bp flanking the miR-196 hairpin into the *Mlu*I and *Nhe*I sites of the bicistronic vector described previously (Das et al., 2006). The pCAGGS-Hoxb8 expression vector was created by cloning the chick Hoxb8 coding sequence (NM_204911) into the *Eco*RI site of the pCAGGS vector (For primer sequences see Table S1). The Hoxb8-IRES-GFP vector was generated by subcloning the Hoxb8 frame from the pCAGGS-Hoxb8 into the *Eco*RI sites of the pMX-IRES-GFP vector. The CMV-GFP-sensor and CMV-GFP-B8UTR vectors were generated by annealing designed oligonucleotides (Table S1) and their subsequent cloning into the *Hin*dIII and *Bam*HI sites of the CMV-EGFP-C1 vector (Clontech). To generate the luciferase sensor, the annealed oligonucleotides were cloned into the *Xho*I and *Not*I sites of the psiCheck double luciferase vector (Promega; for sequence see Table S1). For the miR-196 knockdown the miRIDIAN miR-196 inhibitor was used and as the negative control miRIDIAN microRNA negative control 2 RNA oligonucleotides (Dharmacon) were used, both as 200 pmol stocks in 1× siRNA buffer (Dharmacon). The inhibitor molecules were hairpin RNA oligonucleotides with chemical enhancements to improve efficiency and longevity. The cell lines used include human HEK293T and chick embryonic fibroblast (CEF) cells. HEK293T cells were transfected by FuGENE6 (Roche) and CEF cells by Lipofectamine™ 2000 (Invitrogen), following the manufacturer's instructions. All image analyses were performed on an Olympus BX60 fluorescence microscope or a Leica TCM laser scanning microscope.

Luciferase assay, quantitations, statistical analysis

The luciferase assay was performed using the Dual-luciferase reporter assay kit (Promega) on the Victor™Light 1420 luminescence counter (Perkin Elmer) and relative luciferase activity determined as Renilla/Firefly luciferase values. For quantitation of immunostained sections, the number of cells positive for each marker was counted in the electroporated and the contra-lateral non-electroporated side of the neural tube. The ratios of electroporated:non-electroporated cell