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Isl1 Is required for multiple aspects of motor neuron development

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ABSTRACT

The LIM homeodomain transcription factor Islet1 (Isl1) is expressed in multiple organs and plays essential roles during embryogenesis. Isl1 is required for the survival and specification of spinal cord motor neurons. Due to early embryonic lethality and loss of motor neurons, the role of Isl1 in other aspects of motor neuron development remains unclear. In this study, we generated Isl1 mutant mouse lines expressing graded doses of Isl1. Our study has revealed essential roles of Isl1 in multiple aspects of motor neuron development, including motor neuron cell body localization, motor column formation and axon growth. In addition, Isl1 is required for survival of cranial ganglia neurons.

Introduction

Functional motor circuits are dependent on generation of diverse types of neurons and establishment of precise connections of these neurons with their respective targets. Distinct subclasses of neurons in spinal cord are identified by their soma position, stereotypical axon trajectories and combinatory gene expression (Tsuchida et al., 1994; Appel et al., 1995; Tanabe and Jessell, 1996; Sharma et al., 1998). Combinatorial expression of LIM homeodomain (LIM-HD) transcription factors (LIM code) are required for specification and maintenance of distinct neuronal identities and control coordinated cell migration and axon guidance (Jessell, 2000; Shirasaki and Pfaff, 2002). In ventral spinal cord, motor neurons (MN) and V2 interneurons (IN) are derived from adjacent progenitors that share several components of their genetic programs, such as highly related LIM-HD factors Lhx3 and Lhx4. Lhx3 and Lhx4 are expressed in progenitor cells that give rise to both MNs and V2 INs and play a role in specifying the ventral MN identity (Sharma et al., 1998). Overexpression of Lhx3 alone in chick neural tube promotes the generation of V2 INs, whereas in combination with Isl1, it promotes MN generation (Tanabe et al., 1998; Thaler et al., 2002). LIM-HD factor Isl1 and Homeodomain protein HB9 are among the first MN genes expressed in postmitotic MNs in spinal cord. Hb9 is critical for the consolidation of MN identity by actively suppressing the V2 IN genetic program (Arber et al., 1999; Thaler et al., 1999). Mice deficient in HB9 display aberrant expression of the V2 interneuron marker Chx10, disorganized motor columns and axon pathfinding defects (Arber et al., 1999; Thaler et al., 1999). Recent biochemical and genetic studies have provided further insights into molecular mechanisms regulating fate specification of MN and V2 IN (Thaler et al., 2002; Lee and Pfaff, 2003; Nakano et al., 2005). A MN hexamer composed of 2NLI:2Isl1:2Lhx3 binds to and directly activates the Hb9 enhancer, whereas a V2 IN tetrameric protein complex without Isl1 (2Lhx3;2NLI) drives V2 IN genesis (Thaler et al., 2002). MNs express two V2 IN repressors: LIM only protein LMO4 and Hb9. LMO4 can block V2-tetramer assembly while Hb9 binds directly V2-tetramer response elements and suppresses their activation. Similarly, in V2 INs, V2-tetramer induces Chx10, a repressor that binds MN-hexamer response elements and blocks their activation. Thus, these cross regulatory feedback loops ensure precise assignment of MN and V2 IN fates (Lee et al., 2008).

Isl1 is expressed in all postmitotic MNs and is required for various aspects of MN development (Thor et al., 1991; Ericson et al., 1992; Lundgren et al., 1995; Pfaff et al., 1996; Thor and Thomas, 1997; Segawa et al., 2001). In *Drosophila*, Islet is required for motor axon pathfinding and neurotransmitter expression (Thor and Thomas, 1997). In zebrafish, knockdown of Isl2 leads to abnormal spinal MN soma localization and defects in motor axon projection and

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neurotransmitter expression (Segawa et al., 2001). In mice, ablation of Isl1 results in complete elimination of spinal MNs immediately after cell cycle exit (Pfaff et al., 1996). Reduced levels of total Islet proteins lead to an increase in V2a IN generation at the expense of MN formation (Song et al., 2009). Due to early embryonic lethality and early loss of MNs in Isl1 null mice, the role of Isl1 in later MN development remains unclear.

Isl1 is expressed in forebrain striatum and ablation of Isl1 in brain leads to a loss of cholinergic interneurons in the striatum and loss of cholinergic projection neurons in the nucleus basalis (Wang and Liu, 2001; Elshatory and Gan, 2008). Isl1 is expressed in sensory neurons of dorsal root ganglia and retina and is required for survival and differentiation of these cells (Elshatory et al., 2007; Pan et al., 2008; Sun et al., 2008). In addition, Isl1 is expressed in neurons of cranial ganglia and nucleus, but the role of Isl1 in these cell types has not been investigated (Thor et al., 1991; Inoue et al., 1994).

In the present study, we generated several mouse lines with graded reduction in Isl1 expression. We have shown that Isl1 is required, in a dose dependent manner, for specification and maintenance of spinal MN identity, proper cell soma settling and appropriate axonal trajectories of MNs. We have also shown that reduced Isl1 expression in Isl1 compound mutants leads to the conversion of prospective MNs to V2 INs. In Isl1 hypomorphic embryos, despite proper expression of HB9, MNs fail to form proper motor columns and motor axons which innervate axial muscles and diaphragm muscle are missing or truncated. In addition, we found that Isl1 is required for survival of cranial ganglia neurons.

Results

Generation of Isl1 hypomorphic and compound mutant Mice

Previously we generated an $Isl1^{nLacZ}$ knock-in mouse line in which a nuclear LacZ (nLacZ) gene was introduced into the endogenous mouse Isl1 locus immediately prior to the translation initiation site (ATG) (Sun et al., 2007). Heterozygous $Isl1^{nLacZ/+}$ are fertile and viable, thus utilized as control mice in this study. Homozygous $Isl1^{nLacZ/nLacZ}$ mutant embryos die around E9.5 with phenotypes identical to that described previously for conventional Isl1 null mutation, and no Isl1 immunoreactivity was detected in Isl1 mutant spinal cord (not shown), thus demonstrating that the $Isl1^{nLacZ}$ allele is a null allele. In this study, we generated a floxed Isl1 mouse line. Mice heterozygous for floxed Isl1 ($Isl1^{f/+}$) with or without the neo cassette, and mice homozygous for floxed Isl1 without the neo cassette ($Isl1^{f/f}$) are fertile and viable. However, mice homozygous for floxed Isl1 with the neo cassette ($Isl1^{f;neo/f;neo}$) died soon after birth with significantly reduced Isl1 expression in spinal MNs (Song et al., 2009), suggesting that the neo cassette interferes with Isl1 expression (hypomorphic allele). Although no general morphological defects were found, Isl1 hypomorphic mutant mice exhibited several motor dysfunctions, including an inability to move and breathe. Lungs of Isl1 hypomorphic mice were not inflated, or were barely filled with air (not shown).

To further compromise Isl1 expression, we crossed $Isl1^{f:neo/+}$ mice to $Isl1^{nLacZ/+}$ mice to generate an Isl1 compound mutant with one Isl1 null allele and one hypomorphic allele ($Isl1^{nLacZ/f;Neo}$). Compound mutant mice die embryonically before E12.5, due to cardiac defects (YS, SE, unpublished observation). To better visualize neuronal migration and axon projections in Isl1 hypomorphic mice, we crossed Isl1 hypomorphic mice to Hb9-GFP mice, in which GFP expression is under the control of the mouse Hb9 promoter (Wichterle et al., 2002).

Reduced Isl1 expression in Isl1 compound mutant leads to a reduction in the number of spinal motor neurons and neurons in cranial ganglia

During development, Isl1 is expressed in multiple cell types and tissues and plays essential roles in these cells (Pfaff et al., 1996; Ahlgren et al., 1997; Cai et al., 2003; Laugwitz et al., 2005; Elshatory et al., 2007; Elshatory and Gan, 2008; Pan et al., 2008; Sun et al., 2008). We first examined how Isl1 expression is affected in Isl1 compound mutant embryos by analyzing Isl1 and β-gal stainings. Wholemount β-gal staining showed that Isl1-nLacZ was expressed in a pattern similar to the Isl1 mRNA expression pattern published previously (Fig. 1A) (Pfaff et al., 1996; Cai et al., 2003). In $Isl1^{nLacZ/+}$ control embryos at E11.5, Isl1-nlacZ was expressed in various regions of the central nervous system, including the outer layer of the forebrain striatum, diencephalon, the ventral hindbrain MNs (Figs. 1A–C), the spinal MNs and a subpopulation of dorsal spinal interneurons (dI3 INs) (Figs.1A, D). Isl1-nlacZ was expressed in neurons of the peripheral nervous system, including neurons in the dorsal root ganglia (DRG) (Figs. 1A, D) and the sympathetic ganglia (SG) (Fig. 1D). Isl1-nlacZ was also expressed in most of the cranial ganglia with neurons derived from neural crest and/or placodes (Begbie and Graham, 2001) (Figs. 1A–C, shown are Oculomotor (III), Trigeminal (V), Facial/vestibulocochlear (VII/VIII), glossopharyngeal (IX), Vagal (X), Accessory (XI) ganglia/nerves). In compound mutant embryos, expression of β-gal and the number of β-gal expressing cells in most of these regions in central nervous system was significantly reduced (Figs. 1E–H). This result was confirmed by Isl1 antibody immunostaining (Figs. 1I–P). Compared to controls (Figs. 1I–L), there was a near absence of Isl1 immunoactivity in forebrain striatum and hindbrain MNs (Figs. 1M, O). Isl1 immunoreactivity in the V and VII/VIII cranial ganglia and the spinal MNs was significantly reduced (Figs. 1N, P).

Reduced Isl1 expression leads to malformation of cranial ganglia with increased cell death

To assess the dose dependent effects of Isl1 on cranial ganglia development, we performed wholemount neurofilament antibody staining on E11.5 control and Isl1 mutant embryos with graded reductions in Isl1 expression (Figs. 2A–D). In Isl1 hypomorphic mutant embryos, the size and morphology of cranial ganglia/nerves III, V, VII/VIII, IX, X and XI appeared relatively normal (Fig. 2B). However, axons of cranial nerve XII were significantly thinner and blunted (Fig. 2B, bracket and arrowhead). An ectopic axonal projection was observed that extended from glossopharyngeal ganglion (IX) and connected to vagal (X) and accessory ganglia (XI) (Fig. 2B, white arrow). Further reduction in Isl1 expression in Isl1 compound mutants led to a significant reduction in the size of cranial ganglia V, VII/VIII, IX and X, and axonal projections of these ganglia were significantly thinner (VII, IX, X and IX), blunted (V, VII) or nearly absent (V, XII) (Fig. 2C). Spinal motor axons in Isl1 compound mutant were also significantly thinner or blunted (Fig. 2C). In addition, conditional knockout of Isl1 using Nestin-Cre, which is expressed in neural progenitors (Lendahl et al., 1990; Song et al., 2009), led to a severe loss of cranial ganglia. Cranial nerves and spinal motor axons were blunted or lost (Fig. 2D).

Reduced size of cranial ganglia might be attributed to decreased neurogenesis or increased neuronal apoptosis. To assess apoptosis in cranial ganglia of Isl1 compound mutant embryos, we performed TUNEL staining and Sox10 immunostaining. Sox10 is an HMG-box transcription factor expressed in neural crest and placodal progenitors that give rise to neurons and glial cells in cranial ganglia. In Isl1 compound mutant embryos at E11.5, Sox10 expression, which outlines cranial ganglia, was similar to that of control embryos, suggesting that migration and proliferation of the neural progenitors are not affected in Isl1 compound mutant embryos (Figs. 3E–H). However, significantly increased apoptosis in cranial ganglia V, VII, IX and XI was observed in Isl1 compound mutant embryos (Figs. 3E–H and E′–H′), indicating that Isl1 is required for survival of cranial ganglia neurons. We did not observe significantly increased apoptosis in MNs of the hindbrain and spinal cord of Isl1 compound mutant embryos, despite significantly reduced Isl1 expression in these regions (data not shown).