Biochemical and Biophysical Research Communications 425 (2012) 436–442

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of Wnt1 expression reduces the enrichment of cancer stem cells in a mouse model of breast cancer

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ARTICLE INFO

Article history:
Received 18 July 2012
Available online 28 July 2012

Keywords:
Breast cancer
Cancer stem cells
Wnt1

ABSTRACT

Breast cancer is the leading cause of deaths from cancer in women. Cancer recurrence is the most common cause of mortality in breast cancer patients. The cancer stem cell (CSC) hypothesis proposes that CSCs are the center of cancer development and recurrence. Targeting CSCs, in combination with standard chemotherapy, may prevent cancer recurrence and improve long-term survival. Stem cells can be enriched in non-adherent sphere cultures. To identify molecular targets in breast CSCs, we evaluated the transcription levels of stem cell-related genes in 4T1 mouse mammary cancer cells grown as spheres or in a monolayer culture. The most differentially expressed gene was found to be wingless-type MMTV integration site family member 1 (Wnt1) in the 4T1 sphere culture. Functionally, knockdown of Wnt1 in breast cancer cell lines suppressed the *in vitro* properties of the stem-like cells, including their sphere-forming ability and ALDH activity, whereas the addition of recombinant Wnt1 to breast cancer cell lines enhanced the *in vitro* properties of these stem-like cells. In addition, knockdown of Wnt1 in 4T1 cells affected the properties of the stem-like cells *in vivo*, including their tumorigenic potential and tumor initiation ability. Collectively, these results suggest that Wnt1 expression may give rise to the properties of CSCs in breast tumors. Therefore, targeting Wnt1-associated signaling proteins may provide an effective therapeutic approach for the treatment of advanced breast cancer.

1. Introduction

Breast cancer is the most common malignant disease among women worldwide [1]. Unfortunately, breast cancer can recur at the original site (called recurrence) and spread to other parts of the body (called metastasis) after primary tumor treatment such as chemotherapy, biologics therapy, radiation therapy and surgery [2,3]. Cancer recurrence and metastasis are the most common cause of mortality in breast cancer patients.

Although the cause of recurrence and metastasis of breast cancer remains unclear, the cancer stem cell (CSC) hypothesis suggests that CSCs may be involved in maintaining the primary tumor mass and may also be responsible for resistance to standard therapies, which in many instances leads to cancer recurrence and metastasis. The clinical translation of this hypothesis has resulted in cancer therapies that target the putative breast CSCs [4].

The sphere culture has been widely used to identify stem cells because it enables the evaluation of self-renewal at the single cell level *in vitro*. In addition, stem cells can be enriched in non-adherent sphere culture conditions [5–7]. Recently, applications of sphere culture have been reported to isolate, enrich, maintain or expand potential CSC subpopulations from various types of cancers [6,8,9]. Furthermore, the sphere-forming cell subpopulations from primary tumors, such as breast and prostate cancer, showed increased anti-apoptotic abilities, drug resistance and *in vivo* tumorigenic potential [5,7].

The present study sought to identify breast CSC-specific target genes by comparing the levels of stem cell-related gene expression between sphere and adherent cultures using the best murine breast cancer line for studying human breast cancer progression [10]. Therefore, the purpose of this study was to determine whether an identified gene could be a feasible target for stem cell-specific cancer therapy.

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http://dx.doi.org/10.1016/j.bbrc.2012.07.120

2. Materials and methods

2.1. Cell culture and reagents

The murine breast cancer cell line (4T1) and human breast cancer cell lines (MCF7, BT474 and T47D) were cultured in DMEM (Invitrogen, Grand Island, NY) containing 5% FBS and 1% penicillin/streptomycin (Invitrogen), as previously described [11]. The recombinant human Wnt1 protein was purchased from Peprotech (Rocky Hill, NJ).

2.2. Tumorsphere culture

Single cells were resuspended in serum-free DMEM (Invitrogen) containing B27 (Invitrogen), 20 ng/ml EGF, 20 ng/ml bFGF (Peprotech, Rocky Hill, NJ) and 4 μg/ml heparin (Sigma–Aldrich), as previously described [11]. Primary tumorspheres were derived by plating 50000–100000 cells/well into six-well ultra-low attachment dishes (Corning, Lowell, MA). Secondary tumorspheres were plated at 50000 cells/well. The dishes were cultivated for 7 days to enumerate the spheres. Individual spheres $\geqslant$100 μm from each replicate well ($n \geqslant 9$ wells) were counted under an inverted microscope at 50× magnification using the Image-Pro Plus program (Media Cybernetics). The percentage of cells capable of forming spheres, referred to as the 'tumorsphere formation efficiency (TSFE)', was calculated as follows: [(number of spheres formed/number of single cells plated) × 100], as previously described [12].

2.3. Quantitative reverse-transcription-PCR (RT-qPCR) and the stem cell PCR array

RT-qPCR was performed on the ABI 7300 Real-Time PCR system using SYBR green dye (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RT-qPCR was performed in triplicate. Gene expression levels were normalized to mouse hypoxanthine–guanine phosphoribosyltransferase (HPRT) and human cyclophilin A (PPIA) mRNA. Primer sequences are provided in Supplementary Table 1. The stem cell PCR array (SABiosciences, Frederick, MD) was performed in triplicate according to the manufacturer's instructions.

2.4. Short hairpin RNA (shRNA) and small interfering RNA (siRNA) construction

A lentiviral-based plasmid containing a short hairpin RNA (shRNA) targeting mouse Wnt1 was purchased from Sigma–Aldrich (St. Louis, MO). shRNA infection was performed as previously described [13]. Stable transfectants were selected using puromycin (1 μg/ml; Sigma–Aldrich), and knockdown of Wnt1 expression was determined by RT-qPCR and immunoblotting. The siRNA targeting human Wnt1 and non-targeting siRNA were purchased from Bioneer, Inc. (Daejeon, Korea). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For the RNA interference study, we tested four shRNA plasmids or three siRNA duplexes designed using the target gene sequences. We selected the shRNA or siRNA that resulted in the

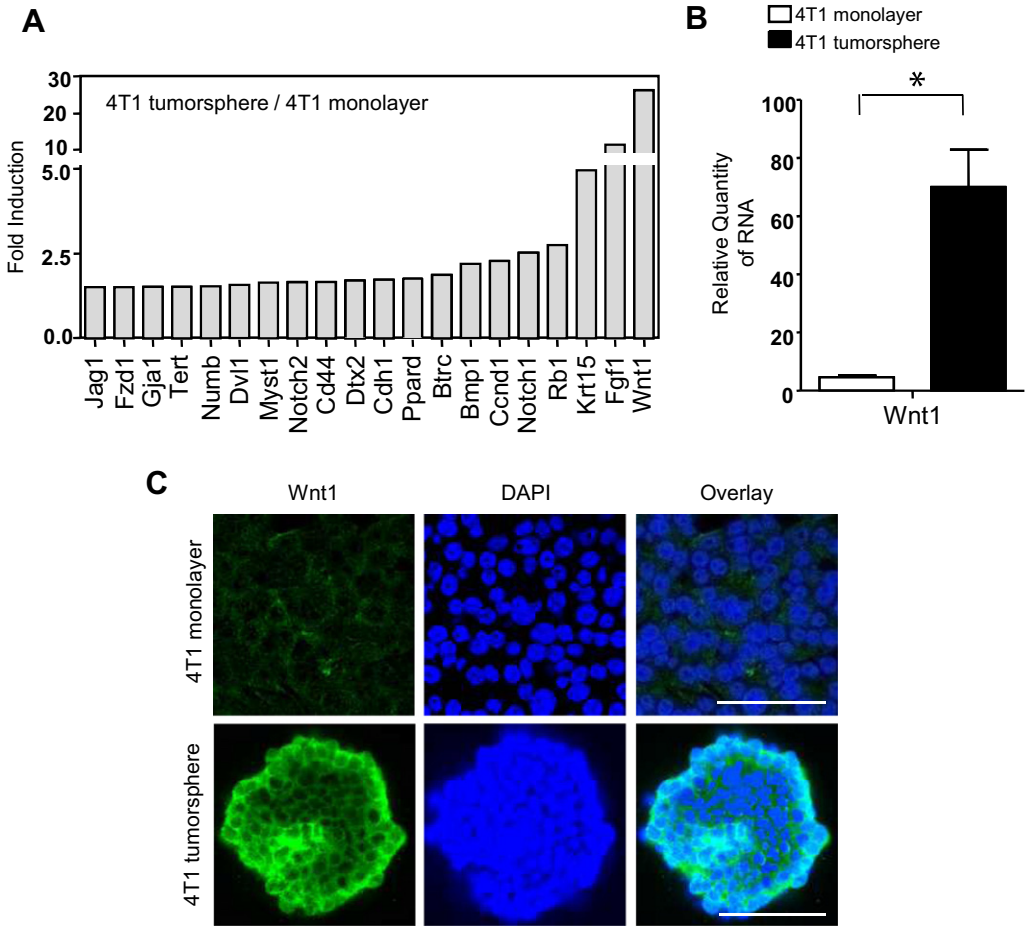


Fig. 1. Wnt1 was overexpressed in 4T1 tumorspheres compared to cells growing in a monolayer. (A) A comparison of stem cell-related gene expression in 4T1 cells grown as tumorspheres or as a monolayer using stem cell PCR array. (B, C) The RT-qPCR and immunofluorescent analysis of Wnt1 expression in 4T1 cells grown as tumorspheres or as a monolayer culture. The values represent the mean ± SD for three independent experiments. *$P < 0.05$. All nuclei were counterstained with DAPI. Scale bar, 100 μm.

greatest inhibition of the target gene mRNA, respectively (Supplementary Table 2 and 3).

2.5. Immunoblotting and immunofluorescence staining

Immunoblotting and immunofluorescence staining were performed as previously described [11]. Immunoblot bands were detected and scanned by the luminescent image analyzer Image Quant LAS-4000 (GE Healthcare, Tokyo, Japan). The immunoblot bands were analyzed using Image J software (NIH, Bethesda, MD) and normalized to β-actin to determine the protein level. Immunofluorescence staining of the tumorspheres was performed as follows: tumorspheres from six-well low attach plates were rinsed in PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. The preparations were permeabilized with 1× Triton X-100 and 0.4 M glycine. The tumorspheres were then centrifuged for 6 min at 800 rpm on glass slides using the cytospin 4 (Thermo Fisher scientific, Waltham, USA) [14,15]. Non-specific antibody binding was blocked with 2% normal swine serum (DAKO, Glostrup, Denmark). The staining was performed as previously described [13] using a primary anti-Wnt1 antibody (dilution 1/200, Abcam, Cambridge, UK). Alexa Fluor 488-conjugated rabbit IgG (Molecular Probes, Eugene, OR) was used to visualize Wnt1. The cells were counterstained with DAPI to identify all nuclei. Samples were examined by fluorescence microscopy (Zeiss LSM 510 Meta). Antibodies against the following proteins were used: Wnt1 (Abcam) and β-actin (Sigma–Aldrich).

2.6. Aldefluor assay

The Aldefluor system (Stem Cell Technologies, Vancouver, Canada) was used to isolate the population with a high aldehyde dehydrogenase (ALDH) enzymatic activity. The cells were stained for ALDH using the Aldefluor reagent according to the manufacturer's instructions and analyzed on a FACSCalibur (Becton Dickinson). As a negative control, separate samples of cell aliquots were treated with 50 μM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. ALDH-positive cells were quantified by calculating the percentage of total fluorescent cells compared with the control stained cells.

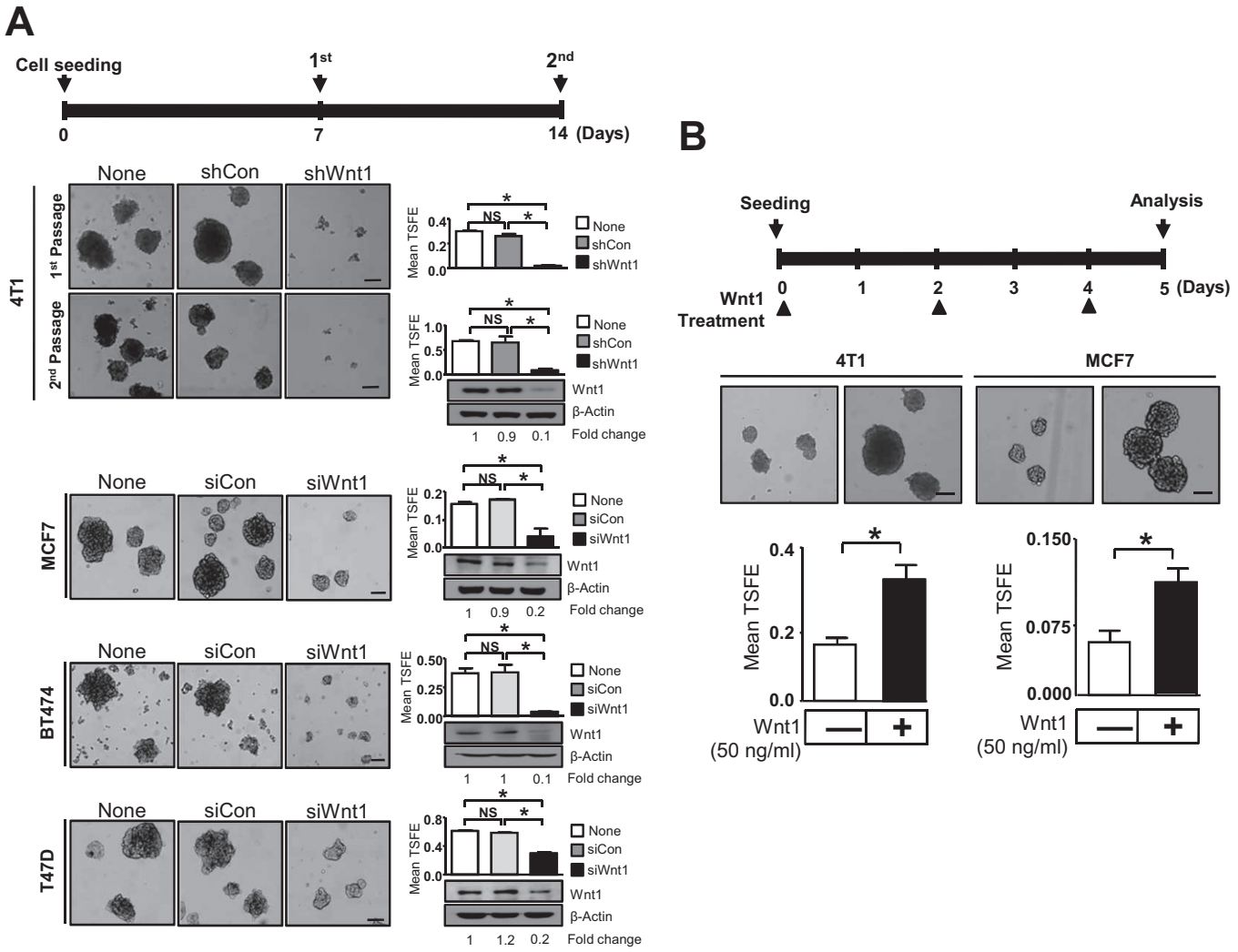


Fig. 2. The effect of Wnt1 expression on tumorsphere formation in breast cancer cells. (A) The analysis of breast cancer tumorsphere formation after knockdown of Wnt1 expression. After Wnt1-targeted shRNA or siRNA transfection under adherent conditions, cells were detached and suspension cultured under serum-free conditions for 7 days. The sphere-forming assay of subsequent passage was derived from 4T1 primary tumorspheres. Immunoblot analysis of Wnt1 expression in breast cancer cell lysates from control cells (None; non-transfected) and cells transfected with non-specific control shRNA or siRNA (shCon or siCon) or Wnt1-targeted shRNA or siRNA (shWnt1 or siWnt1). β-Actin expression was used as a normalization control. Immunoblot data was normalized to β-actin levels using Image J software, and the results are reported as fold changes. (B) The effect of Wnt1 overexpression on the tumorsphere formation of breast cancer cells. The cells were cultured under serum-free conditions with recombinant Wnt1 (50 ng/ml) for 5 days, and the ability to form tumorspheres was determined. The values represent the mean ± SD for three independent experiments. NS, no significance. $^{*}P < 0.05$. Scale bar, 100 μm.

2.7. Animal studies

All mice were maintained according to the IACUC-approved protocols of the Lee Gil Ya Cancer and Diabetes Institute. To analyze tumorigenicity, the left thoracic mammary glands of anesthetized 7-week-old female BALB/c mice (Orient Charles River Technology, Seoul, Korea) were surgically exposed, and cells were resuspended in 50 μl and inoculated into the mouse mammary fat pad (m.f.p.) ($n = 12$ for each group). Mice were euthanized at 21 days, and primary tumors were excised for analysis. The volume of the primary tumor was measured as previously described [11]. For the limiting dilution experiment, primary tumors were minced using scissors and incubated in DMEM (Invitrogen) mixed with collagenase/hyaluronidase (Stem Cell Technologies) at 37 °C for 15–20 min. Primary tumor-derived cells were inoculated into the m.f.p. of mice at varying cell densities ranging from 500 to 10000 cells in a total volume of 50 μl ($n = 10$ for each group). Mice were euthanized at 21 days, and secondary tumors were excised for analysis. The frequency of tumor-initiating cells (TICs) was calculated using ELDA webtool (www.http://bioinf.wehi.edu.au/software/elda), as previously described [12].

2.8. Statistical analysis

All experiments were conducted with a minimum of three samples, and the data are presented as the mean ± SD. Statistical analysis was performed using an unpaired parametric Student's *t* test or a non-parametric Mann–Whitney test, unless otherwise indicated in the text.

3. Results

3.1. Wnt1 was significantly up-regulated in the 4T1 tumorsphere

To identify breast CSC target genes, a stem cell PCR array was performed on 4T1 cells grown as adherent or sphere cultures (Supplementary Table 4). We filtered the data for genes that showed a greater than 1.5-fold change in the average difference between the sphere culture and the adherent culture. The stem cell PCR array revealed that the *Wnt1* gene was expressed at a higher level in the 4T1 sphere culture (4T1 tumorsphere) than in the 4T1 adherent culture (4T1 monolayer) (Fig. 1A). To validate the results of the stem cell PCR array, we performed RT-qPCR and immunofluorescence staining. RT-qPCR analysis confirmed an approximately 15-fold increase in Wnt1 gene expression in the 4T1 tumorsphere compared with the 4T1 monolayer (Fig. 1B). Immunofluorescence staining also showed that Wnt1 expression was higher in 4T1 tumorspheres than in 4T1 monolayer cells (Fig. 1C). These data suggest that Wnt1 may be involved in regulating breast CSCs; therefore, we focused on Wnt1 as a breast CSC target gene.

3.2. Wnt1 expression affected sphere formation by breast cancer cells

To investigate whether the knockdown of Wnt1 expression suppresses breast CSC self-renewal, we used RNA interference technology to knockdown Wnt1 in the 4T1, MCF7, BT474 and T47D breast cancer cell lines. Immunoblotting analysis showed that the expression of the Wnt1 protein was efficiently suppressed in breast cancer cells transfected with Wnt1 shRNA or siRNA (shWnt1 or siWnt1), compared with the parent cells (None, non-transfected cells) and cells transfected with control shRNA or siRNA (shCon or siCon), respectively (Fig. 2A). We found that Wnt1 knockdown significantly reduced tumorsphere-forming efficiency in murine and human breast cancer cell lines, compared with their control counterparts (Fig. 2A). The CSCs can be serially passaged to generate secondary spheres with a cellular composition resembling that of the primary sphere [16]. The secondary tumorspheres generated from the Wnt1 knockdown 4T1 cell-derived tumorspheres were significantly decreased (~3-fold) in number and size, compared with control counterparts. Therefore, these results indicated that Wnt1 knockdown reduced the self-renewal capacity of breast CSCs (Fig. 2A).

To investigate whether, conversely, Wnt1 overexpression enhances tumorsphere formation efficiency, 4T1 and MCF7 cells were cultured in low attachment dishes with recombinant Wnt1 protein (50 ng/ml) for 5 days. The addition of recombinant Wnt1 protein

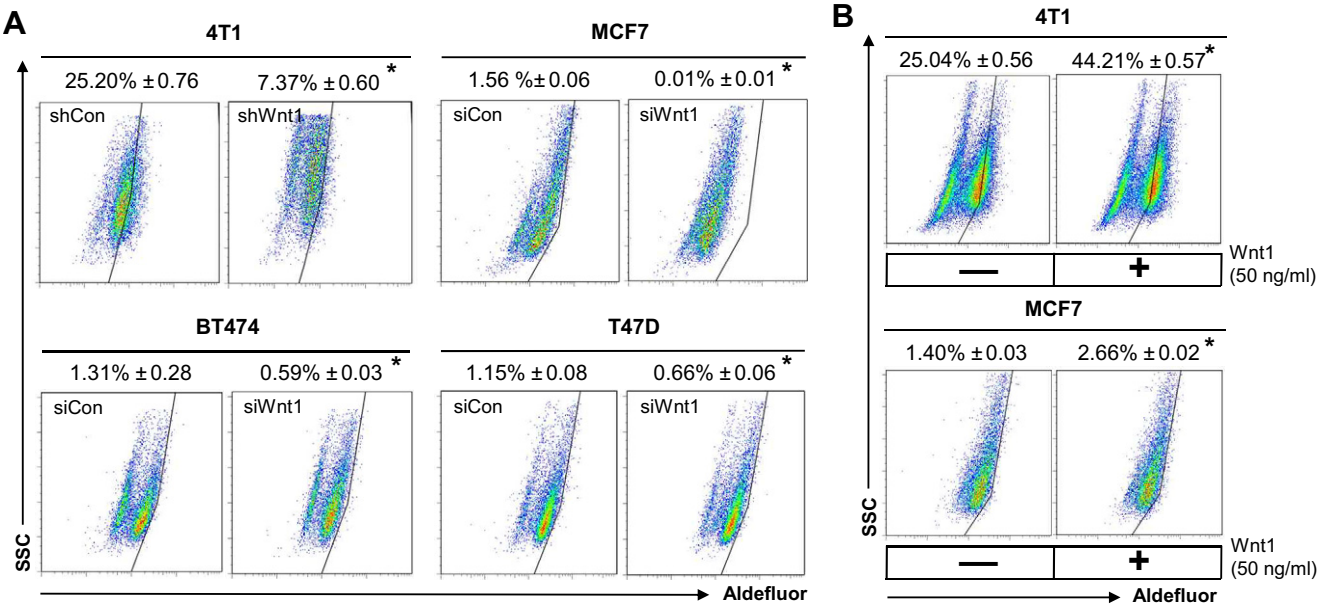


Fig. 3. The effect of Wnt1 expression on ALDH activity in breast cancer cells. (A) The analysis of ALDH activity in breast cancer cells following knockdown of Wnt1 expression. (B) The analysis of ALDH activity in breast cancer cells after treatment with recombinant Wnt1 (50 ng/ml) for 2 days. The size of the Aldefluor-positive population was determined by FACS analysis following knockdown of Wnt1 expression or the addition of recombinant Wnt1 protein. ALDH-positive cells were quantified by calculating the percentage of fluorescent cells compared with a DEAB staining control. The values represent the mean ± SD for three independent experiments. $^*P < 0.05$.

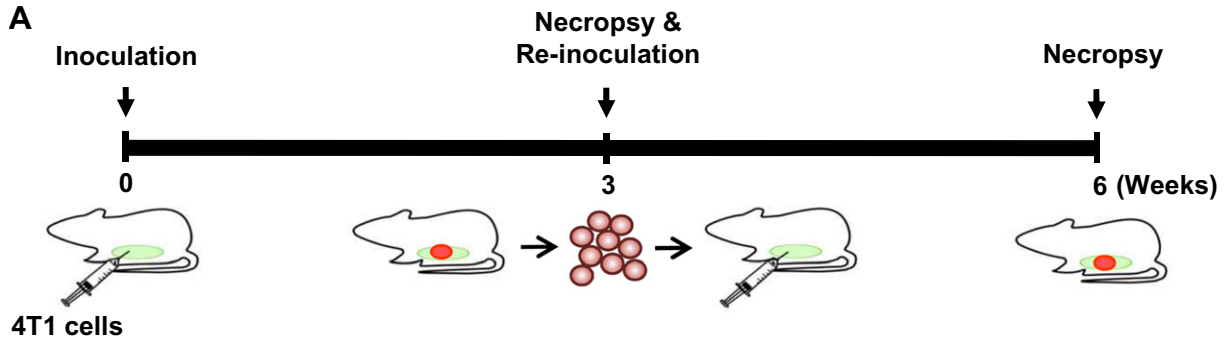
induced the formation of a greater number of tumorspheres that were larger than the control counterparts (Fig. 2B).

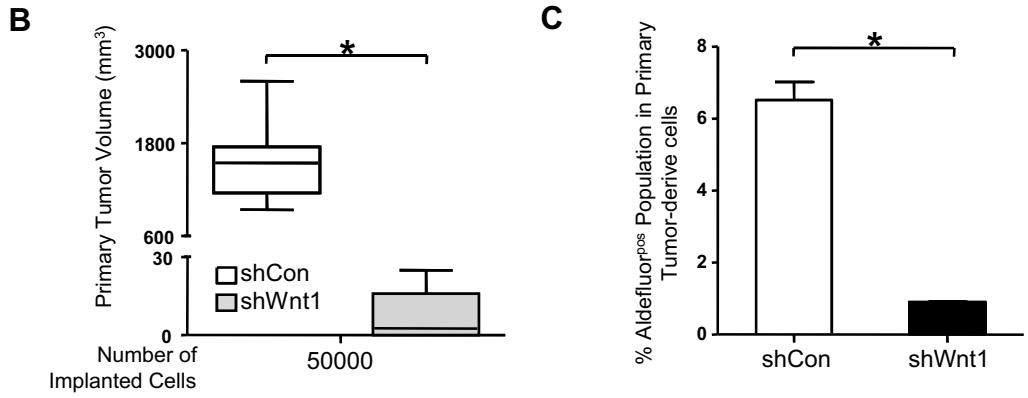
3.3. Wnt1 expression affected the activity of Aldefluor in breast cancer cells

The ALDHs are a group of enzymes that catalyze the oxidation of aldehydes. Several studies have demonstrated that high levels of ALDH activity could serve as a potential marker for CSCs and that the presence of ALDH strongly correlates with tumor malignancy as well as with the self-renewal properties of CSCs in various human cancers, including breast and colon [17,18]. To test whether Wnt1 expression influences ALDH expression in breast cancer cells, we performed an Aldefluor assay. We found that knockdown of Wnt1 expression significantly decreased (~4-fold) Aldefluor activity in 4T1 cells compared with the controls. In addition, we evaluated Aldefluor activity after knockdown of Wnt1 using siRNA in human breast cancer cells, including MCF7, BT474 and T47D cells. Knockdown of Wnt1 also showed a ~1.5–150-fold decrease in Aldefluor activity in human breast cancer cells, compared with the controls (Fig. 3A). Conversely, the addition of recombinant Wnt1 protein increased (~2-fold) the Aldefluor activity in 4T1 and MCF7 cells (Fig. 3B).

3.4. Knockdown of Wnt1 expression resulted in decreased tumorigenic potential and tumor initiation capabilities in vivo

First, to determine whether Wnt1 expression affects tumorigenic potential *in vivo*, shCon- or shWnt1-transfected 4T1 cells were injected into the mammary glands of female BALB/c mice





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2nd generation	Cell type[a]		
Estimated cell frequency	[b]ShCon	[c]ShWnt1	P values[d]
Cell No. of Inoculation, % (n)			
10000	100% (10 of 10)	30% (3 of 10)	3.10E-03
2000	100% (10 of 10)	0% (0 of 10)	<1.00E-4
500	90% (9 of 10)	0% (0 of 10)	1.00E-04
Estimated TIC frequency by ELDA (95% CI)	1/217 (1/484-1/97.7)	1/36439 (1/111825-1/1187.4)	1.42E-19

Fig. 4. The effect of Wnt1 knockdown on stem cell-like properties *in vivo*. (A) The experimental schema. After knockdown of Wnt1 expression, 4T1 cells were injected into the m.f.p. of mice. After 21 days, the primary tumor-derived cells were re-injected into the m.f.p. of a second cohort of mice. (B) Primary tumor growth in the shCon- and shWnt1-transfected 4T1 cells after a 50000 cell inoculation ($n = 12$ for each group). (C) The analysis of ALDH activity in primary tumors derived from shCon- or shWnt1-transfected 4T1 cells. The values (A–B) represent the mean ± SD for three independent experiments. *$P < 0.05$. (D) The effect of Wnt1 knockdown on tumor-initiating ability in the second xenograft mouse model. [a]Primary tumor-derived 4T1 cells were re-injected into the m.f.p. of a second cohort of mice at varying concentrations ($n = 10$ for each group). [b]shCon-transfected cells. [c]shWnt1-transfected cells. The estimated frequency of TICs was determined using ELDA analysis. The *P*-values between cell types for each cell density tested are listed. [d]*P*-values were calculated using Fisher's exact test. ELDA: Extreme limiting dilution analysis; CI: confidence interval.

(Fig. 4A). At the injection dose of 50000 cells, the primary tumors generated by shWnt1-transfected 4T1 cells had ~112-fold less volume than those generated by the shCon-transfected 4T1 cells (Fig. 4B). The Aldefluor activity was significantly decreased (~7-fold) in primary tumors generated by shWnt1-transfected 4T1 cells, compared with the controls (Fig. 4C). Second, to determine whether Wnt1 expression affected tumor recurrence *in vivo*, the primary tumors generated using shCon-, or shWnt1-transfected 4T1 cells were collected, dissociated into single cells and inoculated into the mammary glands of a second cohort of mice at varying cell concentrations, ranging from 500 to 10000 cells (Fig. 4A). The frequency of TICs was determined using the ELDA webtool. ELDA analysis revealed a ~1681-fold lower TIC frequency in secondary tumors generated using shWn1-transfected 4T1-derived tumors, than in shCon-transfected 4T1-derived tumors (Fig. 4D). Therefore, these results suggest that knockdown of Wnt1 expression decreased the frequency of breast CSCs *in vivo*.

4. Discussion

The identification of target molecules involved in the survival and self-renewal of breast CSCs could aid in the development of more effective cancer therapies. To our knowledge, this is the first report to demonstrate that the blockade of Wnt1 signaling is a potentially effective and specific target for breast CSC therapy. A number of data support these findings. First, the knockdown of Wnt1 expression suppressed the stem cell-like properties of CSCs *in vitro* and *in vivo*. Second, Wnt1 overexpression enhanced the stem cell-like properties of CSCs *in vitro*.

Wnt1 (wingless-type MMTV integration site family member 1) belongs to a family of 19 Wnt genes that are involved in Wnt signaling. The first mammalian Wnt gene, *Wnt1*, was originally identified as a gene locus that was activated by retroviral insertion of mouse mammary tumor virus (MMTV), and transgenic *Wnt1* overexpression was subsequently shown to drive the development of mammary tumors in mice [19,20]. Clinical data showed that Wnt1 overexpression was associated with tumor proliferation and a poor prognosis in many types of cancer, including breast and lung [21,22]. In addition, experimental data showed that the blockade of Wnt1 expression with a monoclonal anti-Wnt1 antibody induced tumor-specific apoptosis in colon, liver and breast cancer [23–25]. *In silico* analysis of data obtained from large microarray studies showed that Wnt1 mRNA levels were positively correlated with recurrence of human breast cancer (Supplementary Fig. 1). Therefore, our present work suggests that the contribution of Wnt1 to CSCs may be one of the many mechanisms involved in Wnt1-mediated cancer progression.

Wnt1 stimulates the Wnt/β-catenin signaling pathway, which leads to changes in cell fate and/or cell transformation [26]. The Wnt/β-catenin signaling pathway regulates the transcription of many Wnt-target genes that contain the TCF/LEF1 motif [27]. Consequently, Wnt1 expression could affect biological functions by altering these Wnt-target genes. In our preliminary studies, promoter assays showed that knockdown of Wnt1 expression decreased β-catenin/Tcf4 transcriptional activity in 4T1 cells (Supplementary Fig. 2A). Immunoblotting analysis also showed that endogenous levels of β-catenin/Tcf4 regulated proteins (Cyclin D1, c-Myc and Axin2) were reduced in Wnt1 knockdown 4T1 cells compared with control counterparts (Supplementary Fig. 2B). In embryonic stem cells, overexpression of Wnt1 or overexpression of stabilized β-catenin and the lack of APC results in the inhibition of differentiation and the activation of downstream targets of Wnt signaling, such as cyclins, c-Myc, and BMP [28,29]. Recent reports demonstrated that Wnt signaling has emerged as a critical regulator of Lgr5-positive adult stem cell populations in the intestine and contribute to intestine development and cancer [30]. During development, Wnt signaling plays a critical role in the epithelial-mesenchymal transition (EMT) required for heart cushion development, and aberrant Wnt signaling drives EMT and tumor formation in mouse xenograft models [31,32]. Cells undergoing EMT possess important properties normally found in stem cells, including the acquisition of the $CD44^{+}CD24^{low}$ cell surface marker pattern and the ability to form spheroids in suspension culture [33,34]. Therefore, our present work supports the hypothesis that Wnt1-mediated β-catenin/Tcf4 transcription may affect the maintenance and expansion of CSCs in breast cancer. However, additional studies are necessary to clarify the relative contributions of Wnt1 downstream effectors in CSC maintenance and to obtain a better understanding of how the blockade of Wnt1-associated signaling prevents the maintenance/expansion of CSCs.

Taken together, our results suggest that Wnt1 could enhance tumorigenesis by conferring stem cell properties on breast cancer cells. Therefore, targeting the Wnt1-associated signaling pathway may aid in the development of more effective breast cancer therapies.

Acknowledgment

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the ministry of Education, Science and Technology (2012-0003630).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.120.

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