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Applications of stripe assay in the study of CXCL12-mediated neural progenitor cell migration and polarization

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ABSTRACT

The polarization and migration of neural progenitor cells (NPCs) are critical for embryonic brain development and neurogenesis after brain injury. Although stromal-derived factor-1α (SDF-1α, CXCL12) and its receptor CXCR4 are well-known to mediate the migration of NPCs in the developing brain, the dynamic cellular processes and structure-related molecular events remain elusive. Transwell and microfluidic-based assays are classical assays to effectively study cellular migration. However, both of them have limitations in the analysis of a single cell. In this study, we modified the stripe assay and extended its applications in the study of NPC polarization and intracellular molecular events associated with CXCL12-mediated migration. In response to localized CXCL12, NPCs formed lamellipodia in the stripe assay. Furthermore, CXCR4 and Rac1 quickly re-distributed to the area of lamellipodia, indicating their roles in NPC polarization upon CXCL12 stimulation. Although the chemokine stripes in the assay provided concentration gradients that can be best used to study cellular polarization and migration through immunocytochemistry, they can also generate live imaging data with comparable quality. In conclusion, stripe assay is a visual, dynamic and economical tool to study cellular mobility and its related molecule mechanisms.

1. Introduction

The migration and polarization of neural progenitor cells (NPCs) are critical processes for neural development and neural repair during diseases in the central nervous system (CNS). Understanding the detailed cellular events and intracellular signaling intermediates through gene expressions and protein analyses may provide important information on the mechanisms of neural development and repair [1–3]. Stromal-derived factor-1α (SDF-1α, CXCL12) is a member of the CXC chemokine family that plays an essential role on hematopoiesis, neoangiogenesis, tumorigenesis, immune response and the maintenance of tissue stem cells [4–8]. Notably, CXCL12, its traditional receptor CXCR4 and newly identified receptor CXCR7 are widely express in the CNS, regulating neurogenesis and neural regeneration during the development and pathogenic processes of brain diseases, respectively [9–12]. Although CXCL12/CXCR4 plays crucial roles in various aspects of neurogenesis by regulating NPC migration, proliferation and formation of neuronal circuits, the cellular events and associated molecular mechanisms remain unclear.

Transwell assays, either the Boyden or modified Boyden chamber assay [13], are commonly used to study the migration of cells [14]. In these assays, adherent or nonadherent cells migrate through a permeable filter after triggered by very low chemotactic inducers. Therefore, these assays are appropriate for a variety of cells and quite sensitive. However, these assays are also traditionally being end-point assays, making it difficult to assess cells during migration. Microfluidic-based assays are more recently developed migration assays that connect two chambers with an internal

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channel allowing for cell migration. These assays are generally sensitive and effective. However, they often come with considerable cost for the kits.

Stripe assays was originally developed to investigate the fundamental axonal guidance *in vitro* by F. Bonhoeffer and coworkers [15,16] in the late 1980s [17–20]. By forming stripes containing different factors under investigation, this assay gains its popularity in the research of process outgrowth involving multiple cells, including neural crest cells [21], oligodendrocytes [22,23], and tumor cells [24]. We have previously successfully used stripe assay to explore the surprising role of CXCR7, a newly discovered atypical chemokine receptor, on CXCL12-mediated migration and polarization [12]. The paper generated considerable interest. Therefore, we intend to further characterize the stripe assay in studying cellular migration, polarization, and their associated signaling intermediates in the current paper. We demonstrated that the stripe assay can generate remarkable migration and polarization data with comparable quality comparing with live cell imaging data. In addition, we determined that this assay could be used to investigate signaling intermediates involved in cellular migration and polarization.

2. Materials and methods

2.1. Reagents

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were obtained from Life Technologies. Recombinant mouse CXCL12 was obtained from R&D systems. Primary antibodies included monoclonal rat anti-CXCR4 (R&D Systems, 1:200), polyclonal rabbit anti-Rac1 (Sigma, 1:100), and monoclonal mouse anti-Nestin (Millipore, 1:100). Secondary antibodies included goat anti-mouse IgG (coupled with Alexa Flour 568, Invitrogen), goat anti-rat IgG (coupled with Alexa Fluor 488, Invitrogen), goat anti-rabbit IgG (coupled with Alexa Fluor 647, Invitrogen). AMD3100 was obtained from Sigma–Aldrich.

2.2. Mouse NPCs collection and cell culture

All mice were housed and bred in the Comparative Medicine facilities of the University of Tongji Medical College, China or the University of Nebraska Medical Center, USA. All procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of Tongji Medical College and the University of Nebraska Medical Center.

The mouse forebrain of each embryo at gestational day E13.5 was dissected and dissociated into single cells by triturating the tissue with a 1 ml pipette. Cells from each forebrain were seeded into a 100 mm Petri dish separately at a density of 2×10^5 cells/ml in 10 ml of mouse NeuroCult NSC Proliferation Medium (Stem Cell Technologies) supplemented with epidermal growth factor (20 ng/ml) and basic fibroblast growth factor (10 ng/ml) for neurosphere cultures. Neurospheres were passaged when they reached 100–150 μm in diameter.

2.3. Transwell assay

NPC migration was evaluated using an 8-mm pore size transwell system (Costar) coated with fibronectin (Sigma-Aldirich) at 5 ng/ml and Poly-D-lysine (PDL, Sigma-Aldirich) at 100 μg/ml in PBS overnight. Briefly, NPCs were dissociated into single cells and resuspended in Mouse NeuroCult Proliferation Medium at a density of 10^5 cells/ml. The top chamber of the transwell was loaded with 100 μl of cell suspension and cells were cultured for 12 h to form an adherent monolayer culture. CXCL12 was added to the bottom chamber. After 12 h, the membrane of the transwell inserts was fixed with 4% PFA in PBS, and cells on top of the membrane were removed with a cotton swab. Cells that migrated to the bottom of the membrane were stained with DAPI (Sigma) in PBS at 10 ng/ml. For each insert, 10 fields were randomly selected to be captured under a microscope at 20 × , and cell numbers were quantified using Image-Pro Plus 6.0. The cell number of each treated group was normalized to the cell number of the control group to calculate the migration index.

2.4. PDMS stripe device fabrication

The stripe device was fabricated using mold etched by soft lithography with polydimethylsiloxane (PDMS), modified from the earlier work [25]. To fabricate the PDMS stripe device, the mold was first exposed to chlorotrimethylsilane (Alfa Aesar, Lancs, England) vapor for 3 min in order to promote elastomer release after carrying out the baking steps. A mixture of PDMS was vacuumed for 10 min and then poured onto the mold. After degassing (5 min), the mold was baked for 60 min at 85 °C, after which the PDMS flow layer structure was then peeled from the mold.

2.5. Stripe assay

A silicon wafer was used to generate a template for the PDMS mold and substrates were patterned in parallel stripes of 60 μm width separated by 90 μm gaps. Briefly, PDMS mold was reversibly sealed on PDL-coated glass bottom dishes (35 mm dish with 20 mm bottom well), and microchannels were formed between the PDMS mold and well. The mixture of BSA and CXCL12 or BSA alone was added to one end of the microchannels, and vacuum was applied to the other end of the microchannels to ensure BSA/CXCL12 or BSA filled in all the microchannels. PDMS molds were removed after drying overnight. The stripe-coated dishes were planted with dissociated NPCs to study cell migration or polarization. Cells were then fixed on cover glasses and stained with Nestin and DAPI. Images were taken by a Zeiss 710 confocal microscope and quantified by Image Pro Plus. The percent of cells on stripe versus total cell number was calculated to evaluate cell migration.

2.6. Immunofluorescence and confocal microscopy

For immunofluorescence staining, mNPCs were fixed using 4% paraformaldehyde (PFA), and permeabilized with 0.4% triton-X in PBS. After blocked by 1% BSA in PBS, mNPCs were incubated with primary antibodies overnight. Cultures were washed and then incubated with phalloidin (coupled with rhodamine, Invitrogen) and corresponding secondary antibodies for 1 h at room temperature. Nuclear DNA was labeled with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich) for 2 min after the secondary antibody at room temperature. Cover slips were mounted on glass slides with mounting medium (Sigma–Aldrich). Triple immunostaining was examined by a Zeiss META 710 confocal microscope.

2.7. Transfectiion with LiveAct-RFP plasmid

Lipofectamine® LTX and PLUS™ Reagents (Life Technologies) was utilized for the transfection of LiveAct-RFP plasmid [25]. The experiment was performed according to the manufacturer's instruction. Briefly, NPCs were transfected with LiveAct-RFP plasmid in a 6-well format after optimizing the transfection efficiency. NPCs were incubated at 37 °C in a CO_2 incubator for 18–48 h prior to stripe assays.