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Effect of resveratrol on the metastasis of 4T1 mouse breast cancer cells *in vitro* and *in vivo*

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Abstract

We investigated the effects of resveratrol on metastasis in *in vitro* and *in vivo* systems. 4T1 cells were cultured in the presence of various concentrations (0-30 μmol/L) of resveratrol. For experimental metastasis, BALB/c mice were injected intravenously with 4T1 cells in the tail vein, and were orally administered various concentrations (0, 100, or 200 mg/kg Body weight) of resveratrol for 21 days. After resveratrol treatment, cell adhesion, wound migration, invasion, and MMP-9 activity were significantly decreased in a dose-dependent manner in 4T1 cells ($P < 0.05$). The numbers of pulmonary nodules were significantly decreased in mice fed the resveratrol ($P < 0.05$). The plasma MMP-9 activity was decreased in response to treatment with resveratrol in mice ($P < 0.05$). We conclude that resveratrol inhibits cancer metastasis both *in vitro* and *in vivo*, and this inhibition is likely due to the decrease in MMP-9 activity caused by resveratrol.

Key Words: Resveratrol, metastasis, matrix metalloproteinase-9, 4T1 breast cancer cell, BALB/c mice

Introduction

Resveratrol (trans-3,4',5-trihydroxystilbene) is a naturally occurring polyphenolic phytoalexin that is present in abundance in many fruits, such as grapes, as well as in wine [1]. Resveratrol intake has been reported to have anti-inflammatory and anti-atherosclerosis function and to modulate hepatic alipoprotein and lipid synthesis, platelet aggregation, and production of antiatherogenic eicosanoids by human platelets and neutrophils [2-7]. Therfore, red wine polyphenol resveratrol is believed to be responsible for the well known phenomenon of the French Paradox [8-9]. In addition, resveratrol has recently been found to exhibit anti-cancer properties. Specifically, resveratrol has been found to inhibit the proliferation of a wide variety of tumor cells including breast, lung, colon, liver, pancreas, skin, and prostate cancer cells [10,11]. However, the precise mechanisms by which the anticarcinogenic effects of resveratrol occur remain largely unknown.

Cancer metastasis consists of a complex cascade of events, which ultimately allow for tumor cell escape and seeding of ectopic environments [12]. For breast cancer cells to manifest their malignant potential, they must develop the ability to break through and dissolve extracellular matrix (ECM), particularly the delimiting basement membrane (BM). The degradation of the pericellular BM and ECM is catalyzed by the concerted action of several classes of ECM-degrading enzymes. One important class of ECM-degrading enzymes is the matrix metalloproteinases (MMPs) [13]. MMPs have been implicated as possible mediators of invasion and metastasis in some cancers. Among the human MMPs, gelatinase-B (MMP-9) is the key enzyme that degrades type IV collagen [14]. MMP-9 is overexpressed in invasive tumor and thus it may play an important role in cancer invasion through its enzymatic degradation of the extracellular matrix [15,16].

The purpose of this study is evaluating the effects of resveratrol on the metastasis of the 4T1 mouse breast cancer cell line derived from a spontaneously arising BALB/c mammary tumor, closely resembles breast cancer in humans [17], both *in vitro* and *in vivo*.

Materials and Methods

Chemicals and reagents

Resveratrol was purchased from Sigma (Sigma R5010, St Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO, Sigma D2650) and then diluted in cell culture medium. The mouse breast cancer cell line, 4T1, was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The 4T1 mouse mammary tumor cell line is one of breast cancer models with the capacity to metastasize efficiently to sites

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affected in human breast cancer [17]. The following reagents and chemicals were also utilized in this study: Dulbecco's modified Eagle's medium/Ham's F12 Nutrient Mixture (DMEM/F12), streptomycin, and penicillin (Gibco/BRL, Grand Island, NY, USA); and RIA-grade bovine serum albumin and transferrin (Sigma). In addition, antibodies for MMPs were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA).

Cell culture

4T1 cells were maintained in DMEM/F12 medium containing 100 mL/L of fetal bovine serum (FBS), 100,000 U/L of penicillin and 100 mg/L of streptomycin. To examine the effects of resveratrol, 4T1 cells were plated with DMEM/F12 medium containing 10% FBS. Before the cells were treated with resveratrol, the cell monolayers were rinsed and then serum starved for 24 h in DMEM/F12 medium supplemented with 5 mg/L of transferrin, 0.1 g/L of BSA, and 5 μg/L of selenium. After serum starvation, the cells were supplied with fresh serum-free medium (SFM) containing the indicated concentrations of resveratrol.

Adhesion assay

The adhesion assay was performed as described previously [9]. Briefly, 96-well plates were coated with fibronectin at a concentration of 0.2 g/L phosphate buffered saline (PBS) (BD Biosciences, MA, USA,) and then incubated at 37℃ for 1 h under 5% CO_2. 4T1 cells (8×10^5 cells/ well) were suspended in medium containing 0, 10, 20, or 30 μmol/L resveratrol were seeded into the coated wells. The samples were then incubated for 1 h at 37℃, after which the adherent cells were washed with PBS and reincubated in medium containing 1 g/L of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) for 3 h at 37℃. Finally, the absorbance was measured at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Motility and invasion assay

A wound-healing migration assay, the Boyden chamber motility assay, and the Matrigel invasion assay was performed as described previously [18]. For a wound-healing migration assay, the cells were seeded into 12-well culture plates at a concentration of 5×10^5 cells/mL. The cell monolayers were then incubated with 1 g/mL of mytomycin C for 1 h to stop cell proliferation. The monolayers were carefully wounded using a yellow pipette tip. The wounded monolayers were then incubated for 24 h in SFM containing 0, 10, 20, or 30 μmol/L resveratrol. Photographs of the exact wound areas were taken at the onset of the experiment and after 24 h.

For the Boyden chamber motility assay, PVPF filters (8 μmdiameter pore size) were coated with 0.01% gelatin solution. The lower chamber was then filled with 10% FBS medium. 4T1 cells (2×10^6 cells/mL) that had been resuspended in medium containing 0, 10, 20, or 30 μmol/L resveratrol were carefully transferred into the upper chambers. The Boyden chamber was then incubated at 37℃ under 5% CO_2 for 22 h. The filter was stained with Diff-Quik stain solution, and the cells on the lower surface of the filter were fixed onto a glass slide. The cells in five randomly selected microscopic fields (x400) of the lower slide were then counted.

Wells of a Matrigel chamber (BD Biosciences, MA, USA) were filled with SFM and then allowed to adapt to room temperature. Next, 4T1 cells (1×10^6 cells/ mL) that had been resuspended in medium containing 0, 10, 20, or 30 μmol/L resveratrol were carefully transferred into the upper chambers. The Matrigel chambers were then incubated for 12 h at 37℃ under 5% CO_2. The filters were then stained with Diff-Quik stain solution (Dade Behring, Newark, NJ, USA), after which the cells on the lower surface of the filter were fixed onto a glass slide. Finally, cells in five randomly selected microscopic fields (x400) of the lower slide were counted.

MMP-9 activity (gelatin zymography)

MMP-9 activity was investigated as described previously [18]. Cells were seeded into a 6-well plate at a concentration of 1×10^6 cells/mL. The monolayers were then incubated in serum-free medium containing 0, 10, 20, and 30 μmol/L resveratrol for 16 h. The supernatants were then collected and concentrated using Centricon centrifugal filter devices (Milipore). The supernatant was mixed with 2x sample buffer (Invitrogen), after which zymography was performed using gels (10% polyacrylamide, 1% gelatin). The MMP activity was then visualized by staining the gels with Coomassie blue.

MMP-9 mRNA expressions

Reverse transcriptase polymerase chain reaction was performed as previously described [19]. Total RNA was isolated using TRIzol reagent (Sigma), after which cDNA was synthesized using 2 μg of the isolated total RNA and SuperScriptTM II reverse transcriptase (Invitrogen). Next, primers for MMP-9 (upstream primer, 5'-TGGGCTACGTGACCTATGACCAT-3', downstream primer, 5'-GCCCAGCCCACCTCCACTCCTC-3'), and annealed at 55℃ for 1 min for 35 cycles were used to amplify the cDNA.

Animal and diet

Forty female BALB/c mice (Central Lab. Animal Inc., Korea) that were approximately 5 weeks of age, 15-20 g in weight and housed in groups of 5 were used in this study. The animals were divided into four treatment groups (10/group), namely controls (Con), tumor hosts (Can), tumor hosts + 100 mg/kg BW resveratrol (CanR100), and tumor hosts + 200 mg/kg BW

resveratrol (CanR200). The mice were fed an AIN-96G control diet [20] until the experiments commenced. The food intake was measured twice a weekly and the weight of the mice was measured daily. Animals were maintained at 22 ± 2℃ on a regular light-dark cycle and provided with free access to food and water. All animal studies were conducted in accordance with the Dankook University ethics committee's guidelines for the care and use of laboratory animals.

Tumor inoculation and treatment

The tumor hosts received inoculums of 2×10^5 4T1 cells intravenously. The CanR100 and CanR200 groups were then orally administered a daily dose of resveratrol dissolved in a 2% ethanol at doses of 100 and 200 mg/kg BW, respectively. The Con and Can groups were administered only the 2% alcohol solution. On day 21, the animals were weighed and then anesthetized with ethyl ether, after which they were sacrificed and their blood collected by heart puncture. The plasma was separated and stored at -80℃ until the subsequent analysis of MMP-9 activity. In addition, the lungs were collected after the blood was removed and then visually examined for metastasis. The number of metastases was then determined by staining with Bouin's solution (saturated picric acid:formalin:acetic acid = 15:5:1) after washing the lungs with PBS. The MMP-9 activity in plasma was investigated using gelatin zymography, which was performed using gels (10% polyacrylamide, 1% gelatin). MMP-9 activity was visualized by staining with Coomassie blue.

Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (SAS Institute, Cary, NC). Data were expressed as the means ± standard deviations and then compared among groups by analysis of variance (ANOVA). Statistically significant differences among groups were further tested at $\alpha = 0.05$ using Duncan's multiple range test.

Results

Effects of resveratrol on the adhesion, motility, and invasion in 4T1 cells

As shown in Fig. 1A, resveratrol decreased the adhesion of 4T1 cells in a dose-dependent manner. Treatment of 4T1 cells

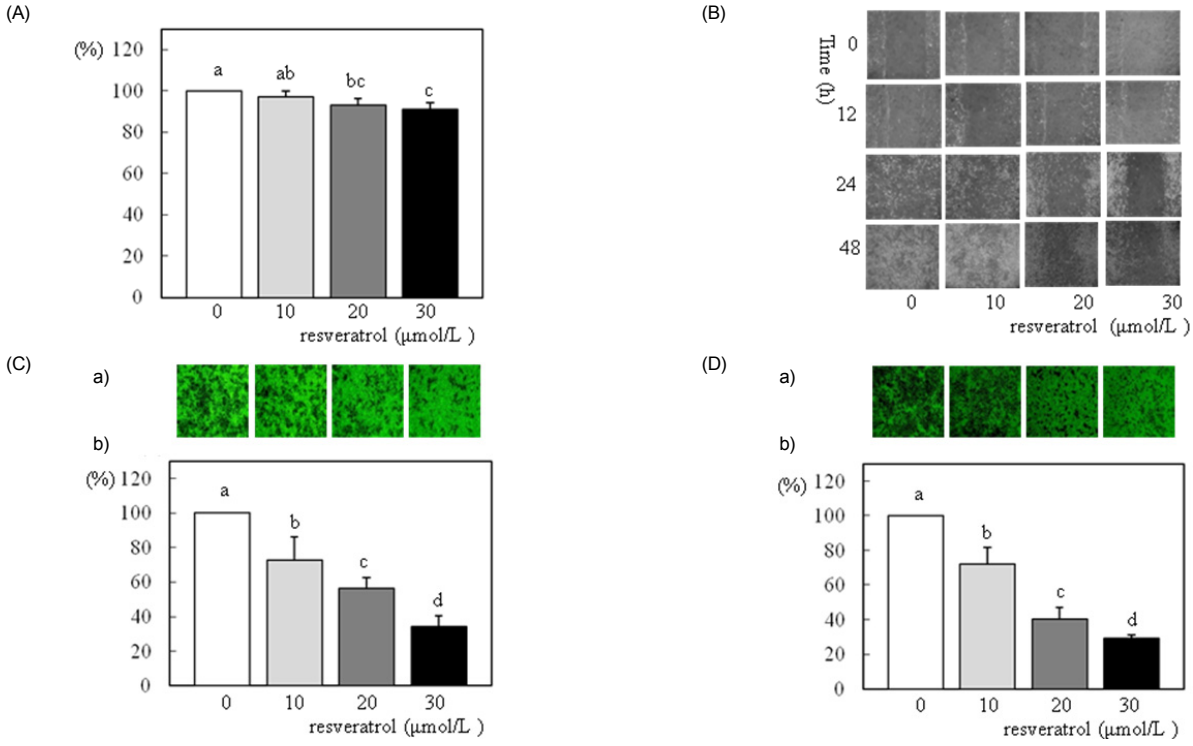

Fig. 1. The effects of resveratrol on the adhesion, motility, and invasion in 4T1 cells. (A) After 8×10^5 cells/mL suspended in DMEM/F12 medium containing 0, 10, 20, or 30 μmol/L resveratrol were plated in each well of a 96 well fibronectin-coated plate for 1 h, the medium was gently removed and the attached cells were subjected to an MTT assay. (B) Cells were plated in a 12-well plate at a density 5×10^5 cells/well in DMEM/F12 supplemented with 10% FBS. Confluent monolayers were then wounded and subsequently incubated in serum free medium in the presence of 0, 10, 20, or 30 μmol/L resveratrol. The cells were then photographed under a phase contrast microscope at 0, 12, 24, and 48 h after being wounded. (C) Cells were cultured in the presence of various concentrations of resveratrol for 8 h in a Boyden chamber. a) Microphotography of cells treated with resveratrol. b) Quantitative analysis of the cell motility assay. (D) Cells were cultured in the presence of various concentrations of resveratrol for 8 h in an invasion chamber a) Microphotography of cells treated with resveratrol. b) Quantitative analysis of the invasion assay. Each bar represents the mean ± SD of three independent experiments. Significant differences ($P < 0.05$) among groups are indicated by different letters above each bar.

with increasing concentrations of resveratrol led to a dose-dependent decrease in wound- healing cell migration (Fig. 1B) and cell motility (Fig. 1C). As shown in Fig 1D, resveratrol exerted a dose-dependent inhibitory effect on cell invasion.

Effects of resveratrol on the activity and mRNA expression of MMP-9 in 4T1 cells

The activity of MMP-9 in 4T1 cells was decreased in response to treatment with resveratrol in a dose-dependent manner (Fig. 2A). Furthermore, the results of RT-PCR analysis suggested that the expression of MMP-9 mRNA was decreased in 4T1 cells in response to treatment with resveratrol (Fig. 2B). These results suggested that resveratrol could play a crucial role in the down-regulation of MMP-9 expression.

Effects of resveratrol on lung metastasis in vivo

We examined the effect of resveratrol on the metastasis of 4T1 cells in BALB/C mice. Tumor burden and the administration of resveratrol had no effects on food intake. However, tumor burden caused a significant reduction in body weight, irrespective of treatment with resveratrol (Table 1). The macroscopic appearance of the lungs from untreated and treated mice clearly showed that treatment with 100 or 200 mg/kg BW resveratrol reduced the number of 4T1 colonies in the lungs of Balb/c mice (Fig. 3A). With no resveratrol, there were 42.6 ± 11.45 colonies ($P < 0.05$). But there were only 29.3 ± 6.09 with 100 mg/kg and 15.2 ± 8.34 with 200 mg/kg resveratrol. As shown in Fig. 3B, plasma MMP-9 activity was decreased in mice that were treated with resveratrol ($P < 0.05$).

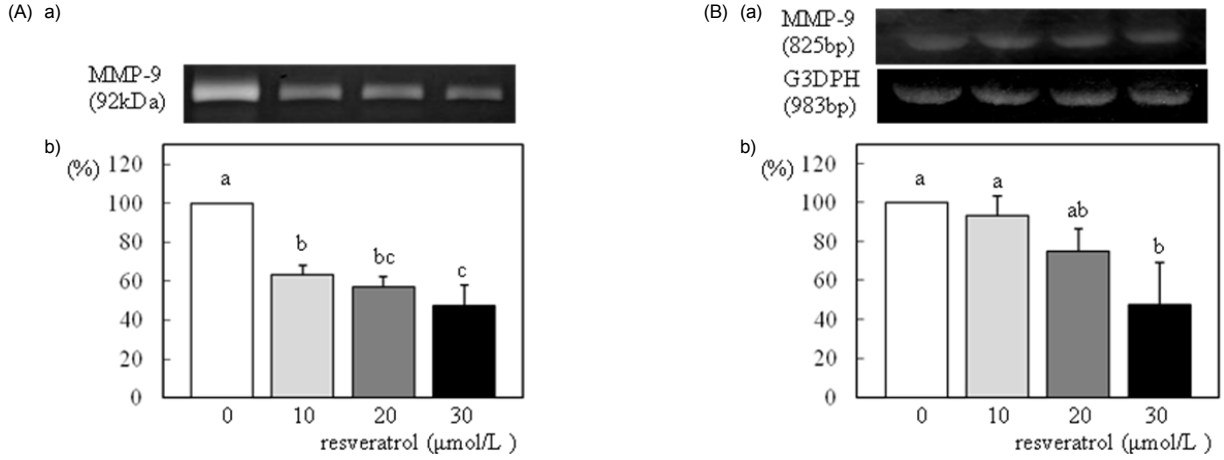

Fig. 2. The effects of resveratrol on MMP-9 activity and mRNA expression in 4T1 cells. Resveratrol decreases activity and mRNA expression of MMP-9. (A) The 4T1 cells were plated in 6 well plates at a density 1×10^6 cells/well, the monolayers were then incubated in serum-free medium containing 0, 10, 20, and 30 μmol/L resveratrol for 16 h. Next, the medium was collected and concentrated for zymography. a) Photographs of MMP-9 bands. b) Densitometric analysis of western blots. (B) For RT-PCR, 4T1 cells were treated with resveratrol and the samples were then separated on a 1% agarose gel. a) Photographs of chemiluminiscent evaluation of the RT-PCR bands. b) Quantitative analysis of RT-PCR. The relative abundance of each band was estimated by densitomertric analysis. Each bar represents the mean ± SD of three independent experiments. Significant differences ($P < 0.05$) among groups are indicated by different letters above each bar.

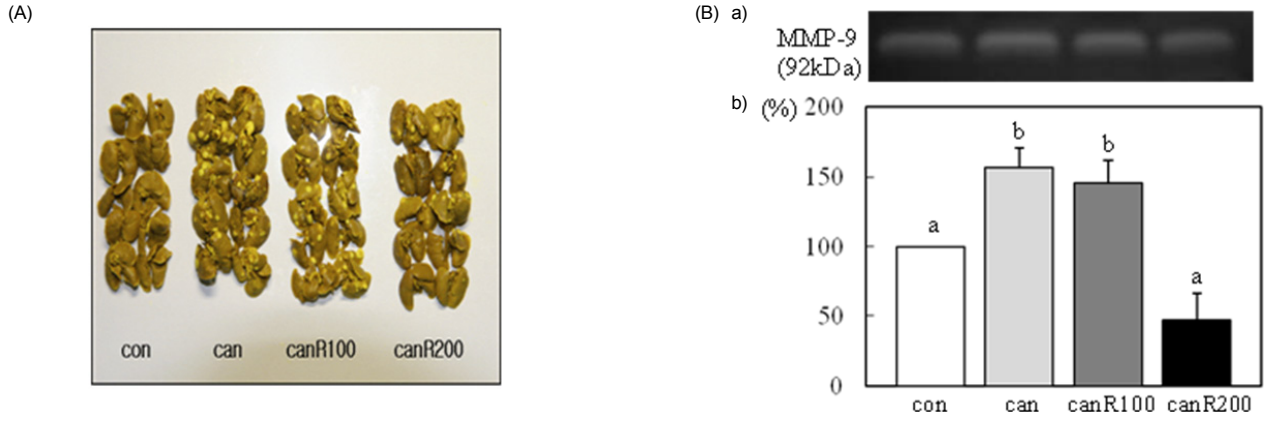

Fig. 3. Resveratrol inhibits experimental metastasis in Balb/c mice. 4T1 cells (2×10^5 cells/0.1 mL) were injected into the lateral tail vein of 40 Balb/c mice. Resveratrol was then administered orally each day at a dose of 100 mg/kg BW (CanR100) or 200 mg/kg BW (CanR200). The colony numbers decreased with the amount of resveratrol increased. With no resveratrol, there were 42.6 ± 11.5 colonies. The number of colony were 29.3 ± 6.1 with CanR100 and 15.2 ± 8.3 with CanR200 (A). MMP-9 activity was measured in plasma (B). a) Photographs of MMP-9 bands. b) Densitometric analysis of western blots. Each bar represents the mean ± SD of three independent experiments. Significant differences ($P < 0.05$) among groups are indicated by different letters above each bar.

Table 1. The effects of resveratrol on body weight and food intake

Group[1)]	Initial body weight (g)	Final body weight (g)	Food intake (g/day)
Con	16.8 ± 0.56[2)NS3)]	19.7 ± 0.69[a4)]	1.93 ± 0.16[NS]
Can	17.0 ± 0.46	16.3 ± 1.24[b]	2.10 ± 0.12
CanR100	17.2 ± 0.51	15.7 ± 1.67[b]	2.08 ± 0.18
CanR200	17.2 ± 0.47	16.8 ± 0.95[b]	1.98 ± 0.19

[1)] Con, control; Can, tumor inoculation; CanR100, tumor inoculation and treated daily with oral supplementation of 100 mg/kg resveratrol; CanR200, tumor inoculation and treated daily with oral supplementation of 200 mg/kg resveratrol. Each group contained six animals.
[2)] Mean ± SD
[3)] NS, not significant
[4)] Different letters within a column represent a significant difference at $\alpha = 0.05$ as determined by Duncan's multiple range test.

Discussion

In this study, the effects of resveratrol on metastasis in 4T1 cells and on experimental metastasis of 4T1 cells in Balb/c mice were evaluated. Cancer metastasis is a complex multi-step process that ultimately enables the escape of tumor cells and the subsequent seeding of ectopic environments [12]. For breast cancer cells to manifest their malignant potential, they must develop the ability to break through and dissolve the ECM, particularly those delimiting the BM. The initial invasive action of metastatic cells involves interactions between tumor cells and the ECM that occur via the process of cell matrix adhesion. Once malignant cells have detached from the primary tumor, they bombard the surrounding BMs and adhere to their meshwork of Type IV collagen, laminin, and fibronectin [21]. As shown in Fig. 1A, treatment with resveratrol inhibited the attachment of 4T1 cells to fibronectin, which is one of the major components of the BM.

The process of tumor cell invasion and metastasis requires degradation of the connective tissue associated with vascular BM and interstitial connective tissue [22,23]. The BM is the largest barrier between a free malignant cell and the bloodstream, and it must be traversed before malignant cells can enter circulating blood [24]. Therefore, invasion through a BM is a critical step in metastasis [25]. To successfully penetrate the filter membrane, cells must successfully adhere, degrade, and traverse the Matrigel-coated insert. The present study demonstrated that resveratrol inhibited the invasiveness of 4T1 breast cancer cells in a dose-dependent manner.

Motility is another property of cancer cells that is required for migration from the primary site to a secondary organ to occur. The results presented here demonstrate that treatment with various concentrations of resveratrol reduced the motility of 4T1 cells.

Degradation of the pericellular BM and ECM is catalyzed by the concerted action of several classes of ECM-degrading enzymes. One important class of ECM-degrading enzymes is the MMPs [13]. MMPs have been implicated as possible mediators of invasion and metastasis in some cancers. MMP-9 is a MMP family enzyme that is related to tumor invasion and metastasis due to its capacity for tissue remodeling via the ECM and its ability to degrade the BM [26,27]. In addition, aberrant overexpression of MMP-9 has been found to be associated with increased cancer invasive potential in breast cancer cells [28-30]. Previous reports have shown that resveratrol prevents tumor progression through the inhibition of MMP-9 expression [16,19, 31,32] and that it suppresses proinflammatory mediators, such as TNF-α, IL-1β, COX-2, and iNOS [33-35]. The results of the present study demonstrated that resveratrol can inhibit MMP-9 activity and the levels of MMP-9 mRNA in 4T1 cells.

Interestingly, the results of the present study demonstrated that oral administration of resveratrol 21 days either inhibited the growth of 4T1 cells or prevented their metastasis to the lungs in mice. Bove *et al*. [36] reported that, while it inhibited 4T1 cell proliferation in a dose-dependent manner *in vitro*, intraperitoneal injection of resveratrol at doses of 1, 3, or 5 mg/kg BW had no effect on *in vivo* metastasis of 4T1 cells in mice. Conversely, Carbó *et al*. [37] and Kimura and Okuda [38] reported that treatment with 1 mg/kg and 2.5-10 mg/kg BW resveratrol induced significant decreases in the occurrence of tumor cells and metastasis to the lungs by hepatoma tumor cells and Lewis lung carcinoma cells, respectively. Weng *et al*. [39] reported that resveratrol suppressed the pulmonary metastasis of BALB/c mice challenged with CT26 colon cancer cells. Furthermore, Busquets *et al*. [40] demonstrated that the administration of resveratrol had no effect on the growth of an intramuscularly implanted experimental tumor in C57B1/6 mice. Taken together, the results of these previous studies indicate that the effects of resveratrol on primary tumor growth may be dependent on the experimental model and doses used.

Several resveratrol studies treating animals, the dose is very various from 100 μg to 100 mg per day [31,41-45]. Comparing with these studies, the concentration of resveratrol of our study is similar to that of Bhat *et al.* [41], Li *et al.* [42], Chen *et al.* [43], and Tseng *et al.* [44] studies and it is 100 to 200 times higher than that of Banerjee *et al*. [31] and Harper *et al*. [45]. Of course, there is a difference on type of treatment that they injected intraperitoneal or subcutaneous, and we supplied orally tube feeding. In our study, there are limitations that we did not set the control group to which we supplied resveratrol without triggering tumor, and that we did not investigate the probability of side effects except food intake and weight induced by high dose of resveratrol. Resveratrol toxicity has been reported at concentration above 10 μmol/L in 549 cells [46]. But other studies showed that had no significant cycotoxic effect of resveratrol was found at a concentration below 100 μmol/L [47]. The deviation between the above results may be related to difference of cell and other experimental conditions or different cytotoxic analysis methods. Therefore, further researches are needed to know whether it would be possible to test the high doses of resveratrol not only on animal but also on human.

This study is the first to report that oral administration of resveratrol inhibits the metastasis of 4T1 cells to the lungs in

a BALB/c murine model of experimentally induced cancer. Based on these findings, we confirmed that resveratrol may inhibit metastasis by decreasing the activity and expression of MMP-9.

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