

# SIRT1 Contributes in Part to Cisplatin Resistance in Cancer Cells by Altering Mitochondrial Metabolism

Xing-Jie Liang,<sup>1,3</sup> Toren Finkel,<sup>2</sup> Ding-Wu Shen,<sup>1</sup> Jun-Jie Yin,<sup>4</sup> Adorjan Aszalos,<sup>1</sup> and Michael M. Gottesman<sup>1</sup>

<sup>1</sup>Laboratory of Cell Biology, National Cancer Institute and <sup>2</sup>Cardiology Branch, National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland; <sup>3</sup>Laboratory of Nanobiomedicine and Nanosafety, Division of Nanomedicine and Nanobiology, National Center of Nanoscience and Technology, Beijing, People's Republic of China; and <sup>4</sup>Instrumentation and Biophysics Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland

## Abstract

Tumors frequently develop resistance to cisplatin, a platinum drug used as a cornerstone of present-day chemotherapy regimens, significantly decreasing its usefulness in the clinic. Although it is known that cisplatin-resistant (CP-r) cancer cells commonly grow more slowly and exhibit reduced uptake of various compounds, including nutrients, the effect of tumor metabolism on cisplatin resistance is unclear. It was found that in CP-r cells, uptake of 2-deoxyglucose was reduced due to dysfunction and altered morphology of mitochondria compared with cisplatin-sensitive parental cancer cells. The CP-r cells overexpressed SIRT1, a histone deacetylase that plays a central role in DNA damage response and transcriptional silencing. Incubation of drug-sensitive cells in low glucose medium induced the expression of SIRT1 and increased cellular resistance to cisplatin. Reduced SIRT1 expression by a SIRT1 SMART small interfering RNA duplex sensitized the >20-fold resistant CP-r cells to cisplatin treatment 1.5- to 2-fold, and SIRT1 overexpression by SIRT1 cDNA transfection increased cisplatin resistance in cisplatin-sensitive cells by 2- to 3-fold. Our findings therefore suggest that reduced glucose use and altered mitochondrial metabolism mediated by SIRT1 is one of several alterations that contribute to cellular resistance to cisplatin. (Mol Cancer Res 2008;6(9):1499–506)

## Introduction

Tumors that recur after an initially positive response to treatment are often resistant to chemotherapeutic drugs.

Cisplatin (*cis*-diamminedichloroplatinum II), which produces adducts between adjacent purines in DNA (1), is one of the most effective and widely used anticancer agents in the treatment of various human malignant solid and metastatic tumors (2). However, the development of cisplatin resistance in cancer cells is a major impediment in clinical treatment. Several mechanisms of cisplatin resistance have been postulated, including decreased drug accumulation, increased levels of intracellular thiols, and increased DNA repair (3). To date, the mechanism of clinical tumor resistance to cisplatin is not entirely understood, despite intensive research to identify the basis of resistance. It is important to explore the mechanism(s) responsible for cisplatin resistance to improve its therapeutic index and overcome the resistance to this drug that emerges in the clinic.

We found that the uptake of various compounds, including nutrients such as glucose, was reduced in cisplatin-resistant (CP-r) cells compared with cisplatin-sensitive (CP-s) cells (4, 5). When nutrients are scarce, a successful survival strategy for tumors requires dynamic alterations in their metabolism. The molecular mechanisms that mediate these alterations are largely unknown. In general, glucose starvation induces a wide range of responses that include altered gene expression and biochemical activities. Recently, the silent information regulator 2 has been linked to extensive metabolic adaptations that render mitochondria dysfunctional. It functions as a histone deacetylase that critically regulates chromatin stability, transcriptional silencing, DNA damage response, and aging under conditions of nutrient restriction (6). SIRT1, the mammalian ortholog of silent information regulator 2, is linked to the energy status of the cell in that the activity of SIRT1 is dependent on the NAD<sup>+</sup>/NADH<sup>+</sup> ratio. SIRT1 plays a pivotal role in regulating cellular or organismal genome stability, metabolism, and resistance to nutritional depletion and is emerging as a key element regulating cell survival in response to various types of stress (7, 8).

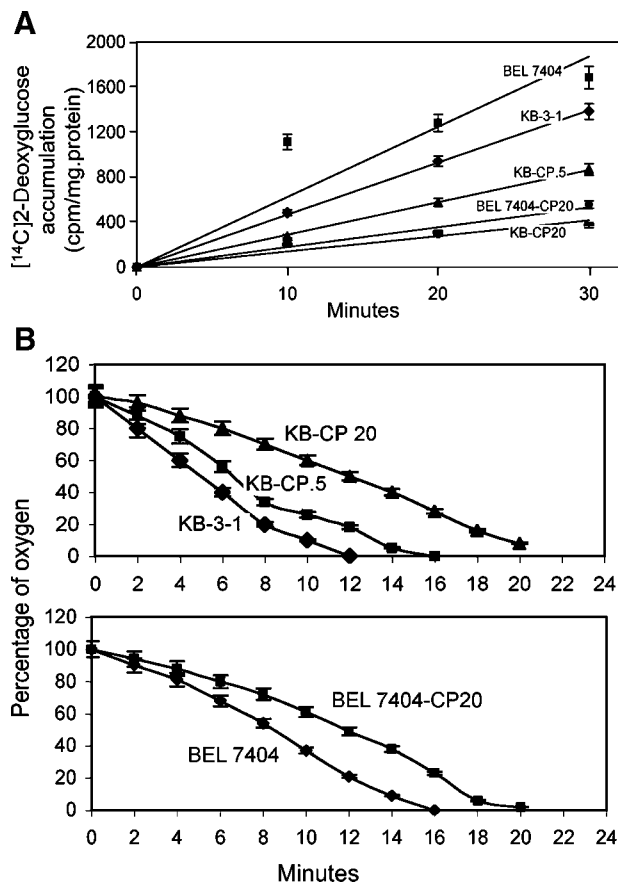
To explore the mechanism of cancer resistance to cisplatin, we isolated two independent cell populations derived from human KB epidermoid adenocarcinoma cells (KB-CP) and human BEL7404 hepatoma cells (7404-CP). Previous work in our laboratory revealed that the growth rate and uptake of different nutrients were significantly reduced in CP-r cells with increasing cisplatin resistance (9, 10). SIRT1, a known nutrient metabolism regulator, was measured in CP-r cells in the current study. Dramatic alterations in mitochondrial membrane

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**Requests for reprints:** Michael M. Gottesman, Laboratory of Cell Biology, National Cancer Institute, NIH, 37 Convent Drive, Room 2108, Bethesda, MD 20892. Phone: 301-496-1530; Fax: 301-402-0450. E-mail: mgottesman@nih.gov or Xing-Jie Liang, Laboratory of Nanobiomedicine and Nanosafety, Division of Nanomedicine and Nanobiology, National Center for Nanoscience and Technology of China, Beijing 100190, People's Republic of China. E-mail: liangxj@nanoctr.cn Copyright © 2008 American Association for Cancer Research. doi:10.1158/1541-7786.MCR-07-2130



**FIGURE 1.** Glucose uptake and oxygen consumption in CP-s and CP-r cells. **A.** Accumulation of [ $^{14}\text{C}$ ]2-deoxyglucose is decreased in CP-r KB-3-1 and BEL7404 cells. Points, mean of three individual experiments; bars, SD. **B.** Oxygen consumption measured by ESR is reduced in CP-r cells. Measurements of oxygen consumption were done five times. Shown are the oxygen consumption rates measured at each time point relative to rates in untreated samples. Bars, SD.

potential and abnormal mitochondrial ultrastructure were found in CP-r cells with reduced oxygen consumption and decreased glucose uptake. SIRT1 was overexpressed in the CP-r cells with high  $\text{NAD}^+$  concentration. Regulation of SIRT1 expression by a SIRT1 SMART small interfering RNA (siRNA) knockdown or SIRT1 full-length cDNA transfection altered the resistance of cancer cells to cisplatin. Incubation of drug-sensitive cells in low glucose medium increased expression of SIRT1 and increased cellular resistance to cisplatin. These data indicate that SIRT1 helps mediate cisplatin resistance when glucose homeostasis is restricted.

## Results

### Reduced Glucose Uptake and Oxygen Consumption in CP-r Cells

With the aim of clarifying the mechanism of cisplatin resistance in cancer, our laboratory found that there is a general defect in uptake of many different nutrients in human adenocarcinoma KB-3-1 and human hepatoma BEL7404 CP-r cells (9, 11). Our previous research showed that the cellular proliferation rate is reduced with increasing cisplatin

resistance in CP-r cells (10). The use of glucose has been recognized as an important feature of tumor development. Glucose uptake was first measured at different time points in KB-3-1 and BEL7404 series of CP-r cells. It was found that uptake of [ $^{14}\text{C}$ ]2-deoxyglucose was reduced in CP-r cells compared with sensitive KB-3-1 and BEL7404 cells (Fig. 1A). The accumulation of [ $^{14}\text{C}$ ]2-deoxyglucose was 5-fold more in KB-3-1 than in KB-CP20 cells and 4-fold more in BEL7404 than in BEL7404-CP20 cells, measured after 30 min.

To determine the effect of reduced glucose uptake on the ability of cells to use glucose through glycolysis, the rate of oxygen consumption was examined. Consistent with the observed decrease in glucose uptake, oxygen consumption measured by electron spin resonance (ESR) oxymetry was reduced in CP-r KB-3-1 and BEL7404 cells with increasing cisplatin resistance. Oxygen consumption was suppressed in CP-r KB-3-1 and BEL7404 cells by 30% to 60% (Fig. 1B). Cancer cells generally maintain a high glycolytic metabolism and use rate despite the presence of limited oxygen in their environment. Reduced oxygen consumption or an altered glycolytic rate in CP-r cells may be an indicator of decreasing tumor sensitivity to anticancer agents.

### Dysfunction of Mitochondria in CP-r Cells

Tumors may harbor lower energy consumption through defective mitochondria after having undergone rounds of anticancer drug selection. Having shown that cellular cisplatin resistance results in reduced glucose uptake and oxygen use (Fig. 1A and B), we sought to measure mitochondrial membrane potential ( $\Delta\psi_m$ ), a component of the overall proton motive force, as it is important for oxidative phosphorylation through use of glucose and oxygen in mitochondria. The  $\Delta\psi_m$ , measured with CBIC<sub>2</sub>(3) by flow cytometry, was found to be higher in the CP-r cells (Fig. 2A), which was consistent with the quantitative confocal microscopic measurements that allowed direct visualization of mitochondria (Fig. 2B). Both KB-3-1 and BEL7404 cells showed increased  $\Delta\psi_m$  (seen as increasing red cytoplasmic staining in Fig. 2B) with increasing cisplatin resistance. The numbers under individual images in panel B indicate the value of  $\Delta\psi_m$  in CP-s and CP-r cells. Combined with the measurement of oxygen consumption, these results indicated a significant difference in metabolism between CP-s and CP-r cells. The mitochondria in the CP-r cells play a different metabolic role compared with their counterparts in the CP-s cells.

### Altered Morphology of Mitochondria in CP-r Cells

Not only do mitochondria show functional alterations in CP-r cells, but electron micrographs show that the mitochondria in the CP-r cells are relatively smaller than those in CP-s cells (Fig. 2C). Consistent with the  $\Delta\psi_m$  measurement, mitochondria showed a typical perinuclear staining pattern and appeared as fibrous, tubule-like structures in most parental cells. However, the mitochondria of CP-r cells are dispersed throughout the cell and have a disordered appearance. In addition, the mitochondria of CP-r cells are much more electron dense than those in CP-s parental cells. The cristae of mitochondria in CP-r cells are thicker and more irregular (Fig. 2C).

### *SIRT1 Overexpression Is Associated with Increased Cisplatin Resistance in KB-3-1 and BEL7404 Cells*

In mammalian cells, SIRT1 levels may change during the progression from normal to cancerous cell growth (12). In addition, reduced energy biogenesis can promote long-term cellular survival by augmenting expression of the SIRT1 deacetylase (13, 14). In addition, SIRT1 overexpression has been associated with increased MDR1 expression in drug-resistant cells and tumors (15). Consistent with reduced bioenergenesis in CP-r cells, there is more SIRT1 expression in whole-cell lysates and the nuclei fraction. SIRT1 expression increases 3- and 5-fold with increasing levels of resistance in the CP-r KB-3-1 and BEL7404 cells (Fig. 3A). The expression of the SIRT1 mRNA was also increased in CP-r cells (2-fold for BEL7404-CP20 cells and 4-fold for KB-CP20 cells) compared with CP-s cells, as determined by semiquantitative reverse transcription-PCR (Fig. 3B). The equal loading of protein or RNA was determined by protein staining of electrophoresis gels and EB staining of RNA agarose gels. Housekeeping genes could not be used as loading controls, owing to the altered expression of standard protein (such as actin, glyceraldehyde-3-phosphate dehydrogenase, and tubulin) in CP-r cells compared with CP-s cells (16, 17). These SIRT1 expression levels were confirmed by confocal microscopy. The images showed higher SIRT1 expression in the nuclei of CP-r cells than in those of CP-s cells (Fig. 3C). In addition,  $\text{NAD}^+$  concentration was significantly higher in CP-r cells that overexpress SIRT1

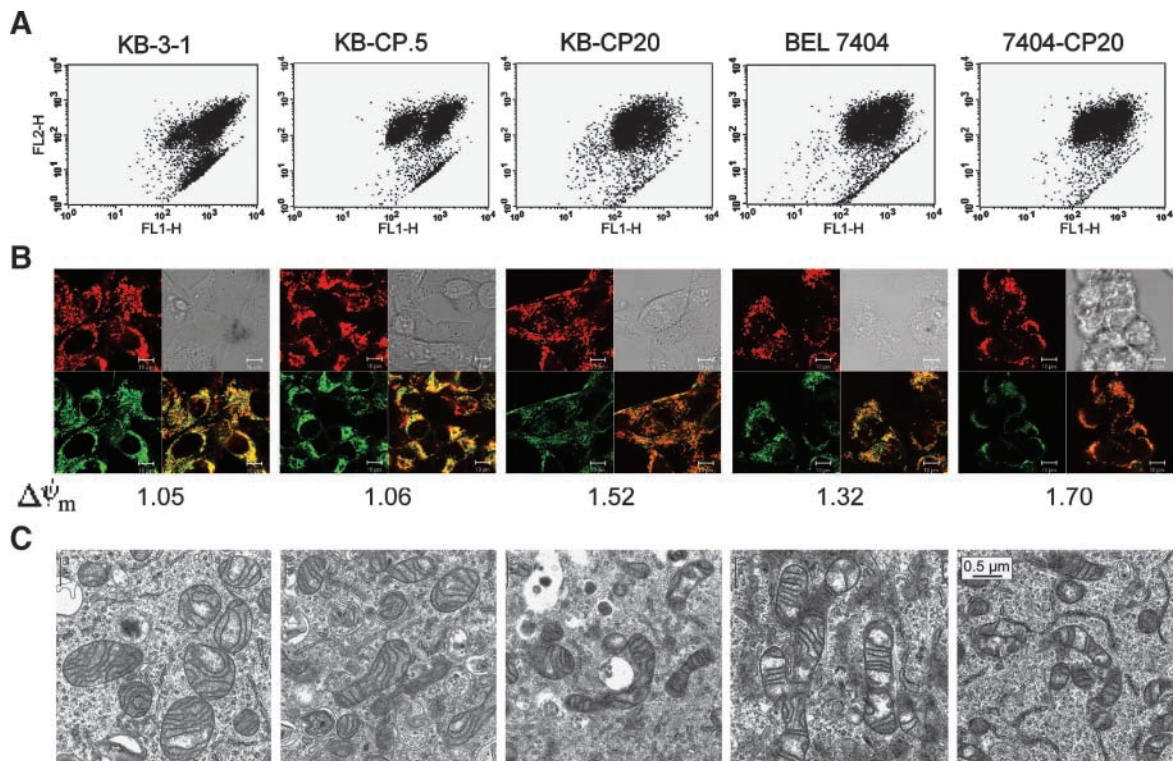
(Fig. 3D), which indicates that SIRT1 requires the presence of  $\text{NAD}^+$  to function. Our results show that SIRT1 was overexpressed in CP-r KB-3-1 and BEL7404 cells, which do not express MDR1. Therefore, overexpression of SIRT1 may be a marker and/or modulator of the drug resistance phenotype in cancer with or without MDR1 expression.

### *SIRT1 Overexpression Due to Limited Glucose Use Increases Cisplatin Resistance*

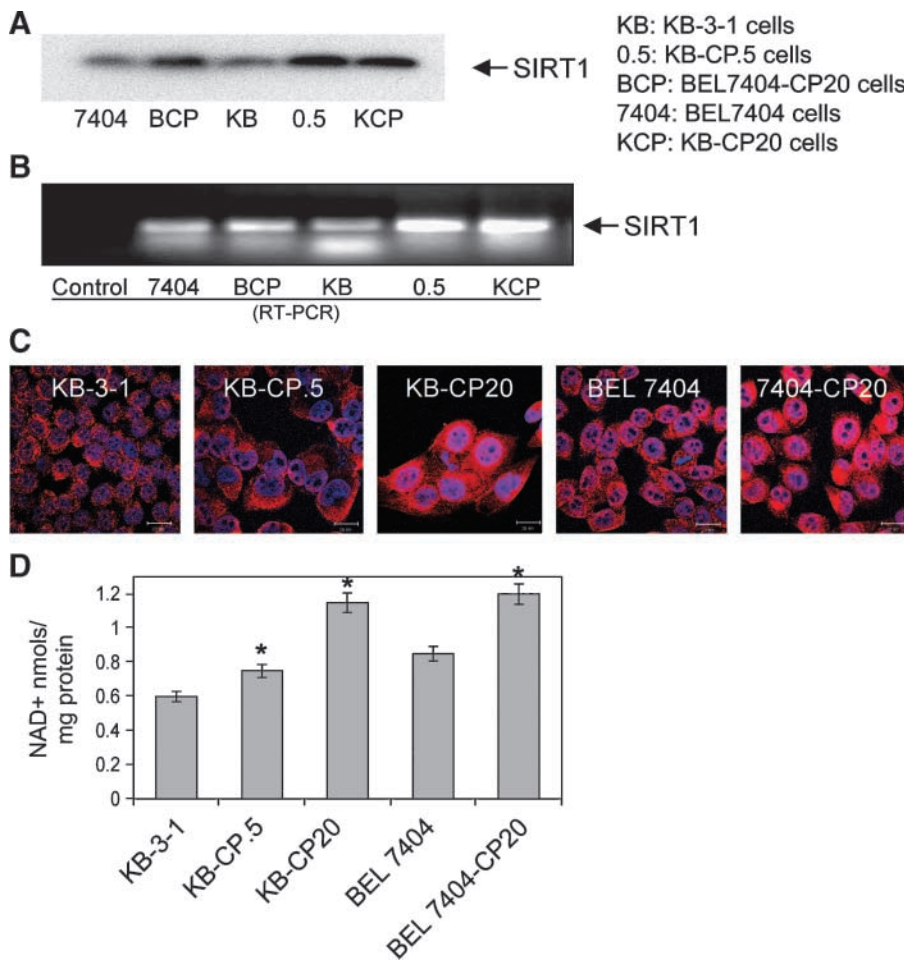
Rodgers et al. (18) showed that impaired glucose homeostasis is associated with SIRT1 expression. Our study of KB-3-1 and BEL7404 cells shows that there is increasing expression of SIRT1 when sensitive cells are incubated with medium as glucose decreases from 4.5 to 0.5 g/L (Fig. 4A). These results are consistent with the increased SIRT1 levels found in PC-12 cells cultured with nutrient limitation (19). In addition, the induced overexpression of SIRT1 due to limited glucose incubation is associated with increased resistance to cisplatin (Fig. 4B).

### *Alteration of SIRT1 Expression Mediates Cisplatin Resistance in Cancer Cells*

To further measure the effect of SIRT1 expression on cisplatin resistance in cancer cells, we established CP-r cells transfected with a SIRT1 SMART siRNA and CP-s cells transfected with a pCruzHA SIRT1 full-length cDNA. It was found that SIRT1 SMART siRNA duplex transfection



**FIGURE 2.** Polarization of mitochondrial membrane potential as determined by flow cytometry and confocal microscopy. **A.** Mitochondrial membrane potential measured by flow cytometry. **B.** Mitochondrial membrane potential measured by confocal microscopy. Orange color indicates the combination of red [CBIC<sub>2</sub>(3) monomer] and green [CBIC<sub>2</sub>(3) aggregate]. Number means the relative arbitrary mitochondrial membrane potential value quantitated by confocal microscopy. **C.** Morphology of mitochondria in CP-s and CP-r cells observed by electron microscopy. Representative images from experiments done in triplicate.



**FIGURE 3.** SIRT1 expression in CP-s and CP-r KB-3-1 and BEL7404 cells. **A.** SIRT1 expression was detected in the whole-cell lysate of CP-s and CP-r KB-3-1 and BEL7404 cells by Western blotting. **B.** SIRT1 expression was detected by semi-quantitative reverse transcription-PCR (RT-PCR). RNA was extracted from each type of cell and reverse transcription-PCR analysis was done in triplicate using specific oligonucleotides. Representative images from experiments done in triplicate. BCP, BEL7404-CP20 cells; KCP, KB-CP20 cells. **C.** SIRT1 expression was detected by confocal microscopy. Red staining indicates the expression of SIRT1 shown with Texas red-conjugated antibody. The nuclei labeled with 4',6-diamidino-2-phenylindole and mounted in antifade medium appear in blue. Scale bar, 20  $\mu$ m. **D.** NAD<sup>+</sup> concentration was increased in CP-r cells compared with CP-s cells. NAD<sup>+</sup> concentration was measured as described in Materials and Methods. Columns, mean; bars, SD. Comparisons of each group were evaluated by one-way ANOVA. When twice the SD was higher than the mean, a nonvariable test was used to evaluate the difference. A significant difference was assumed to exist when  $P < 0.05$ . The results shown are the mean of three individual experiments. Asterisks, there was a statistically significant difference between the CP-r and CP-s cells.

efficiently reduced the SIRT1 expression in CP-r KB-CP20 and BEL7404-CP20 cells compared with the CP-r cells transfected with nontargeting random siRNA used as a control (Fig. 5A). There was a 5-fold decrease in SIRT1 expression in BEL7404-CP20 cells and a 3-fold decrease in KB-CP20 cells. This reduction of SIRT1 expression sensitizes the CP-r KB-CP20 and BEL7404-CP20 cells approximately 1.5- to 2-fold to cisplatin treatment (Fig. 5B). In addition, when SIRT1 was overexpressed in CP-s cells after pCruzHA SIRT1 full-length cDNA transfection, as measured by Western blotting after 72 h (Fig. 5C), the parental CP-s KB-3-1 and BEL7404 cells were 3- and 2-fold more resistant, respectively, to cisplatin treatment (Fig. 5D). Similarly, PC-12 cells transfected with the full-length wild-type SIRT1 vector show a 3-fold increase in cisplatin resistance (data not shown). This shows that the alteration of SIRT1 expression can directly mediate cellular resistance to cisplatin in KB-3-1 and BEL7404 cancer cells. However, it is known that there are multiple, simultaneous alterations frequently encountered in CP-r cells. CP-r cells are 20-fold resistant to cisplatin compared with CP-s cells. Altered expression of SIRT1, one of the variations that affect cellular resistance to cisplatin, seems to circumvent cisplatin resistance 10% to 20% in these carefully designed models.

## Discussion

Cellular resistance to cisplatin is multifactorial and may consist of mechanisms that limit cisplatin uptake and accumulation, altered repair mechanisms, and changes that promote cell survival (20). Our previous study and the work of others have shown that altered uptake of cisplatin occurs because of reduced membrane-binding/transport proteins and reduced endocytosis (9, 21). In this work, we show that SIRT1 up-regulation also contributes to cisplatin resistance in association with altered mitochondrial metabolism.

It is known that cells derive up to 95% of their energy through oxidative phosphorylation carried out in mitochondria. Subtle alterations in the glycolytic metabolic pathway could affect energy biosynthetic reactions and cellular susceptibility in the face of stress. Harper et al. (22) identified and characterized a cellular metabolic strategy that differentiates drug-resistant cells from drug-sensitive cells. According to this strategy, drug-resistant cells use nonglucose carbon sources (fatty acids) for mitochondrial oxygen consumption when glucose becomes limiting, and mitochondria become dysfunctional in resistant cells. Abnormal mitochondrial structure may have a substantial effect on cellular metabolism, and especially on mitochondrial metabolism, which plays an important role in stress resistance, chromatin-dependent gene regulation, and

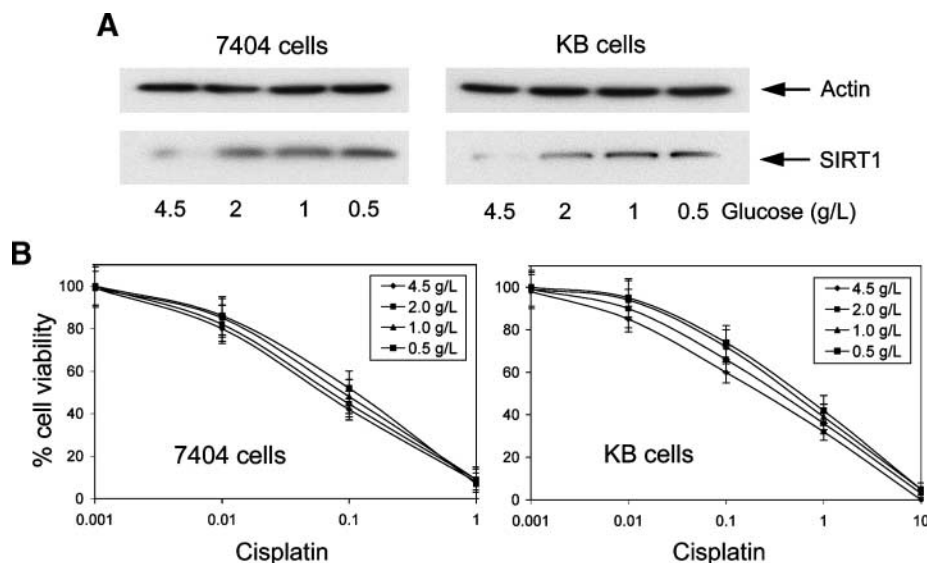
genome stability. Support for mitochondria as a direct target of cisplatin toxicity comes from studies that show that intestinal epithelial IEC-6 rho(0) cells with reduced numbers of mitochondria are more resistant to cisplatin than normal cells (23). This is consistent with our results, which showed that uptake of 2-deoxyglucose was reduced due to dysfunction and altered morphology of mitochondria in CP-r cells.

In addition, glucose transporter 1 (GluT1), a common molecular target of most antidiabetic drugs, is no longer localized to the plasma membrane in CP-r cells (data not shown), which is similar to the recycling defect we have previously reported for a transmembrane protein (multidrug-resistant protein 1) in malignant CP-r cells (5). GluT1, the mediator of basal glucose uptake, is the primary glucose transporter in human cancer and is expressed by most cancer cells. Reduced 2-deoxyglucose uptake in CP-r cells is likely due to the altered localization of GluT1, the main membrane protein transporting glucose into tumor cells, by a mechanism not yet elucidated. Aft et al. (24) found that overexpression of GluT1 transporter protein was associated with increased glucose uptake in breast cancer cells treated with 2-deoxyglucose. The disruption of glucose uptake in CP-r cells is associated with abnormal mitochondrial function, as measured by oxygen consumption and mitochondrial membrane potential. It was found previously that SIRT1 overexpression could reduce oxygen consumption and alter mitochondrial bioenergetics by acting with PGC-1 in PC-12 cells (19). Given that oxygen consumption is linked to the generation of reactive oxygen species and reactive oxygen species levels correlate with cisplatin toxicity (25), these results may have important implications for how SIRT1 regulates cisplatin resistance.

Limitation of glucose use alone results in SIRT1 overexpression in KB-3-1 and BEL7404 cells and increases the

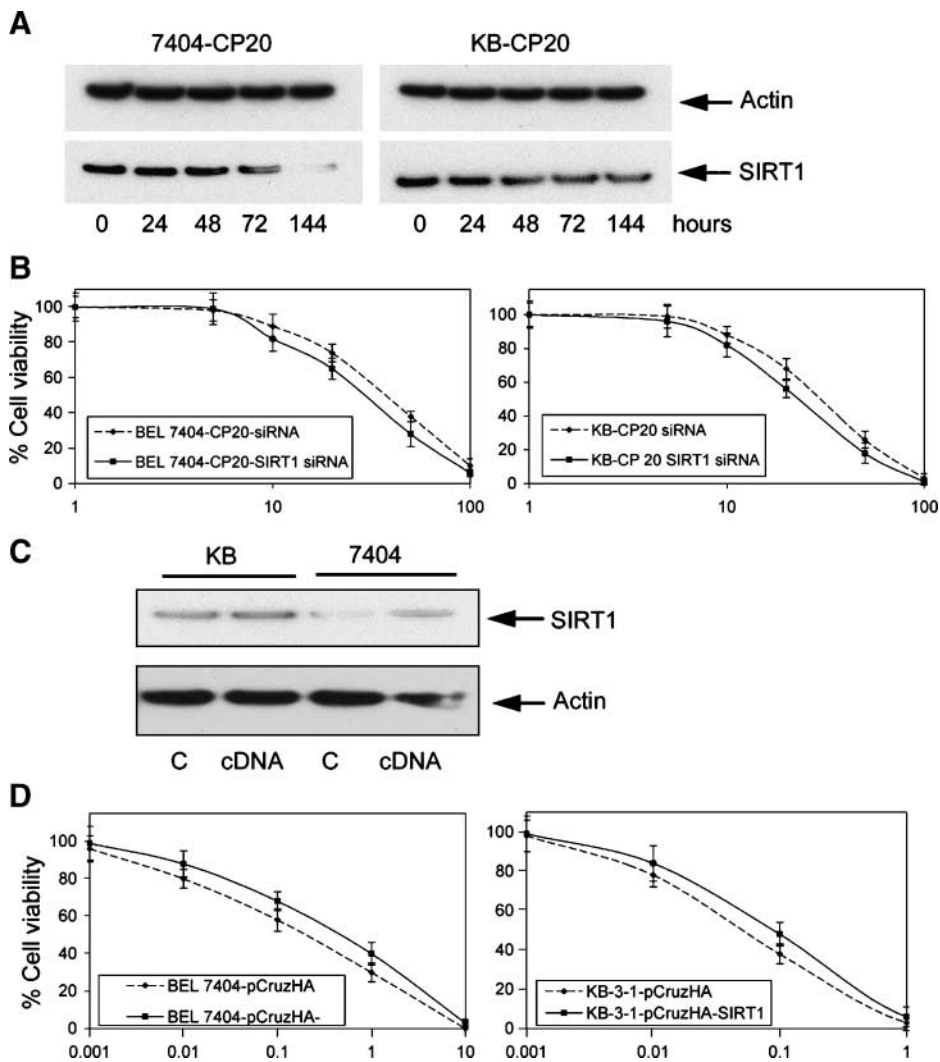
resistance of parental CP-s KB-3-1 and BEL7404 cells to cisplatin. The bioenergetic dysfunction in CP-r KB-CP20 and BEL7404-CP20 cells suggests that reducing glucose metabolism also leads to overexpression of SIRT1, which confers cisplatin resistance to KB-3-1 and BEL7404 cells. How might SIRT1 overexpression contribute to cisplatin resistance? Cisplatin perturbs nucleic acid structure and function when it incorporates into DNA or RNA, thereby causing tumor cells to be killed. SIRT1 is known to deacetylate p53 protein, which recognizes and binds to DNA modified with cisplatin (26). It has been proposed that deacetylation by SIRT1 reduces p53-dependent apoptosis in response to DNA damage (27). Interactions between p53 and SIRT1 after platination might provide a molecular link between DNA damage and p53-mediated DNA repair. The cross-resistance of the CP-r cells studied here to numerous cytotoxic agents, including heavy metals, alkylating agents, and methotrexate (5, 9), could reflect such a general effect on apoptosis mediated by SIRT1.

It is well known that SIRT1 is involved in cellular metabolism, and to function, SIRT1 requires the presence of  $\text{NAD}^+$ . However, there is an active debate about whether  $\text{NAD}^+$  directly controls *in vivo* activity of SIRT1 (28, 29). In this study, we found that  $\text{NAD}^+$  concentration was higher in CP-r cells that overexpress SIRT1 (Fig. 3D). If the cellular  $\text{NAD}^+$  concentration were low, SIRT1 deacetylase activity could be attenuated, thus increasing the chances of a cell becoming senescent or apoptotic through the acetylated form of p53 (30). Lai and colleagues (31) provide evidence of this by showing increased acetylation of wild-type p53 during cisplatin-induced apoptosis. In addition, the requirement for  $\text{NAD}^+$  in the deacetylase activity of SIRT1 suggests that SIRT1 might be involved in metabolism of  $\text{NAD}^+$  as a metabolic



**FIGURE 4.** SIRT1 expression is induced by low glucose incubation, increasing cellular resistance to cisplatin. **A.** KB-3-1 and BEL7404 cells were incubated in medium with various concentrations (4.5, 2, 1, and 0.5 g/L) of glucose for 3 d. Protein extracts were normalized for Western blotting for SIRT1 expression and actin was used as a control. Representative images are shown from experiments done in triplicate. **B.** Low glucose incubation increases cellular resistance to cisplatin in parental CP-s KB-3-1 and BEL7404 cells. Cells seeded in plates were treated with cisplatin at various concentrations. Points, mean; bars, SD. Comparisons of each group were evaluated by one-way ANOVA. When twice the SD was higher than the mean, a nonvariable test was used to evaluate the difference. A significant difference was assumed to exist when  $P < 0.05$ . The results shown are the mean of three individual experiments.





**FIGURE 5.** Alteration of SIRT1-mediated cellular resistance to cisplatin. **A.** SIRT1 expression was knocked down in CP-r KB-CP20 and BEL7404-CP20 cells by SIRT1 SMART siRNA transfection. SIRT1 expression was measured by specific anti-SIRT1 antibody, with actin used as a control, by Western blotting. The experiment is representative of three individual experiments. **B.** Cellular viability was measured in CP-r KB-CP20 and BEL7404-CP20 cells transfected with SIRT1 SMART siRNA. Cell Counting Kit-8 was used for cellular viability assay, as described in Materials and Methods. The data presented represent the mean of at least three experiments. **C.** SIRT1 expression was increased in CP-s KB-3-1 and BEL7404 cells by pCruzHA SIRT1 full-length cDNA transfection. Actin was used as a control. The image is representative of three experiments. **D.** Cellular viability was measured in CP-s KB-3-1 and BEL7404 cells transfected with a pCruzHA SIRT1 full-length cDNA. The data presented represent the mean of at least three experiments.

regulator. SIRT1 may function as a bridge, coordinating metabolic status with regulation of key target genes involved in cancer resistance to cisplatin. Thus, higher  $\text{NAD}^+$  and SIRT1 levels seen in CP-r cells would favor a less effective apoptotic pathway.

Processes that require energy, such as fatty acid synthesis, protein synthesis, and cell growth, are curtailed in CP-r cells (9, 10, 32), possibly through the effects of SIRT1 as a central modulator. Overexpressed SIRT1 in CP-r cells might result in a higher threshold for apoptosis by targeting numerous cellular factors. It is possible that SIRT1 acts as a metabolic sensor, via its  $\text{NAD}^+$  dependence, which links energy consumption to a transcriptional program that modulates response to stress. Deacetylation of transcriptional complexes by SIRT1 is generally associated with diminished transcriptional activation by removing the acetyl group from lysines of certain transcription factors (e.g.,  $\text{NF-}\kappa\text{B}$ , FOXO, and p53; refs. 8, 30, 33). Several transcription factors, such as  $\text{NF-}\kappa\text{B}$ , YB-1, mtTFA, Ets-1, and AP-1, are activated by cisplatin treatment and are involved in drug resistance and DNA repair (34). SIRT1 may act as a scaffold to tether various transcriptional

complexes. Brunet et al. (8) found that SIRT1 conferred resistance to etoposide by augmenting DNA repair, and thymocytes derived from SIRT1 knockout mice were found to be more sensitive to ionizing radiation. Therefore, overexpression of SIRT1 might shift the transcription-dependent response of cisplatin resistance away from cell death and toward cell survival.

Cellular mechanisms of resistance to cisplatin are multifactorial and contribute to severe limitation in the use of cisplatin in the clinic. Our research shows that cisplatin resistance reflects a reduced bioenergenesis associated with decreased glucose uptake in CP-r KB-CP20 and BEL7404-CP20 cells. CP-r KB-CP20 cells originally showed 1,152-fold more resistance to cisplatin than parental cells. Interestingly, the same cells showed 330-fold more resistance to cisplatin after incubation for 94 days without cisplatin selection pressure, which reduced SIRT1 expression.<sup>5</sup> SIRT1 overexpression allowed the parental CP-s KB-3-1 and BEL7404 cells to survive through limited nutrient incubation and caused cellular

<sup>5</sup> D.W. Shen and M.M. Gottesman, unpublished data.

resistance to cisplatin treatment in CP-s cells. This is consistent with the effect of reduced SIRT1 by siRNA, which sensitizes the CP-r cells to cisplatin treatment. Our present data shed light on how SIRT1, regulated by glucose homeostasis, may contribute to the development of tumor cell resistance to cisplatin, and provide insight that may help in the development of treatments to overcome drug resistance in cancer patients.

## Materials and Methods

### Cell Lines

KB-3-1 (epidermoid adenocarcinoma) and BEL7404 cells (hepatoma cells) were the parental CP-s cells used in this study. KB-CP.5 cells were selected by single-step exposure of parental KB-3-1 cells to 0.5  $\mu\text{g}/\text{mL}$  cisplatin. KB-CP20 cells were derived from KB-3-1 cells by stepwise increases in cisplatin concentration up to 20  $\mu\text{g}/\text{mL}$ , as were BEL7404-CP20 cells. The cell lines were all grown as monolayer cultures at 37°C in 5% CO<sub>2</sub> using DMEM with 10% premium FCS (lot 0S010F; BioWhittaker, Inc., Cambrex Bioproducts), L-glutamine, penicillin (50 units/mol), and streptomycin (50  $\mu\text{g}/\text{mL}$ ; Quality Biological, Inc.). BEL7404-CP20 and KB-CP20 cells were maintained in medium containing 5  $\mu\text{g}/\text{mL}$  cisplatin for these experiments.

### Measurement of [<sup>14</sup>C]2-Deoxyglucose Uptake

The uptake of [<sup>14</sup>C]2-deoxyglucose by cell monolayers was measured in glucose-free HBSS containing tracer amounts of [<sup>14</sup>C]2-deoxyglucose. After washing, the monolayers were harvested in 2 mL of 0.1 N NaOH for measurement of radioactivity by liquid scintillation counting and for protein determination.

### Oxygen Consumption of CP-s and CP-r KB-3-1 and BEL7404 Cells as Detected by ESR Oxymetry

The ESR oxymetry measurement is based on bimolecular collision of O<sub>2</sub> with a spin label, causing shorter spin-lattice relaxation time and broadening the line width. Consequently, decreased line width indicates continuous oxygen consumption. In this work, the spin label 4-oxo-2,2,6,6-tetramethylpiperidine-d<sub>16</sub>-1-<sup>15</sup>N-oxy (N-PDT; Cambridge Isotope Laboratories, Inc.) was used, as the line width of this spin probe is very sensitive to O<sub>2</sub> concentration. By measuring line widths obtained in a time span, one can assess respiration of cells positioned in the ESR instrument. For this purpose, an ESR chamber was filled with a suspension of 5 × 10<sup>5</sup> cells/mL and 0.2 mmol/L N-PDT and the chamber was sealed. Decreasing oxygen concentration due to oxygen consumption by cells results in a decreasing line width of N-PDT in the closed chamber system. ESR spectra were recorded at 2-min intervals for 20 min with a Varian E-109 X-band spectrometer with a variable temperature controller accessory. Signals were obtained with 0.5 mW incident microwave power and with 100 kHz modulation. All ESR spectra were recorded at the low field line of N-PDT and at 37°C.

### Measurement of Cellular Sensitivity to Cisplatin

Cellular viability was determined by a sensitive colorimetric assay (Cell Counting Kit-8, Dojindo Molecular Technologies). Cells (5,000) were plated in 96 wells with 100  $\mu\text{L}$  medium

containing serially diluted cisplatin (Sigma Co.). After incubation for about 3 d, the cellular viability at various concentrations of cisplatin was measured at 450 nm absorbance using a microplate reader after 10  $\mu\text{L}$  of the Cell Counting Kit-8 solution added to each well. The IC<sub>50</sub> for each cell line was calculated based on the drug concentration that reduces the cell viability to 50% of those in the control, drug-free medium. A relative resistance factor for each cell line was determined by dividing the IC<sub>50</sub> value of cisplatin for the CP-r cell lines by that for the appropriate CP-s cell line. The values are means of at least triplicate determinations.

### Immunoblotting Detection of Protein Expression

Cells (1 × 10<sup>7</sup>) were harvested at log phase and washed twice with cold PBS. The cells were sedimented by centrifugation at 1,400 × g for 10 min and suspended in ice-cold TD solution buffer (2 mmol/L DTT, 1% aprotinin, 1 mmol/L AEBSF, and DNase) for 5 min on ice. Cells were disrupted by sonication thrice. Samples were checked under a phase-contrast microscope and showed >80% of cells broken. Protein electrophoresis and immunoblotting with antibodies directed to SIRT1 were done. Anti-SIRT1 antibody was purchased from Upstate Biotechnology. Actin was used as a protein loading control detected by monoclonal anti- $\beta$ -actin (Sigma-Aldrich). Dr. Samuel W. Cushman of the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland kindly provided the anti-GluT (GluT1, GluT2, GluT3, GluT4, and GluT5) polyclonal antibodies. Briefly, the samples were separated by SDS-PAGE on a 4% to 12% gradient gel and transferred onto polyvinylidene difluoride membranes. Subsequently, membranes were subjected to immunostaining with monoclonal antibodies against human SIRT1 (1:1,000), actin (1:10,000), and GluTs (1:500) for 1 h at room temperature. Enhanced chemiluminescence reagents were used for developing signals as described by the manufacturer (Pierce Chemical Co.).

### Transfection of SIRT1 SMART siRNA or pCruzHA SIRT1 cDNA into Cells

The SIRT1 SMART siRNA duplex was designed by Dharmacon RNA Technologies. Diluted CP-r cells in antibiotic-free medium were plated for transfection with SIRT1 SMART siRNA based on the Dharmacon protocol. The siCONTROL nontargeting pool was used as siRNA control, which is chemically modified for increased stability and optimized with no perfect matches to known human, mouse, or rat genes. The transfection mixture was prepared by combining the SIRT1 siRNA diluent with GeneSilencer siRNA transfection reagent (Genlantis). This mixture was added directly to plated CP-r cells at 60% confluence at transfection time. SIRT1 expression was measured at different time points. Nontargeting random siRNA was used as a control. In addition, a SIRT1 full-length cDNA was cloned into a pCruzHA mammalian expression vector (Santa Cruz Biotechnology, Inc.). FuGene 6 (Roche Diagnostics) was used as the transfection reagent for pCruzHA SIRT1 plasmid transfection into CP-s cells. SIRT1 expression was measured by Western blotting after 72 h of transfection.

### Immunofluorescence Detection of SIRT1 Expression by Confocal Microscopy

Cells were incubated on sterile 18-mm cover glasses for 3 d and then fixed with 3.5% formaldehyde in PBS for 10 min and followed by 0.1% Triton X-100 treatment for 5 min for permeabilization. After washing, cells were treated with 3% bovine serum albumin in PBS for 30 min and subsequently treated with the primary anti-SIRT1 antibody for 1 h. After three washings, cells were incubated with Texas red-conjugated, affinity-purified secondary antibody (1:100 dilution; Jackson ImmunoResearch Laboratories). The coverslips with the treated cells were mounted on microscope slides with fluorescence mounting medium containing 4',6-diamidino-2-phenylindole for nuclei staining (Dako). Background fluorescence was determined from cells treated only with the secondary antibody but otherwise treated the same way as described with the primary antibody. Confocal images were acquired using a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a 60 × planapochromat lens (Bio-Rad).

### NAD<sup>+</sup> Content Assay

NAD<sup>+</sup> concentration was determined by conversion of NAD<sup>+</sup> to NADH using the alcohol dehydrogenase reaction as previously described (16).

### Statistical Analysis

The experiments were done in triplicate, and all counts obtained from assays were analyzed, averaged, and expressed as mean ± SD. The two-way ANOVA test was used to compare multiple groups of data, whereas the unpaired Student's *t* test was used to compare two groups of data. The level of significance, unless otherwise indicated, was *P* < 0.05.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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