Function of Ezrin-Radixin-Moesin Proteins in Migration of Subventricular Zone-Derived Neuroblasts Following Traumatic Brain Injury

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ABSTRACT
Throughout life, newly generated neuroblasts from the subventricular zone migrate toward the olfactory bulb through the rostral migratory stream. Upon brain injury, these migrating neuroblasts change their route and begin to migrate toward injured regions, which is one of the regenerative responses after brain damage. This injury-induced migration is triggered by stromal cell-derived factor 1 (SDF1) released from microglia near the damaged site; however, it is still unclear how these cells transduce the SDF1 signals and change their direction. In this study, we found that SDF1 promotes the phosphorylation of ezrin-radixin-moesin (ERM) proteins, which are key molecules in organizing cell membrane and linking signals from the extracellular environment to the intracellular actin cytoskeleton. Blockade of ERM activation by overexpressing dominantly-negative ERM (DN-ERM) efficiently perturbed the migration of neuroblasts. Considering that DN-ERM-expressing neuroblasts failed to maintain proper migratory cell morphology, it appears that ERM-dependent regulation of cell shape is required for the efficient migration of neuroblasts. These results suggest that ERM activation is an important step in the directional migration of neuroblasts in response to SDF1-CXCR4 signaling following brain injury. STEM CELLS 2013;31:1696–1705

INTRODUCTION
In the adult brain, spontaneous neurogenesis is maintained in restricted areas, such as the subventricular zone (SVZ) along the lateral ventricle and the dentate gyrus (DG) of the hippocampus. Newly generated neuroblasts in the SVZ migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB), and neuroblasts in the DG migrate to the granule cell layer of the DG along the radial fibers. Normally, neuroblasts in the RMS form a chain-like, neurophilic attachment for efficient migration [1]. Following brain injury, on the other hand, neurogenesis is increased in the neurogenic regions, and the newly produced neuroblasts exit from their normal migratory route and move toward the injured regions [2–4], which may contribute to the functional recovery after brain damage [5–7]. Newly produced progenitors reach the injured cortex by migrating along the extracellular matrix metalloproteinases in migrating neural progenitor cells [8]. Previous reports pointed out the emergence of vimentin-positive astrocytes in the injured brain regions and suggested that radial glia-like cells serve as a scaffold to guide migration to the injury site [4, 9]. Blood vessels also function as a scaffold for migration of progenitor cells toward the injured region [10, 11]. Considering that neuroblasts in the RMS migrate in a chain, neuroblasts are required to detach themselves from the chain and change their mode of migration for injury-dependent rerouting.

Many diffusible and contact-dependent signals are involved in the tight regulation of neuroblast migration [12–15]. Stromal cell-derived factor 1 (SDF1), a CXC chemokine, is well known as a chemoattractant involved in injury-induced stem cell migration [6, 16–19]. CXCR4 is a receptor for SDF1, and neural stem cells and their progenitor cells express CXCR4 [18]. SDF1-CXCR4 signaling promotes the activation of survival signals via ERK activation and cytoskeletal changes through paxillin [16]. Furthermore, SDF1 increases the expression of matrix metalloproteinases in migrating neural progenitor cells [20]. Collectively, these signals may contribute to the rerouting...
of neuroblasts to the SDF1-expressing injury sites. However, intracellular events converting CXCR4 activation into rerouting of the migratory paths are less understood.

ERM-radixin-moesin (ERM) proteins are a family of membrane- cytoskeleton linking proteins [21, 22]. Because ERM proteins play a critical role in the control of cell-surface morphology via regulation of linkages between the plasma membrane and actin networks, they are involved in a wide variety of cellular functions, including signal transduction, cell-cell interaction, and cell migration [23, 24]. The function of ERM activity is mainly regulated by the intramolecular association of the N-terminal FERM/band 4.1 domain with the C-terminal actin-binding domain. Activation of ERM molecules requires at least two modifications. By binding of phosphatidylinositol(4,5)-bisphosphate at their FERM domains, ERM molecules are recruited to the cell membrane. Subsequently, phosphorylation at the C-terminal threonine site (T567 ezrin, T564 radixin, and T558 moesin) stabilizes an open conformation and promotes actin-binding activity [25]. ERM proteins act as essential mediators of various extracellular signals affecting growth cone morphology and neurite development [26–29]. It has been reported that migrating neuroblasts in the RMS also highly express ERM molecules such as radixin [30, 31], raising the possibility that ERM molecules are involved in the regulation of neuroblast migration in the RMS.

In this study, we found that brain injury-induced SDF1 activates ERM signaling through the induction of ERM phosphorylation, which is a requirement for neuroblast migration. These effects appear to be mediated by the regulation of actin remodeling-dependent neuroblast morphology. Our current observation elucidates how extracellular migratory signals influence actin-based cellular morphology.

**Materials and Methods**

**Animals and Cryogenic Traumatic Brain Injury**

Adult male C57BL/6 (2–3-month-old) were obtained from ORIENT BIO (Seongnam, Korea, http://www.orient.co.kr/common/main.aspx). Cryogenic traumatic brain injury (TBI) was performed as described previously [32]. Briefly, under deep anesthesia (pentobarbital 10 mg/kg), a metal probe (5 mm in diameter) chilled by liquid nitrogen was placed on the right side of cranium corresponding to the motor cortex for 1 minute. The animals were allowed to move freely in their cage for 3 days before sacrifice. Immediately after cryogenic TBI, some animals received AMD3100 (5 mg/kg b.wt. in phosphate-buffered saline [PBS]; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com/united-states.html) intraperitoneally twice daily with a 12-hour interval until sacrifice. All experiments were carried out in accordance with the regulations and approval of the Animal Care and Use Committee of Korea University.

**Western Blot**

Brain sections were obtained using a brain mold, and SVZ, RMS, OB tissues, or injured cortex regions were micropunched with a 1-mm micropunch (Ted Pella Inc., Redding, CA, http://www.merckmillipore.com) was directly administered. Cultures were maintained in a humidified incubator at 5% CO2 and 100% humidity. After one day in culture, the adult SVZ explants were seeded onto poly-(L-lysine) coated coverslips. The dilution of EGF (5 mg/kg in phosphate-buffered saline [PBS]; Sigma-Aldrich) was increased to 100 ng/ml, and penicillin-streptomycin was added. After 7 days of culture, the SVZ explants were lysed with a buffer containing 2% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 6.8), phenylmethylsulfonyl fluoride, and protein inhibitor cocktail (Roche Applied Science, Indianapolis, IN, http://www.roche-applied-science.com). Protein contents in the lysates were quantified by the BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, http://www.piercenet.com). Proteins (20–30 μg) were electrophoresed through a 10% SDS polyacrylamide gel and electroblotted onto a polyvinylidene fluoride membrane. The membrane was blocked in Tris-Buffered Saline-Tween 20 (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween-20) containing 5% non-fat milk for 1 hour at room temperature (RT) and then incubated with primary antibodies for 1 hour at RT. The dilutions for primary antibodies were used as follows: rabbit anti-ERM (1:1,000, Abcam, Cambridge, U.K., http://www.abcam.com), mouse anti-erdin (1:1,000, Abcam), rabbit anti-radixin (1:1,000, Abcam), and mouse anti-β-actin (1:4,000, Sigma-Aldrich). After an additional incubation for 1 hour with horseradish peroxidase-conjugated secondary antibodies (1:5,000, Jackson ImmunoResearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com), signals were visualized using ECL reagent (Pierce Biotechnology).

**Plasmid and Retrovirus Preparation**

The pC爾 retroviral vector with enhanced green fluorescent protein (EGFP) open reading frame fragment (EGFP-CL) was kindly provided by Dr. J.H. Park (Hanyang University, Seoul, Korea). The mouse ezrin cDNA was purchased from OriGene (Rockville, MD), and a dominant-negative ERM (DN-ERM) was obtained by polymerase chain reaction amplification of cDNA corresponding to the amino acid residues 1–406 of the mouse ezrin coding sequence and subcloned into the vector. The viral constructs were transfected into 293T packaging cells, and supernatants containing viral particles (SVG-V pseudotyped recombinant retrovirus) were harvested at 3–10 days after transfection. Harvested supernatants were centrifuged at 50,000 × g in a Beckman Type 60 Ti rotor (Beckman Instruments, Palo Alto, CA, http://www.beckman.com). The pellet was resuspended in 1/500 of the original volume of PBS. Viral titer was adjusted to 1 × 10^7 TU/ml. All constructs were confirmed by sequencing and Western blot after HEK29293 cells were transfected.

**SVZ Explant Cultures**

Brains of the EGFP- or DN-ERM retrovirus-infected Crl:CD1(ICR) pups (2–5 postnatal days) were dissected out and immersed in ice-cold Hanks’ balanced saline solution supplemented with d-glucose (Gibco BRL, Carlsbad, CA, http://www.invitrogen.com/site/us/en/home/brands/Gibco.html). Because the litter size of ICR mice is larger than C57BL/6 strain, we used ICR mice. Our preliminary data showed that there was no strain difference in basal chain migration properties (data not shown). The brains were cut into 300–350-μm-thick sections by a vibratome (Campden Instruments Ltd., Loughborough, Leicestershire, U.K, http://www.campden-inst.com), and slices containing SVZ were selected for further manipulations. Under a surgical microscope, the SVZ was dissected along the lateral wall of the lateral ventricles, cut into small pieces, and embedded into 75% Matrigel (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). After polymerization of Matrigel at 37°C for 30 minutes, neurobasal medium containing B27 supplement, t-glutamine (0.5 mM), and penicillin-streptomycin was added. For SDF1 treatment, the adult SVZ explants were seeded onto poly-t-tissue-type (100 μg/ml) and laminin (5 μg/ml)-coated dishes, and human recombinant SDF1 (250 ng/ml; Calbiochem, Rockland, MA, http://www.merckmillepore.com) was directly administered. Cultures were maintained in a humidified incubator at 5% CO2 and 37°C. To analyze cell morphology, images were obtained from a confocal microscope with z-sections of 1 μm, and then the images were stacked to form a single image. The quantification of cell morphology was performed with an LSM Image Browser program.

**In Vivo Viral Infection and Quantification**

EGFP or DN-ERM retrovirus solution (1–1.5 μl) was injected into the SVZ of the adult male C57BL/6 mice using a stereotaxic device [33]. One day after retroviral injection into the right SVZ (AP: +0.2 mm, ML: –1.4 mm, DV: –2.2 mm to Bregma) by a 30-gauge microsyringe at a rate of 0.5 μl/minute, the adult animals were injected intraperitoneally with BrdU (100 mg/kg b.wt. dissolved in 0.1 N NaOH, 0.9% NaCl; Sigma-Aldrich) and www.StemCells.com
sacrificed 3 days after BrdU injection. Six to twelve coronal sec-
tions of the desired regions (SVZ, 0.2 to +1.1 mm; RMS, +2.1
to +5.8 mm; OB, +3.56 to +4.28 mm to Bregma) were used for
quantification of Brd(U)- and GFP-labeled cells. To assess
injury-induced neuronal migration, cryogenic TBI was applied 3
days after viral injection.

Adult Neural Stem Cell Culture
Adult neural stem cells (NSCs) in the SVZ were cultured as
described previously [34]. Briefly, the SVZ area was isolated from
sections of adult mouse brains and then digested with 0.8% papain
and 0.08% dispase II (Roche Applied Science). Dissociated
cells were cultured as neurosphere in an ultra-low attachment sur-
face dish with Dulbecco’s modified Eagle’s medium/F-12 medium
containing 1% N2, 2% B27 supplement, penicillin-streptomycin
(Gibco BRL), basic fibroblast growth factor (20 ng/ml, Invitrogen,
factor (20 ng/ml, Invitrogen), and L-ascorbic acid (20 ng/ml,
Sigma-Aldrich). The neurospheres were dissociated into single
cells by incubation with Accutase (Innovative Cell Technologies,

Micropattern
For stripe assay, a laminin-stripe pattern (line width: 30 μm, spac-
ing: 30 μm) was microprinted by polydimethylsiloxane (PDMS)
microstamp [35]. A PDMS stamp (size: 10 mm × 10 mm) was
fabricated by soft-lithographic technique. Before stamping, it was
cleaned by organic solvents (acetone and isopropyl alcohol) and
rinsed in deionized water. The cleaned stamp was coated with a
10% SDS solution to enhance the protein loading on the surface
of the stamp. The mixture of Cy3-conjugated immunoglobulin (1:500,
Jackson ImmunoResearch Laboratories) and laminin (100 g/ml)
was loaded onto the SDS-coated PDMS microstamp for 30
minutes. After the loading, extra protein solutions were removed
completely by compressed air, and the coated PDMS stamp was
immediately placed on a bare glass coverslip. A constant pressure
(20 g) was applied to the stamp to transfer the protein from the
stamp to the coverslip.

Immunostaining
For immunolabeling, mice were perfused with 4% paraformalde-
hyde (PFA), and the brains were isolated. Following postfixation
with the same fixative, brains were sectioned (40 μm) on a cryo-
stat. Subsequently, immunostaining was performed by the free-
floating method. SVZ explants were fixed with 4% PFA. Samples
were blocked in PBS containing 3% bovine serum albumin and
0.1% Triton X-100 for 1 hour and incubated overnight with primary
antibodies. The following primary antibodies were used: rabbit anti-pERM (1:200, Abcam), anti-pERM (1:200, Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com),
goat anti-DCX (1:500, Santa Cruz Biotechnology, Santa Cruz,
CA, http://www.scbt.com), sheep anti-Tubulin (1:500), Cytoskele-
ton, Denver, CO, http://www.cytoskeleton.com), and chick anti-
GFP (1:4,000, Abcam). For BrdU staining in the in vivo migration
assay, free-floating sections were pretreated with 2 N HCl for 30
minutes at 37°C to denature the DNA, rinsed in 100 mM borate
buffer (pH 8.5), and then serially immunolabeled. Next, appropri-
ate secondary antibodies were applied for 1 hour. Alexa Fluor 488-
invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) or
Cy3- or Cy5-conjugated (1:500, Jackson ImmunoResearch Labora-
tories) secondary antibodies were used. To visualize F-actin,
Alexa594-conjugated phalloidin was used (1:500, Molecular
Probes). Subsequently, sections were washed and observed with a
confocal microscope (LSM 510; Carl Zeiss, Gottingen, Ger-
many, http://corporate.zeiss.com). For colorimetric detection of
immunoreactive signals, 3,3′-diaminobenzidine (DAB) staining
was performed using the Vectastain ABC and DAB kit according
to the manufacturer’s instructions (Vector Laboratories, Burling-

Statistical Analysis
Data are expressed as the mean ± SEM of independent experi-
ments. Comparisons were made using the unpaired t test, one-
way analysis of variance (ANOVA) followed by Tukey’s multiple
comparison tests, or two-way ANOVA followed by Bonferroni
posttests. Statistical tests were carried out using PRISM4.03
(GraphPad Software, LA Jolla, CA, http://www.graphpad.com). A
p-value less than .05 was considered statistically significant.

RESULTS
Phosphorylation of ERM in Migrating Neuroblasts
ERM phosphorylation is a key step in the activation of ERM
proteins. To determine whether ERM proteins play a functional
role in the migration of neuroblasts, we first examined the phos-
phorylation status of ERM proteins in the migrating neuroblasts
(Fig. 1). Immunoreactivity for phosphorylated ERM (pERM)
was prominent in the neuroblasts localized in the SVZ and
RMS. By contrast, pERM signals were markedly reduced in the
OB, where the migration of neuroblasts terminates (Fig. 1A–
1F). Western blot analysis also confirmed the reduction of

Figure 1. Differential expression and phosphorylation of ERM proteins in migrating neuroblasts in the adult RMS. (A–C), Immunofluorescence
labeling of pERM (A–C, green) and the neuroblast marker DCX (D–F,
red) in the adult mouse SVZ-RMS-OB. Nuclei were counterstained with
Hoechst 33342 (blue). The dotted line indicates the border of SE and
GCL. Scale bar = 20 μm. (G), Western blot analyses of ezrin and radixin
proteins and pERM in adult SVZ-RMS-OB tissues. β-Actin was used as a
loading control for total proteins in each lane. (H), Relative pERM protein
levels in the SVZ/RMS and OB by Western blotting and normalized to
β-actin (n = 6). ***, p < .01, unpaired t test comparison. Data represent
the mean ± SEM. Abbreviations: GCL, granule cell layer; OB, olfactory
bulp; pERM, ezrin-radixin-moesin phosphorylation; RMS, rostral migrat-
ory stream; SE, subependymal layer; SVZ, subventricular zone.
pERM in the adult OB (Fig. 1H), whereas the expression levels of two major ERM proteins, ezrin and radixin, were similar in the RMS and OB (Fig. 1G). Although astrocytes surrounding neuroblasts also expressed at least one member of ERM, ezrin, pERM signals were absent in the astrocytes (supporting information Fig. S1). Neuroblasts in neonatal SVZ explants showed the localization of pERM in the leading process and distal tip, where actin remodeling is actively occurring during migration (supporting information Fig. S2).

**Induction of ERM Phosphorylation in Neuroblasts After Cryogenic TBI**

It has been reported that brain damage promotes the migration of neuroblasts to the injury site [2, 3, 36]. Consistent with previous reports, cryogenic TBI enhanced the migration of neuroblasts toward the injured site, resulting in the appearance of DCX-expressing neuroblasts in the injured cerebral cortex (Fig. 2A–2D). Interestingly, phosphorylation of ERM was markedly increased in neuroblasts located at the RMS 3 days after TBI (Fig. 2E, 2G vs. 2F, 2H). Conversely, neuroblasts that had already reached the injury penumbra did not exhibit strong pERM signals (Fig. 2F, 2H insets). By immunoprecipitation analysis, it was revealed that radixin is the major ERM member to be phosphorylated under normal conditions and upon TBI (supporting information Fig. S1). Taken together, these results suggest that the phosphorylation of ERM may be related to an early signal for rerouting and directed migration after brain injury.

**Effect of SDF1-CXCR4 Signaling on ERM Phosphorylation After Cryogenic TBI**

Directional migration of neuroblasts to the injury site after cerebral ischemia is mediated by the increased secretion of SDF1 from the injured area [6, 16, 19]. Consistent with this, SDF1 expression was increased in the activated microglia near the injured cortex following cryogenic TBI (supporting information Fig. S4A–S4F). Furthermore, CXCR4 immunoreactivity was detected in the DCX-expressing neuroblasts that showed increased ERM phosphorylation (supporting information Fig. S4G–S4L), suggesting that neuroblasts can respond to injury-induced SDF1 expression. To assess whether the activation of ERM proteins in neuroblasts is regulated by SDF1-CXCR4 signaling, the effect of SDF1-CXCR4 signaling blockade on ERM phosphorylation was examined (Fig. 3). Intraperitoneal injection of AMD3100 (5 mg/kg in PBS), a CXCR4 antagonist, strongly blunted the induction of ERM phosphorylation by cryogenic TBI (Fig. 3A–3F). Quantification of protein levels further demonstrated an approximately twofold increase in ERM phosphorylation by cryogenic TBI and the blockade of this induction by AMD3100 cotreatment (Fig. 3G, 3H). Collectively, these results suggest that increased ERM phosphorylation is mediated by SDF1-CXCR4 signaling after cryogenic TBI.

To verify whether SDF1 is sufficient to induce ERM phosphorylation, human recombinant SDF1 was directly treated onto the freshly dissociated adult SVZ explants (Fig. 3I, 3J). Immunoblotting showed an approximately twofold increase in
ERM phosphorylation by SDF1 treatment (Fig. 4A–4H), following injection of EGFP- or DN-ERM-expressing retrovirus into the P2–3 mouse lateral ventricle, SVZ explants were collected 24 hours after infection (P3–4) and cultured on matrigel for 2 days. After 2 days in culture, neuroblasts in EGFP-infected groups migrated normally out from the explants in chains (Fig. 4A–4D). In the explants with moderate infection rates, overall outgrowth patterns of DN-ERM-infected explants (Fig. 4E–4G) appeared to be similar to EGFP-infected explants (Fig. 4A–4C). However, the average distance migrated from the core in DN-ERM-infected neuroblasts was significantly shorter than that in the EGFP-infected cells (Fig. 4I). In some explants with high infection rates ([mt]60%) in DN-ERM groups, chain migration was severely impaired, and most neuroblasts stayed in the explant core (Fig. 4D, 4H).

To examine the role of ERM in RMS chain migration in vivo, DN-ERM retrovirus was injected into the adult SVZ with a stereotactic device. Then, we injected BrdU (100 mg/kg) 24-hour after viral infection to trace the migration of infected (GFP+/BrdU) versus noninfected (GFP+/BrdU−) cells that replicated at the same time (Fig. 4J, 4K). In the control EGFP-infected group, the distributions of these two populations were similar, and most of the cells were found in the OB by 3 days after BrdU labeling (Fig. 4I), indicating that GFP expression did not affect normal migration. Conversely, a substantially smaller proportion of DN-ERM-expressing neuroblasts was localized in the OB, while most of them were maintained in the SVZ (Fig. 4K), suggesting that the migration of DN-ERM-expressing neuroblasts was disturbed.

Inhibition of Injury-Induced Neuroblast Migration by SDF1-CXCR4-ERM Blockade

Next, we examined whether increased ERM phosphorylation is involved in the injury-induced directional migration of neuroblasts by infecting neuroblasts with DN-ERM viruses (Fig. 5). After cryogenic TBI, control EGFP virus-infected neuroblasts migrated normally out toward the injured cortex (Fig. 5A). However, DN-ERM led to severe migration defects toward the injured region (Fig. 5B). Quantification of neuroblasts in the injured cortex demonstrated that the directional migration of neuroblasts toward the injury site was reduced by 83.6% after DN-ERM infection compared to the control (Fig. 5C). We also tested the effect of DN-ERM on the proliferation and survival of NSCs or neuroblasts. DN-ERM infection did not change the percentage of S-phase cells as assessed by BrdU incorporation rate following 3-hour incubation (supporting information Fig. 5A–5E). Furthermore, the numbers of cleaved caspase3 positive cells were negligibly small in both animal groups (supporting information Fig. 5F–5I). Collectively, these results indicated that DN-ERM infection primarily interfered with directional migration of neuroblasts.

Similar to DN-ERM, AMD3100 treatment also suppressed the injury-induced migration of neuroblasts (Fig. 5D). Collectively, these results suggest that ERM phosphorylation mediates neuroblast migration controlled by SDF1-CXCR4 signaling.

Effect of ERM Suppression on Morphology of Migrating Neuroblasts

It is well known that a typical morphology during neural progenitor migration is monopolar, with the extension of a single leading process, or bipolar [41, 42]. Although most of the EGFP-expressing neuroblasts were associated with stem cells...
neighboring neuroblasts in chains (Fig. 6A), DN-ERM-expressing neuroblasts appeared to be separated from the chains (Fig. 6B). Furthermore, in the control group infected with EGFP viruses, migrating neuroblasts in chains showed a typical morphology with a single leading process, whereas DN-ERM-expressing neuroblasts showed more complex morphology with multiple branches (Fig. 6C). The average number of processes in DN-ERM-expressing neuroblasts was about three versus typically one in normal neuroblasts (Fig. 6D), and the length of the longest process from neuroblasts infected by DN-ERM virus was also increased by 24.3% (Fig. 6E). Similar changes in neuroblast morphology were also found in vivo (supporting information Fig. S6). These results suggest that ERM-dependent maintenance of cellular morphology may be critical for efficient neuroblast migration.

Effect of DN-ERM on Actin Dynamics of Adult Neural Stem Cells

It is known that ERM plays critical roles in the regulation of actin cytoskeleton [23, 24]. Considering that the marked impairments of chain migration are accompanied by the changes in cell shapes in DN-ERM-infected group, we hypothesized that the changes in intracellular actin structures by perturbation of ERM in turn impairs neuroblast migration. To test this idea, the effect of DN-ERM on the morphology of cultured neural progenitor cells was examined. The morphology of adult SVZ neural stem cells on typical laminin-coated dishes was very diverse (supporting information Fig. S7), and it was difficult to quantify and compare the morphology of cells. Therefore, we cultured the cells on the laminin micropattern which can simplify the cellular morphology [43]. Using the laminin-stripe patterns (30 m lines and 30 m spaces), approximately 60% cells showed typical mono-/bipolar morphology (Fig. 7A–7E). We compared the EGFP versus DN-ERM-infected cell morphology on this micropattern. On the pattern, the cells preferentially attached onto the laminin-coated stripes and most EGFP-infected control progenitors showed nonpolar or bipolar shape. However, a large proportion of DN-ERM-infected neural progenitors showed bipolar or flat morphology (Fig. 7E). Furthermore, even bipolar cells exhibited many varicosities which is associated with F-actin, but not with tubulin (Fig. 7F–7K). These results support the idea that ERM functions to organize actin cytoskeleton, which is required for the maintenance of polarized cell morphology suitable for the neuroblast migration.

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Phosphorylation of ERM in the Migrating Neuroblasts

It is known that ERM functions are dependent on phosphorylation [21, 25]. Phosphorylated ERM is mainly found in actively migrating neuroblasts in the RMS, suggesting that the activation of ERM proteins is involved in neuroblast migration. In particular, phosphorylated ERMs were profoundly localized in leading processes. It is well known that the formation of cellular polarity is required for directional cell migration [44, 45]. Non-neuronal cells, such as lymphocytes, crawl like amoeba on extracellular matrix-coated dishes in vitro. In this amoeba-like migration, ERM proteins are known to be localized in the rear area of the cells, and they are necessary for rear retraction of a migrating cell-mediated by acto-myosin reaction [46]. ERM proteins in fibroblasts are involved in the regulation of cell adhesion at the leading edge during migration [47]. In the peripheral nervous system (PNS), the motility of Schwann cells, myelinating glial cells, is necessary for proper PNS formation. Specific and local activation of ERM proteins at the tip of Schwann cell process and tailing edge is essential for motility and Schwann cell polarity [48, 49]. Migration of neuroblasts in the central nervous system is dependent on the attachment to other cells, such as radial glia (radial migration) or nearby neuroblasts (chain migration). Migrating neuroblasts in the RMS are surrounded in the glial tube. Although the glial tube expresses ezrin proteins [30], we found that phosphorylation of ERM proteins was mainly expressed in migrating neuroblasts. Phosphorylated ERM signals were originated from radixin proteins according to immunoprecipitation experiments using adult RMS tissues. In this respect, the formation of activated ERM polarity in migrating neuroblasts might be involved in proper chain migration of neuroblasts in the RMS.

Figure 5. Inhibition of the injury-induced migration of neuroblasts by blockade of SDF1-CXCR4-ERM signaling in vivo. (A, B): Distribution of GFP- (A) or DN-ERM-transduced (B) neuroblasts in the injured mouse brains. Dotted lines indicate the borders between the corpus callosum and the cerebral cortex. Nuclei were counterstained with Hoechst 33342 (blue). Scale bar = 200 μm. (C): Quantification of transduced neuroblasts in the cortex (n = 5). The number of GFP-positive cells in the cortex area (above dotted line in A and B) was obtained by the summation of all immunoreactive cells in every third section (40 μm) containing entire injury core regions. The counted numbers were corrected by the use of Abercrombie’s correction factor [40]. ***, p < .001 in unpaired t test. Data represent the mean ± SEM. (D): Number of neuroblasts in the cerebral cortex for the control (n = 3), injured (n = 8), and AMD3100-treated groups (n = 4). *, p < .05 compared with the control; *, p < .05 TBI versus TBI+AMD3100, one-way analysis of variance and Turkey’s multiple comparison test. Data represent the mean ± SEM. AMD3100 (5 mg/kg) was applied twice daily with 12-hour interval for 3 days after TBI. Abbreviations: CTL, control; DN-ERM, dominant-negative ezrin-radixin-moesin; EGFP, enhanced green fluorescent protein; TBI, traumatic brain injury.

Figure 6. Morphological alterations of migrating neuroblasts by DN-ERM in subventricular zone (SVZ) explants. Double labeling of GFP and DCX in EGFP- (A) and DN-ERM-transduced (B) groups. Scale bar = 10 μm. Representative images of EGFP- (upper) and DN-ERM-transduced (lower) neuroblasts in SVZ explants (C). Quantification of the number of branches from neuroblasts (n = 10) (D) and the length of the longest processes (n = 10) (E). **, p < .01; *, p < .05, in unpaired t tests. Data represent the mean ± SEM. Abbreviations: DN-ERM, dominant-negative ezrin-radixin-moesin; EGFP, enhanced green fluorescent protein.
Induction of ERM Phosphorylation by SDF1-CXCR4 Signaling After Injury

Brain ischemia promotes regenerative responses, including the recruitment of endogenous neural progenitor cells to the damaged regions [3, 11, 50–52]. Similarly, neuroblasts also migrate toward the injured site following cryogenic TBI, as revealed by this study. Interestingly, neuroblasts exhibited increased ERM phosphorylation, suggesting that ERM activation contributes to the response of migrating neuroblasts to brain injury. SDF1 is the most characterized extracellular signal that induces migration toward the injured site [16, 19, 53]. Cryogenic TBI rapidly promotes microglial activation in the penumbra region, and activated microglia are a major source of SDF1. Two different experimental approaches support our hypothesis that SDF1 signaling is responsible for ERM phosphorylation. First, treatment with an SDF1-CXCR4 inhibitor, AMD3100, markedly blunted injury-induced ERM phosphorylation. Second, direct treatment with SDF1 in vitro also increased ERM phosphorylation. Responses of ERM proteins to SDF1-CXCR4 signaling may be different depending on cellular context. For example, SDF1 stimulation may lead to an inactivation of ERM proteins depending on phospholipase C signaling in lymphocytes [54]. The chemokine CC chemokine ligand 25, on the other hand, induces ERM phosphorylation in T lymphocytes and promotes invasive behaviors [55].

Figure 7. Effect of DN-ERM on morphology of adult neural stem cells. (A–D) Dissociated adult neural stem cells were infected with EGFP (A, C) or DN-ERM (B, D) viruses and seeded onto laminin-stripe patterns. Scale bar = 50 μm. (E): Proportion of different cell morphology grown on laminin-stripe patterns. At least 100 cells were observed in each experiment for assessing the proportion of the cell morphologies (n = 4 for EGFP group and n = 6 for DN-ERM group). **, p < .01; *, p < .05 EGFP versus DN-ERM. Bonferroni post hoc test following two-way analysis of variance. Data represent the mean ± SEM. (F–K): Immunocytochemistry of GFP (F, I), F-actin (G, J), and Tubulin (H, K) in the EGFP (F, G, K) or DN-ERM (I, J, K) infected cells on laminin strip patterns. F-actin was visualized by rhodamine phalloidin. Nuclei were counter stained with Hoechst 33342 (blue). Scale bar = 10 μm. Abbreviations: DN-ERM, dominant-negative ezrin-radixin-moesin; EGFP, enhanced green fluorescent protein.

Function of ERM Proteins in Migrating Neuroblasts

To examine the role of ERM activation in neuroblast migration, retrovirus-expressing DN-ERM was used to transduce SVZ cells. It is known that the amino termini of ERM proteins act in a DN manner to suppress endogenous ERM activity [37, 56]. Perturbation of ERM activation resulted in impairment of neuroblast migration both in vivo and in vitro. In SVZ explants, a large proportion of DN-ERM-expressing cells failed to migrate out from the explant core. Furthermore, even the neuroblasts that migrated out from the core did not migrate efficiently and remained in the proximal region of the chains. Therefore, it appears that neuroblasts with reduced ERM activity have decreased ability to form/maintain chains with neighboring neuroblasts. It is known that cell migration is affected by neighboring cells (community effect); thus, the impairment of neuroblast migration could be non-cell-autonomous. In some explants with [mt]60% infection ratio, DN-ERM transduction perturbed the overall outgrowth of neuroblasts, suggesting that noninfected neighboring neuroblasts also failed to migrate normally. However, the effects of DN-ERM appear to be primarily cell-autonomous. In the explants with moderate levels of infections (20%–50%), migration of noninfected neuroblasts was normal, but the migration of DN-ERM-infected cells was selectively impaired. Similarly, in vivo experiments with <20% infection rate showed normal migration of nontransduced
neuroblasts. Therefore, ERM activation appears to be required for the migration of neuroblasts in a cell-autonomous manner. Conversely, the effect of ezrin overexpression did not influence the migration of neuroblasts in vitro and in vivo (data not shown). Therefore, ERM activation is not sufficient for the modulation of normal neuroblast migration.

The inhibition of ERM phosphorylation also suppressed the migration of neuroblasts toward the injured brain area. Several reports have proposed that SDF1 increases the motility of neuroblasts and stimulates the migration of progenitor cells from vascular niches [57]. Therefore, these results may imply that increased ERM activation in the RMS mediates the directional migration of neuroblasts by SDF1. This hypothesis is directly supported by the fact that transduction of DN-ERM, which interferes with endogenous ERM activation, completely abolished injury-induced neuroblast migration.

Altered Morphology by DN-ERM in Migrating Neuroblasts
ERM proteins regulate actin dynamics and cytoskeleton organization by mediating linkages between the plasma membrane and F-actin [21]. Interestingly, DN-ERM-transduced neuroblasts failed to maintain the typical morphology of migrating neuroblasts and exhibited multiple processes. Especially, it appears that ERM suppression selectively impairs the actin structure revealed by Phalloidin labeling, but relatively spares tubulin, indicating that remodeling of actin cytoskeleton by ERM may be a critical regulator of neuroblast migration. It has been demonstrated that neuroblasts change their morphology dynamically during their migration [58], and morphological defects in the RMS lead to delayed migration [40, 59]. Furthermore, the initiation of migration begins with the extension of actin cytoskeleton at the leading edge [60]. Therefore, increased ERM phosphorylation may be necessary to change direction and reroute migrating cells to the injury site, which requires marked actin remodeling and changes in cell shape.

**Conclusion**

In summary, the present data provide strong evidence that activation of SDF1-CXCR4 signaling after cryogenic TBI leads to the migration of SVZ-derived neuroblasts toward the damaged cortex and that this SDF1 signaling effect is mediated at least in part by the activation of ERM proteins.

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**Disclosure of Potential Conflicts of Interest**

The authors indicate no potential conflicts of interest.

**References**