Trichostatin A inhibits proliferation of triple negative breast cancer cells by inducing cell cycle arrest and apoptosis

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Triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer with poor outcome. Because of lacking therapeutic targets, chemotherapy is the main treatment option for patients with TNBC. Overexpression of HDACs correlates with tumorigenesis, highlighting the potential of HDACs as therapeutic targets for TNBC. Here we demonstrate that trichostatin A (TSA, a HDAC inhibitor) selectively inhibits the proliferation of TNBC cell lines HCC1806 and HCC38 rather than a normal breast cell line MCF10A. The inhibition of TNBC by TSA is via its roles in inducing cell cycle arrest and apoptosis. TSA treatment leads to decreased expression of CYCLIN D1, CDK4, CDK6 and BCL-XL, but increased P21 expression. Moreover, combination of TSA with doxorubicin has synergistic effects on inhibiting proliferation of HCC1806 and HCC38 cells. Our studies identified a promising epigenetic-based therapeutic strategy that may be implemented in the therapy of fatal human breast cancer.

Key words: triple negative breast cancer, Trichostatin A, apoptosis; cell cycle arrest, MAPK

Triple negative breast cancer (TNBC) is one of the most aggressive human cancers associated with early metastasis and death [1–3]. Because TNBC do not express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2), patients with TNBC cannot benefit from the current anti-estrogen and anti-HER2 antibody therapies [4, 5]. Currently, the standard of care is surgery with adjuvant chemotherapy or radiation therapy. Therefore, new therapeutic strategy of TNBC is urgently needed to decrease the side effect and resistance of chemotherapy.

TNBC is a heterogeneous disease and categorized into five major subtypes based on gene expression profile: basal like, immunomodulatory, mesenchymal, mesenchymal stem like and luminal androgen receptor [6]. Basal like TNBC accounting for 47%, is the main type of TNBC. The top gene ontologies for the basal like breast cancer subtype are heavily enriched in cell cycle and cell division components and pathways [7]. Therefore, targeting cell cycle related genes might be effective approach to treat basal like breast cancers.

Epigenetic mechanisms such as DNA methylation and histone modification play important roles in tumorigen-

esis. The histone deacetylase (HDAC) remodel chromatin and regulate genes transcription by removing acetyl groups from histone and further modulate the process of cell proliferation, cell cycle and cell differentiation [8, 9]. Multiple tumors express higher level of HDACs which indicate that HDACs might be potential targets for epigenetic treatment [10]. HDAC inhibitors enhance the acetylation of cellular proteins by blocking HDAC activity and play critical roles against cancer by inducing differentiation, cell cycle arrest and apoptosis [11, 12]. Trichostatin A (TSA) is a pan-HDAC inhibitor and exert anti-tumor effect in multiple cancers, such as colon carcinoma cells, breast adenocarcinoma cells, prostate cancer cells and esophageal squamous cells [13–18]. Yang et al demonstrated that TSA enhance sensitivity of ERα-negative breast cancer to chemotherapy by inducing the expression of ERα [19]. The mechanisms of TSA on TNBC are still under investigation.

In this study, we demonstrate that TSA inhibit proliferation of basal like breast cancer cells through inducing cell cycle arrest and apoptosis. The expressions of CYCLIN D1, CDK4, CDK6 and BCL-XL were down-regulated, while the expression of P21 protein was enhanced. Combination of TSA and doxorubicin showed a synergistically effects on

inhibiting the proliferation of basal like breast cancer cells. Our study will benefit for TNBC therapy in the future.

Materials and methods

Chemicals and antibodies. TSA were purchased from Sigma-Aldrich. Stock solution of TSA was prepared with DMSO. Final concentration of DMSO in assay media was typically kept at 0.1%. Doxorubicin was purchased from Selleck. Antibodies against CDK4, CDK6, CYCLIN D1, P21, p-ERK, ERK, p-AKT, AKT, cleaved caspase3, PARP and β-Actin were all purchased from Cell Signaling Technology.

Cell culture. Basal like TNBC cell lines HCC38 and HCC1806 were cultured in RPMI 1640 supplemented with 10% FBS. The cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO2. Normal mammary epithelial cell line MCF-10A cells were cultured in DMEM/F12 supplemented with 20 ng/ml of epidermal growth factor, 100 ng/ml of cholera toxin, 0.01 mg/ml of insulin, 500 ng/ml of hydrocortisone, and 5% horse serum.

MTT assay. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] dye reduction method was conducted to detect the cell viability as described previously [20]. Briefly, 3 000 cells in the log phase of growth were plated into 96-well plates. Cells were exposed to the indicated doses of TSA for 72 h. Time-dependent effects on cells were tested at 24 h, 48 h and 72 h after treatment. The absorbance value (OD) of the wells was measured with a microplate reader with wavelength of 570 nm. Triplicate samples was performed each test.

Cell cycle assay. Cells (8×10^5) were plated in 6 cm dishes and treated with indicated dose of TSA or vehicle for 24 h. Then cells were harvested and fixed in 70% of cold ethanol overnight. Cells were washed with PBS three times and incubated with RNase at 37 °C for 30 minutes followed by Propidium Iodide (PI) staining for another 30 minutes at 4 °C. Cell suspensions were analyzed by flow cytometry. Data were analyzed with Modifit software.

Immunoblotting analysis. Cells (8×10^5) were plated in 6 cm dishes and treated with indicated dose of TSA or vehicle for 24 h. Cells were washed with cold PBS and lysed in cell lysis buffer (50 mM Tris, pH 7.4, with 150 mM NaCl, 1% NP40, 9 mM ethylenediamine tetra-acetic acid [EDTA], and protease inhibitor cocktail [Roche]), spin down at 12 000 rpm to collect the protein supernatant. The protein concentration was analyzed by a protein assay kit with bovine serum albumin standards according to the manufacturer's instructions (Bio-Rad Laboratories, Hurcules, CA). Cell lysate was separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Following blocking with PBS-Tween-20 containing 5% nonfat dry milk for 1 hour, membranes were incubated overnight at 4 °C with primary antibodies followed by incubation with horseradish peroxidase–conjugated secondary antibody. β-actin was used as loading control. Immunoreactive bands were detected by an enhanced chemiluminescence kit. Grayscale analysis was done by ImageJ software. The control group was set as 1. The ratio of grayscale was calculated vs. control.

Statistical analysis. Statistical analyses were conducted using SPSS 22.0 software package (SPSS Inc., Chicago, IL, USA). Data from experiments were presented as means ± SD, and evaluated by analysis on factorial design of two factors and one-way ANOVA. Differences were considered statistically significant at p<0.05.

Results

TSA inhibited proliferation of basal like TNBC cell lines HCC1806 and HCC38. Side effect is the main hurdle of chemotherapy for clinical use. First of all, it is important to demonstrate that TSA inhibit the proliferation of breast cancer cells with less toxicity on normal breast cells. Here MCF10A, a normal mammary epithelial cell line, was used to detect the toxicity of TSA on normal breast cells. TSA showed a 5%~25% inhibitions on MCF10A cell viability while the inhibition rate of 0.5 μM of TSA on HCC1806 and HCC38 breast cells is ~95% (Figures 1A–1C). Moreover, TSA inhibit HCC1806 and HCC38 cells in dose-dependent and time-dependent patterns (Figures 1D and 1E). It indicates the potential of TSA on TNBC therapy in the near future.

TSA induced cell cycle arrest by down-regulation of CDK4, CDK6 and CYCLIND1 expression and up-regulation of P21 expression. Next, we demonstrate whether TSA inhibit the proliferation of breast cancer cells through inducing cell cycle arrest. 0.13 μM of TSA was used for detecting the cell cycle arrest in TNBC cells. The results showed that both G0/G1 and G2/M arrest were observed in HCC1806 cell line at 24 hours after TSA treatment (Figures 2A and 2B) compared with DMSO control (p<0.01 vs. control). While only G0/G1 arrest was observed in HCC38 cells (Figures 3A and 3B).

In order to explore the molecular basis of cell cycle arrest induced by TSA, we further examined CDK4, CDK6, CYCLIN D1 and P21 protein expression, which are the key regulators participating in G1 to S phase transition. Our data showed that TSA reduced the expression of CDK4, CDK6 and CYCLIN D1, while cyclin-dependent kinase inhibitor, P21 protein, was increased in both HCC1806 and HCC38 cell lines (Figure 2C and Figure 3C).

TSA induced apoptosis by down-regulation of BCL-XL expression. In addition to block cell cycle transition, it has been previously demonstrated that TSA induced cell apoptosis in some tumor cell lines. We further identify whether TSA induces cell apoptosis in HCC1806 and HCC38 cells. Both cells were treated with TSA for 24 hours and western blot was used to check the expression of apoptosis-related proteins. TSA induces cleaved CASPASE3 and cleaved PARP expression in both cells in a dose dependent manner (Figures 4A and 4B). Cleaved CASPASE3 was increased about 35 fold and 12.5 fold in HCC1806 cells and HCC38 cells treated by
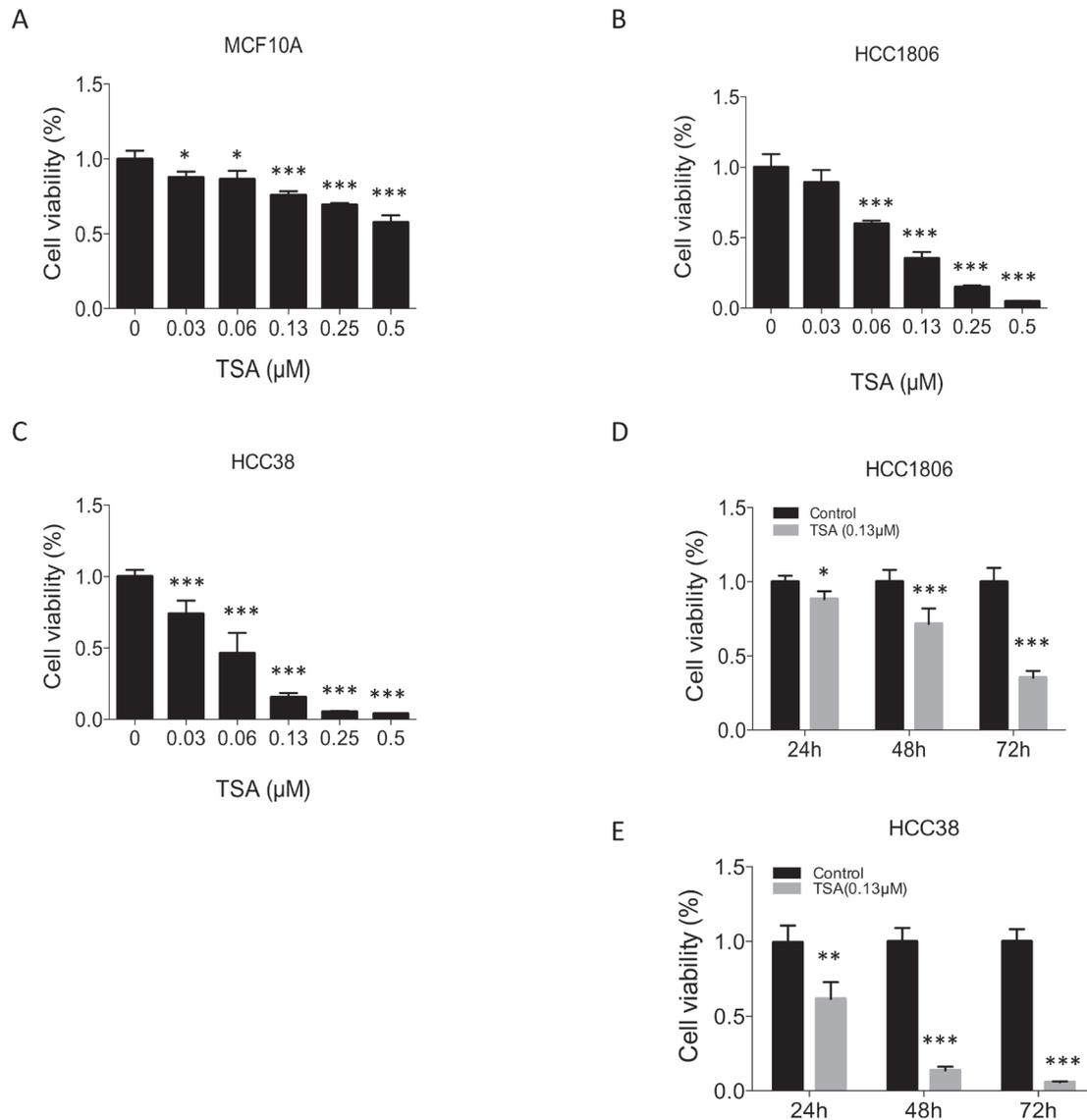
Figure 1. The inhibitory effects of TSA on MCF10A, HCC1806 and HCC38 cells. A–C. MCF10A cells, HCC1806 and HCC38 cells were treated with TSA for 72 h and MTT assay was used to detect the cell viability. D–E. Time-dependent effects of TSA on HCC1806 and HCC38 cells. HCC1806 and HCC38 cells were treated with 0.13 μM of TSA for 24 h, 48 h and 72 h respectively. MTT assay was used to detect the cell viability. Note: n=3, *p<0.05, **p<0.01, ***p<0.005 significantly different compared with control.

0.5 μM of TSA respectively. 0.25 μM of TSA cause more than ten-fold increase of cleaved PARP compared with control in both cell lines (11.02 fold in HCC1806 cells, 10.49 fold in HCC38 cells). Furthermore, we investigated the underlying mechanism of cell apoptosis. The expression of BAX and BCL-XL protein were detected in both HCC1806 and HCC38 cells followed by TSA treatment. Down-regulation of BCL-XL protein was induced in HCC1806 cells by TSA treatment, while no change of BAX protein was observed. But the BCL-XL was down-regulated and BAX protein was increased in HCC38 cells (Figure 4). These results indicate

that apoptosis were induced by TSA treatment in TNBC through distinct mechanisms.

TSA disrupted MAPK pathways. PI3K and MAPK pathways play important roles in cell proliferation and survival. To investigate the underlying mechanism of TSA on basal like breast cancer cells, we detect the expression of p-ERK, ERK, p-AKT and AKT protein in HCC1806 and HCC38 cells treated with TSA. TSA decreased phosphorylation of ERK in both cells (Figures 5A and 5B). The expression of pAKT was decreased only at higher dose of TSA (0.5 μM) treatment. It indicated that down-regulation of MAPK cell
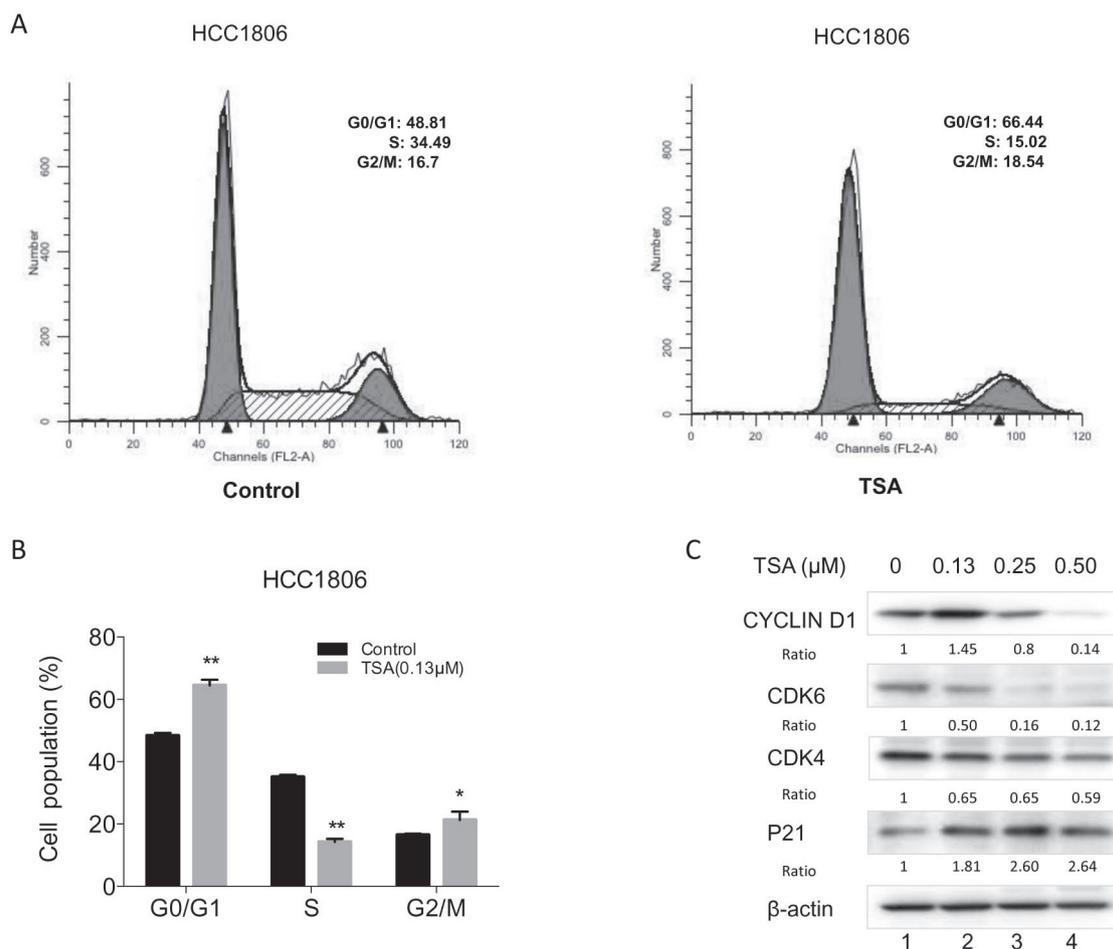
Figure 2. TSA induced cell cycle arrest in HCC1806 cells. A–B. HCC1806 cells were treated with TSA (0.13 μM) and flow cytometry was used to detect the cell cycle of HCC1806 cells 24 hours after treatment. Modifit software was used to analyze the data. G0/G1 arrest was observed in HCC1806 cells treated with TSA, *p*<0.01, vs. control. C. The effects of TSA on cell cycle proteins in HCC1806 cells. HCC1806 cells were treated with TSA and cells were collected at 24 h after treatment and western blot was conducted to detect the expression of proteins. The grayscale value was labeled below the band. Note: n=3, *p<0.05, **p<0.01 significantly different compared with control.

signaling pathway attributed to the suppression of cell proliferation and survival and PI3K cell signaling pathway is not significantly affected by TSA.

TSA and doxorubicin enhanced apoptosis synergistically in HCC1806 cells and HCC38 cells. Doxorubicin (Dox), an anthracycline antibiotic, was routinely used in treating breast cancer patients in clinic. However, it has been limited by the risk of cardiotoxicity and other lethal side effects. Combination therapy is a widely used strategy to increase efficacy and decrease side effects of chemotherapy. Therefore, we assessed the synergistic effect of TSA and doxorubicin on HCC1806 and HCC38 cells proliferation. Herein, we used 0.03 μM of TSA combined with different doses of doxorubicin. Notably, combination of doxorubicin and TSA showed more potential inhibitory effects than either compound alone on proliferation of HCC1806 and HCC38 cells (Figure 6A). The expression of cleaved PARP protein

and cleaved CASPASE3 protein were higher than those of treatment with TSA or doxorubicin alone in HCC1806 cells (Figure 6B). Moreover, the expression of pERK protein is reduced significantly while pAKT has no change (Figure 6C). It indicated more effective in combination group than the single group alone.

Discussion

Here we demonstrated that HDAC small-molecule inhibitor TSA and doxorubicin have a potent synergistic tumor-suppressive effect on human basal like TNBC cell lines. Therapies commonly used in other breast cancer subtypes are ineffective for TNBC. The improvement of efficacy of current chemotherapy for TNBC is urgently needed [20]. Recent studies focus on incorporation of platinum salts and new combinations of conventional chemotherapeutic agents with
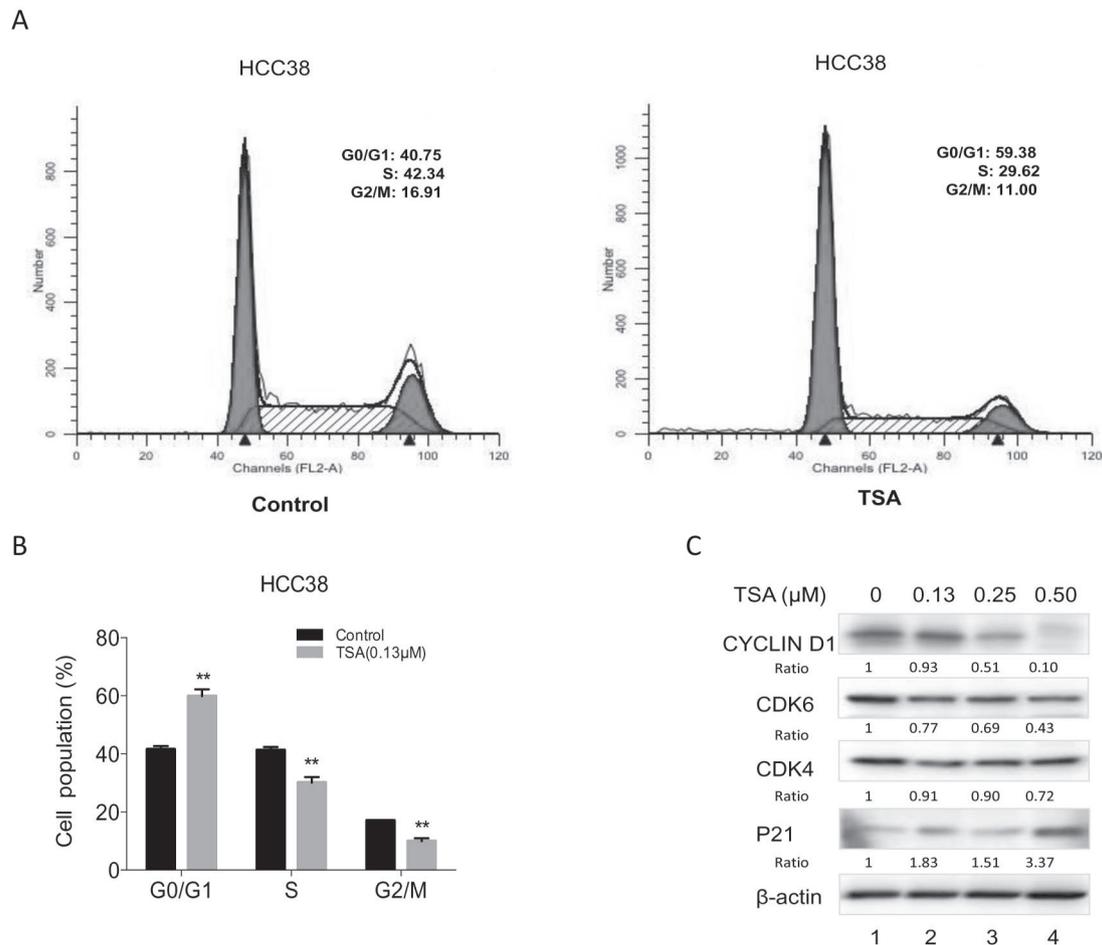
A

B

C

Figure 3. TSA induced cell cycle arrest in HCC38 cells. A–B. HCC38 cells were treated with TSA (0.13 μM) and flow cytometry was used to detect the cell cycle of HCC38 cells 24 hours after treatment. Modifit software was used to analyze the data. G0/G1 arrest was observed in HCC38 cells treated with TSA, *p*<0.01, vs. control. C. The effects of TSA on cell cycle proteins in HCC38 cells. HCC38 cells were treated with TSA as the same as HCC1806 cells. The grayscale value was labeled below the band. Note: n=3, *p<0.05, **p<0.01 significantly different compared with control.

new-targeted agents, such as poly-ADP ribose polymerase inhibitors, antiangiogenic agents, phosphoinositide 3-kinase (PI3K) pathway inhibitors, and androgen antagonist [21].

TSA, a HDAC inhibitor, exerted a potent activity against human colon carcinoma cells, breast adenocarcinoma cells and prostate cancer cells through inducing cell cycle arrest, differentiation and apoptosis [16, 18, 22]. Mitotic cellular division requires the cell to leave the resting state and proceed through phases of DNA synthesis and mitosis. Well-organized progression of dividing cells through the G1, S, G2, and M phases of the cell cycle in eukaryotic cells relies on a series of cell-cycle regulatory proteins, such as CYCLIN A, B, D and E. Cyclins exert their functions via activating a variety of specific cyclin-dependent kinases (CDKs) [5]. The cell cycle in mammals is controlled by interaction of cyclins and cyclin dependent kinases (CDKs), and CDK inhibitors such as P21 and P27. CDK inhibitors inhibited the activity of

cyclin-CDK complexes and blocked cell cycle transition [23]. CYCLIN D1 is a key regulator of G1/S checkpoint control, which forms a holoenzyme complex with CDK4 and CDK6, to phosphorylate pRB (retinoblastoma protein). When RB was phosphorylated, transcription factor E2F was released from pRB to promote cell cycle progress [24, 25]. In this study, we observed that TSA treatment resulted in down-regulation of CYCLIN D1, CDK4 and CDK6 protein expression, but up-regulation of P21 protein expression, which is the underlying mechanism of the subsequent growth inhibition effect of TSA.

In addition, HDAC inhibitors induce apoptosis through mitochondrial-mediated apoptosis pathway [16, 17, 26]. In the mitochondrial pathway, the ratio of BAX to BCL2 or BCL-XL was disrupted in mitochondrial membrane, promoting the release of cytochrome C and other pro-apoptotic molecules into the cytoplasm, which in turn lead
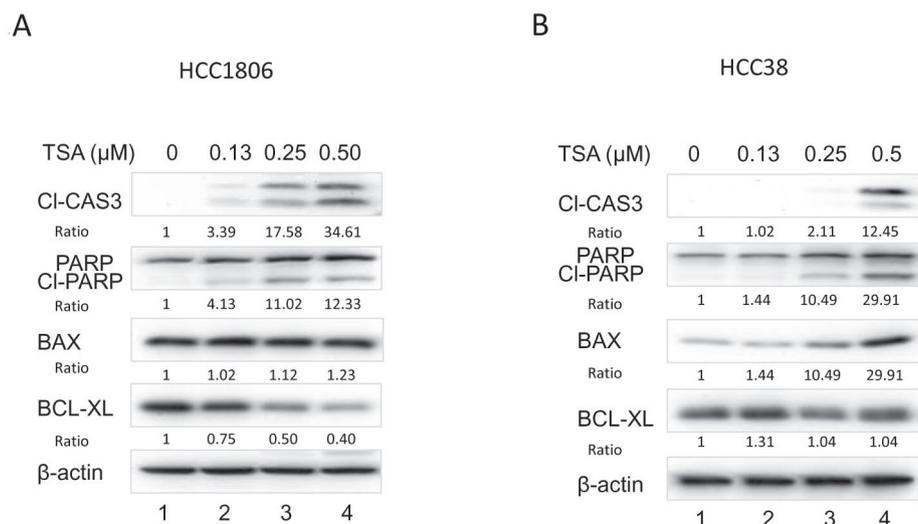
A HCC1806

TSA (μM)	0	0.13	0.25	0.50
CI-CAS3				
Ratio	1	3.39	17.58	34.61
PARP CI-PARP				
Ratio	1	4.13	11.02	12.33
BAX				
Ratio	1	1.02	1.12	1.23
BCL-XL				
Ratio	1	0.75	0.50	0.40
β-actin				
	1	2	3	4

B HCC38

TSA (μM)	0	0.13	0.25	0.5
CI-CAS3				
Ratio	1	1.02	2.11	12.45
PARP CI-PARP				
Ratio	1	1.44	10.49	29.91
BAX				
Ratio	1	1.44	10.49	29.91
BCL-XL				
Ratio	1	1.31	1.04	1.04
β-actin				
	1	2	3	4

Figure 4. TSA induced apoptosis in HCC1806 and HCC38 cells. HCC1806 (A) and HCC38 (B) cells were treated with TSA and cells were collected 24 h after treatment. Cleaved-CASPASE3 (Cl-CAS3), cleaved-PARP(Cl-PARP), BAX and BCL-XL were detected. The grayscale value was labeled below the band.

A HCC1806

TSA (μM)	0	0.13	0.25	0.50
p-ERK				
Ratio	1	0.16	0.05	0.02
ERK				
Ratio	1	1.03	0.95	0.74
p-AKT				
Ratio	1	0.92	0.99	0.72
AKT				
Ratio	1	0.77	0.80	0.74
β-actin				
	1	2	3	4

B HCC38

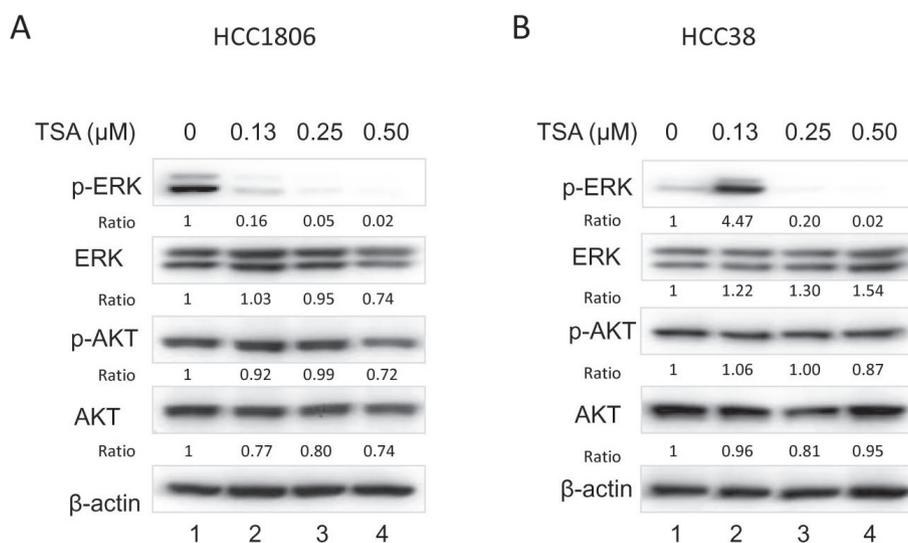| TSA (μM)	0	0.13	0.25	0.50
p-ERK				
Ratio	1	4.47	0.20	0.02
ERK				
Ratio	1	1.22	1.30	1.54
p-AKT				
Ratio	1	1.06	1.00	0.87
AKT				
Ratio	1	0.96	0.81	0.95
β-actin				
	1	2	3	4

Figure 5. TSA inhibited cells growth by disrupting MAPK pathway. HCC1806 (A) and HCC38 (B) cells were treated with TSA for 24 h and cells were lysed with lysis buffer for evaluating the expression of the cell signaling proteins by western blot. The expression of phosphorylated ERK (pERK) represents the activation of MAPK pathway. Phosphorylated AKT (pAKT) is the major regulator of PI3K pathway. The grayscale value was labeled below the band.

to the activation of CASPASE 9 to activate CASPASE 3 and further to cleave PARP. Herein, we demonstrated that TSA treatment promoted the expression of cleaved CASPASE 3 and cleaved PARP, which indicated TSA inducing the cell apoptosis. Furthermore, we demonstrated TSA decreased the expression of anti-apoptotic protein BCL-XL. It suggested that TSA mediated apoptosis through activating the mitochondrial-mediated apoptosis pathway.

It is well known that PI3K and MAPK signaling pathways are closely related to cell proliferation, differentiation and survival [27, 28]. It was reported that HDAC inhibitor decreased phosphorylation of AKT in diffuse large B-cell lymphoma cell lines [5] . We found that TSA decreased the expression of phosphorylation of ERK, but not phosphorylation of AKT in basal like breast cancer cell line. These results indicated that suppressing the MAPK signaling pathways

in basal like breast cancer is responsible for the cell growth inhibition induced by TSA. Crucial preclinical trials on HDAC inhibitors (panobinostat, vorinostat, and entinostat) exert an anti-proliferative effect on TNBC cells and control tumor growth by multiple mechanisms of action, including apoptosis and regulation of the epithelial to mesenchymal transition (EMT). HDAC inhibitors such as suberoylanili-dehydroxamic acid (SAHA), sodium butyrate, mocetinostat, panobinostat, entinostat, YCW1 and N-(2-hydroxyphenyl)-2-propylpentanamide have shown promising therapeutic outcomes against TNBC, especially when they are used in combination with other anticancer agents [29, 30].

Drug combination is a widely used strategy to increase efficacy and decrease side effects of chemotherapy [31]. Doxorubicin is widely used in cancer therapy in clinic. Herein we explored synergistic inhibitory effect of TSA and Doxorubicin on TNBC cell lines. The combination of TSA and doxorubicin exerts synergistic effects in reducing basal like breast cancer cells viability by inducing cleavage of PARP and CASPASE 3 that is associated with cell apoptosis.

In general, HDACs can be divided into Zn^{2+}- dependent classes (class I, II and IV) and NAD-dependent classes (class III). Class I is consisted of HDACs 1, 2, 3 and 8. Class II can be divided further into class IIa (HDAC4, 5, 7, and 9) and class IIb (HDAC6 and 10). Class III members consist of SIRT1-SIRT7. HDAC11 is the only member of Class IV. In this study, we conduct pan- HDAC inhibitor, TSA, and explored the possible inhibitory mechanisms of TSA on TNBC. TSA can effectively inhibit TNBC proliferation and it indicates that TSA inhibit TNBC through regulating the epigenetic process (including histone acetylation and deacetylation) and receptor-independent pathway (including ER, PR and HER2 receptors). Hsieh, et al found HDAC3 linked to CSC homeostasis by increasing β-catenin expression through the Akt/GSK3β pathway [32]. Witt et al demonstrate that clini-cally available HDAC inhibitors (HDACi) targeting HDAC1 and HDAC7 can be used to preferentially target CSCs [33]. Although we observed promising effects of TSA combined with doxorubicin, it is necessary to search for the specific HDAC target and develop the target specific inhibitors.

In conclusion, HDAC inhibitor TSA had an inhibitory effect on basal like breast cancer cell line by inducing cell cycle arrest and apoptosis and inhibiting the MAPK pathway. TSA and doxorubicin combination exerts synergistic effects in reducing basal like breast cancer cells viability. Our study supports the rationale for TSA alone or in combination as
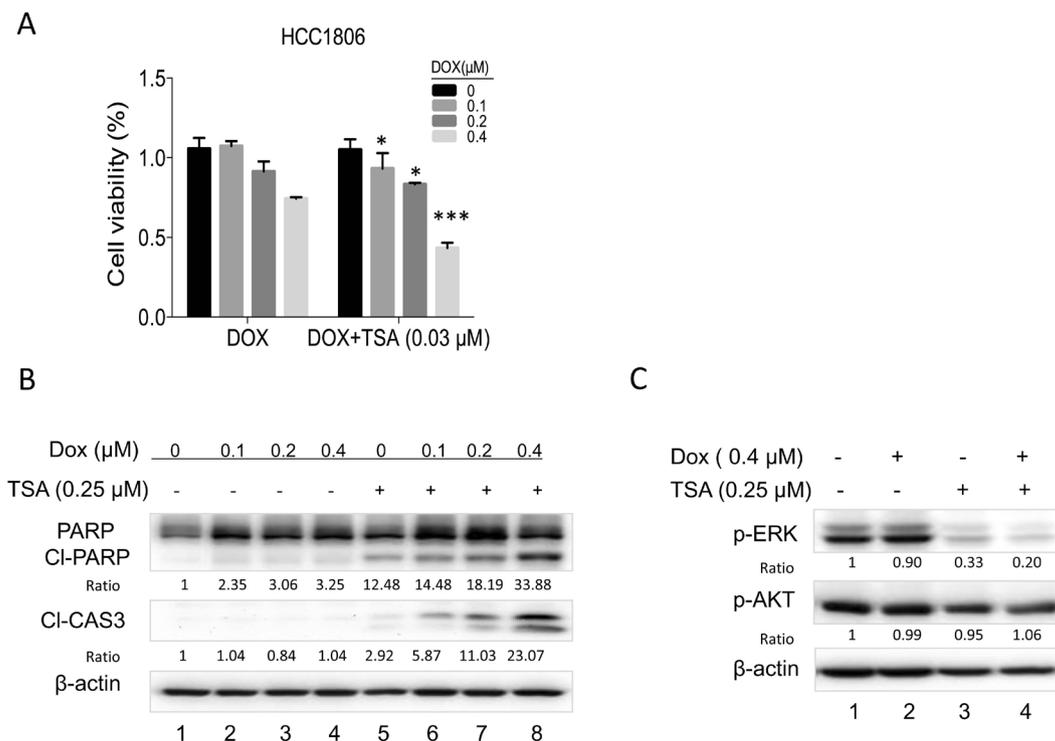

Figure 6. Synergistic effects of TSA with Doxorubicin. A. The effects of TSA combined with doxorubicin on cell viability of HCC1806 cells. HCC1806 cells were treated with doxorubicin (0 μM, 0.1 μM, 0.2 μM and 0.4 μM) and TSA (0.03 μM) for 72 h and MTT assay was used to detect the cell viability. Statistical analysis was done between the group of doxorubicin combined with TSA and the same dose of doxorubicin treatment only group. n=3, *p<0.05, **p<0.01, ***p<0.005. B. The effects of TSA combined with doxorubicin on apoptosis in HCC1806 cells. HCC1806 cells were treated with 0.25 μM of TSA and doxorubicin (0 μM, 0.1 μM, 0.2 μM and 0.4 μM). The grayscale calculation was done as the same as above. C. The effects of TSA on MAPK and PI3K cell signaling pathway. HCC1806 cells were treated with 0.25 μM of TSA and 0.4 μM of doxorubicin for 24 h. Cells were lysed with lysis buffer and western blot was conducted to detect the protein expression. The grayscale value was labeled below the band.

a promising therapeutic approach against basal like breast cancer which are largely refractory to current therapeutic approaches, and will hopefully promote the rapid clinical evaluation of the strategy.

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