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Transforming growth factor-beta increases breast cancer stem cell population partially through upregulating PMEPA1 expression

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Abstract

The prostate transmembrane protein, androgen-induced 1 (PMEPA1) has been previously shown to promote solid malignancies in a variety of cancers, but the role and mechanisms of PMEPA1 in breast cancer has not been fully addressed. Here, we found that PMEPA1 was upregulated in breast cancer cell lines as well as in a set of clinical invasive breast ductal carcinomas. Interestingly, depletion of PMEPA1 decreased breast cancer stem cell (CSC)-enriched populations, while ectopic overexpression of PMEPA1 increased breast CSC-enriched populations. Furthermore, transforming growth factor-β (TGF-β) treatment was also found to upregulate PMEPA1 expression and the CSC-enriched populations in triple-negative breast cancer cell lines. TGF-β-induced PMEPA1 expression partially contributed to TGF-β-induced breast CSC maintenance. These findings suggest that TGF-β-PMEPA1 axis might provide new diagnosis and therapeutic targets for breast cancer treatment.

Key words: PMEPA1, TGF-β, cancer stem cell

Introduction

Several decades of research have consistently shown that the etiology and progression of breast cancer is complex and multifaceted, as are the mechanisms underlying both its origination and progression. Recent evidence suggests that the development of cancer stem cells (CSCs), rare pluoripotent cells with indefinite potential for self-renewal that drive tumorigenesis [1–3], may play critical roles in cancer metastasis, chemo- and radiotherapy resistance, and cancer recurrence [2,4,5], making them a critical target for novel cancer therapeutics.

Transforming growth factor-β (TGF-β) is well known to act as an important regulator of embryo and cancer development, controlling cell growth, differentiation, and migration. Recent studies revealed that TGF-β signaling is also involved in the maintenance of embryonic stem cell identity [6–8]. Brandenberger *et al.* [8] found that the expression profiling of breast epithelial stem cells-enriched population showed transcripts associated with cell motility, adhesion, proliferation, and angiogenesis. Furthermore, the enrichment in transcripts for TGF-β signaling components was striking in stem cells, suggesting a preferential activation and involvement of TGF-β pathway in stem cell biology [9]. Members of the TGF-β family were also found to play key roles in breast CSCs, contributing to several known breast CSC phenotypes [10]. In addition, chemotherapy-induced TGF-β signaling was shown to contribute to drug resistance of triple-negative breast cancers [11]. These results suggest that TGF-β is involved in

regulating breast CSCs, but its underlying mechanisms still remain unclear.

PMEPA1, prostate transmembrane protein, androgen-induced 1 (also named as *TMEPA1*, *STAG1*, *ERG1.2*, or *N4WBP4*), was originally found to be an androgen-induced gene [12]. Aside from androgen, several other growth factors, such as TGF-β [13], epidermal growth factor, platelet-derived growth factor, and insulin-like growth factor [14], were reported to induce *PMEPA1* expression in different cell systems. In 2001, Rae *et al.* [15] found that *PMEPA1* was overexpressed in several malignancies, including renal cell carcinoma, stomach adenocarcinoma, and rectal adenocarcinoma. *PMEPA1* was also found to be overexpressed in other solid tumors, including colon cancer [13], prostate cancer [16], ovarian cancer, and breast cancer [14]. In breast cancer samples, PMEPA1 was found to be upregulated at the mRNA level [15], and in a small number of clinical samples including four triple-negative breast cancer samples, PMEPA1 was found to be upregulated at the protein level [17]. Collectively, these findings suggest that overexpression of *PMEPA1* may play a key role in breast cancer.

Although TGF-β signaling is known to regulate PMEPA1 [13], and TGF-β signaling has also been shown to regulate breast CSC maintenance [11], little is known about the role of PMEPA1 in breast CSCs.

The existing evidence on the roles of CSCs in cancer origination and progression, the confirmed roles of TGF-β signaling in breast CSC maintenance, and the regulation of PMEPA1 by TGF-β [13] collectively suggest that further characterizing the TGF-β-PMEPA1 axis may provide valuable insights into the etiology of breast cancer and help to develop novel therapeutics. In the current study, we found that PMEPA1 showed a higher expression at the protein level in both breast cancer cell lines and a set of clinical breast cancer samples compared with that in the normal controls. The results also demonstrate that PMEPA1 and TGF-β contribute to breast CSC maintenance in breast cancer cells. PMEPA1 could partially mediate the regulation of TGF-β on breast CSC phenotypes. To our knowledge, this is the first report demonstrating the role of PMEPA1 in breast CSCs. Our findings may provide new targets for the diagnosis and treatment of breast cancers.

Materials and Methods

Cells and cell culture

Breast cancer cell lines HCC1806 and HCC1937 used in this study were purchased from the American Type Culture Collection (Manassas, USA). Both cell lines were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM hydroxyethyl piperazineethanesulfonic acid, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C throughout the experiments.

Western blot analysis

Cell lysates were prepared as described previously [18]. Protein concentration was measured using a protein assay kit (Bio-Rad, Hercules, USA). Western blot analysis was performed as described previously [16]. In brief, 40 μg protein samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes. Membranes were blocked and incubated with the specific first antibodies (anti-PMEPA1, 1 : 1000 dilution; and anti-β-actin, 1 : 10,000 dilution) overnight at 4°C. Then membranes were washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) and incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. Membranes were washed with PBST, and the target proteins were then visualized on a Fujifilm LAS-4000 image system by using an enhanced chemoluminescence kit (Sigma, St Louis, USA). The anti-PMEPA1 antibody was purchased from Novus Biologicals, Inc. (Littleton, USA), and the anti-β-actin antibody was from Sigma. All western blot analysis was performed at least in duplicate, and the representative blots were shown.

Stable knockdown and overexpression of *PMEPA1* gene by lentiviral system

A lentiviral pSIH-H1-Puro vector (System Biosciences, Mountain View, USA) was modified to express shRNAs from the H1 promoter. In brief, a pair of oligonucleotides, consisting a sequence-specific 19 nucleotide stretch followed by the loop sequence and the reverse complement of the targeting sequence, was designed following the manufacture's protocols. Luciferase shRNA was used as a negative control. The target sequences of shRNAs for human *PMEPA1* gene are 5′-AGA GCACAGTGTCAGGCAATT-3′ (PMEPA1sh#1) and 5′-GGTTATCA CCACGTTATATTT-3′ (PMEPA1sh#2). The target sequence for Ctrl-sh is 5′-TAGCGACTAAACACATCAATT-3′. PMEPA1 a and b isoforms were constructed into Lenti6 vector as described previously [16].

All lentiviral plasmids were cotransfected into HEK293FT cells along with the packing plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, USA), following the manufacturer's protocols. Lentiviruses were collected at 72 h post-transfection and used to transduce cancer cells in a 6-well plate. At 24-h post-transduction, puromycin (2 μg/ml, for pSIH-H1-Puro system) or blasticidin (5 μg/ml, for Lenti6 system) was added to select the drug-resistant cell populations.

Flow cytometry analysis and mammosphere formation assay of CSCs

Cells were trypsinized with 0.25% trypsin–ethylenediaminetetraacetic acid (GIBCO, Grand Island, USA) and collected with cell culture medium. After being washed once with PBS containing 2% FBS, cells were stained freshly with antibodies against CD24 and CD44 (BD Biosciences, San Diego, USA) according to the manufacturer's protocols. In brief, the cells were incubated with anti-CD24 and anti-CD44 antibodies on ice for 25 min, followed by centrifugation at 500 g for 5 min. Cells were collected and washed twice with PBS containing 2% FBS, and then undergo flow cytometry analysis on a BD Accuri C6 (BD Biosciences).

Aldehyde dehydrogenases (ALDH)-positive cells were identified using the ALDEFLUOR™ Kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's manuals. Briefly, cells were collected as described above and then incubated with Aldefluor in the presence (control) or absence (staining sample) of enzyme inhibitor DEAB at 37°C for 45 min. After incubation, cells were washed, and $ALDH^+$ cells were analyzed using the BD Accuri C6. For the mammosphere formation assay, cells were trypsinized and separated into single cells. Five hundred cells were diluted with MammoCult™ Medium (Stemcell Technologies) and seeded in 24-well ultra-low attachment culture dishes. The number of mammospheres was counted under a Nikon Eclipse Ti microscope on the 14th day after seeding, and the spheres larger than 50 μm in diameter were counted.

siRNA transfection

PMEPA1 siRNAs (Ambion, Inc., Austin, USA) and control luciferase siRNA (Thermo Fisher Scientific Inc., Lafayette, USA) were transfected into different cell lines using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. The final concentration of

siRNAs was 20 nM. The target sequences of PMEPA1 and luciferase were the same as the shRNAs mentioned above. Two PMEPA1 siRNAs were pooled to target PMEPA1.

Immunohistochemistry staining

The anti-PMEPA1 antibody (1 : 100 dilution) was used for immunohistochemistry (IHC) after validation and optimization. In total, 80 samples (20 adjacent breast tissue samples and 60 invasive ductal breast carcinomas) were collected from the First Affiliated Hospital of the Kunming Medical University and stained. IHC was performed as previously described [19] with a standard diaminobenzidine (DAB) staining protocol. Briefly, antigens were first retrieved by boiling the slides in 0.1 M Tris–HCl (pH 9.0) buffer for 5 min. After washing with 0.1 M PBS containing 0.1% Triton X-100, slides were then incubated with the anti-PMEPA1 primary antibody at 4°C overnight. Then the biotinylated anti-mouse secondary antibody and the avidin–biotin complex (Vector Laboratories, Burlingame, USA) were applied to the slides sequentially. Finally, slides were incubated with DAB (Vector Laboratories) until suitable staining developed. Immunostained slides were scored independently by three trained pathologists via the 'Allred Score'. The study was approved by the institutional ethics committees of Kunming Medical University, and all participants provided written informed consent to participate in this study.

Results

PMEPA1 is overexpressed in breast cancer at protein level

To understand the general expression of PMEPA1 in breast cancer cell lines and different molecular subtypes of clinical samples at the protein level, the PMEPA1 protein expression was first examined in seven breast cell lines, including one immortalized cell line (MCF10A), two ER-positive cancer cell lines (MCF7 and T47D), one Her-2-positive cancer cell line (BT474), and three ER-negative cancer cell lines (HCC1937, HCC1806, and SUM149PT). Compared with immortalized breast epithelial cell line MCF10A, PMEPA1 was upregulated in five breast cancer cell lines, but not in T47D (**Fig. 1A**).

To confirm the upregulation of PMEPA1 in breast cancer cells, the expression of PMEPA1 in a subset of clinical samples including 60 invasive breast ductal carcinomas (IDCs) and 20 adjacent breast tissues was also detected via IHC staining. PMEPA1 protein was positively expressed in 15% (3/20) of the adjacent breast tissues, but in ~55% (33/60) of the breast carcinomas (**Fig. 1B** and Table 1). Interestingly, the positive ratio of PMEPA1 protein was significantly increased with the increase of the IDC grades, compared with adjacent tissues (**Fig. 1B** and Table 1). These results suggest that PMEPA1 is overexpressed in breast cancers, especially in high-grade IDCs. Interestingly, when the PMEPA1 expression levels were compared in different

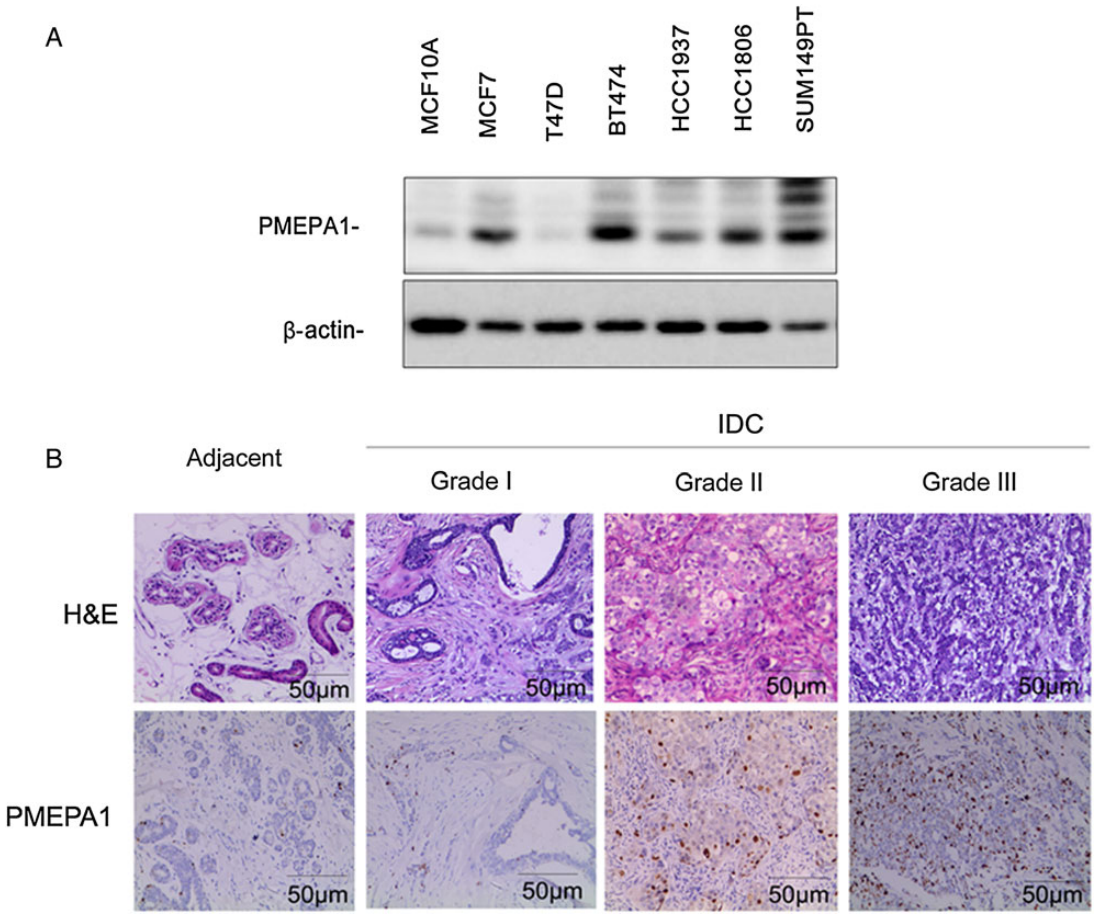

Figure 1. PMEPA1 is overexpressed in breast cancer cell lines and primary tumors at protein level (A) The expressions of PMEPA1 protein in an immortalized breast epithelial cell line MCF10A and six other breast cancer cell lines were measured by western blot analysis. β-Actin was used as the loading control. (B) IHC staining for PMEPA1 in IDC. Twenty normal and 60 IDC sample slides were stained with anti-PMEPA1 antibody, and representative staining images were shown.

Table 1. The results of PMEPA1 IHC

Allred scores[a]	Adjacent	Carcinoma	Grade I	Grade II	Grade III	Luminal A	Luminal B	Her-2	TNBC
Negative (0–2)	17	27	13	9	5	12	12	2	1
Weak (3–4)	2	9	2	3	4	5	2	1	1
Medium (5–6)	1	15	5	4	6	5	4	5	1
Strong (7–8)	0	9	0	4	5	1	1	5	2
All positive	3 (15%)	33 (55%)**	7 (35%)	11 (55%)*	15 (75%)**	11 (48%)	7 (37%)	11 (85%)**	4 (80%)**
Total	20	60	20	20	20	23	19	13	5

[a]The 'Allred score' method has been described previously [16]. Samples with a score >3 were evaluated as positive for PMEPA1 staining. Fisher's exact test compared with adjacent breast tissues. $*P < 0.05$, $**P < 0.01$.

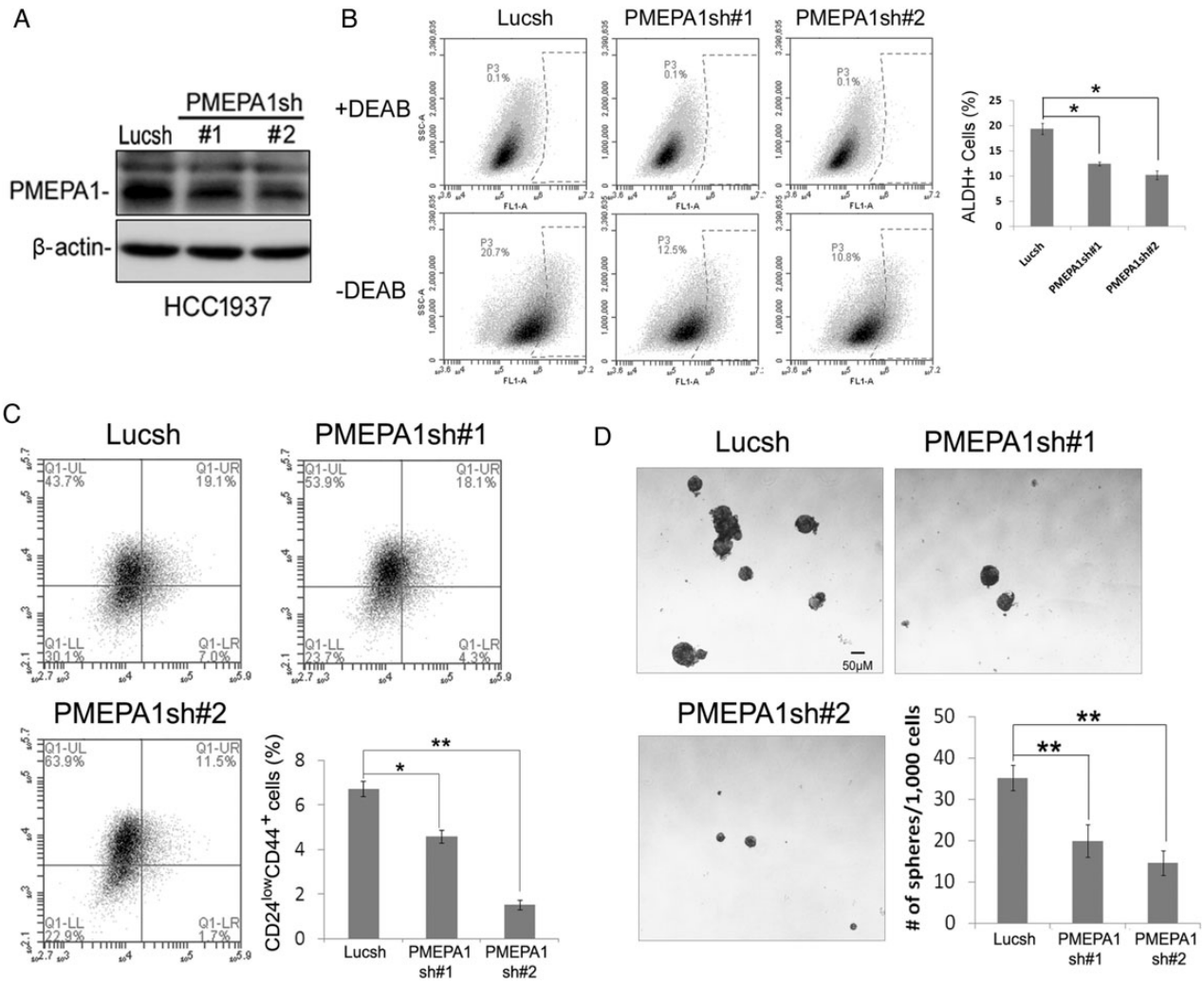

Figure 2. PMEPA1 depletion suppresses breast CSCs (A) PMEPA1 protein expression was downregulated in HCC1937. (B) PMEPA1 knockdown suppressed ALDH$^+$ breast CSC-enriched populations in HCC1937. (C) PMEPA1 knockdown suppressed CD24^{low}/CD44$^+$ cell-enriched populations in HCC1937. (D) PMEPA1 depletion reduced the sphere size and number in HCC1937. Data are presented as the mean ± SD (error bars) from two independent experiments. $*P < 0.05$; $**P < 0.01$.

molecular subtypes (luminal A, luminal B, Her-2, and TNBC) of breast cancers, the Her-2 and TNBC subtypes were found to exhibit significant increases in PMEPA1 expression (Table 1).

PMEPA1 positively regulates breast CSCs

To test whether PMEPA1 regulates breast CSCs that are CD24^{low}/CD44$^+$ cells [20], the CSC populations were analyzed after stable knockdown of PMEPA1 in two triple-negative breast cancer cells, HCC1937 (**Fig. 2**) and HCC1806 (**Supplementary Fig. S1A**), since triple-negative breast cancer is among the most malignant cancers. It was found that two different shRNAs that target PMEPA1 significantly decreased PMEPA1 expression at protein level (**Fig. 2A**). Depletion of PMEPA1 significantly reduced the proportion of CSC-enriched cell population identified by CD24/CD44 marker and ALDH-staining in HCC1937 cell line (**Fig. 2B,C**). These results were further confirmed

by mammosphere formation assay (**Fig. 2D**). After PMEPA1 depletion, both sphere size and number were significantly decreased. This result was further confirmed in another triple-negative cancer cell line HCC1806, which showed similar results (**Supplementary Fig. S1A**). To confirm the role of PMEPA1 in regulating breast CSCs, we ectopically overexpressed PMEPA1 in T47D cells. As shown in **Fig. 3**, PMEPA1a significantly increased the ALDH$^+$ population of T47D cells. Importantly, PMEPA1 expression level is relatively higher in the CSC-enriched population than in the non-CSC populations (**Supplementary Fig. S1B**). Taken together, PMEPA1 plays important roles in regulating breast CSC status.

TGF-β increases CSC population in breast cancer cell lines

TGF-β family plays important roles in breast CSCs. To explore the role of TGF-β in breast CSCs, HCC1937 and HCC1806 cells were treated with either TGF-β or a vehicle control for 48 h before CSC population analysis via CD24/CD44 markers or ALDH method. The results showed that treatment with TGF-β significantly increased CD24^{low}/CD44$^+$ CSC-enriched population in both cell lines (**Fig. 4**) and ALDH$^+$ CSC-enriched population in HCC1937 cells (**Supplementary Fig. S2**).

TGF-β increases breast CSCs partially through upregulating the *PMEPA1* expression

Since TGF-β signaling is known to induce the expression of PMEPA1, and both PMEPA1 and TGF-β play roles in breast CSC maintenance, we further explored whether TGF-β can regulate CSC through PMEPA1. HCC1806 cells were treated with TGF-β or vehicle control for 48 h with or without knocking down *PMEAP1* using PMEPA1 siRNA. Results showed that TGF-β significantly induced *PMEPA1* expression in HCC1806, and PMEPA1 siRNA could block the induction (**Fig. 5A**). CSC population analysis revealed that TGF-β increased

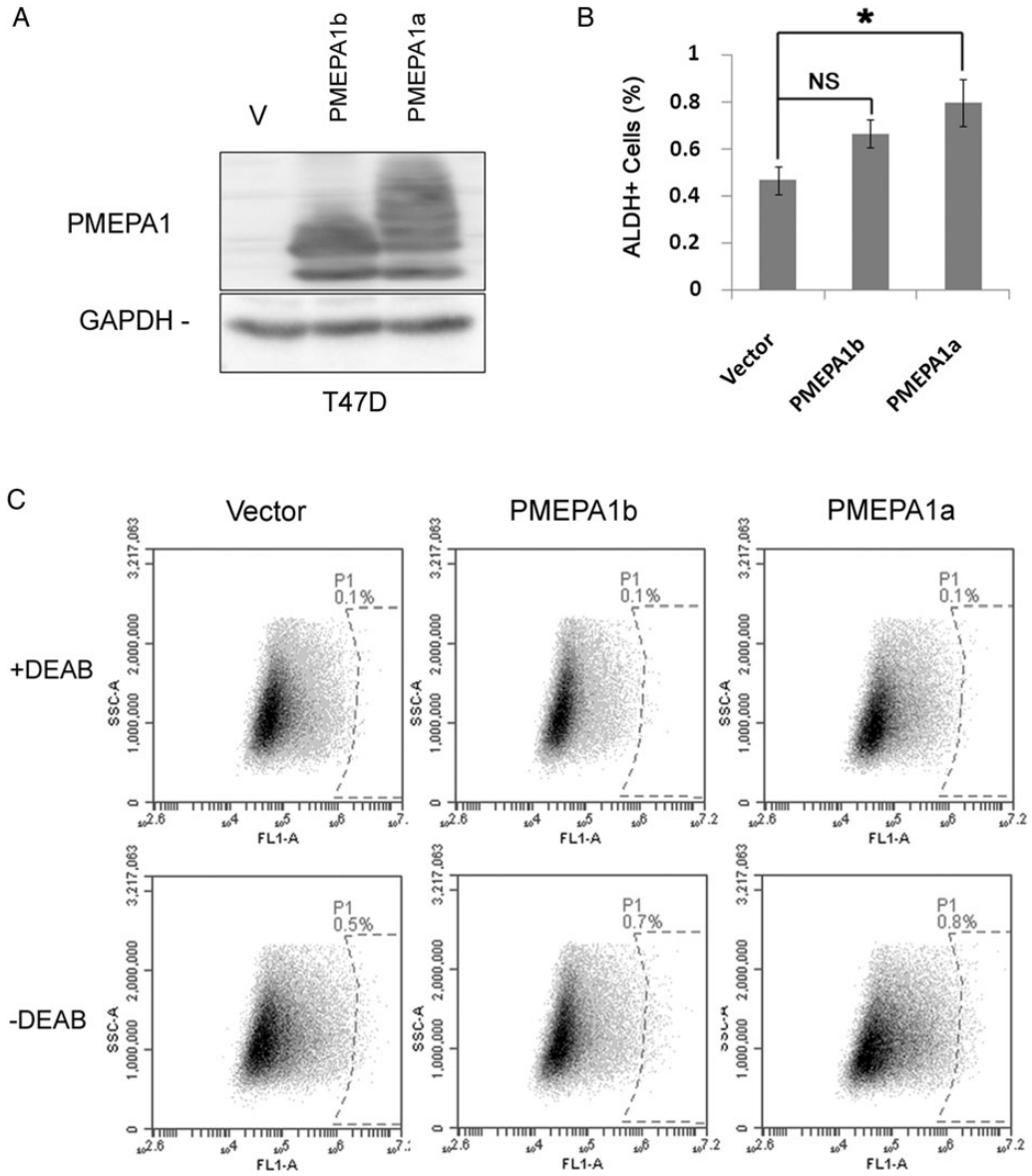

Figure 3. PMEPA1 overexpression increases breast CSCs Ectopic overexpression of PMEPA1 a and b isoforms (A) increased ALDH$^+$ breast CSC-enriched populations in T47D (B and C). *$P < 0.05$.

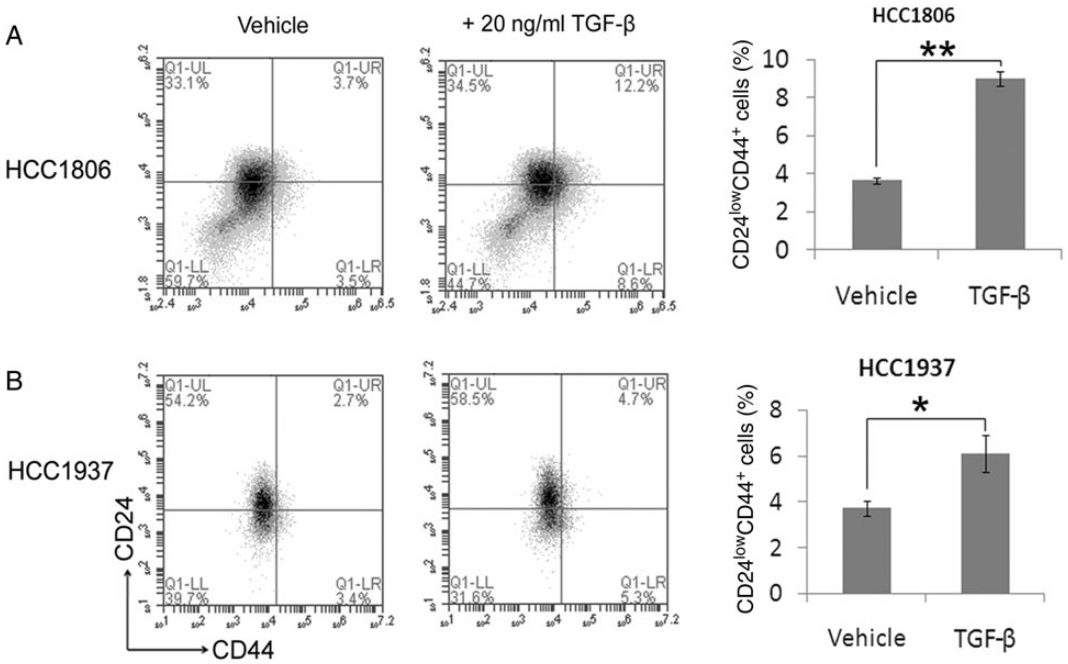

Figure 4. TGF-β induces CSC-enriched population in breast cancer cell lines HCC1806 (A) or HCC1937 cells (B) were plated in 6-well plates. One day after plating, cells were cultured with serum-free medium overnight, followed by either 20 ng/ml TGF-β or vehicle control treatment for 48 h. CSC-enriched population was then analyzed using CD24/CD44 markers. *$P < 0.05$; **$P < 0.01$.

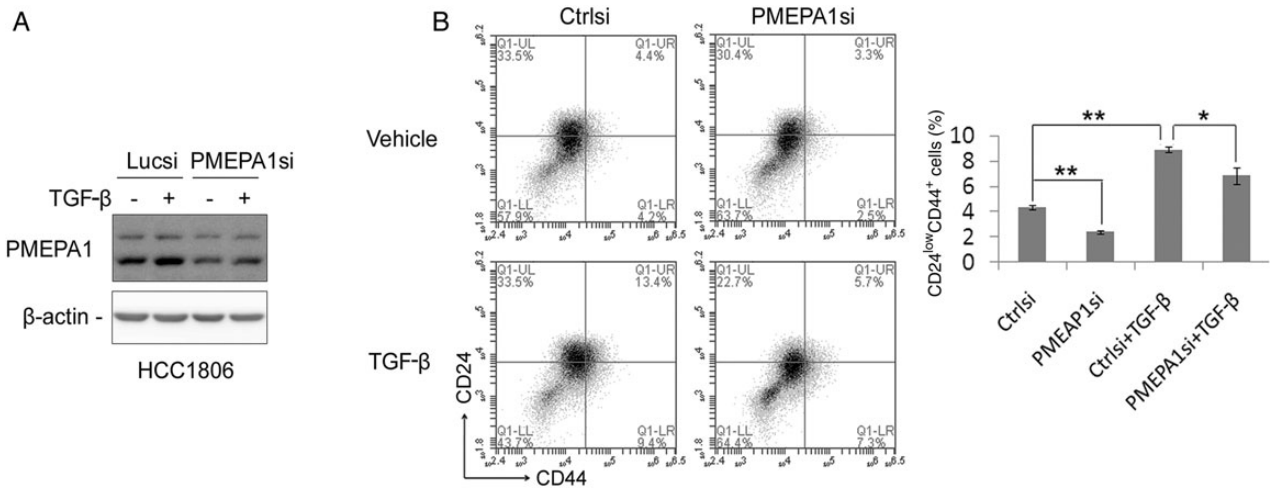

Figure 5. TGF-β induces breast CSCs partially through upregulating PMEPA1 expression (A) TGF-β-induced PMEPA1 expression in HCC1806. HCC1806 cells were seeded in 6-well plate. Twenty-four hours later, cells were transfected with PMEPA1 siRNA or luciferase siRNA control for 24 h followed by culturing in serum-free medium overnight. Serum-starved cells were treated with 20 ng/ml TGF-β or vehicle control for 48 h. Cells were collected for protein extraction and western blot assay. β-Actin was used as loading control. (B) PMEPA1 siRNA partially rescued TGF-β-induced increase of CSC-enriched population in HCC1806. HCC1806 cells were treated as described in (A), and were collected for CD24/CD44 staining and fluorescence-activated cell sorting analysis. *$P < 0.05$; **$P < 0.01$.

CSC-enriched population in HCC1806 and the CSC induction was significantly inhibited by PMEPA1 depletion (**Fig. 5B**). These results suggest that TGF-β, at least partially, regulates breast CSCs via upregulating *PMEPA1* expression.

Discussion

Even though PMEPA1 was previously found to be overexpressed in several solid cancerous malignancies including renal cell carcinoma, rectal adenocarcinoma [15], colon cancer [13], prostate cancer [16], ovarian cancer, and breast cancer [14], the expression patterns of PMEPA1 at the protein level in breast cancer cell lines and different molecular subtypes of clinical samples have not been demonstrated. Among the six cell lines tested in the current study, PMEPA1 was highly expressed in five of the breast cancer cell lines, with the exception of T47D, when compared with the immortalized breast epithelial cell line MCF10A. This result was further confirmed in a series of clinical samples obtained from the First Affiliated Hospital of Kunming Medical

University. We also found that PMEPA1 expression was upregulated in IDCs, and that the expression of PMEPA1 was positively associated with IDC grades (**Fig. 1B** and **Table 1**). Furthermore, the comparison of the PMEPA1 expression levels among several different molecular subtypes with adjacent controls showed that while the PMEPA1 positive percentage was higher in all four molecular subtypes (ranging from 37% to 80%) than that in the adjacent controls (15%), statistically significant increase of PMEPA1 expression was found in Her-2 and TNBC breast cancers (**Table 1**). Unfortunately, the number of clinical sample for Her-2 and TNBC subtypes is not sufficient, and more clinical samples of different subtypes will be needed to further analyze and confirm PMEPA1 expression in different subtypes of breast carcinomas, especially in the TNBC subtype.

Emerging evidence suggests that CSCs play key roles in the development of tumors. The pluoripotent nature and potential for self-renewal make them critical components in the progression of different cancers, but more importantly in the development of novel therapeutics. Although PMEPA1 was previously found to promote tumorigenesis of breast [17] and prostate cancers [16], to the best of our knowledge, no research has been carried out to characterize the role of PMEPA1 in CSCs. In the current study, we found that PMEPA1 depletion dramatically suppressed the ratio of CSC populations in triple-negative breast cancer cell lines HCC1937 and HCC1806 (**Fig. 2** and **Supplementary Fig. S1A**), and on the other hand, PMEPA1 was highly expressed in CSC-enriched cells than in the non-CSC cells (**Supplementary Fig. S1B**), overexpression of PMEPA1 increased the CSC-enriched population (**Fig. 3**). Taken together, our results imply that PMEPA1 plays important roles in regulating breast CSCs. Previous studies have shown that PMEPA1 suppresses the expression of cell cycle-dependent kinase inhibitor $p27^{kip1}$ [17]. Since p27 is important in embryonic stem cell reprogramming and differentiation [21], we tested whether PMEPA1 regulates CSC via p27. As shown in **Supplementary Fig. S3**, knockdown or overexpression of PMEPA1 could suppress or induce p27 expression, respectively. However, in HCC1937, knockdown of p27 did not rescue PMEPA1-depletion-caused reduction of CSC-enriched populations (**Supplementary Fig. S4**). This result suggests that PMEPA1 regulates breast CSCs through other downstream targets.

The role of TGF-β signal in controlling tissue homeostasis and regeneration, immune responses, tumor suppression, metastasis, and embryonic development has been well established, but recent studies have shown that this signal also governs the stem cell properties in numerous types of cell, such as breast cell, neural cell, vascular smooth muscle cell, and cutaneous cell [22–25]. Bhola *et al.* [11] found that both TGF-β receptor inhibitor LY2157299 and smad4 depletion could deplete TGF-β-induced CSC increase in breast cancer cells, indicating that TGF-β signal plays important role in regulating CSC; however, the mechanisms remain unclear. Here, we demonstrated that TGF-β could regulate breast CSC maintenance partially through upregulating PMEPA1 expression (**Fig. 5**). In our study, PMEPA1 depletion was found to only slightly rescue TGFβ-induced increase of CSC-enriched population in HCC1806 (**Fig. 5B**), suggesting that other downstream targets of TGF-β signaling are involved in the CSC control process.

Further studies are need to explain the overall functions of TGF-β signaling in CSC biology, to identify novel targets for further understanding the origination, progression of different types of cancers, and to provide better treatments for cancers.

Supplementary Data

Supplementary Data is available at *ABBS* online.

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