TRIM72 exerts antitumor effects in breast cancer and modulates lactate production and *MCT4* promoter activity by interacting with PPP3CA

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A hypoxic tumor microenvironment (TME) promotes cancer progression, yet its value as a therapeutic target remains underexploited. Tripartite motif-containing 72 (TRIM72) may protect cells against various stresses including hypoxia. Recently, low TRIM72 expression has been implicated in cancer progression. However, the biological role and molecular mechanism of TRIM72 in breast cancer (BC) remain unclear. Herein, we analyzed the TRIM72 expression in BC tissue and cell lines by western blot (WB) and quantitative reverse transcription-PCR. We established the overexpression of TRIM72 using plasmids and lentiviral-mediated upregulation, as well as downregulation of protein phosphatase 3 catalytic subunit alpha (PPP3CA) by siRNA. The tumor-suppressive roles of TRIM72 were assessed on BT549 and MDA-MB-231 cells by MTS, Transwell, and flow cytometry assays *in vitro* and in xenografted tumors *in vivo*. The molecular mechanism of TRIM72 was investigated by luciferase reporter and co-immunoprecipitation (Co-IP) assay. Lactate production was measured by ELISA under hypoxic environments induced by $CoCl_2$. Moreover, the expression of PI3K/Akt/mTOR pathway-associated proteins was detected by WB in BC cells. Results showed that TRIM72 was downregulated in BC. Overexpression of TRIM72 inhibited tumor proliferation and invasion *in vitro* and in a xenograft tumor model. Mechanistically, PPP3CA altered the inhibitory effects of TRIM72 on hypoxia-induced lactate production and monocarboxylate transporter 4-promoter activity, as well as the effect of the PI3K/Akt/mTOR signaling pathway. Our study suggests that TRIM72 modulates the TME and plays tumor-suppressive roles in BC progression. Therefore, TRIM72 may serve as a potential therapeutic target in BC. *Anti-Cancer Drugs* 33: 489–501 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.

Anti-Cancer Drugs 2022, **33**:489–501

Keywords: breast cancer, monocarboxylate transporter 4, PI3K/Akt/mTOR pathway, protein phosphatase 3 catalytic subunit alpha, tripartite motif-containing 72

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Received 14 February 2022 **Revised form accepted** 23 February 2022

Introduction

With almost 1.2 million new cases diagnosed each year, breast cancer (BC) is the most prevalent cancer in women, accounting for over 630 000 annual deaths worldwide [1]. The metabolic shift from oxidative glycolysis toward aerobic glycolysis, even in the presence of oxygen, is a hallmark of cancer cells to rapidly generate energy for growth and is known as the Warburg effect [2]. This shift results in a 20- to 40-fold increase in lactate production and low pH, which constitute a tumor microenvironment (TME) that suppress the immune response and promote BC metastasis [3]. For example, the TME contributes to the heterogeneity in BC subtypes and influences treatment efficacy [4]. As a response to changes in local environmental signals (e.g. hypoxia and decreased pH), several carcinogenesis-associated signaling pathways are activated in the TME, including the phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), nuclear factor kappa B (NF-κB), and hypoxia-inducible factor (HIF) [5,6]. Therefore, TME's related metabolism has been explored as a potential target for BC therapy and still requires further exploration [7].

Tripartite motif-containing 72 (TRIM72), also known as Mitsugumin-53 (MG53), is an E3 ubiquitin ligase that belongs to the TRIM family [8], which is involved in various pathological processes, including immunity, infection, and cancer [9,10]. Originally, TRIM72 deficiency

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website, www.anti-cancerdrugs.com.

was associated with various tissue injuries, including muscular dystrophy, heart failure, lung injury, and kidney disease [11–13]. Later studies have suggested that upregulation of TRIM72 contributes to insulin resistance [14–16]; however, most of the results could not be repeated in more recent investigations [17–19]. New data have suggested that treatment with TRIM72 can repair membrane damage and facilitate tissue regeneration [20–22]. Further studies have also demonstrated that TRIM72 has remarkable protective effects on cells under inflammatory, hypoxic, and oxidative stress conditions [23,24].

Recently, dysregulation of TRIM72 has been associated with oncogenesis and can be a hallmark of cancer progression. Our prior study revealed that reduced serum TRIM72 levels can be a sensitive diagnostic marker for patients with colon cancer. We found TRIM72 levels were negatively correlated with advanced clinical stage, Dukes' stage, distant metastasis, and serum carcinoembryonic antigen levels [25]. Another study has also confirmed that lower TRIM72 expression can act as a relapse predictor of stage II colon carcinoma [26]. Researchers also found that TRIM72 knockdown in SCC25 cells could promote the phosphorylation of Akt^{Ser208} and Akt^{Thr473}, which contribute to tumorigenicity changes in tongue cancer [27]. In non-small cell lung cancer cells, loss of TRIM72 was found to promote lung tumorigenesis by interacting with an oncogenic protein G3BP2 to regulate stress granule (SG) formation under arsenic trioxide (ATO)-induced oxidative stress conditions [28]. As such, TRIM72 could be an effective treatment target to protect against a range of adverse stresses. Additionally, TRIM72 is upregulated in O_2-exposed alveolar epithelial cells (RLE6TN) and human lung carcinoma epithelial cells (A549), implying regulation of TRIM72 expression by oxygen [29]. These findings prompted us to explore possible linkages between TRIM72 and the TME.

Herein, we found that TRIM72 is downregulated in BC cell lines and tissues. Furthermore, TRIM72 is downregulated by hypoxic treatment to promote BC cell proliferation, migration, and invasion *in vitro* and *in vivo*. Additional experiments revealed that TRIM72 represses lactate production and monocarboxylate transporter 4 (MCT4)-promoter activity via interaction with protein phosphatase 3 catalytic subunit alpha (PPP3CA). The interaction between TRIM72 and PPP3CA is implicated in the PI3K/Akt/mammalian target of rapamycin (mTOR) axis – the most important hypoxia-related glycolytic signaling pathway in BC – thereby promoting initiation and progression of tumorigenesis [30]. These findings uncover a TRIM72-associated mechanism for the regulation of the TME and reveal the antitumor role of TRIM72 in BC progression.

Materials and methods

Tissue samples

A total of 24 paired human BC and adjacent nontumor tissues were collected from the Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, Guangdong, China). This study was approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University, and all participants provided their written informed consent.

Cell culture and treatment

BC cell lines (MDA-MB-231, SKBR3, BT474, HS578T, BT549, and MCF7) and the normal breast cell line MCF10A were purchased from the Shanghai Cell Bank (Shanghai, China). The cells were cultured in the indicated medium (listed in Table S1, Supplemental digital content 1, *http://links.lww.com/ACD/A428*) supplied with 10% fetal bovine serum (FBS) (HyClone, Utah, USA) and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2. To simulate hypoxia, MDA-MB-231 and BT549 cells were incubated for 24 h with 125 μM $CoCl_2$ (Sigma, St. Louis, Missouri, USA). For signaling-pathway studies, BT549 cells were treated with 2.5 mM L-lactate purchased from Source Leaf Creature (B21929, Shanghai, China) for 24 h.

Western blotting

Briefly, total protein was extracted from BC cells and quantified. Equal amounts of protein lysates were separated using 8–15% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts, USA). After incubation with 5% nonfat milk, the membranes were hybridized with specific primary antibodies overnight at 4 °C. The membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. The signals were detected using enhanced chemiluminescence detection reagents (Biyuntian Biotech Co., Ltd., Shanghai, China). The anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used as a control for whole-cell lysates. The antibody information is provided in Supplemental Digital Content 2, Supplemental digital content 1, *http://links.lww.com/ACD/A428*.

RNA extraction and quantitative reverse transcription-PCR

Total RNA was extracted from BC cell lines and tissues using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, California, USA). The PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan) was used to synthesize cDNA. Quantitative reverse transcription-PCR (RT-qPCR) was subsequently performed using TBGreen™ PremixExTaq II (Takara) on an ABI Prism 7300 Sequence Detection System. The transcript level of each gene was normalized to that of 18S ribosomal RNA (*18S*) or *GAPDH*. The comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$ and ΔCT) was used to calculate relative expression levels in cells and tissues. All experiments were performed in triplicate. The primers used for RT-qPCR are listed in Table 1.

Table 1 Primers used in this study

Name		Primer sequences used in plasmid construction (5′→3′)
pCDH-GFP+PURO-TRIM72	F	CGCAAATGGGCGGTAGGCGTG
	R	GGACTGTGGGCGATGTGC
Name		Primer sequences used in RT-PCR (5′→3′)
TRIM72	F	ACTACTGGGAGGTGGATGTT
	R	AGCTCAGGTAAAGGCCAATG
PPP3CA	F	TTACTTGGTCCCTTCCATTTGT
	R	TGCTCGGATCTTGTTCCTTATC
LDHA	F	GCCTGTATGGAGTGGAATGAA
	R	CCAATAGCCCAGGATGTGTAG
18S	F	CCTGGATACCGCAGCTAGGA
	R	GCGGCGCAATACGAATGCCCC
GAPDH	F	AACGGATTTGGTCGTATTGGG
	R	CCTGGAAGATGGTGATGGGAT
Name		siRNA Sequences (5′→3′)
NC siRNA		UUCUCCGAACGUGUCACGU
PPP3CA siRNA1		CCAAGUUGUCGACGACCGA
PPP3CA siRNA2		AGUGUUGCAUUGAGAAUAA
PPP3CA siRNA3		GUUCAUACUUCUACAGUUA

18S, 18S ribosomal RNA; GAPDH, anti-glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; LDHA, lactate dehydrogenase A; NC, negative control; PPP3CA, protein phosphatase 3 catalytic subunit alpha; RT-PCR, reverse transcription-PCR; TRIM72, tripartite motif-containing 72.

Plasmids, siRNA, lentivirus construction, and stable transfection

The TRIM72-overexpression plasmids (TRIM72-oe) were generated by cloning PCR-amplified full-length human TRIM72 cDNA into pCDH-GFP-Puro and verified by sequencing. Lentiviral *TRIM72*-oe constructs were generated into lentiviruses. Briefly, $3–4\times10^6$ 293T cells were seeded overnight in 100-mm-diameter dishes with DMEM containing 10% FBS and reached approximately 80% confluency before transfection. Then, 293T cells were co-transfected with the resultant recombinant vector carrying *TRIM72* along with packaging plasmids (i.e. pLP1, pLP2, and pLP/VSVG) using the polyethyleneimine reagent. The supernatant containing the lentivirus was harvested after 48 and 72 h and underwent ultracentrifugation (10 min, 4000*g*, 4 °C). for lentivirus collection. Lentivirus particles produced from the transfected 293T cells were used to transduce BT549 cells to make a stable cell line. The transduced BT549 cells were selected using puromycin (1 μg/ml) for 10–12 days, and the transfection efficiency was evaluated by the detection of green fluorescent protein in more than 95% of the cells. *TRIM72* expression was then determined using RT-qPCR, and an empty vector was used as negative control.

siRNAs targeting *PPP3CA* were synthesized by Hechuang Biotechnology Co., Ltd. (Guangzhou, China). Three *PPP3CA*-targeting siRNA duplexes (siRNA1, siRNA2, and siRNA3) and one NC duplex were tested using RT-qPCR, and siRNA2 was selected after evaluation of PPP3CA downregulation and used in the subsequent experiments. Plasmid and siRNA were transfected into cells with Lipofectamine 2000. Additionally, to assess the interaction between TRIM72 and MCT4 and the effects of TRIM72 on the pathway regulation, TRIM72-overexpressing cells, and control cells were secondarily transfected with PPP3CA-targeting siRNA according to the aforementioned method. All sequences used in this study are shown in Table 1.

Viability, cell cycle, and apoptosis assay

BT549 and MDA-MB-231 cells were transfected with *TRIM72*-oe or NC plasmids in 6-well plates. Approximately 24 h after transfection, the cells (2×10^3 cells) were seeded in a 96-well plate, and cell viability was measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA) every 24 h, thrice. For cell cycle studies, cells transfected with *TRIM72* overexpression plasmid or NC for 24 h were harvested using 0.25% trypsin. Then, 1×10^6 cells were fixed with 70% ethanol for 30 min at 4 °C and stained using a Cell Cycle Kit (Forevergen Biosciences Co., Ltd., Guangzhou, China) in the dark for 30 min. Subsequently, the cells were washed with PBS and subjected to flow cytometry (BD Biosciences, San Jose, California, USA). For cell apoptosis analysis, cells were harvested 24 h after transfection, washed with PBS twice, resuspended in binding buffer, and double-stained using Annexin V and propidium iodide (BD Biosciences). Apoptosis analysis was performed by flow cytometry.

Cell migration and invasion assay

After transfection with TRIM72-oe plasmid or NC for 24 h, 1×10^5 of BT549 and MDA-MB-231 cells were collected, resuspended in 200-μl serum-free medium, and seeded in the upper chamber of a Transwell (Corning, Inc., Corning, New York, USA) for cell migration analysis. The lower chambers were filled with medium containing 10% FBS. During the invasion assay, the cells were treated as described but seeded into a Matrigel-coated chamber (BD Bioscience) instead. After incubation at 37 °C for 24 h, the non-migratory cells were removed, and migratory and invading cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Cells that migrated through the pores were captured and counted in five random fields.

Measurement of lactate production

The BT549 cells were plated and allowed to reach approximately 60% confluency. After TRIM72-oe plasmid transfection, the transfection culture medium was replaced with a medium containing 125 μm $CoCl_2$ to mimic a hypoxic condition. After 24 h, secreted lactate level and intracellular lactate level were measured using a lactate assay kit (Bioassay Systems, Hayward, California, USA). All raw data of intracellular lactate level was measured were normalized against protein concentrations.

Co-IP

BT549 cells in 100-mm culture dishes were transfected with vectors carrying TRIM72 expression using Lipofectamine 2000 and Opti-MEM medium according

to the manufacturer's protocol. After 6 h, the medium was replaced with a complete medium and incubated with $CoCl_2$ for 24 h. Subsequently, the cells were rinsed, lysed, and centrifuged, and the supernatants were collected. The supernatants were divided into three parts, and one part was used as input. The other two parts were preincubated with protein A/G agarose for 1 h. In the IP experiment, 2 μg of TRIM72 antibody and 1 μg of IgG were added to the supernatant and incubated at 4 °C. The Protein A + G Agarose/Salmon was added to the mixture and then centrifuged. Briefly, the bead-*TRIM72* antibody complex (10 μg antibody and 50 μl protein A/G magnetic beads) was prepared, and then the cell lysates and beads were incubated at 4 °C for 12 h. After washing with lysis buffer, the protein complexes were boiled in 1× SDS loading buffer and subjected to western blotting as previously described.

Dual-luciferase reporter assay

The *MCT4* promoter-luciferase reporter harboring 2000 bp of the MCT4 promoter region was amplified by PCR and inserted into the reporter luciferase vector pmirGLO by Hechuang Biotechnology Co., Ltd., and later verified by sequencing. BT549 cells were co-transfected with pGL4.1-luc-*MCT4* vector, pGL4.1, *TRIM72*-oe vector, and *PPP3CA* siRNA, as indicated, into 24-well plates (three replicate wells for each group). After 24 h, luciferase activities were detected using a dual-luciferase reporter assay system (Promega). Relative luciferase activities, reflecting the promotor activity, were determined by normalizing the firefly luciferase activity to that of the corresponding *Renilla* luciferase activity. The primers used to construct the MCT4 promoter are shown in Table 1.

Animal study

The nude BALB/c male mice (6 weeks old; weight: 18–20 g) were purchased from Hechuang Biotechnology Co., Ltd. Ten mice were randomly divided into two groups (n = 5). Equivalent amounts (1×10^7 cells/mouse) of BT549 cells stably transfected with empty vector or TRIM72-*oe* were injected subcutaneously into the flank of male nude BALB/c mice. Tumorigenesis was observed and recorded by measuring solid tumors every 4 days. The mice were euthanized on the 20th day after injection. Following euthanasia, tumor tissues were collected and weighted. Animal studies were approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University and followed the Guideline for Animal Experiments of the National Cancer Center Research Institute.

Hematoxylin and eosin staining

Formalin-fixed tumor tissues were embedded in paraffin and sliced into 4-μm-thick sections. Subsequently, the sections were dewaxed and hydrated. Hematoxylin and eosin (H&E) staining was performed using an H&E staining kit (Servicebio, Wuhan, China). Histopathological changes were observed under a light microscope.

Statistical analysis

Data are expressed as means ± SD. Data were analyzed by unpaired or paired Student's t-test. Multiple comparisons were performed with one-way, two-way, or repeated measure two-way ANOVA using Prism (ver. 8.0.1; GraphPad Software Inc., California, USA). Post hoc multiple comparisons were completed using Turkey's post hoc test or Sidak's multiple comparisons test. P values are lower than 0.05 denoted statistical significance.

Results

TRIM72 expression is downregulated in breast cancer cells and tissues

To identify the role of TRIM72 in BC, we investigated its expression levels in BC tissues and paired adjacent nontumor tissue samples from 24 patients using RT-qPCR (Fig. 1a). Approximately 75% of the paired samples exhibited lower TRIM72 expression in the cancer tissues (a low delta Ct value indicating a high TRIM72 expression level) when compared to adjacent nontumor tissue. Moreover, TRIM72 expression levels were detected using western blotting and RT-qPCR in six BC cell lines (i.e. HS578T, MCF7, BT474, SKBR3, BT549, and MDA-MB-231) and one normal human mammary cell line (i.e. MCF-10A). The results showed that TRIM72 expression was notably lower in all BC cell lines, except for MCF7 (Fig. 1b). Thus, we hypothesized that the low expression of TRIM72 contributes to BC progression.

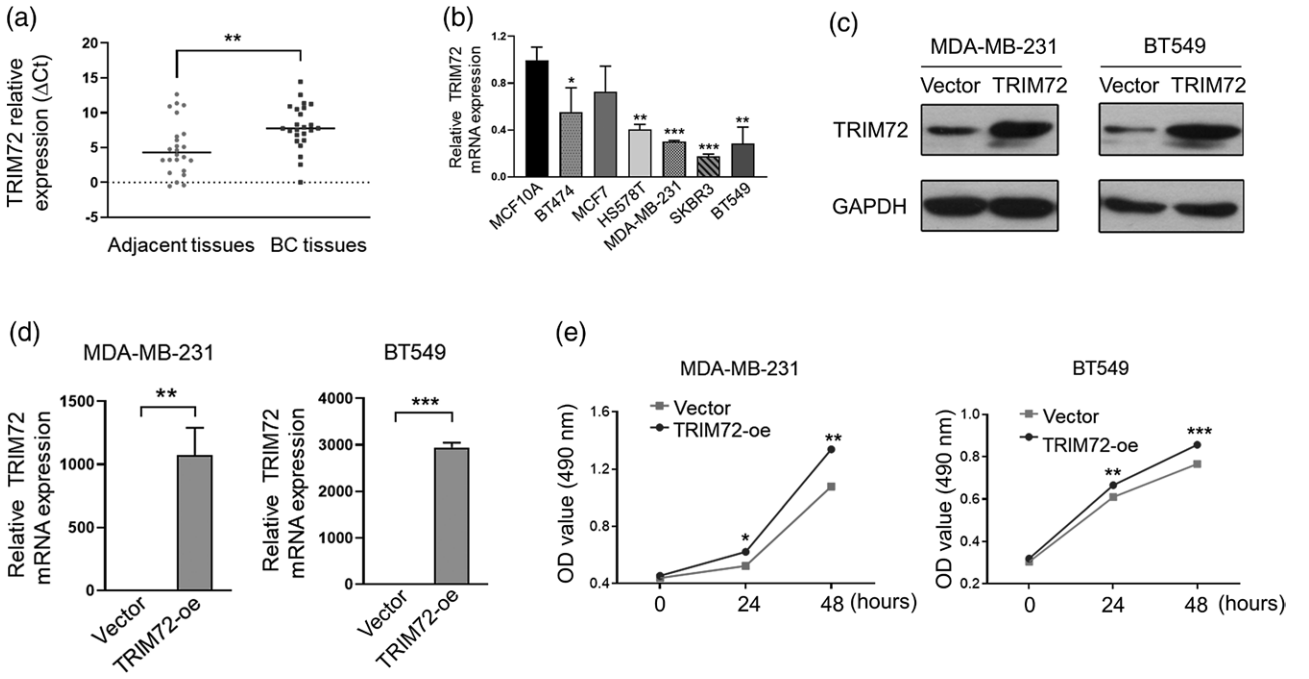
TRIM72 suppresses MDA-MB-231 and BT549 cell proliferation, induces apoptosis, and inhibits the cell cycle

To explore the mechanism of TRIM72 in BC, the triple-negative BC cell lines BT549 and MDA-MB-231 were selected for further functional studies. TRIM72 was overexpressed in the two cell lines and the expression of TRIM72 was verified by western blotting and RT-qPCR (Fig. 1c,d). TRIM72 overexpression significantly inhibited cell proliferation (Fig. 1e) and induced S phase arrest (Fig. 2a). These findings coincided with the viability analysis by flow cytometry. Increased apoptosis was observed in TRIM72-oe MDA-MB-231 and TRIM72-oe BT549 cells (Fig. 2b). Together, TRIM72 overexpression effectively decreases the expansion and viability of MDA-MB-231 and BT549 cells *in vitro*.

TRIM72 inhibits cell migration and invasion ability *in vitro*

Migration and invasion are essential abilities of tumor cells. To determine the effects of TRIM72 on BC cell infiltration, we performed motility experiments using Transwell assays. The results showed that TRIM72 overexpression significantly inhibited the invasion and migration abilities of both MDA-MB-231 and BT549 cells (Fig. 2c).

Fig. 1



TRIM72 is downregulated in BC and inhibits BC cell proliferation. (a) The relative TRIM72 mRNA levels were detected in 24 pairs of BC tissues specimens and adjacent normal tissues using RT-qPCR. (b) The RT-qPCR analysis of TRIM72 expression TRIM72 expression in six BC cell lines in contrast to a normal human mammary cell line ($n = 3$). BT549 and MDA-MB-231 cells were transfected with an empty vector plasmid or TRIM72 overexpression plasmid and TRIM72 expression was verified by WB (c) and RT-qPCR (d) ($n = 3$). (e) Modified BT549 and MDA-MB-231 cells were subjected to an MTS assay to examine the effect of TRIM72 overexpression in the proliferation. The cell proliferation was measured after 24 and 48 h of cell seeding ($n = 3$). GAPDH was used as an internal control. *$P < 0.05$, *$P < 0.01$, ***$P < 0.001$. BC, breast cancer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-qPCR, quantitative reverse transcription-PCR; TRIM72, tripartite motif-containing 72; WB, western blot.

Overexpression of TRIM72 inhibits tumor growth in a xenograft model

To replicate the aforementioned antitumor results *in vivo*, we established a xenograft tumor model. The BT549 cells with stable overexpression of TRIM72 or empty vector were injected into nude mice. The average tumor size in the lentivirus-TRIM72-oe group ($n = 5$) was visibly smaller than in the control group (Fig. 3a). The tumor volume was evaluated at different time points (Fig. 3b), and the tumors formed by TRIM72-oe cells were approximately 60% smaller than those formed by control cells on the 20th day after inoculation (Fig. 3c). The H&E staining experiments showed the respective morphology modifications between the two groups (Fig. 3d). Overall, these results suggest that TRIM72 inhibits tumor growth *in vivo*.

TRIM72 expression decreases in hypoxia-treated breast cancer cells

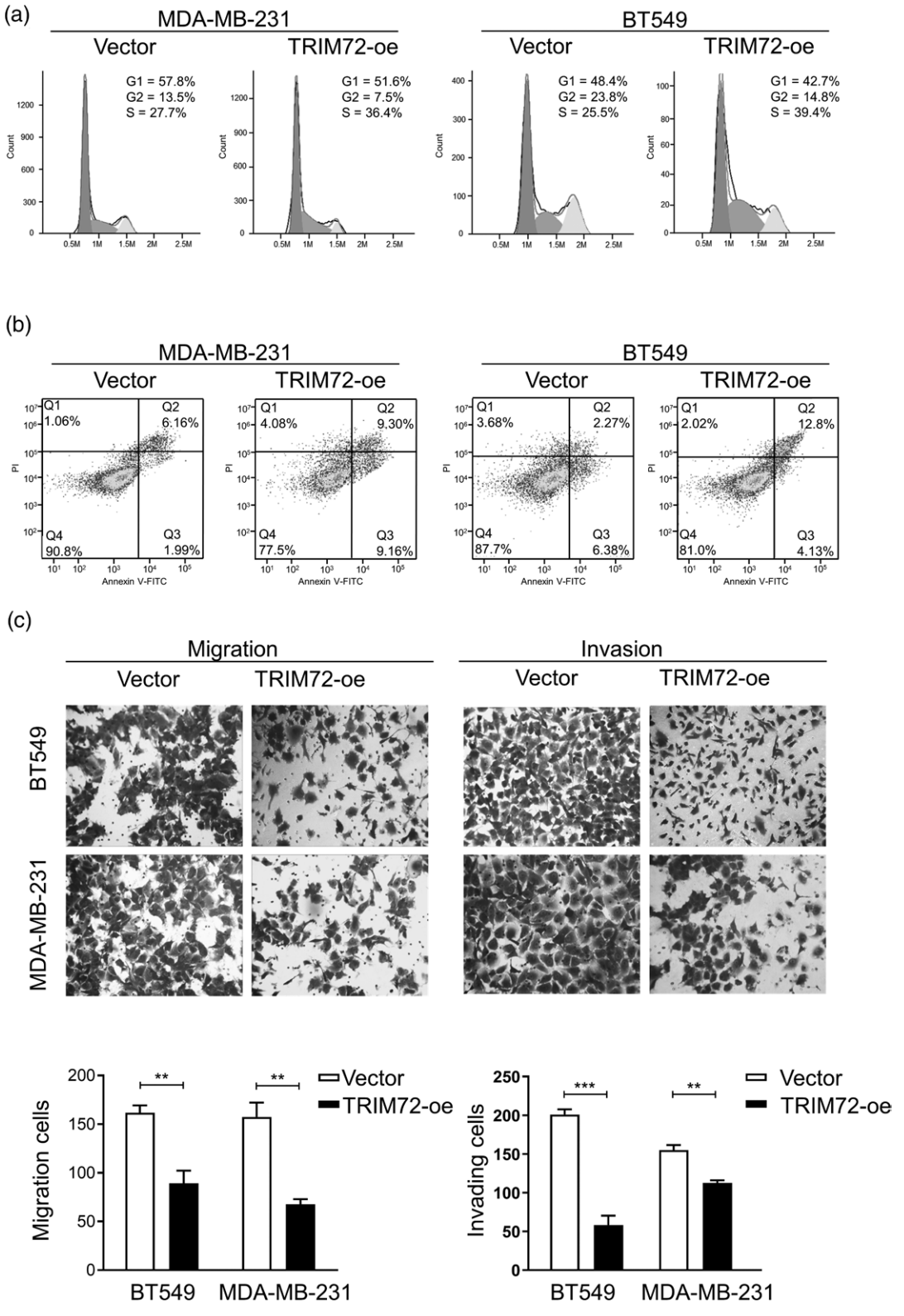
Environmental or metabolic stresses such as hypoxia or nutrient starvation are characteristics of the aggressive TME. To further investigate the relationship between TRIM72 and TME, BC cells were treated with the hypoxia mimetic $CoCl_2$, which largely replicates the hypoxic state. It was found that the expression of TRIM72 was significantly decreased after $CoCl_2$ treatment in BT549 cells (Fig. 4a). These results indicated that hypoxic treatment could decrease endogenous expression of TRIM72 in BC cells.

TRIM72 reverses increased lactate levels observed in the mimic tumor microenvironment and inhibits hypoxia-induced PI3K/Akt/mTOR pathway activation

Considering that TRIM72 could be an effective treatment agent to protect against a range of adverse stresses and could be regulated by oxygen [29,31], we postulated that TRIM72 could modulate TME. To test this hypothesis, we measured the levels of lactate, which is responsible for the acidic TME. When MDA-MB-231 and BT549 cells were treated with $CoCl_2$ for 24 h, a decrease in the intracellular and secreted lactate levels was observed in the TRIM72-oe group (Fig. 4b).

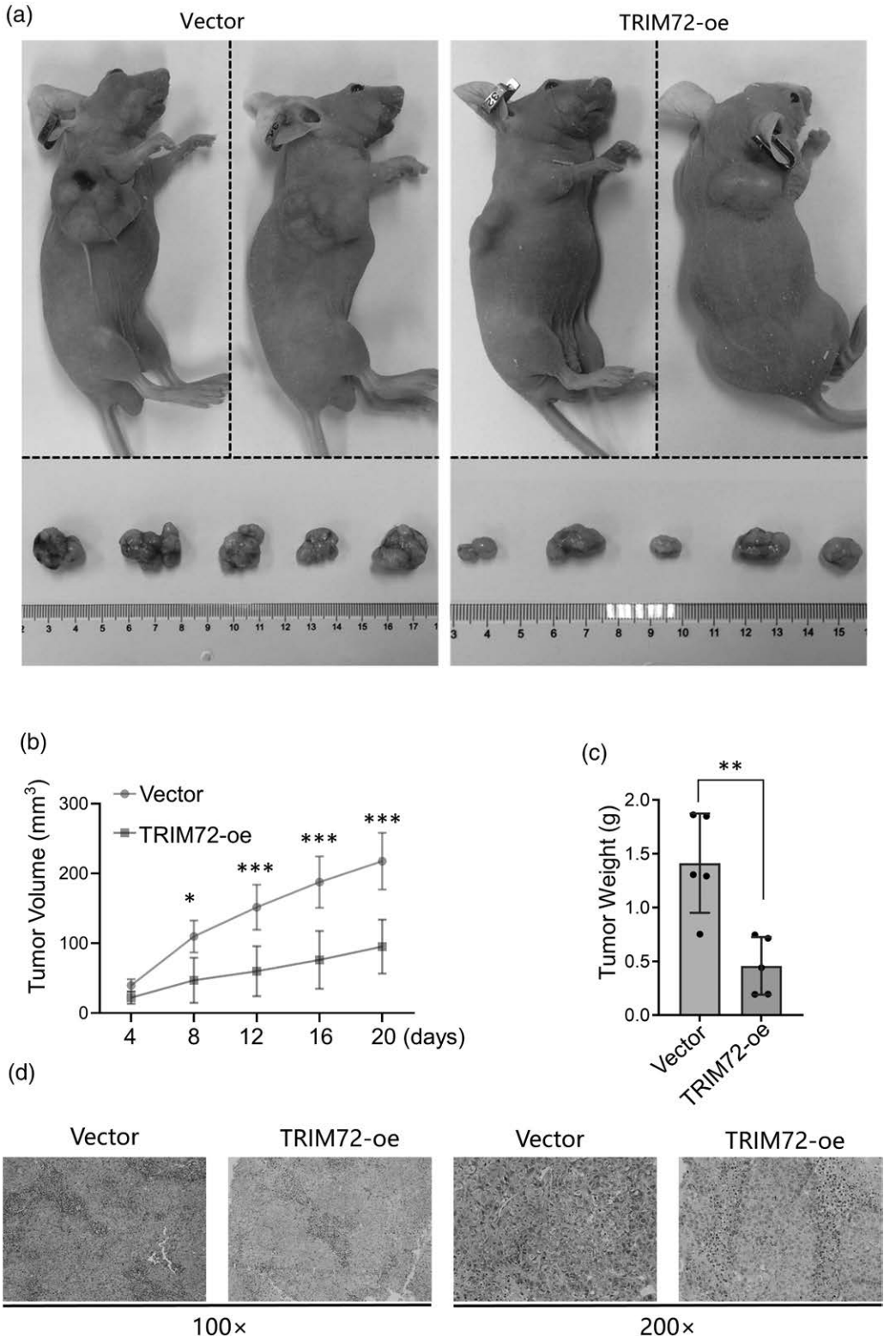
The major isoform of monocarboxylate transporters (MCTs), MCT4, is upregulated by HIF and has been associated with BC aggressiveness and poor prognosis. It is well established that MCTs mediate hypoxia-induced excess lactate efflux to support the acid-resistant phenotype of cancer cells [32]. To evaluate the potential mechanism underlying the effects of TRIM72 on lactate

Fig. 2



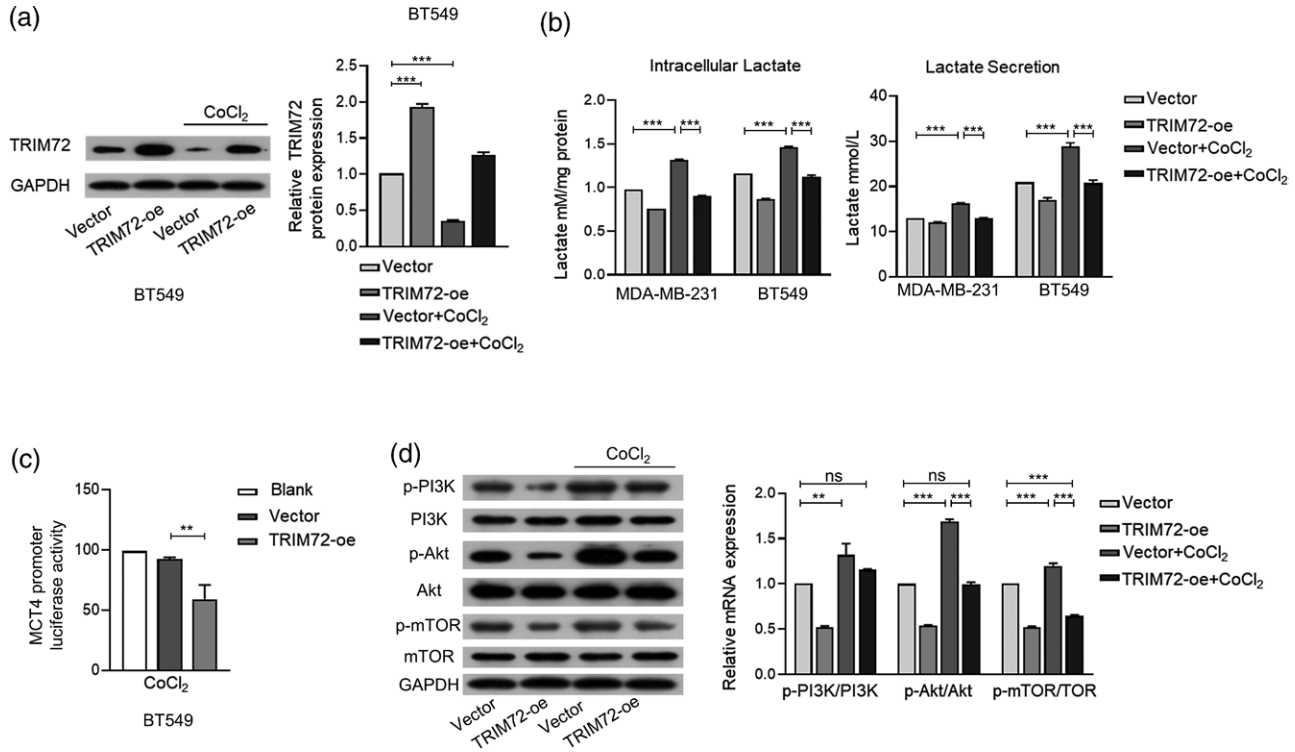
TRIM72 regulates BC cell cycle distribution and inhibits migration and invasion ability. BT549 and MDA-MB-231 were transfected with TRIM72 overexpression plasmid (TRIM72-oe) or empty vector (Vector). Cell cycle assay (a) and cell apoptosis (b) of TRIM72 overexpression and control cells were detected by flow cytometry ($n = 1$). (c) Migration and invasion assay of BC cells ($n = 5$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. BC, breast cancer; TRIM72, tripartite motif containing 72.

Fig. 3



TRIM72 inhibits tumor growth *in vivo*. Lentivirus expressing TRIM72 or empty control stably infected BT549 cells were subcutaneously injected into nude mice. (a) Representative pictures of the xenograft tumors developed after injection of BT549 cells transfected with an empty vector or *TRIM72*-oe-vector ($n = 5$ per group) into BALB/c nude mice. Growth curves (b) and tumor average weight (c) of xenograft tumors ($n = 5$ per group). (d) Representative H&E staining in the Vector control group and the TRIM72-oe group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. H&E, hematoxylin and eosin; TRIM72, tripartite motif-containing 72.

Fig. 4



TRIM72 overexpression attenuates the hypoxia-induced lactate production and PI3K/Akt/mTOR signaling activity. BC cells transfected with TRIM72-oe vector or empty vector were subjected to normoxic (0 μM $CoCl_2$) or hypoxic (125 μM $CoCl_2$) conditions for 24 h. (a) Endogenous TRIM72 expression was downregulated by hypoxic treatment ($CoCl_2$ treated versus control). (b) Impact of *TRIM72* overexpression on lactate production within the cell and secretion in the culture medium of BT549 and MDA-MB-231 cells ($n = 3$). (c) TRIM72 overexpression affects the MCT4 promoter-luciferase activity in BT549 cells under hypoxic conditions, as observed by cell transfection with TRIM72-oe plasmid or an empty vector (as indicated) together with MCT4 promoter-luciferase plasmid. (d) The protein levels of PI3K, Akt, and mTOR and their phosphorylated forms in TRIM72-oe BT549 cells with or without $CoCl_2$ treatment for 24 h. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicates a significant difference among the indicated groups. Akt, protein kinase B; BC, breast cancer; MCT4, monocarboxylate transporter 4; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; TRIM72, tripartite motif-containing 72.

regulation, we monitored the *MCT4* promoter activity in BT549 cells under hypoxic conditions (Fig. 4c). Taken together, these data strongly suggest that TRIM72 restores compromised intracellular pH homeostasis and modulates the acidic TME.

Lactate is also an activator of the PI3K/Akt/mTOR pathway, which is a critical pathway involved in BC glycolytic reprogramming. In addition, lactate-induced reactive oxygen species also activate Akt activity. Consistently, we found that TRIM72 overexpression reversed hypoxia-induced p-PI3K, p-Akt, and p-mTOR upregulation. These results suggest that TRIM72 overexpression could modulate TME and inhibit the PI3K/Akt/mTOR pathway (Fig. 4d).

PPP3CA is a novel candidate of TRIM72-binding protein that regulates lactate production and the PI3K/Akt/mTOR pathway

Recently, in a reference map of the protein interactome, the immune-regulatory PPP3CA was identified to have potential interactions with TRIM72 [33]. PPP3CA, also known as calcineurin A, is a strong candidate as a negative regulator of HIF activity in HeLa cells [34]. Although the role of PPP3CA in hypoxia is well established, whether PPP3CA could regulate the TME remains unknown. However, there are no previous reports on the involvement of these two proteins in cancer progression.

To check this, a Co-IP assay was performed with anti-TRIM72 agarose followed by immunoblotting with PPP3CA antibody. The observed co-precipitation (Fig. 5a, top panel) suggested that TRIM72 interacts with PPP3CA. HIF is a key determinant for hypoxic cancer cellular adaptation to reduce the acidic burden through inducing MCT4 expression under hypoxic conditions [34,35]. As such, we speculated that TRIM72 interaction with PPP3CA might be involved in lactate regulation induced by hypoxic TME. We used siRNA to further assess the role of PPP3CA (Fig. 5a, bottom panel). We observed that PPP3CA knockdown significantly increased lactate dehydrogenase A expression (Fig. 5b) and lactate production (Fig. 5c) in hypoxia treated BT549

cells in the presence or absence of TRIM72 overexpression. Furthermore, we performed a rescue experiment to verify whether the effect of TRIM72 on MCT4 promoter activity requires PPP3CA (Fig. 5d). These results indicate that TRIM72 inhibits MCT4-promoter activity and lactate production via regulation of PPP3CA.

Transfection with a PPP3CA knockdown plasmid antagonized the inhibitory effects of TRIM72 on the PI3K/Akt/mTOR pathway in BT549 cells (Fig. 5e). To this end, we found that additional L-lactate indeed induced a significant increase in phosphorylation of Akt, PI3K, and mTOR in TRIM72-oe cells. These data suggested that lactate has both metabolic and signaling functions. Therefore, the antitumor effect of TRIM72 is at least partly dependent on the PI3K/Akt/mTOR signaling pathway by interaction with PPP3CA (Fig. 6).

Discussion

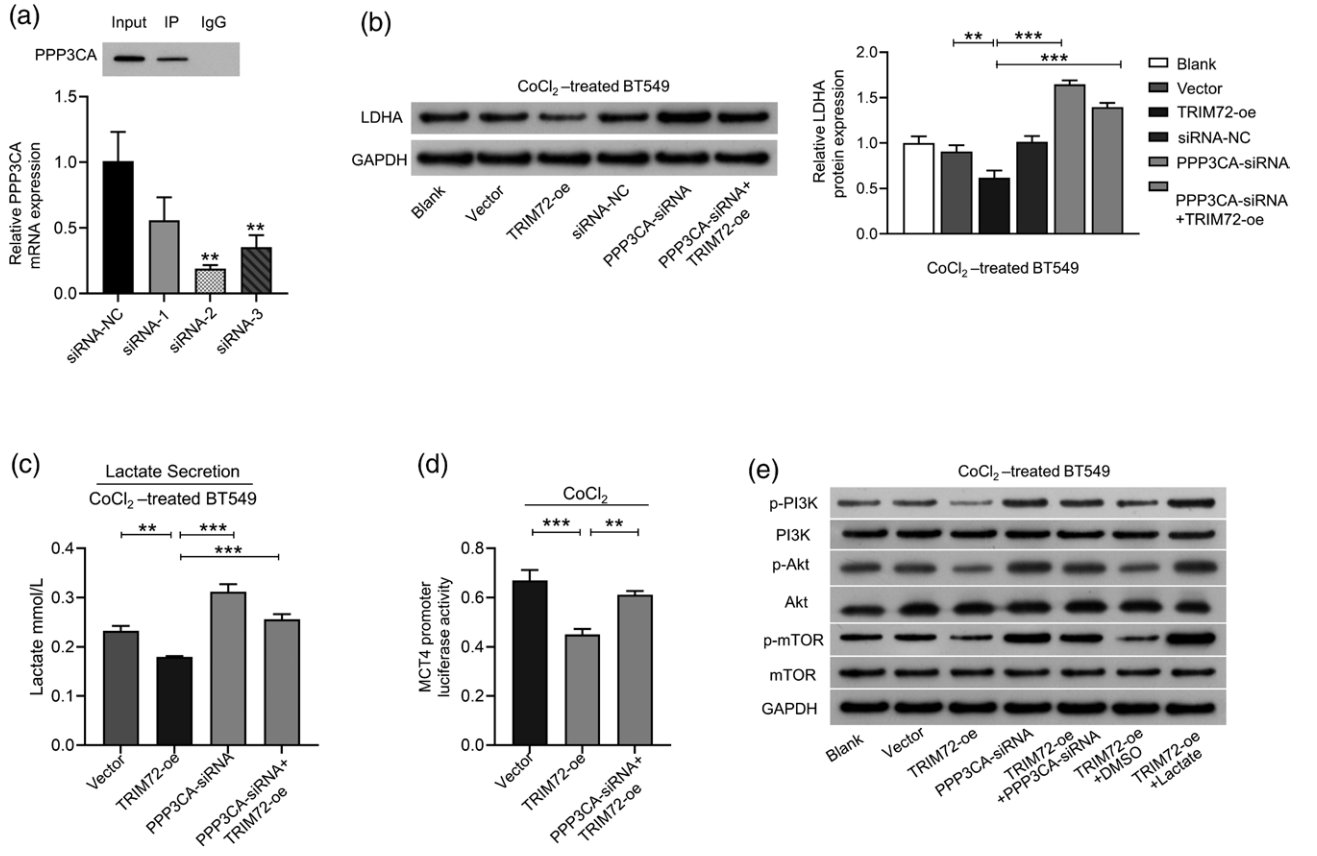
Although emerging evidence has improved our understanding of TRIM72 function and its diagnostic and prognostic value in human cancers, the mechanisms involving TRIM72 and cancer development remain poorly understood. Recent studies have unraveled that reduced TRIM72 expression is implicated in a variety of cancer types and cancer-related processes, including proliferation, invasion, and prognosis [25–28]. Here, we demonstrated that TRIM72 expression was reduced in BC tissues and cell lines; and overexpression of TRIM72 suppressed tumor progression both *in vitro* and *in vivo*. We evaluated the role of TRIM72 in modulating the acidification of TME in BC. Subsequent studies demonstrated that TRIM72 physically interacted with PPP3CA, which can inhibit MCT4-promoter activity and thus suppresses lactate accumulation. Furthermore, the interaction between TRIM72 and PPP3CA could inhibit the PI3K/Akt/mTOR signaling pathway. The results of our study suggested that TRIM72 reduced the acidification of TME and the progression of BC by inhibiting the PI3K/Akt/mTOR axis and MCT4-promoter activity with PPP3CA.

A deteriorating TME accompanies the increased expression of transcription factors to regulate metabolism, proliferation, and inflammation [36]. Recently, treatment with the recombinant human MG53 (TRIM72) could inhibit lung cancer tumor growth by blocking ATO-induced SG formation, suggesting that TRIM72 is a key mediator of cancer cells exposed to adverse environments [28]. Despite the stress-related repressor of TRIM72 having been implicated by previous studies [23,24], the role of TRIM72 in TME-induced pressure and the molecular mechanisms is not well known. Hypoxia was considered a trigger to the glycolytic switch of oxidative phosphorylation to glycolysis. Thus, given that hypoxia and acidification are distinct phenomena that occur in TME, we conducted further experiments using $CoCl_2$ to mimic the hypoxic TME. Lactate is the end-product of glucose consumption; it is a signaling molecule and a key mediator in the metabolic crosstalk between cancer cells and the TME [37]. Here, we showed that the TRIM72 expression level in BC cells subjected to hypoxic conditions was downregulated, whereas TRIM72 overexpression could inhibit lactate accumulation *in vitro*. These results provided evidence that TRIM72 could regulate the phenotypes of BC cells in response to environmental changes. However, more studies are needed to determine the molecular mechanisms involved in TRIM72 expression changes caused by differences in oxygen content. Together, these results reveal that TRIM72 could be regulated by O_2 concentrations and modulate lactate production in TME.

Previously, TRIM72 was reported to negatively regulate the intracellular calcium release, the NF-κB, and the PI3K/Akt pathways [38–40]. Particularly, it has been demonstrated that TRIM72 can directly regulate the Akt pathway in tongue cancer and cardiac disease [27,41]. The PI3K/Akt/mTOR signaling pathway is responsible for aerobic glycolysis and results in lactate accumulation with oncogenic function related to tumor cell adhesion, migration, metabolism, survival, and angiogenesis in TME [42]. In turn, increased lactate leads to PI3K/Akt pathway activation [43]. Therefore, we speculated that TRIM72 could regulate the PI3K/Akt/mTOR signaling pathway in BC. As expected, we observed that the signaling molecules p-PI3K, p-Akt, and p-mTOR were upregulated by hypoxic treatment and downregulated by TRIM72 overexpression. Additionally, p-PI3K, p-Akt, and p-mTOR are major upstream players of HIF expression and are closely associated with the concentration of oxygen in the microenvironment. Hence, the inhibition of PI3K/Akt/mTOR pathway activation induced by TRIM72 may be linked to the TME in BC by negative regulation of HIF-1α and induced immunosuppression TME [44]. Supplementation with additional lactate could stimulate the PI3K/Akt/mTOR signaling pathway, implying positive feedback between the lactate and the PI3K/Akt/mTOR pathway. Both lactate and PI3K/Akt/mTOR pathway inhibition may at least partly explain the antitumor mechanism of TRIM72 overexpression in BC. These results provide evidence that TRIM72 deficiency had a substantial impact on BC progression under adverse conditions.

Regardless of oxygen availability, cancer cells switch metabolically to increased glucose uptake, aerobic glycolysis, and lactate production, reflecting a sort of 'glucose addiction' for intense proliferation [35]. This phenomenon is known as the Warburg effect, which results in a high rate of aerobic glycolysis and rapid production of energy, both of which are beneficial for cancer cells to adapt to the TME. In hypoxia-induced TME, MCT4 is upregulated, which acidifies the extracellular space and

Fig. 5



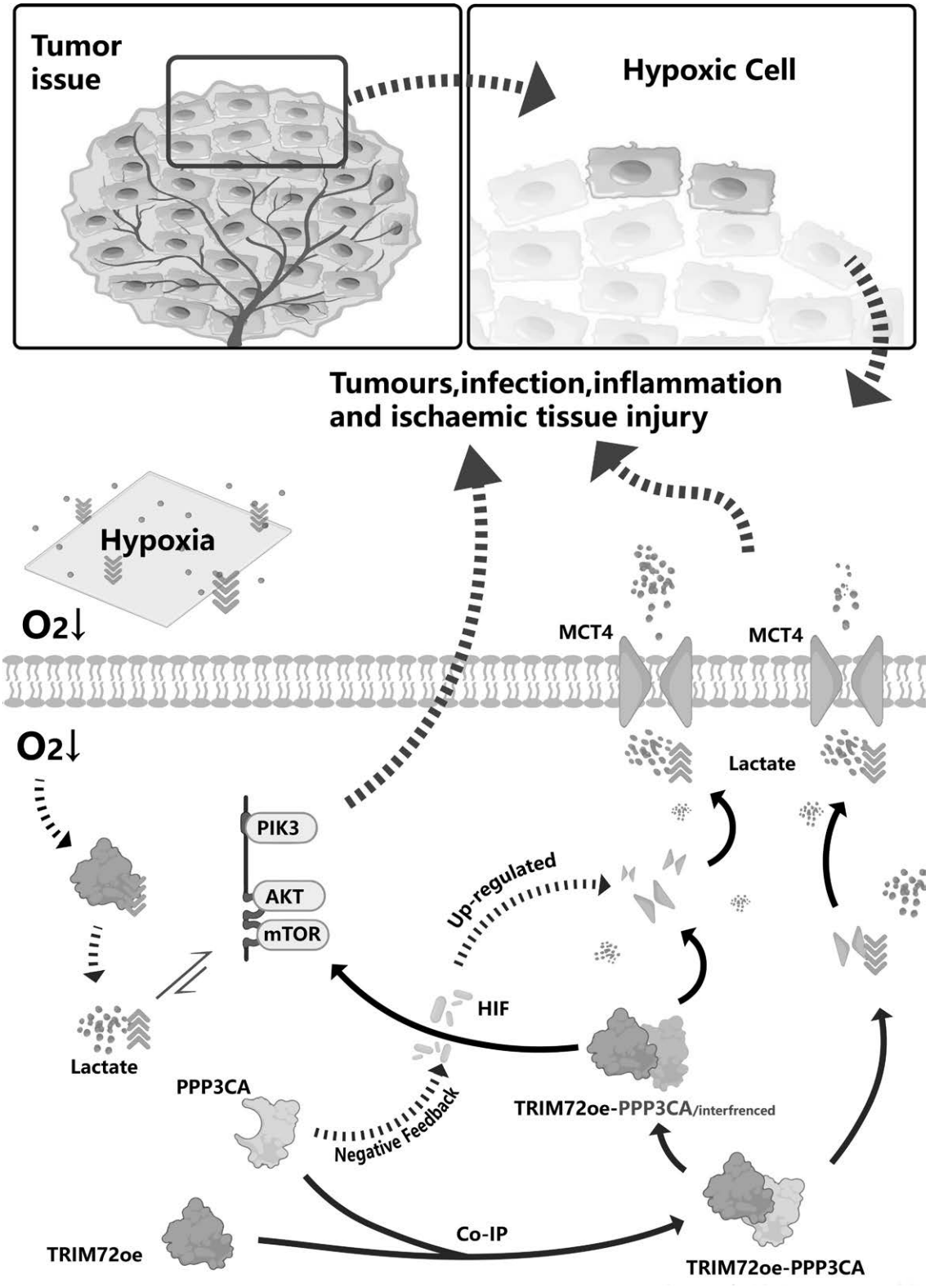
TRIM72 mediates the PI3K/Akt/mTOR pathway and lactate production via interactions with PPP3CA. (a) Top panel showing Co-IP analysis of BT549 cells using a PPP3CA antibody. Lanes from left to right: Input, anti-TRIM72 pull-down, and normal IgG pull-down. Representative panels of $n = 3$ replicates. The bottom panel shows transient PPP3CA knockdown by PPP3CA siRNA (siRNA-NC, siRNA-1, siRNA-2, and siRNA-3) in BT549 cells. The relative mRNA levels of PPP3CA in BT549 cells were determined by RT-qPCR at 24 h after transfection ($n = 3$). (b) The relative protein expression of LDHA in BT549 cells was measured by WB. Cells were transfected either with NC vectors or with TRIM72-oe plasmid, PPP3CA siRNA-2, and cultured with $CoCl_2$ as hypoxia conditioning ($n = 3$). (c) The lactate concentration in the culture medium was measured using ELISA ($n = 3$). (d) The luciferase activities of MCT4 promoter in TRIM72-oe BT549 cells after transfection with PPP3CA siRNA-2 ($n = 3$). (e) Modified BT549 cells were treated with PPP3CA siRNA-2 under hypoxia for 24 h, and the protein levels of PI3K, Akt, and mTOR and their phosphorylated forms were analyzed by western blotting ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicates a significant difference among the indicated groups. Akt, protein kinase B; Co-IP, co-immunoprecipitation; LDHA, lactate dehydrogenase A; MCT4, monocarboxylate transporter 4; mTOR, mammalian target of rapamycin; NC, negative control; PI3K, phosphatidylinositol 3-kinase; PPP3CA, protein phosphatase 3 catalytic subunit alpha; RT-qPCR, quantitative reverse transcription-PCR; TRIM72, tripartite motif-containing 72; WB, western blot.

decreases the intracellular pH by pumping out glycolysis-derived lactate and H^+. This acts as a key player in the maintenance of the Warburg effect [45]. Recent evidence showed that MCT-mediated transport is crucial for the pro-proliferative effect of L-lactate [43]. The congruence between TRIM72 deficiency and MCT4 enabled us to investigate the relationship between these two components. We observed that TRIM72 overexpression could inhibit MCT4 promoter activity and, consequently, lead to lactate suppression.

In addition, calcineurin A, which is coded by PPP3CA, is a signaling enzyme that plays a central role in T-lymphocyte activation. The involvement of PPP3CA in T-cell activity suggests a potential role of PPP3CA in immunomodulation [46]. There is a negative relationship between PPP3CA and lactate. The effects of knockdown of PPP3CA may further influence T-cell proliferation and cytokine production [47]. As mentioned previously, the interaction between PPP3CA and TRIM72 has never been investigated. We first confirmed the direct interaction of TRIM72 and PPP3CA by Co-IP assays. Furthermore, knockdown of PPP3CA markedly attenuated the effects of TRIM72 on MCT4-promoter activity and the PI3K/Akt/mTOR signaling pathway. Based on previous findings and our data, we speculate that the interaction between TRIM72 and PPP3CA could contribute to the progression of BC.

Deciphering the TME is of prime importance for the development of combination therapies against drug resistance and recurrence in BC, and sustaining the hopes for an effective strategy for triple-negative BC [48]. In our study,

Fig. 6



Schematic illustrating the regulatory role of TRIM72 in TME of breast cancer. TME, tumor microenvironment; TRIM72, tripartite motif-containing 72.

we found that TRIM72 deficiency disturbed TME in BC cells by the PI3K/Akt/mTOR signaling pathway. These results indicate the possible use of TRIM72 as an option to control tumor progression. Indeed, the human recombinant TRIM72 protein has been used in animal models to treat various acute injuries [49]. The potential utility of TRIM72 as a therapeutic strategy or a clinically relevant adjuvant for BC treatments is promising and warrants further study. While our study identifies a novel role of TRIM72 in TME, there still are questions that remain to be addressed. For instance, our experiments have not determined the origin of the TRIM72 loss during cancer initiation or progression. This study also did not connect the PI3K/Akt/mTOR signaling pathway with BC cell activities. These issues deserve further exploration in future studies.

Conclusion

Our findings revealed that TRIM72 is downregulated in BC cell lines and tissues, thereby exhibiting an anti-proliferative role by inducing cell cycle arrest at the S phase and apoptosis. Furthermore, we found that decreased TRIM72 expression under hypoxic conditions could induce the production of lactate and may contribute to a tumor-promoting microenvironment, which further activates the PI3K/Akt/mTOR signaling pathways, ultimately leading to tumor progression. Notably, this study demonstrated that TRIM72 regulates lactate production by affecting MCT4 activity via PPP3CA interactions. Therefore, our findings provide novel insights into the anti-oncogenic role of TRIM72 in BC.

Acknowledgements

We thank Professor Cai for his expert advice regarding study design. We also thank Yongqiang Hu for his excellent schematic and LetPub (www.letpub.com) for linguistic assistance and presubmission review.

This work was supported by the National Natural Science Foundation of China (Y.Q., No. 81871712; H.L., No. 82002218), the Natural Science Foundation of Guangdong Province (H.L., No. 2019A1515010103), the President Foundation of Nanfang Hospital, Southern Medical University (H.L., No. 2018Z008), and the Research and Development Projects in Key Areas of Guangdong Province (K.L., No. 2020B0404010002).

Conceptualization: Z.W., H.L., Q.Z., Y.Q., and K.L. Methodology: H.X.W and Q.Z. Software: H.L. Investigation: H.F.W., H.L., and Z.W. Writing – original draft preparation: Z.W. Writing – review and editing: Y.Q. Visualization: X.L. and H.L. Supervision: K.L. and Y.Q. Project administration: Y.Q. All authors have read and agreed to the published version of the manuscript.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University. Animal studies were approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University and followed the Guideline for Animal Experiments of the National Cancer Center Research Institute.

Consent to participate: All participants provided their written informed consent.

Conflicts of interest

There are no conflicts of interest.

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