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Nestin regulates neural stem cell migration via controlling the cell contractility

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ABSTRACT

Neural stem cells (NSCs) migration is essential for neurogenesis and neuroregeneration after brain injury. Nestin, a widely used marker of NSCs, is expressed abundantly in several cancers, where it may correlate with tumor migration and invasion. However, it is not yet known whether nestin participates in NSC migration. Here, we show that nestin down-regulation significantly inhibits the migration and contraction of murine neural stem cells, but does not obviously influence the proliferation, filamentous actin (F-actin) content, distribution or focal adhesion assembly of these cells. Mechanistically, nestin knockdown was found to affect the phosphorylation state of myosin regulatory light chain (MRLC) and regulate the activity of myosin light chain kinase (MLCK). Co-immunoprecipitation experiments showed that it interacts with MLCK and MRLC. Together, our results indicate that nestin may increase NSC motility via elevating MLCK activity through direct binding and provide new insight into the roles of nestin in NSC migration and repair.

1. Introduction

Neural stem cells (NSCs) have the capacity to self-renew and differentiate along multiple lineages, such as neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992). This makes them attractive candidates for cell replacement therapy for treating the neurodegenerative diseases, or repairing brain injury. Numerous studies have revealed that NSCs can proliferate and migrate towards to the sites of injury, such as in animal models of spinal cord injury (Decimo et al., 2011) and ischemic brain injury (Song et al., 2015), where they contribute to functional recovery. These processes are induced by cytokines and their receptors, including stromal cell-derived factor 1 (SDF-1α), and its receptor, chemokine receptor type 4 (CXCR4)/chemokine receptor type 7 (CXCR7) (Dziembowska et al., 2005; Imitola et al., 2004; Ohab et al., 2006; Schonemeier et al., 2008; Takeuchi et al., 2007), epidermal growth factor receptor (EGFR) and connexin 43 (Cina et al., 2009; Kim et al., 2009). However, the exact mechanism underlying NSC migration remains unclear.

Cell migration involves a series of highly coordinated steps, including plasma membrane protrusion, formation of adhesion sites under the protrusion, disruption of older adhesion sites at the cell rear, and contraction of the cytoskeleton to yield cell body

Abbreviations: Bleb, blebbistatin; CXCR4, chemokine receptor type 4; CXCR7, chemokine receptor type 7; EGFR, epidermal growth factor receptor; FAK, focal adhension kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IF, sintermediate filaments; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MRCK, myotonic dystrophy kinase-related CDC42-binding kinase; MRLC, myosin regulatory light chain; NM II, non-muscle myosin II; NSC, neural stem cell; PAK, p21 activated kinase 1; PI3K, phosphatidylinositol 3-kinase; pMRLC, phospho-MRLC; SDF-1α, stromal cell-derived factor 1; shRNA, short hairpin RNA.

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movement (Wehrle-Haller and Imhof, 2003). All of these processes are integrated by extensive signaling networks, like focal adhension kinase (FAK), Rho GTPases, phosphatidylinositol 3-kinase (PI3K), which regulate the dynamic of focal adhesions and cytoskeleton (Devreotes and Horwitz, 2015; Parsons et al., 2010). The contribution of the actin and microtubule cytoskeletal networks in migration is well established (Etienne-Manneville, 2013; Parsons et al., 2010). Protrusion is thought to result from actin filament (F-actin) polymerization against the plasma membrane (Pollard and Borisy, 2003). These protrusions are then stabilized by forming focal adhesions (FAs) that provide a link between the actin cytoskeleton and the extracellular matrix (ECM) and serve as traction points for the cell body to contract and move forward (Parsons et al., 2010). Contraction also promotes the disassembly of the adhesions at the cell rear allowing it to detach (Parsons et al., 2010). Unlike actin and microtubule, the role of intermediate filaments (IFs) is less clear.

Nestin is a class VI IF protein and a well-established marker for NSCs (Reynolds and Weiss, 1992). Its expression has also been detected in other adult stem cells, including hair-follicle bulge stem cells (Amoh et al., 2005), kidney resident mesenchymal stem cells (Jiang et al., 2015), and some tumor cells (Ishiwata et al., 2011; Kleeberger et al., 2007; Matsuda et al., 2011; Rutka et al., 1999). Indeed, nestin has been recently implicated in tumor invasion and metastasis. In many studies, nestin expression appears to be correlated with increased cell motility and invasiveness in a variety of tumor cells types, such as astrocytoma (Rutka et al., 1999), glioma (Ishiwata et al., 2011), prostate cancer (Kleeberger et al., 2007), and pancreatic cancer (Matsuda et al., 2011). This association may not be consistent, however, as nestin knockdown in prostate cancer cells was reported to dramatically increase cell invasion (Hyder et al., 2014). Thus, nestin may play distinct roles in tumor cell migration depending on the involved cell type. Given that nestin-expressing NSCs are capable of migrating under certain circumstances, it seems relevant to examine whether and how nestin contributes to NSC migration.

In this study, we examined the roles of nestin in migration using an immortalized mouse neural stem/progenitor cell line (C17.2) (Ryder et al., 1990). We found that nestin knockdown in C17.2 cells decreased cell migration and contraction, but did not affect cell F-actin content, distribution or FA formation. Moreover, nestin knockdown decreased the activity of myosin light chain kinase (MLCK) but did not affect that of myosin light chain phosphatase (MLCP). Finally, nestin was found to interact with MLCK and myosin light chain (MRLC).

2. Materials and methods

2.1. Cell culture

Immortalized C17.2 cells were grown in 90% Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 5% horse serum (Hyclone), at 37 °C under 5% CO_2. The medium was changed every 2 days. When reaching 80% confluence, cells were detached by incubating with 0.25% trypsin for 1–2 min at 37 °C and replated for continuous passage.

2.2. Lentiviral short hairpin RNA (shRNA) interference-mediated knockdown of nestin

Gene knockdown was accomplished using shRNA and the pLKO.1 lentiviral vector (Addgene). The target sequences, which were selected using Dharmacon software, were 5′-GTGAGACTCTGGAATGCAA-3′ (nestin shRNA #1), and 5′-TAGAATCATTGATAACTTTTAAA-3′ (nestin shRNA #2). The control shRNA sequence was 5′-CCTAAGGTTAAGTCGCCCTCG-3′. For preparation of lentiviruses, subconfluent 293FT cells were cotransfected with pLKO.1 and a compatible packaging plasmid mixture psPAX2 and pMD2.G (Addgene) using X-tremeGENE HD DNA transfection reagent (Roche Diagnostics, IN, USA). Lentivirus-containing supernatants were collected 48–72 h later, and enriched by ultracentrifuged at 50,000*g* for 90 min. Puromycin selection (4 μg/ml) was performed 48 h after infection for 3 days.

2.3. Cell proliferation assay

Cell proliferation was assayed with the Cell Counting Kit (CCK-8, Dojindo, Kumamoto, Japan). Briefly, cells were seeded into 96-well plates (1×10^3 cells/well), and then at indicated time points (day 0–7), the CCK-8 reagent (10 μl) was added to each well and incubated at 37 °C for additionally 2 h. The absorbance at 450 nm was measured under a microplate spectrophotometer (Bio-Tek ELX800, Winooski, VT, USA).

2.4. In vitro wound-healing assay

Cells (2×10^6) were grown to confluence on 60-mm culture dishes, and treated with 0.5 μM mitomycin C (Sigma-Aldrich, Chicago, IL, USA) for 2 h prior to wounding. Monolayers were scratched with a P-20 pipette tip, and washed repeatedly with PBS. Representative images were taken of the scratched areas under each condition at various time points. To estimate the relative migration of the cells, average gap measurements (AG; percentage) were used to quantify the data. The condition at 0 h was considered 100%.

2.5. Time-lapse analysis of cell migration

Tissue culture plates were coated with 5 mg/ml type I collagen, and cells were allowed to adhere for 12 h at 37 °C. Phase-contrast images were acquired every 5 min for up to 24 h using a Zeiss Axio Observer Z1 system (Zeiss, Göttingen, Germany). The Zeiss Axio software was utilized to track cell movement, and the migration speed was calculated by dividing the total distance moved (in microns) by the time.

2.6. Collagen gel contraction assay

Rat tail Type I collagen (Millipore, Billerica, MA, USA) was mixed with DMEM (final concentration, 1.5 mg/ml), the pH was adjusted to 7.4 with 1 M NaOH, and cells that had been treated with 0.5 μM mitomycin C for 2 h were added with a final concentration of 2×10^6 cells/ml. This mixture was dispersed (500 μl per well) to 12-well plates (Costar, Corning, NY, USA) and allowed to solidify for 12 h at 37 °C. Serum-free DMEM was added to the wells, and the collagen gels were gently detached using a pipette tip. The gels were incubated for 12 h at 37 °C, and then the diameter of each gel was measured.

2.7. Immunofluorescence

Cells were fixed with 4% paraformaldehyde, permeabilized for 30 min with 0.2% Triton X-100, and blocked for 30 min at 25 °C with goat serum. The cells were then incubated overnight with primary antibodies at 4 °C: vinculin and nestin were from Millipore (Billerica, MA, USA); pSer19-MRLC and myosin phosphatase target subunit 1 (MYPT1) were from Cell Signaling Technology (Beverly, MA, USA); MLCK was from Abcam (Cambridge, MA, USA), followed by incubation with secondary Alexa Fluor 488- or 594-conjugated antibodies (Invitrogen, USA) for 1 h at 25 °C. For visualization of actin, cells were stained with TRITC-conjugated