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Title: Force-Mediated Proinvasive Matrix Remodeling Driven by Tumor-associated Mesenchymal Stem-like cells in Glioblastoma

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Running Title: tMSLCs promotes proinvasive stiffness in GBM

Keywords: Tumor-associated mesenchymal stem-like cells, proinvasive extracellular matrix remodeling, actomyosin contractility, glioblastoma

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Supplementary Data

Antibodies and inhibitors
Antibodies to p-STAT3 (Y705), JAK1, and p-MLC2 (S19) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to STAT3, p-JAK1 (T1022), and MLC2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemical inhibitors specific to MEK (U0126), p38 (SB203580), PI3K (LY294002), pan-JAK (P6), JAK2/STAT3 (WP1066), STAT5 (STAT5 inhibitor), SRC (pp2), RAF1 kinase (GW5074), PKC (GO6976), and PIM1/2 (SMI-16a) were purchased from Calbiochem (San Diego, CA, USA). The chemical inhibitor specific to ROCK (Y-27632) was purchased from TOCRIS Bioscience (Bristol, UK). Anti-mouse IgG-HRP, anti-goat IgG-HRP, and anti-rabbit Ig-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Culture of GBM cells and tMSLCs
The patient-derived X01 GBM cells were previously established from acutely resected human tumor tissues obtained with written informed consent from a 68-year-old woman with GBM (34). X01 GBM cells were cultured in Dulbecco’s Modified Eagle Medium (Gibco, Seoul, Korea) containing 1% penicillin and streptomycin, supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland). tMSLC0903 (previously referred as KGS-MSC05-04) has been isolated previously from the biopsy of a GBM patient (22). tMSLC0903 was cultured in MEMα (Mediatech) containing 10% FBS (Lonza), 2 mM L-glutamine (Mediatech), and antibiotic–antimycotic solution (Gibco, Seoul, Korea). Upon thawing, tMSLC0903 was cultured no longer than 20 passages.

Organotypic invasion of GBM cells in ECM conditioned by tMSLCs
A collagen-based matrix was prepared in Millicell culture plate insert (12 mm diameter, 0.4 μm pore size; Millipore, Billerica, MA, USA) by mixing collagen type-I (2 mg/ml), matrigel (11% v/v) in a reconstitution buffer (26 mM NaHCO₃; 5 mM NaOH; 20 mM HEPES in Dulbecco’s Modified Eagle Medium). X01 GBM cells (2 x 10⁵) were plated on top of a collagen-based matrix in which tMSLC0903 (2 x 10⁵) were premixed. After 3 days, invasion of X01 GBM cells was visualized by H&E staining after perpendicular section of the gels. Invasion was scored by counting the number of invaded cells from three randomly selected fields. In the other way, tMSLC0903 were seeded into the collagen-based matrix and cultured for 3 days for ECM remodeling. tMSLC0903 were killed by treatment with puromycin, leaving ECM conditioned by tMSLCs. X01 GBM cells were then plated in the conditioned ECM, and their invasion was analyzed as described in above.

Collagen-assembly analysis
Collagen was stained with a 0.1% Sirius red (Sigma, Chennai, India) solution dissolved in aqueous saturated picric acid for 1 hour, washed in acidified water (0.5% acetic acid), and dehydrated. Collagen and non-collagen components were red-stained and orange-stained, respectively.
**Gel-contraction assay**

tMSLC0903 (5 × 10^5) were embedded in an 800 µl collagen-based matrix gel in 24-well. After 1 hour at 37 °C, the matrix was overlaid with growth medium and was carefully detached for gel contraction from the well using syringe needle. The medium was changed every 2 days, and at 4 days, the matrix was photographed. The respective diameters of the well and the gel were measured using ImageJ. The contraction was calculated using the formula 100 x (well diameter – gel diameter)/well diameter.

**Transfection**

Cells were transfected with siRNA using Microporator-mini (Digital Bio Technology, Seoul, Korea) according to the procedure recommended by the manufacturer. All siRNAs were purchased from Genolution Pharmaceuticals, Inc. (Seoul, Korea), and the sequences were as follow;

- si-JAK1 (5’-GCUGUGGAAUAGAUUAUUGUGCUCA-3’)
- si-JAK2 (5’-GCUGGAAACAAUAUGUAAUUCAACG-3’)
- si-STAT3 (5’-CCCGUCAACAAAUAAGAACUGGAAU-3’)
- si-CCL2#1 (5’-GAUCCCCGCAGAGUGGUUCAGGA-3’)
- si-CCL2#2 (5’-AGCUUAAAAGCAGAAGUGGUUCAG-3’)

**Western blot analysis**

Cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris–HCl (pH 8.0), 120 mM NaCl, and 0.1% Nonidet-P40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL, USA). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline and incubated with primary antibodies at 4 °C. Blots were developed with a peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) procedures (Amersham).

**RT-PCR**

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). qRT-PCR were performed using the KAPA SYBR FAST qPCR kit (KAPA Biosystems, Wilmington, MA, USA) in Rotor Gene Q (Qiagen, Seoul, Korea). Fold-changes were calculated by the 2^ΔΔCt method using ACTB as an internal normalization control.

**Kaplan-Meier survival analysis**

The National Cancer Institute’s Repository for Molecular Brain Neoplasia Data (REMBRANDT, http://www.betastasis.com glioma/rembrandt, accessed November 2016) was evaluated for correlations between clinical outcome/survival and CCL2 gene expression in human brain-tumor biopsies.

**Statistical analysis**

All experimental data are reported as means; error bars represent standard deviation (SD). Comparisons between values were performed using unpaired two-tailed Student’s t-test, or
ANOVA for multivariate analysis. All statistical analyses were performed using GraphPad Prism 7.0 and the p values <0.05 were considered significant.
**Fig. S1**

**A**  
- Treatment with P6 (5uM) over 12h and 16h:
  - DMSO control
  - Western blot showing p-STAT3 and STAT3 levels.

**B**  
- Treatment with WP1066 (3 uM) over 4h, 8h, 12h, and 24h:
  - Western blot showing p-STAT3, STAT3, and ACTB levels.

**C**  
- mRNA levels fold change:
  - JAK1
  - JAK2
  - STAT3
  - si-control vs. si-JAK1, si-JAK2, si-STAT3 comparisons with asterisks indicating significance. (*p < 0.05, **p < 0.01).
Fig. S2

si control  si C5  
C5  ACTB

si control  si GROα  
GROα  ACTB

si control  si IL6  
IL6  ACTB

si control  si IL8  
IL8  ACTB

si control  si CCL2  
CCL2  ACTB
Fig. S3

A

B

Invaded cells/field (fold change)

rhCCL2 (ng/ml)

0 10 25 50

Invaded cells/field (fold change)

rhCCL2 (ng/ml)

0 10 25 50

Invaded cells/field (fold change)

rhCCL2 (ng/ml)

0 10 25 50