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Resveratrol significantly inhibits the occurrence and development of cervical cancer by regulating *PLSCR1.*

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Abstract

Cervical cancer is currently the most common female malignancies, and resveratrol is a polyphenol isolated from the skins of grapes that has been reported to significantly alter the cellular physiology of tumor cells, as well as block the process of initiation and progression. Little is known about the role of *PLSCR1* in the occurrence and development of cervical cancer. Here, we demonstrated that resveratrol significantly inhibited both the growth of HeLa cells and expression of *PLSCR1*, which can be recovered by gain of function of *PLSCR1*. These results suggest that resveratrol mediated cell growth inhibition can be regulated by *PLSCR1*.

Keywords: Resveratrol, Cervical cancer, *PLSCR1*.

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Introduction

Cervical Cancer (CC) is currently the most common female tumors 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 tri-hydroxystilbene (resveratrol) is a polyphenol compound found in various nutrients that was found in various nutrients such as peanuts, mulberries, and red wine. It had been shown to have immunomodulatory, anti-cancerogenic, 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 catalyse rapid, bidirectional and non-specific distribution of phospholipids (lipid scrambling) between the inner and outer leaflet 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 cervical cancer. In this study, we evaluated effects of resveratrol as individual agents on the occurrence and development of cervical cancer and explore the *PLSCR1*-related mechanism behind the observed efficacy.

Materials and Methods

Cell lines and cell culture

We specifically used cervical cancer 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 (FBS) at a 37°C humidified atmosphere containing 5% CO_2.

Cell proliferation assay

Cells were plated in 96-well plates and examined at 24, 48, 72, 96 h after plating (n=8). Cells were incubated with CellTiter 96 AQueous (MTS) solution for 3 h. The absorbance at 490 nm was then measured on the microplate reader (BioTek).

Cell viability assay

Cell Counting Kit-8 (CCK-8) assay was used to detect the cell growth status according to 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 h, 10 μl of CCK-8 Solution Reagent was added to each well according to the manufacturer's instructions. After 4 h in culture, cell viability was measured *via* reading the absorbance at 450 nm using a Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA), and the relative cell viability of surviving cells from each group relative to controls, defined as relative cell viability 1.0, was determined by reduction of WST-8.

Cell cycle analysis by flow cytometry

After treatment with resveratrol for 24 h, the Hela cells were trypsinized and fixed with 70 % ethanol. Cells were then stained with a solution of propidium iodide (50 mg/ml) and RNaseA (0.5 mg/ml) in PBS for 30 min at 37°C in the dark. Cell cycle distribution was analysed by flow cytometry (Becton-Dickinson Co.).

Western blot analysis

After treatment with resveratrol for 24 h, 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 was used for normalization of protein loading.

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, USA). For *in vitro* assays, the significance of differences between control and treated cells was measured with the Student's t-test ($P<0.05$ was considered statistically significant).

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 min and then 10% goat serum in PBS for 1 h. Subsequently, samples were incubated with a rabbit polyclonal antibody, diluted at 1:100 in 1% goat serum for 1 h at 37°C. After three washes in PBS, 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.

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-w-old male BALB/C nude mice. The body weight and tumor volumes were measured and recorded every 10 d from 2 w 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 d after inoculation. All mice were housed in the SPF animal facility of the First Affiliated Hospital of Jinan University in a pathogen-free environment with controlled temperature and humidity.

Result

Resv significantly inhibited the growth of HeLa cells

First, we tested resveratrol on cervical cancer cell line (Hela) to measured cell growth inhibition. After application of resveratrol (0, 0.1, 1 and 10 µM) for 3 d, cell proliferation was inhibited in a dose-dependent manner. Hela cells were sensitive to resveratrol and similar with the results from other studies, the effect of resveratrol reached the maximum at concentration of 1 µM. Notably, the effect of Resv did not increase at concentration of 10 µM, suggesting that too high concentration of cell toxicity doesn't suitable for this assay (Figure 1).

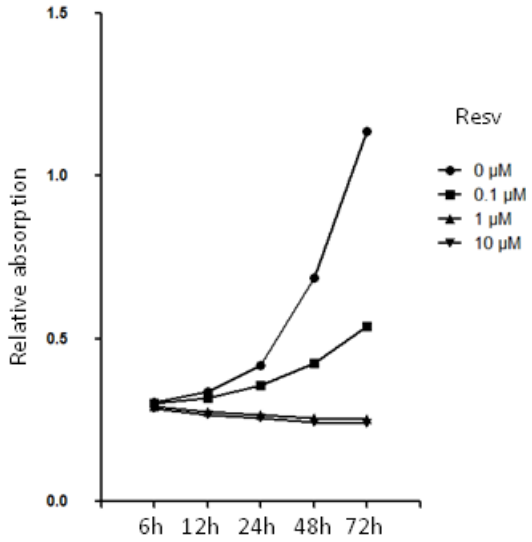


Figure 1. *Resv significantly inhibited the growth of cervical cancer cell line HeLa cells. HeLa cells cultured for 3 d, the cells proliferated at different concentrations of Resv: 0, 0.1, 1 and 10 µM.*

Resv significantly decreases mRNA and protein expression of PLSCR1 in HeLa cells

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

Resv inhibition on HeLa cells after Resv treatment can be recovered by gain of function of PLSCR1

To investigate the role of *PLSCR1* in the cell growth inhibition caused by Resv, CCK-8 assay was performed on cultured Hela cells at various stages 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 Resv treatment can be recovered by gain of function assay *PLSCR1* (Figure 3).

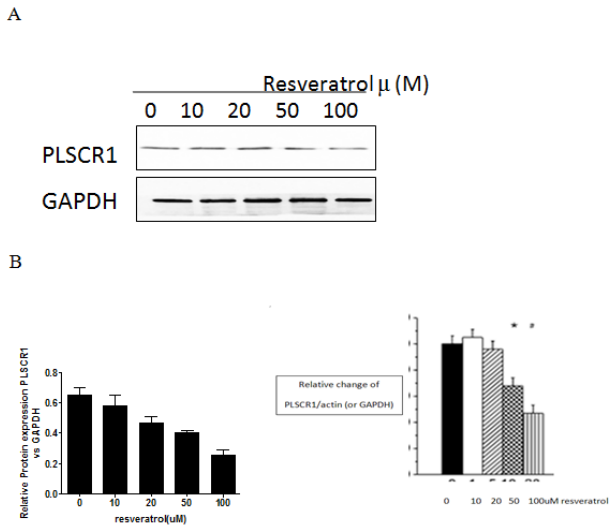


Figure 2. *Resv significantly decrease mRNA level and protein expression of PLSCR1 in HeLa cells. (A) mRNA level of PLSCR1 (B) Statistical analysis of protein expression; (C) mRNA expression of PLSCR1; (d)Statistical analysis of protein expression.*

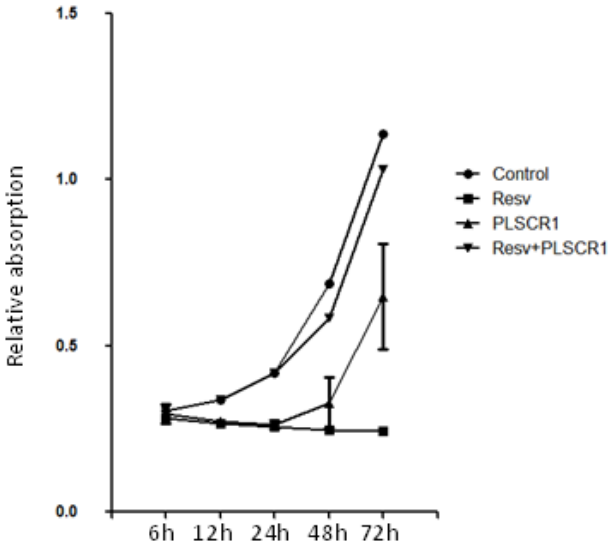


Figure 3. *Resv reduced the growth and proliferation of HeLa cells by downregulation of PLSCR1, HeLa cells can be recovered after Resv treatment by gain of function assay PLSCR1.*

Resv inhibits the tumorigenicity of cervical cancer Hela cells in vivo

To verify the negative role of Resv in cervical cancer cells proliferation, we performed immunochemical staining on Hela cells at d 3, 14 and 28 after Resv treatment. Expectably, the expression of *PLSCR1* in control cells was up-regulated on d 14 and 28. Meanwhile, the expression of *PLSCR1* in Resv-treated cells only showed weak up-regulation on both d 14 and 28 (Figure 4). It implicated *PLSCR1* might contribute to the Resv-mediated cell death.

To verify the negative role of Resv in cervical cancer progression *in vivo*, we performed xenograft tumor assays using Hela cells. The animals were treated with Resv daily respectively. We found that Resv significantly inhibited xenograft tumor growth in nude mice (Figure 5A). The weight of tumors is shown (Figure 5B). These data collectively indicate that Resv acted as a novel tumor-supressing molecule and negatively regulates cervical tumor growth.

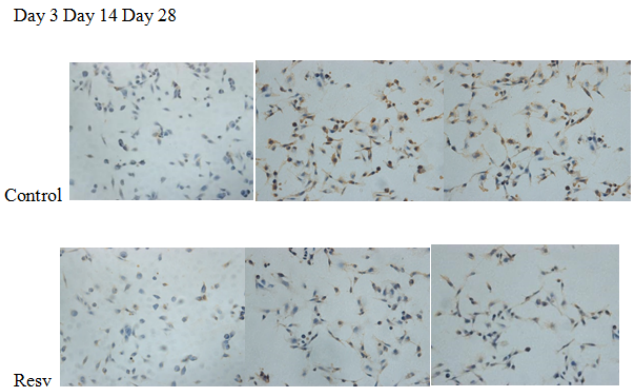
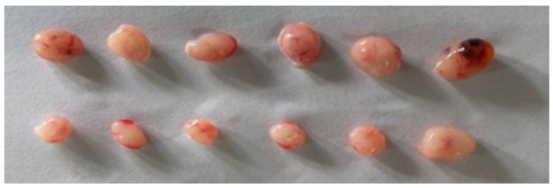


Figure 4. *The PLSCR1 expression was downregulated by Resv treatment during Hela cell proliferation.*



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 () B: The weight of tumors from xenograft models treated with Resv.*

Discussion

In this study, we investigated the inhibition effect of resveratrol on the occurrence and development of cervical cancer and discussed the *PLSCR1*-related mechanism behind the observed efficacy. It's well known that cervical cancer is currently the most common female cancer with an extremely poor prognosis. Cervical cancer occurs frequently in women worldwide and one woman dies because of cervical cancer

every two minutes. Currently, there is no good cure to this cancer, especially to high-risk patients [19-21]. The inhibitory effect of Resv on cervical cancer 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 inhibit the promoter activity of PMA-stimulated MMP-9. Further study showed that resveratrol suppresses the transcription of MMP-9 by the inhibition of both NF-κB and AP-1 transactivation. These results indicate that resveratrol inhibits both NF-κB and AP-1 mediated MMP-9 expression, results in suppression of migration and invasion of cervical cancer cells. Resveratrol has potential for clinical use in preventing invasion by human metastatic lung and cervical cancers [22].

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

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Ethics, Consent and Permissions

Ethical approval was given by the First Affiliated Hospital of Jinan University

Consent to Publish

All of the authors have consented to publish this research.

Author's Contribution

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

Interest Conflict

All of the authors have no conflict of interest in this research.

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