SUPPLEMENTARY MATERIALS AND METHODS

Isolation and identification of $CD133^+CD44^+$ CCSCs

Putative $CD133^+CD44^+$ CCSCs were isolated from cell populations by magnetic bead sorting using a magnetic activated cell sorting (MACS) MicroBead kit (Miltenyi Biotech, Germany). The cells were trypsinized, washed, and resuspended in PBS to prepare single-cell suspensions, which were then incubated with CD133 MicroBeads (Miltenyi Biotech, USA) for 20 min at 2-8°C, after which $CD133^+$ and $CD133^-$ cells were successfully separated using a MACS magnet and MS columns (Miltenyi Biotech, USA). Then, the isolated $CD133^+$ cells were incubated with CD44 MicroBeads (Miltenyi Biotech, USA), which was followed by cleavage of the MicroBeads until $CD133^+CD44^+$ cells were enriched. For $CD133^-CD44^-$ cell isolation, $CD133^-$ cells were used for depletion with a CD44 antibody. Purity assessments for positive and negative separation was performed by flow cytometry after magnetic bead separation. To this end, the samples were then stained with a phycoerythrin (PE)-labeled anti-CD133 antibody (Miltenyi Biotech, Germany) and a fluorescein isothiocyanate (FITC)-labeled anti-CD44 antibody (eBiosciences, USA) and analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA). A mouse IgG1κ antibody conjugated to PE (Miltenyi Biotech, Germany) and a rat IgG2b κ antibody conjugated to FITC (eBiosciences, USA) were used as isotype controls. Three independent experiments were performed.

Sphere formation assay

Isolated control- and shPRDX2-$CD133^+CD44^+$ CCSCs (1×10^4 cells/well) were plated into 6-well Ultra Low Cluster plates (Corning, USA) and cultured in serum-free DMEM-F12 medium (Gibco, USA) supplemented with 20 ng/ml EGF (Peprotech, USA), B27 supplement (1:50, Gibco, USA) and 20 ng/ml basic FGF (Peprotech, USA). The number of cell spheres was counted after three weeks of cultivation.

Animal studies

All animal protocols followed the Institutional Animal Ethics Care of Chongqing Medical University. To generate subcutaneous tumors, single-cell suspensions with >90% survival rates were subcutaneously injected into the lateral wall of 4-week-old female BALB/c nude mice. Tumor growth was observed and recorded every 1 week using the following formula: Volume = $Width^2 \times Length/2$. Subcutaneous tumors were analyzed by histological or flow cytometry analysis. For the orthotopic implantation of tumor cells, mice were anesthetized, and the cecum was exteriorized by laparotomy. Then, control- and shPRDX2-$CD133^+CD44^+$ CCSCs suspended in 30 µl of DMEM F-12 medium and 20 µl Matrigel were injected into the cecal wall.

Transwell migration and invasion assays

For migration assays, isolated control- and shPRDX2-$CD133^+CD44^+$ CCSCs were resuspended to a density of 1×10^4 cells in 250 µl of DMEM-F12 and placed in the upper chamber of transwells with the noncoated membrane (Millipore, USA). Medium supplemented with growth factors was used as a chemoattractant in the lower transwell chamber. After 24 h, the cells were fixed with 4% paraformaldehyde for 30 min, stained for 10 min in 0.5% crystal violet, and then counted under a light microscope at 100× magnification. The invasion assays were performed similarly to the migration assays except that Matrigel-coated membrane was used in the top chamber (Millipore, USA).

Analysis of CCSC adhesion to ECM proteins

Isolated control- and shPRDX2-$CD133^+CD44^+$ CCSCs were plated into 60-mm dishes (5×10^5 cells per dish) coated with type I collagen or fibronectin, respectively, and cultured for 12 h. Nonadhesive and adhesive cells were then collected and counted using a hemocytometer.

Analysis of intracellular ROS levels

Intracellular ROS levels of control- and shPRDX2-HT29-$CD133^+CD44^+$ CCSCs were measured before drug exposure and 24 hours after 500 µg/mL 5-FU or 100 µM oxaliplatin exposure. ROS were detected with a 2,7-dichlorofluorescein diacetate (DCFH-DA) probe (Beyotime, China) according to the manufacturer's instructions. Briefly, the cells were incubated with 10 µmol/L DCFH-DA at 37°C for 20 min, after which they were washed 3 times with PBS to remove excess DCFH-DA, suspended in medium and then immediately analyzed by flow cytometry.

Alkaline comet assay of DNA damage

DNA damage was evaluated using an alkaline comet assay following a previously described procedure with some modifications [1]. Briefly, single-cell suspensions of control- and shPRDX2-HT29-$CD133^+CD44^+$ CCSCs with or without exposure to chemotherapy drugs were prepared. These cells were then embedded in low-melting-point agarose and lysed overnight at 4°C in

lysis buffer (0.1 M EDTA, 1% Triton X-100, 0.01 M Tris base, 2.5 M NaCl, and 5% DMSO, pH 10). Then, the unwinding step was performed in unwinding/electrophoresis buffer (0.3 M NaOH, 2 mM EDTA, pH 13) for 1 h at 4°C. Electrophoresis was conducted for 25 min at 4°C in unwinding/electrophoresis buffer at electric-field strength of 306 mA and 0.6 V/cm. The slides were then neutralized with a neutralization buffer (0.4 Tris-HCl, pH 7.5), rinsed with distilled water, air-dried, and then stained with 20 µl ethidium bromide (2 µg/mL) before being covered with standard cover slips. Subsequently, the slides were analyzed for comets with a Nikon Optiphot microscope attached to a Pulnix video camera and fluorescence image analysis system. DNA single-strand breaks were determined using the tail moment parameter.

REFERENCE

1. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988; 175:184–91.
https://doi.org/10.1016/0014-4827(88)90265-0
PMID:3345800