

Suppression of human breast tumors in NOD/SCID mice by CD44 shRNA gene therapy combined with doxorubicin treatment

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Background: Breast cancer stem cells with a CD44⁺CD24⁻ phenotype are the origin of breast tumors. Strong CD44 expression in this population indicates its important role in maintaining the stem cell phenotype. Previous studies show that CD44 down-regulation causes CD44⁺CD24⁻ breast cancer stem cells to differentiate into non-stem cells that are sensitive to antitumor drugs and lose many characteristics of the original cells. In this study, we determined tumor suppression in non-obese severe combined immunodeficiency mice using CD44 shRNA therapy combined with doxorubicin treatment.

Methods: Tumor-bearing non-obese severe combined immunodeficiency mice were established by injection of CD44⁺CD24⁻ cells. To track CD44⁺CD24⁻ cells, green fluorescence protein was stably transduced using a lentiviral vector prior to injection into mice. The amount of CD44 shRNA lentiviral vector used for transduction was based on CD44 down-regulation by in vitro CD44 shRNA transduction. Mice were treated with direct injection of CD44 shRNA lentiviral vector into tumors followed by doxorubicin administration after 48 hours. The effect was evaluated by changes in the size and weight of tumors compared with that of the control.

Results: The combination of CD44 down-regulation and doxorubicin strongly suppressed tumor growth with significant differences in tumor sizes and weights compared with that of CD44 down-regulation or doxorubicin treatment alone. In the combination of CD44 down-regulation and doxorubicin group, the tumor weight was significantly decreased by 4.38-fold compared with that of the control group.

Conclusion: These results support a new strategy for breast cancer treatment by combining gene therapy with chemotherapy.

Keywords: breast cancer, breast cancer stem cells, CD44, doxorubicin, gene therapy

Introduction

CD44⁺CD24⁻ cells have been identified as a breast cancer stem cell population and the origin of tumors, metastasis, and relapse in breast cancer patients.¹⁻³ Breast cancer stem cell targeting is considered a promising therapy. Thus far, various drugs that are specific to receptors such as Her2/neu and epidermal growth factor receptors have been used to target breast cancer stem cells.⁴⁻¹¹ However, more than 50% of tumors do not express these receptors and are drug resistant.¹²⁻¹⁶ A recent report has shown that triple-negative breast carcinoma contains CD44⁺CD24⁻ breast cancer stem cells.¹⁷ Therefore, it is essential for treatment that new targets be discovered on breast cancer stem cells.

CD44 plays an important role in the phenotype of breast cancer stem cells and is responsible for cancer stem cell-specific characteristics, such as antitumor drug

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resistance in various cancers like colon cancer,¹⁸ salivary gland cancer,¹⁹ and metastasis from the breast to the liver.²⁰ In addition, CD44 has been used to isolate and enrich cells that are capable of forming breast cancer tumors²¹ and numerous other tumors, including head and neck squamous cell carcinoma,^{22–24} esophageal squamous cell carcinoma,²⁵ nasopharyngeal carcinoma,²⁶ and gastric²⁷ and colon cancer stem cells.²⁸

Down-regulation of CD44 using siRNA or shRNA results in metastasis suppression,²⁹ sensitizes cancer stem cells to drugs,³⁰ and causes differentiation of breast cancer stem cells.³¹ Antibodies against survivin also show similar effects.³² CD44 also plays an important role in other cancers. CD44 inhibition suppresses the development of colon tumors in mice³³ and inhibits the proliferation and metastasis of ovarian³⁴ and liver cancer cells.³⁵ This study evaluates breast cancer treatment in mouse models using a CD44 shRNA lentiviral vector to inhibit CD44 expression in combination with doxorubicin chemotherapy.

Materials and methods

Cell culture and establishment of green fluorescent protein (GFP)-expressing breast cancer stem cells

Breast cancer stem cells were isolated and purified as described elsewhere.³⁰ Briefly, tumor biopsies from consenting patients were obtained at hospitals and then transferred to our laboratory. Biopsy samples were washed 3–4 times with phosphate-buffered saline containing $1 \times$ antibiotic-antimycotic (Sigma, St Louis, MO), and then homogenized into small pieces (approximately 1–2 mm³). Homogenized samples were resuspended in M171 medium (Invitrogen, Carlsbad, CA) containing mammary epithelial growth supplement (Invitrogen) and then seeded in 35 mm culture dishes (Nunc, Germany). Cells were incubated at 37°C with 5% CO₂, and medium was replaced every third day. CD44⁺CD24⁻ cells were isolated from the primary cell population by magnetic sorting using a commercial kit (Miltenyi Biotec, Germany). These CD44⁺CD24⁻ cells were named BCSC1. For tracking, we established CD44⁺CD24⁻ cells that stably expressed the *gfp* gene. We used a *gfp* lentiviral vector (Santa Cruz Biotechnology, CA) to transduce isolated CD44⁺CD24⁻ cells. To select and establish GFP-expressing BCSC1, cells were cultured in medium containing 10 µg/mL puromycin dihydrochloride (Sigma-Aldrich, St Louis, MO) for 1 week.

CD44 knockdown of CD44⁺CD24⁻ cells with shRNA using lentivirus particles

In the first assay, we determined a suitable dose of lentiviral particle vector infectious units (IFUs) to apply in the next experiment. CD44 shRNA lentivirus particles (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) were stably transfected according to the manufacturer's instructions. Briefly, BCSC1 cells were seeded on day 1 in a twelve-well plate with complete medium (Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum and $1 \times$ antibiotic-mycotic) and incubated overnight.

Medium was replaced on day 2 with fresh complete medium containing 5 µg/mL polybrene (Sigma-Aldrich, St Louis, MO) for 6 hours, then 20 µL of modified Eagle's medium with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid containing 1×10^5 IFUs of virus was added to the culture. The culture plate was shaken to mix the virus particles and was then incubated overnight at 37°C with 5% CO₂. On day 3, medium was replaced with fresh complete medium without polybrene. Half of the transduced cells were confirmed by CD44 detection using flow cytometry. Half of the transduced cells were selected by culturing in complete medium containing 10 µg/mL puromycin dihydrochloride for 12 hours, followed by 5 µg/mL puromycin dihydrochloride for 1 week.

Flow cytometry

Cells were washed twice in phosphate-buffered saline containing 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO). Fc receptors were blocked by incubation with immunoglobulin G (Santa Cruz Biotechnology, CA) on ice for 15 minutes. Cells were stained with anti-CD44-PE monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ) at 4°C for 30 minutes. After washing, cells were analyzed using a FAC SCalibur flow cytometer (BD Biosciences) and CellQuest Pro software (BD Biosciences) with 10,000 events collected.

CD44 shRNA gene therapy

Female (5–6 weeks old) NOD/severe combined immunodeficiency (SCID) mice (NOD.CB17-Prkdcscid/J; Charles River Laboratories, Wilmington, MA) were subcutaneously injected with BCSC1 cells (2×10^6 cells/mouse). After 2 weeks, tumors were formed and mice were divided into four groups: Group 1 (control) mice ($n = 4$) were used as untreated controls; they received biweekly intratumoral phosphate-buffered saline injections for 6 weeks. Group 2 (doxorubicin [Dox]) mice received intratumoral Dox

injections (2 mg/kg) weekly for 4 weeks. Group 3 (shRNA) mice received intratumoral CD44 shRNA lentiviral vector injections with a dose of IFUs that was doubled compared with that of the tumor cell number. Group 4 (CD44 shRNA in combination with Dox treatment [shRNA + Dox]) mice received intratumoral injections of CD44 shRNA lentiviral vector with IFUs similar to that of Group 3 and, after 48 hours, received intratumoral injections of Dox (2 mg/kg) weekly for 4 weeks. Tumor size was measured as described below. Animals were killed after 7 weeks, and tumors were excised and weighed to record the wet tumor weight. All animal experiments were approved by the Institutional Animal Care and Use Committee of Stem Cell Research and Application Laboratory, University of Science, VNU-HCM.

Tumor size measurement

Tumor size was measured with calipers in two dimensions, and size was calculated using the following formula: $a \times b^2/2$, where “a” is the tumor length and “b” is the diameter.³⁶

Statistical analysis

All experimental procedures were performed in triplicate, except for mouse experiments. The significance of differences between mean values was assessed by a Student's *t*-test and analysis of variance. $P < 0.05$ was considered significant.

Results

Isolation and establishment of breast cancer stem cells expressing green fluorescent protein

We primary-cultured 31 tumor samples from patients; 23 of these samples showed numerous single cells surrounding the tumor tissue. Cells from the 23 samples were allowed to propagate to 80% confluence (Figure 1A). CD44 and CD24

were analyzed and all 23 primary cell samples showed a small population of cells that were positive for CD44 and negative or weakly positive for CD24. This population constituted $3.96\% \pm 1.72\%$ of the total cells derived from primary culture. We isolated two populations of CD44⁺CD24⁻ cells from the 23 primary-culture samples. One cell population, termed “BCSC1,” was used for subsequent experiments (Figure 1B). The BCSC1 cell line was transduced with the *gfp* gene using a lentiviral vector, resulting in 43.12% and 99.9% of BCSC1 cells expressing GFP before and after selection with puromycin, respectively (Figure 1C).

Tumor-bearing mouse models

To establish the tumor-bearing mouse models, we used 5–6-week-old NOD/SCID mice. GFP-expressing BCSC1 cells (2.10^6 cells/mouse) were injected into mammary fat using an insulin needle. This resulted in 100% of mice forming tumors that were apparent after 3 weeks. All tumors contained GFP-expressing cells (Figure 2).

In vitro CD44 down-regulation by the CD44 shRNA lentiviral vector

Next, we evaluated in vitro CD44 down-regulation with CD44 shRNA using a lentiviral vector to determine a suitable dose for in vivo transduction. CD44 down-regulation was dependent on the ratio of IFUs to BCSC1 cells, with a higher ratio of lentiviral vector to BCSC1 cells resulting in higher transduction efficiency. The percentages of CD44 down-regulated BCSC1 cells in the control (1:0), Dox (2:1), CD44 shRNA (1:1), and CD44 shRNA + Dox (1:2) groups were $0.14\% \pm 0.08\%$, $12.21\% \pm 3.30\%$, $37.87\% \pm 5.34\%$, and $47.41\% \pm 3.90\%$, respectively ($P < 0.05$) (Figure 3). Based on these results, the suitable dose of lentiviral vector IFUs was double that of the number of tumor cells. This dose was applied in further experimentation.

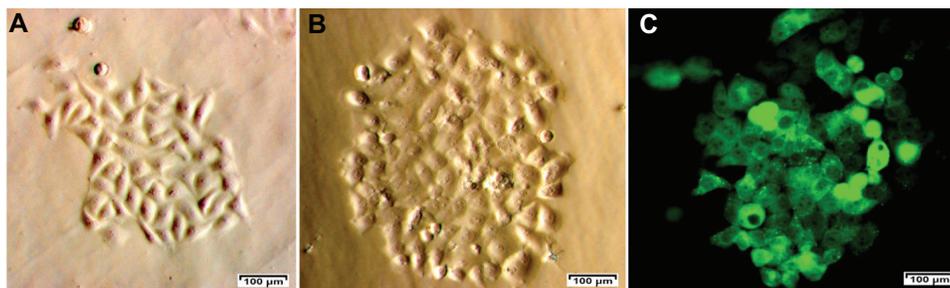


Figure 1 Breast cancer cells from breast tumors (A) were used to isolate CD44⁺CD24⁻ breast cancer stem cell populations (B) for green fluorescent protein expression after transduction with green fluorescent protein using a lentiviral vector and selection with puromycin (C).

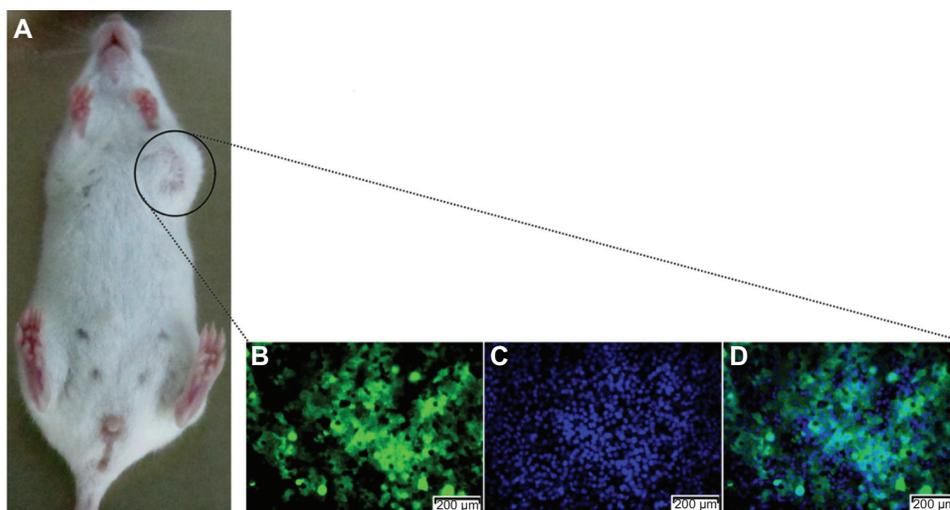


Figure 2 A tumor produced in the mouse model. The tumor (A) was excised and observed by monochromatic fluorescence microscopy (Carl Zeiss AG, Oberkochen, Germany) using fluorescein isothiocyanate (B) and Hoechst 33342 filters (Carl Zeiss AG, Oberkochen, Germany) (C) for a merged image (D).

Tumor size and weight

As shown in Figure 4, the size and weight of tumors were significantly different between the four groups ($P < 0.05$). The average tumor sizes were $246.39 \pm 56.80 \text{ mm}^3$, $142 \pm 25.98 \text{ mm}^3$, $80.89 \pm 11.11 \text{ mm}^3$, and $19.75 \pm 8.50 \text{ mm}^3$ in the control, Dox, CD44 shRNA, and CD44 shRNA + Dox groups, respectively. In comparison with the control group, the tumor sizes were significantly decreased by 1.74-, 3.04-, and 12.47-fold in the Dox, CD44 shRNA, and CD44 shRNA + Dox groups, respectively. Tumor weights also gradually decreased ($0.44 \pm 0.18 \text{ g}$, $0.23 \pm 0.05 \text{ g}$, $0.18 \pm 0.02 \text{ g}$, and $0.1 \pm 0.07 \text{ g}$). In CD44 shRNA + Dox,

the tumor weight was significantly decreased by 4.38-fold compared with that of the control group. These changes in tumor size and weight confirmed the beneficial effects of CD44 down-regulation, Dox treatment, and particularly, the combination of CD44 down-regulation and Dox treatment. Thus, combinatorial therapy of CD44 down-regulation and Dox efficiently suppressed tumor growth in the mouse model.

Discussion

Cancer stem cells are considered the origin of malignant tissues. The existence of cancer stem cells has been recently

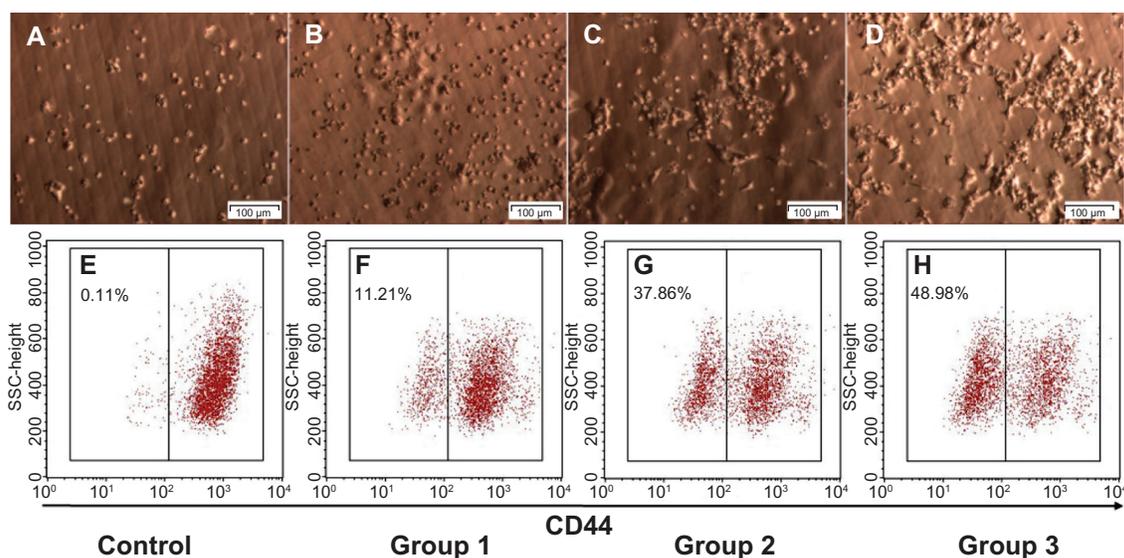


Figure 3 In vitro CD44 down-regulation using the CD44 shRNA lentiviral vector with doses of infectious units to breast cancer stem cells at ratios 1:0 (A and E), 2:1 (B and F), 1:1 (C and G) and 1:2 (D and H).

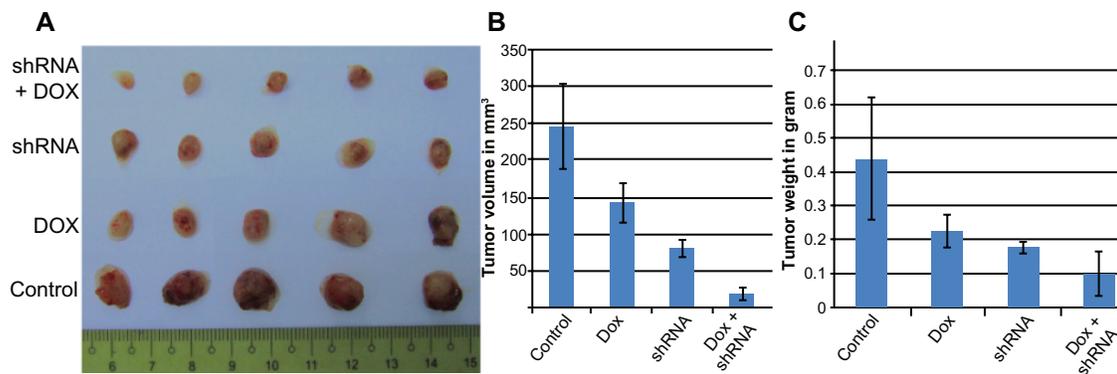


Figure 4 Tumor size and weight in experimental groups (A). Graphs of the differences in the size (B) and weight (C) of tumors in control, Dox, CD44 shRNA, and CD44 shRNA + Dox groups.

Abbreviation: Dox, doxorubicin.

confirmed in solid tumors of the brain, prostate, pancreas, liver, colon, head and neck, lung, and skin.^{36–42} Moreover, CD44⁺CD24⁻ cells have been identified as breast cancer stem cells.²¹

Since the discovery of cancer stem cells, the study of cancer treatment in general, and breast cancer in particular, has gradually focused on targeting cancer stem cells. Thus far, targeting of breast cancer stem cells has been performed using various approaches, but has mainly targeted self-renewal and differentiation of breast cancer stem cells. To influence self-renewal and differentiation, signaling pathways that are important in breast cancer stem cells, such as Wnt, Notch, and Hedgehog, can be targeted.^{43–46} There are numerous methods to target signaling pathways, including gene therapy, immunotherapy, and targeting the cell environment. In our previous study, we found that CD44 down-regulation reduces the drug resistance of breast cancer stem cells to Dox.³⁰ In previous research, we also confirmed that CD44 shRNA lentiviral particles reduced CD44 expression and caused breast cancer stem cell differentiation.³¹ In this study, we used an experimental treatment to target breast cancer stem cells by combining gene therapy targeting CD44 and Dox treatment.

First, we established a breast cancer stem cell line that stably expressed GFP to monitor the xenografted breast cancer tumor in mice. To establish this cell line, breast cancer stem cells were transduced with a lentiviral vector carrying *gfp* and a puromycin resistance gene for selection. Because random insertion of lentiviral DNA into the genome can cause detrimental mutations, we isolated CD44⁺CD24⁻ cells from GFP-breast cancer stem cells using a magnetic cell separation method, and re-analyzed with flow cytometry. Indeed, a study showed that lentiviral vectors demonstrate a low tendency to integrate into genes that cause cancer,⁴⁷ and

another study found no increase in tumor incidence and no earlier onset of tumors in a mouse strain following the use of lentiviral vectors.⁴⁸

These BSCS1 cells were used to evaluate the potential to form tumors in NOD/SCID mice and CD44 knockdown mice using a CD44 shRNA lentiviral vector as well as determination of the optimal dose of lentiviral particles for in vivo analyses. GFP-expressing BSCS1 maintained a tumorigenic capacity and formed malignant tumors in NOD/SCID mice with numerous poorly differentiated and abnormal cells.

Next, we determined the appropriate dose of virus particles to infect tumors, which was considered to be the IFUs that down-regulated CD44 at the highest rate. To determine the appropriate dose, we conducted serial assays with ratios between cells and IFUs at 2:1, 1:1, and 1:2. CD44 down-regulation was highest using double the IFUs compared with that of the cell number. To determine the number of cells in a tumor, we measured the tumor size at the time of treatment. The number of tumor cells is calculated as 1 cm³ tumor contains $\sim 1 \times 10^9$ cells.⁴⁹ Although recent studies have supported this claim,^{50–52} experiments using the same mouse breed under the same conditions are necessary to apply this rule in calculation and comparison among the mice.

Lentiviral vector-injected mice were treated with Dox after 48 hours. This period was chosen because previous study has shown that viruses infect target cells and inhibit CD44 expression after 24 hours.³⁰ The Dox dose used was 2 mg/kg body weight and this was chosen based on a previous study.⁵³

The results showed significant differences in the size and weight of tumors of treated mice compared with those of the controls. Dox treatment and CD44 siRNA therapy alone or in combination inhibited tumor growth. Tumor inhibition with Dox treatment and CD44 shRNA therapy alone was identical,

while a significant difference ($P < 0.05$) was demonstrated between combinatorial therapy with Dox and CD44 shRNA compared with that of single treatments.

CD44 down-regulation also effects adhesion, invasion, and metastasis,^{54–59} and the inhibition of CD44 is also considered as treatment therapy in many cancer targets.^{57–59} In addition, CD44 down-regulation that suppresses the development of tumors has also been shown in in vivo colon cancer tumors,³³ ovarian cancer cells,³⁴ and nasopharyngeal carcinoma cells.^{60–61} In recent research, we recognized that CD44 maintains the stemness of breast cancer stem cells. CD44 knocked-down breast cancer stem cells by CD44 shRNA lentiviral particles can cause differentiation of breast cancer stem cells or loss of stemness can change the tumor formation and metastasis related genes, and can reduce tumor formations in NOD/SCID mice.³¹

CD44 down-regulation using shRNA suppressed xenografted breast tumor growth in a mouse model with or without Dox treatment. However, there are limitations for the clinical application of this therapy. The two most significant issues are the host's immune response to the lentiviral vector and random insertion mutagenesis. The immune response to the lentiviral vector is very low because viral proteins are not translated. Therefore, an immune response occurs only as a primary response to the virus or products of transgenes. In this study, the lentiviral vector was only transcribed into shRNA. Moreover, an immune response occurs only in response to adenoviral vectors or in the nature of the mechanism of adeno-associated viral production of antibodies against them,⁶² while lentiviral vectors possess many traits that enable avoidance of the immune response. As mentioned, insertion mutations caused by lentiviral vectors are fewer and less serious compared with those caused by other vectors. Insertion mutations have been detected in three out of eleven cross-linked SCID children after applying ex vivo therapy using a murine leukemia virus vector.⁶³ Murine leukemia virus vectors are often inserted into promoters and CpG islands that affect transcriptionally active genes.^{64,65} Integrations near transcription start sites may increase oncogenesis, either by influencing the activity of host promoters or producing new full-length transcripts. In contrast, lentiviral vectors that integrate into the entire transcribed region are less likely to disturb the regulation and expression of host genes.⁶⁶ This claim is supported by a Montini et al,⁴⁸ which showed that lentiviral vectors cause insertion mutations related to cancer less often compared with murine leukemia virus vectors in a mouse model. However, these problems can be solved by using site-specific

gene transfer. With the structural advantages of this vector system, cassettes that contain numerous genes can be expressed in the same vector, such as a Cre recombinase in combination with *loxP* sites or a zinc finger nuclease. However, there are some limitations in applying these results in clinical trials. First, the high dose of lentiviral vector can cause some side effects; in particular, lentiviral vectors can migrate into bone marrow to suppress the mesenchymal stem cells and other cells that strongly express CD44. Second, in practice, intratumoral delivery is not generally carried out. However, as many kinds of cells as well as stem cells strongly express CD44, we cannot apply systemic therapy in this case.

Conclusion

Strong CD44 expression in a breast cancer stem cell population with a CD44⁺CD24⁻ phenotype plays a pivotal role in the proliferation and drug resistance of malignant cells. Our data suggest that CD44 down-regulation suppresses tumor growth in a mouse model. Combinatorial therapy of CD44 down-regulation using a CD44 shRNA lentiviral vector and Dox treatment strongly inhibits tumor growth. These results support a new targeted therapy using gene therapy and chemotherapy to eradicate breast cancer stem cells. If this therapy is found to be safe, it may be a promising therapy for breast cancer through the targeting of breast cancer stem cells.

Disclosure

The authors report no conflicts of interest in this work.

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