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Knockdown of Lingo1b protein promotes myelination and oligodendrocyte differentiation in zebrafish

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ABSTRACT

Demyelinating diseases include multiple sclerosis, which is a neurodegenerative disease characterized by immune attacks on the central nervous system (CNS), resulting in myelin sheath damage and axonal loss. Leucine-rich repeat and immunoglobulin domain-containing neurite outgrowth inhibitory protein (Nogo) receptor-interacting protein-1 (LINGO-1) have been identified as a negative regulator of oligodendrocytes differentiation. Targeted LINGO-1 inhibition promotes neuron survival, axon regeneration, oligodendrocyte differentiation, and remyelination in diverse animal models. Although studies in rodent models have extended our understanding of LINGO-1, its roles in neural development and myelination in zebrafish (*Danio rerio*) are not yet clear. In this study, we cloned the zebrafish homolog of the human *LINGO-1* and found that *lingo1b* regulated myelination and oligodendrocyte differentiation. The expression of *lingo1b* started 1 (mRNA) and 2 (protein) days post-fertilization (dpf) in the CNS. Morpholino oligonucleotide knockdown of *lingo1b* resulted in developmental abnormalities, including less dark pigment, small eyes, and a curly spinal cord. The lack of *lingo1b* enhanced myelination and oligodendrocyte differentiation during embryogenesis. Furthermore, immunohistochemistry and movement analysis showed that *lingo1b* was involved in the axon development of primary motor neurons. These results suggested that Lingo1b protein functions as a negative regulator of myelination and oligodendrocyte differentiation during zebrafish development.

Introduction

Demyelinating diseases are medical conditions that are characterized by damage to the myelin sheaths that maintain the conduction of signals in the peripheral nervous system (PNS) or central nervous system (CNS). In turn, deficiencies in movement, sensation, cognition, or other functions are caused by the reduction in nerve conduction. Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS in which the myelin–oligodendrocyte complex of the brain and spinal cord (SC) is affected by T- or B-cell-mediated immune attacks, resulting in a broad spectrum of signs and symptoms, such as demyelination and scarring (Compston and Coles, 2008). Although a spontaneous regeneration of myelin sheaths (remyelination) is followed by demyelination in the early stages of MS, it often fails in many cases (Dubois-Dalcq et al., 2005). Current MS treatments aim to modulate the inflammatory component, whereas recent studies have focused on potential therapies that enhance the neuroprotection and remyelination (Emery, 2010; Martino et al., 2010). Nevertheless, the reasons for remyelination failure in MS have not yet been clarified (Franklin, 2002). Rodent models have allowed the identification of oligodendrocyte precursor cells (OPC) and the regulatory mechanisms of OPC differentiation and remyelination (Chang et al., 2002; Zawadzka and Franklin, 2007).

Leucine-rich repeat (LRR) and immunoglobulin (Ig) domain-containing neurite outgrowth inhibitory protein (Nogo) receptor-interacting protein-1 (LINGO-1) is a CNS-specific single-transmembrane protein. Human LINGO-1 is a 614-amino acid protein encoded on chromosome 15 (15q24.3, GI: 15029689). LINGO-1 contains 12 LRR motifs that are flanked by N- and C-terminal capping domains, one Ig domain, a transmembrane domain, and a short cytoplasmic tail. The cytoplasmic tail contains a canonical epidermal growth factor receptor-like tyrosine phosphorylation site (residue 591) (Mi et al., 2004). LINGO-1 is selectively expressed in both neurons and oligodendrocytes, but it is absent in

Abbreviations: PNS, peripheral nervous system; CNS, central nervous system; MS, multiple sclerosis; SC, spinal cord; OPC, oligodendrocyte precursor cells; pMNs, primary motor neurons; LRR, leucine-rich repeat; Ig, immunoglobulin; NOGO, neurite outgrowth inhibitory protein; LINGO-1, LRR and Ig domain-containing Nogo receptor-interacting protein-1; BDNF, brain-derived neurotrophic factor; TrkB, tyrosine-related kinase B; RNAi, RNA-mediated interference; ORF, open reading frame; SNP, single nucleotide polymorphism; SMART, Simple Modular Architecture Research Tool; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; WISH, whole-mount in situ hybridization; dpf, day post-fertilization; PCR, polymerase chain reaction; Q-PCR, real-time quantitative-polymerase chain reaction; WB, western blots; MOs, morpholino oligonucleotides; UTR, untranslated region; CDS, coding DNA sequence; GFP, green fluorescent protein; 5-MIS, 5-bp mismatch control; WT, wild type; EM, electron microscopy; MAs, Mauthner axons; MBP, myelin basic protein; NF1, neurofibromin 1; Olig2, oligodendrocyte lineage transcription factor 2; OKR, optokinetic response; Acc Dist, accumulation distance; IPP, Image-Pro Plus; hpf, hours post-fertilization; ZFNs, zinc finger nucleases; TALENs, transcription activator-like effector nucleases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline-Tween 20; PBS, phosphate buffered saline; MS-222, tricaine methane-sulfonate; SE, standard error; ANOVA, analysis of variance.

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astrocytes (Mi et al., 2008). In neurons, it functions as an essential component of the NgR1/p75 and NgR1/Taj (Troy) signaling complexes that regulate CNS axon growth through the RhoA pathway (Mi et al., 2004; Nakaya et al., 2012; Park et al., 2005; Shao et al., 2005). In the neuronal survival model (ocular hypertension), LINGO-1 also acts as a negative regulator of brain-derived neurotrophic factor (BDNF) through the tyrosine-related kinase B (TrkB) signaling pathway (Fu et al., 2010). In oligodendrocytes, LINGO-1 has been identified as a key negative regulator of OPC differentiation and myelination with various kinds of molecular genetic techniques. These studies include loss of function and gain of function methods, such as RNA-mediated interference (RNAi), blocking antibodies, null-mutant mice, and transgenic mice (Lee et al., 2007; Mi et al., 2005, 2007, 2009). These data indicate that LINGO-1 plays a key role in neural regeneration, OPC differentiation, and remyelination.

Mammalian and zebrafish (*Danio rerio*) oligodendrocytes and myelin are homologous, and zebrafish provide an increasingly useful myelination model through genetic manipulation and fluorescence transgenesis (Buckley et al., 2008). The newly generated transgenic zebrafish lines Tg(mbp:EGFP-CAAX) or Tg(mbp:mCherry-2A-wt/ca-Fyn) provided superior cellular resolution compared with other myelin transgenic reporter fish lines and revealed the interaction between individual axons with individual oligodendrocytes in vivo (Almeida et al., 2011; Czopka et al., 2013). The zebrafish transparent model of oligodendrocyte development, differentiation, and myelination could be utilized as an efficient tool for screening potential remyelination therapies in vivo (Buckley et al., 2008; Dubois-Dalcq et al., 2008). Despite the fact that evidence regarding the function and regulation of mammalian LINGO-1 has rapidly increased, no reports have been proposed about *lingo1b* in zebrafish. Here, we studied the function of the Lingo1b protein in zebrafish myelination and OPC differentiation during development and gained some insight into the roles of Lingo1 in zebrafish remyelination.

Materials and methods

Animal husbandry

Zebrafish of the WT Oregon AB strain and the *olig2*:EGFP transgenic line (Shin et al., 2003) were reared at 28.5 °C under standard laboratory conditions (Westerfield, 2000). Embryos were collected from natural spawning, which was staged by hpf or dpf according to established criteria (Kimmel et al., 1995) and reared in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM Mg_2SO_4, 10^{-5}% Methylene Blue). Adults were bred to produce AB homozygous or *olig2*:EGFP/AB heterozygous larvae.

All animal manipulations were conducted in strict accordance with the guidelines and regulations set forth by the University of Science and Technology of China (USTC) Animal Resources Center and University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the USTC (Permit Number: USTCACUC1103013). All zebrafish surgery was performed under solution of tricaine methane-sulfonate (MS-222, Sigma, USA) anesthesia, and all efforts were made to minimize suffering.

Plasmid construction

Different fragments of *lingo1a* and *lingo1b* sequences were amplified from the total RNA of the AB strain with the following primers (Supplemental Table S1). The purified polymerase chain reaction (PCR) products were digested and cloned into pCS2+ vector for RNA probes (*lingo1a*-CS2 and *lingo1b*-CS2) and *lingo1b* mRNA rescue (*lingo1b*-res-CS2) or cloned in pCS2+ GFP plasmids for *lingo1b* MO specificity (*lingo1b*-mo-CS2+ GFP).

WISH

WISH was performed as described previously (Thisse and Thisse, 2008). *Lingo1a* and *lingo1b* cRNA probes that were labeled with digoxigenin (Roche, Germany) were synthesized from linearized plasmids by Riboprobe in vitro Transcription Systems (Promega, USA). Stained embryos were imaged with a SZX-16 dissecting microscope and a DP-72 CCD camera (Olympus, Japan).

Morpholino injections and mRNA rescue

Two nonoverlapping anti-sense MOs against the translation-start site of zebrafish *lingo1b* (*lingo1b* MO1: 5′-ACGTCATGCGGTGGAGTCTAAATTT-3′) or the 5′UTR (*lingo1b* MO2: 5′-ATTCTGCCGGAGTGCAGCTTAGATC-3′) and one 5-bp mismatch MO of the *lingo1b* MO1 (5′-ACCTCATC CGCTGGACTGTAAATTT-3′) were synthesized (Gene Tools, USA). All of the MOs were resuspended in embryo medium and injected into embryos at the one-cell stage. For efficient knockdown, the doses of MOs that were injected per embryo were the following: *lingo1b* MO1 (4 ng), *lingo1b* MO2 (4 ng), and *lingo1b* 5-bp mismatch MO1 (4 ng). For the rescue experiments, capped full-length mRNA encoding zebrafish *lingo1b* (*lingo1b*-res-CS2) was synthesized in vitro with the mMessage mMachine SP6 Kit (Ambion, USA). The *lingo1b* mRNA (200 pg) was co-injected with *lingo1b* MO1 (4 ng) at the one-cell stage. For the MO specificity tests, capped MO-target mRNA encoding *lingo1b* and GFP (*lingo1b*-mo-CS2 + GFP) was also synthesized and co-injected with lingo1b MO1 (4 ng) or MO2 (4 ng) at the one-cell stage. Embryos were examined with a SZX-16 dissecting microscope, and the images were captured with a DP-72 CCD camera and DP2-BSW software (Olympus, Japan).

Real-time Q-PCR

Total mRNA was extracted from 4 to 6 groups of 30 WT and MO-injected embryos with RNAiso Plus (TaKaRa, China) and transcribed to cDNA with the PrimeScript RT reagent Kit With gDNA Eraser (TaKaRa, China). Power SYBR Green PCR Master Mix (ABI, USA) was used to amplify *lingo1b*, *mbp*, *nf1a*, *nf1b*, *olig2*, *isl1*, *isl2a* and the endogenous control, *β-actin* (Buckley et al., 2010a), in a MX3005P machine (Stratagene, USA). Primer sequences are listed in Supplemental Table S2. Standard references were used to normalize *lingo1b* or *mbp* levels to those of *β-actin*. The control *lingo1b* or *mbp* relative concentration as a factor was normalized to 1.

Western blots and antibodies

The Lingo1b rabbit polyclonal serum was generated by Abmart (Shanghai, China). Two rabbits were immunized and each was injected with two peptides (GSHKISMKMI and QGKEFKDFPDVL). The MBP rabbit polyclonal antibody was also generated by Abmart using the peptide SRSRSPPKRWSTIF as described previously (Lyons et al., 2005). Both antibodies were used at 1:500 for the western blots.

Embryos were dechorionated and deyolked as described previously (Westerfield, 2000). Three to five groups of 30 embryos were homogenized in sodium dodecyl sulfate (SDS) sample buffer containing the protease inhibitor PMSF (Sigma, USA). Protein lysates were separated on SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare, USA). The membranes were blocked in 5% w/v nonfat dry milk in Tris-buffered saline-Tween 20 (TBST). Signals were detected with rabbit anti-Lingo1b antibody (1:500, Abmart), rabbit anti-MBP (1:500, Abmart), mouse anti-actin (1:1000, LK-TAG, China) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, Abmart) overnight at 4 °C, which was followed by incubation with appropriate secondary horseradish peroxidase conjugated-anti-mouse/rabbit antibodies (1:10,000, Promega, USA) and Pierce ECL substrate (Thermo Scientific, USA), Chemiluminescence was detected with an ImageQuant LAS 4000 machine (GE, USA). Band densities were quantified with