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METASTASIS SUPPRESSOR 1 REGULATES NEURITE OUTGROWTH IN PRIMARY NEURON CULTURES

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Abstract—Metastasis suppressor 1 (MTSS1) or missing in metastasis (MIM) is an actin- and membrane-binding protein with tumor suppressor functions. MTSS1 is important for cell morphology, motility, metastasis. The role of MTSS1 in cell morphology has been widely investigated in non-neuronal tissues; however the role of MTSS1 in neurite outgrowth remains unclear. Here we investigated the effect of MTSS1 on neurite outgrowth in primary cerebellar granule and hippocampal neurons of mouse. We found that overexpression of MTSS1 in cerebellar granule neurons significantly enhanced dendrite elaboration but inhibited axon elongation. This phenotype was significantly reduced by deletion of the Wiskott–Aldrich homology 2 (WH2) motif and point mutation in the insulin receptor substrate p53 (IRSp53) and MIM/MTSS1 homology (IMD) domain. Furthermore, inhibition of Rac1 activity or blocking of phosphatidyl inositol phosphates (PIPs) signaling decreased the effect of MTSS1 markedly. In accordance with the over-expression data, knockdown of MTSS1 in cerebellar granule neurons could increase the axon length but decrease the dendrite length and the number of dendrites. In addition, MTSS1 knock down in embryonic hippocampal neurons suppressed neurite branching and reduced dendrite length. Our findings have demonstrated that MTSS1 modulates neuronal morphology, possibly through a Rac1–PIPs signaling pathway.

Key words: metastasis suppressor 1, neuronal morphology, neurite outgrowth.

INTRODUCTION

The actin cytoskeleton plays important role in the neuronal morphogenesis and structural plasticity (Luo, 2002; Tojima and Ito, 2004; Bouquet and Nothias, 2007; Cingolani and Goda, 2008). The dynamic assembly of local actin cytoskeleton and its interaction with the plasma membrane determine the morphogenesis of neurons (Luo, 2002; Tojima and Ito, 2004; Bouquet and Nothias, 2007). The actin-binding proteins could regulate cytoskeleton remodeling through sequestration of actin monomers, actin nucleation and polymerization, capping or crosslinking of actin filaments (Winder and Ayscough, 2005; Uribe and Jay, 2009; Lee and Dominguez, 2010). Furthermore, the signals originating from plasma membrane could be transmitted to the underlying actin cytoskeleton through small GTPase and phospho-inositides and influence the actin organization (Saarikangas et al., 2010; Hall and Lalli, 2010; Croisé et al., 2014).

MTSS1 is an actin- and membrane-binding protein with roles in cell morphology, carcinogenesis, metastasis and development (Lee et al., 2002; Machesky and Johnston, 2007; Xie et al., 2011). It contains a C-terminal actin monomer binding Wiskott–Aldrich homology 2 (WH2) motif and an N-terminal IRSp53 and MIM homology (IMD) domain, also known as a Bin/amphiphysin/Rvs (BAR) domain (Mattila et al., 2003, 2007; Yamagishi et al., 2004). The IMD domain facilitates fibrous actin bundling, activates Rac1-actin-related protein-2/3 complex mediated lamellipodia formation and induces membrane tubulation as reported for other BAR domains (Yamagishi et al., 2004; Bompard et al., 2005; Mattila et al., 2007). Over-expression of MTSS1 in different cell types induces Cdc42-independent filopodia-like structures, lamellipodia-like structures and loss of stress-fiber structures (Gonzalez-Quevedo et al., 2005; Bompard et al., 2005; Mattila et al., 2007; Machesky and Johnston, 2007).

MTSS1 is ubiquitously expressed during development and becomes more restricted in adulthood. In the central nervous system (CNS), MTSS1 is strongly expressed in developing neurons whereas in adulthood MTSS1 is highly expressed in limited areas of hippocampus and cerebellum (Mattila et al., 2003; Glassmann et al., 2007; Shi et al., 2012; Saarikangas et al., 2015; http://mouse.brain-map.org/experiment/show?id=1777). In addition, it is distributed throughout the cell body, axon and dendrites (Hayn-Leichsenring et al., 2011; Shi et al., 2012; Saarikangas et al., 2015).

The morphological function of MTSS1 in non-neuronal tissues is well described (Machesky and Johnston, 2007; Xie et al., 2011), but its role in neuronal morphogenesis is

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Abbreviations: Bar, Bin/amphiphysin/Rvs domain; GFP, green fluorescent protein; IMD, IRSp53 and MIM/MTSS1 homology domain; IRSp53, insulin receptor tyrosine kinase substrate p53; MTSS1, metastasis suppressor 1; PIPs, phosphatidylinositol phosphates; PIP2, phosphatidylinositol 4,5 bisphosphate.

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not so clear. Gonzalez-Quevedo et al. have speculated that morphogens Shh may cooperate with receptor tyrosine phosphatase-delta to control motor neuron morphogenesis through MTSS1 during spinal cord development (Gonzalez-Quevedo et al., 2005). They also observed that MTSS1 over-expression could induce special actin-based cell projections in Neuro-2a neuroblastoma cells (Gonzalez-Quevedo et al., 2005). Glassmann et al. and our previous study suggested that the transient developmental expression of MTSS1 in cerebellar granule neurons and Purkinje neurons closely parallels their migration and neuritogenesis respectively (Glassmann et al., 2007; Shi et al., 2012). Evidence suggests that MTSS1 may be involved in shaping the membranes of cerebellar Purkinje neurons *in vivo* (Hayn-Leichsenring et al., 2011). Recently, Saarikangas et al. found that MTSS1 might promote dendritic spine initiation through phosphatidylinositol 4,5 bisphosphate (PIP2)-mediated membrane bending to ensure proper synaptogenesis (Saarikangas et al., 2015). With view to the strong expression of MTSS1 between the postnatal 7 days and 21 days in the cerebellum, we speculated that MTSS1 might be also involved in the development of axon and dendrite (Glassmann et al., 2007; Hayn-Leichsenring et al., 2011; Shi et al., 2012).

Here, we examined the role of MTSS1 in the neurite outgrowth of primary neurons. Wild-type MTSS1 overexpression promoted dendrite elaboration but inhibited axonal extension in cerebellar granule neurons, whereas WH2 and IMD mutants did not. Inhibition of Rac1 activity and the use of phosphatidylinositol 3-kinase inhibitor reversed the effects of MTSS1 on dendrite and axon extension. Depletion of MTSS1 by shRNA in cerebellar granule neurons led to increased axon length but decreased dendrite length and the number of dendrites. Similarly, in hippocampal neurons, the knock-down of MTSS1 significantly reduced dendritic arborization. Our findings suggest that MTSS1 plays an important role in neurite outgrowth, which requires the activation of Rac1 and PIPs signaling pathway.

EXPERIMENTAL PROCEDURES

Materials

Poly-L-lysine, Earle's balanced salts, Basal Medium Eagle, Percoll and microtubule-associated protein 2 antibody were from Sigma–Aldrich (St. Louis, MO, USA). Horse radish peroxidase-conjugated secondary antibodies were from Abcam (Cambridge, UK). Trypsin, Dulbecco's modified Eagle's medium, B27 supplemental medium, fetal bovine serum, green fluorescence protein (GFP) polyclonal antibody, species-specific Alexa-Fluor® 488 dye-conjugated secondary antibodies, Prolong Anti-fade Kit, and goat serum were from Invitrogen (Carlsbad, CA, USA). Horse serum and glutathione-Sepharose beads were from GE-healthcare Life Sciences. Rabbit MTSS1 antibody was kind gift from Dr. Xi Zhan's laboratory. Pan-AKT antibody was from Cell Signaling (Danvers, MA, USA) and pAKT (pSer473) was from Santa Cruz (Dallas, TX, USA). Rac1 and growth associated protein-43 (GAP43) antibodies were from Proteintech (Wuhan, China). KOD FX DNA polymerase was from TOYOBO (Osaka, Japan). Restriction enzyme Dpn I was from Thermo Scientific Fermentas (Burlington, ON, Canada). pSuper.Retro.neo vector was from Oligoengine (Seattle, WA, USA). FuGENE® HD transfection reagent were from Promega (Madison, WI, USA). Papain and DNase I were from Worthington (Freehold, NJ, USA). Mouse neuroblastoma Neuro-2a cell line was from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All other chemicals are analytical grade.

Animals

BALB/c mice and Kunming mice were purchased from Hunan SLRC Laboratory Animal Ltd. (Changsha, China). In this study, 160 BALB/c pups (postnatal day 5) and 12 pregnant Kunming mice were used. The housing facility and the care of laboratory animal is in accordance with the 'Regulation for the administration of affairs concerning Experimental animals' issued by Hunan Provincial People's Government (Decree No. 259, 2012). All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Hunan Normal University.

Plasmids preparation

pEGFP-N1-MTSS1, pEGFP-N1-ΔWH2-MTSS1 and pGEX4T-2-p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1)-protein-binding domain (PBD) were kind gifts from Dr. Xi Zhan. pRK5-myc-Rac1-T17N was a kind gift from Dr. Gary Bokoch. pEGFP-N1-MTSS1-IMD mutant (K138A, K139A, K146A, K149, K150A) were prepared by point mutation strategy. MTSS1 shRNA targeting mouse *mtss1* gene (NCBI GenBank NM_144800) were designed by Invitrogen BLOCK-IT™ RNAi Designer (https://rnaidesigner.invitrogen.com/rnai) and constructed into pSuper.Retro.neo vector. The targeting sequences were 5′-GGGAAGACTTCATAAACAAAG-3′ and 5′-GCAATATACCCTCTTCCTTGT-3′ separately and the knock down efficiency has been tested on the MTSS1-GFP over-expression stable cell line before (Mao et al., 2011). The sequence 5′-TTCTCCGAACGT GTCACGT-3′ (QIAGEN) was used as nonspecific negative control.

Primary culture of cerebellar granule neuron and transient transfection

Cerebellar granule neuron cultures were prepared from BALB/c mice as described by Morrison (Morrison and Mason, 1998) with some modification. For any single cerebellar granule neuron purification experiment, 8–12 postnatal day 5 pups were used. Experiments were performed from three separate culture preparations. Briefly, cerebella from pups were pooled and digested with papain (10 U/ml) in Earle's balanced salts medium supplemented with 26 mM $NaHCO_3$, 0.5% glucose and 0.5 mM EDTA at 35.5 °C for 45 min and triturated with