PLOS ONE

RESEARCH ARTICLE

# Tanshinone IIA Inhibits HIF-1α and VEGF Expression in Breast Cancer Cells via mTOR/p70S6K/RPS6/4E-BP1 Signaling Pathway

**Guobing Li[1], Changyu Shan[1], Lei Liu[1], Ting Zhou[1], Jing Zhou[1], Xiaoye Hu[1], Yibiao Chen[2], Hongjuan Cui[2], Ning Gao[1]***

**1** College of Pharmacy, Third Military Medical University, Chongqing, China, **2** State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, China

* gaoning59@163.com





**Citation:** Li G, Shan C, Liu L, Zhou T, Zhou J, Hu X, et al. (2015) Tanshinone IIA Inhibits HIF-1α and VEGF Expression in Breast Cancer Cells via mTOR/p70S6K/RPS6/4E-BP1 Signaling Pathway. PLoS ONE 10(2): e0117440. doi:10.1371/journal.pone.0117440

**Academic Editor:** Xianglin Shi, University of Kentucky, UNITED STATES

**Received:** October 21, 2014

**Accepted:** December 23, 2014

**Published:** February 6, 2015



**Data Availability Statement:** All relevant data are within the paper.

**Funding:** This work was supported by Chongqing Natural Science Foundation (CSTC2013jjB10007), the National Natural Science Foundation of China (No. 81402970), and the Open Project Program of State Key Laboratory of Silkworm Genome Biology, Southwest University (2013024). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

Hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) play important roles in angiogenesis and tumor growth. Tanshinone IIA (T2A) is a novel antiangiogenic agent with promising antitumor effects; however, the molecular mechanism underlying the antiangiogenic effects of T2A remains unclear. In the present study, we provided evidence showing that T2A inhibited angiogenesis and breast cancer growth by down-regulating VEGF expression. Specifically, T2A repressed HIF-1α expression at the translational level and inhibited the transcriptional activity of HIF-1α, which led to the down-regulation of VEGF expression. Suppression of HIF-1α synthesis by T2A correlated with strong dephosphorylation of mammalian target of rapamycin (mTOR) and its effectors ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), a pathway regulating HIF-1α expression at the translational level. In addition, we also found that T2A inhibited the angiogenesis and growth of human breast cancer xenografts in nude mice through suppression of HIF-1α and VEGF. Our study provides novel perspectives and potential targets for the treatment of human breast cancer.

## Introduction

Breast cancer is one of the most common female malignant tumors and the leading cause of cancer death among females [1]. Studies on chemotherapies and identification of novel anticancer agents are highlighted due to the increasing morbidity and mortality of human breast cancer in recent years. Angiogenesis is a common feature of cancers and plays important roles in local tumor growth and distant metastasis of breast cancer [2, 3]. Rapid growth of tumor cells usually causes hypoxia in tumor tissues, which drives angiogenesis [4–6]. Vascular endothelial growth factor (VEGF), a key protein promoting the formation of new blood vessels, has been found to be overexpressed in various human solid tumors [7–9]. The expression of VEGF can be significantly induced by hypoxia [10], in which the transcription factor hypoxia-inducible factor-1 (HIF-1) plays an essential role [11].

**Competing Interests:** The authors have declared that no competing interests exist.

As an oxygen-dependent transcriptional activator, HIF-1α plays an important role in the regulation of a large number of genes involved in angiogenesis, metabolic adaptation to low oxygen, and survival [12, 13]. In the presence of oxygen, HIF-1α is rapidly degraded by proteasomes after post-transcriptional modification [14]. Under hypoxic condition, HIF-1α remains stable and translocates to the nucleus, where it forms heterodimers with HIF-1β to activate the transcription of a large number of genes involved in the survival and growth of cancer cells [12, 15]. It has been reported that overexpression of HIF-1 was associated the high growth rate and metastatic potential of various tumor types [16–18]. The high frequency of HIF-1-positive cells is associated with advanced clinical stages and poor prognosis of breast cancers [16]. Given the critically important role of HIF-1α and VEGF in promoting angiogenesis [19], novel antiangiogenic agents targeting HIF-1α and VEGF are highlighted for the treatment of breast cancer.

Danshen, the dried root of *Salvia miltiorrhiza*, has been widely used for the treatment of coronary artery and cerebrovascular diseases in China [20, 21]. Tanshinone IIA (T2A) is a derivative of phenanthrene-quinone and the major biologically active constituent in Danshen [22]. T2A has been successfully used in clinic for the treatment of coronary heart diseases, angina, myocardial infarction, and cerebrovascular diseases with minimal side effects [21, 22]. Recently, T2A has attracted great attention in cancer therapy due to its potential anticancer activities. For instance, T2A inhibited the growth and induced the apoptosis of breast cancer cells through multiple mechanisms including activation of caspase 3, increasing the Bax to Bcl-xL ratios, as well as epigenetic modification of Aurora A expression [23–25]. It has also been shown that T2A exhibited antiangiogenic effects by inhibiting MMP-2 activity in human umbilical vascular endothelial cells [26]. However, the whether T2A inhibits the growth and angiogenesis of breast cancer and underlying mechanism are largely unknown.

In the present study, we provided evidence showing the antiangiogenic effects of T2A in breast cancer. We found that T2A inhibited angiogenesis and tumor growth through repression of HIF-1α at the translation level. Inhibition of the transcriptional activity of HIF-1 led to the down-regulation of VEGF. We further showed that the interruption of mTOR/p70S6K/4E-BP1 signaling pathway was involved in the inhibition of HIF-1a translation. Our study suggest that T2A may be an effective anticancer agent for the treatment of human breast cancer through inhibition of angiogenesis in tumor tissues.

## Materials and Methods

### Cell lines and cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For normoxic exposure, cells were maintained in a fully humidified atmosphere of 5% carbon dioxide incubator at 37°C. For hypoxia exposure, cells were incubated in a sealed plastic incubation box at 37°C with 5% carbon dioxide, 1% oxygen, and 94% nitrogen.

### Reagents and antibodies

Tanshinone IIA (T2A) was purchased from Sigma (St Louis, MO), cycloheximide (CHX), MG132, and rapamycin were purchased from EMD Biosciences (La Jolla, CA). Antibodies against phospho-mTOR (Ser2448), mTOR, phospho-p70S6 kinase (Thr389), phospho-p70S6 kinase (Thr421/Ser424), p70S6 kinase, phospho-S6 ribosomal protein (Ser235/236), phospho-S6 ribosomal protein (Ser240/244), S6 ribosomal protein, phospho-4E-BP1 (Thr37/46), CD31, Tubulin and 4E-BP1 were purchased from Cell Signaling (Danvers, MA); Antibodies against

HIF-1α, HIF-1β, HIF-2α were from BD Biosciences (Bedford, MA, USA). β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

## Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to measure VEGF protein levels using the Quantikine human VEGF ELISA kit from R&D Systems (Minneapolis, MN). Briefly, MDA-MB-231 cells were cultured in fresh DMEM medium without or with T2A of various concentrations for 24 hours. The relative VEGF concentrations in supernatant were detected using the ELISA kit following the manufacturer's instructions and normalized to the cell number.

## Reporter gene assay

MDA-MB-231 cells were seeded in 6-well plates and co-transfected with a luciferase reporter plasmid (pBI-GL V6L) containing six copies of hypoxia response elements (HRE) from the VEGF reporter, a pRL-SV-40 plasmid encoding a Renilla luciferase as an internal control and a wild-type HIF-1α plasmid. After treatment with T2A or vehicle as indicated, the promoter activity was evaluated using a dual-luciferase reporter assay system (Promega, Madison, WI). Values were normalized to renilla luciferase activity.

## Real-time quantitative RT-PCR

Total RNA was extracted from cells untreated or treated with various concentrations of T2A for 24 hours using the RNeasy mini kit (QIAGEN, Valencia, CA). All real-time RT-PCR reactions were performed in triplicate in a 20 μl mixture containing 0.2 μM of each primer, 2 μl of cDNA templates, and 1x IQ SYBR Green supermix (BioRad, Hercules, CA). Real-time quantitative RT-PCR was performed using the iCycler iQ system (BioRad) according to the manufacturer's instructions. The primers used for PCR are as follows: HIF-1α: 5′-TGGTGACATGATTTACATTTCTGA-3′ (forward), 5′-AAGGCCATTTCTGTGTGTAAGC-3′ (reverse); VEGF: 5′-TCGGGCC TCCGA AACCATGA-3′ (forward), 5′-CCTGGTGAGAGATCTGGTTC-3′ (reverse); Glut1: 5′-TGACAAGACACCCGAGGAGC-3′ (forward), 5′-GTCCAGCCCTACAG ATTA GC-3′ (reverse); EPO: 5′-AACGTCCCACCCTGCTGCTTTT-3′ (forward), 5′-TGTACAGCTTCAGTTTCCCCCGGA-3′ (reverse); β-actin: 5′-GGACTTCGAGC AAGAGATGG-3′ (forward), 5′-AGCACTGTGTTGGCGTACAG-3′ (reverse). The relative expression level of HIF-1α, VEGF, Glut1 and EPO, were normalized to the β-actin gene value.

## HIF-1α pulse-chase

MDA-MB-231 cells were seeded and exposed to the methionine-free DMEM medium for 30 min and then pulse labelled with 100 μCi/ml [$^{35}$S]methionine (ICN, Biomedicals, Inc., Irvine, CA) for 60 min at 37°C. Cells were washed in PBS and transferred to complete medium without or with 20 μM T2A for various times as indicated under normoxic or hypoxic conditions. Subsequently, cells were lysed and incubated with anti-HIF-1α antibody at 4°C overnight for immunoprecipitation assay. Immune complexes were collected with protein A/G-Plus agarose beads (Pierce Biotechnology, Rockford, IL) and washed with lysis buffer. The samples were then separated by SDS-PAGE and examined by autoradiography.

## Western blot analysis

Nuclear proteins were extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's instruction. Whole cell extracts and nuclear extracts were boiled and separated by SDS-PAGE, transferred to a PVDF membrane, and followed by

immunoblotting as described earlier [27]. Densitometric analysis of the blots were performed using Quantity One software (Bio-Rad laboratories, Munchen, Germany).

### Immunofluorescence

MDA-MB-231 cells were plated on Milicell EZ $_{SLIDE}$ glass slides (Millipore, Billerica, MA) and fixed with 3.7% cold formaldehyde for 15 min and blocked with 1% bovine serum albumin dissolved in 0.05% Triton X-100 for 30 min. Cells were incubated with antibodies against HIF-1α (mouse) and Tubulin (rabbit) at 4°C overnight and then incubated for 1 hour with Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 647-conjugated donkey anti-rabbit secondary antibodies (Molecular Probes). Nuclei were counterstained with 0.1 μg/ml DAPI (Sigma, St Louis, MO). Samples were examined under a Leica confocal laser scanning microscope (TCS SP2 AOB; Wetzlar, Germany).

### Ethics statement

The animal experiment was conducted with an approval from and under the supervision of the Laboratory Animal Welfare Ethics Committee of the Third Military Medical University. Animal surgeries were performed under sodium pentobarbital anesthesia with minimal suffering.

### Xenograft experiment

MDA-MB-231 cells ($2\times10^6$) were mixed with a Matrigel basement membrane matrix (BD Biosciences) and subcutaneously injected into the back of nude mice (n = 40, 5-week-old, Vital River Laboratories, Beijing, China). When the subcutaneous tumors grew to 50–150 $mm^3$ (~ 5 days after the injection of tumor cells), the mice were randomly divided into two groups, the T2A and control groups (20 mice per group). Intraperitoneal administration of T2A (50 mg/kg) or saline (equal volume) was conducted for mice of the T2A and control groups for five times per week. Body weight and tumor volume were measured once a week and the tumor volume was calculated according to the formula: $V = (L \times W^2) \times 0.5$, where "L" and "W" were the length and width of a xenograft. Mice were euthanized by $CO_2$ asphyxiation after 8 weeks of drug exposure or when the xenografts reached 30 mm in diameter. Tumors tissues were surgically removed under euthanization, and then subjected to PCR, Western blot and Immunohistochemical analysis as previously described [27]. Tumor tissues from 4 vehicle control mice and 4 mice treated with T2A were harvested and homogenized, supernatant were obtained by centrifugation, and hemoglobin contents were quantified using Drabkin's reagent kit (Sigma, St Louis, MO) according to the manufacturer's instruction.

### Statistical analyses

All data are expressed as mean ±S.D. from three independent experiments. Statistical analyses were performed using Student's *t* test. $P < 0.05$ (∗) or $P < 0.01$ (∗∗) were considered statistically significant.

## Results

### T2A reduced HIF-1α expression and inhibited the transcription of VEGF, Glut-1, and EPO in breast cancer cells

We first examined the effects of T2A on HIF-1α expression in both human breast cancer MDA-MB-231 and MCF-7 cells. Under normoxic condition, high levels of HIF-1α were observed in the whole cell extracts (WCE) of both breast cancer cell lines, and exposure of these cells to T2A resulted in a significant decrease in the expression of HIF-1α in a dose-dependent manner

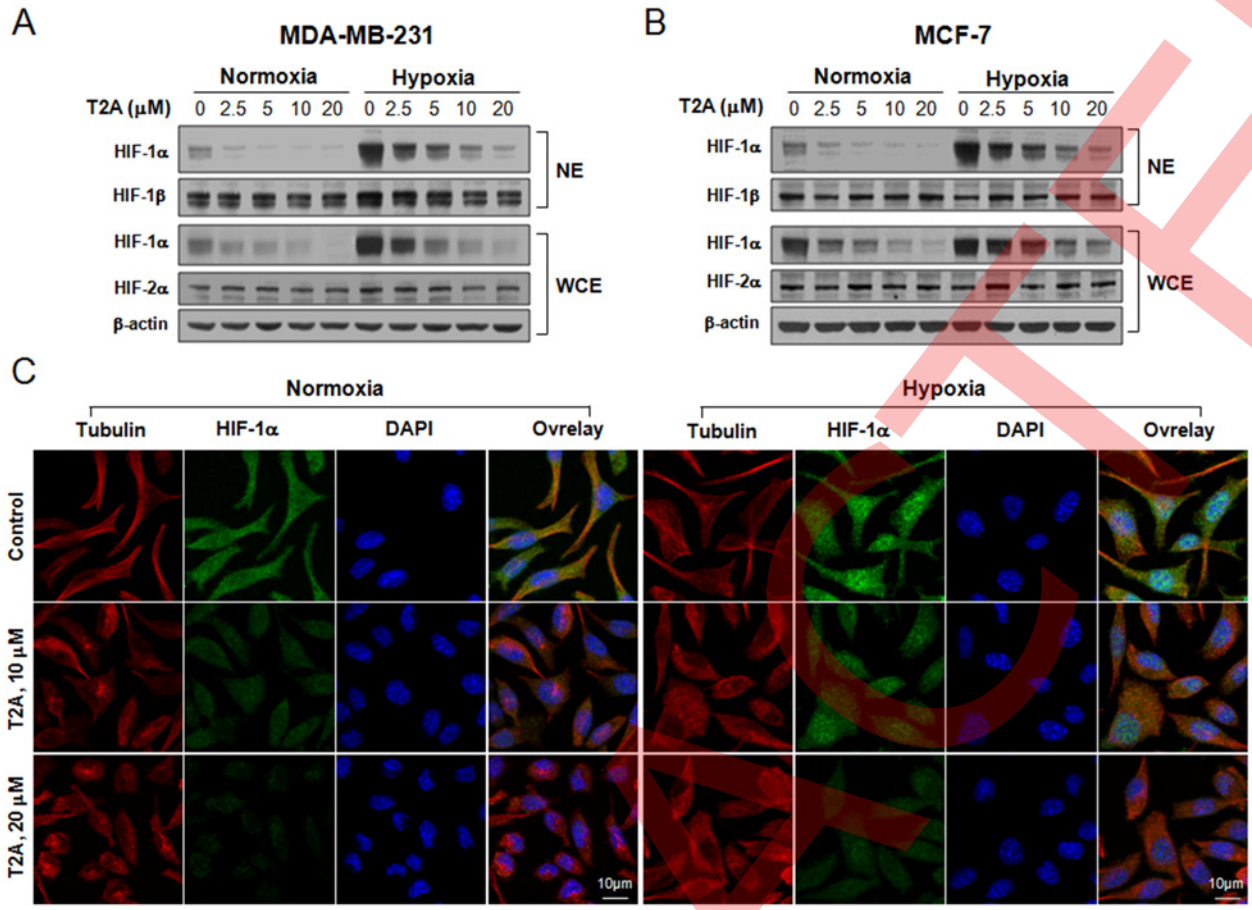


**Fig 1. T2A inhibited HIF-1α expression in MDA-MB-231 and MCF-7 cells.** (A and B) The MDA-MB-231 and MCF-7 cells were treated without or with various concentrations of T2A for 16 hours and then subjected to hypoxia, or remained in normoxia for an additional 8 hours. Whole cell extracts (WCE) and nuclear extracts (NE) were prepared from cells and subjected to Western blot assay using antibodies against HIF-1α, HIF-2α, HIF-1β, and β-actin. (C) Cells were fixed, permeabilized, and processed for immunofluorescence labeling with anti-Tubulin (Red) and anti-HIF-1 (green) antibodies. Nuclei were counterstained with 0.1 μg/ml DAPI (blue). Scale bar represents 10 μm.

doi:10.1371/journal.pone.0117440.g001

(Fig. 1A, B). To assess the effects of T2A on HIF-1α expression under hypoxic condition (1% oxygen), the HIF-1α protein level was determined in cells treated with T2A. Hypoxia markedly induced HIF-1α expression compared with normoxic condition, but T2A treatment inhibited HIF-1α expression in a dose-dependent manner (Fig. 1A, B). In addition, low level of HIF-1α was observed in nuclear extract (NE) under normoxic condition, and the HIF-1α protein level was markedly increased under hypoxic condition in the nuclear extracts of both breast cancer cell lines (Fig. 1A, B). Exposure of these cells to T2A significantly reduced nuclear HIF-1α protein level under both normoxic and hypoxic conditions. In contrast, no significant effects of T2A on the expression of HIF-2α and HIF-1β under both normoxic and hypoxic conditions were observed. We further investigated whether T2A affects HIF-1α accumulation in the nucleus of cells using laser scanning confocal microscopy. Under normoxic condition, low levels of HIF-1α were observed in the cytosol but not in the nucleus. T2A treatment significantly reduced the expression of HIF-1α, but had no effect on the expression of Tubulin (Fig. 1C). On the other hand, cells exhibited increased HIF-1α induction and nuclear accumulation after hypoxia exposure. T2A treatment reduced the HIF-1α level and repressed HIF-1α nuclear accumulation (Fig. 1C).

To determine whether T2A affects the transcriptional activity of HIF-1α, we examined VEGF protein levels in MDA-MB-231 cells treated without or with T2A. T2A significant reduced the VEGF protein levels under both normoxic and hypoxic conditions in a dose-dependent manner, suggesting that T2A treatments inhibited the transcriptional ability of HIF-1α on VEGF (Fig. 2A).

A reporter gene assay was conducted to further confirm whether T2A inhibited the transcriptional activity of HIF-1α. MDA-MB-231 cells were transiently transfected with a hypoxia-responsive reporter construct. Exposure of cells to T2A resulted in significant decreased,

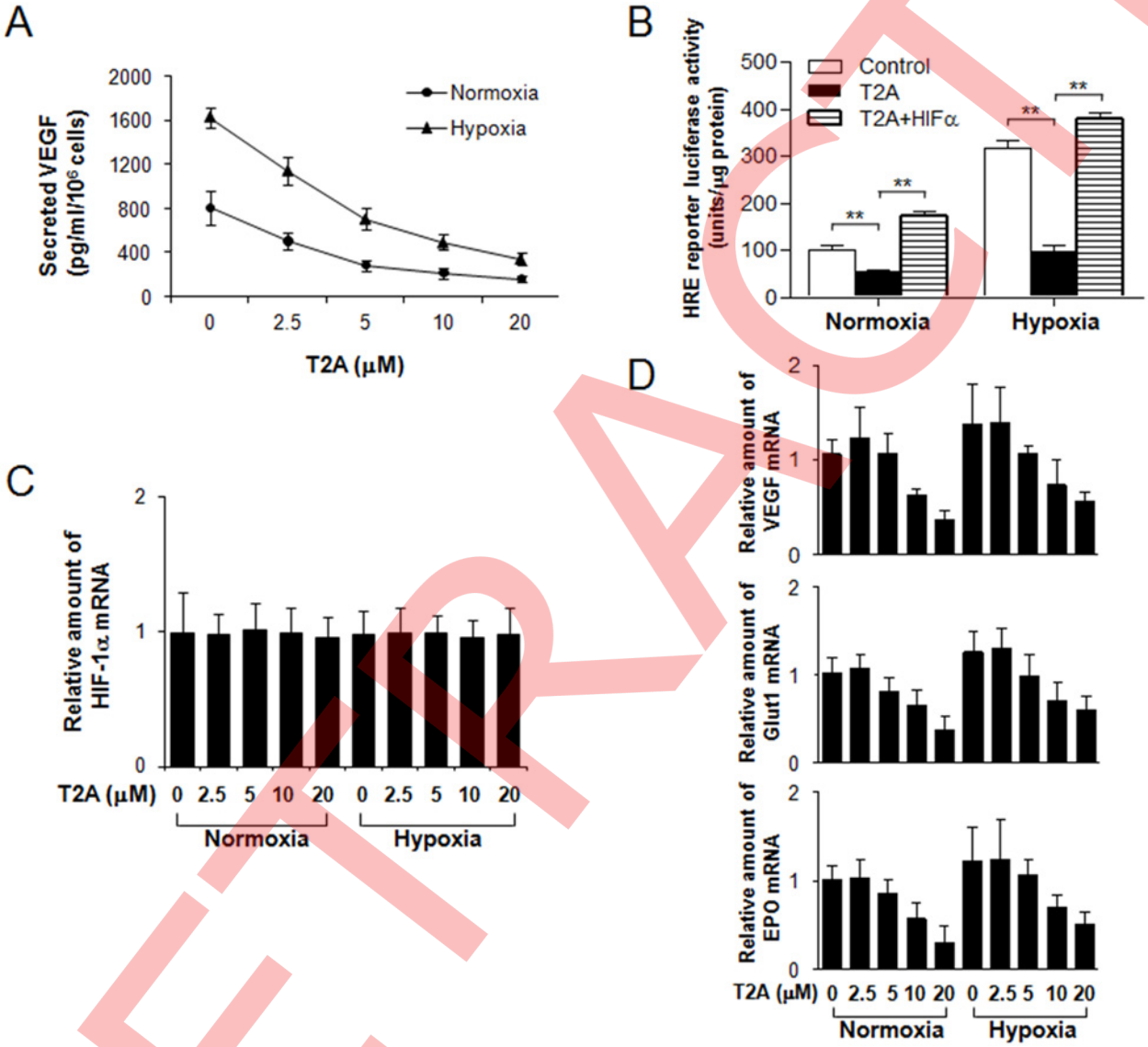


**Fig 2. T2A inhibited the transcriptional activity of HIF-1α, but had no significant effects on the HIF-1α mRNA level.** (A) The MDA-MB-231 cells were treated without or with various concentrations of T2A for 24 hour under normoxic or hypoxic conditions. VEGF in the supernatant was evaluated using ELISA. Error bars represent means ± S.D. (n = 3). (B) The MDA-MB-231 cells were transfected with pBI-GL V6L plasmid, pRL-SV-40 plasmid and HIF-1α plasmid (HIF-1α overexpression group) or an empty vector plasmid (control). Cells were treated with T2A or DMSO. The promoter activity of HIF-1α was analyzed as described in the Methods section. $^{**}P < 0.01$ compared with the control. (C and D) Total cellular RNA was extracted and the HIF-1α, VEGF, Glut1, and EPO mRNA levels were evaluated using real-time PCR. The relative mRNA levels of HIF-1α, VEGF, Glut1, and EPO were normalized according to the β-actin abundance and expressed as percentages of the control. Error bars represent means ± SD (n = 3).

doi:10.1371/journal.pone.0117440.g002

whereas overexpression of HIF-1α restored, the transcriptional activity of HIF-1α under both normoxic and hypoxic conditions (Fig. 2B), suggesting that T2A inhibited the transcriptional activity of HIF-1α.

To determine whether T2A inhibited HIF-1α expression at the transcriptional level, the mRNA levels of HIF-1α were evaluated using quantitative RT-PCR. No significant effects of T2A treatment on HIF-1α mRNA levels were observed under both normoxic and hypoxic conditions (Fig. 2C). To further evaluate whether T2A inhibited the transcriptional effects of HIF-1α on target genes including VEGF, glucose transporter 1 (Glut-1), and erythropoietin (EPO), the mRNA levels of VEGF, Glut-1, and EPO were evaluated using quantitative real-time PCR. Exposure of cells to T2A significantly reduced the mRNA levels of VEGF, Glut-1, and EPO under both normoxic and hypoxic conditions in a dose-dependent manners (Fig. 2D). Taken together, these findings confirm that T2A inhibited the transcriptional effects of HIF-1α on target genes, including VEGF, Glut-1, and EPO through the inhibition of HIF-1α translation and the reduction of HIF-1α protein level.

## T2A inhibited HIF-1α expression by reducing its synthesis rather than increasing its degradation

To understand the potential mechanism underlying HIF-1α inhibition by T2A, we studied the effects of T2A on HIF-1α translational regulation. First, we examined the effects of T2A on HIF-1α protein translation using the protein synthesis inhibitor cycloheximide (CHX). MDA-MB-231 cells were cultured under normoxic or hypoxic conditions respectively, and then treated without or with CHX in the presence or absence of T2A. The expression of HIF-1α were evaluated using Western blot. We found that CHX reduced the HIF-1α levels of T2A-treated and untreated cells under both normoxic and hypoxic conditions (Fig. 3A). However, no significant changes of HIF-1α degradation was detected in cells exposed to T2A and CHX under both normoxic and hypoxic conditions. To assess the effects of T2A on HIF-1α protein stability, cells were pulse-labeled with [$^{35}$S]methionine and chased for the indicated periods (h) in the presence or absence of T2A under normoxic or hypoxic conditions. The HIF-1α protein levels were evaluated using Western blot. As shown in Fig. 3B, the HIF-1α protein level in cells prelabeled with [$^{35}$S]methionine decreased at a similar rate with that in T2A-treated and untreated cells under both normoxic and hypoxic conditions. These findings suggest that T2A inhibited HIF-1α expression at the translation level.

To confirm that T2A inhibited HIF-1α expression through translation inhibition, we examined the effects of T2A on HIF-1α protein synthesis. MDA-MB-231 cells were pretreated with either vehicle or 20 μM T2A for 16 hours and then subjected to hypoxia, or remained in normoxia for an additional 8 hours. The cells were then labeled with [$^{35}$S]methionine in the presence or absence of T2A for the indicated times, and followed by immunoprecipitation of HIF-1α. T2A significantly inhibited the accumulation of [$^{35}$S]labeled-HIF-1α at all times tested under both normoxic and hypoxic conditions (Fig. 3C). This result indicates that T2A primarily inhibited HIF-1α translation rather than directly affected the stability of HIF-1α protein.

A specific proteasome inhibitor MG132 was used to determine whether T2A-mediated inhibition of HIF-1α accumulation occurred through proteasomal degradation of HIF-1α. Pronounced accumulation of HIF-1α proteins of multiple molecular weights and ubiquitinated-HIF-1α proteins of multiple molecular weights, were detected in cells exposed to MG132 alone under both normoxia and hypoxia conditions (Fig. 3D, arrows). However, addition of T2A significantly reduced the ubiquitinated HIF-1α level in cells treated with MG132 under both normoxic and hypoxic conditions. Taken together, these findings suggest that T2A inhibited HIF-1α expression through translation inhibition.

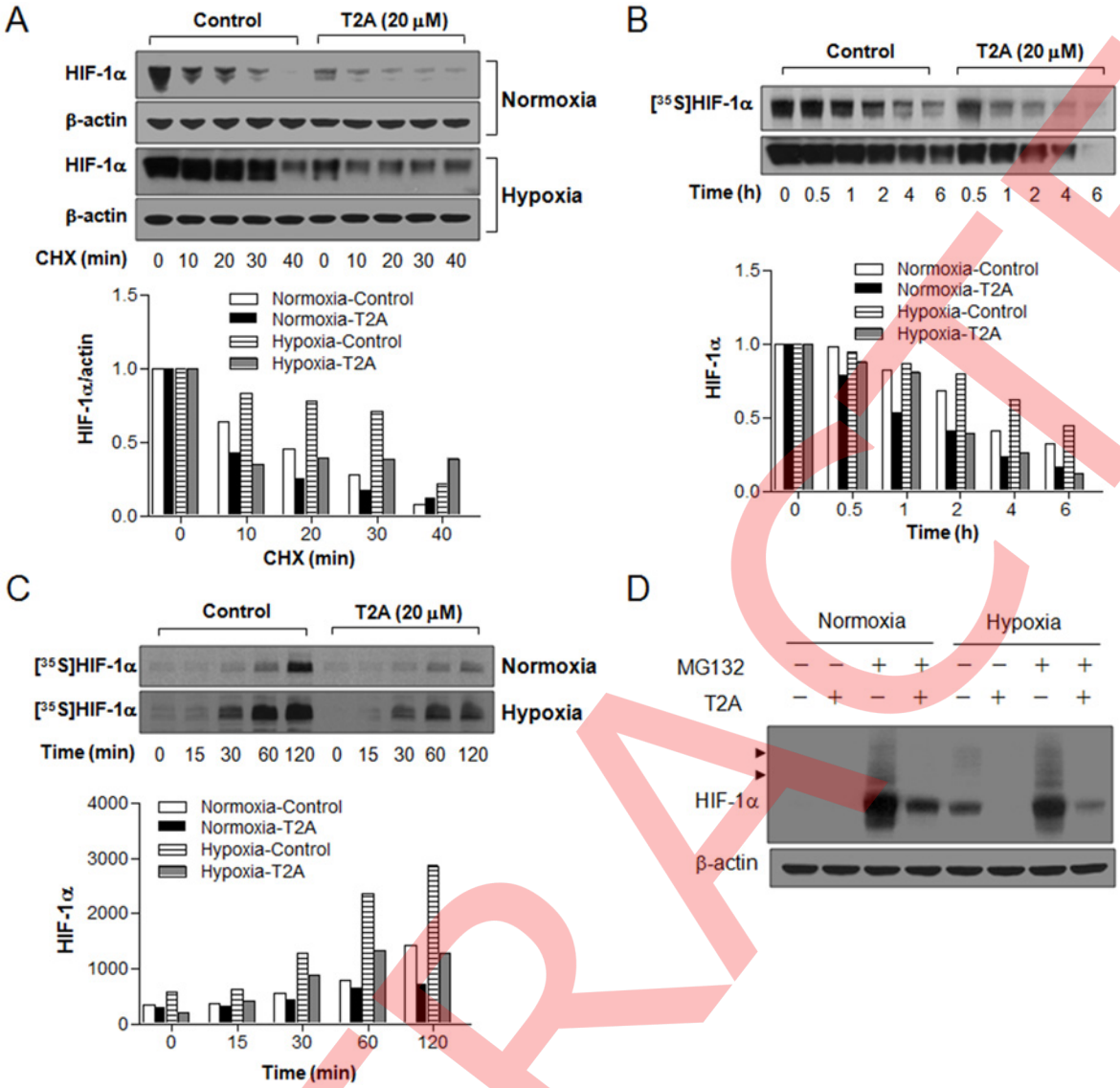


**Fig 3. T2A inhibited HIF-1α expression through the inhibition of protein synthesis rather than the promotion of HIF-1α degradation.** (A) The MDA-MB-231 cells were cultured under normoxic or hypoxic conditions for 8 hours, then pretreated without or with 20 μM T2A for 30 minutes, followed by treatment without or with 20 μM cycloheximide (CHX) for various intervals as indicated. Upper panel: after treatment, whole cell extracts were analyzed by Western blot using antibodies against HIF-1α and β-actin. Lower panel: quantification of the HIF-1α by densitometry following normalization to the β-actin level. The HIF-1α protein levels in untreated or T2A-treated cells were arbitrarily given the value of 100%. (B) Cells were labeled with [$^{35}$S]methionine as described in the Methods section and chased for the indicated periods (h) in the presence or absence of T2A under normoxic or hypoxic conditions. Upper panel: equal amounts of proteins from each cell lysate were subjected to immunoprecipitation and then separated in SDS-PAGE and examined by autoradiography following normalization to the control. Lower panel: quantification of the autoradiographic HIF-1 signal by densitometry. (C) Cells were pretreated with either vehicle or 20 μM T2A for 16 hours and then subjected to hypoxia, or remained in normoxia for an additional 8 hours. Subsequently, cells were labeled with [$^{35}$S]methionine in the presence or absence of 20 μM T2A for the indicated time. Upper panel: Equal amounts of proteins were subjected to immunoprecipitation and then separated in SDS-PAGE and examined by autoradiography. Lower panel: quantification of the autoradiographic HIF-1 signal by densitometry. (D) Cells were pretreated with the proteasome inhibitor MG132 (10 μM) for 2 hours, followed by T2A treatment (20 μM) under normoxic or hypoxic conditions as described above. Whole cell extracts were analyzed by Western blot using antibodies against HIF-1α and β-actin.

doi:10.1371/journal.pone.0117440.g003

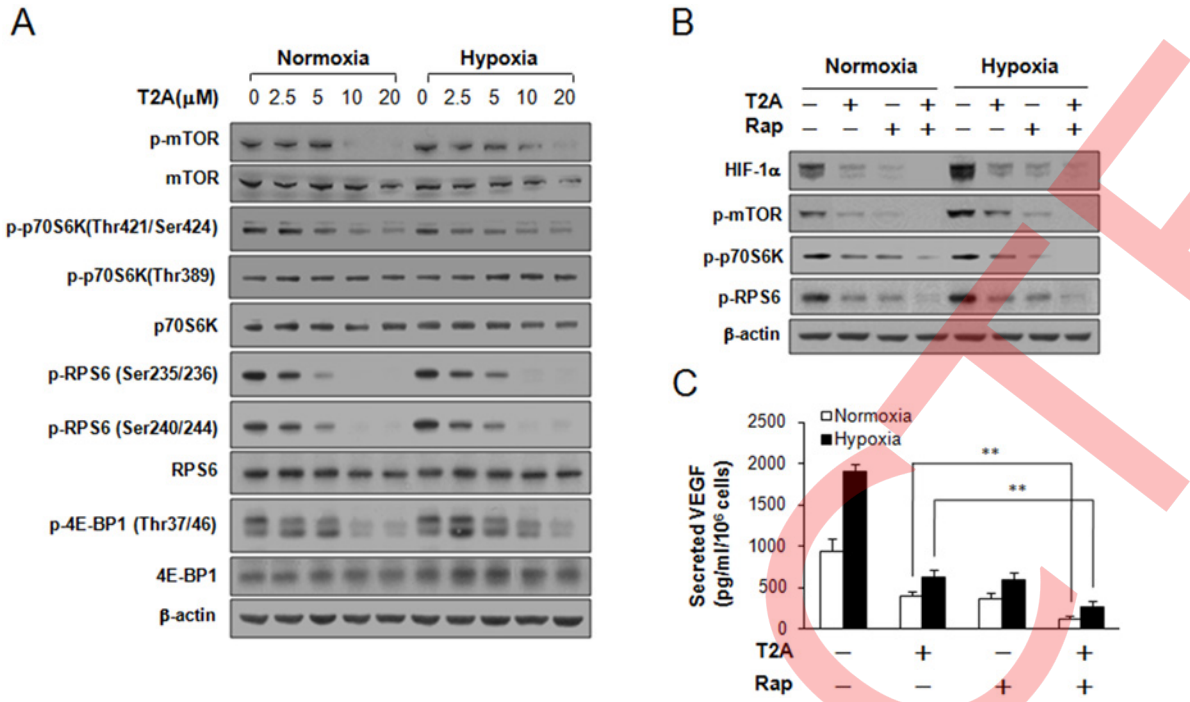


**Fig 4. The mTOR/p70S6K/ RPS6/4E-BP1 signaling pathway was involved in T2A-induced inhibition of HIF-1α.** (A) The MDA-MB-231 cells were treated without or with various concentrations of T2A for 24 hours under normoxic and hypoxic conditions. Whole cell extracts were prepared from cells and subjected to Western blot assay using antibodies against phospho-mTOR (p-mTOR), mTOR, p70S6 Kinase(p70S6K), p-p70S6K (Thr421/Ser424), p-p70S6K(Thr389), S6 Ribosomal Protein (RPS6), p-RPS6(Ser235/236), p-RPS6(Ser240/244), 4E-BP1, and p-4E-BP1(Thr37/46). (B) Cells were pretreated with rapamycin (20 nM) for 2 hours, followed by T2A treatment (20 μM) under normoxic or hypoxic conditions as described above. Whole cell extracts were analyzed by Western blot using antibodies as indicated. (C) VEGF in the supernatant was evaluated using ELISA. Error bars represent means ± SD (n = 3). Values of cells treated with T2A and rapamycin were significantly reduced compared to values obtained from cells treated with T2A alone based on Student's t-test; $^{**}P < 0.01$.

doi:10.1371/journal.pone.0117440.g004

## The mTOR/p70S6K/RPS6/4E-BP1 signaling pathway was involved in T2A-mediated inhibition of HIF-1α

The previous study demonstrated that the mTOR/p70S6K/RPS6/4E-BP1signaling pathway played an important role in regulating HIF-1α expression at the translational level [28]. To determine the potential involvement of this signaling pathway in T2A-mediated inhibition of HIF-1α expression, we examined the effects of T2A on the phosphorylation of mTOR, p70S6K, RPS6, and 4E-BP1. Exposure of cells to T2A of various concentrations inhibited the phosphorylation of mTOR, p70S6K (Thr421/Ser424), RPS6 (Ser235/236 and Ser240/244), 4E-BP1 (Thr37/46) under both normoxic and hypoxic conditions (Fig. 4A). In contrast, T2A treatment had no significant effects on the phosphorylation of p70S6K (Thr389) and the total levels of mTOR, p70S6K, RPS6, and 4E-BP1. These results suggest that inhibition of the mTOR/p70S6K/RPS6/4E-BP1 signaling pathway was involved in T2A-mediated inhibition of HIF-1α.

To further assess the functional significance of the mTOR/p70S6K/RPS6/4E-BP1 signaling pathway in T2A-mediated inhibition of HIF-1α expression, cells were pretreated with the mTOR inhibitor rapamycin (20 nM), and followed by T2A treatment (10 μM). The levels of HIF-1α, VEGF, and related signaling proteins were evaluated using Western blot and ELISA assays. Pretreatment of cells with rapamycin enhanced the inhibitory effects of T2A on HIF-1α expression under both normoxic and hypoxic conditions (Fig. 4B). In addition, rapamycin enhanced the inhibitory effects of T2A on the phosphorylation of mTOR, p70S6K, and RPS6 as well as the expression of VEGF (Fig. 4C). Taken together, these results indicate that the

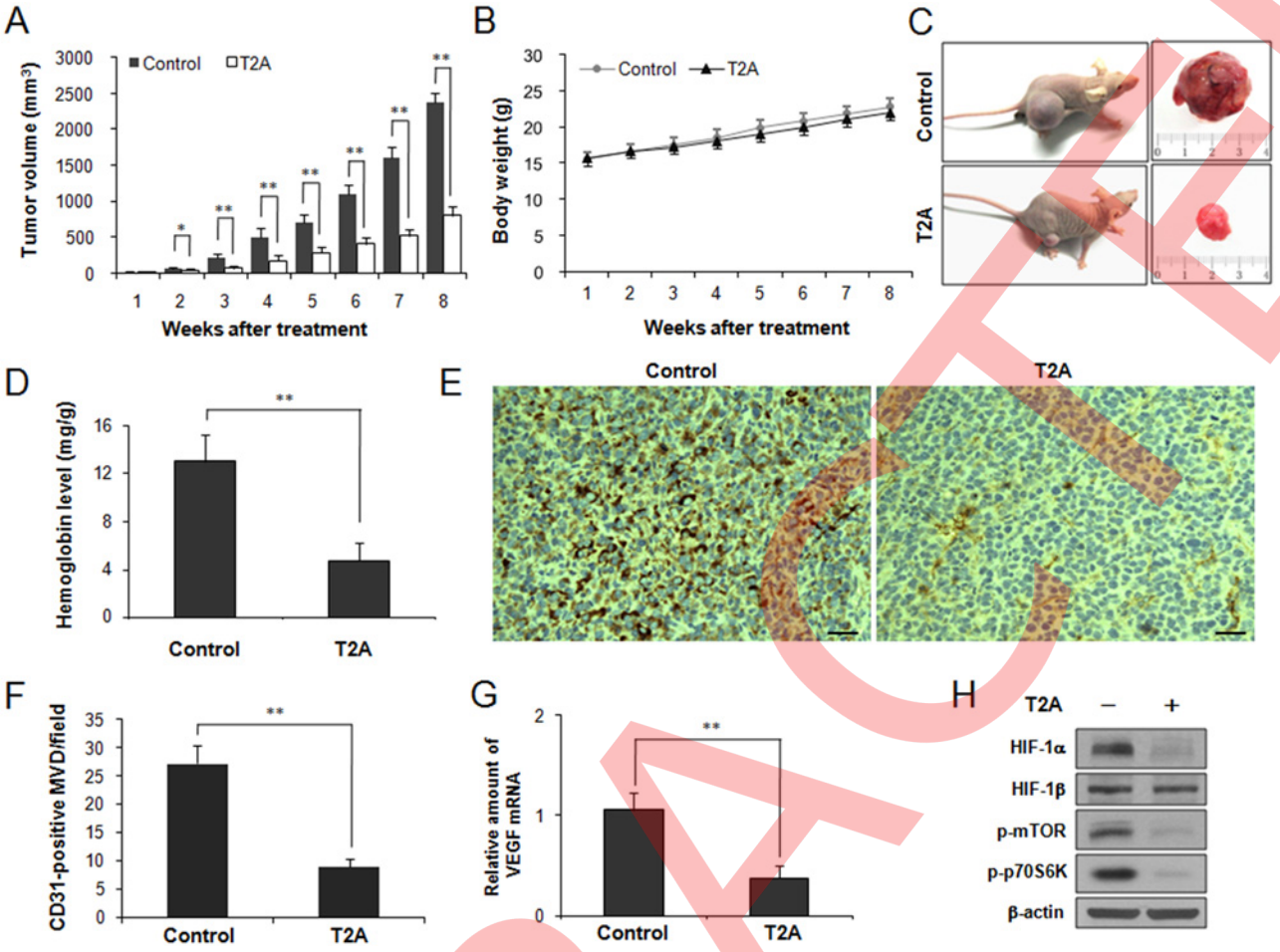


**Fig 5. T2A inhibited angiogenesis and tumor growth *in vivo*.** Xenograft mouse models on the back of nude mice were established using human MDA-MB-231 cells ($2 \times 10^6$). Tumor mice were treated with T2A or vehicle as described in the Methods section. Tumor growth and body weight of mice were measured once a week. (A) Average tumor volume of vehicle control mice and mice treated with T2A. Values of tumor volume from the T2A-treated group were significantly reducted compared with those from mice of the control group based on Student's t test; $*P < 0.05$ or $**P < 0.01$. (B) Body weight of mice during the eight weeks of T2A treatment. (C) Representative tumors from the control and T2A-treated groups. (D) Average hemoglobin levels in tumor tissues from 4 vehicle control and 4 T2A-treated mice. $**P < 0.01$ compared with vehicle control. (E) Representative tumor sections were immunostaining by using CD31 antibody. Scale bar represents 20 μm. (F) Microvessel density (MVD) was counted the CD31-positive blood vessels per field (400 × magnification) from four replicate tumor sections. $**P < 0.01$ compared with vehicle control. (G) Total cellular RNA were extracted from tumors in 4 vehicle control and 4 T2A-treated mice and the VEGF mRNA level was evaluated using real-time PCR. $**P < 0.01$ compared with vehicle control. (H) Tumors from 1 vehicle control mouse and 1 T2A-treated mice were surgically removed and homogenized. Whole tumor lysates were prepared and subjected to Western blot assay using antibodies as indicated.

doi:10.1371/journal.pone.0117440.g005

mTOR/p70S6K/RPS6/ 4E-BP1 signaling pathway was involved in T2A-mediated inhibition of HIF-1α expression.

## T2A inhibited angiogenesis and tumor growth in vivo through suppression of HIF-1α and VEGF

A MDA-MB-231 xenograft mouse model established to assess whether T2A inhibits angiogenesis and tumor growth *in vivo*. As seen in Fig. 5A, a time-dependent reduction of tumor volume was observed in T2A (50 mg/kg) treated xenograft mice compared with the control animals ($*P < 0.05$ or $**P < 0.01$ vs vehicle controls). However, no significant changes in weight and potential toxicity were noted between the T2A-treated mice and control animals (Fig. 5B).

To further analyze the effects of T2A on angiogenesis *in vivo*, we evauated the formation of blood vessels in xenograft. As shown in Fig. 5C, T2A treatment significantly inhibited the formation of blood vessels compared with the control animals. Hemoglobin content in the xenograft was used as an index for the evaluation of angiogenesis and the average hemoglobin content of the control group was 13 mg/g. T2A treatment significantly reduced the hemoglobin level in the xenograft (Fig. 5D). In addition, the CD31 immunostaining of the tumor tissue was performed to determine microvessel density (MVD). The number of CD31-positive vessels was significant decrease in the T2A-treated tumor sections compared with control (Fig. 5E and F), suggesting that treatment of T2A decreased the blood vessel formation in the tumor tissue. Furthermore, given that VEGF plays an important role in angiogenesis, we also evaluated the expression of VEGF at the mRNA level by quantitative real-time PCR. We found T2A treatment significantly reduced the mRNA level of VEGF (Fig. 5G). These results suggest that T2A inhibited tumor growth through the inhibition of angiogenesis.

The inhibition of HIF-1α and interruption of mTOR/p70S6K signaling pathway induced by T2A were also investigated *in vivo*. Western blot was used to evaluate the levels of HIF-1α, phospho-mTOR, and phospho-p70S6K in the extracts from tumor tissues. As shown in Fig. 5H, the expressions of HIF-1α, phospho-mTOR, and phospho-p70S6K in the tumor tissue from MDA-MB-231 xenograft mice were significantly reduced by T2A treatment. These findings confirm that the interruption of mTOR/p70S6K signaling pathway was involved in T2A-induced inhibition of HIF-1α and VEGF expressions, leading to the inhibition angiogenesis and tumor growth in nude mice.

## Discussion

Angiogenesis, characterized by the formation of novel blood vessels, is one of the most important steps in tumor growth and metastasis [7, 29]. It has been shown that HIF-1-mediated VEGF expression plays an important role in the formation of novel blood vessels, therefore, angiogenesis inhibitors that inhibit the expression of HIF-1α and VEGF have been widely studied for the treatment of human cancers [30, 31]. In the present study, we showed that T2A inhibited HIF-1α expression and the transcriptional activity of HIF-1 in breast cancer cells. More importantly, we found that T2A effectively inhibited angiogenesis and tumor growth *in vitro* and *in vivo* through the suppression of HIF-1α and VEGF. The previous study has reported that 2-methoxyestradiol inhibited angiogenesis through the repression of HIF-1α protein synthesis [32]. In the present study, we found that T2A treatment has no significant effects on HIF-1a mRNA expression. However, the degradation of HIF-1α was not significantly increased by T2A based on the experiment using the HIF-1α proteasome inhibitor MG-132. In addition, we found that T2A inhibited HIF-1α expression through a translational-dependent pathway rather than affecting HIF-1α protein stability based on the experiment using the protein translation inhibitor cycloheximide (CHX) and a pulse-chase assay. These results suggest that T2A suppressed HIF-1α expression at the translational level without affecting the stabilization and degradation of HIF-1α.

HIF-1α is an important transcriptional factor regulating the expression of downstream genes such as VEGF, Glut1, and EPO, which promotes angiogenesis in hypoxic tumors [33, 34]. VEGF plays an important role in tumor angiogenesis by inducing the formation of new blood vessels in tumor tissues [35]. Therefore, suppression of VEGF signaling can inhibit both tumor angiogenesis and growth. As expected, T2A inhibited the binding of HIF-1α to the VEGF promoter under hypoxic condition, suggesting that T2A inhibited VEGF expression and angiogenesis through the suppression of HIF-1α. It has also been reported that T2A reduced the mRNA levels of Glut1 and EPO, another two important target genes of HIF-1α [33,

34]. These reports and our results suggest that T2A inhibite the translational activity of HIF-1a and suppreses angiogenesis through the down-regulation of VEGF, Glut1, and EPO.

In this study, we also showed that down-regulation of the mTOR/p70S6K/4E-BP1 pathway was involved in T2A-induced inhibition of HIF-1α and VEGF in breast cancer cells *in vitro*. It has been reported that the mTOR pathway played an important role in the regulation of HIF-1α translation [36]. It has also been showen that mTOR, a central serine/threonine kinase, played a key role in tumor angiogenesis [37]. The mTOR signaling pathway directly or indirectly regulates numerous cellular activities, including translation initiation and protein turnover [28]. The phosphorylation and activation of ribosomal p70S6 kinase and the phosphorylation and inactivation of the eukaryotic initiation factor 4E binding protein-1 (4E-BP1) are involved in mTOR-mediated translation initiation. The phosphorylation of 40S ribosomal protein S6 induced by p70S6K activation ultimately drives the translation of 5'TOP (terminal oligopyrimidine tract) mRNAs [28]. 4E-BP1 is a translation inhibitor binding to the translation initiation factor eIF4E. Upon growth signal, 4E-BP1 is phosphorylated and inactivated, leading to the release of eIF4E from 4E-BP1. Free eIF4E binds to the cap structure and promotes cap-dependent mRNA translation [38, 39]. In the present study, we showed that T2A markedly inhibited the phosphorylation of mTOR, p70S6K, and 4E-BP1, and the expression of HIF-1α in MDA-MB-231 cells. Moreover, pretreatment of MDA-MB-231 cells with rapamycin, an inhibitor of mTOR, significantly enhanced T2A-mediated inhibition of HIF-1α, dephosphorylation of mTOR and p70S6K, and VEGF expression. Taken together, these findings strongly suggest that the mTOR/p70S6K/4E-BP1 pathway plays a critical role in T2A-mediated inhibition of HIF-1α.

Our *in vivo* study showed that T2A inhibited angiogenesis and tumor growth through the inhibition of HIF-1α expression, confirming that HIF-1α inhibition was involved in the antiangiogenic activity of T2A. However, the signaling pathways involved in these events are not fully understood. Recent studies demonstrated that mTOR was highly activated and continuously upregulated during human breast cancer progression [40, 41], suggesting that mTOR activation serves as a predictive marker in breast cancer entity, as well as a potential molecular target for anticancer therapy [8, 42]. Our *in vitro* experimental results demonstrated that the mTOR/p70S6K/4E-BP1 pathway played an important role in the T2A-induced inhibition of HIF-1α. To further investigate the role of the mTOR/p70S6K/4E-BP1 pathway in angiogenesis and tumor growth *in vivo*, Western blot was used to evaluate the levels of HIF-1α, phospho-mTOR, and phospho-p70S6K in the protein extracts prepared from tumor tissues in nude mice. We found that T2A-induced inhibition of HIF-1α was mediated by the mTOR/p70S6K pathway in xenograft tumors in nude mice, which is consistent with the *in vitro* experimental results.

## Conclusions

The present study demonstrated, for the first time, that T2A inhibited the angiogenesis and growth of breast cancer *in vitro* and *in vivo* through the suppression of HIF-1α protein synthesis and VEGF expression, in which the mTOR/p70S6K/4E-BP1 signaling pathway was involved. Our results provide novel perspectives and potential targets for the development of anticancer therapies against breast cancers.

## Author Contributions

Conceived and designed the experiments: GBL TZ HJC NG. Performed the experiments: GBL TZ CYS JZ YBC. Analyzed the data: GBL TZ CYS LL. Contributed reagents/materials/analysis tools: LL JZ XYH. Wrote the paper: GBL HJC NG.

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