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Slit and Semaphorin signaling governed by Islet transcription factors positions motor neuron somata within the neural tube

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ABSTRACT

Motor neurons send out axons to peripheral muscles while their cell bodies remain in the ventral spinal cord. The unique configuration of motor neurons spanning the border between the CNS and PNS has been explained by structural barriers such as boundary cap (BC) cells, basal lamina and radial glia. However, mechanisms in motor neurons that retain their position have not been addressed yet. Here we demonstrate that the Islet1 (Isl1) and Islet2 (Isl2) transcription factors, which are essential for acquisition of motor neuron identity, also contribute to restrict motor neurons within the neural tube. In mice that lack both Isl1 and Isl2, large numbers of motor neurons exited the neural tube, even prior to the appearance of BC cells at the ventral exit points. Transcriptional profiling of motor neurons derived from *Isl1* null embryonic stem cells revealed that transcripts of major genes involved in repulsive mechanisms were misregulated. Particularly, expression of *Neuropilin1* (*Npr1*) and *Slit2* mRNA was diminished in *Islet* mutant mice, and these could be target genes of the Islet proteins. Consistent with this mechanism, *Robo* and *Slit* mutations in mice and knockdown of *Npr1* and *Slit2* in chick embryos caused motor neurons to migrate to the periphery. Together, our study suggests that *Islet* genes engage Robo-Slit and Neuropilin–Semaphorin signaling in motor neurons to retain motor somata within the CNS.

Introduction

Motor neurons that control locomotion are a unique population of the CNS whose cell bodies lie in the neural tube but whose axons exit the neural tube and project toward muscles in the periphery. Numerous studies have investigated the initial acquisition of motor neuron identity and axon pathfinding. However, the key mechanisms that direct axons toward the motor exit points (MEPs) located in the ventral spinal cord while retaining the motor somata within the CNS are still unclear. Around embryonic day 9 (E9.0) in mice, motor neurons are born in the pMN domain of the ventral spinal cord (Nornes and Carry, 1978). Subsequently, they migrate laterally to occupy ventro-lateral positions, and their axons converge and pass through MEPs. Although several structural barriers such as neural crest-derived boundary cap (BC) cells, radial glia and basal lamina have been proposed to prevent motor neuron somata from escaping the neural tube, motor neurons are frequently found outside the neural tube when the specification of motor neurons is defective (Lee and Song, 2013; Niederlander and Lumsden, 1996; Thaler et al., 2004; Vermeren et al., 2003). Thus, mechanisms that retain cell bodies within the neural tube appear to include factors located within the motor neurons themselves.

Members of the LIM homeodomain (LIM-HD) transcription factor family play a role in numerous aspects of motor neuron development, including the initial acquisition of motor neuron identity and the diversification of motor columns (Ericson et al., 1992; Kania and Jessell, 2003; Song et al., 2009; Thaler et al., 2004; Tsuchida et al., 1994). The LIM-HD proteins Isl1 and Isl2 are very similar with 72% protein identity (98% in HD domain and 82% in the LIM domains). Isl1 first appears in all motor neurons when motor neurons exit the cell cycle, and the expression of Isl2 follows (Pfaff et al., 1996; Thaler et al., 2004). Later, expression of Isl1 and Isl2 becomes restricted to some of the motor columns, and this is important for assigning motor columnar identity. Consistent with an important function in motor neuron identity, conditional elimination of *Isl1* in the CNS results in a loss of motor neurons and the formation of ectopic V2a interneurons (Song et al., 2009). In *Isl2* null mice, however, only visceral motor neurons are affected and mis-positioned on the dorsal side of the spinal cord (Thaler et al., 2004). Thus, Isl1 and Isl2 may serve only partially overlapping functions in motor neuron development.

In this study, we identify a new function of Isl1 and Isl2, which allows axons but not cell bodies to penetrate MEP. In *Islet* mutant mice,

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many motor neurons exit from the neural tube regardless of their subtype, and this exit begins even before BC cells appear at the MEP. We find that the emigration of motor neurons in these animals is accompanied by downregulation of *Neuropilin1* and *Slit2* transcripts, raising the possibility that the latter genes may be targets of Islet proteins and that they may be responsible for preventing neuronal migration. Consistent with this hypothesis, we demonstrated that *Robo* and *Slit* mutant mice, as well as chick embryos with depleted *Nrp1* and *Slit2* transcripts, have motor neuron cell bodies that emigrate out of the neural tube. Our results suggest that repulsive activity in motor neurons controlled by the Islet proteins is a key mechanism maintaining the boundary between CNS and PNS.

Materials and methods

Mice

Isl1 hypo, *Isl2* null mice and *Hb9::GFP* mice were described previously (Lee et al., 2004; Song et al., 2009; Sun et al., 2008; Thaler et al., 2004). The Robo and Slit mutant strains were gifts of Dr. Marc Tessier-Lavigne, Rockefeller University (Long et al., 2004). Wild type C56BL/6 and CD-1 mice (6–8 weeks old) were purchased from Damul Science and Charles River Laboratories, respectively. Robo and Slit PCR genotyping was performed as previously described (Grieshammer et al., 2004; Long et al., 2004; Plump et al., 2002). All experiments used protocols approved by the Animal Care and Ethics Committees of the Gwangju Institute of Science and Technology (GIST), or by the University of Nevada, Reno Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The day when a vaginal plug was detected was designated embryonic day 0.5 (E0.5).

Immunohistochemistry, immunocytochemistry and in situ hybridization

Embryos were obtained and processed for immunohistochemistry or in situ hybridization as described previously (Song et al., 2009). The following antibodies were used: rabbit and guinea pig anti-Hb9 (Thaler et al., 1999), guinea pig anti-Lhx3 (Sharma et al., 1998), guinea pig anti-Chx10 (Thaler et al., 2002), rabbit anti-Foxp1 (Abcam), rabbit anti-Krox20 (Covance), rabbit and guinea pig anti-Isl1/2 (Ericson et al., 1992), mouse anti-Neurofilament (DSHB), mouse anti-GFP (Sigma), rabbit anti-β-III-tubulin (Covance), and rabbit anti-β-galactosidase (Cappel) antibodies. For immunocytochemistry, dissociated cultured cells were fixed and immunostained with antibodies including rabbit anti-Robo1 and Robo2 (kind gift of Dr. Elke Stein, Yale) and mouse anti-Isl1 (DSHB). Previous characterization of the Robo1 and Robo2 antisera confirmed that specific labeling was lost in homozygous mutants. For in situ hybridization, embryonic cDNA at E10.5 or E12.5 was used to generate riboprobes using an Advantage cDNA PCR kit (Clonetech).

Chick in ovo electroporation

Nrp1 siRNA/scrambled siRNA (Bron et al., 2004), *Slit2* morpholino/control morpholino (Giovannone et al., 2012) and siRNAs/scrambled siRNA against Semaphorin ligands (see Supplementary methods) were electroporated with GFP into the chick spinal cord at Hamburger and Hamilton (HH) stages 10 to 12 and harvested at HH stages 20 to 25. Electroporation was carried out using a square wave electroporator (BTX) with 5 pulses of 25 V, 50 ms at 1 s intervals.

Microarray analysis

Embryonic stem (ES) cells were derived from littermate blastocysts of *Isl1* heterozygous intercrosses (also containing *Hb9::gfp* transgenes) and cultured in standard ES cell conditions as described (Macfarlan et al., 2011). To induce motor neuron differentiation, ES cells were adapted to gelatinized dishes for two passages, and 10^6 trypsinized cells were seeded in mDiff medium (1:1 knockout DMEM:DMEM/F12 (Invitrogen), 5% Knockout™ Serum Replacement (Invitrogen), 1× NEAA (Mediatech), 2 mM L-glutamine, 14.3 mM 2-mercaptoethanol) in bacterial grade 10 cm^2 dishes, and the medium was changed every 2 days. RA (1 μM) and smoothened agonist (1 μM, Calbiochem) were added to induce motor neuron differentiation after 2 days of EB formation. *Hb9::gfp* positive cells were then collected on day 6 by FACS. RNA was prepared from mES-derived MNs using an RNEasy kit (Qiagen) with on-column DNAse digestion. dscDNA was generated from 100 ng–1 μg of total RNA using a GeneChip3′ IVT Express Kit (Affymetrix), fragmented, and hybridized to Affymetrix Mouse Genome 430 2.0 expression arrays. Differentially expressed genes in four wild type ES cell lines (heterozygous and homozygous) and three knockout lines (independent replicates from a single ES cell line) were identified using Vampire and the default settings (http://genome.ucsd.edu/microarray/).

Quantification and statistics

At least 6 sections from 3 embryos were analyzed for each genotype and axial level. Limb levels were determined by *Raldh2* expression in adjacent sections. To measure % of sections with ectopic motor neurons in the chick spinal cord, the proportion of sections that contained ectopic motor neurons on either side of the neural tube was calculated. Statistical significance was analyzed by unpaired Student's *t*-test and the Kruskal–Wallis test for multiple comparisons.

Results

Soma emigration of motor neurons occurs when the level of Islet protein expression is low

Since Isl1 and Isl2 co-exist in spinal motor neurons, they may play redundant roles that might be revealed only when both gene products were eliminated (Hutchinson and Eisen, 2006; Thaler et al., 2004). To test this idea, we investigated motor neuron formation in *Isl1* hypomorphic (*Isl1* hypo) mice and *Isl2* null (*Isl2* KO) mice (Sun et al., 2008; Thaler et al., 2004). *Isl1* hypo mice make low level of Isl1 due to the insertion of a neo cassette that reduces the splicing efficiency of *Isl1* transcripts (Sun et al., 2008). We examined expression of Isl1 and Isl2 in these mice by immunohistochemistry using antibodies against Isl1 at subsaturating concentrations (Sup. Fig. 1A–D, H). Isl1 protein was reduced to as little as 19.8% of normal in *Isl1* hypo mice and to 6.2% in *Isl1* hypo; *Isl2* KO mice. In *Isl1* hypo mice, Isl2 expression was reduced and it was almost undetectable in *Isl2* KO and *Isl1* hypo; *Isl2* KO mice (Sup. Fig. 1E–G, I). Thus, the level of total Islet proteins decreased in the order *Isl2* KO, *Isl1* hypo and *Isl1* hypo; *Isl2* KO.

When we examined the motor columns in the spinal cords of E11.5 *Islet* mutant mice, we found significant numbers of motor neurons labeled by Hb9, a transcription factor present in postmitotic motor neurons, outside the neural tube (Fig. 1). A few ectopic motor neurons were detected at the cervical level in *Isl2* KO and *Isl1* hypo mice (about 1 cell per quadrant of the spinal cord in *Isl2* KO and 4 cells in *Isl1* hypo mice). This is in stark contrast to littermate controls in which virtually no cells escaped from the neural tube. Remarkably, more than 15 ectopic motor neurons were found in *Isl1* hypo; *Isl2* KO mice, indicating that Isl1 and Isl2 cooperate to confine motor neurons. We also examined motor neurons in *Isl1* conditional *Nestin-Cre* knockout mice that selectively remove *Isl1* expression in the CNS (Song et al., 2009). *Isl1* conditional *Nestin-Cre* knockout mice had lower numbers of ectopic motor neurons (2 cells per section), indicating that elimination of *Isl1* is not sufficient to fully emigrate motor neurons (Sup. Fig. 2). Similar results were obtained at other segmental levels of the spinal cord (Fig. 1Q). Somatal emigration was accompanied by corresponding