CD166 Promotes the Cancer Stem-like Properties of Primary Epithelial Ovarian Cancer Cells

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Abbreviations: A2780-SP, A2780-derived sphere-forming cells; ALCAM, Activated leukocyte cell adhesion molecule; ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; EOC, Epithelial ovarian cancer

Running title: Role of CD166 in cancer stem cells

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Supporting Information

Supplementary Methods

Analysis of sphere forming ability

For measurement of sphere-forming ability, cancer cells were plated in Ultra-Low Attachment 24-well plates at a density of 100-10,000 viable cells/well and grown in sphere culture medium. Cells were grown for 10 days, and the numbers of spheres were counted under a microscope.

Fluorescence activated cell sorting analysis

Cells were incubated with PE-labeled anti-CD166 antibody at 4 ˚C for 15 min in 100 μL of HBSS. The incubated cells were washed twice with 1 mL of HBSS, followed by analysis with FACS Canto II (BD Bioscience) or sorted by FACS Aria/Aria III (BD Bioscience). 7-AAD was added at a 1:250 dilutions to distinguish live cells from dead cells.

Western blot analysis

For western blot analysis, cells were harvested and lysed in lysis buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, and 1% Triton X-100; pH 7.4). Lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and then stained with 0.1% Ponceau S solution. After blocking in 5% fat milk, the membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence Western Blot kit (ECL, Amersham Biosciences).

Reverse transcription-polymerase chain reaction (RT-PCR) and RNA extraction

Total RNA was extracted from 80 - 90% confluent cultures using TRIzol reagent (Sigma-Aldrich) and reverse transcribed into cDNA using the Reverse Transcription cDNA Kit (#RT50KN; NanoHelix Co, Ltd). cDNA in 1 μL of the reaction mixture was

amplified using the Ready-2×-Go pre-mix PCR kit (#PMD008L; NanoHelix, Co Ltd) and 10 pmol each of sense and antisense primers. The thermal cycle profile was as follows: denaturation at 95 °C for 30 s, annealing at 52–58 °C for 30 seconds depending on the primers used, and extension at 72 °C for 30 s. Each PCR reaction was carried out for 25–30 cycles and PCR products were analyzed by 1% agarose gel with gel-RED electrophoresis and visualized under UV transillumination.

The primer pairs were as:

GAPDH: 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′;
CD166: 5′-CAGAACACGATGAGGCAGAC-3′, 5′-AGCAAGGAGGAGACCAACAA-3′;
OCT4A: 5′-GATCGGATCCATGGCGGGACACCTGGCT-3′, 5′-CCTTCCCAAATAGAACCC-3′;
SOX2: 5′-CAACATGATGGAGACGGAGC-3′, 5′-GTGCATCTTGGGGTTCTCCT-3′;
ALDH1: 5′-CTCGAAATTA AGTACACCAA-3′, 5′-TCAGTAGA CCCTGTGAATGC-3′;
ABCB1: 5′-CCATCAGTCCT GTTCTTG-3′, 5′-C TGCTCCTCTTGCATTT-3′;
ABCG2: 5′-TTCAGCCGTGGAACTCTTTG-3′, 5′-CCACACTCTGACCTGCTGCT-3′;
ABCC6: 5′-AGACAGACGCTGGGACCC-3′, 5′-ACCATCTTGGCTTTGAAGAGTG -3′;

Proliferation and cytotoxicity analyses

To measure cell proliferation rate, $CD166^{-}$ and $CD166^{+}$ populations of A2780 cells were seeded into a 24-well culture plate at a density of 1×10^{4} cells/well, respectively. MTT assay was used to determine relative cell growth rate every 24 h for cell growth curves. To assess viability of cells under the treatment of doxorubicin, 10^{4} cells were plated into a 96-well plate in 100 μL RPMI 1640 medium with 2% FBS. Following removal of culture medium at different time point, the cells were washed twice with HBSS and incubated with 10 μL of MTT (Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) for 2 h at 37 °C. After the incubation, formazan granules generated by the cells were dissolved in 100 μL of dimethyl sulfoxide, and the absorbance of the solution at 570 nm was determined using a Sunrise™ Absorbance Reader (Tecan Trading AG, Switzerland) after dilution to a linear range. Cell proliferation rate and cell viability rate of sphere cells were estimated by the CCK-8 assay (Dojindo molecular technologies Inc), Approximately A2780-SP cells were seeded into a 24-well low attachment cell culture plate at a density of 1×10^{4} cells/well with 100 μl medium

each well. CCK-8 assay was used to determine relative cell growth rate every 24 h for cell growth curves. To assess viability of sphere cells under the treatment of doxorubicin, 1×10^5 cells were plated into a 24-well plate. Each well was incubated with 10 μg CCK-8 solution for 2 h away from light before measuring the absorbance at 470 nm by Sunrise™ Absorbance Reader (Tecan Trading AG, Switzerland).

Cell migration assay

Ovarian cancer cells were harvested with 0.05% trypsin containing 0.02% EDTA, and suspended in a RPMI medium with 10% FBS at a concentration of 1×10^5 cells/mL. Membrane filters (8-μm pore size) in disposable 96-well chemotaxis chambers (Neuro Probe, Gaithersburg, MD) were pre-coated for 6 h with 20 μg/mL rat-tail collagen at room temperature. Aliquots (50 μL per well) of the cell suspension were loaded into the upper chambers, and RPMI medium with 10% FBS or experimental medium was placed in the lower chamber. After incubation for 12 h at 37 ˚C, the filters were disassembled, and the upper surface of each filter was scraped free of cells by wiping with a cotton swab. The numbers of cells that had migrated to the lower surfaces of each filter were determined by counting the cells in three different places under the microscope (×100 magnification) after staining with Hoechst 33342 (10 μM).

Cell adhesion assay

Adhesion assay was performed on 96-well plates coated with 20 μg/mL Collagen for 1 h at 37 ˚C. To block nonspecific binding, the plates were incubated with 0.1% BSA (bovine serum albumin) for 1 h at 37 ˚C or room temperature. A2780 cells (1×10^5/100 μL) sorted using the PE-CD166 antibody were added into each well and placed for 30~60 min at 37 ˚C in 5% CO2 humidified air incubation. Non-adherent cells were removed by gently washing the wells three times with PBS (phosphate-buffered saline) or HBSS. Adherent cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by rinsing with HBSS, and stained with Hoechst 33342 for 10 min. After extensive rinsing, the number of adherent cells was determined by microscopic counting of cells in three places (×100 magnification).