

Original Article

Antiproliferative and antiangiogenic effects of metformin on multidrug-resistant MCF-7 cells

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Abstract: Epidemiological analyses have demonstrated that there is a positive correlation between increased risk of malignant neoplasms and long-term diabetes. Human studies have showed that metformin might have activity against several types of neoplastic diseases including breast cancer. Therefore, we investigated the mechanisms underlying the anti-cancer effects of metformin. The effect of metformin treatment on the growth of multi-drug resistant breast cancer cell *in vitro* was evaluated first. The effect of metformin treatment on tumor growth and tumor angiogenesis *in vivo* was further investigated using Xenograft breast tumor models, which were established by injecting MCF-7/ADR' cells into 6-week-old female BALB/c nude mice. We found that 1 or 2 μ M, but not 0.1 or 0.5 μ M, metformin inhibited MCF-7/ADR' cells proliferation *in vitro*. Furthermore, systemic injections of metformin dose-dependently inhibit tumor growth *in vivo*, which was associated with increased tumor necrosis and decreased tumor angiogenesis. Finally, systemic injections of 120 mg/kg metformin increased caveolin-1, but not caveolin-2 expression in tumor cells. Taken together, these results suggested that metformin likely inhibits the proliferation of MCF-7/ADR' cells, and suppress the tumor angiogenesis via promoting the expression of caveolin-1, which has been indicated in the process of tumor angiogenesis.

Keywords: Caveolar, metformin, breast cancer, tumor angiogenesis

Introduction

Epidemiological analyses have demonstrated that there is a positive correlation between increased risk of malignant neoplasms and long-term diabetes [1]. In particular, patients with preexisting type II diabetes exhibit a higher risk of cancer development and cancer-related mortality, as compared to non-diabetic cancer patients. Given such a correlation between type II diabetes and cancer, human studies have revealed that metformin, a commonly used medicine for the pharmacotherapy of type II diabetes, might have activity against several types of neoplastic diseases including breast cancer [2-4]. However, the biological mechanisms underlying the anti-cancer effects of metformin have not been well investigated.

Metformin is one of the most efficacious and safe anti-diabetic medications for type II diabetes. The benefits of metformin treatment for breast cancer patients were first reported in 2005 [5]. Ever since then, an increasing num-

ber of studies have been conducted to evaluate the anticancer effects of metformin. Among various cancers, breast cancer is highly heterogeneous. It has been well known that different breast cancer subtypes have distinct molecular profiles and variable responses to different anti-cancer treatments [6-8]. Based on the differential expression of various genes, breast cancer has been categorized into five major distinct molecular subtypes with prognostic significance: luminal A; luminal B; overexpression of HER2, also known as ErbB2; breast-like; and basal-like/triple negative [6]. Triple negative breast cancers have been further classified into six distinct subtypes: immunomodulatory, mesenchymal, mesenchymal stem-like, luminal androgen receptor, basal-like 1, and basal-like 2 [9]. In addition, there are at least seventeen rare subtypes [10]. Response to therapy is dependent on the pathology and classification of the breast tumor. The most predominant subtype, luminal A, is known to have the best prognosis with HER2 and the basal-like triple nega-

tive subtype has the worst outcome [11]. Nevertheless, many breast cancers recur and acquire resistance to conventional treatments. Strikingly, various studies have shown that metformin can inhibit breast cancer subtypes [12, 13]. These results suggested that metformin may provide anti-cancer effects through common biological mechanisms regardless of the distinct molecular profiles of the subtypes of breast cancer.

Indeed, anti-proliferative effects of metformin have been reported in multiple tumor cell lines via multiple molecular pathways, including the adenosine monophosphate kinase (AMPK) pathway, the insulin receptor cascade, and the AMPK-independent RagGTPase-dependent 3mTORC1 signaling network [1, 14]. Additionally, metformin has been reported to play an anti-inflammatory role in suppressing cancer progression by inhibiting cancer stem cells [15]. In contrast, other studies have shown no association between metformin treatment and cancer-related mortality [16]. In fact, studies using epidemiological analysis recently showed that there is no direct association between metformin treatment and cancer outcome [17]. These controversies regarding the use of metformin as potential anticancer treatment may be due to the complex pharmacological mechanisms of metformin. In fact, beside the anti-proliferative effects, previous studies have also demonstrated that metformin can inhibit the formation of capillary-like networks by human umbilical vein endothelial cells (HUVEC), and decrease microvessel density in tumor-free mice [18]. Moreover, previous studies have shown that metformin targeted *in vitro* and *in vivo* breast cancer cells, resulting in profound effects on breast cancer angiogenesis and local and metastatic cancer cell growth that are likely due to additive effects on both tumor and microenvironment cells [19]. However, the mechanisms underlying the effects of metformin treatment on breast cancer angiogenesis have not been well elucidated.

Therefore, in the present study, we examined the effects of metformin treatment on the growth of multi-drug resistant breast cancer cell *in vitro*, and on tumor growth and tumor angiogenesis *in vivo*.

Materials and methods

Cell culture and viability assay

The human breast carcinoma cell line MCF-7 was supplied by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin (50 IU/mL and 50 µg/mL, respectively) at 37°C in an atmosphere of 5% CO₂. Given the well-known issue of the original MCF-7/ADR cell line, a new adriamycin (ADR)-resistant cell line MCF-7/ADR' was generated using the same procedure as described previously [20]. Cells were seeded at a density of ~5000 cells per well in 96-well plates and maintained at 37°C under standard culturing conditions. Cells were exposed to a series of concentrations of metformin (Sigma-Aldrich, Beijing, China) continuously and cell viability was determined at the end of 24 h or 72 h using a cell counting kit-8 (CCK-8; Dojindo, Japan).

In vivo assessment

Xenograft breast tumor models were established by injecting MCF-7/ADR' cells into 6-week-old female BALB/c nude mice (Charles River Laboratories, Shanghai, China). Once the tumor size reached ~100-150 mm³, mice were randomly assigned to either control group or metformin-treated group. Intraperitoneal injection of metformin (60 or 120 mg/kg body weight) or sterile phosphate buffered saline (PBS; 1 ml/kg) was administered for 14 consecutive days. Body weight was monitored and tumor volumes were measured and corrected.

Histology

Two weeks after metformin treatment, tumors were dissected and fixed in 4% paraformaldehyde before being paraffin embedded. Consecutive sections (thickness, 5 µm) were sectioned onto microscope slides. Haematoxylin and eosin (H&E) staining was used to assess tumor morphology. Immunofluorescent staining using an antibody against von Willebrand factor (vWF; 1:200 dilution; Abcam, Shanghai, China) was also conducted to evaluate the formation of capillary. The staining was further analyzed with a fluorescent microscope (Leica, Germany)

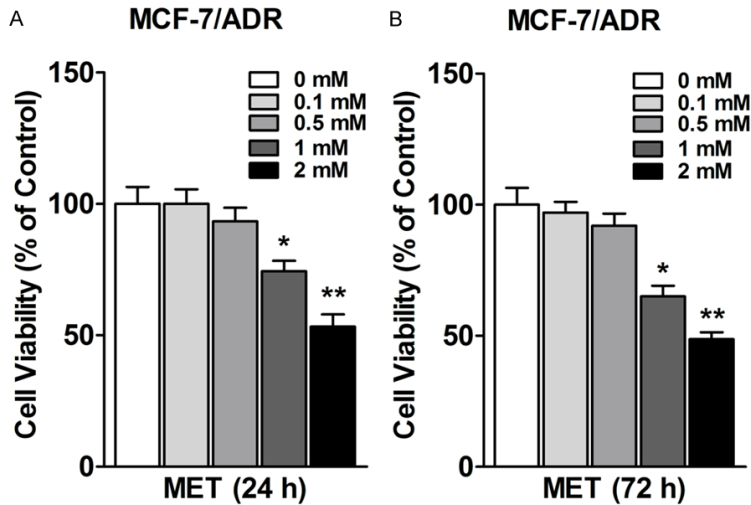


Figure 1. Effects of metformin treatment on the proliferation of MCF-7/ADR cells *in vitro*. After (A) 24 h or (B) 72 h treatment, the cell proliferation assay was conducted using CCK-8 kit. Cell numbers were evaluated by measuring the absorbance at 450 nm. Five replicates for each treatment were conducted on the plate. Asterisks ($p < 0.05$) represent the ANOVA simple main effect, as compared to control.

and the fluorescent intensity was then quantified using NIH Image J software based on published protocol [21].

Western blotting

The expression of Caveolin-1 (21-24 kDa) and Caveolin-2 were determined by Western blot following metformin treatment for 14 days. Tumor tissues were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 mM EDTA) containing a EDTA-free protease inhibitor cocktail (Abcam, Shanghai, China), 1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitors (5 mM sodium orthovanadate). Each sample was then added into 20 μ l 2 \times sample loading buffer (0.125 M of 5 M Tris-HCl, amresco; 20% glycerol, usb; 4% of 10% sodium dodecyl sulfate, amresco; 1% β -mercaptoethanol, amresco; 0.2% of 0.05% (w/v) bromophenol blue, sigma). The samples were boiled for 5 min before loading. 10% running gel (25% of 40% acrylamide stock, Beyotime; 0.375 M of 1.5 M Tris-HCl, pH 8.8; 1% of 10% sodium dodecyl sulfate; 1% of 10% ammonium persulfate; 0.1% Tetramethylethylenediamine) was utilized. The gel was transferred to a same size Nitrocellulose transfer membrane (Thermo Scientific, Waltham, MA, USA) within transfer buffer (25 mM Tris

base, 192 mM glycine, 0.037% sodium dodecyl sulfate, and 20% methanol) under 45 V for 40 min, and probed with the first antibody against caveolin-1 (ab2910; Abcam, Shanghai, China) or caveolin-2 (ab-97476; Abcam, Shanghai, China) with a 1/1000 dilution in blocking buffer (50 mM Tris base; 100 mM NaCl; 0.02% Tween 20; and 3% BSA) overnight. The membrane was washed by TTBS (0.1% Tween 20, 10 mM Tris base, 100 mM NaCl, pH 7.5) for three times before adding secondary antibody (ab6721, Abcam, Shanghai, China) with 1/5000 dilution in blocking buffer for 2 hours. Background color was reduced carefully by washing with TTBS. The results were visualized using ECL kit (Abcam, Shanghai, China), and protein levels were normalized to GAPDH and quantified using NIH Image J software.

Statistical analysis

All the data were presented as means \pm standard error of means (SEM). The data were analyzed using either t-test, one-way ANOVA, or multi-factor ANOVA followed by Tukey's *post hoc* test with GraphPad PRISM software package (GraphPad Software, Inc.), wherever appropriate. Data were determined to be statistically different when $p < 0.05$.

Results

Effects of metformin on MCF-7/ADR' cell growth *in vitro*

The effects of metformin on MCF-7/ADR' cell viability were assessed using MTT assay. After incubation with 0.1 mM or 0.5 mM metformin for 24 hours, metformin did not alter MCF-7/ADR' cell viability as compared with control. However, 1 mM or 2 mM metformin decreased MCF-7/ADR' cell viability as compared with control (**Figure 1A**). Furthermore, 0.1 mM or 0.5 mM metformin did not alter MCF-7/ADR' cell viability after 72 hours, as compared with

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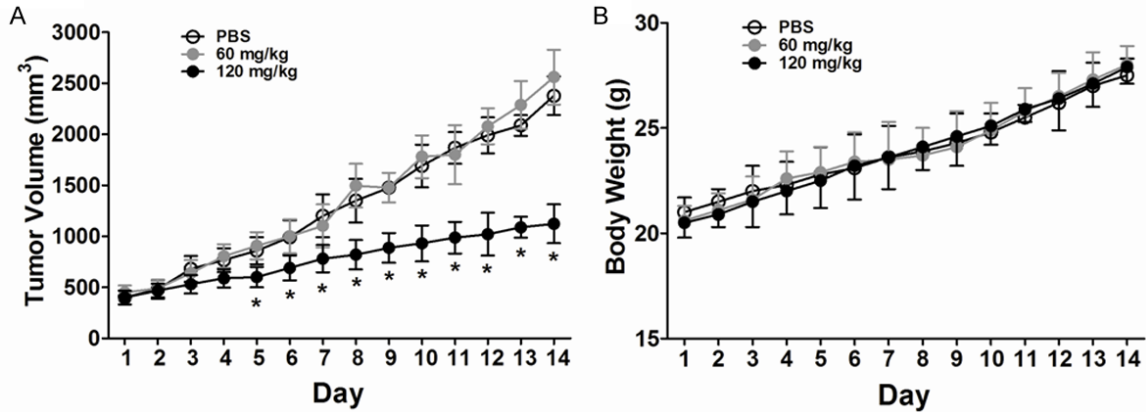


Figure 2. Effects of metformin treatment on tumor growth *in vivo*. Sterile PBS (1 ml/kg) or Metformin (60 or 120 mg/kg) was injected intraperitoneally to mice with breast carcinoma for 14 consecutive days. (A) Average tumor size and (B) body weight were monitored and plotted against time. Data were presented as means \pm SEM. Asterisks ($p < 0.05$) represent the ANOVA simple main effect, as compared to PBS control.

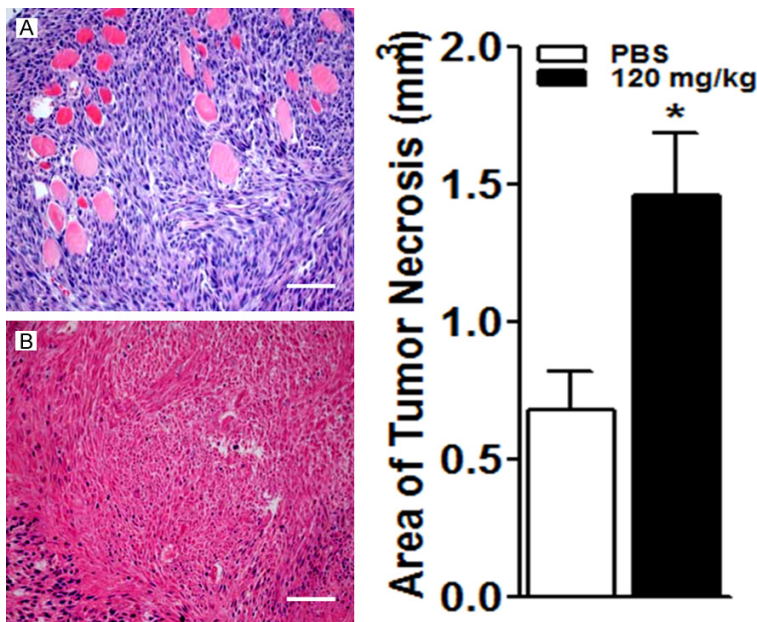


Figure 3. Effects of metformin treatment on tumor necrosis. Representative Haematoxylin and eosin (H&E) staining images of tumor morphology was obtained from (A) Control groups and (B) Metformin (120 mg/kg). Scale bar: 100 μ m. Area of tumor necrosis was quantified. Data are presented as means \pm SEM. Asterisk ($p < 0.05$) represents significant difference, as compared to PBS control.

control (Figure 1B). However, after incubation with 1 mM or 2 mM metformin for 72 hours, metformin decreased MCF-7/ADR' cell viability as compared with control (Figure 1B).

Effect of metformin on tumor growth *in vivo* and tumor necrosis

Based on the *in vitro* cytotoxicity assay, which demonstrated that metformin can inhibit breast

cancer cell growth *in vitro*, we further evaluated the effects of metformin on tumor growth *in vivo*. We first established human xenograft breast tumor mouse models and then treated animals with intraperitoneal injections of either PBS or metformin once daily for 14 consecutive days. We found that 60 mg/kg metformin treatment did not alter tumor volume as compared with PBS treatment. However, 120 mg/kg metformin treatment decreased the growth of tumor volume over time as compared with PBS treatment (All main and interaction effects, $F_{(2,16)} = 12.19-24.56$, $p = 0.001-0.0001$; Figure 2A). In addition, neither 60 mg/kg or 120 mg/kg metformin treatment altered animal body weight over 14 days as compared with PBS treatment (Figure 2B). Subsequent histological analy-

ses revealed that 120 mg/kg metformin treatment significantly increased tumor necrosis in mice ($t_{(14)} = 9.21$, $p < 0.01$; Figure 3).

Effect of metformin on tumor angiogenesis

To examine the effects of metformin treatment on tumor angiogenesis, immunofluorescence staining of von Willebrand factor (vWF), a microvascular endothelial marker, was conduct-

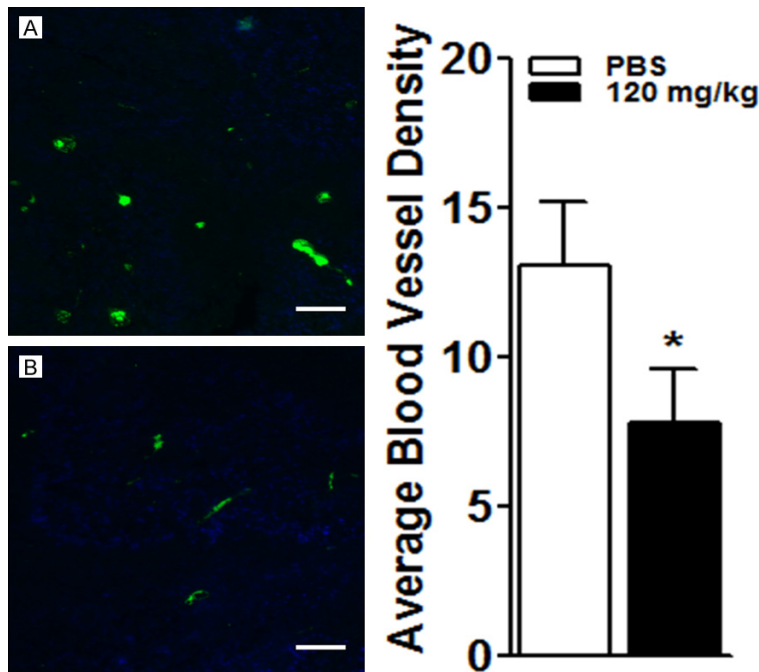


Figure 4. Effects of metformin treatment on tumor angiogenesis. Representative Immunofluorescent staining images of vWF (green) in tumors was obtained from (A) Control groups and (B) Metformin (120 mg/kg). Nuclei were counter-stained with DAPI (blue). Scale bar: 100 μ m. Fluorescent intensity was quantified using ImageJ software. Asterisk ($p < 0.05$) represents significant difference, as compared to PBS control.

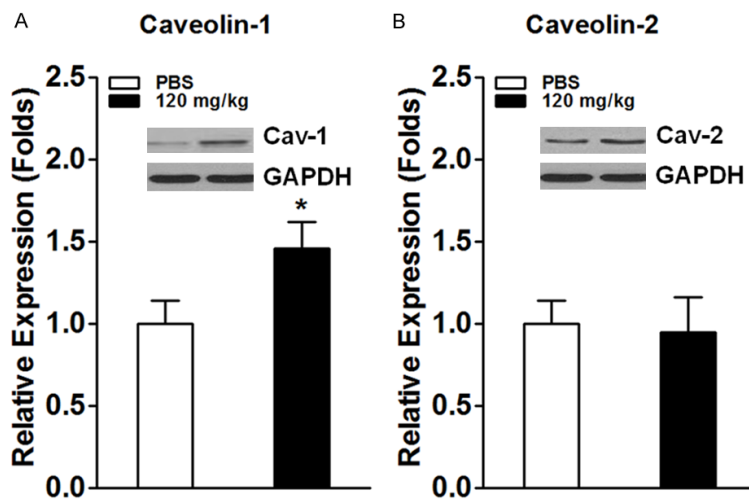


Figure 5. Effects of metformin treatment on the protein expression of caveolin-1 and caveolin-2. After the treatment of (A) PBS (1 ml/kg) and (B) Metformin (120 mg/kg), the expression of caveolin-1 and caveolin-2 in tumors was measured using Western blot. The relative expression of caveolin-1 and caveolin-2 was shown as folds of GAPDH (i.e., loading control). Asterisk ($p < 0.05$) represents significant difference, as compared to PBS control.

ed. We found that 120 mg/kg metformin treatment reduced average blood vessel density in tumors in animals, as compared with PBS treatment ($t_{(14)}=10.04$, $p < 0.01$; **Figure 4**).

Effect of metformin on the expression of caveolin-1 and caveolin-2 in tumor

It has been shown that Caveolar constituents play a critical role in angiogenesis. Thus, we further investigated that the effects of metformin treatment on the expression of caveolin-1 and caveolin-2 in tumors. We found that 120 mg/kg metformin treatment significantly enhanced the expression of caveolin-1 ($t_{(14)}=11.17$, $p < 0.01$; **Figure 5A**), but not caveolin-2 in tumors (**Figure 5B**).

Discussion

The present study was designed to examine the putative effects of metformin on the breast cancer angiogenesis. We have demonstrated that 1 or 2 μ M, but not 0.1 or 0.5 μ M, metformin inhibited MCF-7/ADR' cells proliferation *in vitro*. Furthermore, systemic injections of metformin dose-dependently inhibited tumor growth *in vivo*. Such an effect was associated with increased tumor necrosis and decreased tumor angiogenesis after repeated systemic injections of 120 mg/kg metformin. Finally, we found that systemic injections of 120 mg/kg metformin increased caveolin-1, but not caveolin-2 expression in tumor cells. Taken together, our results suggested that metformin likely inhibits the proliferation of multi-drug resistant breast cancer cells, and suppresses the tumor angiogenesis via promoting the expression of caveolin-1, which has been indicated in the process of tumor angiogenesis.

While the present study has shown that systemic injections of metformin inhibited tumor growth in the human MCF-7/ADR' multidrug-resistant breast tumor xenograft model, previ-

ous studies have shown no effects of metformin on tumor growth in human MCF-7 breast tumor xenograft model [22]. Specifically, this study showed no detectable tumor reduction after local injection of metformin (20 mg/kg body weight) for 15 days. Besides the difference in the route of metformin injections, our study had a significantly higher dose of metformin used in the treatment. While the anti-tumor growth effects metformin may depend on the dose, it is also likely that the mechanisms underlying the anti-tumor growth effects of metformin in MCF-7/ADR' and MCF-7 cell lines may involve different signaling pathways. In fact, the effect of metformin against breast cancer has been extensively studied [23]. Most of the previous studies have reported that metformin treatment can decrease the incidents and severity of mammary cancer in rodent models after long-term oral or intravenous administration of metformin. Similarly, high dose of metformin treatment has been shown to attenuate tumor progression in humans [23]. However, a few studies have shown that metformin has no anti-tumor growth effects. Such a discrepancy may be due to the lower dose of metformin used in the experiment. Besides the anti-tumor growth effects of metformin, the present study has also demonstrated that long-term treatment of metformin could induce tumor necrosis. Therefore, the present study suggested that metformin has not only short-term anticancer ability *per se*, but can induce long-term effects on tumor physiology.

Additionally, we have shown that systemic injections of metformin could attenuate the formation of capillary in MCF-7/ADR' multidrug-resistant breast tumor xenograft model. Our results were consistent with a previous study, which has shown an AMPK/mTOR-dependent anti-angiogenic effect of metformin on ovarian cancer [24]. In fact, a few studies have already shown the anti-angiogenic effects of metformin on non-tumor cells and tissues, suggesting common signaling pathways might be involved in the anti-angiogenic effects of metformin. However, given the multiple pharmacological mechanisms of metformin, the exact mechanisms underlying the anti-angiogenic effects of metformin on cancers are still needed to be elucidated.

Importantly, the present study has found that systemic metformin treatment can increase

the expression of caveolin-1, but not caveolin-2, in tumor tissues in MCF-7/ADR' multidrug-resistant breast tumor xenograft model. Caveolins are key components of detergent resistant cholesterol lipid rich membranes including lipid rafts and caveolae. While caveolin-3 is expressed exclusively in muscles, caveolin-1 and caveolin-2 are ubiquitously expressed and interact with each other [25, 26]. Notably, the amino acid sequence between caveolin-1 and caveolin-2 is only 38% identical [27], indicating distinct functional roles [28]. However, in contrast to well studied caveolin-1, much less is known about caveolin-2, although most recent studies suggest that caveolin-2 could be involved in regulating angiogenesis [28, 29]. Adding to the literature, the present study suggested that the expression of caveolin-1 is critical for angiogenesis in breast tumor tissues.

While the present study has demonstrated that metformin treatment can promote the expression of caveolin-1, the exact role of caveolin-1 in angiogenesis is still not clear. In fact, using either Matrigel or tumor models, it has been shown that caveolin-1 may have both pro-angiogenic and anti-angiogenic effects. Specifically, in a Matrigel model, overexpression of caveolin-1 can enhance endothelial tube formation, implicating a proangiogenic potential of caveolin-1 [30]. Additionally, it has been showed that B16-F10 tumors implanted in Cav-1 KO had impaired angiogenesis, leading to smaller tumors [31]. In contrast to these studies, it has been reported that caveolin-1 overexpression in endothelial cells decreased VEGF-induced migration and prevented endothelial tube formation in Matrigel [32]. Furthermore, cationic lipid-based transfection of caveolin-1 increased caveolin-1 expression in the tumor vasculature and resulted in decreased tumor growth [32]. The apparent discrepancy between the different studies may arise from the different tumor models. Matrigel models do not mimic a tumor microenvironment where tumor secreted cytokines and growth factors as well as the host inflammatory responses during tumor invasion may influence the angiogenic profile of these tumors. Furthermore, Matrigel models do not take into account permeability aspects of angiogenesis and cavtratin, an antipermeability peptide, which is capable of attenuating tumor permeability, angiogenesis, and growth, but did not influence angiogenesis in Matrigel plugs [33].

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In summary, besides that short-term benefit of metformin in suppressing tumor growth *in vivo*, the present study has indicated that metformin may play a preventive role against breast cancer, which is at least partially attributed to the attenuation of tumor angiogenesis. Such an effect of metformin on tumor angiogenesis likely involves the expression of caveolin-1. While the present study did not explore the role of caveolin-1 in the process of angiogenesis in MCF-7/ADR' multidrug-resistant breast tumor xenograft model, future studies might be necessary. Such a line of research may not only help understand the tumor physiology, but shed more light on the development of novel and effective pharmacological therapies to treat breast cancer.

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Disclosure of conflict of interest

None.

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