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ORIGINAL ARTICLE

The functional role of the EZH2 gene in controlling breast cancer stem cells

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Summary

Purpose: *Breast cancer is caused by rare populations of self-renewing cancer stem cells that might also play a role in tumor relapse. Genes that regulate cancer stem cells are, therefore, of great interest in controlling cancer. EZH2 gene expression is reported to be elevated during breast cancer progression and it plays a role in expanding breast stem cell populations. In the current study, we analyzed the correlation between the silencing effect of EZH2 and breast cancer stem cell expansion.*

Methods: *We used $CD44^{+}/CD24^{-/low}$ cells to develop initial-, moderate-, and advanced-stage breast cancers in female NOD/SCID mice. Immunohistochemistry and western blotting were used to study the expression of aldehyde dehydrogenase 1 (ALDH1) and EZH2 in different stages of breast cancer.*

Results: *Histology showed that as tumors progressed, the pathological condition changed exhibiting enlarged nuclei, higher cell proliferation, and more invasive cells. In EZH2-silenced mice histopathology also showed enlarged cell nucleus, lesion formation and cell aggregation. Immunohistochemistry and western blotting analyses of EZH2 and ALDH1 demonstrated elevated expression as tumors progressed to the next level. Interestingly, the expression of ALDH1 in EZH2-silenced breast cancer tissue showed prolonged overexpression.*

Conclusions: *We conclude that the normal expression of EZH2 in cancer tissue controls cancer stem cell expansion, because it is highly elevated in EZH2-silencing cancer tissue.*

Key words: *breast cancer, EZH2, ALDH1, cancer stem cells, cell proliferation, $CD44^{+}/CD24^{-/low}$*

Introduction

Breast cancer is among the most prevalent malignancies in women and its incidence may increase over the next 20 years despite preventive measures [1-3]. Despite small scientific advances, including improvements in managing breast cancer, several clinical problems like disease metastasis, recurrence, and treatment resistance have practically remained constant [4]. Breast cancer stem cells play a major role in tumor metastasis [5], disease recurrence [6], and resistance to treatment [7]. The identification of the various factors that regulate breast cancer stem cells may help in the quest to develop novel therapeutic drugs.

Following puberty and pregnancy, a woman's breast cells undergo proliferation, demonstrating the presence of stem cells within the breast cell population [8]. Cumulative evidence suggests that breast cancer cells, which possess stem cell-like properties, are responsible for breast cancer progression [9]. Studies of breast cancer stem cells have shown that dysregulation of the Hedgehog and Notch pathways contributed to maintaining the cell phenotype [10]. The expression of stem cell-specific transcription factors like Oct4, Sox2, and Nanog has also been observed in breast cancer stem cells [11-13].

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The derivation of breast cancer stem cells remains unclear; they may originate from mutations that accumulate within breast stem cells or from dedifferentiated cells [14]. Targeting these small populations of breast cancer stem cells helps stop disease progression. In this context, identifying the genes that negatively regulate breast cancer stem cells is of great interest. The polycomb group protein enhancer of zeste homolog 2 (EZH2) is a type of transcriptional repressor protein involved in regulating the transcriptional memory of a cell [15]. The EZH2 gene has been found to be overexpressed in many cancers including breast and prostate cancer [15,16]. The cancer-inducing mechanism of EZH2 works via the epigenetic silencing of many tumor suppressor genes [17]. Other studies of EZH2 have determined that its presence is required for controlling the differentiation of mammary epithelium during pregnancy [18]. In the current study, we investigated EZH2 and ALDH1 expression levels at different stages of breast cancer using a mouse model.

Methods

Mouse model with breast cancer

To initiate a mouse model with breast cancer, female NOD/SCID mice (n=16; 3 months) were purchased from the Jackson Laboratory and their mammary fat pads were injected with $CD44^{+}/CD24^{-/low}$ cells (2×10^{8} cells). Initially, CD44+/CD24-/low enriched cells are isolated from MDA-MB-231 (ECACC Cat no: 92020424) cell lines using flow cytometry by following the procedure as already described [19]. Following injection, one group of mice (n=4) was euthanised at 4 weeks post-injection for initial tumor development study. In another set of mice (n=4) moderate stage of tumors developed after 6 weeks of $CD44^{+}/CD24^{-/low}$ cells post-injection and then euthanised. The remaining mice were maintained for up to 10 weeks to develop advanced-stage breast cancer and then euthanised. Control mice were injected with DMEM medium only. For euthanization, we administered multiple dose of 5% isoflurane until breath stops and we physically examine the heart functioning to determine the end point. The experimental mice were monitored twice per day and the loss of appetite along with signs of pain and weakness were endpoint conditions dictating termination of the painful procedure.

Silencing EZH2 gene expression in mouse breasts

EZH2 expression was suppressed in mouse breasts using siRNA, which was purchased from Dharmacon (GeneID:14056); we used Dharmacon siEZH2 [5'- GAAUGGAAACAGCGAAGGA-3'] for experimental positive control, while for the negative control non-targeted siRNA [Dharmacon siRNA Control, 5'-UAGCGACUAAACACAUCAA-3'] was used. The standardised dosage of siRNA injection to silence the EZH2 gene was 20 µM. The obtained siRNA was resuspended in 1×siRNA buffer and injected directly into the mammary fat pads 1 week after $CD44^{+}/CD24^{-/low}$ injection. The normal mice breast tissue played the role of a non-injected control. Tumor development and EZH2 expression patterns were analysed at 4, 6, and 10 weeks post-injection and compared with those of positive controls.

Histology to assess tumor development

Dissected breast tissue samples were fixed by immersing them in 10% paraformaldehyde solution for 48 h at 40°C. After fixation, the samples were dehydrated with ethanol solutions of continuously increasing concentration. The samples were then transferred to xylene solution, which helped impregnate the tissue with wax. Finally, the paraffin-embedded tissue samples were subjected to thin sectioning (4 µm) using a microtome. The sections were then deparaffinised by dissolving the wax in xylene solution. To perform staining, the sections were dehydrated by dipping the slides in ethanol and stained with hematoxylin and eosin.

Immunohistochemistry staining

EZH2 and ALDH1 expression in the breast tissue samples was analysed by immunohistochemistry staining. The prepared tissue blocks were cut into thin sections (4 µm) and placed on glass slides. The slides were then incubated in blocking solution, 4% bovine serum albumin (BSA) in 1× tris buffered saline with tween (TBST) solution for 6 h at room temperature, and then incubated with anti-EZH2 (Abcam, ab186006; 1:500 dilution) or anti-ALDH1 (Abcam, ab56777; 1:300 dilution) antibody for 6 h at 4°C. After incubation, the slides were washed three times with 1×phosphate-buffered saline (PBS) and overlaid with suitable HRP-conjugated secondary antibody (Abcam, ab6721; 1: 3500 dilution or Abcam, ab6789; 1:5000 dilution) for 2 h at room temperature. The slides were then washed with 1× PBS and mounted permanently using mounting medium.

Western blotting analyses

Following dissection, the tissue samples were immediately transferred to ice-cold 1× PBS solution and then crushed in lysis buffer to prepare the protein samples. Immediately after lysis, the samples were heated at 100°C for 5 min and then stored at -20°C. The protein concentration of the samples was estimated using Bradford assay and loaded into 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, applying equal loading (70µg). The separated proteins in the 12% gel were transferred to nitrocellulose membrane and, after blocking, were incubated overnight with primary antibody, anti-EZH2, or anti-ALDH1 or anti-beta Actin antibody (Abcam, ab8227; 1:3000 dilutions, loading control) in a rocker at 4°C. The membrane was then washed three times with 1×PBS and incubated with suitable HRP-conjugated secondary antibody (Abcam, ab6721; 1: 3500 dilution or Abcam, ab6789; 1:5000 dilution) at room temperature for 1 h. After washing, secondary antibody non-specific binding signals were detected using a DAB kit.

Statistics

The experiments performed were repeated more than three times to obtained statistically significant data. Correlation analysis was performed using SPSS (Statistical Package for Social Sciences) software version 21.0. The data were evaluated using one-way ANOVA, followed by Tukey's *Post Hoc* test for multiple data comparisons. The obtained data are shown as mean ± SD and was considered statistically significant when the p value was less than 0.05.

Results

Mice injected with CD44^{+}/CD24$^{-/low}$ developed breast cancer

To develop the characteristic features of breast cancer during the initial, moderate, and advanced stages of breast cancer, female NOD/SCID mice were injected with CD44^{+}/CD24$^{-/low}$ cells and euthanised at 4, 6, and 10 weeks post-injection as

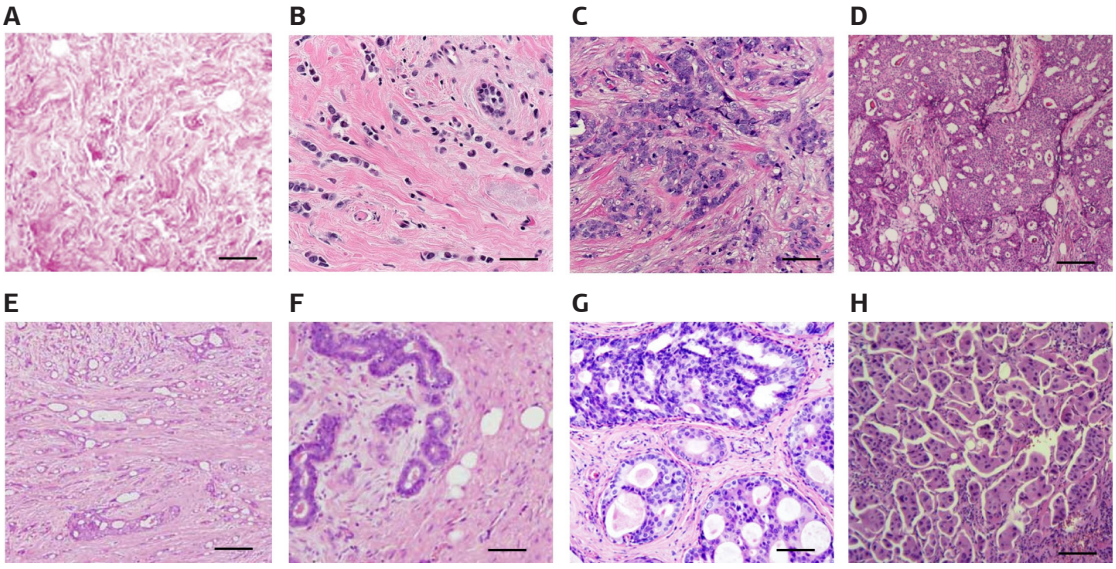

Figure 1. Histology of normal and EZH2-silenced breast cancer progression. **A:** Normal breast tissue with evenly spaced cells. **B:** Breast tissue with initial-stage tumor and enlarged cells. **C:** Moderate-stage tumor with more proliferating cells. **D:** Advanced-stage breast cancer with more invading cells. **E:** EZH2-silenced normal breast tissue. **F:** EZH2-silenced initial breast cancer tissue with more enlarged cells. **G:** More lesions observed in EZH2-silenced moderate-stage tumour. **H:** Aggregate mass of cells developed in EZH2-silenced advanced-stage breast cancer. Haematoxylin and eosin staining. Scale bar: 50 µm.

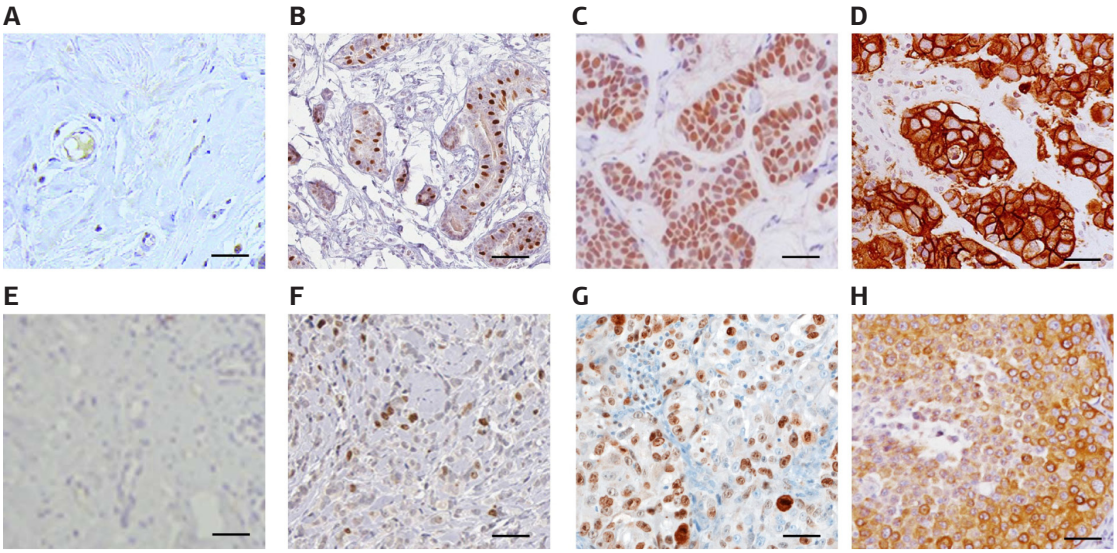

Figure 2. EZH2 and ALDH1 expression in different stages of breast cancer. **A:** EZH2 expression in normal breast tissue. **B:** EZH2-positive cells in initial-stage tumor. **C:** Progression of EZH2 signalling in moderate-stage tumor. **D:** High-strength EZH2 signalling in advanced-stage breast cancer. **E:** ALDH1 showed minimal or no expression in normal breast tissue. **F:** ALDH1-positive cells observed in initial-stage tumour. **G:** More ALDH1-positive signalling observed in moderate-stage tumor. **H:** Higher ALDH1 expression in advanced-stage breast cancer. Haematoxylin staining. Scale bar: 50 µm.

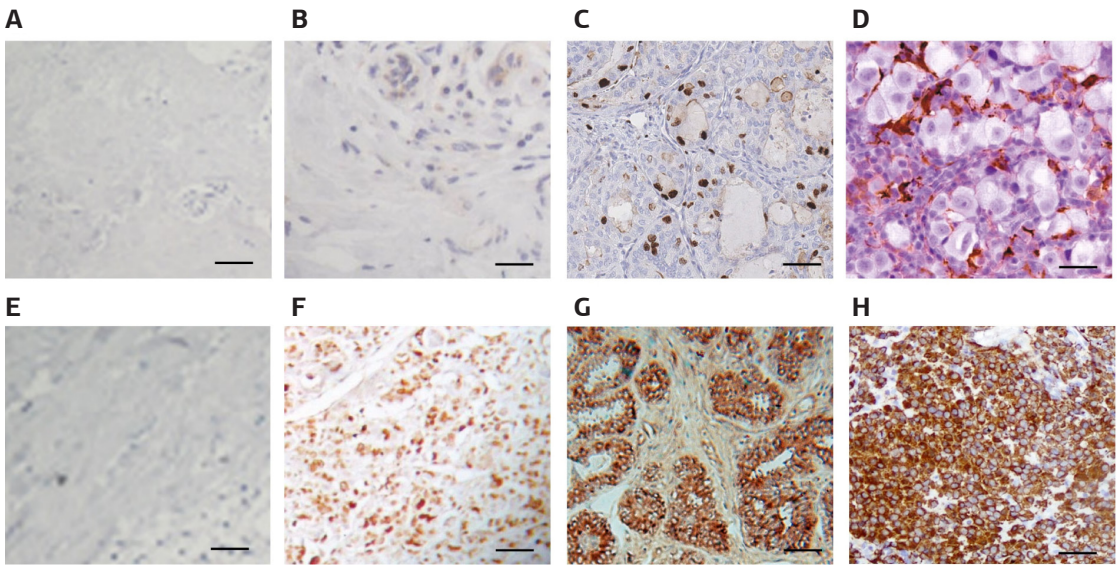

Figure 3. EZH2 silencing and its correlation with ALDH1 expression. **A:** EZH2 silenced in normal breast tissue. **B:** EZH2 silenced in initial-stage tumor. **C:** Downregulated expression of the EZH2 signal in moderate-stage tumor. **D:** Effect of EZH2 siRNA in advanced-stage breast cancer. **E:** EZH2-silenced tissue showed less ALDH1 expression in normal breast tissue. **F:** Enriched ALDH1-positive cells in EZH2-silenced initial-stage breast tumor. **G:** Progressive upregulated ALDH1 signalling in EZH2-silenced moderate-stage tumor. **H:** Overexpression of ALDH1 in EZH2-silenced advanced-stage breast cancer. Haematoxylin staining. Scale bar: 50 μm.

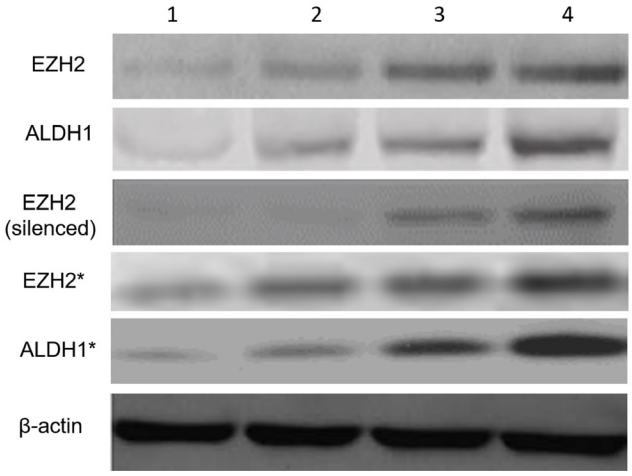

Figure 4. Detection of EZH2 and ALDH1 in various breast cancer tissues by Western blotting. Expression of EZH2 and ALDH1 shows gradual upregulation as breast cancer progresses. In EZH2 silenced breast cancer tissue the ALDH1 expression shows abnormal overexpression pattern. Lane 1: control breast tissue. Lane 2: initial-stage breast cancer. Lane 3: moderate-stage breast cancer. Lane 4: advanced-stage breast cancer. EZH2*: expression of EZH2 in non-target siRNA injected breast tissue; ALDH*: ALDH1 expression in EZH2-silenced breast tissue.

described in the Methods section. The collected breast tissue samples were histologically studied and their pathological response was documented (Figure 1A-F). Mice euthanised 4 weeks after $CD44^{+}/CD24^{-/low}$ cell injection exhibited microscopic characters (Figure 1B) that were different from those of the control tissue (Figure 1A). In the control tissue, cell integrity between cells was evenly distributed (Figure 1A); however, as in the initial cancer stage, the evenly spread cells were interrupted by enlarged cells, which formed common foci at certain locations (Figure 1B). Moderate stage of tumor was developed after 6 weeks of $CD44^{+}/CD24^{-/low}$ cells post-injection with more proliferative cells; thus, cell interaction occurred more closely (Figure 1C). Mice euthanised 10 weeks post-injection exhibited more invading and poorly differentiated cells (Figure 1D).

Silencing EZH2 expression enhanced tumor progression

We injected siRNA for EZH2 into mouse fat pads during the week following $CD44^{+}/CD24^{-/low}$ cell injection. The silencing effect of EZH2 increased the aggressive behavior of the breast cancer during the initial (Figure 1F), moderate (Figure 1G), and advanced stages (Figure 1H). The lesions formed were more complex and overfilled by proliferative cells; particularly in the advanced stages of breast cancer, irregular masses of cells were observed.

EZH2 and ALDH1 expression increased as tumors progressed

EZH2 expression was assessed at different cancer sub-stages; during the initial stage, expression was less prominent in the enlarged cells (Figure 2B), but the control tissue exhibited mild expression (Figure 2A). As tumors advanced, EZH2 expres-

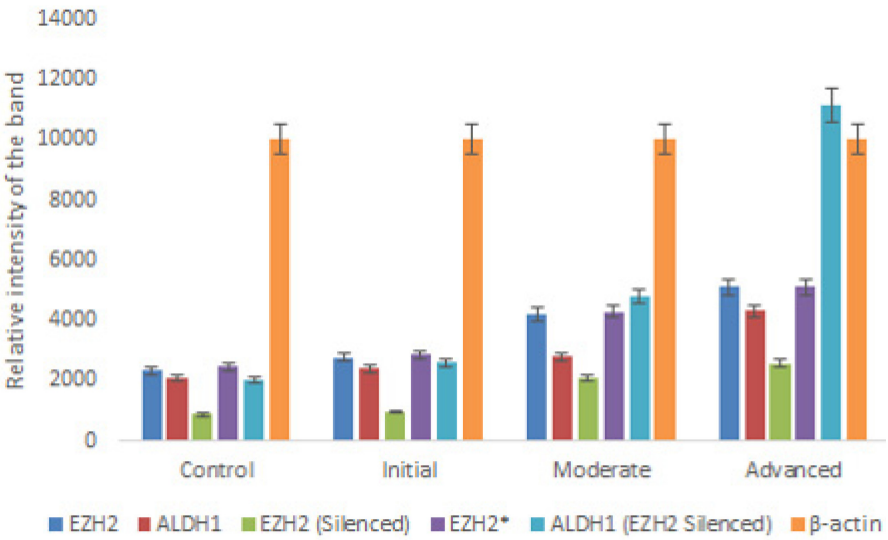

Figure 5. Quantification of EZH2 and ALDH1 expression in different stages of breast cancer. Based on the band intensity the expression of EZH2 and ALDH1 in different conditions of breast cancers are analyzed and represented in bar diagram. The experiments were performed more than three times to obtain concordant data which are presented as mean±SD. EZH2*: expression of EZH2 in non-target siRNA injected breast tissue, shown as violet color.

sion increased, as shown in moderate (Figure 2C) and advanced-stage cancers (Figure 2D). ALDH1 expression was determined during the initial, moderate, and advanced stages of breast cancer (Figure 2E–H), indicating that expression was upregulated as tumors progressed.

Link between EZH2 silencing and ALDH1 expression

siRNA for EZH2 was injected into the mice fat pads 1 week after inducing breast cancer and it was observed that EZH2 expression was downregulated (Figure 3A-D). In the initial stage, cancer was developed after 4 weeks of $CD44^{+}/CD24^{-/low}$ cells post-injection and in the control group EZH2 expression was almost blocked (Figure 3A and 3B) and exhibited only minimal expression in moderate- (Figure 3C) and advanced-stage breast cancers (Figure 3D). The relationship between EZH2 silencing and ALDH1 showed that its expression was increased as tumors progressed (Figure 3E-H). The experimental data characterising different tissue patterns associated with EZH2 and ALDH1 expression were cross-verified by western blotting. These experiments yielded similar results (Figure 4), significantly validating our EZH2 and ALDH1 expression data which showed statistically significant expression as shown in Figure 5.

Discussion

EZH2 is a type of histone methyl transferase that targets genes associated with cell cycle regulation, proliferation, and differentiation, thereby effecting transcriptional silencing [20]. EZH2 overexpression has been associated with breast cancer progression and metastasis [21]. Pharmacological suppression of EZH2 using ZLD1039 has demonstrated antitumor activity against breast cancer cell lines [22]. In many cell culture studies, the inhibition of EZH2 expression was found to inhibit breast cancer cell metastasis and proliferation [23,24]; however, related studies using *in vivo* models are lacking. One study of EZH2 showed that it expands breast stem cells through NOTCH1 signalling [25]. There are also some reports which show that long noncoding RNAs, like LINC-UBC1, are influencing tumor metastasis by regulating EZH2 [26]. EZH2 expression was also used to define aggressive phenotype of breast adenocarcinoma [27].

However, in the current study we observed tumor progression as EZH2 was silenced in the initial stages of cancer, while its potency was partially suppressed during the later stages.

Initially, the injection of $CD44^{+}/CD24^{-/low}$ cells at a dose of approximately 2×10^{8} cells was sufficient to initiate early-stage tumors 4 weeks post-injection (Figure 1B). Higher cell proliferation and pleomorphism were observed in moderate- (Figure 1C) and advanced-stage (Figure 1D) breast cancer. Unlike the previously reported effect of tumor suppression, we observed tumor progression in EZH2-silenced mice (Figure 1E-F). This contradictory result may be due to tumor recurrence, since we provided only a single dose of EZH2 siRNA during the initial stage of cancer.

The EZH2 gene regulates tumor-initiating cells [28] and is upregulated in women up to 12 years of age, at which point breast stem cell expansion is

promoted [29]. Breast cancer recurrence activates the proliferation of cancer stem cells in treatment-resistance conditions, thereby initiating aggressive tumor regeneration [30]. Our investigation supports this theory that the initial silencing of EZH2 promotes tumor progression when compared with the normal cancer expression pattern detected via ALDH1 expression (Figures 2 & 3). ALDH1 acts as a breast cancer stem cell marker; this function has been exploited to characterize its phenotype in many studies [31,32]. Our results suggest that higher ALDH1 expression in EZH2-silenced tissue is due to breast cancer relapse. Immunohistochemistry evidence from western blotting analysis further validated our findings (Figure 4). However, absence of data from qPCR, gene overexpression and co-immunoprecipitation experiments to study the correlation between EZH2 and ALDH1 are limitations of our study.

The results of this study demonstrate that the transition of normal breast to breast cancer developed in non-EZH2 silenced tissue to a relapse stage through partial suppression of EZH2 that promotes cancer stem cell proliferation. This study provides direct evidence that the progressive expression of EZH2 in breast cancer developed in non-EZH2 silenced tissue may influence breast cancer stem cells, because its silencing exacerbates the tumor condition via over-proliferation of cancer stem cells.

Conflict of interests

The authors declare no conflict of interests.

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