Received: 20 March 2018 | Accepted: 28 June 2018

DOI: 10.1002/jcb.27335

RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

Resveratrol significantly inhibits the occurrence and development of cervical cancer by regulating phospholipid scramblase 1

Yanhua Zhao[1] | Xiying Yuan[1] | Xiang Li[2] | Yi Zhang[1]

[1]Department of Obstetrics and Gynecology, Xiangya Hospital of Central South University, Changsha, Hunan, China

[2]Department of Pathology, Xiangya Hospital of Central South University, Changsha, Hunan, China

Correspondence
Yi Zhang, Department of Obstetrics and Gynecology, Xiangya Hospital of Central South University, No. 87 Xiangya Road, Changsha 410008, China.
Email: zhangyi_zh2015@sina.com

Abstract

Cervical cancer (CC) is one of the most common female malignancies, and resveratrol is a polyphenol isolated from the skins of grapes, which has been reported to significantly alter the cellular physiology of tumor cells. However, little is known about the role of phospholipid scramblase 1 (PLSCR1) in pathogenesis of CC. Here, we demonstrated that resveratrol could significantly inhibit both the growth of HeLa cells and expression of PLSCR1. These results suggest that resveratrol-mediated cell growth inhibition can be regulated by PLSCR1.

KEYWORDS

cervical cancer, phospholipid scramblase 1 (PLSCR1), resveratrol

1 | INTRODUCTION

Cervical cancer (CC) is currently the most common female tumor worldwide with an extremely poor prognosis, accounting for more than 60% of the gynecological cancer burden in developing countries. Every year, more than 500,000 women are diagnosed with CC, and CC accounts for more than 275,000 deaths globally.[1,2]

The antioxidant 3,4′,5 trihydroxystilbene (resveratrol) is a polyphenol compound found in various nutrients such as peanuts, mulberries, and red wine. It is shown to have immunomodulatory, anticancerogenic, and cardioprotective effects.[3] It has been increasingly recognized that resveratrol possesses cancer-preventive and suppressive activities. More importantly, resveratrol has little cytotoxic effect on normal tissues in vitro and in vivo at effective anticancer doses, reflecting its potential value in cancer treatments when administered appropriately.[4-8]

Phospholipid scramblase 1 is a calcium-dependent endofacial plasma membrane protein. The first function ascribed to PLSCR1 was to catalyze rapid, bidirectional, and nonspecific distribution of phospholipids (lipid scrambling) between the inner and outer leaflets of the plasma membrane, resulting in collapse of the phospholipid asymmetry.[9-18] However, little is known about the role of PLSCR1 in the occurrence and development of CC. In this study, we evaluated the effects of resveratrol as individual agents on the occurrence and development of CC and explore the PLSCR1-related mechanism behind the observed efficacy.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

We specifically used CC cell lines Hela. All the cell lines used in this study were purchased from the Shanghai Cell Bank, Shanghai Institute for Biological Sciences, China Academy of Sciences. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at a 37°C humidified atmosphere containing 5% CO_2.

2.2 | Cell proliferation assay

Cells were plated in 96-well plates and examined at 24, 48, 72, 96 hours after plating ($n = 8$). The cells were

incubated with CellTiter 96 AQueous (MTS) solution for 3 hours. The absorbance at 490 nm was then measured on the microplate reader (BioTek Instruments, Inc., Winooski, VT).

2.3 | Cell viability assay

Cell Counting Kit-8 (CCK-8) assay was used to detect the cell growth status according to the manufacturer's instruction. Cells were cultured at a density of 5×10^4 cells per well in flat-bottomed 96-well plates with various concentrations of resveratrol. After 72 hours, 10 μL of CCK-8 Solution Reagent was added to each well according to the manufacturer's instructions. After 4 hours in culture, cell viability was measured via reading the absorbance at 450 nm using a Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA), and the relative cell viability of surviving cells from each group relative to controls, defined as relative cell viability 1.0, was determined by the reduction of WST-8.

2.4 | Cell cycle analysis by flow cytometry

After treatment with resveratrol for 24 hours, the Hela cells were trypsinized and fixed with 70% ethanol. Cells were then stained with a solution of propidium iodide (50 mg/mL) and RNase A (0.5 mg/mL) in phosphate-buffered saline (PBS) for 30 minutes at 37°C in the dark. Cell cycle distribution was analyzed by flow cytometry (Becton-Dickinson Co).

2.5 | Western blot analysis

After treatment with resveratrol for 24 hours, the Hela cells were harvested and lysed. Equal amounts of cell lysates were resolved by SDS/PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with specific primary antibodies, washed with PBS containing 0.1% (v/v) Tween 20, and then incubated with horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL). GAPDH (Monoclonal Anti-GAPDH −Peroxidase antibody produced in mouse) was used for normalization of protein loading.

2.6 | Statistical analysis

The data are expressed as means ± standard errors of means (SEM) of at least three independent experiments. Statistical analysis was performed with GraphPad Prism5.0 (GraphPad Software, San Diego, CA). For in vitro assays, the significance of differences between control and treated cells was measured with the Student t test ($P < 0.05$ was considered statistically significant).

2.7 | Immunohistochemical staining experiment

HeLa cells were subjected to a heat-induced epitope retrieval step in 0.01 M sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in distilled water. The sections were then incubated with 0.3% Triton X-100 in PBS for 15 minutes and then 10% goat serum in PBS for 1 hour. Subsequently, samples were incubated with a rabbit polyclonal antibody, diluted at 1:100 in 1% goat serum for 1 hour at 37°C. After three washes in PBS, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:500). The stain was developed with the GTVision III Immunohistochemistry Detection Kit (GeneTech Inc, Shanghai, China) according to the manufacturer's protocols. Sections were counterstained with hematoxylin.

2.8 | In vivo xenograft assay

Cell suspensions (1×10^6 cells) of cancer cells in a total volume of 100 μL mixed with matrigel at a 1:1 ratio were injected subcutaneously into the right flanks of 4-week-old male BALB/C nude mice. The body weight and tumor volumes were measured and recorded every 10 days from 2 weeks after inoculation. Tumor volume was calculated with the following formula: volume = 0.5 × tumor length × tumor width^2. Tumors were collected and photographed at 50 days after inoculation. All mice were housed in the SPF animal facility in a pathogen-free environment with controlled temperature and humidity.

3 | RESULTS

3.1 | Resveratrol significantly inhibited the growth of HeLa cells

First, we tested resveratrol on CC cell line (Hela) to measure cell growth inhibition. After application of resveratrol (0, 0.1, 1, and 10 μM) for 3 days, cell proliferation was inhibited in a dose-dependent manner. Hela cells were sensitive to resveratrol and results similar to other studies were obtained; the effect of resveratrol reached the maximum at a concentration of 1 μM. Notably, the effect of resveratrol did not increase at a concentration of 10 μM, suggesting that too high a concentration of cell toxicity is not suitable for this assay (Figure 1).

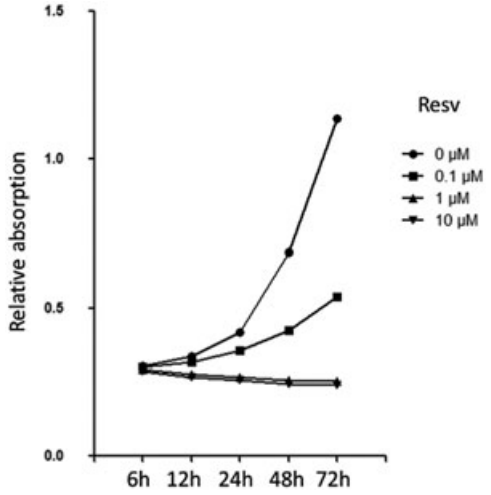


FIGURE 1 Resv significantly inhibited the growth of cervical cancer cell line HeLa cells. HeLa cells cultured for 3 days, the cells proliferated at different concentrations of Resv: 0, 0.1, 1 and 10 μM. Resv, resveratrol

3.2 | Resveratrol significantly decreases mRNA and protein expression of PLSCR1 in HeLa cells

To gain further insights into the underlying mechanism of resveratrol in tumor cells, we detected the mRNA and protein expression of PLSCR1 in resveratrol-treated Hela cells. Resveratrol dramatically reduced PLSCR1 expression at both mRNA and protein levels (Figure 2A and 2B).

3.3 | Resveratrol inhibition on HeLa cells after resveratrol treatment can be recovered by the gain of function of PLSCR1

To investigate the role of PLSCR1 in the cell growth inhibition caused by resveratrol, CCK-8 assay was performed on cultured Hela cells at the various stage with PLSCR1 plasmid transfection. Resveratrol (1 μM) significantly reduced the growth and proliferation of HeLa cells by downregulation of PLSCR1(Figure 2A). Strikingly, the growth inhibition of HeLa cells after resveratrol treatment can be recovered by the gain of function assay PLSCR1 (Figure 3).

3.4 | Resveratrol inhibits the tumorigenicity of cervical cancer HeLa cells in vivo

To verify the negative role of Resveratrol in CC cells proliferation, we performed immunochemical staining on Hela cells at days 3,14, and 28 after Resveratrol

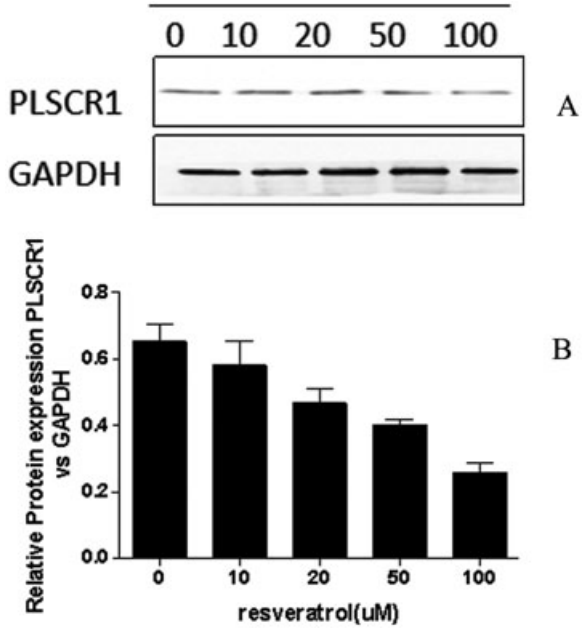


FIGURE 2 Resv significantly decreases mRNA level and protein expression of PLSCR1 in HeLa cells. A, mRNA level of PLSCR1; B, Statistical analysis of protein expression; C, mRNA expression of PLSCR1; D, Statistical analysis of protein expression. PLSCR1, phospholipid scramblase 1; mRNA, messenger RNA; Resv, resveratrol

treatment. Expectably, the expression of PLSCR1 in control cells was upregulated on days 14 and 28. Meanwhile, the expression of PLSCR1 in resveratrol-treated cells only showed weak upregulation on both days 14 and 28 (Figure 4). It implicated that PLSCR1 might contribute to the resveratrol-mediated cell death.

To verify the negative role of resveratrol in CC progression in vivo, we performed xenograft tumor assays using HeLa cells. The animals were treated with

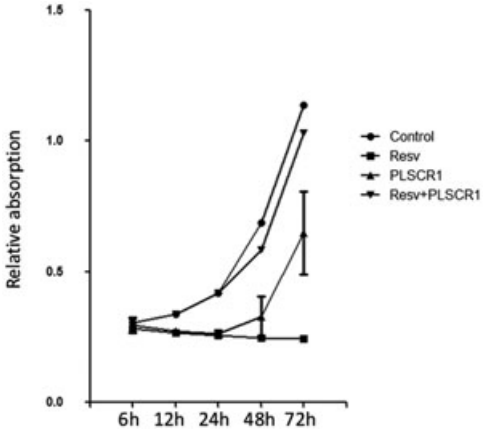


FIGURE 3 Resv reduced the growth and proliferation of HeLa cells by downregulation of PLSCR1, HeLa cells can be recovered after resv treatment by gain of function assay PLSCR1. PLSCR1, phospholipid scramblase 1; Resv, resveratrol

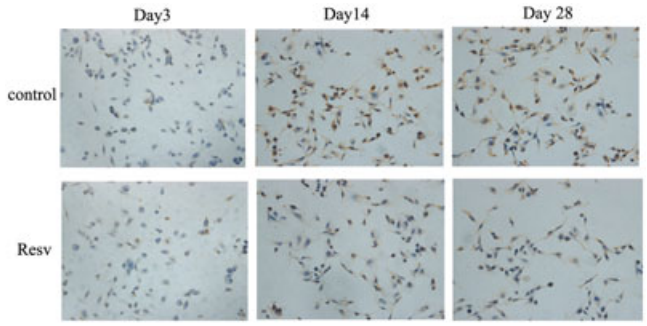


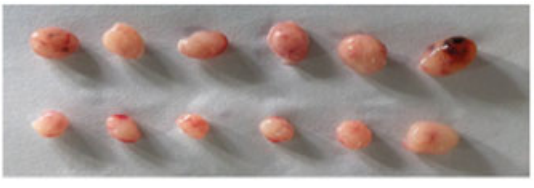
FIGURE 4 The PLSCR1 expression was downregulated by Resv treatment during HeLa cell proliferation. PLSCR1, phospholipid scramblase 1; Resv, resveratrol

resveratrol at a concentration of 10 mg/kg daily. We found that resveratrol significantly inhibited xenograft tumor growth in nude mice (Figure 5A). The weights of tumors are shown (Figure 5B). These data collectively indicate that resveratrol acts as a novel tumor-suppressing molecule and negatively regulates cervical tumor growth.

4 | DISCUSSION

In this study, we investigated the inhibition effect of resveratrol on the occurrence and development of CC and discussed the PLSCR1-related mechanism behind the observed efficacy. It is well known that CC is currently the most common female cancer with an extremely poor prognosis. CC occurs frequently in women worldwide and one woman dies because of CC every 2 minutes. Currently, there is no effective cure to this cancer, especially to high-risk patients.[19-21]

(A)



(B)

Group	Weight of tumor (g)					
Control	0.1641	0.1537	0.1442	0.1893	0.2253	0.2954
Resv	0.0725	0.0828	0.0607	0.0726	0.1047	0.2075

FIGURE 5 Tumor formation and weight of tumors in the serial xenograft model using HeLa cells. A, Upper: tumors treated with PBS. Bottom: Tumors treated with Resv at concentration of (0.0725, 0.0828, 0.0607, 0.0726, 0.1047, 0.2075). B, The weight of tumors from xenograft models treated with Resv. PBS, phosphate-buffered saline; Resv, resveratrol

Resveratrol is a natural polyphenol found in many fruits and vegetables, such as grapes, peanuts, berries, and red wine.[22] Some plants also produce resveratrol to fight off invasive bacteria. So, resveratrol was originally considered as an antibacterial drug. In recent years, studies have found that resveratrol has a strong role in cancer prevention and treatment, and its regulatory effect can occur in various processes of tumor cell growth, division, and apoptosis.[23] Resveratrol can play a significant role in inhibiting the progression of various cancers, including skin cancer, breast cancer, lung cancer, pancreatic cancer, and gastric cancer.[24] In addition, the anticancer effect of resveratrol has been extensively studied at the molecular and cellular levels, such as cell signaling pathway, enzyme pathway, and p53 interface, leading to apoptosis.[25]

The inhibitory effect of Resveratrol on CC has been reported before, including a variety of experiments. Resveratrol shows cancer preventive activities, including inhibition of migration and invasion of some metastatic tumors. Resveratrol also decreases both the expression and the enzymatic activity of matrix metalloproteinase-9 (MMP-9), and inhibits the promoter activity of para-Methoxyamphetamine-stimulated MMP-9. A further study showed that resveratrol suppresses the transcription of MMP-9 by the inhibition of both NF-κB and activator protein 1 (AP-1) transactivation. These results indicate that resveratrol inhibits both NF-κB and AP-1 mediated MMP-9 expression, resulting in suppression of migration and invasion of CC cells. Resveratrol has potential for clinical use in preventing invasion by human metastatic lung and CCs.[26]

Resveratrol can inhibit the occurrence and development of CC through PLSCR1 regulation. This result has not been reported before. This is the first time we have investigated the role of PLSCR1 in this process. Taken together, these results suggest that resveratrol mediated cell gowth inhibition can be regulated by PLSCR1. The study demonstrates a potential molecular mechanism underlying the inhibitory effect of resveratrol on CC, and indicates that PLSCR1 might act as a potential prognostic biomarker and therapeutic target in patients with CC.

5 | CONCLUSION

Resveratrol significantly inhibited the growth of HeLa cells. The effect of resveratrol reached the maximum at concentration of 1 μM. Resveratrol significantly decreases mRNA and protein expression of PLSCR1 in HeLa cells. In a word, there was obviously an effect of resveratrol on inhibiting the occurrence and development of CC by regulating PLSCR1. This study will provide novel therapeutic targets for CC, especially based on the signal

pathway, and provide a theoretical basis for basic research.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Yi Zhang http://orcid.org/0000-0002-9400-0057

REFERENCES

1. Bychkovsky BL, Ferreyra ME, Strasser-Weippl K, et al. Cervical cancer control in Latin America: a call to action. *Cancer*. 2016;122(4):502-514.
2. Kilic S, Cracchiolo B, Gabel M, Haffty B, Mahmoud O. The relevance of molecular biomarkers in cervical cancer patients treated with radiotherapy. *Ann Trans Med*. 2015;3(18):261.
3. Kulkarni SS, Cantó C. The molecular targets of resveratrol. *Biochimica Et Biophysica Acta*. 2015;1852(6):1114-1123.
4. Carter LG, D'Orazio JA, Pearson KJ. Resveratrol and cancer: focus on in vivo evidence. *Endocr Relat Cancer*. 2014;21(3):R209-R225.
5. Singh CK, Ndiaye MA, Ahmad N. Resveratrol and cancer: challenges for clinical translation. *Biochim Biophys Acta*. 2015;1852(6):1178-1185.
6. Xu Q, Zong L, Chen X, et al. Resveratrol in the treatment of pancreatic cancer. *Ann NY Acad Sci*. 2015;1348(1):10-19.
7. Zhong LX, Zhang Y, Wu ML, et al. Resveratrol and STAT inhibitor enhance autophagy in ovarian cancer cells. *Cell Death Discov*. 2016;2:15071.
8. Feng Y, Zhou J, Jiang Y. Resveratrol in lung cancer- a systematic review. *J BUON*. 2016;21(4):950-953.
9. Huang Y, Zhao Q, Zhou CX, et al. Antileukemic roles of human phospholipid scramblase 1 gene, evidence from inducible PLSCR1-expressing leukemic cells. *Oncogene*. 2006;25(50):6618-6627.
10. Wu DJ, Liu TT, Zhou QH, et al. Significance of PLSCR1 in matrine induced differentiation of ATRA resistant APL cells. *Chin J Integ Tradi West Med*. 2015;35(11):1345-1350.
11. Bailey K, Cook HW, McMaster CR. The phospholipid scramblase PLSCR1 increases UV induced apoptosis primarily through the augmentation of the intrinsic apoptotic pathway and independent of direct phosphorylation by protein kinase C delta. *BBA*. 2005;1733(2-3):199-209.
12. Berghold VM, Gauster M, Hemmings DG, et al. Phospholipid scramblase 1 (PLSCR1) in villous trophoblast of the human placenta. *Histochem Cell Biol*. 2015;143(4):381-396.
13. Chen Y, Hui H, Yang H, et al. Wogonoside induces cell cycle arrest and differentiation by affecting expression and subcellular localization of PLSCR1 in AML cells. *Blood*. 2013;121(18):3682-3691.
14. Cusick JK, Mustian A, Jacobs AT, Reyland ME. Identification of PLSCR1 as a protein that interacts with RELT family members. *Mol and Cell Biochem*. 2012;362(1-2):55-63.
15. Frasch SC, Henson PM, Nagaosa K, Fessler MB, Borregaard N, Bratton DL. Phospholipid flip-flop and phospholipid scramblase 1 (PLSCR1) co-localize to uropod rafts in formylated Met-Leu-Phe-stimulated neutrophils. *J Biol Chem*. 2004;279(17):17625-17633.
16. Kodigepalli KM, Nanjundan M. Induction of PLSCR1 in a STING/IRF3-dependent manner upon vector transfection in ovarian epithelial cells. *PLoS One*. 2015;10(2):e0117464.
17. Rami A, Sims J, Botez G, Winckler J. Spatial resolution of phospholipid scramblase 1 (PLSCR1), caspase-3 activation and DNA-fragmentation in the human hippocampus after cerebral ischemia. *Neurochem Int*. 2003;43(1):79-87.
18. Song G, Fleming JAGW, Kim J, Spencer TE, Bazer FW. Pregnancy and interferon tau regulate DDX58 and PLSCR1 in the ovine uterus during the peri-implantation period. *Reproduction*. 2011;141(1):127-138.
19. Brucker SY, Ulrich UA. Surgical treatment of early-stage cervical cancer. *Oncol Res Treat*. 2016;39(9):508-514.
20. Hillemanns P, Soergel P, Hertel H, Jentschke M. Epidemiology and early detection of cervical cancer. *Oncol Res Treat*. 2016;39(9):501-506.
21. Vordermark D. Radiotherapy of cervical cancer. *Oncol Res Treat*. 2016;39(9):516-520.
22. Saiko P, Szakmary A, Jaeger W, Szekeres T. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad [J]. *Mutat Res*. 2008;658(1/2):68-94.
23. Kang NH, Hwang KA, Kim TH, Hyun SH, Jeung EB, Choi KC. Induced growth of BG-1 ovarian cancer cells by 17β-estradiol or various endocrine disrupting chemicals was reversed by resveratrol via downregulation of cell cycle progression [J]. *Mol Med Rep*. 2012;6(1):151-156.
24. Yi CO, Jeon BT, Shin HJ, et al. Resveratrol activates AMPK and suppresses LPS-induced NF-κB-dependent COX-2 activation in RAW 264.7 macrophage cells [J]. *Anat Cell Biol*. 2011;44(3):194-203.
25. A. kroon P, Iyer A, Chunduri P, Chan V, Brown L. The cardiovascular nutrapharmacology of resveratrol: pharmacokinetics, molecular mechanisms and therapeutic potential [J]. *Curr Med Chem*. 2010;17(23):2442-2455.
26. Kim YS, Sull JW, Sung HJ. Suppressing effect of resveratrol on the migration and invasion of human metastatic lung and cervical cancer cells. *Mol Biol Rep*. 2012;39(9):8709-8716.

How to cite this article: Zhao Y, Yuan X, Li X, Zhang Y. Resveratrol significantly inhibits the occurrence and development of cervical cancer by regulating phospholipid scramblase 1. *J Cell Biochem*. 2018;1-5.
https://doi.org/10.1002/jcb.27335