The Role of Versican in Modulating Breast Cancer Cell Self-renewal

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Abstract

Versican is highly expressed during the early stages of tissue development and its expression is elevated during wound repair and tumor growth. There is little literature on the potential role of breast cancer stem cells on the cellular–extracellular matrix interactions involving versican. An anti-versican short hairpin RNA (shRNA) was used to observe the effect of reduction of versican on breast cancer self-renewal. A versican G3 construct was exogenously expressed in breast cancer cell lines. Colony formation and mammosphere formation assays were conducted; flow cytometry was applied to analyze the prevalence of side population cells. The versican G3- and vector-transfected 66c14 cells were injected transdermally into BALB/c mice as a 10-fold dilution series from $1 \times 10^2$ to $1 \times 10^5$ cells per mouse. Versican G3 domain enhanced breast cancer self-renewal in both experimental in vitro and in vivo models. Versican G3–transfected cells contained high levels of side population cells, formed more mammospheres when cultured in the serum-free medium, and formed a greater number and larger colonies. Reduction of versican’s functionality through anti-versican shRNA or knocking out the EGF-like motifs reduced the effect of versican on enhancing mammosphere and colony formation. Versican-enhanced self-renewal played a role in enhanced chemotherapeutic drug resistance, relating partly to the upregulated expression of EGF receptor (EGFR) signaling. Versican is highly expressed in breast cancer progenitor cells and was maintained at high levels before cell differentiation. Overexpression of versican enhanced breast cancer self-renewal through EGFR/AKT/GSK-3β (S9P) signaling and conferred resistant to chemotherapeutic drugs tested. Mol Cancer Res; 11(5); 1–13. ©2013 AACR.

Introduction

Experimental and clinical evidence support the understanding that tumorigenesis is sustained by a small population of cells that function as tumor stem/progenitor cells (1, 2). In breast cancer, these cells arise from mutated mammary stem/progenitor cells, which have been characterized by the expression of key cell surface markers (e.g., CD24, CD44, CD29, or Sca-1) as well as dye efflux assays based on the differential release of incorporated Hoechst dyes or rhodamine-121 through overexpressed or overactivated drug transporters in the cell membrane (3–5). These tumor progenitor cells are characterized by their ability to form new serially transplantable tumors in mice and to display stem/progenitor cell properties such as competence for self-renewal and the capacity to reestablish tumor heterogeneity (6, 7). In addition to flawed regulation of the self-renewal pathways, the number of cells within a tumor that have the ability to self-renew is constantly expanding, resulting in a continuous expansion of tumorigenic cancer cells (8). Thus, the identification of mechanisms in which cancer cells regenerate through self-renewal is critical to our understanding of tumorigenesis (9). A number of developmental signaling pathways, such as Wnt, Notch, and Hedgehog, have been observed to play pivotal roles in governing both self-renewal and the process of malignant transformation (10, 11).

Tumor stem cells, as well as other cells in the tissue, do not function autonomously as independent decision-making entities. Increasing evidence shows the influence of the cellular microenvironment on tumor development and progression (2, 12). Cellular activity is closely regulated through interactions with adjacent cells that create well-defined microenvironments in which tumor stem cells reside. However, tumor cells also possess the ability to interact with and influence their surrounding environment. Examples of these properties include neo-angiogenesis, recruitment of epithelial cells, and modification of tissue architecture. Interestingly, certain extracellular matrix (ECM) and cell-surface
receptor molecules, such as Wnt, Notch, and TGF-β have been shown to be involved in stem cell self-renewal and tumor development (2, 10, 11, 13).

Versican, a chondroitin sulfate (CS) proteoglycan, is one of the main components of the ECM and provides a loose and hydrated matrix during key events in development and disease (14). To date, 4 isoforms of versican (V0, V1, V2, and V3) have been identified in various tissues (15). Versican is structurally composed of an N-terminal G1 domain, a CS attachment region, and a C terminus (or G3) selectin-like domain. The G3 domain interacts with different ECM proteins and binds to certain cell surface proteins including EGF receptor (EGFR; ref. 16). The G3 domain is composed of 2 EGF-like repeats, a lectin-like motif, and a complement binding protein-like motif.

Versican is highly expressed in the early stages of tissue development, and its expression decreases after tissue maturation. It is also highly expressed in the interstitial tissues at the invasive margins of breast carcinoma (17). Expression levels of versican seem to be related to the metastatic potential of breast cancer and are predictive of relapse and overall survival (18). Relapse in women with node-negative breast cancer seems related to the level of versican deposited in peritumoral stroma by mammary fibroblasts (18, 19). Neoplastic remodeling of the ECM through increased versican deposition may facilitate local invasion and subsequent metastasis (20). The G3 domain of versican has been previously shown to be important in both local and systemic tumor invasiveness of breast cancer and may enhance the interactions between tumor cells and their surrounding stromal components. Furthermore, the G3 domain of versican has been shown to enhance neo-vascularization through interactions with VEGF and fibronectin (17, 21). Thus, versican participates in cell adhesion, proliferation, migration, aggregation, and angiogenesis and seems to play a central role in normal tissue morphogenesis (14, 22–24). Given recent scientific interest in the biology of cancer stem cells, the role of versican in the regulation of breast cancer cell self-renewal has not been well characterized.

To investigate the effects of versican expression on breast cancer cell self-renewal, we exogenously expressed a versican G3 construct in mouse mammary tumor cell lines 4T1, 66c14, 4T07, and human breast cancer cell lines MT-1, MCF-7, and MDA-MB-468 were cultured in RPMI-1640 media. Cells were supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/mL), and maintained at 37°C in a humidified atmosphere of 5% CO2. A pcDNA1-G3 (G3) construct and pcDNA1-G3 fragment lacking the EGF-like motifs (G3ΔEGF) construct were stably expressed in 66c14, 4T07, 4T1, and MT-1 cells as described previously (25, 26).

A monoclonal antibody 4B6 that recognizes an epitope in the leading peptide engineered to the G3 and G3ΔEGF construct was used to confirm expression of the G3 products (25, 26).

A short hairpin RNA (shRNA) construct targeting versican was generated by inserting the sequence 5'-ccgtgagctctgcccagggagagcgattctgcctctctcagtttcagaagaggagactcctgactcactgggtgagacgtacgatc into the plasmid pSuper. This construct targeted the sequence of versican at nucleotides 9453-9471 (5'-gtggccgcagaaattcg in NM 004385.4).

**Side population cell analysis**

Cells were removed from tissue culture dishes with trypsin and EDTA, washed, suspended at 106 cells/mL in DMEM containing 2% FCS, and preincubated at 37°C for 10 minutes, then cultured for 90 minutes with 2.5 µg/mL Hoechst 33342 dye, either alone or in combination with 50 µmol/L verapamil. Cells were analyzed by flow cytometry using a dual-wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350-nm UV light. Propidium iodide–positive dead cells (<15%) were excluded from the analysis.

**Western blot analysis**

Samples were subjected to SDS-PAGE using 7% to 10% acrylamide. Separated proteins were transferred onto a nitrocellulose membrane in 1× Tris/glycine buffer containing 20% methanol at 60 V for 2 hours in a cold room. The membrane was blocked in TBST (10 mmol/L Tris–Cl, pH 8.0, 150 mmol/L NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk powder (TBSTM) for 1 hour at room temperature, and then incubated with primary antibodies at 4°C overnight. The membranes were washed with TBST.
(3 × 30 minutes) and then incubated with appropriate HRP-conjugated secondary antibodies in TBSTM for 1 hour. After washing, the bound antibodies were visualized by ECL detection.

Mammosphere culture

Harvested G3- and vector-transfected cells were mechanically and enzymatically dissociated in 0.05% Trypsin–EDTA for 10 minutes at 37°C, followed by resuspension in serum-free DMEM/F12 medium containing 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL EGF, 1-glutamine (1:400), and B27 supplement (1:50). Single cell suspensions were plated onto 60-mm Petri dishes at a density of 1,000 cells/mL. After culturing for 3 days, sphere cells were collected by gentle centrifugation, dissociated to a density of 1,000 cells/mL. After culturing for 3 days, single cells were collected by gentle centrifugation, dissociated to a density of 1,000 cells/mL. After culturing for 3 days, single cells were considered 'large' spheres, whereas small spheres contained less than 30 cells per sphere.

Colony formation in soft agarose gel

A total of 10^3 cells were mixed in 0.3% low-melting agarose in DMEM supplemented with 10% FBS and plated onto 60-mm Petri dishes at a density of 1,000 cells/mL. After culturing for 3 days, sphere cells were collected by gentle centrifugation and photographed. Complexes containing 30 or more cells were considered 'large' spheres, whereas small spheres contained less than 30 cells per sphere.

Cell viability assays

The G3- and vector-transfected 66c14 cells (2 × 10^5) were cultured in 10% FBS/DMEM medium in culture dishes and maintained at 37°C for 12 hours. After cell attachment, culture media was changed into serum-free DMEM medium or 10% FBS/DMEM medium containing different concentrations of chemotherapeutical chemicals including C2-ceramide, docetaxel, doxorubicin, or epirubicin. Cells were harvested daily and cell number was analyzed by Coulter counter. Cell survival assays were conducted using a colorimetric proliferation assay (Cell Proliferation Kit; Basel, Switzerland). Versican G3- and vector-transfected breast cancer cells (1 × 10^4 cells/well) were inoculated and cultured in 10% FBS/DMEM medium in 96-well culture dishes and maintained at 37°C with 5% CO2. At 70% confluence, the cells were re-fed with fresh 10% FBS/DMEM medium. Inoculated cells were cultured for 12 hours. After cell attachment, culture media was changed to serum-free DMEM medium or 10% FBS/DMEM medium containing different concentrations of C2-ceramide, docetaxel, doxorubicin, or epirubicin. Cells were harvested, washed, and counted using a Coulter counter.

RT-PCR

A total of 2 × 10^5 66c14 or MT-1 cells were harvested and total RNA was extracted using the Qiagen RNeasy Mini Kit. Two micrograms of total RNA were used to synthesize cDNA, a portion of which (0.2 μg RNA) was used in a PCR with 2 appropriate primers. PCR products were measured using quantitative real-time PCR (qRT-PCR) incorporating TaqMan chemistry (Applied Biosystems). Sequences of primers used in this study were: mouse integrin-β1: forward, 5'-tgatttgcaactggtttcgcg; reverse, 5'-taattctgacagagctct; mouse Sca-1: forward, 5'-ttggatcactcttctacaatagt; reverse, 5'-cagacagagctctcagagag; human Oct-4: forward, 5'-gctctctccccgagcggg; reverse, 5'-tgactgcatccccccccatcc; human Gapdh: forward, 5'-aaggctgggcctcatttgcag; reverse, 5'-gatgttctggagagcccggc; mouse Cdc42: forward, 5'-tgcctgtgtttctgcgtct; reverse, 5'-ttggactctccctttgacagagag.

Immunofluorescence staining

Cultured cells were fixed with cold 3.5% paraformaldehyde for 15 minutes, washed 3 times with PBS and permeabilized with 1% Triton X-100 in PBS for 30 minutes. After washing 3 times with PBS, cells were blocked in 1% bovine serum albumin (BSA) in PBS for 30 minutes, and then incubated with primary antibodies (1:200) at 4°C overnight. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:400) for 1 hour. Control samples were treated with either secondary antibody alone or preimmune mouse serum. The samples were examined under a Nikon Eclipse E600 upright microscope equipped with fluorescent devices.

Flow cytometry

Cells were washed once with PBS and harvested in 0.05% trypsin/0.025% EDTA. Detached cells were washed with PBS containing 1% FCS and 1% penicillin–streptomycin (wash buffer) and resuspended in wash buffer (10^6 cells/100 μL). Cells were then incubated with Sca-1 monoclonal antibodies and FITC-conjugated secondary antibodies for 30 minutes on ice. The labeled cells were washed in wash buffer and then analyzed by flow cytometry.

In vivo tumorigenicity in BALB/c mice

The G3- and vector-transfected 66c14 cells were cultured in 10% FBS/DMEM medium in 37°C with 5% CO2. At 70% to 80% subconfluency, the cells were re-fed with fresh 10% FBS/DMEM medium 24 hours before inoculation into mice. Cell viability was determined by Trypan blue exclusion and cells were suspended with greater than 95% viability without cell clumping. Ethics approval was received from the institutional animal care committee. Four-week-old BALB/c mice were injected subcutaneously with the G3- and vector-transfected 66c14 cells (1 × 10^5, 1 × 10^4, 1 × 10^3, and 1 × 10^2 cells in 150 μL 10% FBS/DMEM medium). Injection was administered into the fourth (inguinal) mammary fat pad using a 1 mL syringe with a 26 G needle. Each experimental group contained 8 randomly chosen mice. Tumors were measured weekly with a vernier caliper. All mice were sacrificed when the tumor measured 1 cm, when acceptable tumor endpoints in animal care where reached, or at 21 weeks posttransplantation. Primary tumors were harvested and fixed in 10% formalin.
Figure 1. Expression of versican in mammary tumor stem/progenitor cells. A, prevalence of Hoechst<sup>low</sup> side population (SP) cells in the 4 mouse mammary cell lines. Compared with 66c14 cells; *, P < 0.05; **, P < 0.01; n = 4; analyzed with t test. B, cells of the 4 mouse mammary cell lines were cultured in DMEM medium with 10% FBS and cell lysates were prepared and subjected to immunoblotting with antibodies to versican and β-actin. C, 4T1 cells were cultured in DMEM medium with 10% FBS or cultured as mammospheres in suspension with serum-free medium for 3, 7, and 14 days. Cell lysates were prepared and subjected to immunoblotting with antibodies to versican and β-actin. D, 4T1 cells were cultured as mammospheres in suspension with serum-free medium for 14 days. The mammosphere cells were also recovered with 10% FBS/DMEM for 4, 8, 16, 24, 48, and 72 hours. The differentiated cells were cultured with 10% FBS/DMEM for 4, 8, 16, 24, 48, and 72 hours. The differentiated cells were cultured with 10% Du et al.

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Tissue slide H&E staining and immunohistochemistry

Freshly excised primary tumors were fixed in 10% formalin overnight, immersed in 70% ethanol, embedded in paraffin, and sectioned. Sections were deparaffinized with xylene and ethanol and boiled in a pressure cooker. After washing with TBS containing 0.025% Triton X-100, the sections were blocked with 10% goat serum and incubated with primary antibody in TBS containing 1% BSA overnight. The sections were washed and labeled with biotinylated secondary antibody, followed by avidin-conjugated HRP provided by the Vectastain ABC Kit (Vector, PK-4000). The slides were then counterstained with Mayer’s Hematoxylin and slide-mounted.

Results

Versican is highly expressed in mammary tumor stem/progenitor cells

The Hoechst 33342 dye-based side population technique facilitated isolation of mouse mammary stem/progenitor cells. To investigate the prevalence of side population cells in breast cancer cells, we stained the 4 mouse mammary tumor cell lines with Hoechst 33342 dye and identified the side population by its characteristic fluorescent profile in dual-wavelength analysis. The 4 mouse mammary tumor cell lines contained side population cells ranging from 0.4% to 3.2% of the total viable cells (67NR, 0.62%; 4T07, 0.12%; 66c14, 0.42% ± 0.05%; 4T1, 0.96% ± 0.11%; 4T1, 2.73% ± 0.53%). The proportion of side population cells in 4T1 cell lines were substantially higher than observed in the other 3 cell lines, with a median of 2.56% and a range of 1.98% to 3.34% (Fig. 1A). Immunoblotting also showed that 4T1 cells expressed higher levels of the versican V1 isoform than other cell lines (Fig. 1B). We compared the expression of versican in mammary/progenitor cells with that of differentiated mammary tumor cells by using mammosphere-derived cells grown in suspension culture with serum-free medium versus regularly cultured 4T1 cells with 10% FBS medium. Immunoblotting showed that the expression of versican is greater in stem/progenitor cells contained within mammospheres than in differentiated cells cultured in serum medium (Fig. 1C). The mammosphere 4T1 cells expressed higher levels of versican compared with the differentiated cells and approached that of differentiated cells once cultured in serum medium for 72 hours (Fig. 1D). The mammosphere 4T1 cells also contained a high percentage of side population cells when compared with differentiated cell cultures. The prevalence of side population cells declined rapidly once cultured with serum medium and cells were also observed to be differentiating (Fig. 1E). Cells containing a high percentage of side population cells, possessing stem cell-like properties, also produced high levels of versican before cell differentiation. Immunostaining showed that mammosphere 4T-1 cells not only expressed high levels of versican, but also expressed high levels of characteristic breast cancer stem cell markers Sca-1, Sox2, and ALDH1 (Supplementary Fig. S1). These results indicated that versican was highly expressed in mammary tumor stem/progenitor cells and its expression seemed to be downregulated during cell differentiation.

Versican G3 enhances breast cancer cell mammosphere and colony formation

To investigate the effect of versican on breast cancer self-renewal, we exogenously expressed a versican G3 construct in MT-1, 66c14, 4T07, and 4T1 cell lines. Flow cytometry assays showed that the proportions of side population cells were higher in versican G3-transfected MT1, 66c14, and 4T07 cells than in vector control groups (Fig. 2A and B). Cultured in suspension with serum-free medium, versican G3-transfected cells formed larger mammospheres than vector control cells (Fig. 2C). The number of mammospheres reflected the quantity of cells capable of in vitro self-renewal, whereas the number of cells/sphere indicated the self-renewal capacity of each sphere-generating cell. Dissociated G3-transfected cells from primary mammospheres generated an equivalent proportion of secondary and tertiary spheres, which highlighted their potential for in vitro self-renewal (Fig. 2D and E). Versican G3-expressing breast cancer cells showed enhanced colony formation capacity on soft agarose gel (Supplementary Fig. S2A and S2B).

Versican enhances breast cancer cell resistance to chemotherapy and influences markers associated with self-renewal

We treated versican G3- and vector-transfected 66c14 cells with 8 μmol/L doxorubicin or 10 μmol/L epirubicin for 24 hours. We observed that versican G3-transfected 66c14 cells showed resistance to doxorubicin and epirubicin treatment (27). To investigate whether these remaining viable cells were of tumor stem cell origin, we carried out immunofluorescence staining with antibodies to Sca-1, which was highly expressed in mouse mammary tumor progenitor/stem cells. Remaining viable cells showed a high level of Sca-1 expression (Supplementary Fig. S3A). An immunofluorescence staining experiment carried out on 66c14 cells cultured in 10%FBS/DMEM medium also showed that versican G3-transfected cells expressed higher levels of Sca-1 than the vector cells (Supplementary Fig. S3B). In studying the relationship between versican-enhanced breast cancer cell self-renewal and chemotherapy resistance, we also cultured the 4 mouse mammary tumor cell lines 67NR, 66c14, 4T01, and 4T1 (containing different proportions of side population cells and differential versican expression) in 8 μmol/L doxorubicin for 4 days. We observed that although the 4T1
Figure 2. Expression of versican G3 domain enhanced breast cancer cell mammosphere formation. A, the typical prevalence of Hoechst\textsuperscript{low} side population (SP) cells in versican G3- and vector-transfected MT-1, 66c14, 4T07, and 4T1 cell lines as shown by flow cytometry. B, prevalence of Hoechst\textsuperscript{low} side population cells in versican G3- and vector-transfected MT-1, 66c14, 4T07, and 4T1 cell lines. Compared with vector control group; \( ^{\ddagger} \), \( P < 0.05 \); \( ^{\ddagger\ddagger} \), \( P < 0.01 \); \( n = 4 \); analyzed with \( t \) test. C, a typical mammosphere of versican G3- and vector-transfected 66c14 cells cultured in suspension with serum-free medium. D, typical mammospheres of versican G3- and vector-transfected 66c14 cells formed primarily, secondarily (generated from dissociated primary spheres), and tertiary. E, numbers of primary, secondary, and tertiary mammospheres. Compared with vector control group; \( ^{*} \), \( P < 0.05 \); \( ^{**} \), \( P < 0.01 \); \( n = 6 \); analyzed with \( t \) test.
cells, which had a high proportion of progenitor cells and expressed higher levels of versican compared with other mouse mammary tumor cell lines, showed evidence of cellular survival (Supplementary Fig. S3C).

**Versican-expressing cells expressed high levels of breast cancer self-renewal markers**

RT-PCR and immunoblotting experiments showed that the versican G3-transfected mouse mammary tumor cells expressed high levels of Sca-1 and integrin-β1 (Supplementary Fig. S3D–S3F). Immunoblotting showed that G3-expressing human breast cancer cells expressed increased ALDH1, CD44, and integrin-β1 (Supplementary Fig. S3G). These cells expressed high levels of Oct-1 as evaluated by RT-PCR (Supplementary Fig. S3H). The evaluated breast cancer cell self-renewal markers seemed upregulated in versican G3-transfected breast cancer cells, supporting G3’s influence on breast cancer self-renewal.

**Effect of versican on promoting breast cancer cell self-renewal is related to its EGF-like motifs**

The 4T07 cells were stably transfected with a G3 construct, a G3 fragment lacking the EGF-like motifs (G3AEGF), and a vector control. We observed that G3AEGF expression did not enhance mammosphere formation to the degree observed with G3-transfected cells (Fig. 3A and D). Colony formation assays showed that both G3 and G3AEGF-transfected cells formed more colonies than vector control cells. However, G3AEGF-expressing cells formed smaller colonies when compared with G3-expressing cells (Fig. 3B and E). The prevalence of side population cells in G3AEGF-expressing cells was lower than in G3-expressing cells (Fig. 3F). Immunoblotting showed that G3AEGF-expressing cells did not show enhanced pEGFR, Sca-1, Sox2, ALDH1, and integrin-β1 as was observed with G3-transfected cells (Fig. 3C). Flow cytometry indicated that without the EGF-like motifs, G3-expressing 4T07 cells did not significantly enhance Sca-1 expression (Fig. 3G).

**Versican promoted breast cancer self-renewal through enhanced EGFR signaling**

To investigate the role of EGFR signaling in G3-enhanced self-renewal, the G3- and vector-transfected 4T07 cells were cultured in suspension culture conditions using serum-free medium. G3 cells were also cultured with or without EGF, with selective EGFR inhibitor AG 1478, selective ERK inhibitor PD 98059, selective c-jun-NH2-kinase (JNK) inhibitor side population 600125, or selective AKT inhibitor triciribine for 14 days. The G3 cells formed more mammospheres than the vector control cells, however, the G3 cells were observed to form very few mammospheres without EGF in the medium (Fig. 4A). Both selective EGFR inhibitor AG 1478 and selective AKT inhibitor triciribine prevented versican G3-enhanced mammosphere formation, which indicated that observed G3-enhanced breast cancer self-renewal effects were due, at least in part, to its effect on influencing the EGFR/AKT pathway (Fig. 4A). Immunoblotting showed that the versican G3-expressing cells expressed high levels of pEGFR, pAKT, GSK-3β (S9P), Sca-1, Sox2, and ALDH1. These effects were blocked by inhibitor AG 1478 and selective AKT inhibitor triciribine (Fig. 4B). To observe whether versican enhanced breast cancer cell self-renewal through EGFR-AKT-GSK-3β (S9P)–mediated pathways, we cultured the versican G3-expressing cells in 40 μmol/L c2-ceramide or 2 μmol/L docetaxel. We observed inhibited expression of GSK-3β (S9P) in breast cancer cells (27). Immunoblotting also showed decreased expression of GSK-3β (S9P) with c2-ceramide and docetaxel. Previously observed effects of versican G3-expressing cells on enhanced expression of stem cell markers—Sca-1, Sox2, and ALDH1—were blocked (Fig. 4D).

Overexpression of versican in breast cancer cells enhanced expression of pEGFR and its downstream effects on pERK, pSAPK/JNK, and pAKT. pAKT seemed to enhance the expression of GSK-3β (S9P) and some stem cell markers—Sca-1, Sox2, and ALDH1—resulting in enhanced tumor cell resistance to chemotherapy, enhanced cellular survival, and enhanced self-renewal (Fig. 4C). On the other hand, enhanced expression of pEGFR/pJNK in cancer cells seemed to enhance cell sensitivity to the chemotherapeutic drugs evaluated (27).

**Silencing versican’s functionality reduces its observed effects on breast cancer cell self-renewal**

To investigate the effect of silencing versican’s function on breast tumor cell self-renewal, we first transfected the human breast cancer cell line MDA-MB-231 with anti-versican shRNA. These transfected cells formed fewer and smaller mammospheres when cultured in suspension culture conditions using serum-free medium (Fig. 5A and B). Flow cytometry showed that anti-versican shRNA cells possessed lower proportions of tumor progenitor/stem cells compared with vector control cells (Fig. 5C). Immunoblotting showed that anti-versican shRNA-expressing MDA-MB-231 cells decreased expression levels of versican V1, pEGFR, pAKT, GSK-3β (S9P), and stem cell markers Sca-1, Sox2, and ALDH1 (Fig. 4D). Anti-versican shRNA-expressing cells also showed reduced colony formation ability when compared with the vector control cells (Fig. 5E and F).

**Expression of versican promoted breast cancer self-renewal in vivo**

We injected versican G3- and vector-transfected 66c14 cells into the fourth mammary fat pad of BALB/c mice as a 10-fold dilution series from 1 × 10² to 1 × 10⁵ cells per mouse. With as few as 1,000 inoculated cells, G3-transfected 66c14 cells could form tumors in BALB/c mice, whereas the vector control cells were unable to show the same ability at the same dilution (Table 1). Although tumors could form if greater than 10,000 vector control cells were injected, the incidence of tumors in the vector control group was much lower than that observed of the G3 group using the same number of injected cells.

Immunohistochemical staining showed that primary tumor tissues of versican G3-treated mice not only expressed...
Figure 3. Knocking out the EGF-like motifs in the versican G3 construct (G3ΔEGF) resulted in reduced effects on breast cancer cell self-renewal. A, typical mammospheres of vector-, versican G3-, and G3ΔEGF-transfected 4T07 cells formed in serum-free DMEM medium after culture for 14 days. B, typical colonies of vector-, versican G3-, and G3ΔEGF-transfected 4T07 cells formed in soft agarose gel after culture for 4 weeks. C, vector-, versican G3-, and G3ΔEGF-transfected 4T07 cells were cultured in 10% FBS/DMEM medium. Cell lysates were prepared and subjected to immunoblotting with antibodies to versican G3, pEGFR, Sca-1, Sox2, ALDH1, integrin-β1, and β-actin. D, the numbers of mammospheres of vector-, versican G3-, and G3ΔEGF-transfected 4T07 cells counted at 7, 14, and 21 days. Compared with vector control group; *, P < 0.05; **, P < 0.01; n = 6; analyzed with t test. E, the numbers of colonies of vector-, versican G3-, and G3ΔEGF-transfected 4T07 cells formed on soft agarose gel. Compared with vector control group; *, P < 0.05; **, P < 0.01; n = 3; analyzed with t test. F, prevalence of Hoechst™️ side population (SP) cells of vector-, G3-, and G3ΔEGF-transfected 4T07 cells. Compared with 4T07 cells; *, P < 0.05; **, P < 0.01; n = 4; analyzed with t test. G, typical flow cytometry of vector-, G3-, and G3ΔEGF-transfected 4T07 cells stained with antibodies against Sca-1 and FITC-conjugated secondary antibodies.
high levels of 4B6, pEGFR, pAKT, and GSK-3β (S9P), all of which were related with tumor invasiveness, but also expressed high levels of tumor stem cell markers Sox2, Sca-1, and ALDH1. Our in vivo studies complemented observed in vitro effects on breast cancer self-renewal (Fig. 6).

Discussion

In human breast carcinoma, versican is detected in the interstitial tissues at the invasive margins and in the elastic tissues associated with tumor invasion (25, 28). The high expression of versican in human breast tumors is prognostic, being predictive of relapse, and negatively impacting overall survival rates (18, 19). The mechanisms by which versican facilitates tumor growth and metastatic transformation in breast cancer are still not clear. Our study highlights the understanding that versican expression is greater in more invasive breast cancer cell lines, such as 4T1. In our experiments using the Hoechst 33342 dye-based side population technique, we observed that invasive breast cancer cell lines contained a high proportion of side population cells, an enriched source of stem cells or primitive and undifferentiated cells (29, 30). This was concordant with high levels of versican expression. Mammary stem/progenitor cells were reported in these enriched sources of primitive and undifferentiated cells (29, 30). This was concordant with high levels of versican expression. Mammary stem/progenitor cells were reported in these enriched sources of primitive and undifferentiated cells (29, 30).
are spherical colonies formed by epithelial cells when cultured on nonadherent surfaces, were enriched in stem/progenitor cells (30). Increasing expression of versican, as well as Sca-1, Sox2, and ALDH1, was observed in cultured mammospheres. Sca-1, the first identified mouse stem cell marker, is an anchored membrane protein expressed by different progenitor populations including the mammary gland progenitors (32). Sox2 and ALDH1 are also reported to be stem cell markers in human breast cancer cells. The mammosphere 4T1 cells contained a high percentage of side population cells when compared with differentially cultured cells. An interesting observation included the high expression of versican in mammospheres. Both the expression of versican as well as the prevalence of side population cells were reduced as cellular differentiation occurred over time in serum medium culture.

Structurally, all versican splice forms include an N-terminal G1 domain and a C-terminus containing a selectin-

![Image](image-url)

**Table 1.** Tumor formation of versican G3- and vector-transfected 66c14 cells in vivo

<table>
<thead>
<tr>
<th>Cell number injected and tumors formed</th>
<th>1 × 10^2</th>
<th>1 × 10^3</th>
<th>1 × 10^4</th>
<th>1 × 10^5</th>
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<td>Vector</td>
<td>0/8</td>
<td>0/8</td>
<td>3/8</td>
<td>8/8</td>
</tr>
<tr>
<td>G3</td>
<td>0/8</td>
<td>4/8*</td>
<td>7/8*</td>
<td>8/8</td>
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NOTE: Versican G3- and vector-transfected 66c14 cells were injected into the fourth mammary fat pad of BALB/c mice as a 10-fold dilution series from 1 × 10^2 to 1 × 10^5 cells per mouse. All mice were sacrificed after 6 weeks (*, P < 0.05). The overall test for differences in stem cell frequencies between the groups were calculated the website: http://bioinf.wehi.edu.au/software/elda/, P = 0.00132).
like (G3) domain (33, 34). Given their ubiquitousness and high degree of conservation, it is likely that G1 and G3 play vital roles in proteoglycan functionality (35). The effects of versican were greatly reduced when the G3 domain or EGF-like motifs within the G3 domain were deleted (36–38).

This study showed that the versican G3 domain not only enhanced breast cancer cell mammosphere formation and colony formation but also promoted tumor formation in vivo, consistent with observations from our in vitro experiments. With as few as 1,000 cells, G3-transfected 66c14 cells could form tumors in BALB/c mice, whereas the vector control cells required more than 10,000 cells before tumor formation. Versican G3-expressing human breast cancer cells expressed higher levels of CD44, Sox2, ALDH1, Sca-1, and integrin-β1, whereas the G3-expressing mouse mammary tumor cells also showed increased expression of integrin-β1 and Sca-1. Increased expression of breast cancer cell self-renewal markers was associated with the increased expression of versican G3. This was supported by studies silencing versican or the versican G3 domain’s functionality. Using shRNA or G3 untranslated region (UTR) reduced the effects on mammosphere and colony formation corroborating versican G3 domain’s role in enhancing breast cancer cell self-renewal.

Given the frequency at which abnormalities in EGFR signaling are present in human breast cancer, EGFR has been an attractive target for therapeutic manipulation (39–42). Versican-promoted breast tumor invasion is influenced by its enhanced expression of EGFR signaling (25). In our study of stem cell renewal, we observed that negating the effects of
EGF-like motifs using a versican G3 fragment (G3ΔEGF) reduced versican G3 effects on breast cancer cell mammosphere formation and colony formation. Mammosphere formation assays showed that versican G3-enhanced breast cancer self-renewal was related to enhanced expression of key markers in the EGFR/AKT/GSK-3β (S9P) pathway. Both selective EGFR inhibitor AG 1478 and selective AKT inhibitor triciribine could block this signaling pathway and prevent the versican G3-observed effects on mammary cancer cell mammosphere formation and expression of GSK-3β (S9P), Sox2, ALDH1, and Sca-1. Moreover, reducing the expression of GSK-3β (S9P) using C2-ceramide or doxetaxel also inhibited versican-enhanced expression of stem cell markers—Sox2, ALDH1, and Sca-1. G3’s effect on in vivo tumor formation was associated with changes to both EGFR/AKT/GSK-3β (S9P) signaling and the aforementioned tumor cell self-renewal markers. EGFR/AKT/GSK-3β (S9P) signaling and breast cancer cell self-renewal markers—Sox2, ALDH1, and Sca-1—were observed to be much higher in primary tumors of G3-treated mice as compared with those of the vector control group.

In this study, we also observed that versican G3-promoted breast cancer cell self-renewal conferred enhanced resistance to chemotherapeutic drug therapy using clinical agents such as doxorubicin and epirubicin. Recent studies have proposed that cancer-initiating cells may be more resistant to irradiation than other cells in the tumor population (1, 43, 44). There are also reports that cancer-initiating cells can be more resistant to chemotherapeutic drugs because of increased expression of antiapoptotic proteins or increased expression of the ATPase drug efflux pump ABCG2/5 (1, 45–47). It has been reported that when EGFR targeting therapy was coadministered with cytotoxic chemotherapy agents or radiotherapy, additive or even synergistic antitumor effects were observed (26, 27, 48). The study of versican as a potential treatment target merits further preclinical study.

Versican is highly expressed in breast cancer progenitor cells and its expression is maintained at high levels before cellular differentiation. Silencing versican expression was shown to inhibit breast cancer self-renewal. Overexpression of the versican G3 domain enhanced breast cancer self-renewal through EGFR/AKT/GSK-3β (S9P) signaling and conferred enhanced resistance to the chemotherapeutic drugs. Findings from our mechanistic study complement clinical literature recognizing the importance of versican in cancer invasiveness and metastasis. Strategies designed to target versican-mediated breast cancer self-renewal may lead to therapies and/or adjuncts benefiting patients with breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: W.W. Du, L. Fang, X. Yang, W. Sheng, B.L. Yang, A. Seth, Y. Zhang, B.B. Yang, A. J. Yee
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.W. Du, L. Fang, A. Seth, Y. Zhang, B.B. Yang, A. J. Yee
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