Title

Subpopulation of small-cell lung cancer cells expressing CD133 and CD87 show resistance to chemotherapy

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Summary (220 words)

Tumors are presumed to contain a small population of cancer stem cells (CSCs) that initiate tumor growth and promote tumor spreading. Multidrug resistance in CSCs is thought to allow the tumor to evade conventional therapy. This study focused on expression of CD133 and CD87 because CD133 is a putative marker of CSCs in some cancers including lung, and CD87 is associated with a stem-cell-like property in SCLC. Six SCLC cell lines were used. The expression levels of CD133 and CD87 were analyzed by real-time quantitative reverse transcription–polymerase chain reaction and flow cytometry. CD133+/− and CD87+/− cells were isolated by flow cytometry. The drug sensitivities were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Non-obese diabetic/severe combined immunodeficiency mice were used for the tumor formation assay.

SBC-7 cells showed the highest expression levels of both CD133 and CD87 among the cell lines. CD133−/CD87−, CD133+/CD87−, and CD133−/CD87+ cells were isolated from SBC-7 cells; however, CD133+/CD87+ cells could not be obtained. Both CD133+/CD87− and CD133−/CD87+ subpopulations showed a higher resistance to etoposide and paclitaxel and greater re-populating ability than the CD133−/CD87− subpopulation. CD133+/CD87− cells contained more G0 quiescent cells than CD133−/CD87− cells. By contrast, CD133−/CD87− cells showed the highest tumorigenic potential.

In conclusion, both CD133 and CD87 proved to be inadequate markers for CSCs; however, they might be beneficial for predicting resistance to chemotherapy.

Introduction

Small-cell lung cancer (SCLC) is highly sensitive to chemotherapy. More than 80% of patients achieve an objective response; however, most responders eventually relapse because of drug resistance. Less than 30% of patients with limited disease and 1–2% of patients with extensive disease survive to 5 years (1).

Cancer stem cells (CSCs) have been proposed as one of the causes of treatment resistibility. CSCs are a rare population of undifferentiated cells that are responsible for tumor initiation, maintenance, and spreading. They are resistant to anticancer agents and can self-renew and generate progeny in the form of differentiated cells that constitute most of the cells in tumors (2, 3). Because a surviving population of CSCs after conventional treatment might be responsible for tumor regrowth, identifying and eradicating the CSC population are very important.

CSCs were isolated initially from leukemia and subsequently from solid tumors, including brain, breast, prostate, colon, and liver cancer (2-6). The methods used to isolate CSCs include cell surface marker analysis (2-6), side-population analysis (7), and the sphere-formation assay (5, 8). Putative CSC markers were reported to be CD34-positive/CD38-negative for acute myeloid leukemia, CD44-positive/CD24-negative/α2β1-low/Lin-negative for breast cancer, CD44-positive/α2β1-high/CD133-positive for prostate cancer, and CD133-positive/nestin-positive for brain cancer (9). The present study focused on expression of CD133 and CD87 as putative cell-surface markers. CD133 is reported to be a marker of CSCs in some cancers, such as brain, prostate, and colorectal

cancer (3-5). Freshly dissociated human SCLC and non-small-cell lung cancer contain CD133-positive cells, which could generate long-term lung tumor spheres *in vitro* that could both differentiate and preferentially form tumors *in vivo* (8). However, CD133 was reported to be both a positive and a negative marker of CSCs in lung cancer (10, 11). Meanwhile, in human SCLC cell lines, a small population of urokinase plasminogen activator receptor (uPAR/CD87)-positive cells were identified, of which a subset demonstrated enhanced clonogenic activity *in vitro* (12). CD87 has been implicated in the growth, metastasis, and angiogenesis of several solid and hematologic malignancies, and its increase was associated with a poor clinical outcome (13). Targeting CD87 can have broad-spectrum antitumor effects (14).

We hypothesized that both CD133 and CD87 might be useful as CSCs markers in SCLC. To test this hypothesis, we investigated the expression levels of CD133 and CD87 using six SCLC cell lines. Additionally, we examined whether amrubicin might be effective for such cancer stem-like cells because it was demonstrated to be effective for refractory SCLC patients (15).

Material and Methods

Drugs

Drugs were obtained from the following sources: cisplatin and amrubicinol from Nippon Kayaku (Tokyo, Japan); etoposide and paclitaxel from Bristol-Myers Squibb (Tokyo, Japan);

7-ethyl-10-hydroxy-campthothecin (SN-38), an active metabolite of irinotecan, from Yakult Honsha Co. Ltd. (Tokyo, Japan); and 3-[4,5-dimethyl-thizol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

The SBC-3, 4, 5, 6, 7, and 9 cell lines were established in our laboratory from SCLC patients (16). The SBC-3 cell line was derived from bone marrow aspirates of an untreated patient (17). The other cell lines were established from pleural effusion or pericardial effusion of patients who had received chemotherapy. All cell lines were characterized by Tsuchida *et al*. (18), and some were stored at the Japanese Collection of Research Bioresources (http://cellbank.nibio.go.jp/cellbank.html). These cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a tissue culture incubator at 37°C under 5% CO_2.

Reverse transcription (RT)–polymerase chain reaction (PCR)

RNA samples were prepared for RT–PCR using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol, and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Duplex TaqMan real-time PCR was used to analyze the CD133 and CD87 expression levels in each cell line using an ABI PRISM 5700 Sequence Detection

System (Applied Biosystems, Foster City, CA, USA). Sequences of the Taqman probe and primers for CD133, CD87, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: CD133: Taqman probe (5′-FAM-TGGCATCGTGCAAACCTGTGGCC-TAMRA-3′), forward primer (5′-AGTGGATCGAGTTCTCTATCAGTG-3′), reverse primer (5′-CAGTAGCTTTTCCTATGCCAAACC-3′); CD87: Taqman probe (5′-FAM-ACAGCCCGGCCAGAGTTGCCCT-TAMRA-3′), forward primer (5′-CCACTCAGAGAAGACCAACAGG-3′), reverse primer (5′-GGTAACGGCTTCGGGAATAGG-3′). GAPDH was co-amplified in the same reaction mixture as an endogenous reference gene. Sequences of the probe and primers for GAPDH were as follows: Taqman probe: 5′-FAM-CGTCGCCAGCCGAGCCACATCG-TAMRA-3′; forward primer: 5′-CGACAGTCAGCCGCATCTTC-3′; and reverse primer: 5′-CGACCTTCACCTTCCCCATG-3′. The average levels of CD133 and CD87 expression were determined from differences in the threshold amplification cycles between CD133 and CD87 and GAPDH.

Flow cytometry

Cells were harvested and re-suspended at 1×10^6 cells/ml of staining buffer. Fluorescent-labeled monoclonal antibodies were added in concentrations recommended by the manufacturer. After washing, the labeled cells were analyzed and sorted using a FACS Aria flow cytometer (Becton Dickinson,

Mountain View, CA, USA). The antibodies used were allophycocyanin (APC)-conjugated mouse anti-human CD133 (Clone AC 133; Miltenyi Biotec, Auburn, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human uPAR (CD87; American Diagnostica, Inc., Stamford, CT, USA) and phycoerythrin (PE)-conjugated mouse anti-human MDR1 (eBioscience, Inc., San Diego, CA, USA). Gating was implemented on the basis of negative-control staining profiles. The sort was performed in four-way purity mode (the purity was >98%). The cell-cycle analysis was performed after staining with Hoechst 33342 and Pyronin Y (Sigma-Aldrich, St. Louis, MO, USA). Cells were stained according to the manufacturer's instructions.

Limiting dilution assay

To determine the clonogenicity and regenerative ability of single cells, a limiting dilution assay was carried out. The cells were resuspended in fresh medium, diluted to 3 cells/ml, and seeded at approximately 0.3 cells/well with 100 μl of medium into 96-well plates. Wells containing no cells or more than one cell were excluded after careful microscopic examinations, and those containing a single cell were marked and monitored daily under a microscope. After colony formation, the colonies were counted, dissociated, harvested, and cultured again.

Cell proliferation assay

Cell proliferation was examined on days 1, 2, 3, and 4. Isolated cells (1×10^5) were seeded in a cell culture flask at a final volume of 5 ml. After incubation, proliferation was evaluated by enumerating cells. Growth inhibition was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction assay with Cell Counting Kit-8 (Dojindo, Tokyo, Japan). Briefly, cells were plated on 96-well plates at a density of 3,000 cells per well with RPMI 1640 with 10% FBS. Several concentrations of each drug were added to wells, and incubation was continued for 72 h. MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was then added to all wells, and incubation was continued for a further 2 h. After the dark blue crystals had dissolved, the absorbance was measured with a microplate reader. The percentage of growth is shown relative to that of untreated controls. Each assay was performed in triplicate or quadruplicate. The mean ± standard error of the 50% inhibitory concentration (IC_{50}) of the drugs in cells was determined.

Immunoblotting

Proteins were extracted from each cell line and incubated in lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol phosphate, 10 mM NaF, and 1 mM Na-orthovanadate] containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) and centrifuged at 15,000 rpm (20,630 g) for 20 min at 4°C. Proteins were separated by SDS-PAGE using 5–15% precast gels (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes. Specific

proteins were detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) using the antibodies to aldehyde dehydrogenase 1A1 (1:100 dilution; Abcam, Cambridge, MA) and β-actin (1:1,000 dilution; Cell Signaling Technology, Danvers, MA). The secondary antibody; anti rabbit IgG (HRP-linked, species-specific whole antibody) (GE Healthcare), was used at a 1:5,000 dilution.

Xenograft model

Sorted cells were injected subcutaneously into the backs of 7-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Charles River, Yokohama, Japan). Groups of mice were inoculated with CD133+/CD87−, CD133−/CD87+, or CD133−/CD87− cells at 5×10^3 and 2×10^3 cells. Tumor growth was monitored twice per week, and tumor volume ($\text{width}^2 \times \text{length}/2$) was determined periodically. A lack of tumor formation at 8 weeks after sorted-cell injection was described as 'no tumor formation'.

Statistical analysis

The differences between the groups were compared using Student's t-test and chi-square test. $P < 0.05$ was considered statistically significant. All data were analyzed using Microsoft Office Excel 2007 (Microsoft Japan Corporation, Tokyo, Japan).

Results

SBC-7 cells showed high expression levels of both CD133 and CD87

Expression levels of CD133 and CD87 mRNA by real-time quantitative RT–PCR were determined. SBC-7 cells showed the highest expression of both CD133 and CD87 among the six cell lines. SBC-9 cells also showed both CD133 and CD87 expression, and SBC-4 and SBC-5 cells showed expression of only CD133 and CD87, respectively. SBC-3 cells demonstrated neither CD133 nor CD87 expression (Fig. 1A). We confirmed expression of CD133 and CD87 in each cell line by flow cytometry (Fig. 1B, C). SBC-7 cells displayed some subpopulations: CD133+/CD87− (41.1%), CD133−/CD87+ (10.1%), and CD133−/CD87− (48.3%); however, CD133+/87+ double-positive cells were very rare (0.6%). The cell-surface expression of CD133 was confirmed in SBC-7 and SBC-9, and that of CD87 was in SBC-5 and SBC-7, respectively. Although there seemed to be a correlation between the mRNA levels and cell surface expressions, cell surface expression was not detected at moderate mRNA levels, such as CD133 in SBC-4 and CD87 in SBC-9. Because only SBC-7 cells showed both CD133 and CD87 expressions in flow cytometry analysis, we selected SBC-7 cells and investigated their characteristics as CSCs.

CD133+/CD87− and CD133−/CD87+ subpopulations showed re-populating ability

We used SBC-7 cell lines and examined the properties of each subpopulation. To compare the re-populating ability of each subpopulation, we sorted the CD133+/CD87−, CD133−/CD87+,

CD133−/CD87−, and CD133+/CD87+ cells by flow cytometry (Supplementary Fig. S1), cloned the sorted cells with limiting dilutions, and cultured them separately under the same conditions for 6 weeks. Although we attempted to select CD133+/CD87+ cells several times, no double-positive cells could be obtained for further examination, including *in vivo* study. Therefore, we investigated the characteristics of three subpopulations: CD133+/CD87−, CD133−/CD87+, and CD133−/CD87−. We then re-stained the cultured cells with CD133 and CD87 antibodies and analyzed them by flow cytometry. The CD133+/CD87− population generated both CD133+/CD87− and CD133−/CD87− subpopulations, and the CD133−/CD87+ population generated both CD133−/CD87+ and CD133−/CD87− subpopulations. However, the CD133−/CD87− population produced only CD133−/CD87− cells. CD133+/CD87+ cells were not obtained from any cultured subpopulation (Fig. 2).

Drug sensitivity, cell cycle and aldehyde dehydrogenase 1A1 expression in the subpopulations

Next, we examined the sensitivity of each subpopulation to the chemotherapeutic drugs cisplatin, etoposide, paclitaxel, and 7-ethyl-10-hydroxycamptothecin (SN-38: active metabolite of irinotecan). Cells expressing either CD133 or CD87 were more resistant to etoposide and paclitaxel than were double-negative cells (Table 1). In addition, CD133+/CD87− cells showed the highest resistance to etoposide among the three groups ($p < 0.05$). The IC_{50}s (μM) to cisplatin were 5.19 ± 0.19 in CD133−/CD87−, 3.49 ± 0.68 in CD133+/CD87−, 4.72 ± 0.64 in CD133−/CD87+, and 2.14 ± 0.22 in

parent SBC-7 (Table 1). Although CD133- and CD87-positive cells tended to be more sensitive to cisplatin than double-negative cells, there was no significant difference among the cell lines tested. When compared with SBC-7 parental cells, CD133+/CD87− cells showed more resistance to etoposide (p = 0.01) and paclitaxel (p = 0.02), and CD133−/CD87+ cells were more resistance to paclitaxel (p = 0.03).

Additionally, we analyzed the cell cycle of each subpopulation by flow cytometry. The CD133+/CD87− subpopulation contained more G0 quiescent cells than did CD133−/CD87+ and CD133−/CD87− subpopulations (Fig. 3). Aldehyde dehydrogenase 1A1 levels seemed similar among the three subpopulations (Supplementary Fig. S2).

The growth rate and MDR1 expression in the subpopulations

We also investigated the cell proliferation rates of each subpopulation (Supplementary Fig. S3). The growth rate of CD133−/CD87+ cells was greater than that of CD133−/CD87− and CD133+/CD87− cells. The growth rates of CD133−/CD87− and CD133+/CD87− cells were similar. Although rapid proliferation makes a cell line appear more drug-sensitive compared with a more-slowly growing cell line, the drug sensitivity of the SBC-7 subclones could not be explained by the growth rate alone. Next, we examined the expression levels of MDR1 on each subpopulation by flow cytometry. The expression of MDR1 was higher in CD133−/CD87+ cells than that in CD133−/CD87− cells (8.1% vs. 3.1%) (Supplementary Fig. S4).

Drug exposure did not induce CD133 or CD87 expression

We investigated whether the expression levels of CD133 and CD87 were up-regulated in cells resistant to chemotherapeutic drugs. We used the SBC-3 cell line as a parent cell, which expressed neither CD133 nor CD87, and its resistant cell lines to cisplatin, SN-38, or etoposide (SBC-3/CDDP, SBC-3/SN-38, or SBC-3/ETP, respectively) (19-21). The CD133 mRNA levels in SBC-3/CDDP and CD87 in SBC-3/ETP were slightly up-regulated compared with those in SBC-3 (Fig. 4A). However, in flow cytometry analysis, there was no significant up-regulation of CD133 or CD87 expression in the resistant cells (Fig. 4B). Thus, the surface expression of CD133 or CD87 at least was unlikely to be induced by the chronic exposure of chemotherapeutic drugs *in vitro*.

CD133−/CD87− subpopulations showed high tumor formation ability *in vivo*

The tumorigenic potential of each subpopulation through subcutaneous injection of each sorted cell line in NOD/SCID mice was evaluated. We monitored tumor growth twice per week. As shown in Table 2, when 5,000 sorted cells were injected, each subpopulation could initiate new tumors. However, when 2,000 cells were injected, the CD133−/CD87− subpopulation showed the highest tumor initiating capability, and the CD133−/CD87+ subpopulation could not produce new tumors. When parental SBC-7 cells were injected, tumor formation was confirmed as in the CD133–/CD87– subpopulation. The pathological feature of the

tumors with hematoxylin-eosin staining was similar to parental SBC-7 xenograft tumors (Supplementary Fig. S5). Re-analysis of each derived tumor using CD133 and CD87 antibodies in flow cytometry showed that the surface markers of the tumor cells were similar to those of each subpopulation cultured *in vitro* (data not shown).

CD133-positive cells were also resistant to amrubicinol

Although CD133- and CD87-positive cells could not satisfy the requirements for CSCs, these cells showed chemoresistant characteristics. Additionally, CD133+/CD87− cells had higher tumorigenicity and higher resistance to chemotherapeutic drugs than CD133−/CD87+ cells. The IC_{50}s of amrubicinol in CD133-positive and -negative cells were 0.732 ± 0.119 μM and 0.172 ± 0.038 μM, respectively (p = 0.009).

Discussion

The need to target therapies at the self-renewal capacity of the stem-cell compartment, effectively interrupting the source of recurrence in tumors sensitive to conventional therapeutic approaches, has also evolved under the CSC hypothesis in the lung cancer field (9). However, identifying a phenotypic marker in lung CSCs has been unsuccessful. In this study, we investigated whether CD133 or CD87 might be putative marker of CSCs. At first, we examined the expression levels of CD133 and CD87 mRNA by

real-time quantitative RT–PCR. And then, we confirmed the expression of CD133 and CD87 on cell surface by flow cytometry. Although there were discrepancies between the expression levels of mRNA and protein in some cell lines, such as SBC-4 and SBC-9, only SBC-7 cells displayed both CD133 and CD87 cell-surface markers. The ambivalence might be explained by following reasons. 1) Although mRNA was induced, the protein might not be detected because of small quantity. 2) The protein might be subject to degradation easily. 3) It might stay in the cytoplasm and could not appear on the cell surface.

Both CD133- and CD87-positive cells showed higher resistance to chemotherapeutic drugs and a higher re-populating ability and contained more G0 quiescent cells than did the double-negative subpopulation *in vitro*. However, the double-negative subpopulation showed the highest tumor-initiating capability *in vivo*. Thus, CD133 and CD87 did not satisfy the requirements for CSCs in SCLC cells. The reason that double-negative cells showed the highest tumor-initiating capability remains unclear. We used SCLC cell lines to examine the characteristics of CD133- and CD87-positive cells. In cell lines, the characteristics of tumor cells can be changed from primary cultured cells or fresh cells; thus, the double-negative subpopulations might acquire some specific ability to initiate new tumors. In addition, Meng et al. previously reported that lung cancer cell lines regardless CD133 expression could initiate new tumors in nude mice (11). Thus, CD133 alone might not be useful as a stem cell marker for lung cancer.

Particularly, because CD133-positive cells showed a higher tumor-initiating capability than CD87-positive cells, we investigated the strategy to overcome the resistance to conventional

chemotherapy in CD133-positive cells. Amrubicin, a synthetic 9-aminoanthracycline, is converted to the active metabolite amrubicinol via reduction of its C-13 ketone group to a hydroxyl group by carbonyl reductase (22). Adriamycin-resistant cells show partial resistance to amrubicin *in vitro* (23). Phase II studies of previously treated SCLC patients showed that amrubicin was effective in both sensitive and refractory relapse (16). Unfortunately, CD133-positive cells were 4.3 times more resistant to amrubicinol than were CD133-negative cells.

In the present study, both CD133 and CD87 proved to be inadequate markers for CSCs; however, they seemed to predict resistance to chemotherapy. We could not clarify the mechanism why CD133- or CD87-positive cells showed higher resistance to etoposide and paclitaxel. Etoposide targets the cells in S/G2/M phase. CD133+/CD87− fraction, which harbored 16.2% of S/G2/M fraction, showed higher level of IC_{50} in etoposide than CD133−/CD87− containing 29.7% of that fraction. However, CD133−/CD87+ fraction which harbored higher levels S/G2/M phase was also more resistant against etoposide compared with CD133−/CD87−. Therefore, the resistant mechanism of CD133 or CD87 was not clarified only by cell cycle analysis. Gutova *et al*. reported that CD87-positive cells showed higher expression of MDR1 (12). In our study, the expression level of MDR1 was higher in CD133−/CD87+ subpopulation. However, the expression rate of MDR1 (8.1%) was lower than that (10–40%) in their report (12). Chen *et al*. indicated that CD133-positive cells were highly co-expressed with ABCG2 transporter and were significantly resistant to conventional treatment methods compared with CD133-negative non-small-cell

lung cancer cells (24). Thus, the CD133- or CD87-positive subpopulation in SBC-7 might be related to drug resistance. Meanwhile, cisplatin seemed effective irrespective of the CD133 or CD87 status because cisplatin resistance was not associated with MDR1 or ABCG2 overexpression (25, 26). The surface expressions of both CD133 and CD87 were not increased after chronic exposure of SBC-3 cells to chemotherapeutic drugs, resulting in acquisition of resistance. The up-regulation of CD133 or CD87 expression might be a part of a complicated chemoresistance mechanism.

Increased levels of urokinase plasminogen activator and its receptor CD87 were strongly correlated with poor prognosis and unfavorable clinical outcome in patients with acute myeloid leukemia and breast cancer (13). In many solid tumors, such as glioblastoma, the presence of CD133 was correlated with poor survival (3). In patients with non-small cell lung cancer, CD133 was indicative of a resistance phenotype, but did not represent a prognostic marker for survival (27). Although the clinical outcome of CD133 or CD87 expression in SCLC patients remains unclear, our data suggested that the tumors expressing CD133 and/or CD87 might be resistant to conventional chemotherapy. To prove the hypothesis, the relationship between CD133 and/or CD87 expression levels on human SCLC materials and corresponding chemosensitivity should be investigated. The drugs should be screened for their ability to overcome the resistant SCLC cells.

The limitation of our study was that we were unable to generate CD133+/CD87+ double-positive cells, which might have true CSC characteristics. Thus efficient sorting of a small population of

double-positive cells for *in vivo* experimentation is necessary. Characterization of the CD133+/CD87+ cells might be relevant for this study and could reveal some remarkable properties of this subset (for example, an enhanced tumorigenic ability) compared with single-positive CD133 or CD87 fractions. In addition, we extensively examined the SBC-7 line, which was the only cell line that exhibited surface expression of both CD133 and CD87 among the cells we used. We tried to confirm that CD133 or CD87 positive cells showed higher chemoresistance than negative cells using the SBC-9 cells. SBC-9 cells were divided into CD133+/CD87− and CD133−/CD87− subpopulations. Unfortunately, CD87 positive cells in the SBC-9 cells were not obtained because it might be due to the small amount of the cells (0.4%). We investigated cell viability of both subpopulations after 96h exposure to cisplatin, etoposide and paclitaxel at the IC_{50} of each drug for the SBC-9 cells. CD133+/CD87− cells were resistant to only etoposide than CD133−/CD87− cells (Supplementary Fig. S6). We should further examine using the cell lines which could be clearly divided into CD133-positive/negative cells or CD87-positive/negative cells. Furthermore, a second tumorigenic assay using CD133+ and CD87+ cells sorted from an alternate SCLC cell line could confirm our results, such a cell line could be generated.

In conclusion, both CD133 and CD87 in the SBC-7 line proved to be inadequate markers of CSCs; however, they might be beneficial for prediction of resistance to chemotherapy.

Disclosure Statement

We report no conflict of interest.

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Figure Legends

Figure 1.

A. The mRNA expression levels of CD133 and CD87 in each cell line using real-time quantitative reverse transcription–polymerase chain reaction. SBC-7 cells showed the highest expression levels of both CD133 and CD87 among the six cell lines. SBC-4 cells expressed only CD133, and SBC-5 cells expressed only CD87. SBC-3 cells expressed neither CD133 nor CD87. Bars indicate the standard deviation.

B. Flow cytometry analysis of SBC-7 cells stained with CD133 and CD87 antibodies. SBC-7 cells showed CD133+/CD87−, CD133−/CD87+, and CD133−/CD87− subpopulations; however, a CD133+/CD87+ subpopulation was not obtained.

C. Flow cytometry analysis of SBC-3, 4, 5, and 9 cells stained with CD133 and CD87 antibodies. SBC-5 showed a CD133–/CD87+ subpopulation. SBC-9 cells showed a CD133+/CD87– but not a CD133–/CD87+ subpopulation.

Figure 2.

Re-analysis of each subpopulation after limiting dilatation by flow cytometry. CD133+/CD87− and CD133−/CD87+ subpopulations in SBC-7 cells showed re-populating ability. However, the CD133−/CD87− subpopulation could produce only CD133−/CD87− cells.

Figure 3.

Cell-cycle analysis of each subpopulation with Hoechst 33342 and Pyronin Y. The CD133+/CD87− subpopulation contained more G0 quiescent cells than did CD133−/CD87+ and CD133−/CD87− subpopulations.

Figure 4.

A. CD133 and CD87 mRNA levels in parental (SBC-3) and resistant (SBC-3/CDDP, SBC-3/SN38, and SBC-3/ETP) cell lines using real-time quantitative reverse transcription–polymerase chain reaction. CD133 in SBC-3/CDDP and CD87 in SBC-3/ETP were more highly expressed than those in SBC-3.

B. Flow cytometry analysis of SBC-3/CDDP cells stained with CD133 and CD87 antibodies. The expression of CD133 or CD87 was not increased in resistant cells.

Supporting information

Supplementary Figure 1.

CD133 and CD87 expression and sort position in SBC-7 cell line.

Supplementary Figure 2.

The expression levels of aldehyde dehydrogenase 1A1 (ALDH1A1) in each subpopulation by western blotting.

Supplementary Figure 3.

Growth curves of each subpopulation.

Supplementary Figure 4.

The cell surface expression levels of MDR1 on each subpopulation by flow cytometry.

Supplementary Figure 5.

Hematoxylin-eosin staining of xenograft tumors.

Supplementary Figure 6.

The cell viability of CD133+/CD87− cells and CD133−/CD87− cells in the SBC-9 after treatment with cisplatin, etoposide or paclitaxel.

Fig. 1A

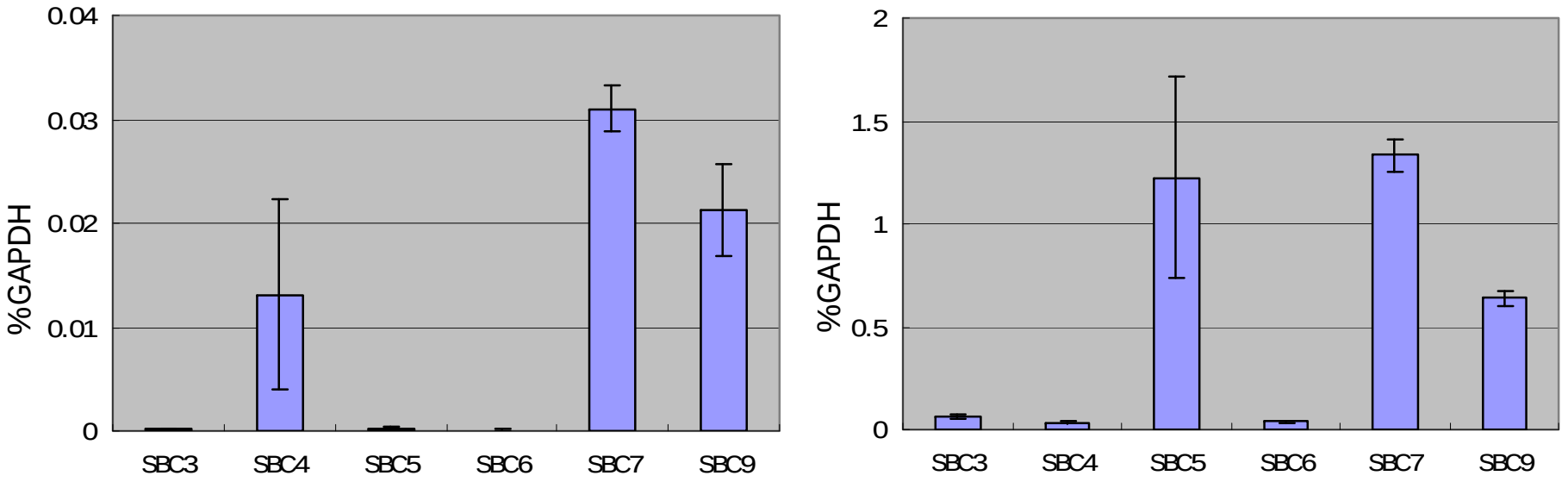

CD133
0.04
0.03
0.02
0.01
0
%GAPDH
SBC3
SBC4
SBC5
SBC6
SBC7
SBC9
CD87
2
1.5
1
0.5
0
%GAPDH
SBC3
SBC4
SBC5
SBC6
SBC7
SBC9

Fig. 1B

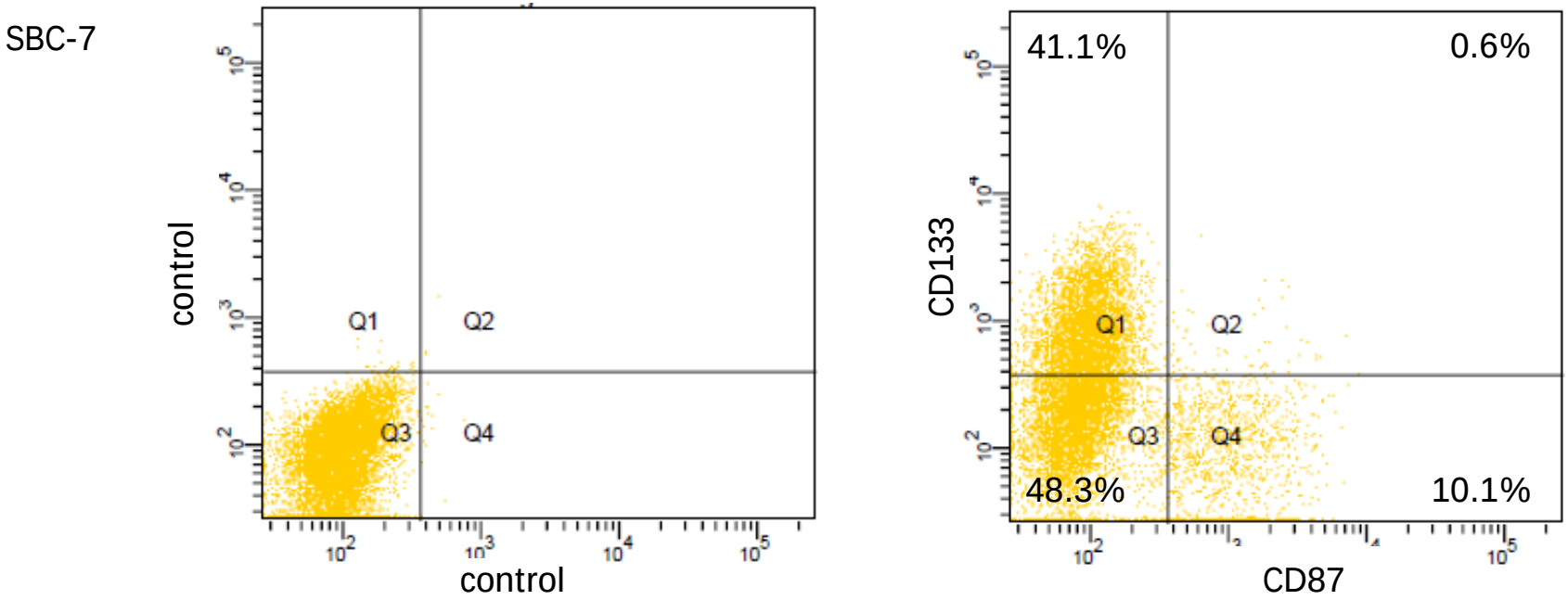
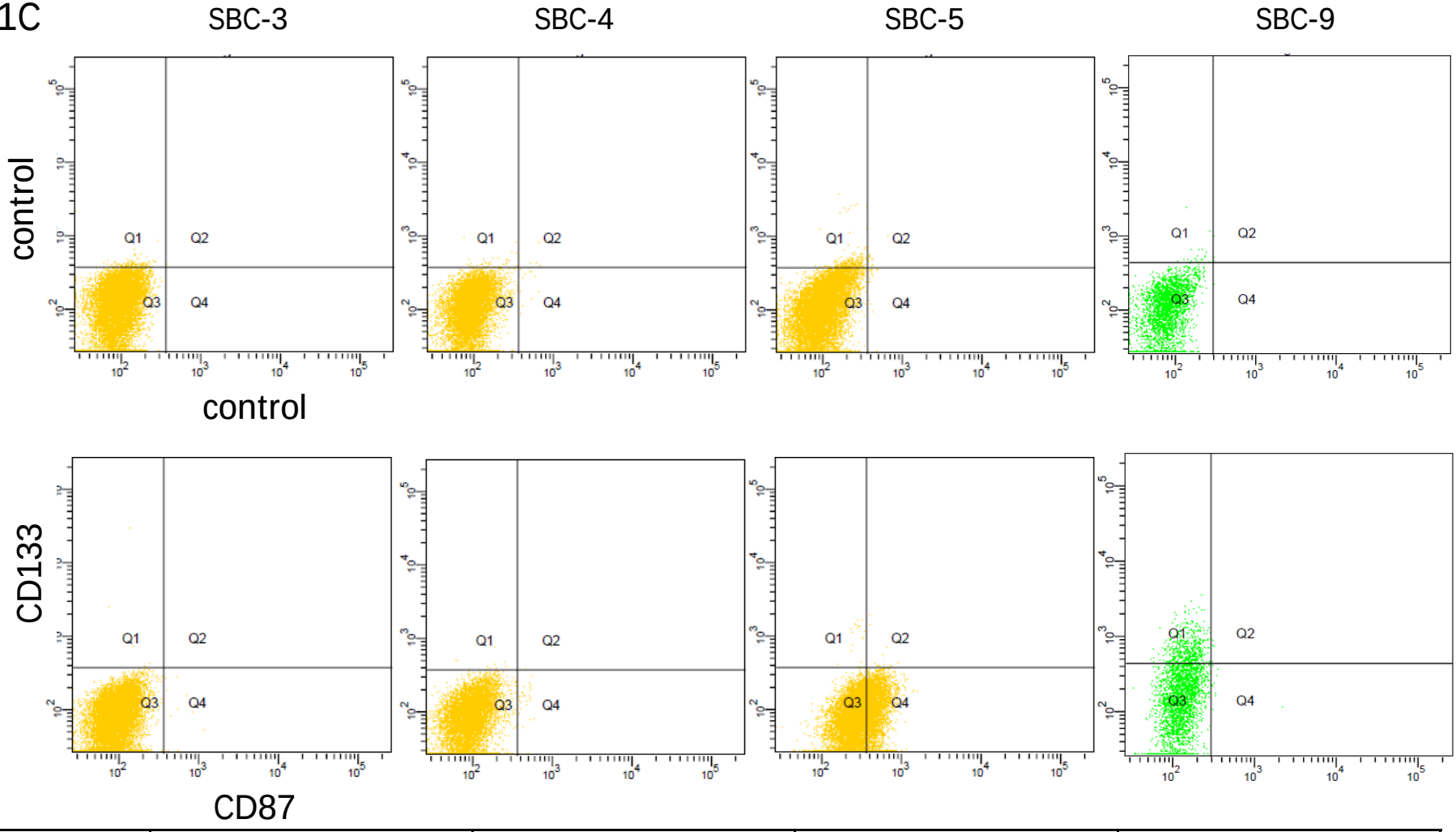

SBC-7
control
Q1
Q2
Q3
Q4
control
CD133
41.1%
0.6%
Q1
Q2
Q3
Q4
48.3%
10.1%
CD87

Fig. 1C



	SBC-3	SBC-4	SBC-5	SBC-9
CD133+ / 87+	0 %	0.1 %	0.1 %	0.1 %
CD133+ / 87-	0 %	0 %	0.1 %	14.4 %
CD133- / 87+	0.1 %	0.3 %	26.5 %	0.3 %
CD133- / 87-	99.9 %	99.6 %	73.4 %	85.2 %

Fig. 2

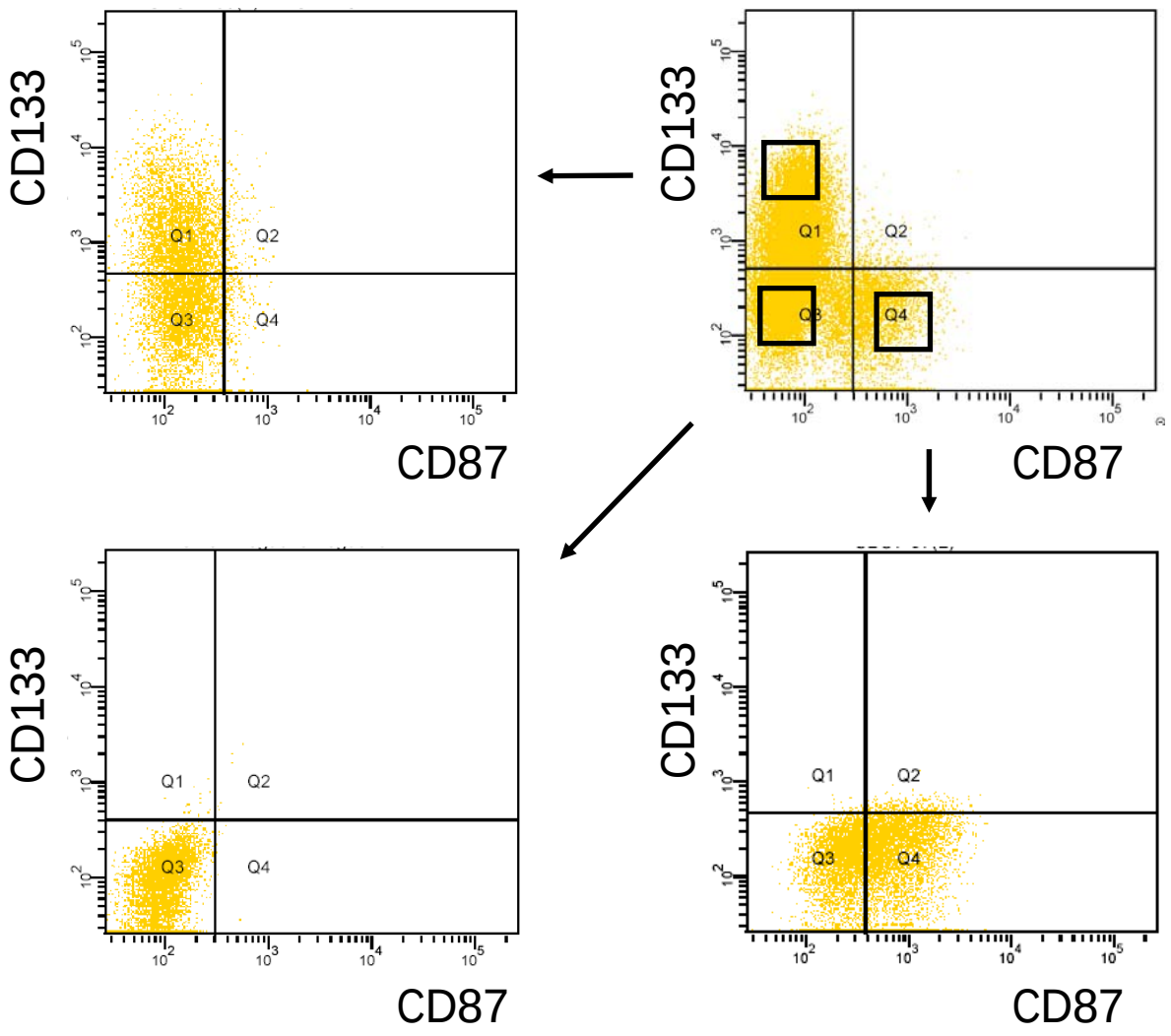
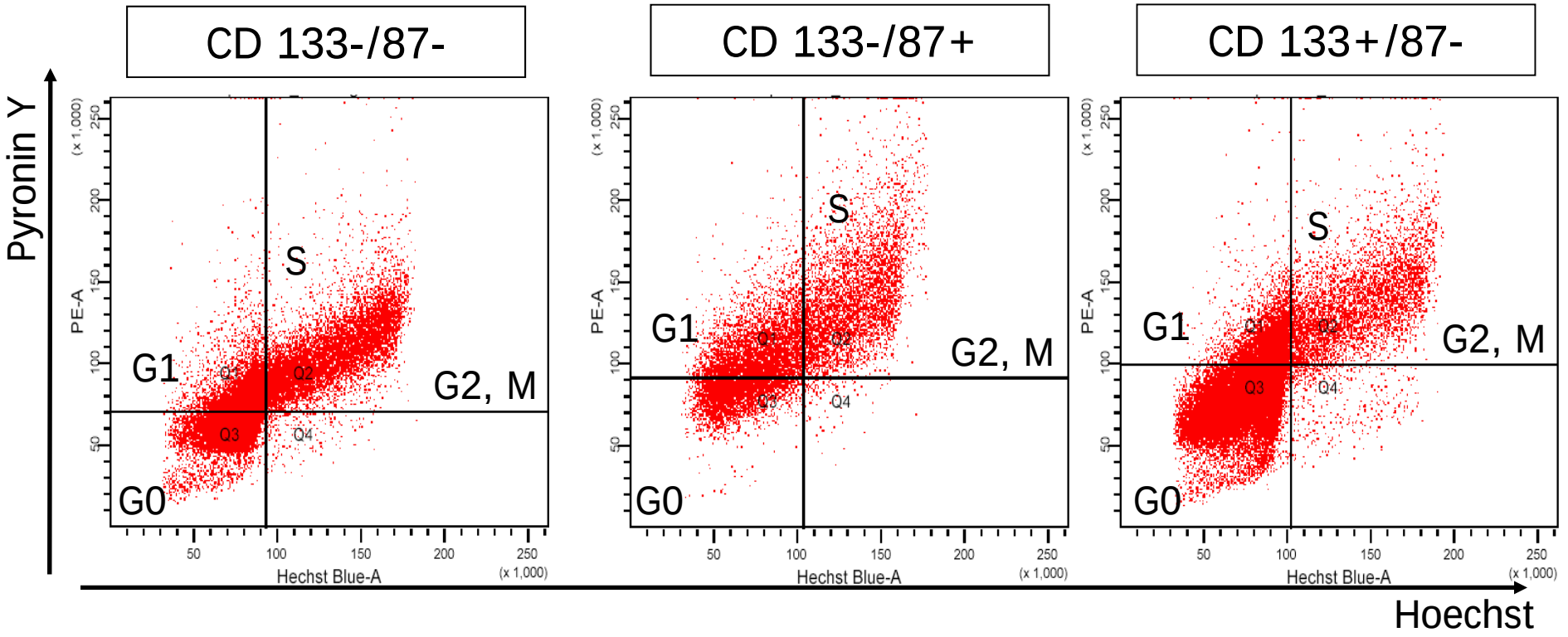


Fig. 3A



G0	44.5 %
G1	25.0 %
S/G2/M	29.7 %

G0	27.8 %
G1	34.6 %
S/G2/M	36.6 %

G0	65.5 %
G1	16.6 %
S/G2/M	16.2 %

Fig. 4A

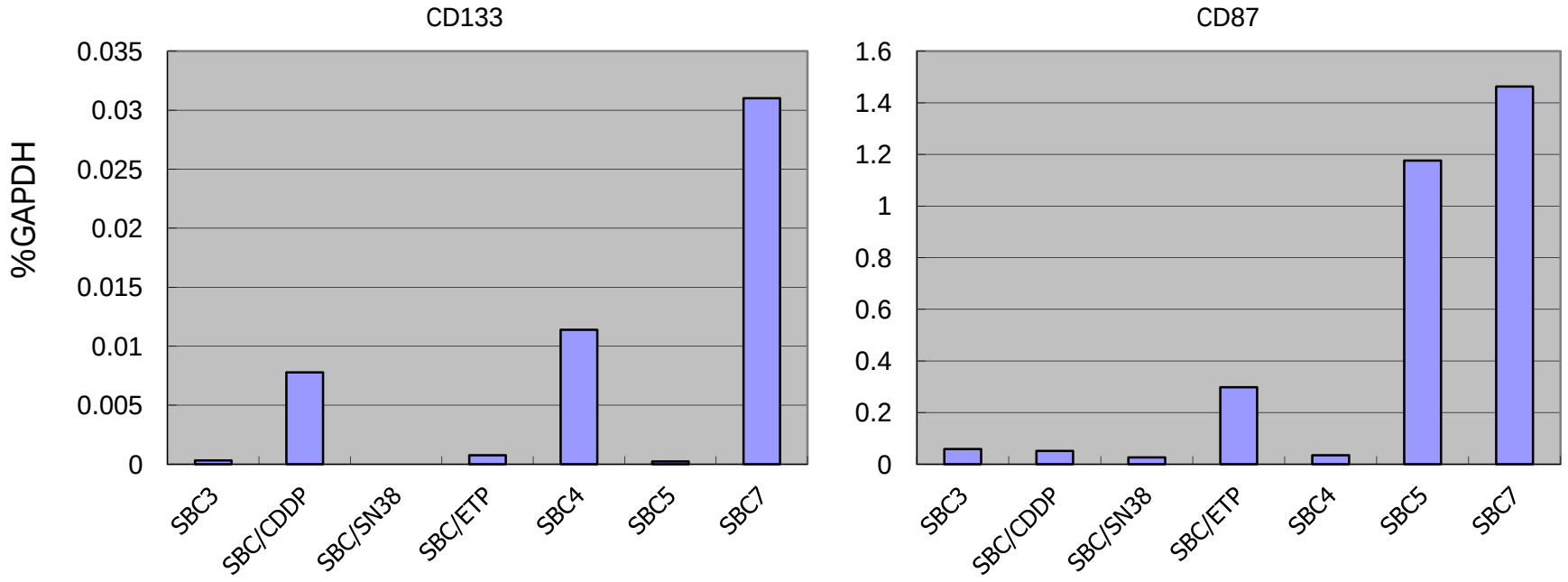
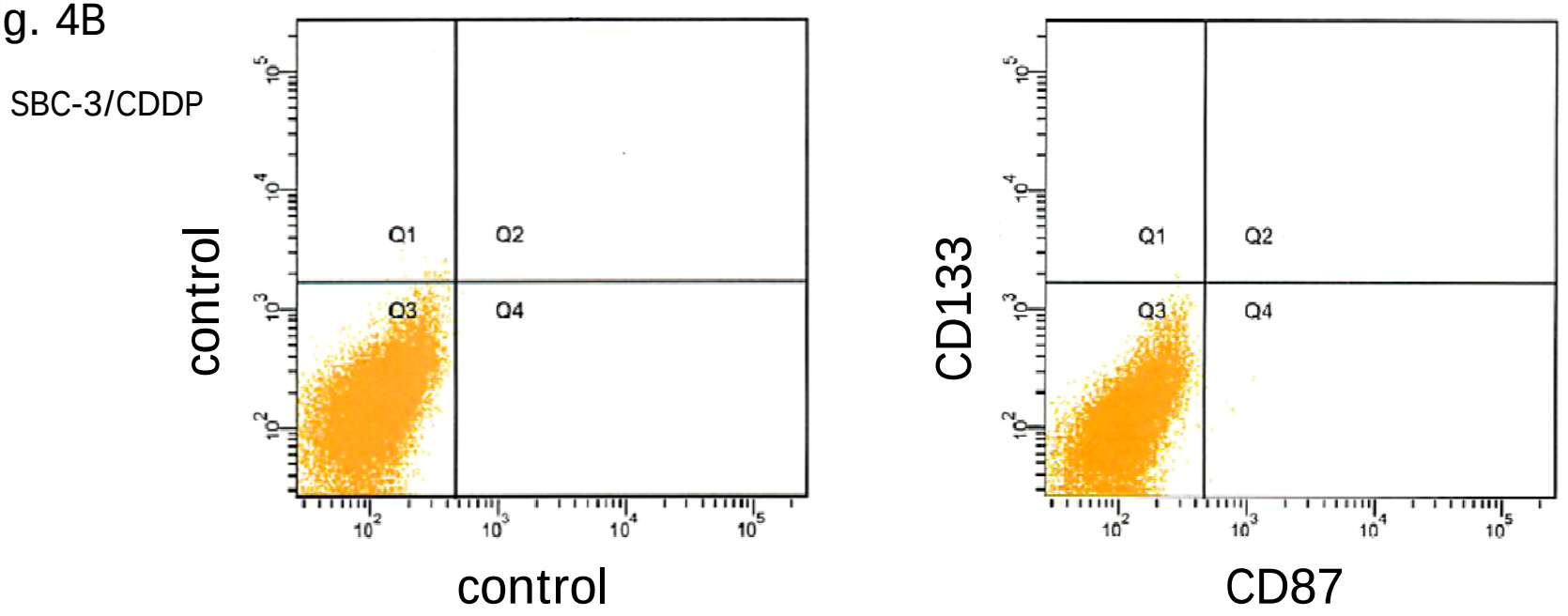
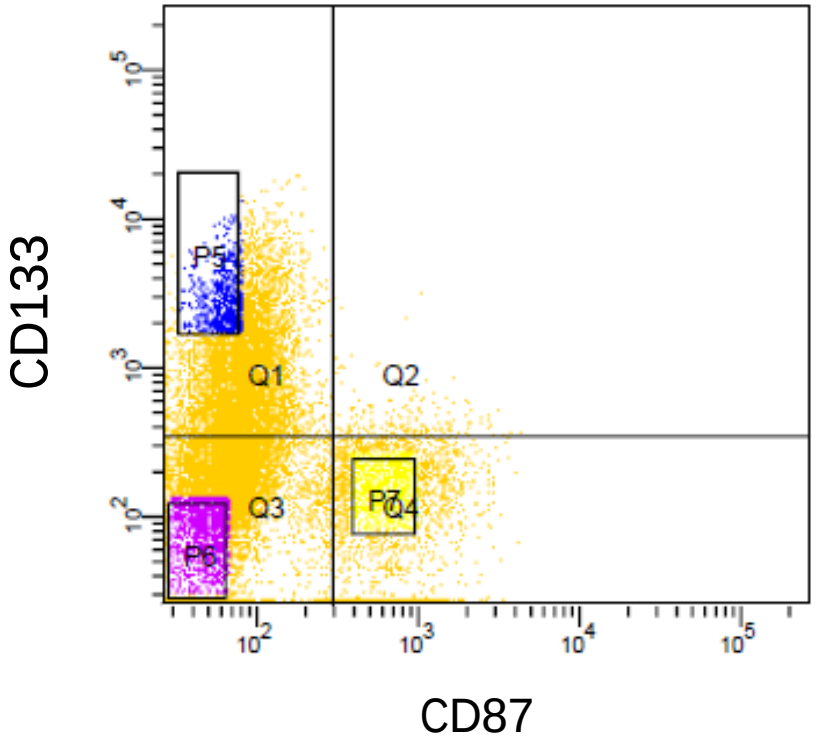

CD133
%GAPDH
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
SBC3
SBC/CDDP
SBC/SN38
SBC/ETP
SBC4
SBC5
SBC7
CD87
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SBC3
SBC/CDDP
SBC/SN38
SBC/ETP
SBC4
SBC5
SBC7

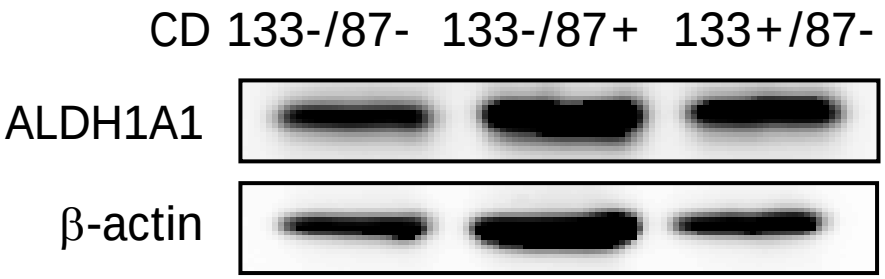
Fig. 4B


SBC-3/CDDP
control
Q1
Q2
Q3
Q4
control
CD133
Q1
Q2
Q3
Q4
CD87

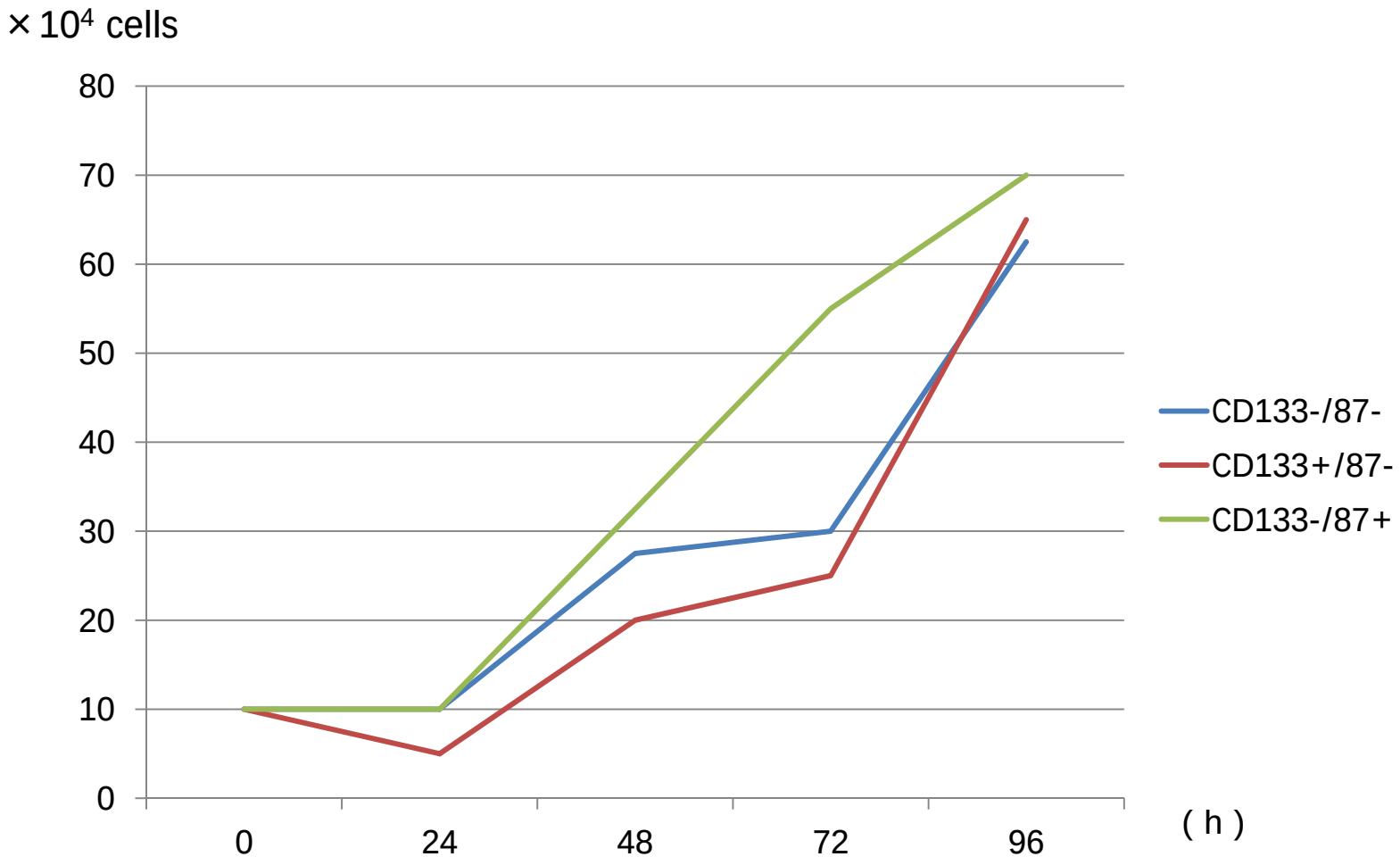
Supplementary Fig. S1



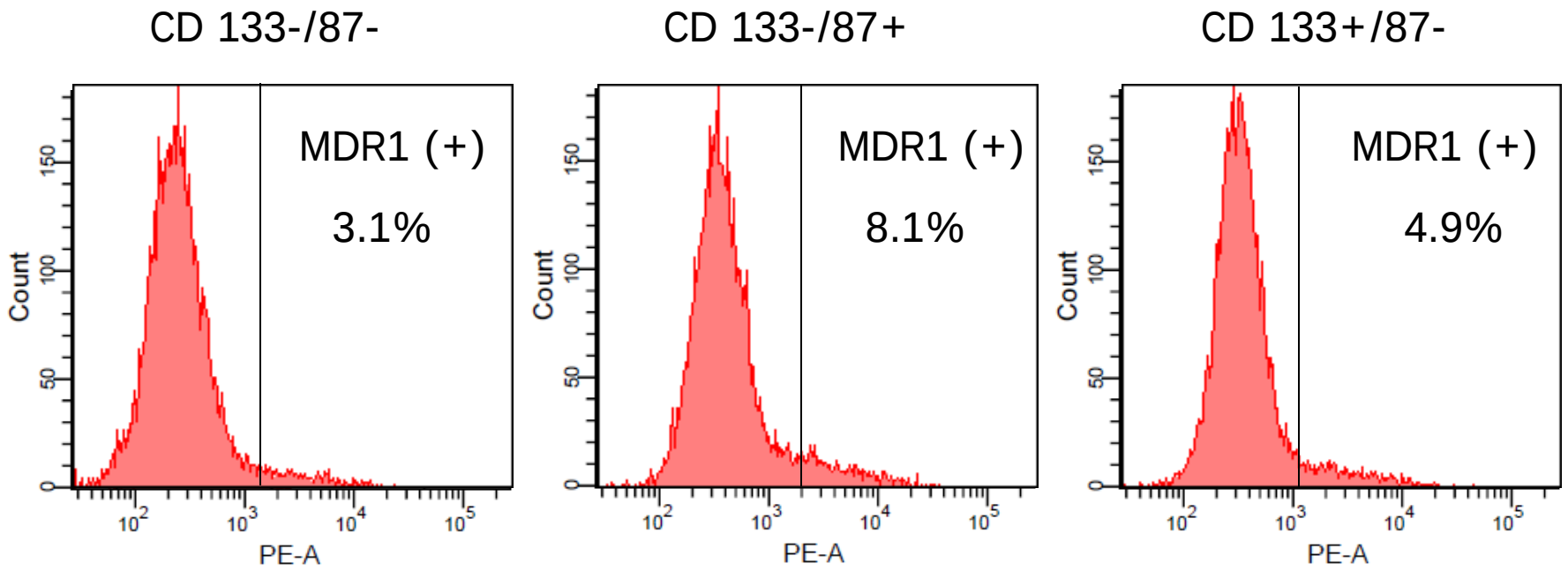
Supplementary Fig. S2



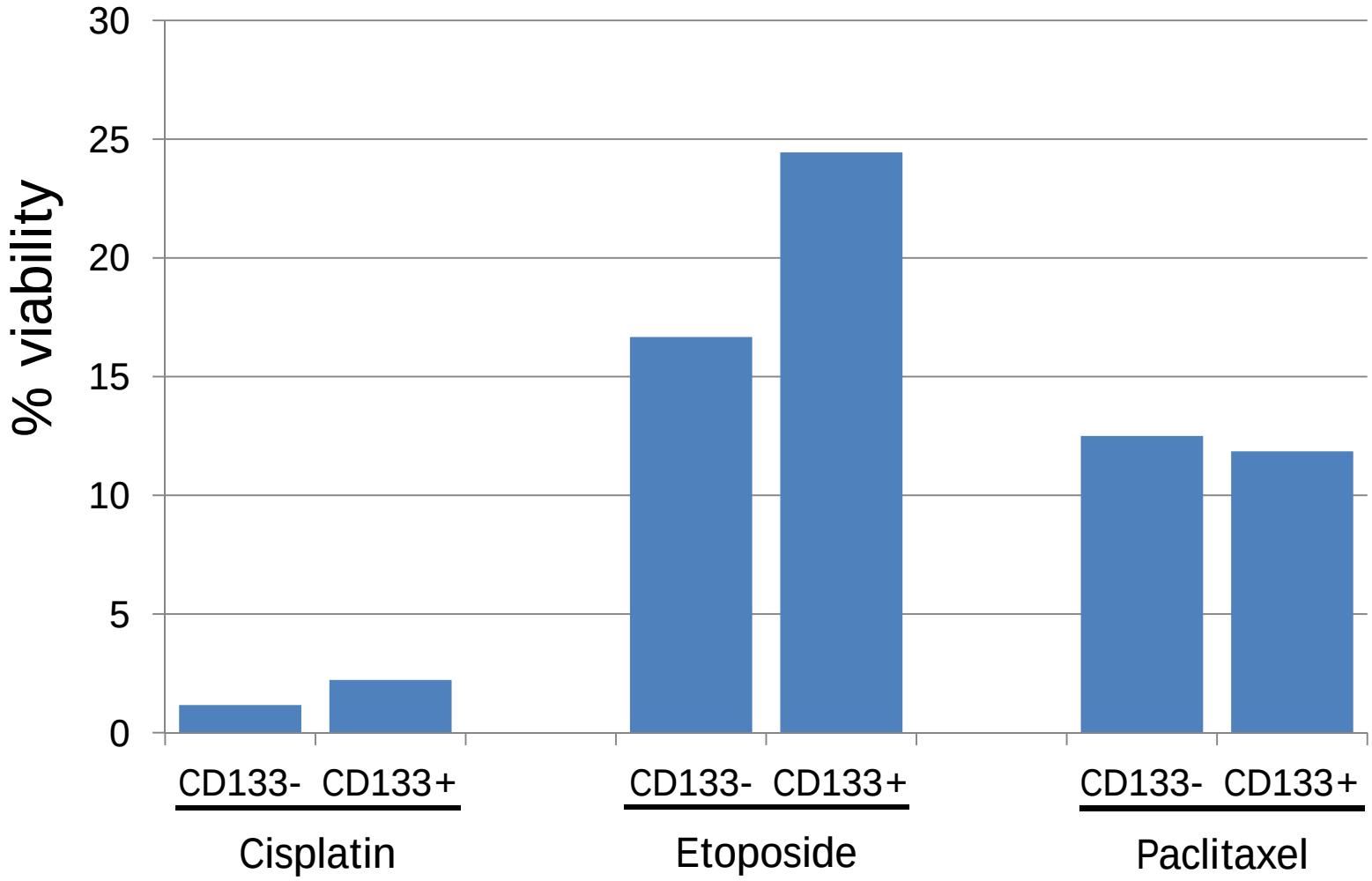
Supplementary Fig. S3



Supplementary Fig. S4



Supplementary Fig. S5



Supplementary Fig. S6