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Simultaneous Color-Coded Imaging to Distinguish Cancer "Stem-Like" and Non-Stem Cells in the Same Tumor

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ABSTRACT

In this study, we demonstrate that the differential behavior, including malignancy and chemosensitivity, of cancer stem-like and non-stem cells can be simultaneously distinguished in the same tumor in real time by color-coded imaging. $CD133^+$ Huh-7 human hepatocellular carcinoma (HCC) cells were considered as cancer stem-like cells (CSCs), and $CD133^-$ Huh-7 cells were considered as non-stem cancer cells (NSCCs). $CD133^+$ cells were isolated by magnetic bead sorting after Huh-7 cells were genetically labeled with green fluorescent protein (GFP) or red fluorescent protein (RFP). In this scheme, $CD133^+$ cells were labeled with GFP and $CD133^-$ cells were labeled with RFP. CSCs had higher proliferative potential compared to NSCCs in vitro. The same number of GFP CSCs and the RFP NSCCs were mixed and injected subcutaneously or in the spleen of nude mice. CSCs were highly tumorigenic and metastatic as well as highly resistant to chemotherapy in vivo compared to NSCCs. The ability to specifically distinguish stem-like cancer cells in vivo in real time provides a visual target for prevention of metastasis and drug resistance. J. Cell. Biochem. 111: 1035–1041, 2010.

KEY WORDS: CANCER STEM CELLS; CD133; HEPATOCELLULAR CARCINOMA; NUDE MICE; CHEMOTHERAPY; GFP; RFP; COLOR-CODED IMAGING

The cancer stem cell hypothesis of solid tumors, has thus far not been fully substantiated since stem and non-stem cells have not been simultaneously visualized and compared in real time in tumors of live animals.

There are two general models of heterogeneity of cells in solid cancers. One model is that among the heterogeneous cells in a tumor, most have only limited malignant potential, but a subset can be highly malignant and tumor initiating. This model therefore predicts that a distinct subset of cells has greater ability to form new tumors and metastasis compared to other cancer cell types in the tumor. This type of cell is termed a cancer stem cell (CSC) and the other types of cancer cells in the tumor are non-stem cancer cells (NSCC). The alternative model is that any given cell in a tumor has an equal probability and ability to form a tumor and metastasis [Bonnet and Dick, 1997; Reya et al., 2001; Al-Hajj et al., 2003; Abraham et al., 2005; Dean et al., 2005; Bao et al., 2006; Jordan et al., 2006].

Expression of the surface protein CD133 (also known as AC133 and prominin-1) is a marker that may identify CSCs in solid tumors including brain [Reya et al., 2001; Bjerkvig et al., 2005]; lung [Donnenberg et al., 2007]; melanoma [Monzani et al., 2007]; prostate [Miki et al., 2007]; kidney [Bussolati et al., 2005]; and colon [O'Brien et al., 2007; Ricci-Vitiani et al., 2007]. CD133 is also expressed in developing epithelia and differentiated cells [Jaszai et al., 2007; Mizrak et al., 2008]. CD133 was originally identified as a marker for stem and progenitor cells of the hematopoietic system [Yin et al., 1997; Toren et al., 2005]. We and others previously reported that the CD133-expressing cells play a critical role in the self-renewal of a range of hepatic stem cells [Suetsugu et al., 2006; Ma et al., 2007; Yin et al., 2007].

Our laboratory has pioneered the use of fluorescent proteins to visualize in vivo phenomena, including primary tumor growth, tumor cell motility and invasion, metastatic seeding and colonization, angiogenesis, and the interaction between the tumor and its microenvironment in live animals in real time [Chishima et al., 1997; Yang et al., 2000; Hoffman, 2005; Hoffman and Yang, 2006a,b,c]. Fluorescent proteins of different colors have now been characterized, and these can be used to color-code cancer cells of a

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specific genotype or phenotype. For example, the interaction of highly metastatic cancer cells labeled with green fluorescent protein (GFP) and low metastatic cancer cells labeled with red fluorescent protein (RFP) can be directly compared in vivo in the same tumor [Tome et al., 2009]. Real-time imaging with fluorescent proteins is especially important when evaluating the efficacy of therapeutics on metastasis and tumor recurrence [Hoffman, 2005; Hoffman and Yang, 2006a,b,c]. A set of multicolor fluorescent proteins can be used simultaneously for multifunctional in vivo imaging. These properties make fluorescent proteins optimal for cellular imaging in vivo [Hoffman, 2005].

In the present study, we utilized real-time, color-coded imaging with fluorescent proteins for real-time imaging of the comparative behavior of CSCs and NSCCs in the same tumor including malignancy and drug sensitivity.

MATERIALS AND METHODS

CELL LINE AND CULTURE CONDITION

Huh-7, a human hepatocellular carcinoma (HCC) cell line, was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin and streptomycin. The cells were cultured at 37°C in a 5% CO_2 incubator.

GENE TRANSDUCTION OF FLUORESCENT PROTEINS TO HUH-7 CANCER CELLS

Huh-7 cells were labeled with RFP or GFP as previously reported [Bussolati et al., 2005] using retroviruses expressing GFP or RFP. Huh-7 cells were transfected with retroviruses. Clones expressing RFP or GFP were established by subculture in selective medium containing G418.

ISOLATION OF CSC FROM HUH-7 CELLS

The expression of CD133 was determined by standard flow cytometry analysis using a phycoerythrin (PE)-labeled antibody against human CD133/2 (Miltenyi Biotech, Bergisch Gladbach, Germany) with an FACS-vantage flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Approximately 49% of Huh-7 cells expressed CD133. In this study,

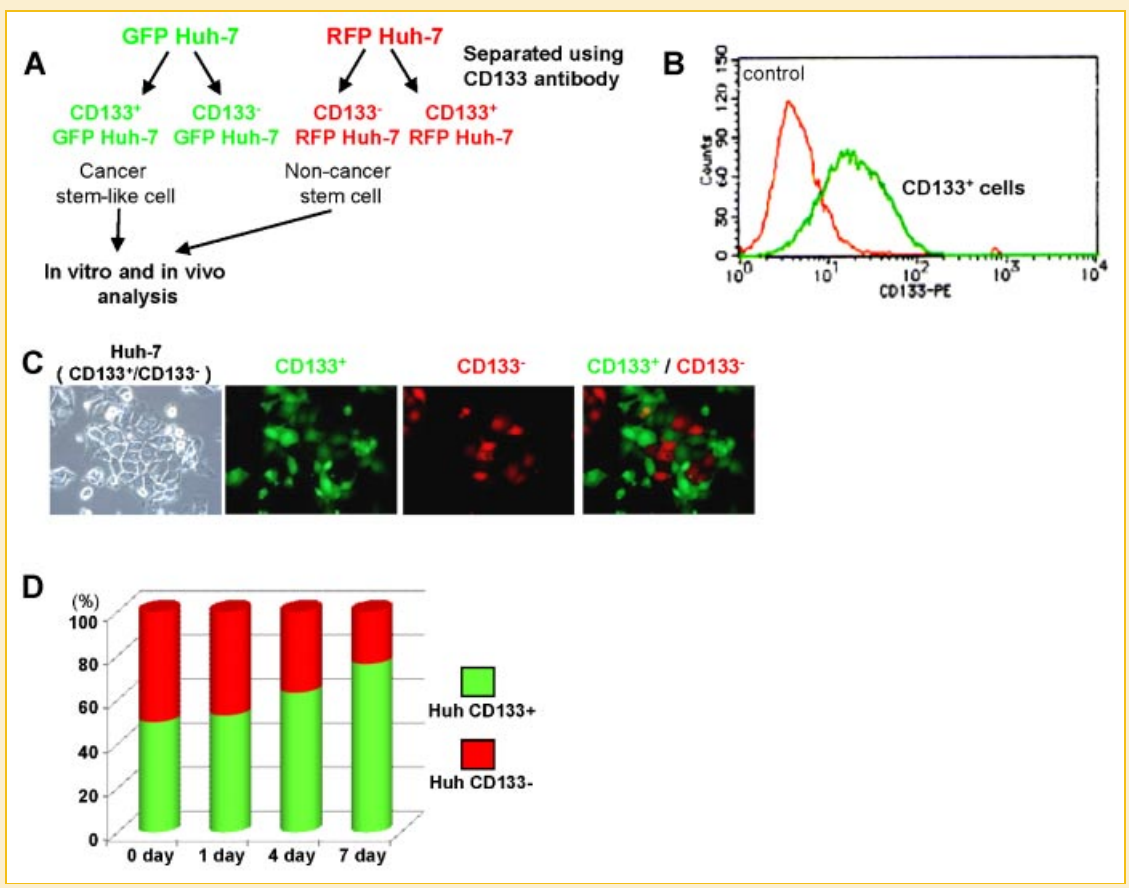

Fig. 1. A: Schematic representation of experimental protocol. CD133$^+$ cells and CD133$^-$ cells were isolated, respectively, from the established GFP and RFP Huh-7 cells by magnetic bead sorting. GFP-expressing CD133$^+$ Huh-7 cells were used as CSCs (GFP-CSCs), and RFP-expressing CD133$^-$ Huh-7 cells were used as NSCCs (RFP-NSCCs). B: CD133 expression in Huh-7 cells was examined by FACS analysis. The results shown are representative of at least three independent experiments. C: The same number of the GFP-CSCs and RFP-NSCCs were mixed and co-cultured. GFP-expressing and RFP-expressing cells were observed at 24 h after plating. D: The total intensities of GFP and RFP were compared in the wells after plating at the indicated time points. The results are representative of at least three independent experiments.

$CD133^+$ Huh-7 cells were considered as CSCs and $CD133^-$ Huh-7 cells were as NSCCs. $CD133^+$ Huh-7 cells were isolated by magnetic bead sorting using the MidiMACS system (Miltenyi Biotech) with monoclonal CD133 antibody (Miltenyi Biotech) according to the manufacturer's instructions. A schematic representation of the experimental protocol is shown in Figure 1A. The same number of the CSCs labeled with GFP and the NSCCs expressing RFP were mixed and co-cultured in dishes or co-implanted in mice.

IN VIVO IMAGING

We used color-coded in vivo imaging [Hoffman, 2005; Hoffman and Yang, 2006a,b,c] to visualize CSCs or NSCCs in vivo, using the Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan).

The Olympus OV100 which contains an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used for cellular imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging as well as microimaging with high light-gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 10^5-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has the lenses mounted on an automated turret with a high magnification range of 1.6× to 16× and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell^R (Olympus Biosystems) [Yamauchi et al., 2006].

The viable cell area was measured using National Institutes of Health (NIH) Image J analysis software program (available at http://rsb.info.nih.gov/nih-image/).

NUDE MOUSE CANCER MODELS

Nude mice were anesthetized with a ketamine mixture (10 μL ketamine HCl, 7.6 μL xylazine, 2.4 μL acepromazine maleate, and 10 μL H_2O) injected into the peritoneal cavity. To simultaneously compare CSCs and NSCCs in the same tumor, the same number of liver GFP-CSCs and RFP-NSCCs were mixed, and injected into the spleen (1×10^6 cells/50 μL matrigel) during open laparotomy or injected subcutaneously (1×10^6 cells) in non-transgenic nude mice.

All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals under assurance A3873-1. Animals were kept in a barrier facility under HEPA filtration.

CHEMOTHERAPY

Nude mice were subcutaneously implanted with GFP-CSCs and RFP-NSCCs as described above. The mice were treated in the following groups: (1) saline (vehicle/control) (intraperitoneally), (2) CDDP (10 mg/kg, intraperitoneally) (Cisplatin, Nippon Kayaku CO., Japan), (3) bevacizumab (BEV) (5 mg/kg, intraperitoneally) (Avastin, Roche, South San Francisco, CA), and (4) combined treatment with CDDP and BEV (CDDP/BEV). Control, CDDP and BEV injections were performed on a weekly basis from day 7 after tumor implantation. By this time, the HCC tumors reached approximately 20 mm. Each treatment arm involved 10 HCC-bearing mice. No significant effects on body weight, morbidity, or severe toxicities were observed in any treatment arm. Tumor growth was monitored weekly by measuring the long tumor axis and body weights were recorded. Animals were sacrificed at 6 weeks, and tumors were harvested for analysis. The

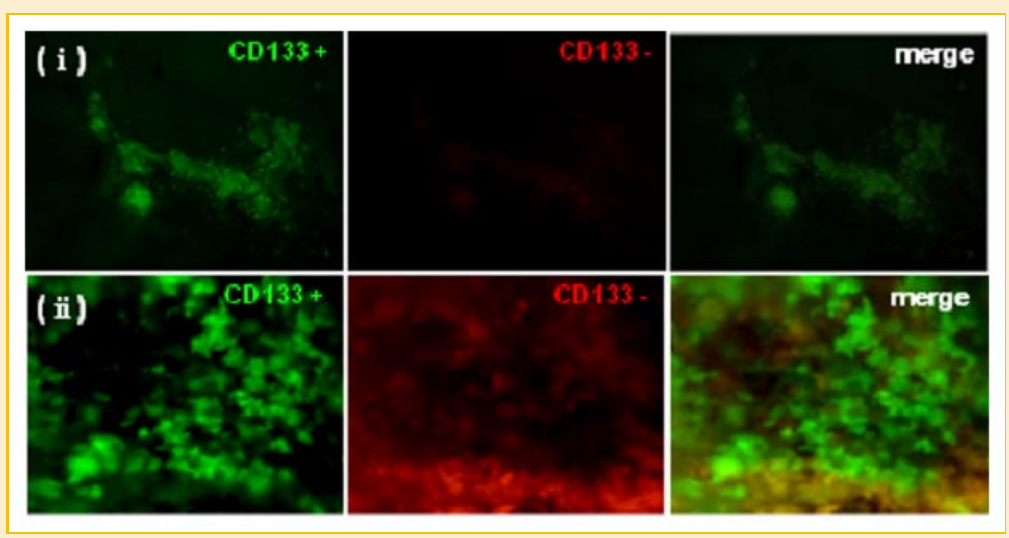

Fig. 2. A mixture of GFP-CSCs and RFP-NSCCs (1×10^6/50 μl) cells was injected into the spleen of nude mice during open laparotomy. On day 28 after cell injection, GFP and RFP fluorescence was observed in tumor colonies in the liver. (i) A diffuse metastasis and (ii) a nodular metastasis.

GFP and RFP fluorescence in tumors was imaged as described above. For some experiments, chemotherapy-resistant cancer cells were isolated by collagenase, and the isolated cells were cultured.

STATISTICAL ANALYSIS

All data represent the mean of at least three independent experiments ± SD. For the determination of statistical significance, the unpaired Student's *t*-tests, was used. *P*-values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

COMPARATIVE BEHAVIOR OF CO-CULTURED COLOR-CODED CD133$^+$ AND CD133$^-$ HUH-7 HCC CELLS IN VITRO

Co-cultures of CD133$^+$ (CSC) and CD133$^-$ (NSCC) Huh-7 cells are shown in Figure 1. Approximately 49% of Huh-7 cells expressed CD133 (Fig. 1B). To compare the properties of Huh-7 CSCs and NSCCs, the same number of isolated GFP-CD133$^+$ and RFP-CD133$^-$ cells were co-cultured in dishes, and then GFP and RFP fluorescence was imaged. On days 0 and 1 after plating, both CD133$^+$ and CD133$^-$ Huh-7 cells showed similar morphology (Fig. 1C). From day 3, however, GFP CD133$^+$ cells became elongated and stretched (data not shown). In contrast, RFP CD133$^-$ cells showed minimal morphological change. Moreover, GFP CD133$^+$ cells showed higher proliferative activity than RFP CD133$^-$ cells 7 days after plating (Fig. 1D).

COMPARATIVE BEHAVIOR OF COLOR-CODED CD133$^+$ AND CD133$^-$ HUH-7 HCC CELLS IN THE SAME LIVER METASTASIS

To simultaneously image the properties of Huh-7 CD133$^+$ and CD133$^-$ cells in a tumor, an equal mixture of the two cell types was injected into the spleen of nude mice in order to obtain experimental liver metastasis. On day 1 after cell injection, GFP$^-$ and RFP$^-$

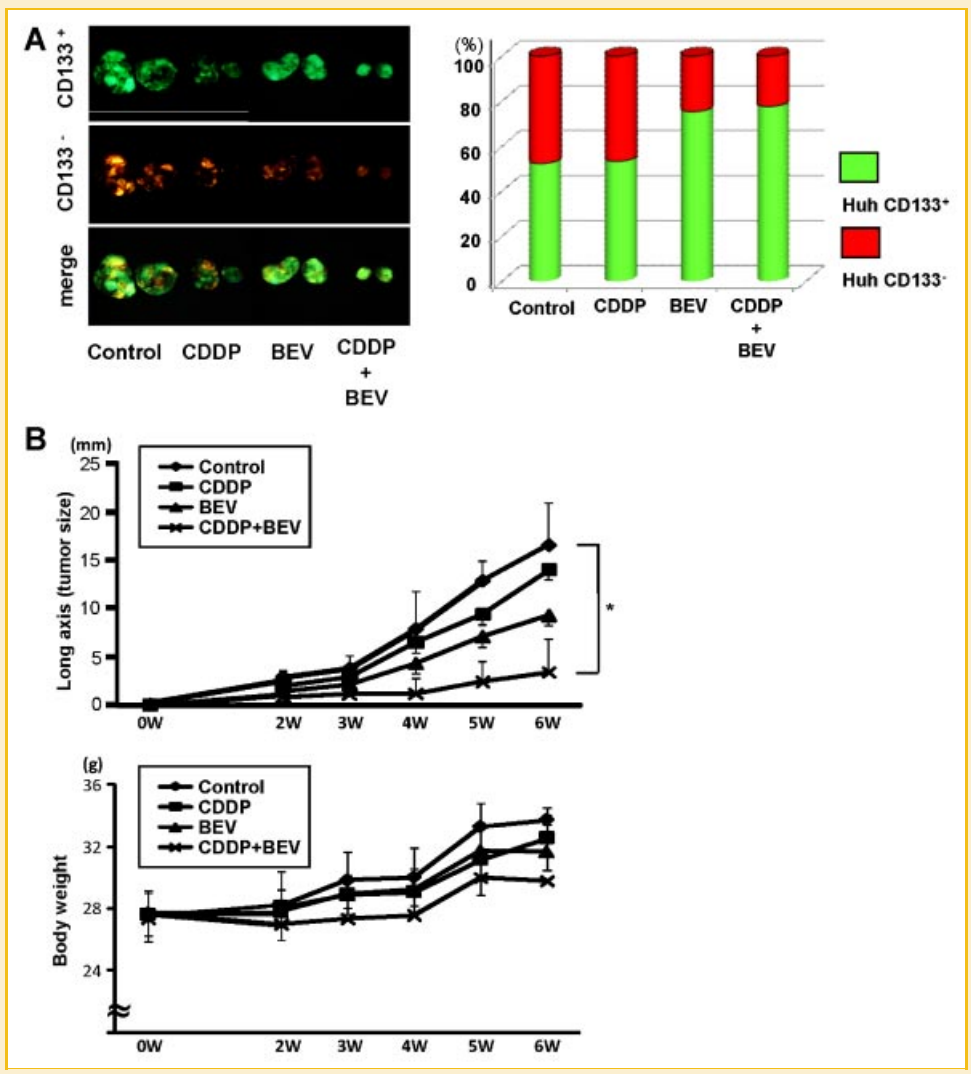

Fig. 3. The same number of isolated GFP-CSCs and RFP-NSCCs (1×10^6) were implanted subcutaneously in nude mice. The mice were treated with CDDP, BEV, or CDDP + BEV for 5 weeks from day 7 after implantation. A: The fluorescent areas of RFP and GFP were compared in the excised tumor. CD133$^+$ GFP-CSCs were more treatment resistant. B: Tumor size (longer axis) and body weight were measured. Data are means ± SD from at least 10 mice. $^*P < 0.01$ using the Student's *t*-test.

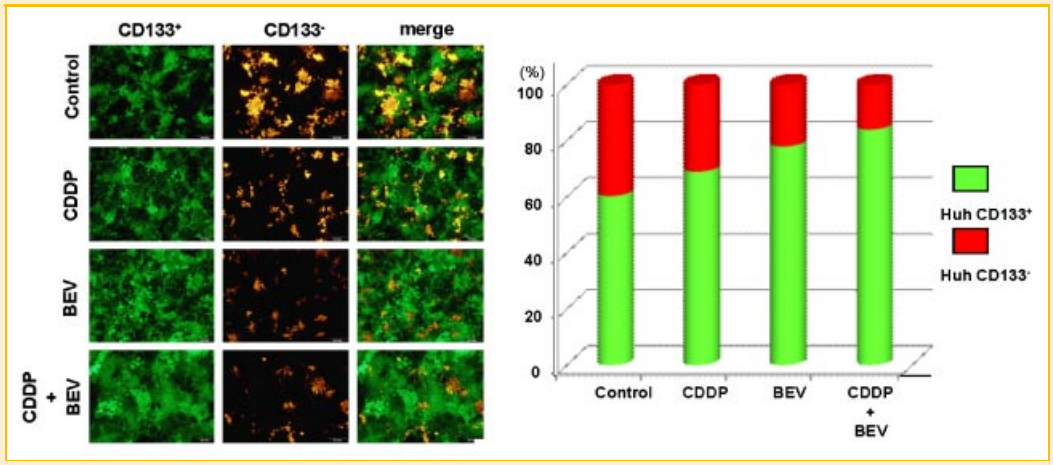

Fig. 4. The same number of isolated GFP-CSCs and RFP-NSCCs (1×10^6) were implanted subcutaneously in nude mice. The mice were treated with CDDP, BEV, or CDDP + BEV for 6 weeks from day 7 after implantation. The tumors were excised and treated with collagenase. The isolated cells (1×10^4) were cultured on plastic 64 well dishes. The total intensities of GFP and RFP fluorescence were compared in the wells 4 days after plating.

expressing cells were observed in the liver indicating the cancer cells began metastasizing to the liver. By day 28 after cell injection, very large multiple tumors were observed in the liver. GFP-expressing $CD133^+$ cells showed diffuse proliferation in the liver (Fig. 2). In contrast, RFP-expressing $CD133^-$ cells formed nodules on the liver (Fig. 2). The majority of the cells in the liver metastases were GFP $CD133^+$ cells, suggesting their greater ability to colonize the liver (Fig. 2). These results indicate that the $CD133^+$ showed enhanced migration and proliferation suggesting they are more malignant than the $CD133^-$ cells.

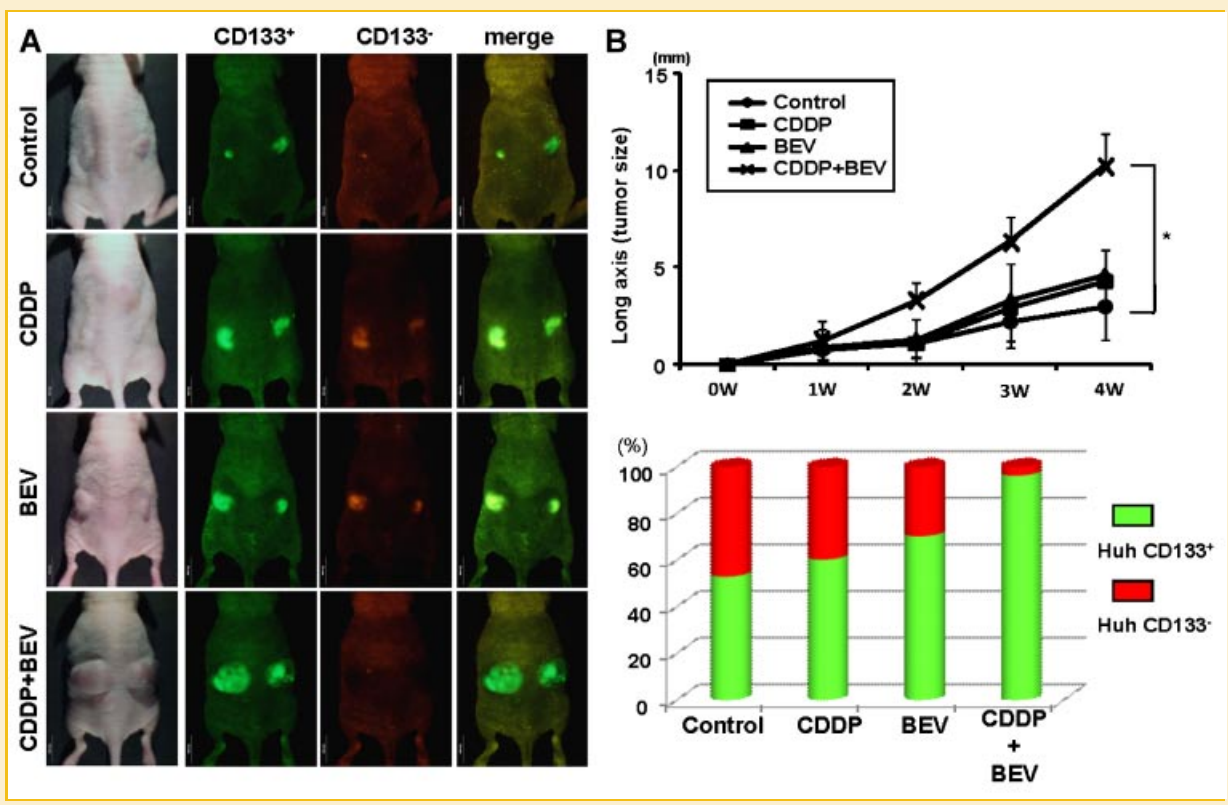

Fig. 5. The same number of the isolated GFP-CSCs and RFP-NSCCs (1×10^6) were implanted subcutaneously in nude mice. The mice were treated with CDDP, BEV, or CDDP + BEV for 6 weeks from day 7 after implantation. The tumors were excised and treated with collagenase. The isolated cells were cultured in plastic dishes. Four days after plating, the cultured cells (1×10^4) were subcutaneously implanted again into nude mice. A: Whole-body fluorescence images of animals were taken 4 weeks after implantation. The fluorescent areas of RFP and GFP were compared. B: Tumor size (longer axis) was measured at the indicated time points. Data are means ± SD from at least 10 mice. $^*P < 0.01$ using the Student's *t*-test.

COMPARATIVE CHEMOSENSITIVITY OF COLOR-CODED HUH-7 CD133$^+$ AND CD133$^-$ CELLS IN THE SAME TUMOR

To examine differential chemo-sensitivity of CSCs and NSCCs, the same number of isolated GFP CD133$^+$ and RFP-CD133$^-$ cells were implanted subcutaneously in nude mice. The mice were treated with CDDP, BEV, or CDDP + BEV beginning from day 7 after implantation. Chemotherapy reduced tumor growth, especially combination therapy with CDDP + BEV (Fig. 3A,B). The majority of the remaining cells in the tumors of CDDP + BEV treated mice were GFP-expressing CD133$^+$ suggesting that the CSCs were chemo-resistant.

Cells were isolated from the chemotherapy-treated subcutaneous tumors and cultured on plastic dishes. The ratio of GFP-expressing to RFP-expressing cells isolated from the non-treated group was $60.3 \pm 2.5\%$ to $39.6 \pm 2.5\%$ at 4 days after plating. In contrast, the ratio of GFP-expressing to RFP-expressing cells in CDDP + BEV treated group was $84 \pm 4\%$ to $16 \pm 4\%$ at 4 days after plating (Fig. 4) indicating that CD133$^+$ cells were highly resistant to the CDDP + BEV combination. GFP-CD133$^+$ also showed higher proliferative activity than RFP CD133$^-$ cells after isolation from CDDP + BEV-treated tumors. In contrast, the RFP-CD133$^-$ cells started to die and detach from the dish (data not shown) after plating, probably due to effects of the in vivo CDDP + BEV treatment.

To further investigate the properties of the chemotherapy-resistant cells, the same number of control or chemotherapy resistant cells isolated and cultured from subcutaneous tumors were implanted again into nude mice. The CDDP + BEV-resistant cells formed the largest tumors (Fig. 5A). In these tumors, GFP-CD133$^+$ cells were found to be more predominant than RFP-CD133$^-$ cells. The ratio of GFP-CD133$^+$ to RFP-CD133$^-$ in the tumors formed from the cells isolated and cultured from the control group was $59.6 \pm 5.1\%$ to $40.3 \pm 5.1\%$. In contrast, the ratio of GFP-CD133$^+$ to RFP-CDD133$^-$ cells isolated and cultured from the CDDP + BEV-treated groups was $96.33 \pm 1.5\%$ to $3.66 \pm 1.5\%$ (Fig. 5B). These results demonstrate that the viable CDDP + BEV-resistant cells were almost all CD133$^+$.

In the present study, we could differentiate between CSCs and NSCCs in the same tumor using different colored fluorescent proteins to label CSCs and NSCCs and then perform color-coded imaging. We demonstrated striking differences in cell proliferation, metastasis, and drug sensitivity between CD133$^+$ and CD133$^-$ cells. Our results demonstrate that Huh-7 CD133$^+$ have at least some properties of cancer stem cells.

Labeling CSC with GFP and NSCC with RFP allows imaging of CSC and NSCC interaction as well as interaction of each cell type with the host.

The present report now enables cancer stem-like cells to be characterized and distinguished from non-stem cells in the same tumor in real time with regard to the most important features of malignancy. Our results confirm that tumors can contain two very different populations with regard to malignancy and drug sensitivity. However, the malignant cell set (stem-cell like) is not a rare population in Huh-7 tumors. Imageable CSC can be a visual target to prevent metastasis, which would be of great importance for adjuvant chemotherapy. Future studies will focus on selective in vivo labeling of CSC visual targets of chemotherapy [Kishimoto et al., 2009a,b].

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