Dietary Resveratrol Prevents Development of High-Grade Prostatic Intraepithelial Neoplastic Lesions: Involvement of SIRT1/S6K Axis

Guiming Li1, Paul Rivas1, Roble Bedolla1, Dinesh Thapa1, Robert L. Reddick2, Rita Ghosh1,3, and Addanki P. Kumar1,3,4

Abstract

SIRT1 (mammalian ortholog of the yeast silent information regulator 2) is a NAD-dependent histone deacetylase belonging to the multigene family of sirtuins. Anecdotal and epidemiologic observations provide evidence for beneficial effects of the calorie restriction mimetic resveratrol (RES), a SIRT1 activator in preventing cardiovascular diseases and cancer. Although SIRT1 possesses both tumorigenic and antitumorigenic potential, the molecular mechanisms underlying SIRT1-mediated tumor progression or inhibition are poorly understood. In this study, we investigated the role of SIRT1 in multiple human prostate cancer cell lines and prostate-specific PTEN knockout mouse model using resveratrol. Androgen-independent prostate cancer cell lines (C42B, PC3, and DU145) express higher levels of SIRT1 than androgen-responsive (LNCaP) and nontumorigenic prostate cells (RWPE-1). Resveratrol enhanced this expression without any significant effect on SIRT1 enzymatic activity. Inhibition of SIRT1 expression using shRNA enhanced cell proliferation and inhibited autophagy by repressing phosphorylation of S6K and 4E-BP1. These biologic correlates were reversed in the presence of resveratrol. Analysis of prostates from dietary intervention with resveratrol showed a significant reduction in prostate weight and reduction in the incidence of high-grade prostatic intraepithelial neoplastic (HGPIN) lesions by approximately 54% with no significant change in body weight. Consistent with the in vitro findings, resveratrol intervention in the PTEN knockout mouse model was associated with reduction in the prostatic levels of mTOR complex 1 (mTORC1) activity and increased expression of SIRT1. These data suggest that SIRT1/S6K-mediated inhibition of autophagy drives prostate tumorigenesis. Therefore, modulation of SIRT1/S6K signaling represents an effective strategy for prostate cancer prevention. Cancer Prev Res; 6(1); 27–39. ©2012 AACR.

Introduction

SIRT1 (mammalian ortholog of the yeast silent information regulator 2) is a NAD-dependent histone deacetylase belonging to a multigene family of sirtuins that contains 7 members with distinct and diverse functions. There is increasing evidence that SIRT1 is a longevity protein that mediates the process of aging (1). SIRT1 has been shown to be involved in: (i) mediating cellular metabolism and energy production through regulation of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-α), FOXO1 (Forkhead box protein O1) activity, and insulin sensitivity; (ii) modulation of inflammatory responses through deacetylation of NF-κB subunit p65 and modulation of T-cell tolerance in response to various stimuli; (iii) regulation of cell growth through inhibition of mTOR activity; and (iv) protection from apoptosis in response to genotoxic stresses (1–5). Collectively, these data suggest that SIRT1 regulates a wide variety of cellular processes including cell growth, survival, proliferation, apoptosis, and autophagy. Consistent with these findings, elevated expression of SIRT1 has been observed in a number of cancers and higher levels of SIRT1 are associated with poor prognosis (6–11). Overexpression of SIRT1 has been shown to be oncogenic in cell culture models (12). On the other hand, some preclinical data indicate that SIRT1 possesses strong tumor suppressor activity (13–18): (i) increased SIRT1 expression delays development of sarcoma and lymphoma in p53-heterozygous mice; (ii) SIRT1 transgenic mice are protected from metabolic syndrome-driven liver...
cancer; (iii) whole body or tissue-specific expression of SIRT1 in genetically engineered animal models established protection from various abnormalities including cancer, cardiovascular, and metabolic syndrome–associated diseases; and (iv) disruption of SIRT1 in the prostate results in the formation of prostatic intraepithelial neoplastic (PIN) lesions. Interestingly, although SIRT1 expression has been reported to be elevated in prostate tumors, it is not entirely clear whether SIRT1 is functionally active in this situation. Overall, these data suggest that SIRT1 could function to either promote or suppress tumorigenesis and indicate a strong need for clarification of its role in tumor development.

Resveratrol (3,4′-trihydroxystilbene), a natural product from grapes that is present in significant concentrations in red wine, has the ability to inhibit growth and induce apoptosis in wide variety of tumor cell lines and has been reported to activate SIRT1 (19). The potential efficacy of resveratrol as a chemopreventive agent for cancer has attracted considerable interest because of studies showing its antitumorigenic activity both in vitro and in vivo in various tumor models, including prostate. Using the transgenic adenocarcinoma of a mouse prostate (TRAMP) model that develops spontaneous prostate tumors, dietary administration of 625 mg/kg resveratrol (TRAMP) model that develops spontaneous prostate tumors to animals showed reduction of adenocarcinoma by 7.7-fold (20). Another study examined the efficacy of liposomal-encapsulated resveratrol (50 mg/kg) in a limited number of PTEN knockout mice and showed reduction of adenocarcinoma (21). However, the rationale for using encapsulated resveratrol is not clear, and none of these studies addressed the ability of resveratrol to prevent the development or progression of high-grade PIN (HGPIN) lesions. Given the high frequency of HGPIN lesions in men in their sixth and seventh decades (41% and 61%, respectively), a high frequency of HGPIN lesions in men in their sixth and seventh decades (41% and 61%, respectively), a better strategy may be the use of resveratrol to prevent the progression of HGPIN lesions, which are putative precursors of prostate cancer (22).

However, to the best of our knowledge, no studies have explored the efficacy of resveratrol for preventing or delaying the development of PIN lesions. Given the preponderance of PTEN mutations in both primary (~30%) and advanced metastatic prostate tumors (~60%)–70%), we explored the efficacy of resveratrol intervention using a prostate-specific PTEN knockout mouse model that develops PIN and prostate cancer (23). We provide the first demonstration that resveratrol intervention reduces the incidence of HGPIN lesions and prostate weight with no significant change in body weight, suggesting that SIRT1 might be a novel therapeutic target for prostate cancer management. In addition, we show that resveratrol inhibits proliferation of both androgen-responsive and androgen-independent prostate cancer cells, primarily through induction of SIRT1-mediated autophagy via inhibition of the phosphorylation of S6K and 4E-BP1, thus implicating the Akt/mTOR signaling pathway in the function of SIRT1 as a tumor suppressor.

Materials and Methods

Chemicals

Resveratrol was purchased from Sigma-Aldrich, dissolved in dimethyl sulfoxide as 10 mmol/L stock, and stored in aliquots at –20°C. Resveratrol purchased from LaliLab, Inc was used in the preparation of diet for animal studies.

Cell culture studies

Human prostate cell lines RWPE-1, LNCaP, PC3, and DU145 were purchased from American Type Culture Collection (ATCC). RWPE-1 cells were cultured in keratinocytes serum-free media (K-SFM) supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL EGF plus 100 units penicillin and 100 μg streptomycin (hereafter referred to as antibiotics); LNCaP and DU145 cells were grown in RPMI-1640 media containing 10% FBS and antibiotics; PC3 cells were grown in F12-K media containing 10% FBS plus antibiotics; C42B cells obtained from Dr. Thambi Dorai (Department of Biochemistry and Molecular Biology, New York Medical College, NY) were grown in T-media containing 5% heat-inactivated FBS plus antibiotics. Cells were treated with the indicated reagents when they were approximately 80% confluent as described previously (24). The authors did not authenticate RWPE-1, LNCaP, PC3, and DU145 cells obtained from ATCC and C42B from Dr. T. Dorai.

qPCR analysis

Total cellular RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s recommendations. A 2-step real-time (RT)-PCR method was used to synthesize single-strand cDNA with a superscript VILO cDNA synthesis kit (Invitrogen). Target genes were analyzed using 7300 Applied Biosystems with SYBR Green 1 dye. The primers used were as follows: SIRT1 (NM_012238), forward ACCCGAACATAGACCCGTGAA and reverse TCTCCTCGTACACCTCACAGTCA and β-actin (NM_001101), forward GGGCAACACGCAAATGAAACTGAA and reverse TAGAAGCATTGCGGTGGACGATG. PCR reactions were conducted in triplicate, and relative mRNA expression was normalized to β-actin and control sample mRNA levels, respectively. The specific amplification of target genes was validated using a dissociation curve. Quantitative PCR (qPCR) was performed 3 times independently.

Establishment of stable DU145 and PC3 SIRT1 knockdown cells

Puromycin and lentiviruses expressing control or SIRT1 shRNA pool were purchased from Santa Cruz Biotechnology. The optimal concentration of puromycin for selection and maintenance of SIRT1 knockdown cells was titrated before use following the manufacturer’s instructions. The procedures for lentivirus transduction were as follows: On day 1, 2.5 × 10⁶ DU145 or PC3 cells and 1 mL of growth media were plated in 12-well plates and incubated overnight. On day 2, the cells were gently washed with 1× Dulbecco’s PBS (DPBS). Lentiviruses diluted in 0.5 mL of growth media (MOI 10) plus 5 μg/mL polybrene were
added to the monolayer of cells. After 24-hour infection, the viruses were removed and the cells were washed twice with 1× DPBS and incubated with fresh growth media for another 24 hours, followed by addition of 1 μg/ml puromycin for SIRT1 knockdown cell selection and maintenance. In every 3 days, the media was replaced with fresh media containing 1 μg/ml puromycin. The efficiency of SIRT1 knockdown was verified using Western blotting and q-PCR.

**SIRT1 activity assay**

Cell lysates were prepared as described previously (23). Briefly, the cells were lysed with lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 0.5% Triton X-100, and 150 mmol/L NaCl] containing 1% protease inhibitor cocktail on ice and centrifuged at 12,000 × g for 15 minutes. The supernatants were taken as cell lysates. Ten micrograms of the cell lysate from each sample were mixed with the Cyto-ID autophagy detection kit (Enzo Life Science Inc) following the manufacturer’s instructions. Briefly, cells were plated in 24-well plates. After treatment, the medium was carefully removed, 100 μL dual detection reagent was added, and the cells were incubated at 37°C for 30 minutes in the dark. The cells were then carefully washed twice with 1× assay buffer, and stained cells were imaged under a Nikon fluorescence microscope installed with NIS element software.

**Immunoblot analysis and Akt kinase activity assay**

Whole-cell lysates were prepared and immunoblot analysis was conducted as described previously (24). Primary antibodies against phospho-PDK1, phospho-Akt (S473), phospho-mTOR, phospho-S6K, phospho-FOXO3α, phospho-4E-BP1 (Ser65), total FOXO3α, and LC3 were obtained from Cell Signaling Technology and used at a dilution of 1:500. Monoclonal antibody against β-actin was purchased from Sigma-Aldrich and used at a dilution of 1:5,000. Antibody against SIRT1 was obtained from ABCAM Inc and used at a dilution of 1:1,000. An ECL method was used for detection of specific proteins and the images were processed and analyzed by SYNGENE gel imaging system (SYNGENE Inc.).

**Colony formation and MTT assay**

For colony formation, PC3 and DU145 cells stably expressing scrambled or SIRT1 shRNA were seeded in 12-well plates at a density of 200 cells per well in 2 mL of growth media and incubated for 12 days. On day 12, the colonies were stained with crystal violet and the visible colony numbers were counted. MTT assay was conducted as follows: prostate cancer (PCA) cells were seeded in triplicate in 96-well plates at a density of 2 × 10^3 cells per well in 100 μL growth media and incubated overnight. The cells were treated with increasing doses of resveratrol for different time periods and cell proliferation was measured as previously described (24).

**Cell survival and growth analyses**

Cells were plated in 6-well plates at a density of 5 × 10^4 cells per well in triplicate and the cell number was counted at 24, 48, 72, and 96 hours using the Trypan blue exclusion method. Apoptosis was measured using Annexin V staining and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay. DNA fragmentation, and cell-cycle distribution changes were determined as described previously (24).

**Autophagy analysis**

Autophagy was measured by fluorescence microscopy with the Cyto-ID autophagy detection kit (Enzo Life Science Inc) following the manufacturer’s instructions. Briefly, cells were plated in 24-well plates. After treatment, the medium was carefully removed, 100 μL dual detection reagent was added, and the cells were incubated at 37°C for 30 minutes in the dark. The cells were then carefully washed twice with 1× assay buffer, and stained cells were imaged under a Nikon fluorescence microscope installed with NIS element software.

**Animal studies**

Animals were handled in accordance with established humane guidelines and protocols approved by the Institutional Animal Care and Use Committee University of Texas Health Science Center at San Antonio (San Antonio, TX). Homozygous male prostate-specific PTEN knockout mice were generated by custom breeding and obtained from Jackson Laboratories (The Jackson Laboratories). All mice were maintained in a climate-controlled environment with a 12-hour light/dark cycle. Diet and water were supplied ad libitum. For in vivo experiments, a pelleted AIN 76A diet containing different doses of resveratrol (0.1% and 2%) was prepared at Dyets, Inc. and administrated to 4- to 5-week-old animals. Standard AIN 76A without resveratrol was used as the control diet. Animals were monitored for body weight changes weekly and food consumption was measured. After 14 weeks of treatment, the animals were euthanized and necropsy was conducted. At necropsy, animals were examined for gross organ abnormalities. Prostate tissue or tumors were harvested, weighed, and fixed in 10% neutral-buffered formalin. Tumors were analyzed using a light microscope in a blinded fashion.

**Immunohistochemical analysis**

Immunohistochemical analysis was conducted according to established protocols. Briefly, sections from paraffin-embedded tissues were deparaffinized and rehydrated with hematoxylin and eosin (H&E) to visualize cell nuclei and cytoplasm. Prostate lesions were scored using an established grading system based on the studies of Park and colleagues and Shappell and colleagues (25, 36). The slides were reviewed using a light microscope in a blinded fashion.
Carpenteria). Slides were developed using a polymer detection system (Biocare Medical Concord) and a DAB Chromogen System (DAKO).

**Immunohistochemical evaluation**

Immunohistochemical staining was evaluated using a semiquantitative scoring method based on proportion (assessment based on number of cells staining none to 100% positive) and intensity of staining (negative to strong) as described by Ganapathy and colleagues before (24).

**Statistical analysis**

All numerical results are expressed as mean ± SD derived from 3 independent experiments, unless otherwise stated. Statistical analyses were conducted using one-way ANOVA and statistically significant differences were established as $P < 0.05$.

**Results**

**Expression of SIRT1 in human prostate cancer cell lines**

To investigate the role of SIRT1 in prostate cancer, we investigated SIRT1 expression in the human prostate cancer cell lines RWPE-1, LNCaP, C42B, PC3, and DU145. Comparison of SIRT1 mRNA expression among these cell lines showed that SIRT1 mRNA levels were higher in both androgen-responsive LNCaP and androgen-independent PC3 and DU145 cells than in the nontumorigenic RWPE-1 cell line (Fig. 1A). Following treatment with resveratrol, the expression of SIRT1 mRNA significantly increased as a
function of time up to 48 hours in all cell lines, with DU145 cells showing the greatest level of induction (10-fold; Fig. 1B). Consistent with this observation, androgen-independent PC3 and DU145 cells also showed higher expression levels of SIRT1 protein (more than 20-fold compared with RWPE-1; Fig. 1C). However, resveratrol treatment decreased the protein levels of SIRT1 selectively in PC3 and DU145 cells with no significant change in LNCaP, RWPE-1, and C42B cell lines (Fig. 1D and data not shown). Interestingly, although we did not observe any significant change in the levels of SIRT1 protein in DU145 cells at 48 hours, we saw an increase at 24 hours time point under these experimental conditions. On the other hand, resveratrol treatment resulted in decreased levels of SIRT1 protein in PC3 cells both at 24- and 48-hour time points. The precise reasons for the observed discrepancy between RNA expression and protein levels is not clear; however, we speculate that this could be due to increased mRNA stability or reduced translation efficiency, for example, through a block in the protein translation machinery. Next, we investigated whether the observed alterations in SIRT1 expression are reflected by changes in SIRT1 enzymatic activity using PC3 and DU145 cells, which showed the highest expression of SIRT1. Although previous studies had shown that resveratrol is an activator of SIRT1, we found that resveratrol did not activate SIRT1 activity under our experimental conditions (Fig. 1E). A recent study indicates that resveratrol may not be a direct SIRT1 activator (27). Taken together, our data show that: (i) expression levels of SIRT1 mRNA are elevated in androgen-independent prostate cancer cell lines; (ii) resveratrol further enhances SIRT1 mRNA expression; (iii) resveratrol selectively influences SIRT1 protein expression in PC3 cells; and (iv) resveratrol had no effect on SIRT1 enzymatic activity.

Effect of resveratrol on prostate cancer cell proliferation

Given the different expression levels of SIRT1 in various prostate cancer cell lines, we tested whether the antiproliferative effects of resveratrol depend on SIRT1 status. Nontumorigenic RWPE-1 cells were the most sensitive to resveratrol, followed by DU145 and PC3 (Fig. 2A–C). Among the cell lines tested, androgen-responsive LNCaP cells and an androgen-independent subline C42B were relatively resistant, requiring resveratrol treatment for 72 hours to achieve an antiproliferative effect (Fig. 2D and E). However, 50% growth inhibition could not be achieved in LNCaP cells even with resveratrol concentrations as high as 100 µmol/L. Interestingly, resveratrol showed a biphasic effect in lower concentration (<5 µmol/L) induced proliferation, whereas higher concentrations had an inhibitory effect. Specifically, proliferation of RWPE-1 cells was increased approximately 30% by 5 µmol/L resveratrol (Fig. 2A) and DU145 and PC3 cells displayed a 30% and 80% increase in cell proliferation, respectively, after 72-hour resveratrol treatment compared with no treatment (Fig. 2B and C). At concentrations higher than 50 µmol/L, resveratrol induced 40% to 60% inhibition of cell proliferation. However, LNCaP cells exhibited resistance and even showed a marginal increase in cell proliferation (Fig. 2D) at all concentrations tested. Resveratrol had no effects on proliferation of C42B cells up to 48 hours but showed significant inhibition at 72 hours (Fig. 2E). It is noteworthy that cell lines that express higher basal levels of SIRT1 (PC3, DU145, and LNCaP) were either resistant to resveratrol or required higher concentrations (up to 100 µmol/L) to inhibit cell proliferation. On the other hand, in cell lines with low levels of SIRT1 (RWPE-1), resveratrol showed significant dose-dependent inhibition of cell proliferation at concentrations less than 25 µmol/L. It is noteworthy that low concentrations of resveratrol (<10 µmol/L) have been shown to activate AMPK without decreasing the AMP/ATP ratio, whereas higher concentrations of resveratrol activate AMPK by decreasing energy and increasing the AMP/ATP ratio. Therefore, the observed concentration-dependent biphasic effects of resveratrol under our experimental conditions could be because of alteration in the AMP/ATP ratio (28).

Resveratrol inhibits prostate cancer cell growth through activation of SIRT1

To gain insight into the relationship between SIRT1 and resveratrol-mediated inhibition of cell proliferation, we used pharmacologic (nicotinamide) and genetic approaches (shRNA) to inhibit SIRT1. As shown in Supplementary Fig. S1, nicotinamide treatment alone had no significant effect on proliferation of PC3 cells; however, it caused a significant reduction of 13% in DU145 cells. Resveratrol alone inhibited proliferation at concentrations of 50 µmol/L and higher (Fig. 3A). However, after pretreatment with 300 µmol/L nicotinamide, a similar level of inhibition was achieved with lower concentrations of resveratrol (10 µmol/L) suggesting that inhibition of SIRT1 sensitized these cells. shRNA efficiently and consistently knocked down the expression of SIRT1 by more than 50% and 90% in PC3 and DU145 cells, respectively (Fig. 3B and C). Cell viability assays showed that the growth rate of SIRT1 knockdown cells was enhanced and resveratrol treatment had no effect on growth inhibition, compared with control cells transfected with scrambled shRNA (Fig. 3D). Cells transfected with control-scrambled shRNA showed inhibition of proliferation in response to resveratrol treatment (Fig. 3E), consistent with data presented in Fig. 2. However, SIRT1 knockdown resulted in a higher proliferation rate in both PC3 and DU145 cells, and resveratrol had an inhibitory effect in the knockdown cells (Fig. 3E). Interestingly using the anchorage-independent growth assay, we did not observe any significant difference in the number of colonies between scrambled and SIRT1 shRNA–transfected cells. However, the important observation was that there was an increase in the size of colonies formed in SIRT1 knockdown PC3 and DU145 cells compared with cells transfected with scrambled shRNA (Fig. 3F), although DU145 SIRT1 knockdown cells formed larger colonies than PC3 cells. Resveratrol treatment (50 µmol/L) completely
abolished colony formation in both cell lines independent of SIRT1 status (data not shown).

These data suggest that resveratrol-induced growth inhibitory effects are mediated both by SIRT1-dependent and independent pathways. The observed increase in resveratrol-induced inhibition of proliferation in the presence of nicotinamide could be due to synergistic or additive inhibitory activity of resveratrol and nicotinamide. However, the role of other SIRT family members in mediating resveratrol-induced growth inhibitory effects cannot be ruled out. Taken together, our results and published findings on the prostate model indicate a tumor suppressive role for SIRT1 and suggest that activation of SIRT1 could be a therapeutic strategy for the treatment of prostate cancer.

Resveratrol induces autophagy in prostate cancer cells

Numerous mechanisms have been shown to mediate the antiproliferative effects of resveratrol in various cell types (29–32). Resveratrol has been shown to modulate the expression of genes involved in various processes including apoptosis, survival, and inflammation [including survivin, Akt, NF-κB, and mitogen-activated protein kinase (MAPK)]. Despite numerous studies, the precise mechanism of resveratrol-mediated growth inhibition and the role of SIRT1 remain unclear. We first investigated whether the observed growth inhibitory effects of resveratrol are associated with induction of apoptosis, autophagy, or both. DU145 cells that were stably transfected with SIRT1 shRNA or scrambled control were treated with different doses of...
Resveratrol for 24 hours. Resveratrol treatment resulted in a marginal increase in apoptosis (3%–8%) irrespective of the presence or absence of SIRT1, suggesting that resveratrol-induced apoptosis is independent of SIRT1 (Supplementary Fig. S2). Similar results were obtained using independent approaches including DNA fragmentation and TUNEL assay (data not shown). In addition to apoptosis, cancer cells can die through the process of autophagy. Because we observed only a marginal increase in apoptosis, we investigated whether resveratrol inhibits prostate cancer cell proliferation through induction of autophagy using a CytoID autophagy detection kit. Autophagosome formation was observed under the fluorescence microscope as dot-like structures distributed within the cytoplasm and in the perinuclear region (Fig. 4A). The number of these structures increased in the presence of resveratrol, indicating induction of autophagy. Resveratrol-induced autophagosome formation was higher in DU145 cells than in any other cell line tested. In addition, we analyzed the expression levels of LC3 II and I using immunoblotting. As shown in Fig. 4B, RWPE-1 and C42B cells contained only the cleaved, faster-migrating form of LC3 II, indicating high

Figure 3. Effect of pharmacologic and genetic inhibition of SIRT1 on proliferation of PC3 and DU145 cells. A, androgen-independent PC3 and DU145 prostate cancer cells were plated in 96-well plates at a density of 4 × 10³ cells per well. Following overnight incubation at 37°C, cells were pretreated with 300 μmol/L nicotinamide for 24 hours followed by treatment with the indicated concentrations of RES. Cell proliferation was measured after an additional 24 hours as described in Fig. 2. Asterisk represents statistically significant difference between nicotinamide plus RES and RES alone. B–E, stable clones were generated using a scrambled or SIRT1 shRNA pool as described in the Methods sections. The efficiency of SIRT1 knockdown was verified using qPCR (B) and Western blot analysis (C). PC3 (A) and DU145 (B) cells stably expressing scrambled or SIRT1 shRNA were plated into 6-well plates at a density of 5 × 10⁴ cells per well and incubated for the indicated time. Cell numbers were counted using the Trypan blue exclusion method (D). Cell proliferation was measured as in Fig. 2E (E). PC3 and DU145 cells stably expressing scrambled or SIRT1 shRNA were plated on 12-well plates at a density of 200 cells per well and incubated for 12 days with media changes every 48 to 72 hours. Colonies were visualized by crystal violet staining (F). Data shown are representative of 3 independent experiments (mean ± SD).
basal levels of autophagy. We observed increased formation of cleaved LC3 II in LNCaP cells in response to resveratrol treatment, indicating induction of autophagy. On the other hand, DU145 cells expressed only LC3 I, the intensity of which decreased following treatment with resveratrol (Fig. 4B). It is, however, possible that we did not detect cleaved LC3 II in these cells despite autophagosome formation because of conjugation of LC3 to the autophagosome membrane, which in turn could be targeted for degradation. Knockdown of SIRT1 in DU145 cells significantly attenuated resveratrol-induced autophagy as evidenced by decreased autophagosome formation (Fig. 4C). These data suggest that SIRT1 plays an important role in resveratrol-induced autophagy in prostate cancer cells.

**Resveratrol induces autophagy through SIRT1/S6K**

Given the known roles of Akt/mTOR signaling in mediating autophagy (33–36), we examined the impact of resveratrol on Akt/mTOR signaling by analyzing the levels and activity of Akt/mTOR signaling components in prostate cancer cells. Resveratrol treatment had no effect on Akt phosphorylation (T308 or S473) in LNCaP and C42B cells. Consistent with the wild-type status of PTEN, we did not detect phosphorylated Akt in RWPE-1 or DU145 cells (Fig. 4D). Most significantly, we observed inhibition of phosphorylated S6K in response to resveratrol treatment in all cell lines tested (Fig. 4D). We next explored whether knockdown of SIRT1 expression in DU145 cells enhanced the level of phosphorylated S6K. To test the relationship between SIRT1 and pS6K, we analyzed levels of S6K and pS6K in DU145 cells in which SIRT1 was silenced. As shown in Fig. 4E, resveratrol treatment decreased the level of pS6K in control scrambled knockdown cells and SIRT1 knockdown partially (~43% inhibition) rescued the resveratrol-induced downregulation of pS6K. These data show that the reduced production of pS6K in response to resveratrol treatment is mediated by SIRT1 and implicate pS6K as a key player in resveratrol-mediated growth inhibitory effects. It is not clear how the deacetylase SIRT1 reduces pS6K expression, although S6K can be regulated by acetylation/deacetylation (37). Moreover, inhibition of SIRT1 by nicotinamide enhances S6K acetylation, suggesting a potential role for SIRT1 in mediating S6K deacetylation. Resveratrol has also been shown to function as an acetylation inhibitor (38). Although we have not directly shown that S6K can undergo acetylation under our experimental conditions, we speculate that SIRT1-mediated deacetylation could trigger dephosphorylation of S6K. These observations suggest that resveratrol inhibits prostate cancer cell growth through induction of programmed cell death (a combination of apoptosis and autophagy) via SIRT1-mediated inhibition of S6K phosphorylation. We also detected phosphorylated 4E-BP1, GSK3α/β, and FOXO3α in LNCaP and C42B cells, levels of which were not altered in response to resveratrol treatment (Supplementary Fig. S3). Inactivated (phosphorylated) FOXO3α and GSK3α/β promote cell survival by preventing the upregulation of genes involved in apoptosis and autophagy. This could explain why LNCaP and C42B cells were resistant to resveratrol-mediated inhibition of cell proliferation.

**Resveratrol intervention prevents development or progression of HGPIN lesions in prostate-specific PTEN knockout mice**

The PI3K signaling pathway that mediates cell survival plays a central role in tumorigenesis. The levels of...
phosphoinositide-3 (PI3) phosphates in a cell are regulated through the activity of dual specificity tyrosine-threonine/PI3 phosphatase MMAC/PTEN (mutated in multiple advanced cancers/phosphatase and tensin homologue detected in chromosome 10). PTEN prevents accumulation of PI3 phosphates and thus attenuates PI3 kinase (PI3K) signaling. Studies have shown that PTEN is an important tumor suppressor protein that is lost in a large number of human cancers including prostate; for example, approximately 40% of primary and 70% of metastatic prostate tumors have genetic alterations in PI3K signaling pathway through loss of PTEN. Given the connection between Akt/NF-κB and SIRT1, we tested the efficacy of resveratrol in inhibiting prostate cancer development using the prostate-specific PTEN knockout mouse model (23). PTEN knockout mice were generated by deleting PTEN specifically in the prostate epithelium and develop prostate tumors akin to human prostate carcinogenesis (23). These mice develop murine PIN (mPIN) lesions by 6 weeks of age that progress to invasive cancer by 9 to 29 weeks and metastatic cancer by 12 to 29 weeks (23). Treatment of 4- to 5-week-old PTEN knockout mice with resveratrol for 14 weeks resulted in significant histopathologic alterations in the prostate (Fig. 5A). Specifically, prostate from 11 of 15 (73%) animals receiving the control diet showed abnormal histologic features including: (i) enlarged and dilated acini lined by multilayered nuclei of variable size; (ii) mild to moderate nuclear pleomorphism and increased mitotic activity; (iii) presence of cribriform areas; and (iv) thinned stroma with scattered inflammatory cells. These characteristics are consistent with high-grade mPIN lesions (26). In contrast, only 6 of 15 mice receiving low-dose resveratrol and 5 of 12 mice receiving high-dose resveratrol showed such lesions (Table 1). Prostate from these animals showed dilated acinar, often lined by a single layer of cells with bland nuclei with small nucleoli. In addition, mitotic, necrotic, and apoptotic activity was absent in these lesions. Cribriform patterns were present in some areas and the adjacent stroma was loosely cellular. These features are consistent with low-grade mPIN lesions. Cumulative analysis of these data indicated that resveratrol intervention resulted in a decreased incidence (~54%) of grade 3 HGPIN lesions irrespective of the dose (Table 1). In addition, resveratrol intervention resulted in a significant decrease in the weight of the genitourinary complex at the high dose (2%), but not at the low dose (0.1%) despite pathologic changes at both doses (Fig. 5B). Importantly, resveratrol intervention had no toxic effects on these mice, as evidenced through negligible changes in body weight (Supplementary Fig. S4). Furthermore, we did not observe any significant differences in their food consumption (Supplementary Fig. S4). This is the first demonstration that resveratrol can inhibit PIN lesions in an animal model that is highly relevant to human pathogenesis.

**Resveratrol inhibited formation of PIN lesions in vivo through the Akt/mTOR signaling pathway**

To examine whether resveratrol inhibited PIN lesion formation as a result of alterations in the Akt/mTOR signaling pathway, we analyzed prostate tissues from resveratrol-treated PTEN knockout mice and age-matched controls using immunohistochemical analysis. We observed decreased staining for pS6K and increased staining for
SIRT1 (changes were more prominent at high dose) with no change in the expression of pAkt, pmTOR, cleaved caspase-3, pStat3, or androgen receptor following resveratrol treatment of PTEN knockout mice (Fig. 6A and data not shown). Semiquantitative analysis of these data show that there was about 29% and 57% decrease in pS6K staining in low- and high-dose resveratrol, respectively, compared with control. On the other hand, SIRT1 staining showed marked increase only in the high-dose resveratrol (75% increase) compared with control. We also did not observe any significant changes in the proliferation as evidence by Ki67 staining. These data suggest that the in vivo mechanism of resveratrol action involve SIRT1-mediated inactivation of pS6K. A hypothetical model of this mechanism is shown in Fig. 6B.

Discussion

Sirtuins are histone deacetylases that play a key role in the regulation of life span of lower organisms and in post-translational modifications of proteins including p53 and NF-κB, thereby regulating diverse cellular processes (1–18). Elevated expression of SIRT1 has been reported in a number of primary cancers as well as in chemotherapy-resistant cancers (39, 40). SIRT1 has been shown to possess both tumor promoting and tumor suppressor activities (1–18). Recently, a screen for modulators of SIRT1 activity identified the polyphenolic compound resveratrol as the most potent activator of SIRT1 in human cells (41). Anecdotal observations coupled with epidemiologic studies provide evidence that resveratrol has beneficial effects in lowering the risk of cardiovascular diseases and cancer (42). Resveratrol has been reported to exhibit antitumorigenic activity both in vitro and in vivo and chemopreventive activity in a variety of tumor models including skin, liver, colon, breast, lung, and esophageal cancers (29). Resveratrol was also reported to inhibit tumor growth in a TRAMP model (20), although a limitation of that study was the selection of adenocarcinoma as the end point rather than PIN lesions, which are putative precursor lesions for prostate cancer. Given the high frequency of HGPIN lesions in men in their sixth and seventh decades of life (41% and 61% respectively), a better aim might be the development of resveratrol as a preventive agent against the progression of HGPIN lesions (22). To the best of our knowledge, no studies have explored the efficacy of resveratrol in preventing or delaying the development of PIN. It should be mentioned that liposomal-encapsulated resveratrol (50 mg/kg) has been reported to reduce adenocarcinoma in PTEN knockout mice (21) although the lack of information on the potential of nonencapsulated resveratrol and small sample size (n = 3) preclude drawing any firm conclusions.
underlying mechanism of action using in vitro cell culture models and tissues generated from in vivo models.

We found that SIRT1 expression was significantly higher in more aggressive cancer cells than in normal or less aggressive prostate cancer cells (Fig. 1) and that resveratrol treatment induced SIRT1 expression at the RNA level. Interestingly, our data showed differential growth inhibitory effects of resveratrol in prostate cancer cells (Fig. 2); androgen-responsive LNCaP and androgen-independent C42B cells were relatively resistant to resveratrol treatment. Strikingly, we observed that at low doses (5 μmol/L), resveratrol treatment stimulated, rather than inhibited, cell proliferation (Fig. 2). Mitogenic effects of resveratrol were also reported in breast cancer cells (43). The mechanism by which resveratrol stimulates cell proliferation at low doses involves activation of NF-κB or possibly alteration in AMP/ATP ratios (28, 43). Recent studies also indicated that resveratrol might not be a direct activator of SIRT1 (27).

Consistent with these data, we did not observe SIRT1 activation by resveratrol under our experimental conditions despite alterations in SIRT1 RNA and protein levels. In contrast, stable knockdown of SIRT1 in DU145 and PC3 cells revealed a tumor suppressive role, with knockdown cells exhibiting enhanced proliferation and viability and decreased autophagy compared with control cells transfected with scrambled shRNA (Fig. 3 and 4). Treatment with resveratrol reversed these end points, presumably through induction of SIRT expression. Our findings are in disagreement with those of Kojima and Jun-Hynes and colleagues, who showed a tumor-promoting role for SIRT1 in DU145 cells using pharmacologic and genetic inactivation (39, 44).

Although the exact reason for this discrepancy is not clear, one possible explanation could be differences in experimental conditions. However, our observations are in agreement with published findings showing that overexpression of SIRT1 inhibits growth and proliferation of androgen-responsive prostate cancer cells and that activation of SIRT1 inhibits hormone-induced activation of androgen receptor (45). Furthermore, SIRT1 knockdown using shRNA has been shown to accelerate tumor formation in a colon cancer model (46).

Mechanistically, SIRT1 mediates a broad spectrum of functions through deacetylation of NF-κB, p53, and FOXO3, leading to the suppression of apoptosis. Previous studies indicated that resveratrol mediates its anticancer roles through induction of cancer cell apoptosis. However, our results indicate that resveratrol treatment induced only up to 10% apoptosis in DU145 cells but resulted in a significant increase in autophagy (Fig. 4). In addition, SIRT1 knockdown in DU145 attenuated the autophagy induced by resveratrol treatment, implying that SIRT1 is a positive regulator of autophagy in DU145 cells (Fig. 4). Recent studies indicate that inhibition of the Akt signaling pathway not only induces apoptosis, but also enhances autophagy (47). Our analysis of the effects of resveratrol on components of the Akt signaling pathway indicated that resveratrol inhibits multiple phosphorylation events, including S6K and 4E-BP1, with little to no significant effect on other Akt/mTOR components (Fig. 4). Our results have clearly shown that resveratrol-induced cell death is primarily mediated by autophagy with a minor contribution from apoptosis. It is noteworthy that inhibition of SIRT1 by the pharmacologic inhibitor nicotinamide enhances S6K acetylation, suggesting a potential role for SIRT1 in mediating S6K deacetylation (38). Although we have not shown that S6K can undergo acetylation under our experimental conditions, we speculate that SIRT1-mediated deacetylation could trigger dephosphorylation of S6K. These observations suggest that resveratrol inhibits prostate cancer cell growth through induction of programmed cell death (a combination of both apoptosis and autophagy) via SIRT1-mediated inhibition of S6K phosphorylation.

This is the first demonstration that the natural compound resveratrol inhibits the incidence of PIN lesions, a known precursor lesion of prostate cancer, without any significant toxicity (Fig. 5). The biologic activity of resveratrol largely depends on the serum and tissue concentrations and toxicity-associated effects. Although we have not measured the tissue or serum levels of resveratrol, based on its ability to decrease the incidence of HGPIN, we assume that it is reaching the target tissue at an effective concentration. Resveratrol has been shown to be minimally toxic in animal models; consistent with this, we did not observe any significant changes in the body weight of treated animals.

It is possible that SIRT1 plays a dual role in tumorigenesis; in the early stage of cancer development when mutations are relatively low and tissue architecture is normal, activation of SIRT1 prevents the development of cancer. However, in advanced stages, where tissue architecture is altered and numerous mutations have occurred, SIRT1 may have a tumor promoting role. One of the biggest challenges in PCA management is the lack of effective therapeutic options for castrate-resistant disease. In this regard, our data that show the ability of resveratrol to induce autophagy through SIRT1 in the androgen receptor-negative cell line (DU145) is noteworthy. We are also aware that one of the limitations of translating resveratrol to clinic applications is its low bioavailability, resulting from a half-life of approximately 9 hours due to rapid metabolism and elimination (48).

Therefore, our future studies will focus on identifying or developing more stable forms of resveratrol or analogs that cannot be rapidly metabolized or enhancing its bioavailability through combinatorial approaches. The present studies clearly warrant further in-depth investigation to determine whether the stage of intervention determines the functional ability (tumor suppressor vs. tumor promoting) of SIRT1. Nonetheless, we have identified SIRT1/S6K as a novel therapeutic target that could be developed for the management of prostate cancer, and possibly other malignancies in which Akt/mTOR signaling is deregulated.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Dietary Resveratrol Prevents Development of High-Grade Prostatic Intraepithelial Neoplastic Lesions: Involvement of SIRT1/S6K Axis

Guiming Li, Paul Rivas, Roble Bedolla, et al.


**Updated version**
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-12-0349

**Supplementary Material**
Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2012/11/20/1940-6207.CAPR-12-0349.DC1

**Cited articles**
This article cites 48 articles, 19 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/6/1/27.full.html#ref-list-1

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.