Original Article
The TGF-β induced gene, PMEPA1, attenuates antitumor efficacy of aspirin in triple-negative breast cancer by regulating TGF-β1

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Abstract: Prostate transmembrane protein, and androgen-induced 1, originally found in prostate tissue, is over-expressed in a variety of cancers. The gene is mainly regulated by TGF-β and androgen receptor signaling. Recently, aspirin is regarded as a promising candidate for cancer treatment. Mechanisms of cancer repression acted by aspirin include cell-cycle arrest, apoptosis induction and cancer stem cells inhibition. In this study, we found out that aspirin upregulated TGF-β1 and PMEPA1 in a dose-dependent manner and TGF-β1 is a key mediator of aspirin in inducing apoptosis of 4T1 cells. When PMEPA1 was silenced, TGF-β1 levels increased in cell supernatants and more apoptosis of 4T1 cells was induced compared to dealt with aspirin alone. Our results indicated that high expression of PMEPA1 feedback repressed TGF-β1, suggested that up-regulation of PMEPA1 may be a protective mechanism of cancer cells against aspirin treatment. Therefore, silencing PMEPA1 might be a novel approach for improving the effects of aspirin therapy.

Keywords: Aspirin, PMEPA1, TGF-β1, apoptosis, triple-negative breast cancer

Introduction

Breast cancer is one of the most frequently diagnosed cancers in the world [1]. It is expected to account for 30% of all new cancers in American women in 2017, and is the leading cause of cancer-related death [2]. Since 1991, breast cancer-related mortality has been declining due to early detection and more effective treatments [3, 4]. However, some patients inevitably experience recurrence and metastases, which negatively impact the length and quality of the lives of breast cancer patients. Therefore, further studies into breast cancer biology and therapies are needed. Recently, studies have shown that aspirin, a classic anti-inflammatory drug, exhibits good antitumor efficacy in many types of cancers, including colorectal, pancreatic, and breast cancers [5-7].

Aspirin, a non-steroidal anti-inflammatory drug, is extensively applied for the treatment of numerous diseases due to its various properties, which include analgesic, antipyretic, and anti-inflammatory activity [8]. COX is the main target of aspirin, especially COX2. COX2 repression leads to decreases in prostaglandins (PGs) [9], which play crucial roles in many physiological and pathological processes. PGs have long been known to help cancer cells survive, proliferate, and invade [10]. Preclinical research has elucidated the antitumor mechanisms of aspirin that make it a promising candidate for cancer treatment.

Prostate transmembrane protein, androgen-induced 1 (PMEPA1, also called TMEPA1, STAG1, ERG1.2, or N4WBP4) is a gene that is located at chromosome 20q13 [11]. It is over-expressed in a variety of cancers, such as prostate, breast, and colon cancers [12-14]. Studies show that PMEPA1 contributes to cell proliferation, migration, and invasion [15-17]. There are two distinct regulatory mechanisms of PMEPA1 expression. In prostate cancer, PMEPA1 is mainly regulated by androgen recept-

or signaling [18], whereas, in other types of cancers, many cytokines can induce PMEPA1 expression, including transforming growth factor-β (TGF-β) [19], epidermal growth factor [20], and granulocyte-macrophage colony stimulating factor [21], among which TGF-β is the most important factor.

TGF-β1 is a versatile cytokine of the TGF-β superfamily. TGF-β plays paradoxical roles in the development of cancers. On one hand, TGF-β1 induces epithelial-to-mesenchymal transition in cancer cells which promotes invasive and metastasis in advanced cancer. On the other hand, TGF-β regulates diverse cell processes, including cell-cycle arrest and promote apoptosis in a pre-malignant stage, which are crucial function mechanisms of anti-cancer drugs [22, 23].

Aspirin is a well-accepted chemo-prevention drug for cancers, but its effect is less than satisfactory in breast cancer treatment [24]. And the molecular mechanism is complex and poorly understood. In this study, we sought to characterize the relationships between aspirin, PMEPA1 and TGF-β1, and tried to explain the complex antitumor mechanism of aspirin in triple-negative breast cancer.

Material and methods

Cell culture

The mice breast carcinoma 4T1 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured with Dulbecco's Modified Eagle's Medium (Gibco-BRL, Grand Island, NY, USA) in 75 cm^2 flasks or six and ninety-six well plates, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 units/ml streptomycin and maintained at 37°C in a humidified condition of 95% air and 5% CO_2 atmosphere throughout the experiments.

Cell proliferation assay

The effect of aspirin on 4T1 cells was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 4T1 cells (5 × 10^3 cells/well) were seeded into 96-well plates and after cultured for 24 hours, we added different concentration of aspirin and continuously culture for 48 hours, following with addition of MTT solution to the cells for 4 hours. After removing the medium, the remaining MTT formazan crystals were solubilized in DMSO and measured at 570 nm using a microplate reader (Benchmark Electronics, Angleton, TX, USA).

Flow cytometry analysis of apoptosis

4T1 cells were treated with aspirin at 0, 4, and 8 mM concentration for 48 hours and then collected, washed in cold phosphate buffered saline (PBS), double stained with fluorescein isothiocyanate (FITC) conjugated Annexin V and Propidium Iodide (KeyGene Biotech, Jiangsu, China) and analyzed by flow cytometry (FACS Aria SORP, BD Biosciences, Erembodegem, Belgium).

Western blot

The cells were collected, lysed, and total protein was quantified with Micro BCA Protein Assay Kit (Pierce, USA). Western blot analysis was performed as described previously. In brief, total protein (30 μg) from each sample was separated by electrophoresis using 12% SDS-PAGE gels, and transferred onto PVDF membranes, blocked with 5% skim milk (Merck), and incubated using the primary antibodies (1:1000) overnight at 4°C. Then, membranes were washed with phosphate-buffered saline (PBS) containing 0.1% tween-20 (PBST) and incubated with corresponding secondary antibodies (1:10000) for 1 h at room temperature. Membranes were washed with PBST, and the target protein signals were developed on X-ray films following exposure to ECL advanced luminescence. All western blot analysis was performed at least in duplicate, and the representative blots were shown.

siRNA transfection

PMEPA1 siRNAs (GenePhama, Shanghai, China) and negative control siRNA were transfected into 4T1 cell line using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. The final concentration of siRNAs was 20 nM. The target sequences of the three PMEPA1 siRNAs were pooled to target PMEPA1 and the sequences of siRNAs are as follows: siRNA 1105 (5'-GCUCCAUCACUC-GCACAUUTT-3', 5'-AAUGUGCGAGUGAUGGAGC-TT-3'); siRNA 1141 (5'-GGAGAAGGAGAAACAG-
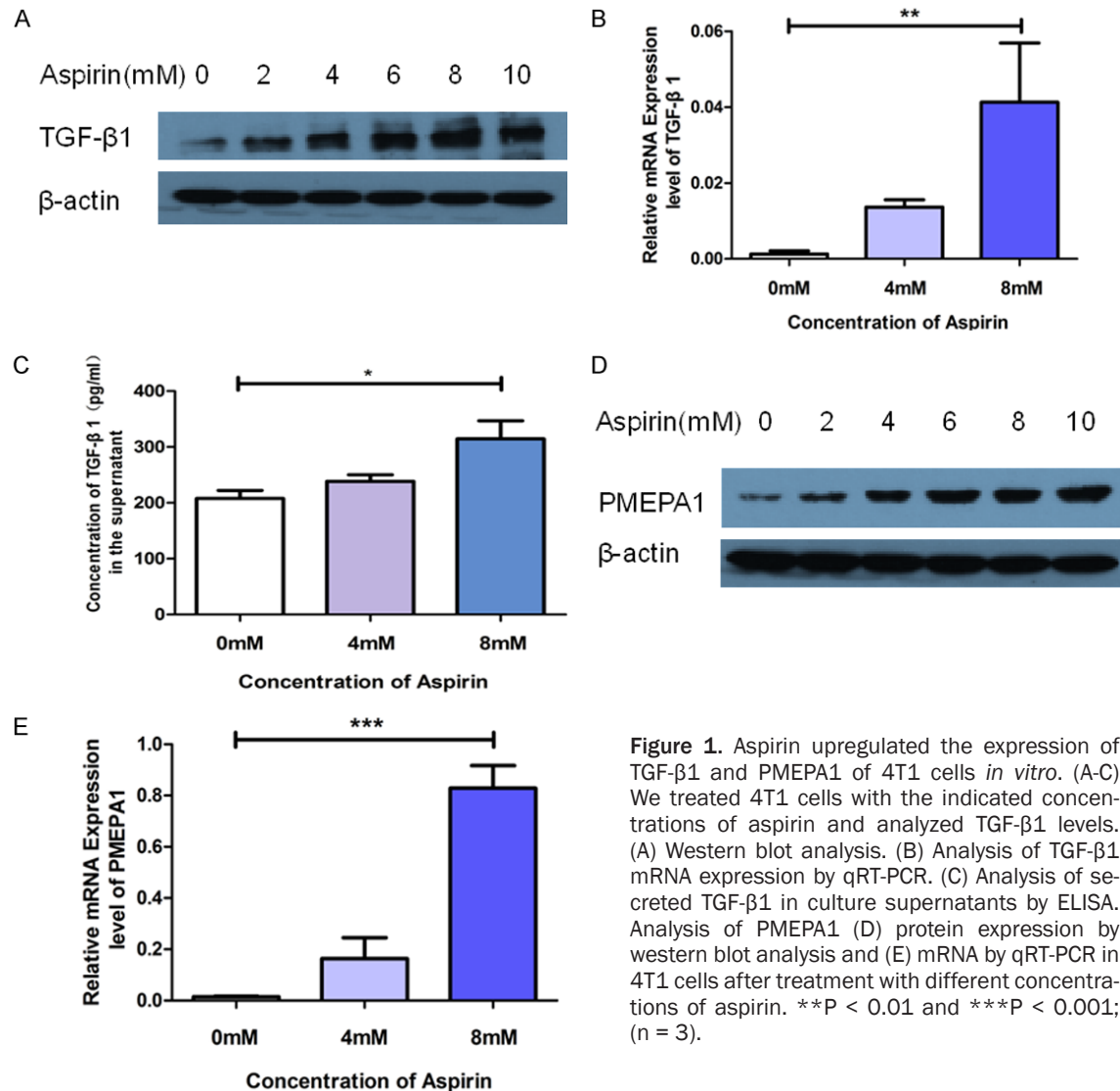
Figure 1. Aspirin upregulated the expression of TGF-β1 and PMEPA1 of 4T1 cells *in vitro*. (A-C) We treated 4T1 cells with the indicated concentrations of aspirin and analyzed TGF-β1 levels. (A) Western blot analysis. (B) Analysis of TGF-β1 mRNA expression by qRT-PCR. (C) Analysis of secreted TGF-β1 in culture supernatants by ELISA. Analysis of PMEPA1 (D) protein expression by western blot analysis and (E) mRNA by qRT-PCR in 4T1 cells after treatment with different concentrations of aspirin. **P < 0.01 and ***P < 0.001; (n = 3).

AAATT-3', 5'-UUUCUGUUUCUCCUUCUCCTT-3'); siRNA 902: (5'-GACAGUGACCUUAUAGA CATT-3', 5'-UGUCUAUAAGGUCACUGUCTT-3'); Negative control siRNA: (5'-UUCUCCG AACGUGUCACGU-TT-3', 5'-ACGUGACACGUUCGGAGAATT-3').

Enzyme-linked immunosorbent assay

4T1 cells were added to six-well plates with the same amount. After 24 h, silencing PMEPA1 by siRNA as described earlier, and then incubated the cells for another 48 h. The culture supernatants were collected. The levels of TGF-β1 in the samples were assessed by mouse ELISA kits (eBioscience, Minneapolis, MN, USA) according to the manufacturer's instructions, and the colorimetric reaction was measured at 450

nm using a microplate reader (Benchmark Electronics, Angleton, TX, USA).

Quantitative real-time PCR

Total RNA was extracted from cultured cells with Trizol reagents (Invitrogen) according to the manufacturer's protocol. Briefly, total RNA was reverse transcribed to cDNA by using Prime Script RT reagent Kit (Takara, Dalian, China). The quantitative real-time RT-PCR analysis was performed by using Takara SYBR Premix ex-taqtm (Takara Biotechnology, Dalian, People's Republic of China). The reaction mixtures containing SYBR Green were composed following the manufacturer's protocol. The sequences of the primers used were as follows: β-actin
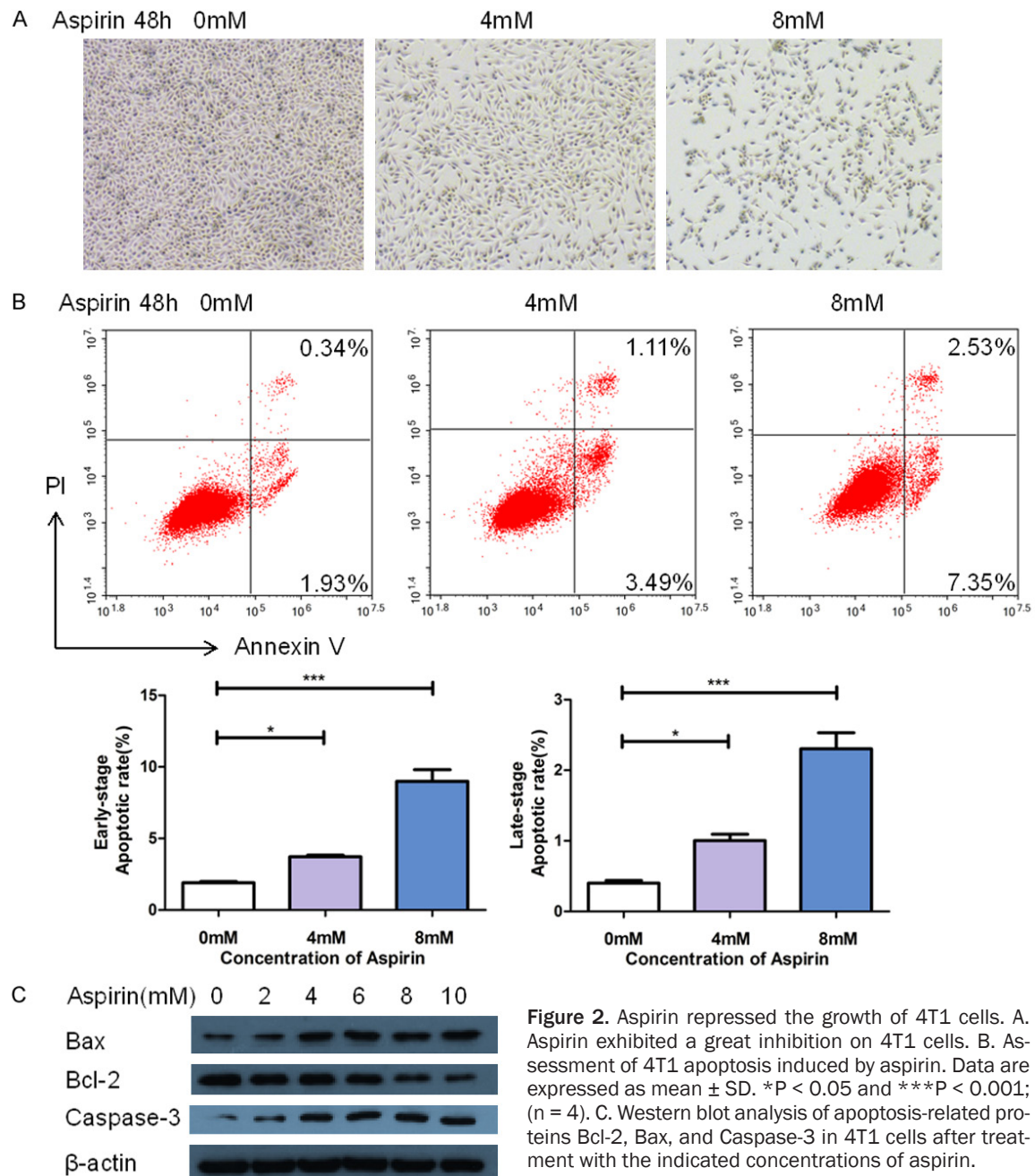
Figure 2. Aspirin repressed the growth of 4T1 cells. A. Aspirin exhibited a great inhibition on 4T1 cells. B. Assessment of 4T1 apoptosis induced by aspirin. Data are expressed as mean ± SD. *P < 0.05 and ***P < 0.001; (n = 4). C. Western blot analysis of apoptosis-related proteins Bcl-2, Bax, and Caspase-3 in 4T1 cells after treatment with the indicated concentrations of aspirin.

(5'-CCTCTATGCCAACACAGTGC-3', 5'-ACATCTGC-TGGAAGGTGGAC-3'); TGF-β1 (5'-AGGGCTACCA-TGCCACTTC-3', 5'-GCGGCACGCAGCACGGTGAT-3'); PMEPA1 (5'-TGGAGTTCGTGCAAATCGTG-3', 5'-TCCGAGGACAGTCCATCGTC-3').
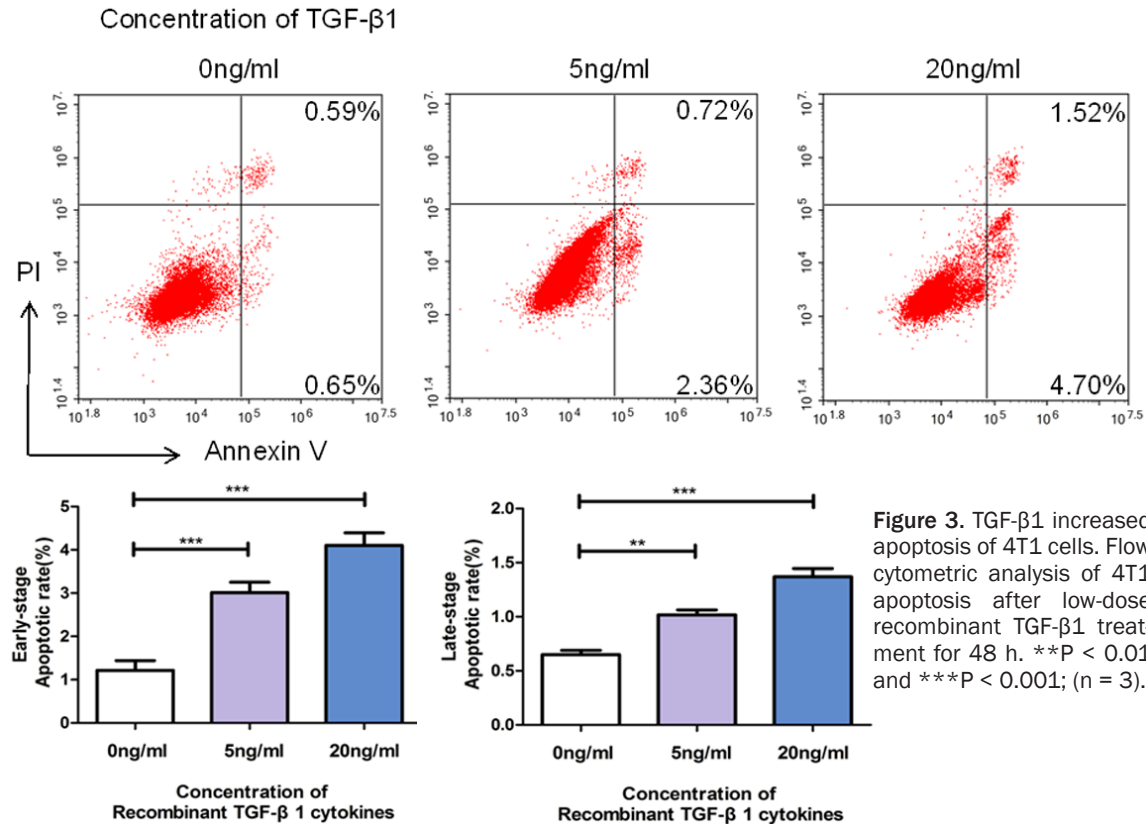
Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM Software, New York, NY, USA). All data are expressed as means ± SD. Comparisons between groups were performed by Student's *t*-test and one-way analysis of variance (ANOVA). The level of significance was set at *P* < 0.05.

Results

Aspirin upregulated the expression of TGF-β1 and PMEPA1 of 4T1 cells in vitro

We tested expression of TGF-β1 of 4T1 cells seeded in six-well plates by western blot, ELISA and qRT-PCR after treated the cells with differ-

Figure 3. TGF-β1 increased apoptosis of 4T1 cells. Flow cytometric analysis of 4T1 apoptosis after low-dose recombinant TGF-β1 treatment for 48 h. **P < 0.01 and ***P < 0.001; (n = 3).

ent concentration of aspirin for 48 h. The results of western blot and qRT-PCR shown that levels of TGF-β1 were increased in a dose-dependent manner (**Figure 1A** and **1B**). The secretion of TGF-β1 in the supernatants tested by ELISA shown that total TGF-β1 decreased in a dose-dependent manner, but we also observed a decrease in the amount of 4T1 cells. We counted the number of cells in different groups and calculated levels of TGF-β1 secreted by 1×10^6 4T1 cells and discovered that higher concentration of aspirin induced more TGF-β1 secretion by the same number of 4T1 cells (**Figure 1C**). we next tested expression of PMEPA1 by western blot and qRT-PCR. The results showed that PMEPA1 was up-regulated by aspirin also in a dose-dependent manner (**Figure 1D** and **1E**). We concluded that aspirin could improve expression of TGF-β1 and PMEPA1 of 4T1 cells *in vitro*.

TGF-β1 played a key role in inducing apoptosis of 4T1 cells

Since we could observe a decrease in the amount of 4T1 cells (**Figure 2A**) after aspirin treatment and according to our previous study

that aspirin could induce apoptosis of CT26 cells by TGF-β1 [25], we speculated that aspirin might lead to apoptosis or cell-cycle arrest of 4T1 cells. Then, we tested the apoptosis and cell cycle status of 4T1 cells treated with aspirin for 48 h. As shown in **Figure 2B**, the highest concentration of aspirin induced more 4T1 cells apoptosis. However, the effects of aspirin on 4T1 cell cycle inhibition were inconsistent. Next, we tested if aspirin modulated the levels of Bcl-2, Bax, and Caspase-3, the prime regulators of apoptosis. The results shown that 4T1 cells had increased expression of Bax and Caspase-3 in a dose-dependent manner, whereas Bcl-2 expression decreased (**Figure 2C**).

To determine if TGF-β1 played a role in apoptosis inducing act of aspirin, we treated 4T1 cells with recombinant TGF-β1 and tested apoptosis, the results is that recombinant TGF-β1 promoted the apoptosis of 4T1 cells (**Figure 3**). Next, we pretreated 4T1 cells with 20 μM SB431542, a selective TGF-β/Smad inhibitor, for 24 hours, then treated with aspirin or recombinant TGF-β1 cytokines for 48 h. We found no significant differences in apoptosis among the aspirin-
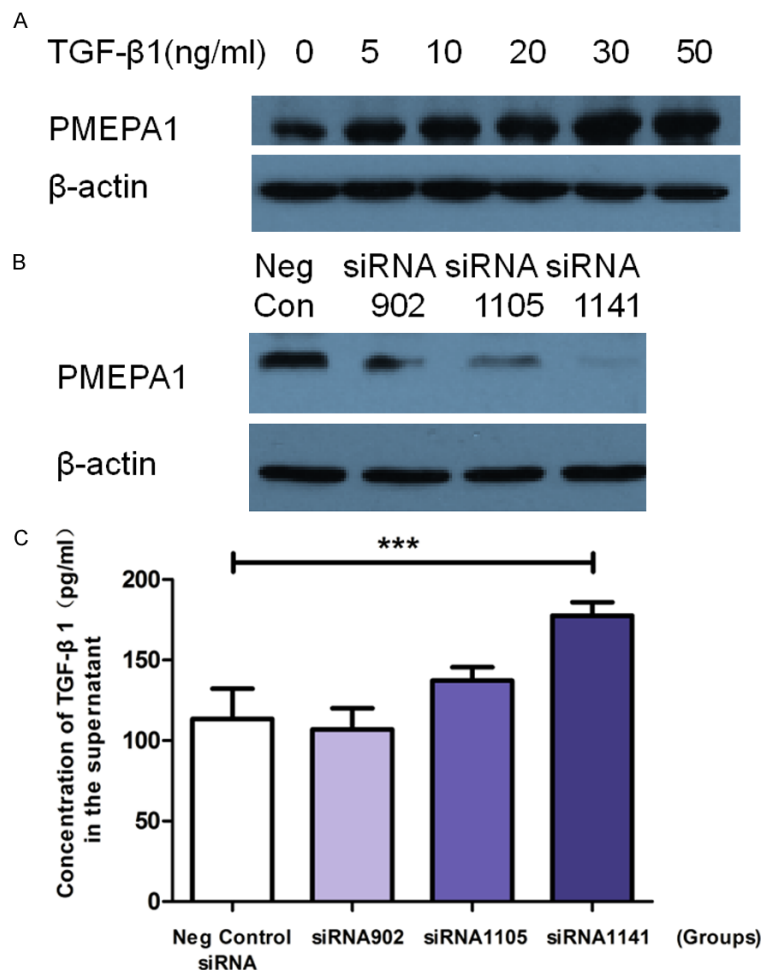
Figure 4. The combination of aspirin treatment and PMEPA1 silencing enhances antitumor activity over aspirin alone by increasing TGF-β1. (A) Analysis of PMEPA1 expression by western blot analysis (B) Western blot analysis of PMEPA1 in 4T1 cells after treatment with siRNAs for 48 h. (C) Analysis of secreted TGF-β1 in the culture supernatants of 4T1 cells after PMEPA1 silenced and treated with aspirin for 48 h by ELISA. ***P < 0.001; (n = 3).

PMEPA1 through TGF-β1. We tested the expression of PMEPA1 by western blot after 4T1 cells were treated with recombinant TGF-β1. The results shown that PMEPA1 was up-regulated by recombinant TGF-β1 (**Figure 4A**). Previous study has shown that PMEPA1 is also a regulator of TGF-β [26]. We hypothesized that silencing PMEPA1 might improve the function of aspirin in increasing TGF-β1. Then, we applied siRNA to interference expression of aspirin, and the effect was tested by western blot. The results shown that siRNA 1141 had the strongest inhibition of PMEPA1 (**Figure 4B**). And we tested TGF-β1 in the supernatants after applied siRNA for 48h. The result indicated that TGF-β1 levels were higher compared with the control group, especially the siRNA 1141 group (**Figure 4C**). Finally, we tested the apoptosis of PMEPA1-silenced 4T1 cells after treatment with aspirin. The combination of aspirin treatment and PMEPA1 silencing led to the accumulation of more early and late apoptotic cells than in the negative control group, and siRNA 1141 inhibited PMEPA1 most effectively (**Figure 5**). Taken together, these results indicated that in combination with silencing PMEPA1, aspirin induced more 4T1 cell apoptosis by increasing TGF-β1 and PMEPA1 is a potential target to improving antitumor activity of aspirin.

Discussion

Although breast cancer mortality is declining, it remains the leading cause of cancer-related death in women globally [2, 3]. It is, therefore, critical to search for new therapeutic targets. In the present study, we discovered that aspirin upregulated TGF-β1 and PMEPA1 in a dose-dependent manner and TGF-β1 is a key mediator of aspirin in inducing apoptosis of 4T1 cells.

treated and TGF-β1-treated groups pretreated with the inhibitor and the non-treated controls (Figure S1A, S1B). These results suggested that TGF-β1 played a key role in apoptosis-inducing activity of aspirin, and the apoptosis-inducing activity of aspirin and recombinant TGF-β1 were inhibited by the TGF-β/Smad inhibitor.

In combination with silencing PMEPA1, aspirin induced more 4T1 cell apoptosis by increasing TGF-β1

Previous studies verify that PMEPA1 is a gene which is mainly regulated by TGF-β and androgen receptor signaling [18, 19]. we speculated that aspirin might up-regulated expression of
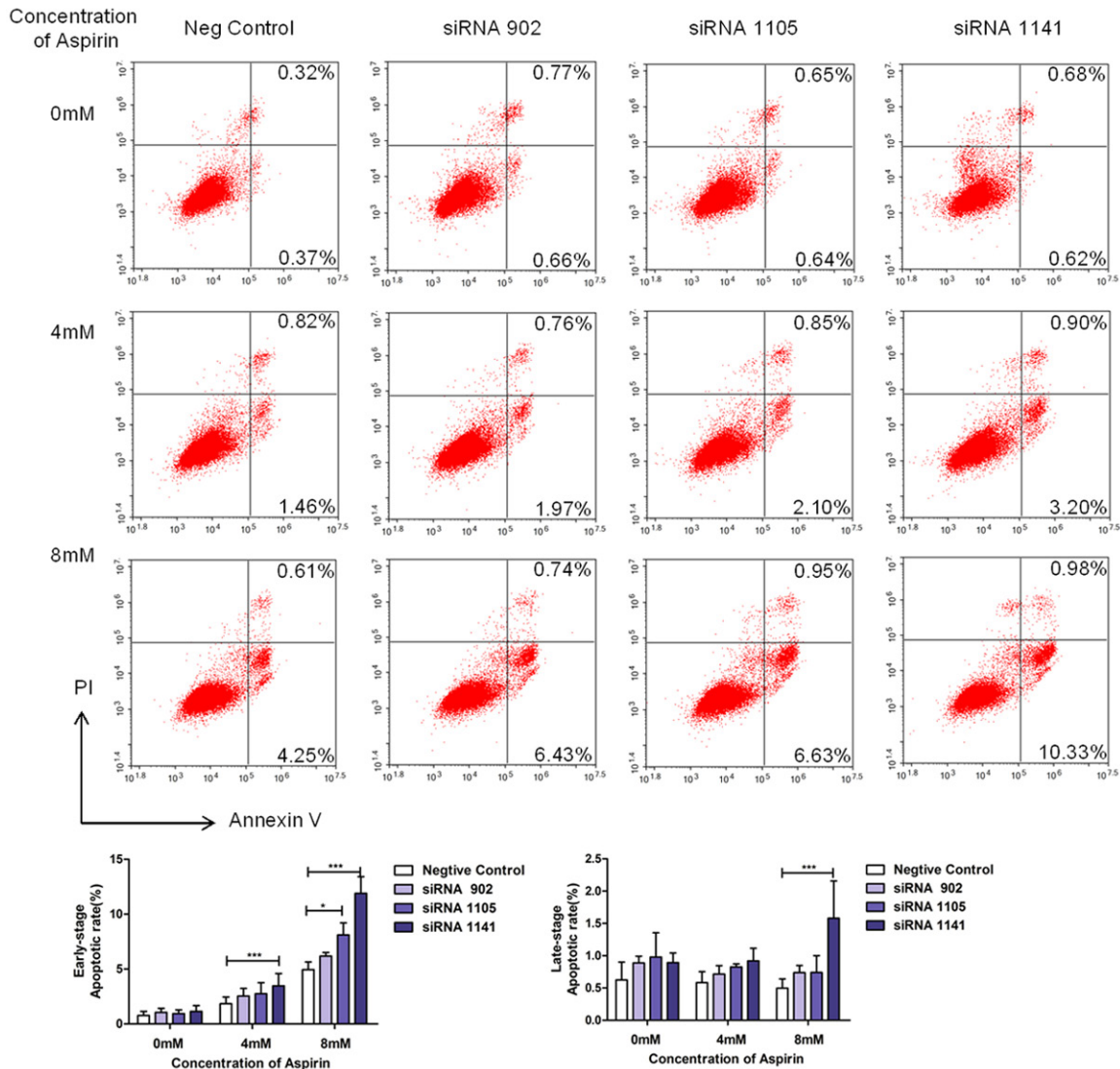
Figure 5. The combination of aspirin treatment and PMEPA1 silencing led to the accumulation of more early and late apoptotic cells. Flow cytometric analysis of 4T1 apoptosis upon PMEPA1 silencing combined with aspirin treatment. *P < 0.05 and ***P < 0.001; (n = 3).

And when PMEPA1 was silenced, TGF-β1 levels increased more in cell supernatants and more apoptosis of 4T1 cells was induced compared to dealt with aspirin alone. Our results indicated that high expression of PMEPA1 feedback repressed TGF-β1, suggesting that up-regulation of PMEPA1 may be a protective mechanism of cancer cells against aspirin treatment. Therefore, silencing PMEPA1 might be a novel approach for improving the effects of aspirin therapy.

Recently, aspirin has been regarded as a promising candidate for cancer treatment. It is now recommended for chemo-prevention of color-

ectal cancer by the U.S. Preventive Services Task Force [24]. Clinical studies have shown that aspirin prolongs the overall survival and disease-free survival of breast cancer patients [27]. Preclinical studies indicate that aspirin uses various mechanisms to repress malignant tumors, including inhibiting proliferation [28], inducing apoptosis [29], decreasing cancer stem cells [30], and relieving inflammation in the tumor microenvironment [6].

Apoptosis is one of the most important cell death mechanisms. It is recognized as a critical activity of anticancer agents [31, 32]. The rate of apoptosis affects the growth of tumors by
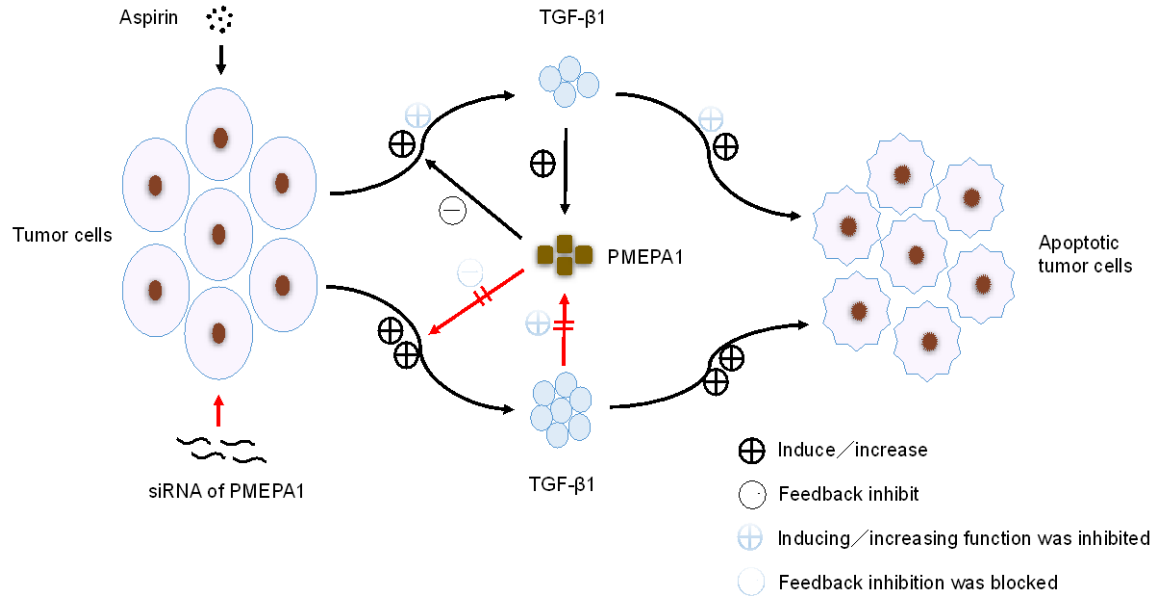
Figure 6. The relationships of aspirin, TGF-β1 and PMEPA1 in apoptosis process of 4T1 cells. Aspirin inhibited 4T1 cells partially through inducing apoptosis, and TGF-β1 plays a key role in the process. PMEPA1 was up-regulated by aspirin also through TGF-β1 at the same time. Then, the up-regulated PMEPA1 feedback repressed the TGF-β1 increasing activity of aspirin. Silencing PMEPA1 reinstated the TGF-β1 increasing activity of aspirin and thus, aspirin induced more apoptosis of 4T1 cells.

shortening the lifespan of cancer cells in a living system [33]. Aspirin induces apoptosis through both COX-dependent and COX-independent pathways. In COX-dependent pathway, inhibition synthesis of prostaglandin, which helps cancer cells growth and invasion, is the main mechanism [34]. The COX-independent mechanisms include modulation of the IL-6-STAT3 signaling pathway [35], activation of p38 MAP kinase [36], and repression of NF-κB signaling [37]. Aspirin also suppresses the growth of PI3K-mutant breast cancer by activating AMPK and inhibiting mTORC1 signaling [38]. The COX-dependent and -independent pathways can work cooperatively to drive the apoptosis of tumor cells [39, 40].

TGF-β1, a versatile member of the TGF-β superfamily, can inhibit the proliferation of many epithelial cell types [41], such as intestinal epithelial cells, whereas it stimulates the growth of mesenchymal cells. It can also induce apoptosis of various cancer cells, but the reactions of different malignant cells to TGF-β1 are different. Patients with breast cancer characterized by high levels of TGF-β1 appear to have a longer disease-free interval with a better probability of survival [42]. A study has elucidated that TGF-β1 inhibits the growth of breast cancer by

repressing tumor-initiating cells [43]. Our results firstly demonstrated that TGF-β1 is a key mediator of the aspirin-induced growth inhibition of breast cancer by inducing apoptosis.

PMEPA1 expression is mainly regulated by TGF-β and androgen receptor signaling [18, 19]. Microarray analysis identified PMEPA1 as the gene most increased in PC-3 cells by TGF-β treatment, and the study verifies that PMEPA1 affects bone metastasis of prostate cancer through regulating TGF-β [26]. Our study shows, for the first time, that PMEPA1 plays a role in apoptosis induced by aspirin. There is a known feedback loop between TGF-β and PMEPA1, wherein TGF-β induces PMEPA1 expression and knockdown of PMEPA1 increases the expression of TGF-β [19, 26]. In our experiment, when we silenced PMEPA1, the levels of TGF-β1 increased in the culture supernatants. This regulation mechanism is consistent with those described in previous studies in other cell lines. Therefore, we propose that aspirin-mediated up-regulation of PMEPA1 may be a self-protection mechanism in 4T1 cells. PMEPA1 silencing partially suppressed this mechanism, and reinstated the ability of aspirin to increase TGF-β1. Thus, PMEPA1 silencing in combination with aspirin treatment could induce greater tumor cell apoptosis.

Although much more experiments were needed to clarify the precise relationship of PMEPA1, TGF-β1 and aspirin in 4T1 cell line, it could be explained as follows (**Figure 6**): aspirin inhibited 4T1 cells partially through inducing apoptosis, and TGF-β1 plays a key role in the process. PMEPA1 was up-regulated by aspirin also through TGF-β1 at the same time. Then, the up-regulated PMEPA1 feedback repressed the TGF-β1 increasing activity of aspirin. Silencing PMEPA1 reinstated the TGF-β1 increasing activity of aspirin and thus, aspirin induced more apoptosis of 4T1 cells.

In conclusion, aspirin is regarded as a promising candidate for cancer treatment. In this study, we have demonstrated that aspirin represses 4T1 cells growth by inducing apoptosis in vitro and TGF-β1 is a key mediator of aspirin in inducing apoptosis of 4T1 cells. Aspirin also increased the expression of PMEPA1 by TGF-β1. When PMEPA1 was silenced, TGF-β1 levels increased more in cell supernatants and more apoptosis of 4T1 cells was induced compared to dealt with aspirin alone. Our results indicated that high expression of PMEPA1 feedback repressed TGF-β1, suggesting that up-regulation of PMEPA1 may be a protective mechanism of cancer cells against aspirin treatment. Thus, combined aspirin treatment with PMEPA1 silencing might improve the antitumor efficacy of aspirin against breast cancer via cooperatively increasing TGF-β1 expression and silencing PMEPA1 might be a novel approach for improving the effects of aspirin therapy.

Acknowledgements

Disclosure of conflict of interest

None.

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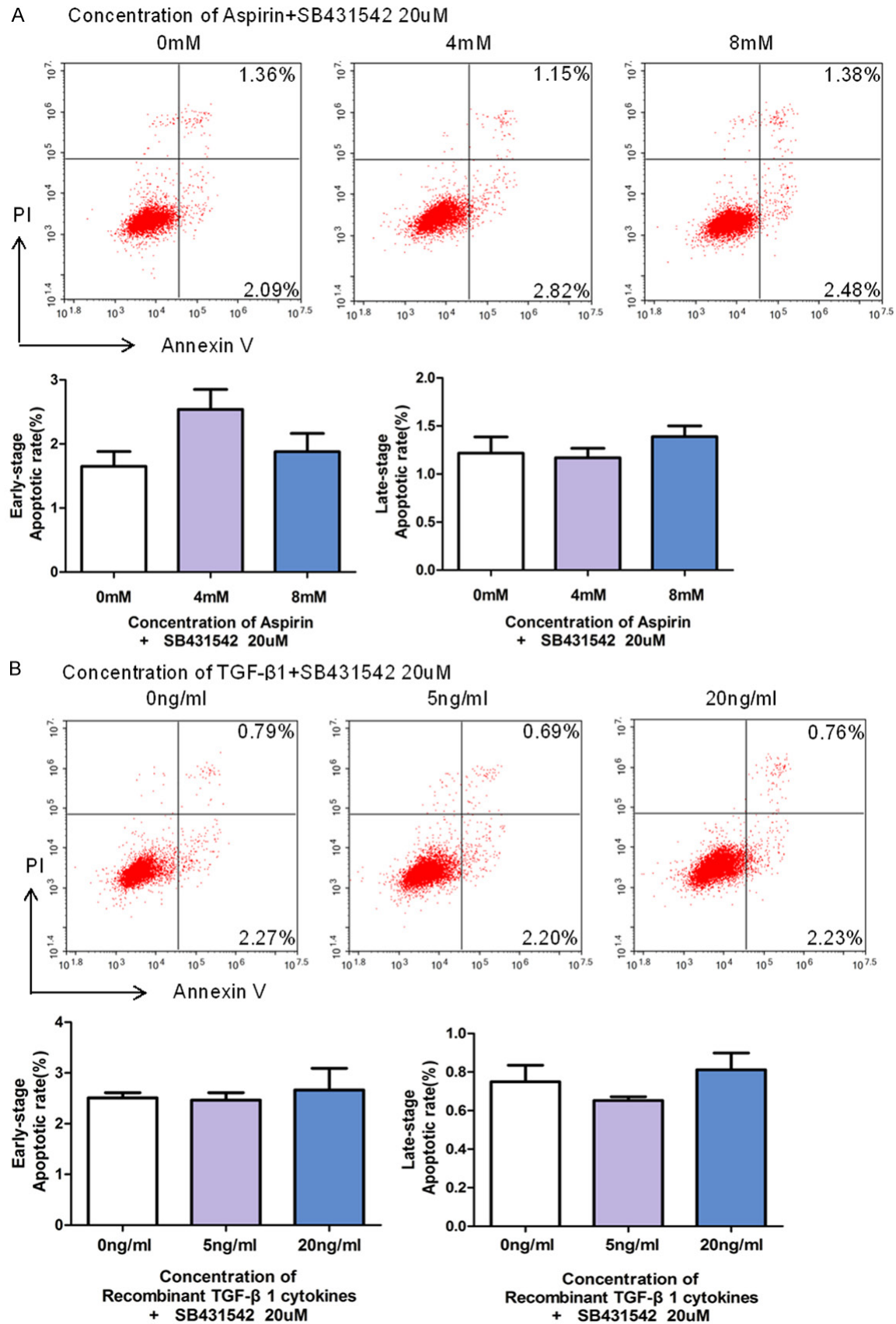
Figure S1. The apoptosis inducing function of aspirin and TGF-β1 could be inhibited by TGF-β1/Smad inhibitor SB431542. Flow cytometric analysis of apoptosis in 4T1 cells pretreated with SB431542 for 24 h, then treated with (A) different concentrations of aspirin, or (B) low-dose TGF-β1.