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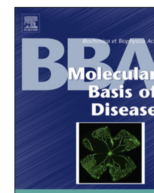

Role of p38γ MAPK in regulation of EMT and cancer stem cells

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ABSTRACT

p38γ is a member of p38 MAPK family which contains four isoforms p38α, p38β, p38γ, and p38δ. p38γ MAPK has unique function and is less investigated. Recent studies revealed that p38γ MAPK may be involved in tumorigenesis and cancer aggressiveness. However, the underlying cellular/molecular mechanisms remain unclear. Epithelial-mesenchymal transition (EMT) is a process that epithelial cancer cells transform to facilitate the loss of epithelial features and gain of mesenchymal phenotype. EMT promotes cancer cell progression and metastasis, and is involved in the regulation of cancer stem cells (CSCs) which have self-renewal capacity and are resistant to chemotherapy and target therapy. We showed that p38γ MAPK significantly increased EMT in breast cancer cells; over-expression of p38γ MAPK enhanced EMT while its down-regulation inhibited EMT. Meanwhile, p38γ MAPK augmented CSC population while knock down of p38γ MAPK decreased CSC ratio in breast cancer cells. MicroRNA-200b (miR-200b) was down-stream of p38γ MAPK and inhibited by p38γ MAPK; miR-200b mimics blocked p38γ MAPK-induced EMT while miR-200b inhibitors promoted EMT. p38γ MAPK regulated miR-200b through inhibiting GATA3. p38γ MAPK induced GATA3 ubiquitination, leading to its proteasome-dependent degradation. Suz12, a Polycomb group protein, was down-stream of miR-200b and involved in miR-200b regulation of EMT. Thus, our study established an important role of p38γ MAPK in EMT and identified a *novel* signaling pathway for p38γ MAPK–mediated tumor promotion.

1. Introduction

p38 Mitogen Activated Protein Kinases (p38 MAPKs) play an important role in the cellular response to environmental stress. There are four p38 MAPK isoforms in mammalian cells encoded by different genes: p38α (*MAPK14*), p38β (*MAPK 11*), p38γ (*MAPK 12*), and p38δ (*MAPK 13*) [1]. The expression pattern of p38 MAPKs vary in different tissues. p38α is ubiquitously expressed in all cell types and tissues while p38β is highly expressed in the brain, thymus, and spleen; its expression is lower in the adrenals, lung, kidney, liver, pancreas, and heart, and it is not expressed in skeletal muscle [1]. p38γ is very abundant in skeletal muscle and its expression is lower in most other tissues is [1]. p38δ levels are high in the pancreas, intestine, adrenal gland, kidney, and heart [1]. All p38 MAPKs are Serine/Threonine kinases and activated by a wide variety of environmental and cellular stresses or inflammatory cytokines. p38α was the first p38 MAPK family member identified, therefore the most studied and best-characterized isoform; most of the literature on p38 MAPK refers to p38α.

Recently, p38γ MAPK has emerged as an oncogenic MAPK (MAPK) [2–6]. It was also found to be highly expressed in some human cancer cells [4,5,7,8]. p38γ MAPK is shown to be involved in the regulation of cancer stem cells (CSCs), migration/invasion, tumorigenesis, and cell transformation [2–6,9,10]. However, the underlying mechanisms and cell signaling pathways are unclear.

Breast cancer is the most prevalent cancer in women worldwide and distant site metastasis is the main cause of death in breast cancer patients. Epithelial-mesenchymal transition (EMT) defined by the progressive loss of epithelial cell characteristics and the acquisition of mesenchymal features is a fundamental mechanism occurring during embryonic development and tissue differentiation. EMT also plays a

Abbreviation: EMT, epithelial-mesenchymal transition; CSC, cancer stem cell; p38 MAPK, p38 Mitogen Activated Protein Kinases; ALDH, aldehyde dehydrogenase; miRNA, micro RNA; shRNA, short hairpin RNA; RT-PCR, real-time polymerase chain reaction

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crucial role for cancer progression, and contributes to the dissemination of cancer cells from solid tumors and the formation of detectable metastases [11]. EMT has also been implicated in therapy resistance, relapse, immune escape, and maintenance of cancer stem cell properties, such as self-renewal capacity [12,13]. Understanding the complex events that lead to the EMT will therefore help to design new therapies against metastatic breast cancer. In this study, we investigated the *novel* role of p38γ MAPK in EMT and delineated a signaling pathway which may mediate the action of p38γ MAPK.

2. Materials and methods

2.1. Materials

Anti-E-cadherin and Suz12 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Protein A/G beads were obtained from Santa Cruz Biotechnology (San Diego, CA). Anti-Vimentin, p38α and p38γ antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-GAPDH antibody was obtained from Research Diagnostics, Inc. (Concord, MA). Anti-GATA3 antibody was obtained from Abcam Inc. (Cambridge, MA). Anti-E-cadherin monoclonal antibody was purchased from BD Transduction Laboratories (San Jose, CA). ALDEFLUOR kits and MammoCult™ Human Medium Kit were purchased from Stemcell Technologies (Vancouver, Canada). Ultra-low cluster plates were obtained from Corning Incorporated (Corning, NY). p38γ MAPK shRNA, control shRNA, GATA3 siRNA, and control siRNA were purchased from Santa Cruz Biotechnology (San Diego, CA). Wild type and mutated p38γ MAPK plasmids (p38WT and p38D179) were gifts from Dr. Oded Livnah (Hebrew University of Jerusalem, Jerusalem, Israel) [14]. Human GATA3 plasmid was obtained from Sino Biological Inc. (Beijing, China). miRNA mimic and inhibitors were purchased from Ambion (Thermo Fisher, Waltham, MA). Antibiotic-Antimycotic (Anti-Anti) and cell culture mediums were obtained from Gibco (Thermo Fisher, Waltham, MA). Cyclohexamide was obtained from Biovision (Milpitas, CA). MG132 was purchased from EMD Millipore (Burlington, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture and treatment

MCF7 cells were grown in DMEM medium containing 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic. MCF7 cells overexpressing ErbB2 (MCF7-ErbB2) were cultured in full DMEM medium with hydrocortisone (1 μg/ml) and insulin (10 μg/ml). BT474 cells were cultured in full RPMI medium with insulin. All cell lines were grown at 37 °C with 5% CO_2. For cyclohexamide treatment, culture medium was changed to serum free and treated with cyclohexamide (50 μg/ml) for indicated times. Cells were treated with chloroquine (100 μM) or MG132 (10 μM) for 6 h followed by the collection of cell lysates.

2.3. Cell transfection and generation of stable cell lines

Transient transfection of mimics or inhibitors miR-200b and miR-34c, GATA3 siRNA (GATA3 si), control siRNA (con si) (San Cruz Biotech), GATA3 plasmid (GATA3 P), and control plasmid (con P) was performed using a Neon Transfection System (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol. Briefly, cells were electroporated and incubated with indicated miRNAs, siRNAs, and plasmids. Experiments were initiated 48 h after the transfection.

For establishing stable transfectants, the plasmids of p38WT, p38D179, and control plasmids were transfected into MCF7 cells using a Neon Transfection machine (Life Technologies). Positive colonies were selected in standard cell culture media containing G418 (400 μg/ml). Short hairpin RNA (shRNA) of p38γ MAPK (p38γsh) or scrambled control shRNA (Santa Cruz Biotechnology) was transfected into MCF7, MCF7-ErbB2, and BT474 cells using a Neon Transfection machine (Life Technologies). Positive colonies were selected in standard cell culture media containing 4 μg/ml puromycin. Cell lysates were collected and analyzed by immunoblotting for the verification of the overexpression or silencing of p38γ MAPK.

2.4. ALDEFLUOR assay (stem-like cell population assay)

The cancer stem-like cells (CSCs) were identified by measuring aldehyde dehydrogenase (ALDH) activity [12,15]. The ALDEFLUOR assay (Stemcell Technologies) was performed according to the manufacturer's protocol and the high ALDH enzymatic activity in cells were tested using a flow cytometer as described previously [4,5]. Briefly, 10^6 cells were incubated in ALDEFLUOR assay buffer containing ALDH substrate (1 μmol/l per 1×10^6 cells) for 40 min at 37 °C. Meanwhile, an aliquot of cells was treated under identical conditions with a specific ALDH inhibitor [50 mmol/l, diethylaminobenzaldehyde (DEAB)] as a negative control. CSCs (cells expressing high levels of ALDH) were identified by a FACSCalibur (Becton Dickinson) flow cytometer. The percentage of CSC population was analyzed and calculated using the WINMDI software.

2.5. Tumorsphere assay

Tumorsphere assay was performed as described previously [16,17]. Briefly, cells were plated as a single cell suspension in ultra-low attachment 24-well plates (Corning) at 1000 cells/well. Cells were grown in serum-free MammoCult™ Human Medium (Stemcell Technologies) for 10 days. The number of tumorspheres (mammospheres) in each well that were 60 μm or larger in size were counted according to the manufacturer's protocol (MammoCult™ Human Medium, Stemcell Technologies).

2.6. Immunoblotting and immunoprecipitation

Cells were lysed in modified RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate) containing 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 μg/ml of aprotinin, and 2 μg/ml of leupeptin. The procedure for immunoblotting has been previously described [18]. Briefly, protein samples were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to nitrocellulose membranes. The membranes were probed with indicated primary antibodies, followed by the appropriate horseradish peroxidase-conjugated secondary antibodies, and developed by enhanced chemiluminescence. The intensity of specific proteins was quantified using Carestream Molecular Image Software.

For immunoprecipitation, equal amount of protein (500–800 μg) was incubated with anti-Ubiquitin antibody overnight at 4 °C, followed by treatment with Protein A/G beads conjugated to agarose for 4 h at 4 °C. Immunoprecipitates were collected by centrifugation at 5000*g* for 5 min at 4 °C. Samples were washed 5× with RIPA buffer, 1× with cold-TBS, and boiled in sample buffer (187.5 mM Tri-HCl, pH 6.8, 6% SDS, 30% glycerol, 150 mM DTT and 0.03% bromophenol blue). Proteins were resolved in SDS-PAGE and analyzed by immunoblotting.

2.7. Immunofluorescence microscopy

The procedure for immunofluorescent microscopy has been previously described [19,20]. Briefly, cells were seeded on coverslips precoated with fibronectin (10 μg/ml). Cells were fixed with 3.7% paraformaldehyde for 10 min, washed 3 times in PBS, and permeabilized with 0.5% Triton X-100 for 5 min. Cells were blocked with 5% BSA, incubated with primary antibodies for 1 h, and washed then treated with Alexa Fluor-labeled secondary antibodies. After rinsing with PBS, coverslips were mounted with Prolong Gold anti-fade reagent, and

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