

# *In vivo* monitoring of CD44<sup>+</sup> cancer stem-like cells by $\gamma$ -irradiation in breast cancer

MI HYUN KIM<sup>1,4</sup>, MIN HWAN KIM<sup>1</sup>, KWANG SEOK KIM<sup>2</sup>, MYUNG-JIN PARK<sup>2</sup>,  
JAE-HOON JEONG<sup>3</sup>, SEUNG WOO PARK<sup>3</sup>, YOUNG HOON JI<sup>3</sup>, KWANG IL KIM<sup>1</sup>,  
TAE SUP LEE<sup>1</sup>, PHIL YOUL RYU<sup>4</sup>, JOO HYUN KANG<sup>1</sup> and YONG JIN LEE<sup>1</sup>

<sup>1</sup>Molecular Imaging Research Center, <sup>2</sup>Division of Radiation Cancer Research/Research Center for Radio-Senescence,

<sup>3</sup>Research Center for Radiotherapy, Korea Institute of Radiological and Medical Sciences, Seoul;

<sup>4</sup>Department of Microbiology, Chonnam National University College of Medicine, Gwangju, Republic of Korea

Received February 15, 2016; Accepted March 22, 2016

DOI: 10.3892/ijo.2016.3493

**Abstract.** There is increasing evidence that cancer contains cancer stem cells (CSCs) that are capable of regenerating a tumor following chemotherapy or radiotherapy. CD44 and CD133 are used to identify CSCs. This study investigated non-invasive *in vivo* monitoring of CD44-positive cancer stem-like cells in breast cancer by  $\gamma$ -irradiation using molecular image by fusing the firefly luciferase (fLuc) gene with the CD44 promoter. We generated a breast cancer cell line stably expressing fLuc gene by use of recombinant lentiviral vector controlled by CD44 promoter (MCF7-CL). Irradiated MCF7-CL spheres showed upregulated expression of CD44 and CD133, by immunofluorescence and flow cytometry. Also, gene expression levels of CSCs markers in irradiated spheres were clearly increased. CD44<sup>+</sup> CSCs increased fLuc expression and tumor growth *in vivo* and *in vitro*. When MCF7-CL was treated with siCD44 and irradiated, CD44 expression was inhibited and cell survival ratio was decreased. MCF7-CL subsets were injected into the mice and irradiated by using a cobalt-60 source. Then, *in vivo* monitoring was performed to observe the bioluminescence imaging (BLI). When breast cancer was irradiated, relative BLI signal was increased, but tumor volume was decreased compared to non-irradiated tumor. These results indicate that increased CD44 expression, caused by general feature of CSCs by irradiation and sphere formation, can be monitored by using bioluminescence imaging. This system could be useful to evaluate CD44-expressed CSCs in breast cancer by BLI *in vivo* as well as *in vitro* for radiotherapy.

## Introduction

Breast cancer is the most common cause of death in females among all malignant tumors. Currently, surgical treatment is mainly directed at primary treatment, chemotherapy, and radiotherapy, whereas targeted treatment aims to eliminate the residual tumor cells and thus mitigate the risk of recurrence and metastasis. Some patients, however, still relapse or metastasize after chemotherapy, radiotherapy, and targeted therapy (1).

Cancer cell propagation in tumor can ultimately acquire a capacity for self-renewal and can have multi-lineage potency for cancer organization. A very small population in cancer has these characteristics (2-4). The existence of breast cancer stem cells (BCSCs) in malignant breast tumors has been demonstrated in many previous studies (5,6). BCSCs exhibit a range of phenotypes, including CD44<sup>+</sup>/CD24<sup>-/low</sup> in breast cancer cells (7). The radio-resistance of breast cancer cells have more increased spheres than in monolayer, and the spheres have demonstrated enrichment of CD44<sup>+</sup>/CD24<sup>-</sup> cells (8,9). The radio-resistance of CSCs has also been demonstrated in mouse mammary progenitor cells, with an increase of progenitor cells with the characteristic stem cell surface markers following radiation of primary BALB/c mouse mammary epithelial cells (7,10). Discovery of BCSCs has thus opened up several potential approaches for breast cancer treatment and diagnosis. Thus, it is becoming increasingly important to identify, track, and target BCSCs *in vivo*. CSCs have been identified based on the expression, their lack of surface markers such as CD44, CD24, CD29, Lin, CD133, and Sca-1 (11).

CD44, ubiquitous multi-structural and multifunctional adhesion molecule, is a cell surface transmembrane glycoprotein in cell-cell and cell-matrix interactions (5). Previous studies on CD44's role in breast tumor invasion demonstrated that CD44-mediated adhesion and signaling are required for cell growth, and the dissemination of breast tumors and CD44s can promote breast tumor cell invasion *in vitro* and *in vivo* (12-16).

Bioluminescence imaging (BLI) is a commonly-used method to measure transgene expression *in vivo* (17-19). BLI application to high throughput screening and imaging of cell

---

*Correspondence to:* Dr Yong Jin Lee, Molecular Imaging Research Center, Korea Institute of Radiological and Medical Sciences (KIRAMS), 75 Nowon-gil, Gongneung-Dong, Nowon-Gu, Seoul 139-706, Republic of Korea  
E-mail: yjlee@kirams.re.kr

**Key words:** CD44, cancer stem cells, irradiation, breast cancer, molecular imaging

functions in intact animals makes the technique particularly versatile and attractive (20). *In vivo* imaging for CSCs, by using molecular imaging methods such as BLI, will provide information on CSCs populations, tracking, or characteristics in cancer. Evaluation of CSCs in cancer therapy will give significant help to overcome the recurrence or metastasis of the cancer, or failure of cancer treatment.

In this study, we investigated non-invasive *in vivo* monitoring of CD44-positive cancer stem-like cells in breast cancer model by  $\gamma$ -irradiation using molecular imaging by fusing the firefly luciferase (fLuc) gene with the CD44 promoter.

## Materials and methods

Animal care and the experimental procedures in this study were approved by the Animal Care and Ethics Committee at Korea Institute of Radiological and Medical Sciences (Seoul, Korea).

**Cell culture.** Human breast cancer cell lines, MCF7 and MCF7-CL were maintained in RPMI-1640 (WelGENE Inc., Korea) containing 10% fetal bovine serum (FBS) and 1% antibiotics in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

**Lentivirus reproduction.** Recombinant lentivirus (pWPXL-CD44p-luc) vectors were produced with pWPXL and CD44 promoter pGL3 vector (21) (Addgene, USA). The pWPXL lentiviral vector was deleted of GFP site by digesting with *Sall* and *SpeI*, and was inserted CD44p-luciferase site from CD44p pGL3 vector by digesting with *Sall*, *XbaI*. The recombinant vector was packaged using packaging plasmids, pMD2.G and psPAX2, by a Calcium Phosphate Transfection Kit (Invitrogen Co., USA) according to the manufacturer's protocol. Briefly, cultured cells for transfection were prepared in 100-mm dishes, then transfection mixture (2 M CaCl<sub>2</sub>, 20  $\mu$ g DNA, HEPES-buffered saline and sterile water) was added to the media and incubated overnight. After changing media in the cells, the supernatant was harvested in 48-72 h. Breast cancer cells were infected with recombinant lentivirus using 8  $\mu$ g/ml polybrene (Sigma-Aldrich Co., USA). Stable transfectants were selected by single-colony pick-up and bioluminescence assay, according to manufacturer's protocol (Bright-Glo Luciferase assay system; Promega, USA). Briefly, a volume of reagent equal to that of the culture medium was added to the cells in each well, and it was mixed and measured in a luminescence microplate reader (SpectraMax L, Molecular Devices, USA).

**Cell irradiation assay.** MCF7-CL was exposed to  $\gamma$ -rays from a Caesium (Cs)-137  $\gamma$ -ray source (BioBeam 8000; STS, Germany) at 0-10 Gy irradiation at a dose rate of 2.67 Gy/min for the time required to apply a prescribed dose. Irradiated cells were incubated for 4 or 7 days. We performed bioluminescence assay and flow cytometry analysis (BD Biosciences, flow cytometry, USA).

The transfection of interfering RNA (siRNA) against CD44 (Dharmacon, GE Healthcare, Co., USA) was carried out using Lipofectamine 2000 transfection reagent (Invitrogen Co., USA) according to the manufacturer's protocol (22). The cells were irradiated using a Cs-137  $\gamma$ -ray source (Gammacell 3000

ELAN, Atomic Energy of Canada, Ltd., Canada) at a dose rate of 3.81 Gy/min for the time required to apply a prescribed dose. Each subset was collected and subjected to clonogenic assay by following standard protocol (23).

**Primary sphere formation assay.** Spheres were grown in sphere media (SFM). The SFM condition was serum-free DMEM/F12 (WelGENE Inc.) supplemented with 10 ng/ml fibroblast growth factor (bFGF, R&D Systems, USA), 10 ng/ml epidermal growth factor (EGF, R&D Systems), B27 (Invitrogen) and 2.75 ng/ml selenium (insulin-transferrin-selenium solution; ITS, Invitrogen). SFM were added every 2-3 days, the media were supplemented with fresh growth factors, and the cells were allowed to form spheres for 7-14 days. Morphological changes were also observed by microscopy (Olympus DP71 digital microscopic camera, Olympus, Japan).

**Isolation of cancer stem-like subsets.** Magnetic-activated cell sorting system (MACS cell separator, Miltenyi Biotec Inc., USA) was carried out using CD44 or CD24-microbead (Miltenyi Biotec GmbH, Germany) according to the manufacturer's protocol. We prepared PBS, 0.5% BSA, and 2 mM EDTA by diluting MACS BSA stock solution with washing buffer. For MACS, the single cells were resuspended with 80  $\mu$ l of 0.5% BSA and 2 mM EDTA buffer/10<sup>7</sup> cells, and 20  $\mu$ l of microbeads/10<sup>7</sup> cells was added for 15 min at 4°C. After washing with buffer, the cells were resuspended with  $\leq 10^8$  cells/500  $\mu$ l of buffer, cell suspension were applied onto the column into the magnetic bars and were washed 3 times. Columns were removed from the magnetic bar and were immediately pipetted with the buffer. Then, we obtained CD44-positive cells or CD24-negative cells. Isolated CD44-positive expression subset (cancer stem-like subset; CD44<sup>+</sup>) and CD44 low/negative expression subset (non-cancer stem-like subset; CD44<sup>low</sup>) were irradiated by dose and were cultured.

**Flow cytometry analysis.** The cultured cells were detached from the dish by adding a solution of 0.25% trypsin and 0.02% EDTA (Gibco, USA), and then by washing with PBS. A cell pellet was collected after centrifugation at 1,500 rpm for 3 min and then resuspended in test tubes containing PBS (1x10<sup>6</sup> cells/ml). Fluorescence conjugated antibodies, CD24-PE, CD44-PE, and CD133-FITC (eBioscience, USA), were added to single cells for 30 min at 4°C. All samples were washed with PBS, and cells were fixed in 1% paraformaldehyde. Phenotypic analysis was performed with a flow cytometer (FACSCalibur, BD Biosciences, USA).

**Immunofluorescence staining.** Cells were cultured in 2-well chamber slides and fixed with 4% paraformaldehyde for 1 h at 4°C and acetone for 5 min at room temperature (RT). Fixed cells were washed with PBS and were blocked with 10% normal goat serum and 1% bovine serum albumin (BSA) for 1.5 h in RT. Slides were incubated with mouse anti-human CD44 antibody (monoclonal, Abcam Co., UK), rabbit anti-human CD133 antibody (polyclonal, Biorbyt Co., UK), and rabbit anti-human IgG antibody (monoclonal, Abcam Co., UK) for 1.5 h in RT. After rinsing with PBS, FITC (goat anti-rabbit, polyclonal) or cyanine 3 (Cy3, goat anti-mouse, monoclonal) conjugated secondary antibody (Abcam Co.) was added to

Table I. Primers used in RT-PCR analysis.

| Locus          | Gene       | Primer sequence                                            |
|----------------|------------|------------------------------------------------------------|
| NM_013230.2    | CD24       | 5'-GCTCCTACCCACGCAGATTT-3'<br>5'-GAGACCACGAAGAGACTGGC-3'   |
| NM_000610.3    | CD44       | 5'-CCCCAGCAACCCTACTGATG-3'<br>5'-CCAGTTTTCTTGCCTCTTGG-3'   |
| NM_001145848.1 | CD133      | 5'-AACAGTTTTGCCCCCAGGAAA-3'<br>5'-GGTTTGCACGATGCCACTTT-3'  |
| JN542721.1     | Luciferase | 5'-GGCCTTTATGAGGATCTCTCT-3'<br>5'-CGCCTTGATTGACAAGGATGG-3' |
| NM_008084.2    | GAPDH      | 5'-AGGCCGGTGCTGAGTATGTC-3'<br>5'-TGCCTGCTTCACCACCTTCT-3'   |
| NM_002701.4    | OCT4       | 5'-TGATCCTCGGACCTGGCTAA-3'<br>5'-AACCACACTCGGACCACATC-3'   |
| NM_0024865.2   | Nanog      | 5'-GGATCCAGCTTGTCCCCAAA-3'<br>5'-TGCACCAGGTCTGAGTGTTC-3'   |

slide and incubated for 2.5 h. After rinsing, the slides were mounted with mounting solution (Vectashield Mounting Medium with DAPI, Vector Labs, USA) and observed using an Olympus DP71 digital microscopic camera system (Olympus). All images were processed with Olympus analySIS Five software (Olympus Soft Image Solutions).

**Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.** Total RNA was extracted from the cultures using TRI reagent (Molecular Research Center, USA). Five micrograms of total RNA were reverse transcribed, using superscript III reverse transcriptase (Invitrogen) and random hexamers to generate complementary DNA (cDNA). PCR was performed using a 20- $\mu$ l reaction volume, 3 ng of final concentration of all reagents in the reactions and 30 cycling conditions (GeneAmp PCR System 9700, Applied Biosystems, USA) with primers (Table I). Signals of the PCR products were visualized with a gel documentation system (Uvitec, UK). The fold change in the ratio of each gene mRNA to total GAPDH mRNA was measured in ImageJ software (National Institutes of Health, USA) by boxing each band per representative image with the rectangular selection tool, and calculating the total area of the band in pixels. The total area of each gene mRNA band in pixels was normalized to the total area of the total GAPDH mRNA band in pixels.

**Generation of tumor xenografts in nude mice.** Female BALB/cJ/nu/nu nude mice (6-week-old) were purchased from Nara Biotech (Korea). Mice were treated with 17 $\beta$ -estradiol tablets (Innovative Research of America, USA) before injection with cells for 48 h. MCF7-CL subsets were injected into the fourth mammary fat pad and MACS sorted cells were injected into the thigh of the hind legs. Subcutaneous or orthotopic tumors were generated from implanting 5x10<sup>6</sup> cells. Irradiation into mice was a single dose of 6 Gy using a cobalt-60 source (Cobalt-60

Teletherapy Unit; Theratron T-780, Theratronics, Canada, dose rate 0.6 Gy/min). The mammary fat pad of the anesthetized mice bearing the tumor cells was placed in a 2x30-cm radiation field of a cobalt-60 source, whereas the rest of the body was shielded (19). The tumor volume was measured by a caliper.

**In vivo imaging of CD44 promoter-bioluminescence labeled breast cancer cells.** Bioluminescence imaging was performed with CCD camera mounted in a light-tight specimen chamber (IVIS200, Xenogen, USA). Mice were injected intraperitoneally with a 100- $\mu$ l of 2.5 mg/100  $\mu$ l aqueous solution of D-luciferin potassium salt and anesthetized with 2% isoflurane before the imaging. Imaging acquisition time was from 1 sec to 1 min, depending on the bioluminescence signal. Imaging and quantification of signals were obtained using the acquisition and analysis software Living Image V. 2.50 (Xenogen Corp.). To measure the intensities of the emitted light, the regions of interest (ROI) were drawn over the emitted region of target signal (total p/s/cm<sup>2</sup>/sr).

**Immunohistochemistry.** The xenograft tumors were isolated and frozen. All tissue samples were sectioned (Cryotome, Leica CM 1950, Leica Biosystems Nussloch GmbH) at 5  $\mu$ m thickness and were attached to a slide. Sections had been dried and routinely fixed in 70% ethanol for 5 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min. The slides were washed with Tris-buffered saline (TBS). The 10% FBS was used for 1 h in order to block non-specific binding. Cells were cultured in 2-well chamber slides and fixed with 4% paraformaldehyde for 1 h at 4°C and acetone for 5 min at RT. Fixed cells were washed with PBS and were blocked with 10% normal goat serum and 1% BSA for 1.5 h at RT. Slides were incubated with the primary antibodies, mouse anti-human CD44 antibody, mouse anti-human luciferase antibody (monoclonal, Abcam Co.), and anti-mouse

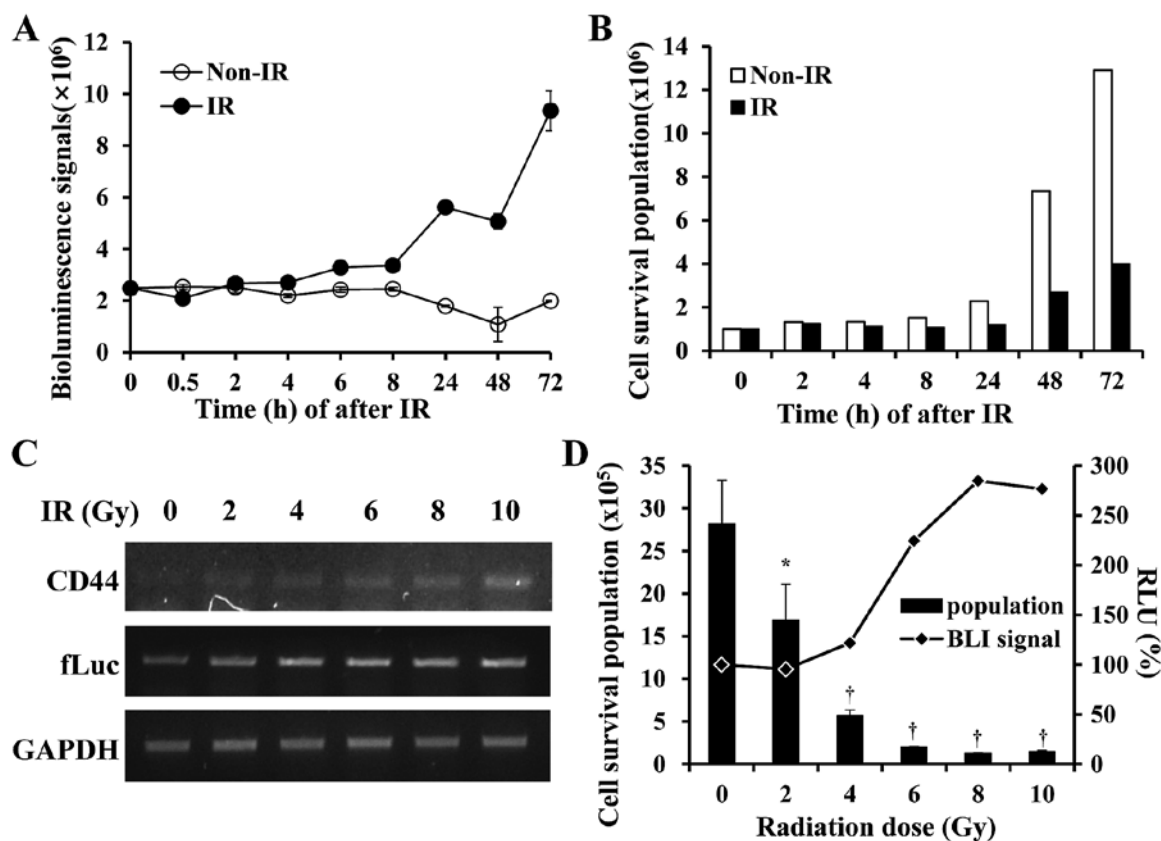


Figure 1. CD44 expression by  $\gamma$ -irradiation (IR). (A) Bioluminescence signals observed time-dependently after irradiation (B) cell survival population in IR and non-IR condition. (C) Confirmation of CD44, fLuc and GAPDH gene expression levels with RT-PCR at day 4. (D) Cell survival population and RLU (relative bioluminescence unit) by dose irradiation. \*Significant difference ( $P < 0.05$ ); †significant difference ( $P < 0.0001$ ).

IgG antibody (monoclonal, Abcam Co.), in 1% BSA overnight at 4°C. After rinsing with TBS, horseradish peroxidase (HRP) conjugated secondary antibody (Dako Denmark, Denmark) was added to slide and incubated for 30 min. The slides were incubated and developed with Liquid DAB (3,3-diaminobenzidine tetrahydrochloride) substrate solution (Liquid DAB+Substrate Chromogen System, Dako North America, Inc. USA) for 2 min. After washing with tap water for 10 min, light Mayer's hematoxylin (Vector Laboratories, Inc., USA) was applied as a counterstain. After washing with tap water for 30 min, the slides were then dehydrated in a series of ethanol and mounted with permount mount media (Gel Mount aqueous mounting medium, Sigma, USA). The cell slides were observed using an Olympus DP71 digital microscopic camera system (Olympus). All images were processed with Olympus analysis Five software (Olympus Soft Image Solutions).

**Statistical analysis.** Data are presented as means-standard errors of the mean. Statistical significance was performed using GraphPad PRISM (GraphPad Software, Inc., USA). Statistical significance was tested by using a Student's t-test, one-way ANOVA and two-way ANOVA.  $P < 0.05$  was considered to indicate a significant difference.

## Results

**Increased CD44 expression by dosed  $\gamma$ -irradiation.** We generated MCF7-CL stably expression of CD44 promoter-luciferase gene

using bioluminescence assay. When cell population increased, BLI signals and bioluminescence signals were increased (data not shown). After irradiation, bioluminescence signals were increased (Fig. 1A) and cell survival populations were decreased time-dependently (Fig. 1B). According to the increment of irradiation dose, cell survival population was decreased, but CD44 and fLuc gene expression was increased (Fig. 1C). The irradiated cells were recorded as 98% at 4 Gy, 31% at 6 Gy, 11% at 8 Gy and 4.5% at 10 Gy of survival populations to compared with non-irradiated cells (Fig. 1D). Whereas, bioluminescence activity of irradiated cells was 122% at 4 Gy, 224% at 6 Gy, 285% at 8 Gy and 276% at 10 Gy with dramatic increase compared to non-irradiated cells. After 6-Gy dosed irradiation, a small number of cells survived and slowly repopulated after irradiation at 4 days (Fig. 2A, left side). In sphere-formed condition (SC), sphere numbers were similar between irradiated and non-irradiated cells, but the sphere sizes of irradiated cells were larger than non-irradiated cells (Fig. 2A, right side).

**Comparison of CD44 and CD24 expression with irradiated subset and CD44 rich subset by sphere formation.** In irradiated condition, the CD24 presented cell population was decreased, but CD44 presented cell population was increased. The sphere formed cells and irradiated sphere formed cells were decreased in CD24 presented cells and increased in CD44 presented cells. Especially, irradiated sphere formed cells were 15% of the CD24 presented population decrease and 12% of the CD44 increased population (Fig. 2B). The

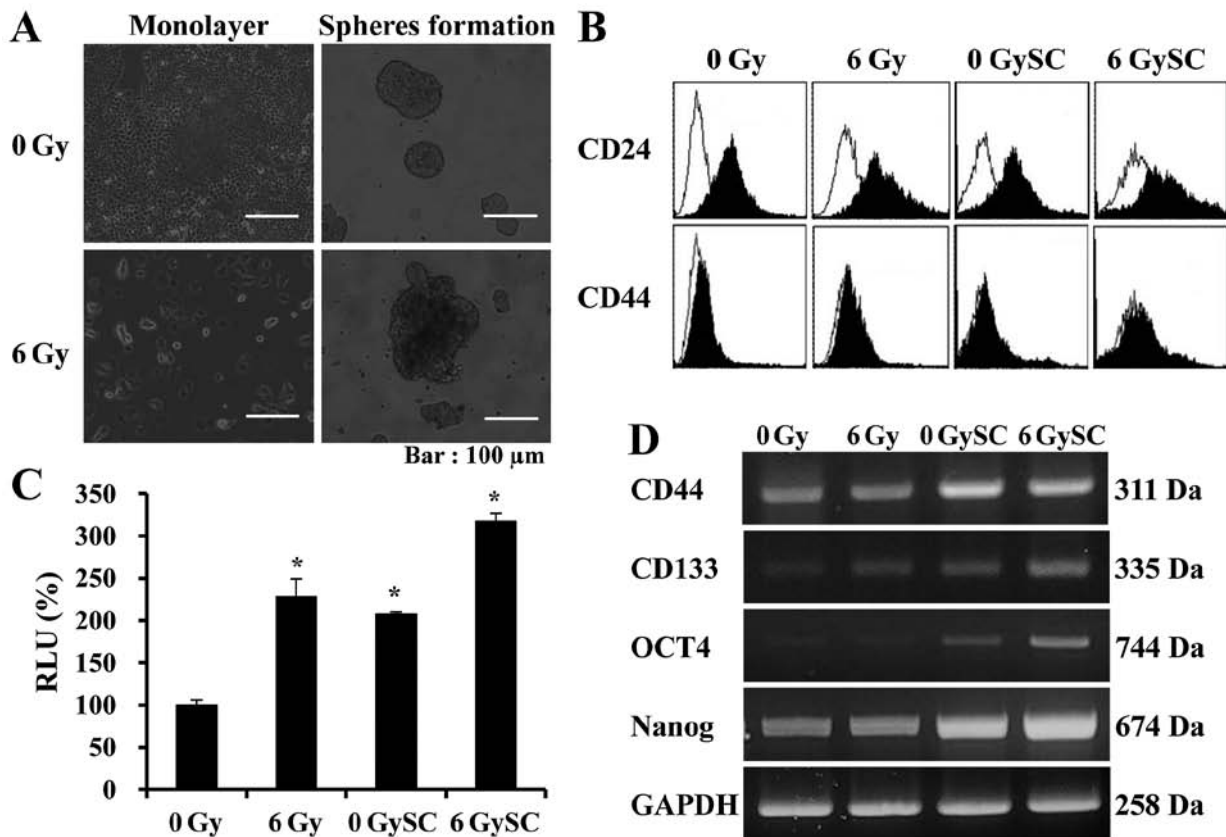


Figure 2. Characteristics of CD44-rich population by IR. (A) Cell morphology. Upper panel, non-IR and Lower panel, IR (bar, 100  $\mu$ m). (B) Flow cytometry analysis. Upper, CD24-FITC, and lower, CD44-FITC. (C) RLU on four types of MCF7-CL. \*Significant difference ( $P < 0.0001$ ). (D) Confirmation of each gene expression in MCF7-CL cells by RT-PCR. Identification of CD44, CD133, OCT4, Nanog and GAPDH gene expression.

bioluminescence activity of treated cells demonstrated an increased pattern (Fig. 2C,  $P < 0.0001$ ), the irradiated spheres were 3.2-fold increased compared to non-treated cells.

*Induced CD44 subset in spheres by irradiation and similarity between these subsets and CSCs.* CD44 expression of MCF-CL cells under irradiation, sphere formation culture or both treated conditions showed higher fluorescence intensity than non-treated cells. In particular, irradiated and sphere formed cells (cancer stem-like cells) had the strongest signals of CD44-Cy3 conjugated antibodies (Fig. 3A and C; 2.4-fold increase,  $P < 0.0027$ ). Also, CD133 expression was similar to CD44 (Fig. 3B and D; 3.2-fold increase,  $P < 0.0001$ ). The CD44 and fLuc gene expression levels were slightly increased, and CD133, OCT4 and Nanog were increased in treated cells in RT-PCR (Fig. 2D).

*In vivo monitoring of increased CD44 expression by irradiation.* Initial BLI signal with MCF-CL cell injected mouse (non-IR) was  $\sim 5 \times 10^5$  p/s/cm<sup>2</sup>/sr. The bioluminescence signals in MCF-CL group ( $2.15 \times 10^6$  p/s/cm<sup>2</sup>/sr) were higher than in non-irradiated group ( $2 \times 10^5$  p/s/cm<sup>2</sup>/sr) immediately after irradiation (day 0). The BLI signals of irradiated group gradually became stronger than non-irradiated group and were shown to be relatively strong for 3 days. After 4 days, BLI signal intensity of irradiated group was higher  $\sim 3$ -fold ( $1.2 \times 10^7$  p/s/cm<sup>2</sup>/sr) than non-irradiated group ( $4.4 \times 10^6$  p/s/cm<sup>2</sup>/sr) (Fig. 4B,  $P < 0.0001$  on Student's t-test).

*Radio-resistance of Stem-like subsets and effect of CD44 depletion to radio-resistance in vitro.* CD44<sup>+</sup> subset showed higher bioluminescence activity ( $\sim 2$ -fold) than CD44<sup>low</sup> subset (Fig. 5B). Two subsets showed different aspects by irradiation in survival population. As the irradiation dose increases, the surviving fraction of CD44<sup>+</sup> or CD44<sup>low</sup> subsets was similarly decreased, but in CD44<sup>low</sup> subset, the width of decrease of surviving fraction was more than CD44<sup>+</sup> subset (Fig. 5A,  $P < 0.0001$ ). After 6 Gy irradiation, CD44<sup>+</sup> subset survival was 20%, and CD44<sup>low</sup> subset was survived 13% (data not shown). The bioluminescence activity of CD44<sup>+</sup> subset was increased by about twice the CD44<sup>low</sup> subset with 6 Gy dose of irradiation (Fig. 5B). For the radio-resistance by CD44 expression, survival fraction of CD44 siRNA treated MCF7-CL was measured after irradiation of each dose. Survival fraction of CD44 siRNA treated MCF7-CL was 3-fold decreased compared to that of non-treated MCF7-CL at 4 Gy dose of irradiation ( $P < 0.0089$ ) (Fig. 5C).

*In vivo monitoring of CD44 expression and tumor growth from stem-like subset, and BLI after irradiation in breast cancer mouse model.* Initially, there was a significant difference between the two groups, and the difference was gradually increased with time elapse. In CD44<sup>+</sup> group, tumor growth started to be recognized by the naked eye after 7 days (Fig. 5D). After 28 days, we observed a total of five tumors in the CD44<sup>+</sup> mice and two tumors in the CD44<sup>low</sup> mice (n=5 transplants

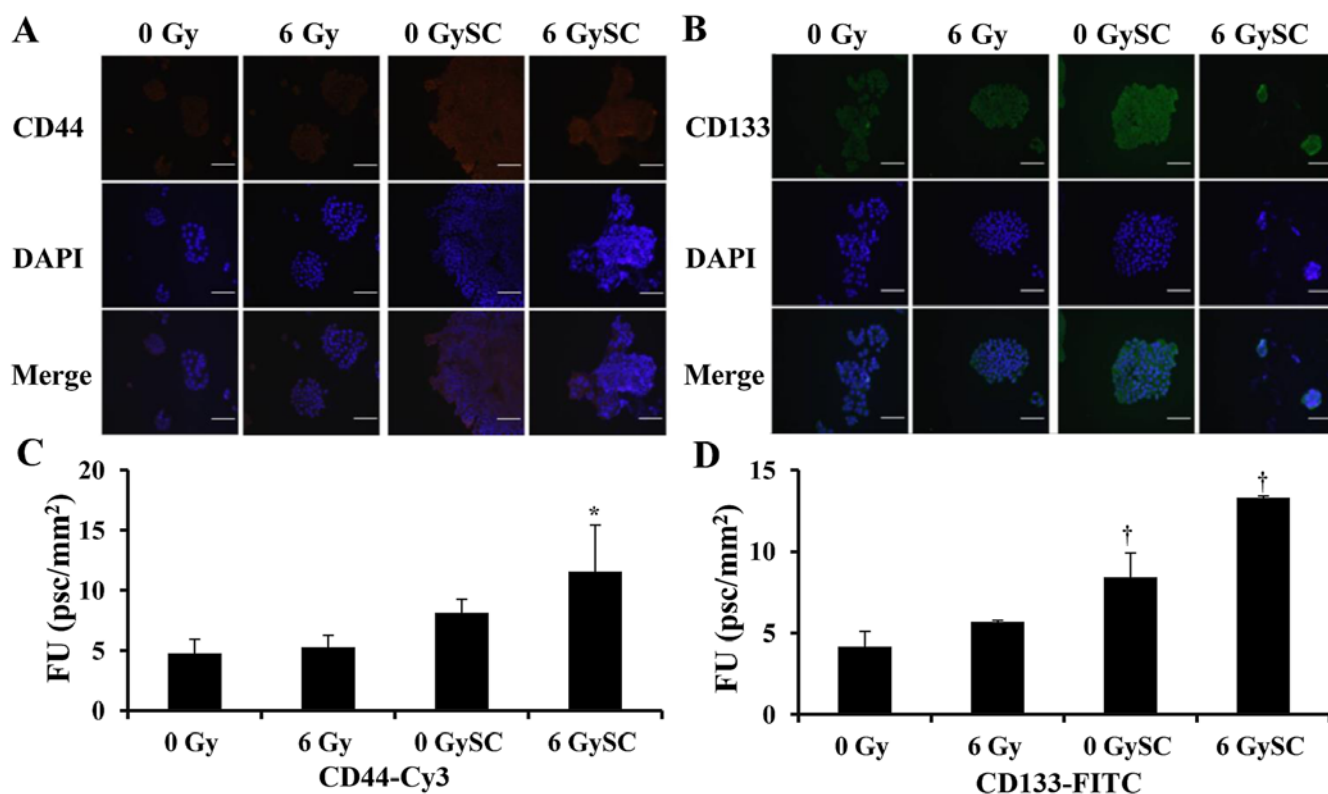


Figure 3. Increase of stem-like subsets by IR. Optical images of CD44 (A) and CD133 expression (B) as CSC marker on MCF7-CL by fluorescence microscopy. Bar, 50  $\mu$ m. (C and D) Graphs of (A and B) fluorescence signals. Fluorescence unit (FU). \*Significant difference ( $P < 0.0027$ ); †significant difference ( $P < 0.0001$ ).

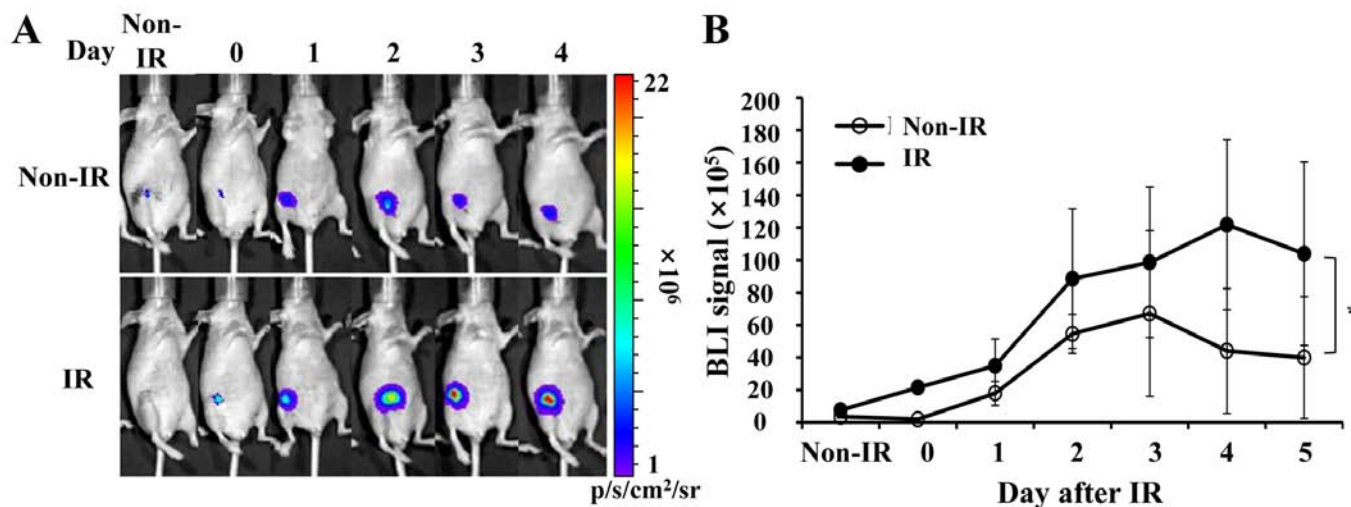


Figure 4. Analysis of CD44 expression in cells after IR *in vivo*. Monitoring fLuc expression by induced CD44 expression with BLI *in vivo* is shown. (A) Irradiated (IR) mice ( $n=3$ , lower) or non-IR mice ( $n=3$ , upper). The presented images are sequential BLIs of the same animal. (B) BLI signals graph, the lower is the non-irradiated group ( $\circ$ ) and the upper the radiated group ( $\bullet$ ). Signal intensity is represented by  $p/s/cm^2/sr$ . \*Significant difference ( $P < 0.0001$ ).

for both conditions). The bioluminescence activity and tumor volume in CD44<sup>+</sup> group was 3.3-fold (Fig. 5E,  $P < 0.017$ ) and ~44-fold (Fig. 5F,  $P < 0.0001$ ) higher than those of CD44<sup>low</sup> group, respectively. In order to assess the BLI signal change by irradiation, we compared the irradiated and non-irradiated MCF7-CL tumor mouse models (Fig. 6A). After irradiation to the CD44<sup>+</sup> tumor, BLI signal was decreased initially, but

it was restored and started to increase time-dependently, compared to the non-irradiated group (Fig. 6B), whereas BLI signal of irradiated CD44<sup>low</sup> tumor changed only slightly. In immunohistochemical study, the expression of CD44 and luciferase showed high correlation in CD44<sup>+</sup> MCF-CL tumor (Fig. 6C). Also, irradiated tumor usually had a higher CD44 and luciferase expression level than non-irradiated tumor.

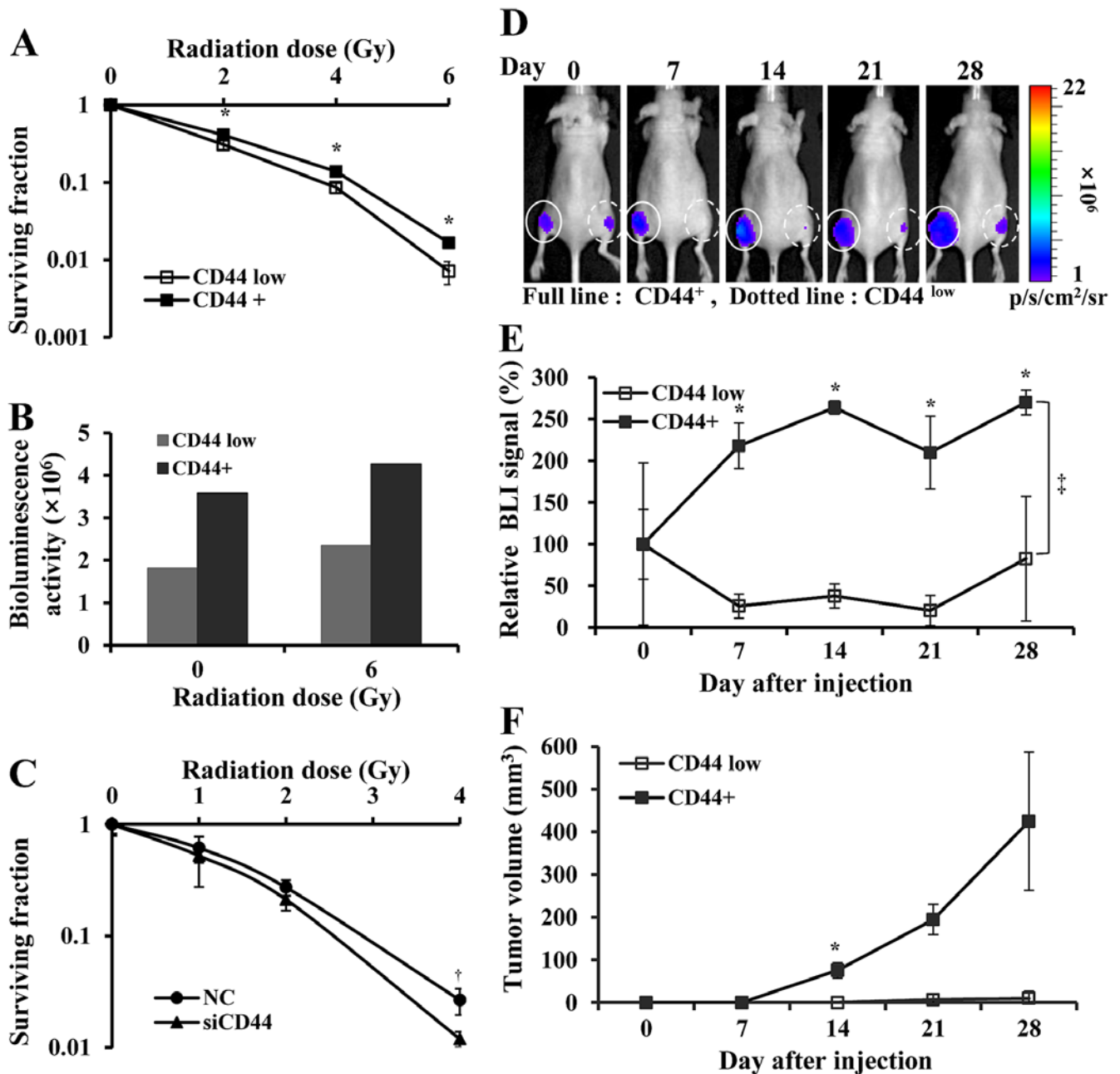


Figure 5. Increased radio-resistance of CD44<sup>+</sup> stem-like subsets *in vivo* and *in vitro*. (A) Cell surviving fraction with clonogenic assay in CSCs (CD44<sup>+</sup>, ■) and non-CSCs (CD44<sup>low</sup>, □) by irradiation. \*Significant difference ( $P < 0.0001$ ). (B) Bioluminescence activity in CSCs and non-CSCs after irradiation. (C) Cell surviving population (●) and treated siCD44 cells (▲) after irradiation. †Significant difference ( $P < 0.0089$ ). (D) BLI. CD44<sup>+</sup> (left leg, full line) and CD44<sup>low</sup> (right leg, dotted line) were observed for 28 days ( $n=5$ ). Signal intensity is represented as  $p/s/cm^2/sr$ . (E) Graph of RLU (%). Non-stem-like subset (□) and stem-like subset (■). Signal intensity is represented as  $p/s/cm^2/sr$ . ‡Significant difference ( $P < 0.017$ ); \*significant difference ( $P < 0.0001$ ). (F) Tumor volumes of two subsets for the various days of injection. \*Significant difference ( $P < 0.0001$ ).

## Discussion

A recent study discovered that CD44 is a marker which is significantly correlated with response to radiotherapy both at the mRNA and protein levels, in the early stage larynx cancer patients (24,25). In agreement with the previous research, this study found correlation between the breast cancer cell CD44 expression and  $\gamma$ -irradiation. We also concluded that CD44 mRNA level of breast cancer cells is closely connected with  $\gamma$ -irradiation. Additionally, increase

in irradiation caused reduction in cell survival population, but the luciferase activity was accelerated by increasing BLI signal (26). It is foreseeable that it induces other genes associated with cancer stem cells (CSCs) as well. Also, we found an increased expression of CD133, OCT4 and Nanog (27). According to these results, the irradiation guided the cells to have similar characteristics as CSCs or survival cells which had similar characteristic to CSCs (6,14,15).

We hypothesized that CD44 depletion was affected by radio-resistance similarly to CSC. To identify the correla-

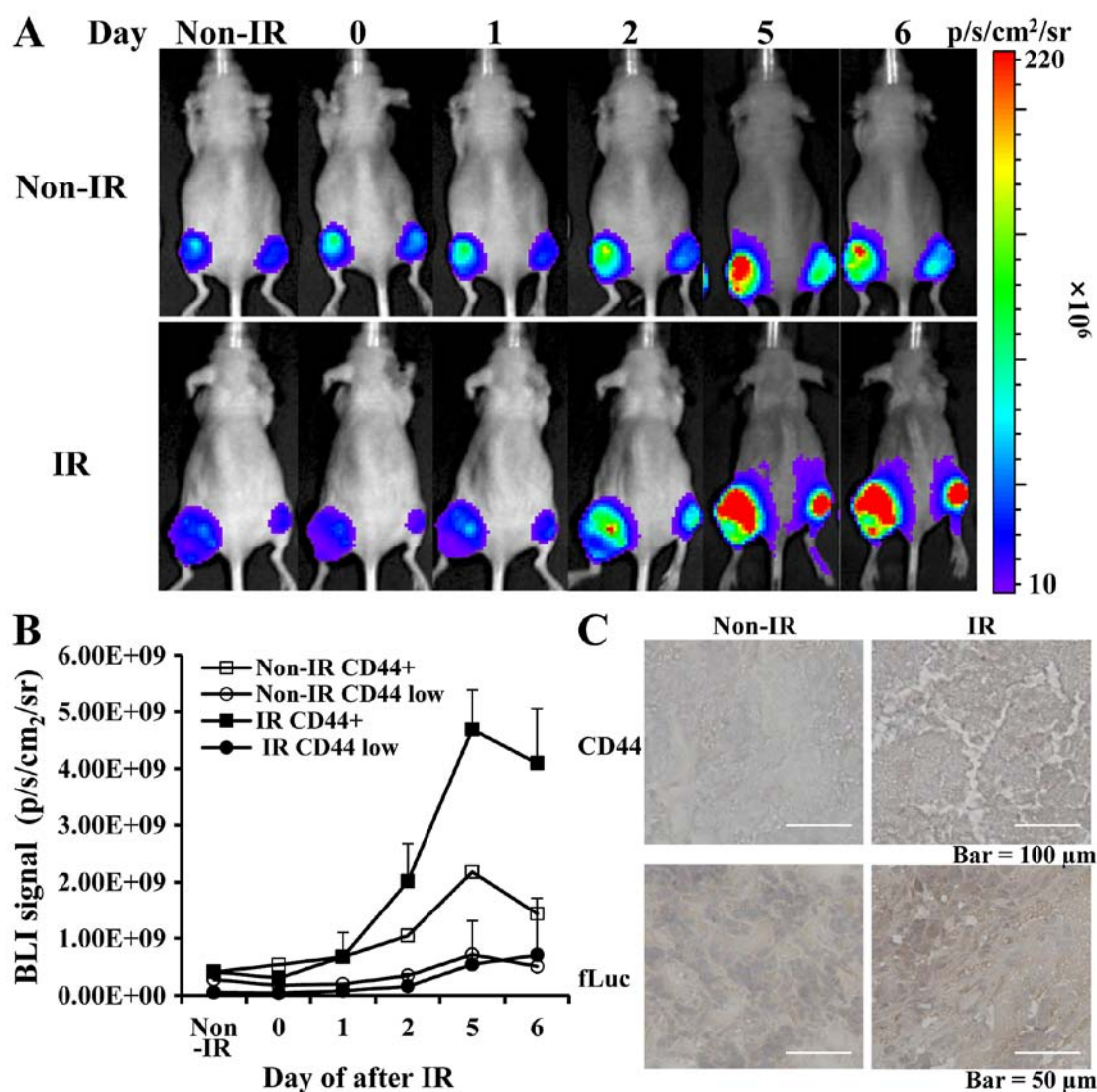


Figure 6. Analysis of bioluminescence in CD44<sup>+</sup> and CD44<sup>low</sup> cells xenografted tumor model by irradiation. (A) Monitoring of bioluminescence signals change after IR. Irradiated CD44<sup>+</sup> (left leg) and CD44<sup>low</sup> tumor were observed for 6 days. The upper panel shows non-irradiated mice (non-IR, n=2), and the lower irradiated mice (IR, n=3). (B) Graph of BLI signal (p/s/cm<sup>2</sup>/sr) by ROI. Non-IR CD44<sup>low</sup> (○), non-IR CD44<sup>+</sup> (□), IR CD44<sup>low</sup> (●) and IR CD44<sup>+</sup> (■). (C) IHC of CD44 is correlated with luciferase expression in primary breast tumor models (upper panels: bar, 100  $\mu$ m; lower panels: bar, 50  $\mu$ m).

tion between radio-resistance and CD44, one group of cells containing plenty of CD44, and another group with shortage of CD44 were evaluated. We confirmed the increase in survival rate in the CD44<sup>+</sup> cells and also, a decreased survival ratio on normal cells, which were intentionally treated with shortage of CD44 using siRNA (22).

We also verified changed expression of CD44, by irradiation *in vivo*, using mouse tumor model with fLuc. The use of bioluminescence as an optical marker for gene expression is a rapidly evolving technique of biomedical research, and it has been recently extended to non-invasive, real-time analysis of molecular events in intact cells and living animals (19,28,29). The bioluminescence signal gradually increased and reduced as time passed, in implant and irradiation. According to this clearer data, increase in CD44 expression, is strongly correlated with irradiation *in vivo* (15,21). These results suggest that the irradiated condition could actually enrich the cells with a CD44<sup>+</sup> phenotype (19,28,29).

In recent studies, the cells in the CD44<sup>+</sup>/CD24<sup>-low</sup> subpopulation have been shown to express higher levels of pro-invasive genes and have highly invasive properties (16). The solid tumor contains CD44<sup>+</sup> expression tissue similar to breast cancer, pancreatic cancer, gastric cancer, and colorectal cancer, in the tumor formation probability in a mouse model and characterization is similar to CSCs (30). Accordingly, successful cancer treatment would need to detect and eliminate these CSCs (34). We have observed that CD44<sup>+</sup> cells from breast cancer cells are significantly enriched in sphere-formed culture or through irradiation, suggesting that CD44<sup>+</sup> cell subsets are more likely CSCs and to undergo cancer formation than CD44<sup>low</sup> cells (31-33). Tumor initiation and growth are more rapid in CD44-positive cells. In a previous study (20), bioluminescence imaging was used to observe differences between CSC and non-CSC growth. Consequently, we found that breast cancer cells containing plenty of CD44 had more rapid formation and growth of tumors, compared to CD44<sup>low</sup> cells. However, BLI



signal of CD44<sup>low</sup> tumors increased by tumor formation and growth. We concluded that the tumor formation and growth could be predicted by monitoring of CD44 expression. In *in vivo* imaging data, we visualized phenomena that had been previously appreciated with irradiation, BLI and tumor volume in MCF7-CL tumor model. The tumors commonly increased BLI signal and growth, but after irradiation, they showed rapid increase of BLI signal, and reduced tumor volume (4). The CD44 expression in MCF7-CL tumor model was rapidly increased until 5 days after irradiation, and the reduced volume of tumor was also sustained. Therefore, we verified an increased CD44 expression correlated with increase in radio-resistance (7,27). It is possible that assessment of curability of a cancer may not only be the existing therapy, but also novel radiotherapy-dependent on the radio-sensitivity of intrinsic and induced BCSCs.

The limitation of luciferase imaging system is that it is not approved for clinical use. However, reporter gene is like near-infrared fluorescence gene, non-toxic to human, it is more widespread in clinical application of optical imaging.

Despite hundreds of published studies on CD44 in the past, no consensus of opinion has been reached as of today, apart from that it plays some role in tumor progression, and that the overwhelming majority of studies failed to take CD44 into consideration. In conclusion, we developed a CD44 monitoring system which enables to inducible CD44 expression in breast cancer *in vivo*. This CD44 promoter-fLuc imaging system could be useful for monitoring of breast cancer stem cells in breast cancer during radio- or chemotherapy.

### Acknowledgements

This study was supported by the Korea Science and Engineering Foundation (KOSEF) grant (grant nos. NRF-2012M2A2A7013480 and 2011-0030162) and funded by the Korean government (Ministry of Education, Science and Technology; MEST).

### References

- Gaffan J, Dacre J and Jones A: Educating undergraduate medical students about oncology: A literature review. *J Clin Oncol* 24: 1932-1939, 2006.
- Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. *Nature* 414: 105-111, 2001.
- Lagasse E: Cancer stem cells with genetic instability: The best vehicle with the best engine for cancer. *Gene Ther* 15: 136-142, 2008.
- Vlashi E, Kim K, Lagadec C, Donna LD, McDonald JT, Eghbali M, Sayre JW, Stefani E, McBride W and Pajonk F: *In vivo* imaging, tracking, and targeting of cancer stem cells. *J Natl Cancer Inst* 101: 350-359, 2009.
- Ouhtit A, Abd Elmageed ZY, Abdraboh ME, Lioe TF and Raj MHG: *In vivo* evidence for the role of CD44s in promoting breast cancer metastasis to the liver. *Am J Pathol* 171: 2033-2039, 2007.
- Naor D, Sionov RV and Ish-Shalom D: CD44: Structure, function, and association with the malignant process. *Adv Cancer Res* 71: 241-319, 1997.
- Yin H and Glass J: The phenotypic radiation resistance of CD44<sup>+</sup>/CD24<sup>(-or low)</sup> breast cancer cells is mediated through the enhanced activation of ATM signaling. *PLoS One* 6: e24080, 2011.
- Phillips TM, McBride WH and Pajonk F: The response of CD24<sup>(-low)</sup>/CD44<sup>+</sup> breast cancer-initiating cells to radiation. *J Natl Cancer Inst* 98: 1777-1785, 2006.
- Lagadec C, Vlashi E, Della Donna L, Meng Y, Dekmezian C, Kim K and Pajonk F: Survival and self-renewing capacity of breast cancer initiating cells during fractionated radiation treatment. *Breast Cancer Res* 12: R13, 2010.
- Woodward WA, Chen MS, Behbod F, Alfaro MP, Buchholz TA and Rosen JM: WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc Natl Acad Sci USA* 104: 618-623, 2007.
- Kruger JA, Kaplan CD, Luo Y, Zhou H, Markowitz D, Xiang R and Reisfeld RA: Characterization of stem cell-like cancer cells in immune-competent mice. *Blood* 108: 3906-3912, 2006.
- Khan SA, Cook AC, Kappil M, Günthert U, Chambers AF, Tuck AB and Denhardt DT: Enhanced cell surface CD44 variant (v6, v9) expression by osteopontin in breast cancer epithelial cells facilitates tumor cell migration: Novel post-transcriptional, post-translational regulation. *Clin Exp Metastasis* 22: 663-673, 2005.
- Naor D, Nedvetzki S, Golan I, Melnik L and Faitelson Y: CD44 in cancer. *Crit Rev Clin Lab Sci* 39: 527-579, 2002.
- Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, Goulet Jr R, Badve S and Nakshatri H: CD44<sup>+</sup>/CD24<sup>-</sup> breast cancer cells exhibit enhanced invasive properties: An early step necessary for metastasis. *Breast Cancer Res* 8: R59, 2006.
- Bánkfalvi A, Terpe HJ, Breukelmann D, Bier B, Rempe D, Pschadka G, Krech R and Böcker W: Gains and losses of CD44 expression during breast carcinogenesis and tumour progression. *Histopathology* 33: 107-116, 1998.
- Hill A, McFarlane S, Mulligan K, Gillespie H, Draffin JE, Trimble A, Ouhtit A, Johnston PG, Harkin DP, McCormick D, *et al*: Cortactin underpins CD44-promoted invasion and adhesion of breast cancer cells to bone marrow endothelial cells. *Oncogene* 25: 6079-6091, 2006.
- Herring PJ: Species abundance, sexual encounter and bioluminescent signalling in the deep sea. *Philos Trans R Soc Lond B Biol Sci* 355: 1273-1276, 2000.
- Wilson T and Hastings JW: Bioluminescence. *Annu Rev Cell Dev Biol* 14: 197-230, 1998.
- Contag CH and Ross BD: It's not just about anatomy: *In vivo* bioluminescence imaging as an eyepiece into biology. *J Magn Reson Imaging* 16: 378-387, 2002.
- Liu H, Patel MR, Prescher JA, Patsialou A, Qian D, Lin J, Wen S, Chang YF, Bachmann MH, Shimono Y, *et al*: Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. *Proc Natl Acad Sci USA* 107: 18115-18120, 2010.
- Godar S, Ince TA, Bell GW, Feldser D, Donaher JL, Bergh J, Liu A, Miu K, Watnick RS, Reinhardt F, *et al*: Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* 134: 62-73, 2008.
- Pongcharoen P, Jinawath A and Tohtong R: Silencing of CD44 by siRNA suppressed invasion, migration and adhesion to matrix, but not secretion of MMPs, of cholangiocarcinoma cells. *Clin Exp Metastasis* 28: 827-839, 2011.
- Franken NAP, Rodermond HM, Stap J, Haveman J and van Bree C: Clonogenic assay of cells in vitro. *Nat Protoc* 1: 2315-2319, 2006.
- de Jong MC, Pramana J, van der Wal JE, Lacko M, Peutz-Kootstra CJ, de Jong JM, Takes RP, Kaanders JH, van der Laan BF, Wachtors J, *et al*: CD44 expression predicts local recurrence after radiotherapy in larynx cancer. *Clin Cancer Res* 16: 5329-5338, 2010.
- Gomez-Casal R, Bhattacharya C, Ganesh N, Bailey L, Basse P, Gibson M, Epperly M and Levina V: Non-small cell lung cancer cells survived ionizing radiation treatment display cancer stem cell and epithelial-mesenchymal transition phenotypes. *Mol Cancer* 12: 94, 2013.
- Krause M, Yaromina A, Eicheler W, Koch U and Baumann M: Cancer stem cells: Targets and potential biomarkers for radiotherapy. *Clin Cancer Res* 17: 7224-7229, 2011.
- Lagadec C, Vlashi E, Della Donna L, Dekmezian C and Pajonk F: Radiation-induced reprogramming of breast cancer cells. *Stem Cells* 30: 833-844, 2012.
- Contag CH and Bachmann MH: Advances in *in vivo* bioluminescence imaging of gene expression. *Annu Rev Biomed Eng* 4: 235-260, 2002.
- Luker GD, Pica CM, Song J, Luker KE and Piwnicka-Worms D: Imaging 26S proteasome activity and inhibition in living mice. *Nat Med* 9: 969-973, 2003.

30. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF and Simeone DM: Identification of pancreatic cancer stem cells. *Cancer Res* 67: 1030-1037, 2007.
31. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, Hilsenbeck SG, Pavlick A, Zhang X, Chamness GC, *et al*: Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst* 100: 672-679, 2008.
32. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ and Wicha MS: In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* 17: 1253-1270, 2003.
33. Gupta PB, Chaffer CL and Weinberg RA: Cancer stem cells: Mirage or reality? *Nat Med* 15: 1010-1012, 2009.
34. Fábán Á, Vereb G and Szöllösi J: The hitchhikers guide to cancer stem cell theory: Markers, pathways and therapy. *Cytometry A* 83: 62-71, 2013.