Control of Multidrug Resistance Gene *mdr1* and Cancer Resistance to Chemotherapy by the Longevity Gene *sirt1*

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Abstract

Irreversible growth arrest (also called senescence) has emerged recently as a tumor suppressor mechanism and a key determinant of cancer chemotherapy outcome. Previous work from our laboratory suggested that the cellular ability to undergo or to escape senescence dictates its fate to become drug-sensitive or drug-resistant, respectively. In the present study, we made the hypothesis that longevity genes, by virtue of their ability to inhibit senescence, may contribute to the onset of drug resistance. We report that expression of the longevity gene *sirt1* increased both at the RNA and protein levels in all the five drug-resistant cell lines tested when compared with their drug-sensitive counterparts. In addition, biopsies from cancer patients treated with chemotherapeutic agents also expressed high levels of this molecule. These changes were specific for *sirt1* because the expression of other members of its family was not affected. More importantly, small interfering RNA–mediated down-regulation of *sirt1* significantly reversed the resistance phenotype and reduced expression of the multidrug resistance molecule P-glycoprotein. This was further confirmed by ectopic overexpression of *sirt1*, which induced expression of P-glycoprotein and rendered cells resistant to doxorubicin. Collectively, these findings uncovered a novel function for the longevity gene *sirt1* as a potential target for diagnosis and/or treatment of cancer resistance to chemotherapy. They also describe a proof of principle that signaling pathways implicated in longevity may share similarities with those leading to development of drug resistance in cancer. (Cancer Res 2005; 65(22): 10183-7)

Introduction

Cellular senescence, traditionally associated with organismal aging, has emerged recently as a key determinant of cancer chemotherapy outcome (1–5). Our laboratory and others have shown that the stress level required for induction of senescence is significantly lower than those necessitated for other cellular toxic responses, such as apoptosis or necrosis (6–8), and that forcing cancer cells to undergo senescence was sufficient for reversal of their resistance phenotype (9). These findings suggested that senescence deficiency may regulate the early events that control development of resistance to chemotherapy. Thus, identification and targeting of antisenescence genes may have potential therapeutic utility for the prediction and/or prevention of drug resistance. In an effort to identify such genes, we directed our focus to research in the field of aging. Recently, enhanced activity of the silent information regulator (*sir2*) gene was found to be associated with increased longevity in yeast (10, 11), worm (12), flies (13), and rodents (14). Its human homologue, *sirt1*, was also described to be associated with longevity and was believed to act primarily by inhibiting cellular senescence (15–19). This later characteristic of *sirt1* prompted us to investigate its potential role in mediating resistance to chemotherapy in cancer. Our results indicate that this gene was overexpressed in all the drug-resistant cell lines tested in our laboratory as well as in tumor specimens from cancer patients treated with chemotherapy. Moreover, a positive regulatory relationship was identified between *sirt1* and the multidrug resistance gene *mdr1*, suggesting that this longevity gene was not only associated with but also plays an active role in this phenomenon.

Materials and Methods

Cell lines, biopsies, and reagents. Human neuroblastoma SKN-SH, osteosarcoma SaOS2, breast cancer MCF7, and 293 cell lines were purchased from American Type Culture Collection (Rockville, MA). Drug-resistant cells were generated by continuous incubation of parental cell lines with stepwise increases in drug concentration over a period of 3 to 6 months. The ovarian cancer cell lines A2780 and IGROV1 and corresponding cisplatin-resistant cells were a generous gift from Dr. Mary C. Hendrix. Tumor biopsies were obtained at surgery and frozen at −70°C until use. DMEM and fetal bovine serum (FBS) were obtained from BioWhittaker (Walkersville, MD). The following drugs and reagents were obtained from the companies cited: doxorubicin, etoposide, and cisplatin (Sigma, St. Louis, MO); antibody to Sirt1 (Novus Biological, Littleton, CO); antibody to β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA); secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA); enhanced chemiluminescence reagents (ECL, Amersham, Arlington Heights, IL); and Immobilon-P transfer membrane for Western blots (Millipore, Bedford, MA).

Western blot. Cells were seeded in DMEM containing 10% FBS, cultivated for 48 hours, then lysed in a lysis buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 100 mmol/L NaF, 1 mmol/L $MgCl_2$, 1.5 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1 μg/mL leupeptin, 1 mmol/L phenyl-methyl-sulfonyl-fluoride]. Tumor biopsies were homogenized in the lysis buffer described above and centrifuged to remove insoluble material. Equal quantities of protein were separated by electrophoresis on a 12% SDS-PAGE gel and transferred onto Immobilon-P membranes. Proteins of interest were identified by reaction with specific primary and secondary antibodies linked to horseradish peroxidase. Reactive bands were detected by chemiluminescence.

Reverse transcription-PCR. RNA extraction and reverse transcription-PCR (RT-PCR) reactions were done using the GeneAmp RNA PCR kit (part no. N808-0017, Applied Biosystems, Foster City, CA) according to the procedure of the manufacturer. PCR products were visualized on 2% agarose gel with 10% ethidium bromide.

Small interfering RNA design and transfection. The human Sirt1 small interfering RNA (siRNA; ref. 14) was synthesized by Dharmacon (Lafayette, CO). On the day before transfection, 3×10^5 doxorubicin-resistant osteosarcoma cells were seeded into six-well plates and grown in 2.5 mL of DMEM supplemented with 10% FBS. After 24 hours in culture, 25 μL of 20 μmol/L stock solution of siRNA duplexes were transfected into

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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cells using GeneSilencer siRNA Transfection Reagent kit according to the protocol of the manufacturer (Gene Therapy Systems, San Diego, CA). After 24 hours of incubation, cells were treated with doxorubicin and maintained in culture for an additional 48 hours before measuring cell viability and expression of the silenced molecules with Western blot or RT-PCR.

Assay of the mdr1 promoter activity. Cells were cotransfected either with Sirt1 siRNA or the plasmid PGL3 containing the promoter sequence for the *mdr1* gene (20). After 48 hours of incubation, cells were lysed and the luciferase activity measured using the Luciferase Assay System kit (Promega, Madison, WI).

Transfection with *sirt1*. *sirt1* transfectants were produced using 293 cells maintained in DMEM medium supplemented with 10% FCS in six-well plates. The pYSIR2 plasmid (a generous gift from Dr. R. Weinberg; ref. 17) was introduced alone or in combination with the PGL3 plasmid containing the mdr1 promoter region using LipofectAMINE Reagent 2000 following the protocol of the manufacturer (Invitrogen, Carlsbad, CA). After 48 hours, expressions of *sirt1* and *mdr1* were detected by RT-PCR. Expressions of these two genes at the protein level were determined by Western blot. Activation of the mdr1 promoter was assayed as described above.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were incubated with doxorubicin at concentrations varying between 1×10^{-9} and 1×10^{-6} mol/L for 96 hours. Viable cells were quantitatively estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (10 μL of 5 mg/mL solution) was added to each well of the titration plate and incubated for 4 hours at 37°C. The cells were then solubilized by the addition of 100 μL of 10% SDS/0.01 mol/L HCl and incubated for 15 hours at 37°C. The absorbance of each well was determined in an ELISA plate reader using an activation wavelength of 570 nm and a reference wavelength of 650 nm. The percentage of viable cells was determined by comparison with untreated control cells.

Results and Discussion

To establish whether Sirt1 could be associated with development of drug resistance in cancer, we used various drug-sensitive and drug-resistant cancer cell lines representing neuroblastoma, osteosarcoma, mammary, and ovarian cancinomas (9). As shown in Fig. 1*A*, Western blot analysis using a polyclonal antibody to this molecule indicated that its expression was up-regulated in all drug-resistant cells tested when compared with their drug-sensitive counterparts. The validity of this observation seemed to be independent of cell type or the nature of drugs used to generate resistant cells, suggesting that increased Sirt1 expression may represent a general phenomenon associated with resistance to chemotherapy. To verify whether Sirt1 could be associated with treatment response in cancer patients, matching biopsy specimens obtained at either diagnosis or after chemotherapy were analyzed by Western blot (Fig. 1*B*). Although the number of specimen studied was limited ($n = 4$), the data clearly indicate that elevated amounts of Sirt1 were invariably detected following treatment with chemotherapeutic agents. To verify or rule out the possibility that increased Sirt1 amounts occurred at the gene expression or posttranslational

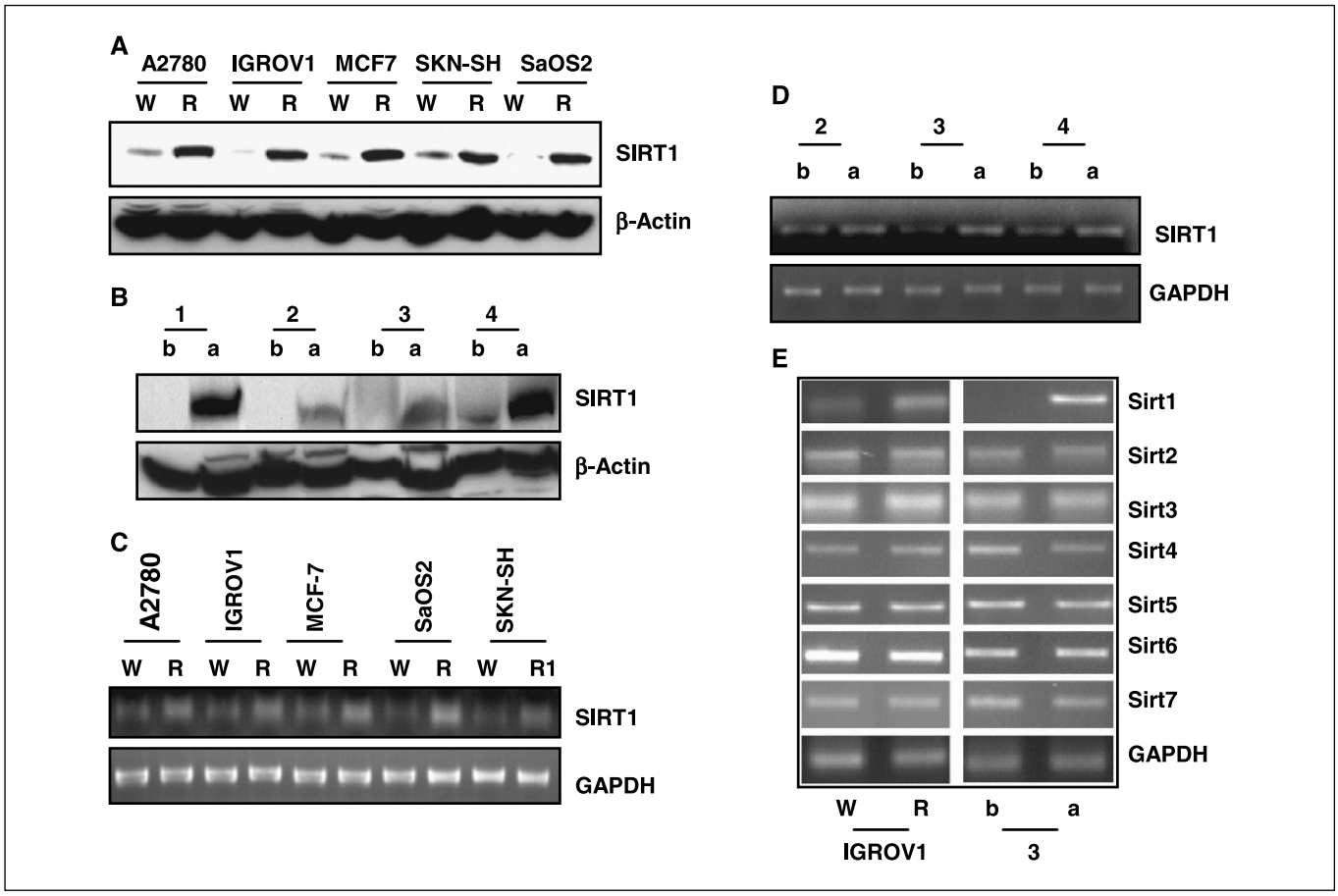

Figure 1. Differential expression of Sirt1 in drug-sensitive and drug-resistant tumors. *A,* Western blot detection of Sirt1 in wild-type (*W*) and drug-resistant (*R*) cancer cell lines. *B,* detection of Sirt1 expression in tumor specimen obtained before (*b*) and after (*a*) chemotherapy treatment from four patients (*1-4*). Anti-β-actin was used in (*A* and *B*) as a loading marker. *C* and *D,* RT-PCR analysis of Sirt1 expression in cancer cell lines and tumor specimen. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. *E,* comparison of Sirt1 expression to other sirtuins by RT-PCR in IGROV1 wild-type cells, their resistant counterparts, and tumor specimen from patient #3 before and after chemotherapy. GAPDH was used as control.

level, pairs of drug-sensitive and drug-resistant cell lines (Fig. 1*C*) and biopsy specimens from untreated and drug-treated patients (Fig. 1*D*) were analyzed for expression of this molecule by RT-PCR. The results were similar to those obtained by Western blot using a polyclonal antibody (Fig. 1*A* and *B*), suggesting that changes in the amount of Sirt1 occurred at the gene expression level.

Sirt1 belongs to a sirtuin family of seven members (21); therefore, we investigated the possibility that other sirtuins may also be associated with drug resistance. The analysis of RT-PCR profiles in IGROV1 cells and their cisplatin-resistant counterpart as well as in specimen from patient #3 (Fig. 1*E*) revealed that except for Sirt1, no other sirtuin seemed to be affected by exposure of cancer cells to therapeutic agents *in vitro* or *in vivo*. These findings suggest that Sirt1 may represent a stress-responsive molecule and, with regards to chemotherapy, this molecule may serve as marker for prognosis.

To determine the stress level and time required for Sirt1 expression, neuroblastoma cells, SKN-SH, were subjected to treatment with 0.5×10^{-8} mol/L doxorubicin, which inhibits proliferation, or 10^{-6} mol/L, which induces apoptosis in these cells (8), and expression of this molecule was measured over a period of 96 hours. The results (Fig. 2*A*) show that increased amounts of Sirt1 were observed at low, but not at high, drug concentrations, suggesting that only the stress levels that inhibit proliferation may activate the Sirt1 pathway. This cellular response was quite rapid because increased amounts of Sirt1 were detected as early as 24 hours of doxorubicin treatment and culminated at 48 hours of incubation. Sirt1 expression in response to drug treatment was also transient and after 72 hours of exposure, a noticeable decrease was observed, perhaps due to reduced drug activity and/or availability. Of particular interest is the correlation of expression of Sirt1 with that of the cell cycle inhibitor p21/WAF1 but not with caspase-3 activation (Fig. 2*A*), which suggests that Sirt1 may exert its protective function only in response to sublethal stress levels. A similar relationship was observed in the ovarian cancer cell line A2780 treated with cisplatin (Fig. 2*B*) and, as in the neuroblastoma cell line, there was also an inverse correlation between the activation of caspase 3 and expressions of Sirt1 and p21/WAF1. This puzzling observation prompted us to hypothesize that Sirt1 may be a substrate for caspase 3. Indeed, we have found that when recombinant Sirt1 was incubated with purified caspase 3, its degradation could be detected as early as 30 minutes (Fig. 2*C*). Together, these findings indicate that Sirt1 is expressed in response to antiproliferative stress levels; however, its protective function may be lost if cells are exposed to apoptosis-inducing stimuli. The relationship between Sirt1 and p21/WAF1 seems to be in favor of the view that Sirt1 may exert its protective function by signaling for a reduction in proliferation rate to allow cellular adaptation and survival in a cytotoxic environment.

To gain further insight into the mechanisms that govern drug resistance through Sirt1, we investigated the possibility that this molecule may regulate, directly or indirectly, the expression and/or activity of genes known to be implicated in drug resistance. The most widely described among these are the drug efflux pump P-glycoprotein (22), which is encoded by the *mdr1* gene, superoxide dismutase (SOD) (23, 24), and glutathione *S*-transferase-π (GST-π), which catalyzes drug inactivation (25). We used siRNAs specific for Sirt1 to knockdown the expression of this molecule in drug-resistant cells and to determine whether this affects expression of these genes. After transfection, the amount of Sirt1 was markedly reduced (Fig. 3*A*); in addition, sensitivity to doxorubicin increased in the transfected cells when compared with their nontransfected or vehicle-treated counterparts (Fig. 3*B*). We have shown previously

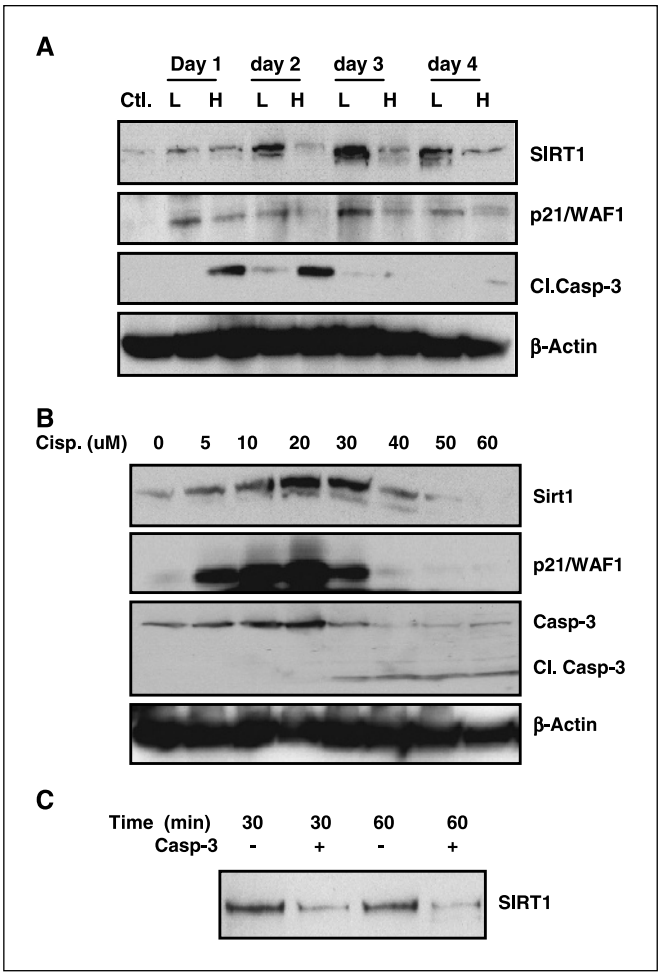

Figure 2. Effect of stress level and duration of drug incubation on Sirt1 expression: correlation with p21/WAF1 expression and caspase-3 activation. *A,* Western blot analysis of expressions of Sirt1, p21/WAF1, cleaved (activated) caspase 3, and β-actin following incubation of SKN-SH cells with low (5×10^{-8} mol/L; *L*) or high (10^{-6} mol/L; *H*) doxorubicin for the indicated times (in days). *B,* ovarian cancer cells A2780 were incubated with cisplatin for 24 hours at the indicated concentrations and expressions of Sirt1, p21/WAF1, caspase 3, activated caspase 3, and β-actin were detected by Western blot. *C, in vitro* cleavage of Sirt1 by caspase 3. Recombinant Sirt1 (50 ng) was incubated at room temperature with purified caspase 3 (50 ng) for 30 minutes or 1 hour and samples were subjected to Western blot using anti-Sirt1 antibody.

that a 1 μmol/L drug concentration leads to the death of 80% of drug-sensitive SaOS2 cells and only 20% of their drug-resistant counter parts (9). Sirt1 siRNA enhances cellular response in the drug-resistant cells by an additional 25% to 30%, resulting in a total cell death of 45% to 50%. Although this is not a complete reversal of drug resistance, it represents a significant enhancement of cellular response to the drug.

These findings indicate that Sirt1 is not only associated with but may also play an active role in the development of resistance to drugs. Significantly, siRNA-mediated down-regulation of Sirt1 was associated with decreased expression of P-glycoprotein but it did not affect the expression of SOD or GST-π (Fig. 3*C*). Similar findings were obtained by RT-PCR (Fig. 3*D*), indicating that regulation of P-glycoprotein expression by Sirt1 occurred at the transcriptional level rather than at the posttranslational level. Further confirmation of the modulatory action of Sirt1 on P-glycoprotein expression is illustrated by the reduction in *mdr1* promoter activity on cotransfection with Sirt1 siRNA (Fig. 3*E*). Cellular proliferation was not significantly altered during the time of the experiment (data

not shown), suggesting that the effect of Sirt1 siRNA on the activity of mdr1 promoter was specific and not due to difference in growth rate.

Gain of Sirt1 function was also investigated to determine whether it may influence *mdr1* expression, and for this we used two approaches: First, we used resveratrol, a phenol contained in red wine that has been shown to induce Sirt1 activity and stimulate its protective function (26, 27). The data indicate that on cellular exposure to this compound for 48 hours, *mdr1* gene expression increased relatively compared with untreated cells as measured by semiquantitative PCR (Fig. 4*A*) and, as expected, the activity of the corresponding promoter was also significantly enhanced (Fig. 4*B*). In the second approach, ectopic overexpression of the *sirt1* gene was carried out in 293 cells and, as expected, this was accompanied with a strong expression of the *mdr1* gene (Fig. 4*C*). Activity of the *mdr1* promoter was also increased (Fig. 4*D*), as was the induction of P-glycoprotein expression (Fig. 4*E*), in the Sirt1-transfected cells. More importantly, cells stably transfected with the Sirt1 gene displayed resistance to doxorubicin (Supplementary data 1). Taken together, these findings provide conclusive evidence that expression of the *mdr1* gene can be controlled by the histone deacetylase Sirt1 and highlight the importance of chromatin remodeling in the regulation of drug resistance development.

In conclusion, the present study has uncovered a novel function of the longevity molecule Sirt1 as a potential marker and modulator of the drug resistance phenotype in cancer. As this molecule seemed to control the expression of a major drug resistance molecule, P-glycoprotein, new avenues may be open for further understanding of the causes leading to the onset of drug resistance. With regards to the relationship between aging and cancer, the present study sheds light on common signaling pathways that may be used by somatic and cancer cells to overcome toxic stress.

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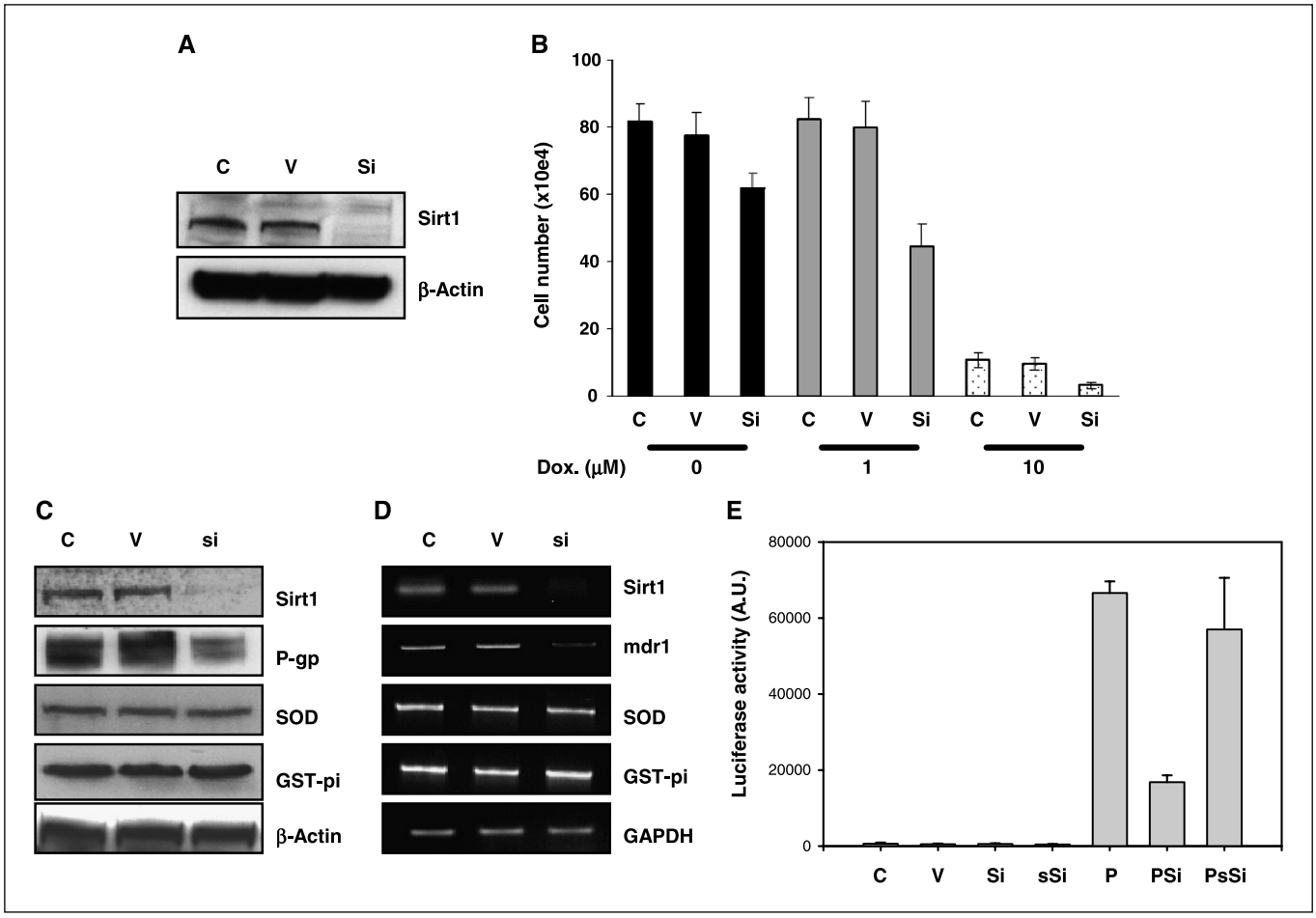

Figure 3. siRNA-mediated knockdown of Sirt1 expression reverses drug resistance and inhibits P-glycoprotein expression. *A,* Sirt1 expression by Western blot in drug-resistant SaOS2 cells nontransfected (*C*) or transfected with the vehicle (*V*) or with Sirt1 siRNA (*Si*) and incubated for 48 hours. β-Actin was used as a control. *B,* cells were treated as in (*A*) and doxorubicin was added at the indicated concentrations, incubated for an additional 48 hours, and then viable cells were counted. *C,* comparison of expression of Sirt1 with those of P-glycoprotein (*P-gp*), SOD, GST, and β-actin by Western blot using specific antibodies in drug-resistant SaOS2 cells treated as in (*A*). *D,* RT-PCR comparing expressions of the corresponding genes to that of GAPDH used as control. *E,* effect of Sirt1 knockdown on activity of the mdr1 promoter. Wild-type SaOS2 cells were transfected with Sirt1 siRNA alone (*Si*), siRNA with scrambled sequence (*sSi*), or mdr1 promoter alone (*P*), or cotransfected with either one of the siRNAs. Luciferase activity was measured and compared with that in cells nontransfected or transfected with the vehicle.

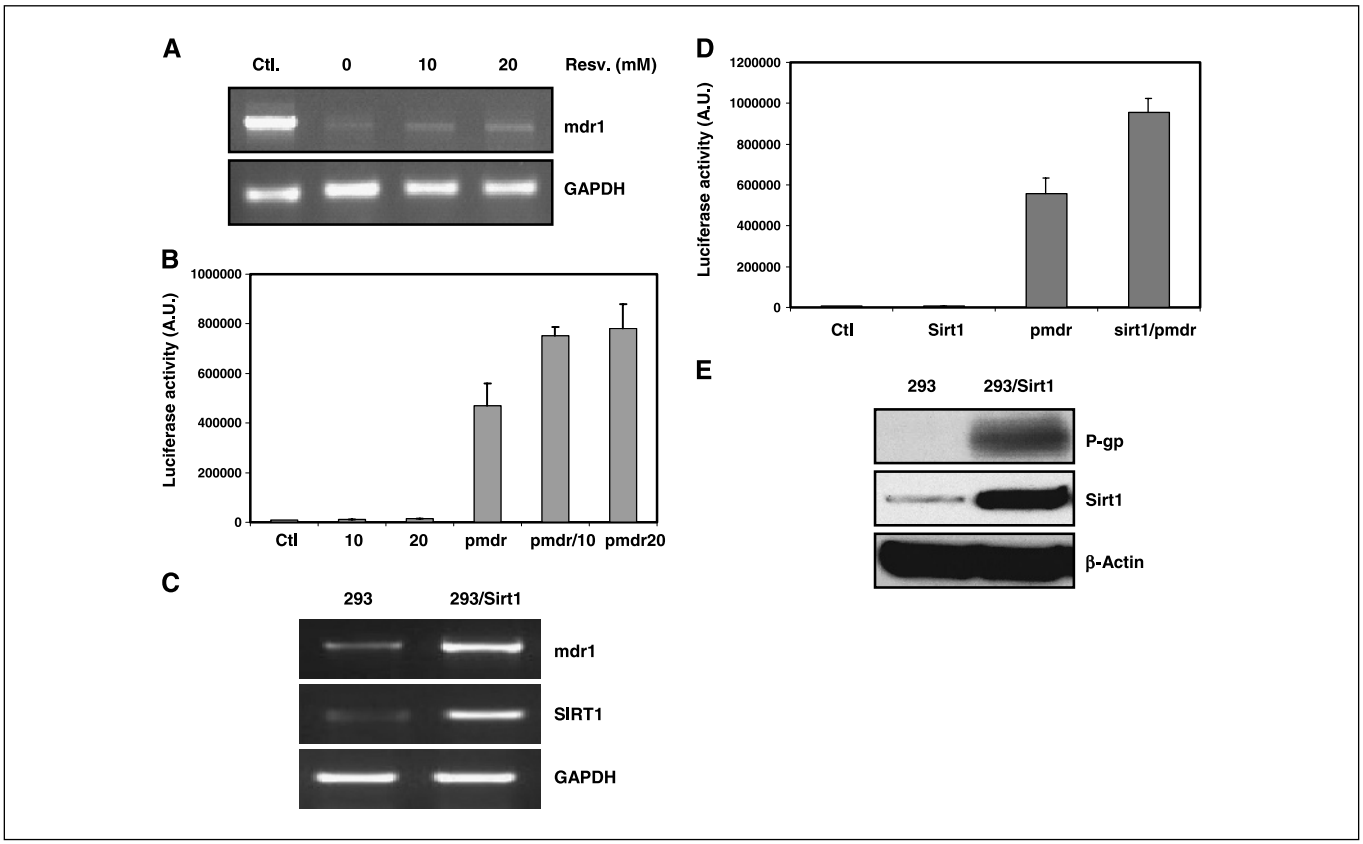

Figure 4. Sirt1 gain of function up-regulates expression of the *mdr1* gene. *A,* 293 cells were incubated with nontoxic concentrations (micromoles per liter) of resveratrol for 2 days and expression of the *mdr1* gene was measured by RT-PCR. *B,* 293 cells transfected with the *mdr1* promoter (*pmdr*) were incubated in the absence or presence of resveratrol as in (*A*) for 48 hours. The luciferase activity was then measured and compared with that of nontreated cells (*Ctl*). *C,* expressions of *sirt1* and *mdr1* genes determined by RT-PCR after transfection of 293 cells with the pYSIR2 plasmid. *D,* luciferase activity of the *mdr1* promoter in nontransfected and cells transfected with *sirt1*, *pmdr*, or both. *E,* expression of P-glycoprotein detected by Western blot in 293 cells nontransfected or transfected with *sirt1* gene. β-Actin is used as a loading control.

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