Research Article

Cancer Prevention Research

Mammary Cancer Chemoprevention by Withaferin A Is Accompanied by *In Vivo* Suppression of Self-Renewal of Cancer Stem Cells

Su-Hyeong Kim and Shivendra V. Singh

Abstract

Current dogma favors elimination of therapy-resistant cancer stem cells for chemoprevention of breast cancer. We showed recently that mammary cancer development in a transgenic mouse model (mouse mammary tumor virus-*neu*; MMTV-*neu*) was inhibited significantly upon treatment with withaferin A (WA), a steroidal lactone derived from a medicinal plant. Herein, we demonstrate that the mammary cancer prevention by WA is accompanied by *in vivo* suppression of breast cancer stem cells (bCSC). *In vitro* mammosphere formation was dose-dependently inhibited by WA treatment in MCF-7 and SUM159 human breast cancer cells. Other markers of bCSC, including aldehyde dehydrogenase 1 (ALDH1) activity and $CD44^{high}/CD24^{low}$/epithelial-specific antigen-positive (ESA+) fraction, were also decreased significantly in the presence of plasma achievable doses of WA. However, WA exposure resulted in cell line–specific changes in *Oct4*, *SOX-2*, and *Nanog* mRNA expression. WA administration to MMTV-*neu* mice (0.1 mg/mouse, 3 times/week for 28 weeks) resulted in inhibition of mammosphere number and ALDH1 activity *in vivo*. Mechanistic studies revealed that although urokinase-type plasminogen activator receptor overexpression conferred partial protection against bCSC inhibition by WA, Notch4 was largely dispensable for this response. WA treatment also resulted in sustained (MCF-7) or transient (SUM159) downregulation of Bmi-1 (B-cell–specific Moloney murine leukemia virus insertion region-1) protein. Ectopic expression of Bmi-1 conferred partial but significant protection against ALDH1 activity inhibition by WA. Interestingly, WA treatment caused induction of Kruppel-like factor 4 (KLF4) and its knockdown augmented bCSC inhibition by WA. In conclusion, this study shows *in vivo* effectiveness of WA against bCSC. *Cancer Prev Res; 7(7); 738–47. ©2014 AACR.*

Introduction

Breast cancer affects thousands of families each year worldwide. Nearly 40,000 women succumb to this disease every year in the United States alone (1). Substantial reduction in mortality and morbidity from breast cancer and improvement in quality of life for women diagnosed with this disease is possible with nontoxic preventive interventions. Currently available preventive interventions, including selective estrogen receptor (ER) modulators (e.g., tamoxifen and raloxifene) and aromatase inhibitors (e.g., exemestane), have undoubtedly demonstrated clinical benefit against ER-positive breast cancers (2–4). These preventive interventions, however, are not perfect for several reasons, including (i) a subset of ER-positive breast cancer is not responsive to some of these interventions (2, 3); (ii) these agents are ineffective against ER-negative or triple-negative breast cancers (2–4), and (iii) selective ER modulators as well as aromatase inhibitors have some side effects (2–6).

Phytochemicals derived from edible and medicinal plants are attractive for chemoprevention of breast and other cancers because of their efficacy in preclinical models and favorable safety profile (7, 8). Protective effect of some of these plants or their constituents against cancer (e.g., isothiocyanates from cruciferous vegetables) is substantiated by population-based epidemiological studies as well as preclinical data in experimental animals (7–9). It is interesting to note that a majority of naturally occurring phytochemicals exhibit selectivity towards cancer cells, which likely contributes to their favorable safety profile (7, 8).

Withania somnifera plant is a key ingredient of the Ayurvedic remedies used in Indian subcontinent for alleviation of different chronic health problems (10, 11). Root extract of *W. somnifera* was shown to be effective for prevention of chemically induced cancer in experimental animals (12, 13). Alleviation of cancer chemotherapy-induced toxicity and fatigue and improvement in quality of life in patients with cancer by administration of *W. somnifera* were also shown (14, 15). Health promoting effects of *W. somnifera* are attributed to steroidal lactones collectively

Authors' Affiliation: Department of Pharmacology and Chemical Biology, University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Corresponding Author: Shivendra V. Singh, 2.32A Hillman Cancer Center Research Pavilion, University of Pittsburgh Cancer Institute, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-3263; Fax: 412-623-7828; E-mail: singhs@upmc.edu

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referred to as withanolides (16). Withaferin A (WA) is one of the withanolides that has been studied extensively for its anticancer properties using cultured cancer cells and xenograft models (8). We showed recently that WA administration resulted in significant inhibition of mammary tumor burden as well as pulmonary metastasis incidence in mouse mammary tumor virus-*neu* (MMTV-*neu*) transgenic mice without any apparent side effects (17). The chemopreventive effect of WA in MMTV-*neu* mice was associated with tumor cell apoptosis induction and inhibition of glycolysis (reversal of Warburg effect; ref. 17). A similar dosing regimen was also effective in retarding growth of MDA-MB-231 human breast cancer xenografts in athymic mice (18). Previous studies have also identified novel targets of WA in breast cancer cells, including FOXO3a (18), complex III of the electron transport chain (17, 19), estrogen receptor-α (20), signal transducer and activator of transcription 3 (21), and Notch family of transcription factors (22).

Recent studies suggest that a small subset of tumor initiating cells or breast cancer stem cells (bCSC), which were first identified by Al-Hajj and colleagues (23), may be responsible not only for tumor initiation and progression but also for treatment failure (24, 25). Consistent with this notion, removal of both therapy-sensitive tumor cells constituting bulk of the tumor mass and bCSC may be necessary to achieve chemopreventive response (24, 25). In this study, we have determined the effect of WA on bCSC using cellular and *in vivo* models of breast cancer.

Materials and Methods

Ethical considerations for animal studies and *in vivo* effect of WA on bCSC fraction

Freshly dissected breast tumor samples from our previous study on mammary cancer chemoprevention by WA in MMTV-*neu* mice (17) were used for *in vivo* analysis of bCSC. Care of animals was consistent with the Institutional Animal Care and Use Committee guidelines. Briefly, mammary cancer incidence and burden were determined in female MMTV-*neu* mice after 28 weeks of intraperitoneal treatment with 0.1 mg WA/mouse (3 times per week) or vehicle (control). The overall tumor incidence was not different between the control and the WA treatment groups (17). However, the palpable tumor size was decreased by 50% upon WA administration in comparison with control ($P = 0.03$ by 2-sided Student *t* test; ref. 17). The mean area of microscopic invasive carcinoma was lower by >95% in the WA treatment group compared with control (17). Mechanistic studies revealed increased apoptosis, inhibition of complex III activity of the electron transport chain, and reduced levels of glycolysis and tricarboxylic acid cycle intermediates in the tumors of WA-treated mice when compared with those of control mice (17).

Reagents and cell lines

WA (purity 99%) was purchased from Enzo Life Sciences and dissolved in dimethyl sulfoxide (DMSO). Final concentration of DMSO for the *in vitro* experiments did not exceed 0.1%. Cell culture medium, fetal bovine serum, and antibiotics were purchased from Invitrogen-Life Technologies. Antibodies against B-cell–specific Moloney murine leukemia virus insertion region-1 (Bmi-1) and Kruppel-like factor 4 (KLF4) were from Cell Signaling Technology, whereas anti-actin and anti-cleaved Notch4 antibodies were purchased from Sigma-Aldrich. Small interfering RNA (siRNA) targeted against Notch4 was purchased from Santa Cruz Biotechnology; KLF4-targeted siRNA was from Abnova, and a control (nonspecific) siRNA was from Qiagen. MCF-7 cell line was purchased from the American Type Culture Collection and last authenticated in February 2012. Frozen stocks of the authenticated MCF-7 cells were used in this study. Monolayer cultures of MCF-7 cells were maintained in MEM supplemented with 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum, and antibiotics. SUM159 cell line was purchased from Asterand and authenticated by the provider. The SUM159 cultures were maintained in Ham's F-12 media supplemented with 5% fetal bovine serum, 1 μg/mL hydrocortisone, 5 μg/mL insulin, and 10 mmol/L HEPES. MCF-7 cells stably transfected with a plasmid encoding for urokinase-type plasminogen activator receptor (uPAR) were generously provided by Dr. S.L. Gonias (University of California, San Diego, CA), and maintained as recommended by the provider (26). MCF-7 cells were stably transfected with empty pcDNA3.1 vector or the same vector encoding for Bmi-1 using FuGENE6. The pcDNA3.1-Bmi-1 plasmid was a generous gift from Dr. M.H. Yang (National Yang-Ming University, Taipei, Taiwan). Clones with stable overexpression of Bmi-1 were selected in the presence of 800 μg/mL of G418 over a period of 8 weeks. Each cell line was maintained at 37°C.

Mammosphere formation assay

Mammosphere assay was performed as described by us previously (27). The first-generation mammospheres of >50 μm in size were scored under an inverted microscope after 5 days of cell seeding. The first-generation mammospheres were disaggregated and single-cell suspensions were replated without further treatment with DMSO or WA for the second-generation mammosphere formation. The second-generation mammospheres were scored after 7 days of cell plating.

Flow cytometric analysis of aldehyde dehydrogenase 1 (ALDH1) activity and $CD44^{high}/CD24^{low}$/epithelial-specific antigen-positive (ESA^+) population

The ALDH1 activity was quantified by using the ALDEFLUOR Assay Kit from Stem Cell Technologies and by following the supplier's instructions. Diethylaminobenzaldehyde (DEAB), a specific ALDH1 inhibitor, was used as a control. The $CD44^{high}/CD24^{low}/ESA^+$ population was analyzed as previously described (27).

Real-time quantitative polymerase chain reaction

Total RNA from cells was isolated using RNeasy Kit (Qiagen). First-strand cDNA was synthesized using superscript reverse transcriptase (Invitrogen-Life Technologies) with

oligo $(dT)_{20}$ primer. Primers for stemness-related genes, including *Oct4*, *Nanog*, and *SOX-2*, are described in Kim and colleagues (27). Relative gene expression was calculated by the method described by Livak and Schmittgen (28).

RT^2 profiler PCR array

MCF-7 cells were treated with 0.5 μmol/L WA or DMSO for 24 or 48 hours. Total RNA was extracted using RNeasy Kit (Qiagen) followed by reverse transcription with 1 μg of total RNA and RT^2 First-Strand Kit (Qiagen) as suggested by the supplier. To evaluate the effect of WA treatment on expression of a panel of genes involved in cancer stemness, Human Cancer Stem Cell RT^2 Profiler PCR Array (Qiagen) was used. Briefly, 25 μL reaction mixture containing cDNA and RT^2 SYBR GREEN ROX qPCR master mix was prepared immediately before the real-time PCR and loaded into each well of the PCR array plate (96-well). Real-time PCR was performed using an ABI StepOne PLUS instrument with 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Change in gene expression was quantitated using web-based software provided by the manufacturer. Gene expression with Ct value above 35 was considered undetectable. The cut-off was at least 1.5-fold change (up- or downregulation) and $P \leq 0.05$ (by Student *t* test).

In vivo analysis of bCSC from MMTV-*neu* tumors

Primary tumors from control and WA-treated MMTV-*neu* mice after their sacrifice (17) were washed with phosphate-buffered saline and digested in DMEM supplemented with 300 units/mL collagenase and 100 units/mL hyaluronidase for 3 to 4 hours at 37°C. The resultant cells were suspended in Hank's balanced salt solution supplemented with 2% fetal bovine serum and ammonium chloride. The cell suspension was resuspended in 0.25% Trypsin-EDTA, 5 mg/mL dispase, 0.1 mg/mL DNase1 in Hank's balanced salt solution and passed through a 40-μm strainer. The cells were used for mammosphere formation assay and determination of ALDH1 activity.

Western blot analysis

Details of cell lysate preparation and Western blotting have been described by us previously (29, 30).

Ectopic expression of Bmi-1 by transient transfection

SUM159 cells were transiently transfected at ~50% confluence with pcDNA3.1 empty vector or the same vector encoding for Bmi-1 using FuGENE6. Twenty-four hours after transfection, the cells were treated with DMSO (control) or WA for 24 hours and then processed for flow cytometric analysis of ALDH1 activity.

RNA interference

Cells were transiently transfected with Notch4-targeted siRNA, KLF4-targeted siRNA, or a control (nonspecific) siRNA using Oligofectamine. After 24 hours, cells were treated with DMSO (control) or WA for 48 (for Notch4) or 72 hours (for KLF4). Subsequently, the cells were collected and processed for flow cytometric analysis of ALDH1 activity or mammosphere formation.

Statistical analyses

One-way ANOVA with Dunnett's adjustment or multiple comparisons tests were used to determine statistical significance of difference between groups. Student *t* test was used for binary comparisons. Difference was considered significant at $P < 0.05$.

Results

WA treatment inhibited self-renewal of bCSC *in vitro*

MCF-7 (ER-positive) and SUM159 (triple-negative) human breast cancer cell lines were used to determine the *in vitro* effect of WA on bCSC population. Figure 1A shows representative first-generation mammospheres resulting from MCF-7 and SUM159 cells after 5 days of cell seeding and treatment with DMSO or WA. Of note, the WA concentrations used herein were well within the plasma achievable level of 1.8 μmol/L based on a pharmacokinetic study in mice (31). WA treatment resulted in a dose-dependent and statistically significant decrease in first- and second-generation mammosphere frequency in both cell lines (Fig. 1B). These results indicated inhibition of bCSC self-renewal by WA treatment.

Inhibitory effect of WA treatment on bCSC fraction was confirmed by flow cytometric analysis of ALDH1 activity and $CD44^{high}/CD24^{low}/ESA^{+}$ fraction. The ALDH1 activity was decreased significantly in the presence of 0.5 μmol/L of WA in both cell lines when compared with control (Fig. 1C). In comparison with DMSO-treated control, the $CD44^{high}/CD24^{low}/ESA^{+}$ fraction was significantly lower in the WA-treated MCF-7 (0.5 μmol/L WA) and SUM159 (0.25 and 0.5 μmol/L WA) cultures (Fig. 1D).

Expression of stemness-related genes, including *Oct4*, *SOX-2*, and *Nanog*, was determined after 24- or 72-hour treatment of MCF-7 and SUM159 cells with DMSO or WA (0.25 or 0.5 μmol/L). The expression of only *SOX-2* mRNA was reduced dose dependently after 24-hour treatment with WA in MCF-7 cells; but this effect was abolished at the 72-hour time point (Fig. 2A). In SUM159 cells, reduced expression of *Oct4*, *SOX-2*, and *Nanog* was clearly evident at the 72-hour time point with 0.5 μmol/L of WA (Fig. 2A).

Because of cell line–specific differences, targeted PCR array was performed for a panel of cancer stemness-related genes using MCF-7 cells (Fig. 2B). Expression of several genes was affected significantly ($P \leq 0.05$ by 2-sided Student *t* test) by 1.5-fold upon 24- or 48-hour treatment with WA (Fig. 2B). In agreement with data shown in Fig. 2A, *SOX-2* expression was significantly decreased after 24-hour treatment of MCF-7 cells with WA when compared with control. Collectively, these results provided evidence for *in vitro* inhibition of bCSC by WA treatment in MCF-7 and SUM159 cells.

WA administration suppressed bCSC in tumors of MMTV-*neu* mice

We have shown previously that WA administration significantly reduces mammary tumor burden in MMTV-*neu*

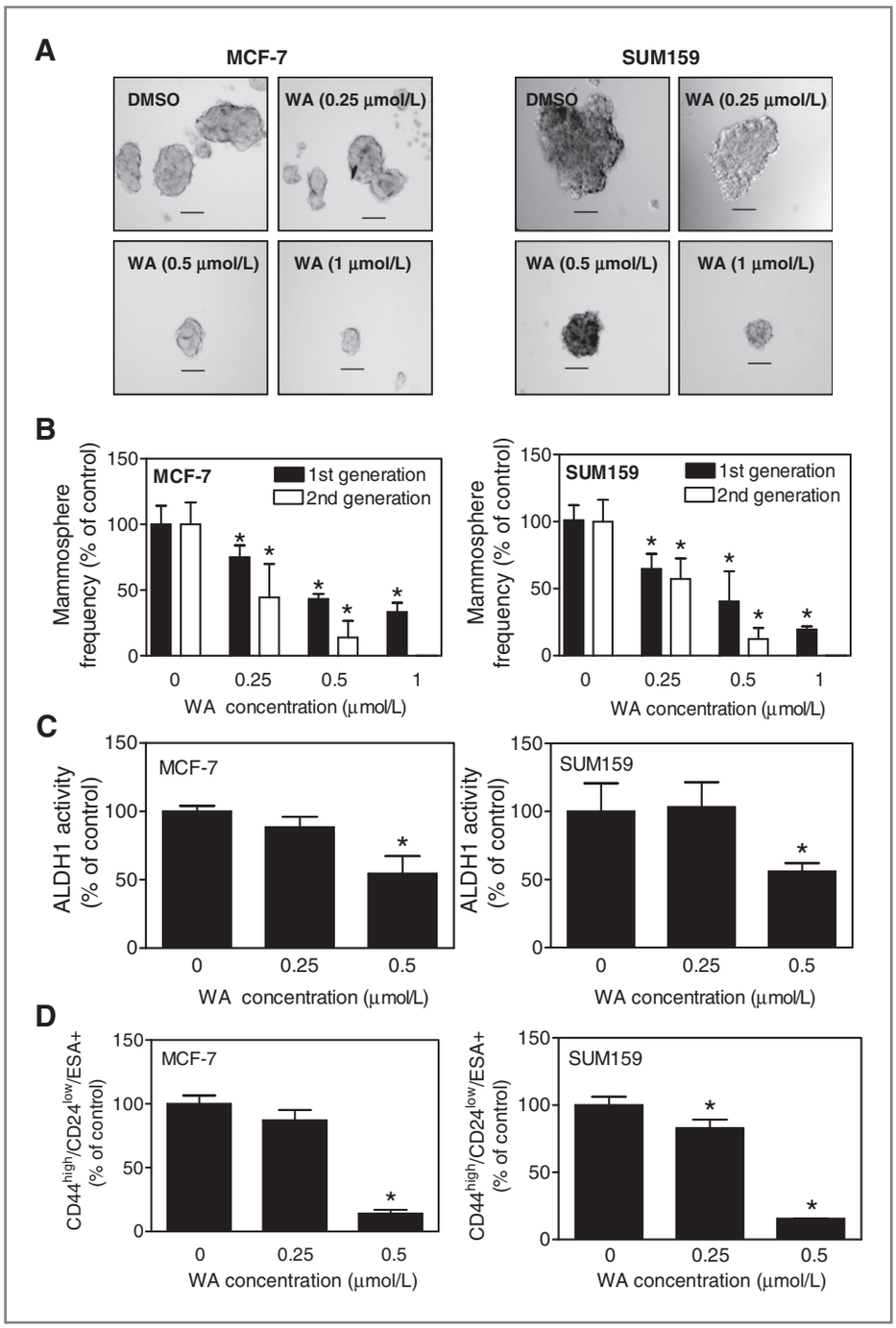

Figure 1. WA treatment inhibited bCSC *in vitro* in MCF-7 and SUM159 human breast cancer cells. A, representative images of first-generation mammospheres after treatment with DMSO or WA (×50 magnification; scale bar = 100 μm). B, percentage of first-generation and second-generation mammospheres relative to DMSO-treated control. C, percentage of ALDH1 activity relative to DMSO-treated control (72-hour treatment with DMSO or WA) in MCF-7 and SUM159 cells. D, quantitation of $CD44^{high}/CD24^{low}/ESA^{+}$ population relative to DMSO-treated control (72-hour treatment with DMSO or WA) in MCF-7 and SUM159 cells. The results shown (mean ± SD, $n = 3$) are representative of at least 2 independent experiments. *, significantly different ($P < 0.05$) compared with DMSO-treated control by one-way ANOVA with Dunnett's adjustment.

mice (17). Freshly collected tumors from 5 mice of each group (at the time of sacrifice) from this study were used to determine the *in vivo* effect of WA on bCSC. Figure 3A depicts primary mammospheres resulting from representative tumor of a control mouse and that from a WA-treated MMTV-*neu* mouse after 7 days of cell plating. The Number of mammospheres from tumors of WA-treated mice was lower compared with control at each cell density, but the difference was significant only at 5,000 cell density (Fig. 3B). The mean mammosphere size from tumors of WA-treated mice was also smaller by about 30% compared with control; although the difference was not significant because of large data scatter especially in the control group (Fig. 3C). Five tumors from each group were also used for measurement of ALDH1 activity. The ALDH1 activity was unusually very high in one tumor sample from the control group, and this

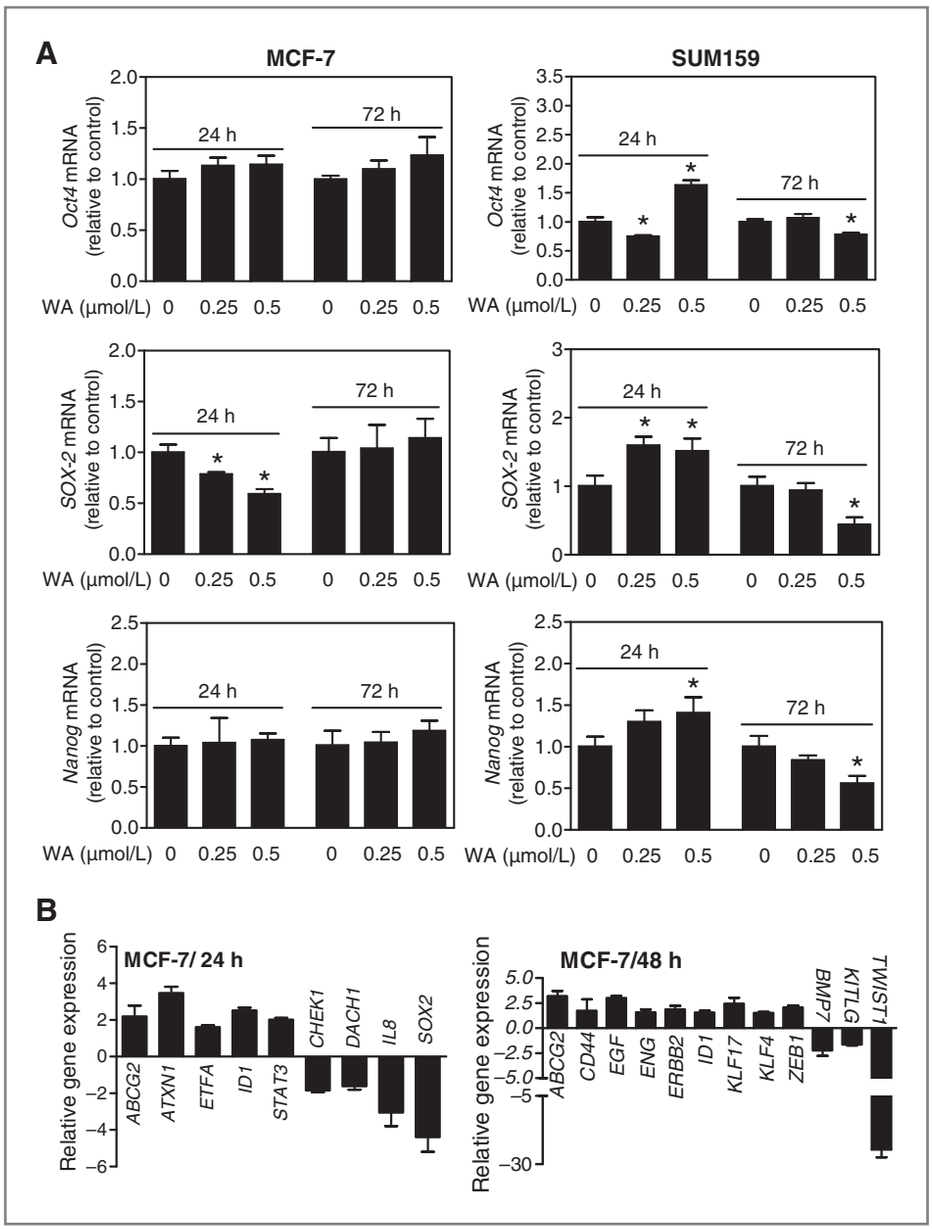

Figure 2. WA treatment decreased mRNA expression of stemness-related genes in MCF-7 and SUM159 cells. A, quantitation of *Oct4*, *SOX-2*, and *Nanog* mRNA expression by qPCR in MCF-7 and SUM159 cells relative to DMSO control. The results shown are mean ± SD ($n = 3$). *, significantly different ($P < 0.05$) compared with control by one-way ANOVA with Dunnett's adjustment. Representative data from replicate experiments are shown. B, changes in expression of genes related to cancer stemness in MCF-7 cells after treatment with 0.5 μmol/L WA when compared with DMSO-treated control (cut-off 1.5-fold change and $P \leq 0.05$). The results shown are mean ± SD ($n = 3$).

sample was not included in the statistical analysis. The ALDH1 activity was lower by about 44% ($P < 0.05$ by Student *t* test) in the tumors from WA treatment group in comparison with the control (Fig. 3D). Together, these results provided evidence for *in vivo* inhibition of bCSC by WA.

Effect of uPAR overexpression on WA-mediated inhibition of bCSC

Overexpression of uPAR alone is sufficient to drive stemness in MCF-7 cells (26). Overexpression of uPAR in MCF-7 cells was confirmed by Western blotting (results not shown). Consistent with published findings (26), mammosphere number was markedly higher from uPAR overexpressing MCF-7 cells compared with vector control (Fig. 4A and B). WA treatment inhibited mammosphere number in both empty vector transfected control cells and uPAR overexpressing MCF-7 cells (Fig. 4B). However, the difference in mammosphere number in the presence of WA between empty vector transfected cells and uPAR overexpressing MCF-7 cells was not significant except at 500 and 2,000 cell density with 0.5 μmol/L WA. WA treatment caused about 69% decrease in $CD44^{high}/CD24^{low}/ESA^{+}$ fraction compared with DMSO control in empty vector transfected MCF-7 cells (Fig. 4C). Percent decrease in $CD44^{high}/CD24^{low}/ESA^{+}$ fraction after WA treatment in uPAR overexpressing MCF-7 cells was about 51% and the difference was significant from empty vector transfected MCF-7 cells

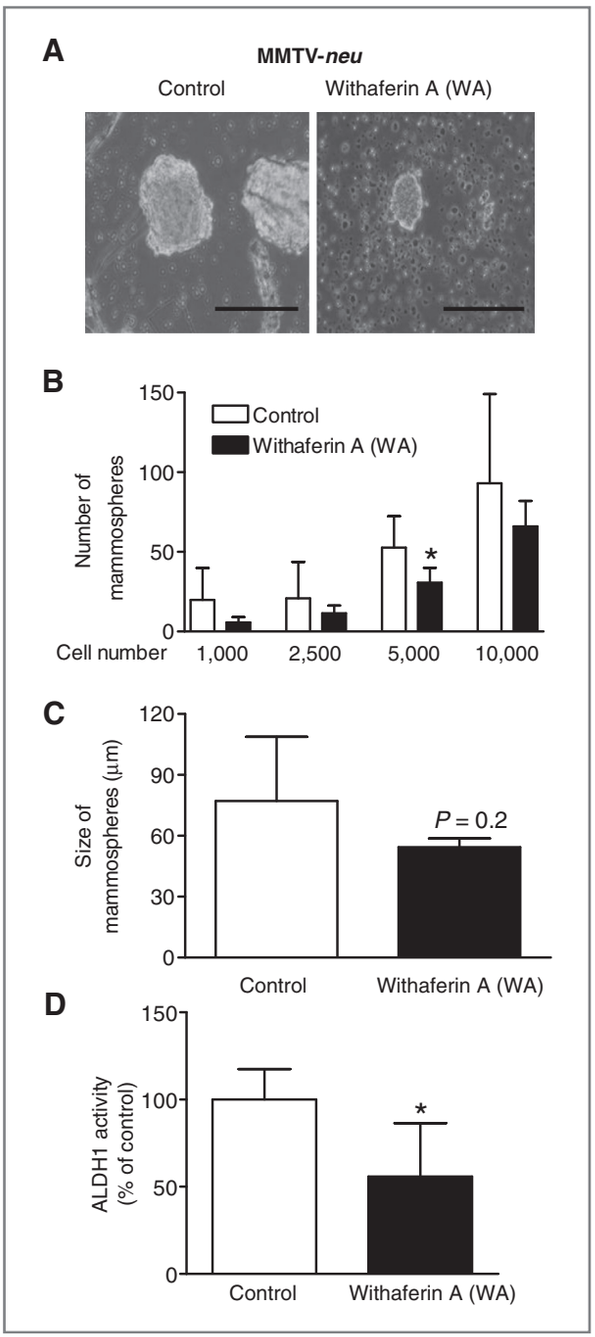

Figure 3. Mammary cancer chemoprevention by WA administration in MMTV-*neu* mice was accompanied by *in vivo* inhibition of bCSC. A, representative photomicrographs showing mammospheres formed by isolated primary tumor cells from MMTV-*neu* transgenic mice (×100 magnification, scale bar = 200 μm). B, mammosphere number from tumor cells of control and WA-treated MMTV-*neu* mice. Results shown are mean ± SD ($n = 5$, except for 1,000 cells where mammospheres were observed only from 2 to 3 samples). C, the bar graph shows quantitation of mammosphere size from the 5,000 cell density group. Mammosphere size was determined from at least 5 nonoverlapping regions of each sample. D, flow cytometric quantitation of ALDH1 activity in tumor cells (relative to control) from control and WA-treated ($n = 4$–5) MMTV-*neu* mice. The results shown are mean ± SD. *, significantly different ($P < 0.05$) compared with control by Student *t* test.

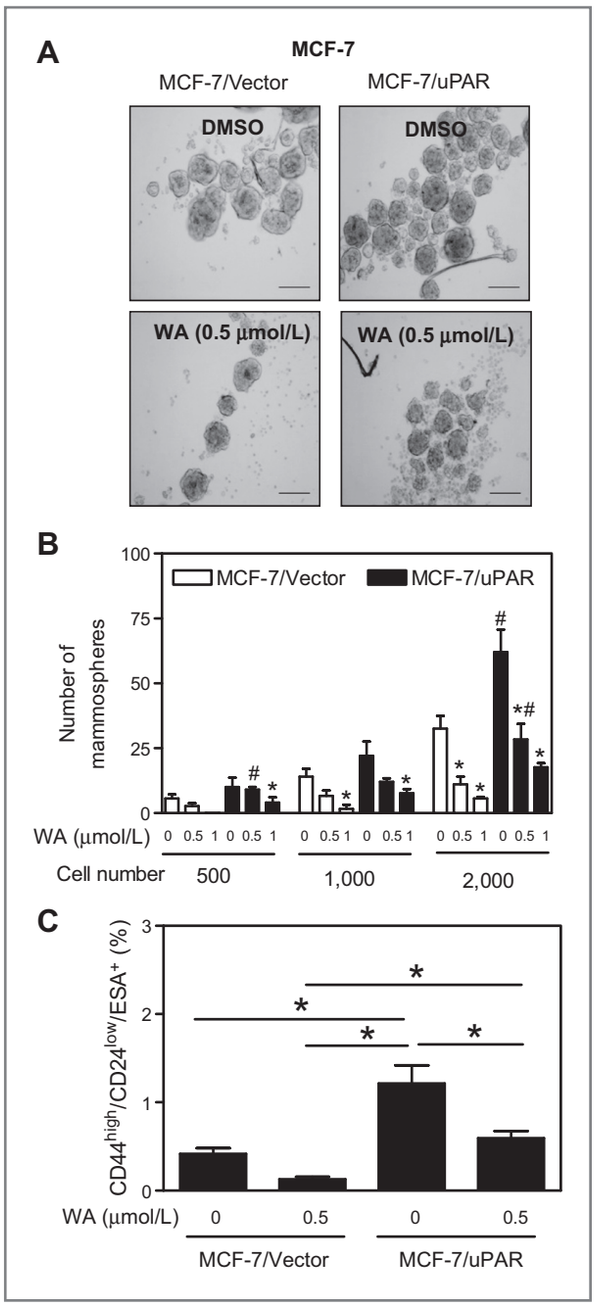

Figure 4. uPAR overexpression conferred partial protection against bCSC inhibition by WA. A, representative photomicrographs depicting first generation mammospheres from MCF-7 cells stably transfected with empty vector (MCF-7/Vector) or uPAR plasmid (MCF-7/uPAR) after 5 days of cell seeding and treatment with DMSO or WA (×50 magnification; scale bar = 100 μm). B, mammosphere number in MCF-7/Vector or MCF-7/uPAR cells. Significantly different ($P < 0.05$) compared with (*) respective DMSO control and (#) between MCF-7/Vector and MCF-7/uPAR cells by one-way ANOVA followed by the Bonferroni test. C, percentage of $CD44^{high}/CD24^{low}/ESA^{+}$ fraction in MCF-7/Vector and MCF-7/uPAR cells after 72-hour treatment with DMSO or 0.5 μmol/L WA. The results shown are mean ± SD ($n = 3$). *, significantly different ($P < 0.05$) between the identified groups by one-way ANOVA followed by the Bonferroni test. The experiments were repeated twice with comparable results.

(Fig. 4C). Based on these results, we conclude that uPAR overexpression confers partial protection against bCSC inhibition by WA.

The role of Bmi-1 in WA-mediated inhibition of bCSC

The polycomb group protein Bmi-1 has been implicated in self-renewal of bCSC (32). The level of Bmi-1 protein was decreased markedly after treatment of MCF-7 cells with WA (Fig. 5A). Stable overexpression of Bmi-1 protein in MCF-7 cells was confirmed by Western blotting (Fig. 5B). Treatment with 0.5 μmol/L WA for 72 hours resulted in a significant decrease (64% decrease compared with DMSO-treated control; $P < 0.05$) in ALDH1 activity in empty vector transfected MCF-7 cells (Fig. 5C). The ALDH1 activity was about 1.6-fold higher in Bmi-1 overexpressing MCF-7 cells compared with the empty vector transfected cells in the absence of WA treatment (Fig. 5C). The ALDH1 activity was decreased by only about 31% compared with DMSO control after 72-hour treatment of Bmi-1 overexpressing MCF-7 cells with 0.5 μmol/L WA (Fig. 5C). The difference in ALDH1 activity in the presence of WA was statistically significant between empty vector transfected cells and Bmi-1 overexpressing MCF-7 cells (Fig. 5C).

WA-mediated decrease in Bmi-1 protein level was transient in SUM159 cells (24 hours only; Fig. 5A). However, a 2-fold overexpression of Bmi-1 protein in SUM159 cells after transient transfection (Fig. 5B) resulted in significant abrogation of ALDH1 activity inhibition by WA (Fig. 5C). Collectively, these results indicated that Bmi-1 suppression was partly responsible for bCSC inhibition resulting from WA exposure.

Notch4 activation by WA was dispensable for its inhibitory effect on bCSC

Signaling through the Notch4 receptor has been implicated in regulation of bCSC activity (33). Previous work

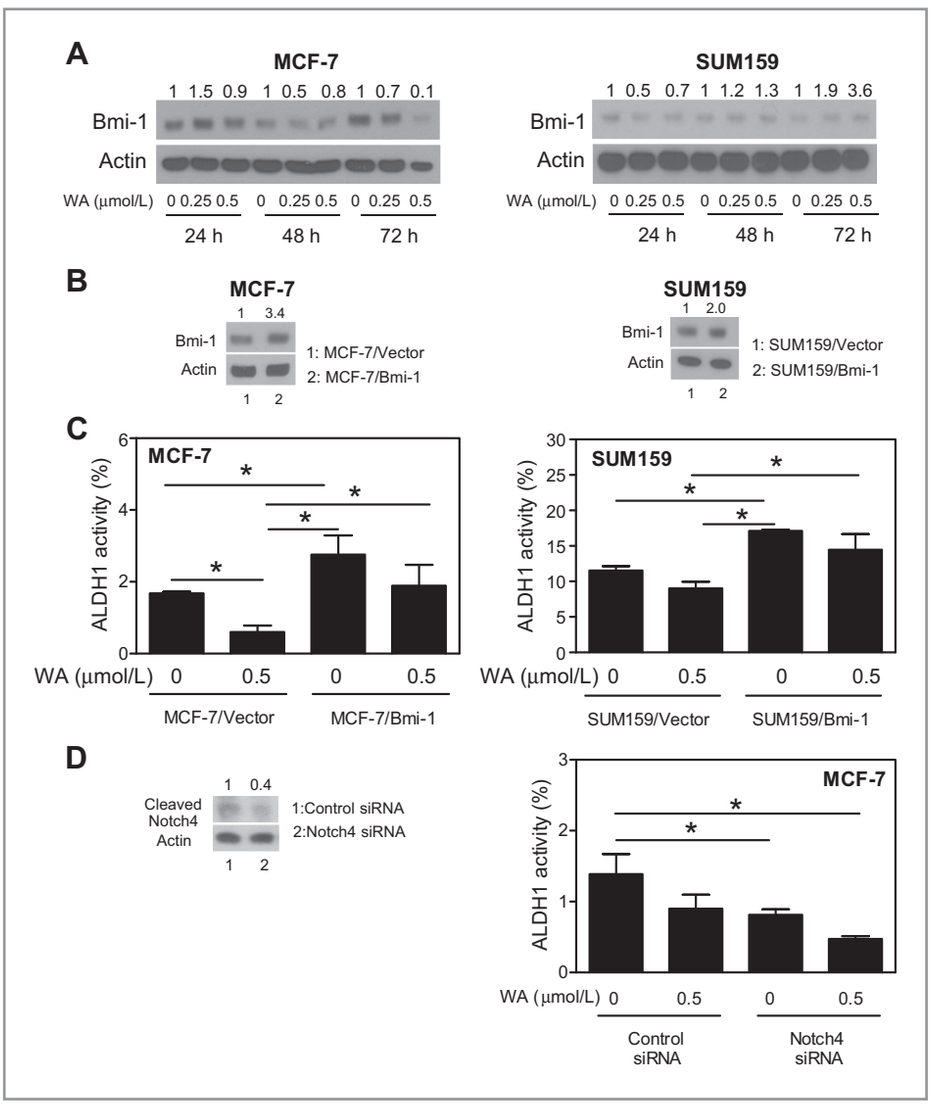

Figure 5. Ectopic expression of Bmi-1 conferred partial protection against ALDH1 activity inhibition by WA. A, Western blotting for Bmi-1 protein using cell lysates from MCF-7 and SUM159 cells after treatment with DMSO or WA. Densitometric quantitation relative to respective DMSO control and after normalization for protein loading (actin) is shown above the band. B, Western blotting for protein levels of Bmi-1 in MCF-7 (stable transfection) or SUM159 cells (transient transfection) transfected with empty vector (lane 1) or Bmi-1 plasmid (lane 2). C, percentage of ALDH1 activity in empty vector transfected or Bmi-1 overexpressing MCF-7 and SUM159 cells after 72 (MCF-7) or 24 hours (SUM159) treatment with DMSO or 0.5 μmol/L WA. The results shown are mean ± SD ($n = 3$). *, significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA with Tukey *post hoc* analysis. D, ALDH1 activity in MCF-7 cells transfected with a control siRNA or a Notch4 targeted siRNA after 48-hour treatment with DMSO or 0.5 μmol/L WA. The Western blot analysis shows knockdown of cleaved Notch4. The results shown are mean ± SD ($n = 3$). *, significantly different ($P < 0.05$) between the indicated groups by one-way ANOVA followed by the Bonferroni multiple comparison test. Comparable results were observed in 2 independent experiments.

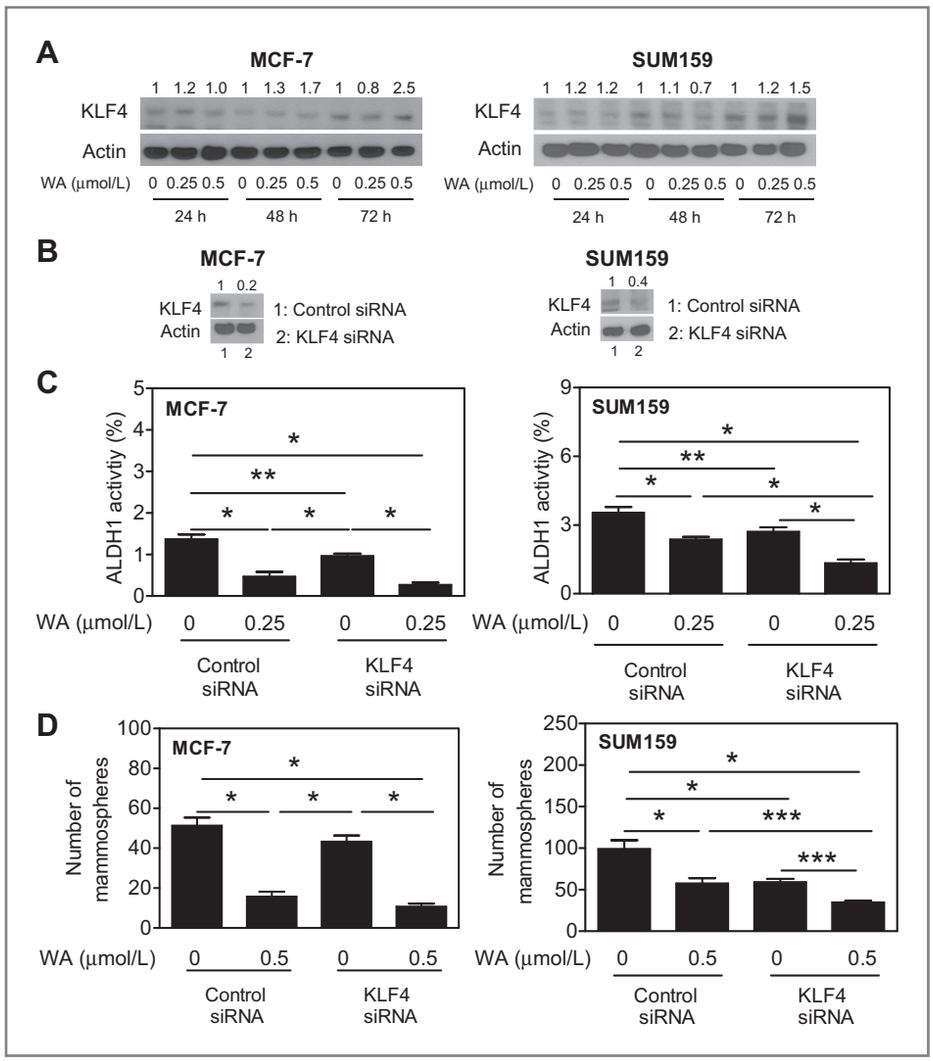

Figure 6. KLF4 status affected WA-mediated inhibition of bCSC in SUM159 cells. A, Western blotting for KLF4 protein using cell lysates from MCF-7 and SUM159 cells after treatment with DMSO or WA. Densitometric quantitation relative to respective DMSO control and after normalization for protein loading (actin) is shown above the band. B, Western blotting for KLF4 protein expression in MCF-7 and SUM159 cells transfected with a control siRNA (lane 1) or KLF4 siRNA (lane 2). C, percentage of ALDH1 activity in MCF-7 and SUM159 cells transiently transfected with a control siRNA or a KLF4-targeted siRNA after 72-hour treatment with DMSO or WA. D, number of primary mammospheres (after 5 days of cell seeding and treatment with DMSO or WA) from MCF-7 and SUM159 cells transfected with a control siRNA or KLF4 siRNA. The results shown are mean ± SD ($n = 3$). Significantly different (*, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$) between the indicated groups by one-way ANOVA followed by the Bonferroni multiple comparison test. Each experiment was repeated twice with comparable results.

from our own laboratory has indicated cleavage (activation) of Notch4 upon treatment of breast cancer cells with WA (22). Thus, it was of interest to determine if Notch4 activation by WA affected its activity against bCSC. Knockdown of Notch4 itself inhibited ALDH1 activity by about 41% in MCF-7 cells (Fig. 5D). However, knockdown of Notch4 did not significantly augment WA-mediated inhibition of ALDH1 activity.

KLF4 knockdown augmented WA-mediated inhibition of bCSC

KLF4 was required for maintenance of bCSC and mammary cancer cell migration and invasion (34). Because expression of *KLF4* was modestly but significantly increased after 48-hour treatment of MCF-7 cells with WA (Fig. 2B), we first determined the effect of WA on KLF4 protein level using MCF-7 and SUM159 cells (Fig. 6A). WA treatment resulted in induction of KLF4 protein in MCF-7 and SUM159 cells especially at the 72-hour time point with 0.5 μmol/L dose (Fig. 6A). Expression of KLF4 was decreased by 60% to 80% in MCF-7 and SUM159 cells transfected with the KLF4-targeted siRNA compared with corresponding control siRNA transfected cells (Fig. 6B). Knockdown of KLF4 itself significantly inhibited ALDH1 activity in both MCF-7 and SUM159 cells (Fig. 6C). In addition, KLF4 knockdown significantly augmented WA-mediated inhibition of ALDH1 activity at least in the SUM159 cells; a similar trend was discernible in the MCF-7 cells but the difference was not significant (Fig. 6C). Mammosphere inhibition by WA treatment was also significantly augmented by RNA interference of KLF4 in the SUM159 cell line (Fig. 6D). Together, these results indicated that KLF4 induction by WA treatment modestly impeded its inhibitory effect on bCSC.

Discussion

An emerging hypothesis argues for removal of tumor cells constituting bulk of the tumor mass as well as bCSC for effective prevention and treatment of breast cancers (24, 25). The role of bCSC in mammary carcinogenesis has

been reviewed extensively (24, 25), but a few examples to support its role in cancer metastasis follow: (i) presence of CD44-positive/CD24$^{-/low}$ fraction in primary human breast tumors may favor distant metastasis (35), and (ii) the ALDH1 positivity has been shown to correlate with metastasis and poor clinical outcome in inflammatory breast cancers (36). This study demonstrates, for the first time, that WA administration inhibits self-renewal capacity of bCSC *in vivo* in HER-2–driven mammary cancer. Mechanistic studies demonstrate a role for uPAR and the polycomb group protein Bmi-1 in bCSC inhibition by WA.

We show that WA can overcome Notch4 activation for inhibition of bCSC. These observations have clinical ramifications as Notch4 is implicated in regulation of bCSC (33). Notch4 activity is significantly higher in bCSC in comparison with differentiated cancer cells (33). Pharmacologic as well as genetic inhibition of Notch4 decreases stem cell activity *in vitro* and reduces tumor formation *in vivo* (33). In addition, Notch4 overexpression has been observed in triple-negative human breast cancer specimens (37). This study reveals decrease in ALDH1 activity after Notch4 knockdown in MCF-7 cells. However, knockdown of Notch4 protein does not synergize with WA for inhibition of ALDH1 activity.

KLF4, a zinc finger transcription factor, plays an important role in regulating various cellular processes, including apoptosis, migration, and caner stem cells (34, 38). Earlier studies have shown that KLF4 can either function as a tumor suppressor or can be oncogenic depending on type of cancer (38). However, many studies point to an oncogenic role for KLF4 in breast cancer. For example, >70% of breast cancers exhibit elevated KLF4 expression and increased nuclear staining for KLF4 is associated with an aggressive phenotype in early-stage breast cancer (39, 40). Second, KLF4 knockdown inhibits mammary tumor development *in vitro* and *in vivo* as well as suppresses migration and bCSC fraction (34). In agreement with these published findings, results shown herein demonstrate an oncogenic role for KLF4 in breast cancer cells. Knockdown of KLF4 itself inhibits stemness in MCF-7 and SUM159 cells, but only SUM159 cells exhibit augmentation with WA for inhibition of ALDH1 activity and formation of mammospheres. Even though the molecular basis for differential sensitivity of MCF-7 versus SUM159 cells to WA-mediated augmentation of bCSC inhibition by KLF4 knockdown is still unclear, these observations have clinical implications. SUM159 is a triple-negative cell line and triple-negative breast cancers have worse prognosis (41). We are tempted to speculate that WA likely exhibits greater efficacy against triple-negative breast cancers. Although validation of this contention requires further experimentation, our more recent studies have revealed that the SUM159 cell line (IC_{50} after 48-hour treatment—about 1 μmol/L) is relatively more sensitive to cell-proliferation inhibition by WA in comparison with MCF-7 cells (IC_{50} after 48-hour treatment—>2 μmol/L; ref. 42).

A clinically viable preventive intervention targeting both ER-positive and ER-negative breast cancers is still not available. This study together with our previous observations (17) suggest that WA may be useful for prevention of hormone-dependent as well as hormone-independent breast cancers because: (i) WA administration significantly inhibits ER-negative mammary cancer development in MMTV-*neu* mice (17); (ii) incidence and multiplicity of methylnitrosourea-induced rat mammary cancer, which is ER-positive, is reduced by gavage with *W. somnifera* root extract (13); (iii) WA functions as an anti-estrogen and proapoptotic effect of WA is partially but significantly attenuated by overexpression of ER-α in MDA-MB-231 cells (20); and (iv) WA treatment inhibits self-renewal of bCSC in both ER-positive (MCF-7) and triple-negative (SUM159) breast cancer cells (this study).

This study reveals cell line–specific differences in the mechanisms by which WA may inhibit bCSC. A molecular target approach (43) may be necessary to gain insights into the mechanisms underlying cell line–specific differences. As suggested by Lee and colleagues (43), the overall approach for identification of molecular target(s) of phytochemicals may involve RNA interference screen followed by validation using *in vitro*, *ex vivo*, and/or *in vivo* models. *In silico* virtual screening based on natural product libraries and diverse ligand databases, including the ZINC database (which contains "ready-to-dock" searchable libraries of 4.6 million 3-dimensional compounds) and the Asinex database, is also useful for identification of potential chemopreventive phytochemicals.

In conclusion, the results of this study indicate that: (i) WA treatment inhibits bCSC *in vitro* in MCF-7 and SUM159 cells; (ii) mammary cancer prevention by WA administration in MMTV-*neu* mice is associated with inhibition of self-renewal of bCSC *in vivo*; and (iii) Bmi-1 plays a role in WA-mediated inhibition of ALDH1 activity in MCF-7 and SUM159 cells, whereas KLF4 status affects bCSC inhibition by WA in SUM159 cell line. The results shown herein together with our previously published *in vivo* efficacy data (17, 18) warrants clinical investigation of WA for prevention and/or treatment of human breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.-H. Kim, S.V. Singh
Development of methodology: S.-H. Kim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-H. Kim
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.-H. Kim, S.V. Singh
Writing, review, and or revision of the manuscript: S.-H. Kim, S.V. Singh
Study supervision: S.V. Singh

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