Roles of SIRT1 and AMPK in hypoxia-induced resistance of non-small cell lung carcinoma to cisplatin and doxorubicin

Dong Hoon Shin, Yong-Joon Choi, Jong-Wan Park*

Department of Pharmacology, Department of Biomedical Science, Ischemic/hypoxic disease institute, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-799, Korea

Running title: Roles of SIRT1 and AMPK in hypoxia-induced drug resistance

Grant Support: This work was supported by a grant (2010-0029948) funded by the National Research Foundation of Korea.

*Correspondence: Jong-Wan Park, M.D., Ph.D. Department of Pharmacology, Seoul National University College of Medicine, 103 Daehang-ro, Jongno-gu, Seoul 110-799, Korea. Tel: +82-2-740-8289, FAX:+82-2-745-7996, E-mail: parkjw@snu.ac.kr

Disclosure of Potential Conflicts of Interest: All authors declare no potential conflict of interest.

Key words: non-small cell lung carcinoma, hypoxia, drug resistance, SIRT1, AMPK

Abbreviations: AMPK, AMP-activated protein kinase; CP, cisplatin; Cyt-c, cytochrome c; DOXO, doxorubicin; HIF, hypoxia-inducible factor; LKB1, liver kinase B1; NSCLC, non-small cell lung carcinoma; SIRT1, sirtuin 1.

Word count: 5478

Total number of figures and tables: 7
Abstract

Sirtuin 1 (SIRT1) is a NAD⁺-dependent protein deacetylase used to cope with metabolic imbalances under nutrition- or oxygen-deficient conditions. Although the roles of SIRT1 in aging and metabolic disorders have been established, its role in tumor progression remains controversial, particularly concerning its role in tumor response to chemotherapy. As hypoxia occurs often within most tumors and provokes drug resistance, this study addressed the involvement of SIRT1 in hypoxia-induced chemoresistance. SIRT1 was down-regulated in five non-small cell lung carcinoma (NSCLC) cell lines exposed to hypoxic conditions for 48 hours. AMP-activated protein kinase (AMPK) was inactivated in NSCLC cells during hypoxia, likely to the attenuation of the SIRT1-activated, LKB1-mediated AMPK activation process. The hypoxic inactivation of the SIRT1-AMPK pathway was associated with the acquisition of cisplatin and doxorubicin resistance. Molecular biology- and pharmacology-based experiments suggested the following mechanism. The SIRT1-AMPK pathway promotes the tumor-killing actions of cisplatin and doxorubicin by ensuring a mitochondriogenic apoptosis. However, the drug-mediated apoptosis is attenuated under hypoxia due to mitochondrial suppression following inactivation of the SIRT1-AMPK pathway. The involvement of this pathway in NSCLC response to cisplatin was also confirmed in a xenograft tumor model. A SIRT1 activator SRT1720 augmented the anticancer effect of cisplatin, which was blocked by an AMPK inhibitor compound C. The SIRT1-AMPK pathway could be a potential target to overcome the hypoxia-induced chemoresistance in NSCLC.
Introduction

Solid tumors almost always contain hypoxic areas when they are radiologically detected. In tumors, oxygen consumption is increased by proliferating cancer cells and infiltrating immune cells, and also oxygen delivery is impaired due to the abnormal vasculature network and the high interstitial pressure (1, 2). When cancer cells are challenged intermittently or constantly by hypoxic stress, they set their metabolism to an energy-saving mode and refrain from proliferating. Many clinical and experimental studies have demonstrated that cancer cells exposed to hypoxia acquire resistance to anticancer drugs. Basically, drugs are poorly delivered to hypoxic areas with circulation disturbance, and cancer cells are better able to survive after adapting to the hypoxic insult (3). Many molecules have been suggested to be responsible for the hypoxia-induced drug resistance (4). However, the mechanisms underlying chemoresistance are quite different according to cancer contexts and drugs, and complicated even in a cell-line treated with single drug.

Sirtuins belong to the class III histone deacetylase (HDAC) family that includes seven isoforms (SIRT1-7) in mammalian cells. They require NAD$^+$ as a substrate to deacetylate the lysine residues in histones and non-histone proteins (5, 6). SIRT1 is considered as the prototype of mammalian sirtuins because its structure is most similar to that of the yeast Sir2. SIRT1, which is mainly located in the nucleus, regulates gene expressions by changing the chromatin structures and by modulating the activities of transcription factors (7, 8). As its activity depends on the ratio of NAD$^+/$/NADH, SIRT1 acts as a redox sensor to cope with metabolic imbalance under nutrition- or oxygen-deficient conditions (9). As a whole, SIRT1 helps cells utilize glucose and survive under harmful conditions. In addition, SIRT1 is critical in diverse biological processes, such as cell division, differentiation, senescence, and tumorigenesis (10, 11).
Cumulative evidence has supported the involvement of SIRT1 in cancer progression. SIRT1 exerts its tumor suppressive activity by inhibiting oxidative stress, inflammation, proliferation, and angiogenesis; by counteracting genotoxic stress; and by inducing apoptosis and autophagy (12-15). In contrast, SIRT1 also provides cancer cells with survival and expansion advantages, which are achieved through p53 inactivation, Myc activation, and epithelial-to-mesenchymal transition (16-18). Besides these Janus-faced roles in cancer promotion, SIRT1 might determine tumor responses to anticancer drugs. Although SIRT1 has not been intensively explored in terms of cancer chemotherapy, previous studies have supported the notion that SIRT1 plays some positive roles in tumor acquisition of chemoresistance (19). Whether SIRT1 participates in the hypoxia-induced chemoresistance is unclear.

During hypoxia, the cellular ratio of NAD⁺/NADH declines because the dehydrogenation of NADH to NAD⁺ in the mitochondrion is impaired, which results in decreased functionality of SIRT1 (20). In addition, the increased NADH triggers the repressor C-terminal-Binding Protein-1 (CtBP) to associate with Hypermethylated In Cancer 1 (HIC1) on the SIRT1 promoter, reducing the expression of SIRT1 (21). Taken together, SIRT1 action is generally regarded to be inhibited under hypoxia.

Based on the previous concept about the SIRT1-mediated chemoresistance, theoretically SIRT1 inhibition under hypoxia may sensitize cancer cells to anticancer drugs. However, in reality chemoresistance is induced under hypoxia. To clarify this dichotomy, the present study was designed and performed. In particular, we focused on the role of SIRT1 in the hypoxia-induced resistance against cisplatin or doxorubicin in non-small-cell lung carcinoma (NSCLC).
Materials and Methods

Reagents and antibodies

SRT1720, EX527, A769662, cisplatin, and doxorubicin were purchased from Selleck Chemicals (Houston, TX); compound C from EMD Millipore (Billerica, MA). Antibodies against SIRT1, PGC-1α, cytochrome c, Lamin B, Caspase-9, PARP, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against AMPKα, pAMPKα, mTOR, pmTOR, Caspase-3, and Beclin-1 from Cell Signaling Technology (Beverly, MA); antibodies against Bcl-2 family (Bad, Bak, Bid, Bax, Bim, Bcl-x, Mcl-1, Bcl-2 and Bag-1) from Abcam (Burlingame, CA). Anti-HIF-1α antiserum was raised in rabbits, as previously described (22).

Cell culture and hypoxic incubation

NSCLC (H1299 and A549), Fibrosarcoma (HT1080), Gastric cancer (MKN28), Ovarian cancer (SKOV3), Breast cancer (MDAMB-231), Brain cancer (SHSY5Y and SKNMC), Hepatoma (Hep3B and HepG2), Renal cancer (786O and RCC4), Cervical cancer (HeLa and SiHa), Colon cancer (HT29, HCT116 and CT26) and Head and neck cancer (YD10B) cell-lines were obtained from the American Type Culture Collection (Manassas, VA); other NSCLC cell lines (HCC15, HCC366 and HCC827) from Korea Cell Line Bank (Seoul, Korea). The cells were cultured in MEM, DMEM, or RPMI1640, supplemented with 10% heat-inactivated fetal bovine serum. Cells were incubated in a humidified atmosphere at 37°C at 20% O2/5% CO2 for normoxic condition or at 1% O2/5% CO2 for hypoxic condition.
Cell viability assay

After cells (2 x 10^3) were cultured in 96-well plates under normoxia or hypoxia for 2 days, cell viability was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye. Quadruplicate wells were used for each analysis and at least three independent experiments were done.

Immunoblotting and immunoprecipitation

Cell lysates were prepared in a lysis buffer containing 1% NP40, 5 mM sodium orthovanadate and protease inhibitors. After being boiled in a denaturing SDS sample buffer, samples were subjected to SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4°C with a primary antibody, incubated for 1 hour with a HRP-conjugated secondary antibody, and visualized using the ECL Plus kit (Thermo Fisher scientific, MA). For immunoprecipitation, cell lysates were incubated with 5 μl of anti-LKB1 or anti-AMPK antiserum, or preimmune serum at 4°C for 2 hours, and immune complexes were further incubated with protein A/G-Sepharose beads (GE Healthcare) at 4°C for 4 hours. Immunocomplexes were eluted by 10-minute boiling in a denaturing SDS sample buffer and subjected to immunoblotting with anti-SIRT1, anti-LKB1, or anti-acetyl-lysine antibody.

Analysis of mitochondrial morphology

Cells were rinsed with 0.1 M cacodylate buffer and fixed in 3.5% glutaraldehyde (pH 7.4) for 24 hours at 4°C. After being washed in the cacodylate buffer containing 5% sucrose, cells were
post-fixed in 1% osmium tetroxide for 1 hour at 4°C. Cells were gradually dehydrated in ethanol (50% to 100%) and infiltrated with Spurr’s resin (Electron Microscopy Sciences, PA) over 2 hours. The resin was replaced with fresh Spurr’s resin poured in inverted BEEM embedding capsules (TED PELLA, INC., CA) and allowed to be polymerized at 60°C for 36 hours. Beam capsules were snapped off, and attached cells were analyzed by electron microscopy. Parameters of mitochondrial morphology (Area and perimeter) were quantified using the ImageJ program (NIH).

**Tumor xenograft**

All animal procedures were performed in accordance with a protocol approved by the Seoul National University Animal Care and Usage Committee (Approve number, SNU-130104). Nude mice (BALB/cAnNCrj-nu/nu) from Charles River Japan Inc. (Shin-Yokohama, Japan) were injected at a dorsal flank site with 1x10⁶ cells in saline. Tumor volume was measured with calipers (volume = axb²x0.52, where “a” is the widest width and “b” is the perpendicular width) once three days. When tumors reached a volume of 80-100 mm³ (termed day 0 in this study), mice were injected i.p. three times a week with DMSO, cisplatin, SRT1720, or compound C. On day 21, mice were sacrificed by cervical dislocation. Each tumor was cut into two parts, which were fixed with 4% formalin or frozen in liquid nitrogen.

**Statistical analysis**
Each result is expressed as the mean and standard error (SE) or standard deviation (SD), which was calculated using Microsoft Excel 2010. We used unpaired, two-sided Student’s t test for all tests, and statistical difference was considered significant when p < 0.05.

Results

Hypoxia-induced resistance to cisplatin and doxorubicin are due to SIRT1 down-regulation in NSCLC cells.

Six cancer cell-lines were treated with cisplatin or doxorubicin under normoxic (21% O₂) or hypoxic (1% O₂) conditions for 48 hours. The IC₅₀ value for cisplatin or doxorubicin significantly increased under hypoxia in H1299, A549, Hep3B, and HT1080, but did not in HCT116 and MCF7 (Fig. 1A). We checked SIRT1 in 21 different cancer cell lines and also hypoxia inducible factor-1 alpha (HIF-1α) to verify cellular responses to hypoxia. SIRT1 expression in hypoxia was variable in cell lines (Supplementary Fig. 1). Notably, five NSCLC lines (H1299, A549, HCC15, HCC366, and HCC827) displayed hypoxic repression of SIRT1 at the mRNA and protein levels (Figs. 1B and 1C). Given that HIFs are crucial in hypoxia-induced gene regulation, we examined the regulation of SIRT1 expression by HIF-1α or HIF-2α. SIRT1 expression under either normoxia or hypoxia was not affected by overexpression or silencing of HIF-1α and HIF-2α (Supplementary Fig. 2), suggesting that SIRT1 is expressed independently of HIFs. We next examined the role of SIRT1 in chemoresistance by manipulating SIRT1 expression. Under either normoxic or hypoxic conditions, the IC₅₀ values for cisplatin and doxorubicin in H1299 and
A549 cells were significantly reduced by SIRT1 expression, whereas these were raised by SIRT1 knock-down (Fig. 1D and Supplementary Fig. 3A). These results indicate that SIRT1 overexpression sensitizes NSCLC cells to the anticancer drugs whereas its knock-down confers drug resistance. Given that the effects of SIRT1 were shown in normoxic and hypoxic conditions, SIRT1 may sensitize cancer cells to the drugs regardless of the oxygen tension. Moreover, even in HCT116 cells which did not show drug resistance and SIRT1 down-regulation during hypoxia (Fig. 1B), the IC₅₀ values for both drugs were decreased or increased by SIRT1 expression or knock-down, respectively (Supplementary Fig. 3B). The chemosensitization by SIRT1 may not be a hypoxia-specific event. To confirm the role of SIRT1 in response to chemotherapy, we examined the effects of a SIRT1 activator, SRT1720, and a SIRT1 inhibitor, EX527, on cisplatin-or doxorubicin-induced cell death. Hypoxia-induced chemoresistance in both cell-lines was attenuated by SRT1720, but was augmented by EX527 (Supplementary Fig. 4). To test whether SIRT1 determines the cytotoxic effects of cisplatin and doxorubicin in naturally occurring hypoxia, a colony formation assay was carried out because typically the core part of a colony is hypoxic due to limited oxygen diffusion (23). As was expected, colony numbers substantially decreased in the presence of cisplatin or doxorubicin. The anticancer effects were potentiated by SRT1720, but diminished by EX527 (Fig. 1E). All of these results suggest that the SIRT1 down-regulation during hypoxia induces tumor resistances against cisplatin and doxorubicin, and that SIRT1 restoration sensitizes hypoxic NSCLC cells to the drugs.

**AMPK is inactivated in NSCLC cells during hypoxia, leading to tumor resistance to cisplatin and doxorubicin.**

SIRT1 and AMPK act as metabolic sensors to revise the energy metabolisms according to nutritional states. They play this role independently, or do so cooperatively by regulating each
other and by sharing common target molecules (24, 25). Therefore, we measured phospho- Thr172 AMPKα (an active, catalytic subunit of the AMPK complex) levels in NSCLC cells subjected to hypoxia (Supplementary Fig. 1). As was shown for SIRT1, phosphorylated AMPKα (pAMPKα) was diminished under hypoxia in NSCLC cells (Fig. 2A). pAMPKα level correlated well ($r^2=0.94$) with SIRT1 level in NSCLC cells (Supplementary Fig. 5A). This result encouraged us to check the dependence of AMPKα phosphorylation on SIRT1. AMPKα phosphorylation was regulated SIRT1-dependently under both normoxic and hypoxic conditions (Fig. 2B). In contrast, the mTOR phosphorylation at Ser2448 was negatively regulated by SIRT1, suggesting that the SIRT1-activated AMPK inhibits mTOR phosphorylation. An in vitro assay of AMPK activity also demonstrated that SIRT1 activates AMPK (Fig. 2C). In seeking to determine how SIRT1 activates AMPK, we were prompted by a previous report (26) to check the possibility that SIRT1 deacetylates and activates LKB1, which functions to activate AMPK. When SIRT1 was down-regulated during hypoxia or knocked-down using siRNA, the acetylated form of LKB1 noticeably increased, but was reduced in hypoxia by SIRT1 expression (Fig. 2D), suggesting that SIRT1 activates AMPK in NSCLC cells by deacetylating LKB1. Moreover, AMPKα sensitized NSCLC cells to cisplatin (Fig. 2E) and doxorubicin (Supplementary Fig. 5B).

**The SIRT1-AMPKα pathway underlies hypoxia-induced chemoresistance in NSCLC cells.**

To confirm that the hypoxic inactivation of the SIRT1-AMPK pathway confers drug resistance to NSCLC cells, NSCLC cells were treated with SIRT1 and AMPK modulators in combination (Fig. 3A). A SIRT1 inhibitor (EX527) induced drug resistances under normoxia, which was reversed by an AMPK activator (A769662). In contrast, a SIRT1 activator (SRT1720) sensitized cancer cells to cisplatin and doxorubicin under hypoxia, which was reversed by an AMPK inhibitor.
Likewise, another SIRT1 activator, resveratrol augmented the anticancer actions of both drugs in H1299 and A549 cells under hypoxia, but such effects of resveratrol were abolished by compound C (Supplementary Fig. 6). To rule out off-target effects of small-molecules, we checked cell viabilities using the plasmids and siRNAs for SIRT1 and AMPKα (Fig. 3B). Consistent with the results shown in Fig. 3A, SIRT1 knock-down significantly induced resistance to cisplatin and doxorubicin under normoxia, and this effect was abolished by AMPKα overexpression. Under hypoxia, SIRT1 overexpression augmented the anticancer effects of cisplatin and doxorubicin, but the SIRT1 effects were attenuated by AMPKα knock-down. Moreover, the EX527-induced chemoresistance in normoxia was overcome by AMPKα expression, whereas the SRT1720-induced chemosensitization in hypoxia was attenuated by AMPKα knock-down (Fig. 3C). To determine whether the SIRT1 deacetylase function determines tumor responses to drugs, a deacetylase-defective, dominant-negative mutant of SIRT1 (H363Y) was expressed (27). SIRT1\_H363Y induced cisplatin and doxorubicin resistance under normoxia, which was reversed by AMPKα expression (Fig. 3D). These results suggest that the SIRT1-AMPK pathway determines the sensitivities of NSCLC cells to cisplatin and doxorubicin.

**SIRT1-AMPK inactivation under hypoxia protects tumor cells from apoptosis in the presence of cisplatin or doxorubicin.**

To understand how the SIRT1-AMPK pathway determines tumor responses to cisplatin and doxorubicin, various apoptotic markers in cisplatin- or doxorubicin-treated NSCLC cells were measured. The number of TUNEL-positive cells was reduced by SIRT1 knock-down, but was increased by AMPKα expression. The TUNEL-positive cell population was significantly reduced
under hypoxia. This effect of hypoxia was attenuated by SIRT1 expression, which was reversed by AMPKα knock-down (Fig. 4A and Supplementary Fig. 7A). Likely, caspase-9 in NSCLC cells treated with the drugs was activated in a SIRT1- and AMPK-dependent manner (Fig. 4B and Supplementary Fig. 7B). As the release of mitochondrial cytochrome c (Cyt-c) is the initial event of apoptosis, we evaluated the Cyt-c release by co-staining Cyt-c (green) with Mitotracker (red) (Supplementary Fig. 8). Normally, Cyt-c co-localizes with Mitotracker, which produces yellow fluorescence due to a green-red overlap. However, the yellow areas under normoxia were reduced in cancer cells treated with the drugs, but increased with siSIRT1, which was reversed by AMPKα expression. Under hypoxia, however, Cyt-c still localized within mitochondria even in the presence of the drugs, but it was dispersed by SIRT1 expression, which was reversed by AMPKα knock-down. The localization of Cyt-c was also rechecked in cytoplasmic and mitochondrial fractions by Western blotting (Supplementary Fig. 9). To confirm the involvement of SIRT1 in drug-induced apoptosis, we measured the cleaved forms of caspase-3 and poly-ADP ribose polymerase (PARP). Caspase-3 and PARP were cleaved under normoxia in the presence of cisplatin or doxorubicin. The drug-induced cleavages were attenuated under hypoxia, which was reversed by SIRT1 expression (Fig. 4C). Moreover, the hypoxia-induced resistance to cisplatin was attenuated by SIRT1 expression, which was reversed by a caspase inhibitor (Fig. 4D). These results indicate that the hypoxic inactivation of the SIRT1-AMPK pathway renders cancer cells resistant to caspase-mediated apoptosis induced by anticancer drugs.

Hypoxic inactivation of the SIRT1-AMPK pathway reduces apoptosis by inhibiting mitochondrial activity.
Mitochondria play a central role in apoptotic cell death under genotoxic stress induced by anticancer agents (28, 29). As both SIRT1 and AMPK promote mitochondrial biogenesis (25), we hypothesized that they determine tumor sensitivities to cisplatin and doxorubicin by ensuring mitochondrial biogenesis. To examine mitochondrial structure, transmission electron microscopy was used to examine NSCLC cells in normoxic and hypoxic conditions (Fig. 5A, left). Mitochondrial parameters like area, number, and perimeter were measured using the ImageJ program (Fig. 5A, right). Mitochondria decreased in number and size during hypoxia. These changes were prevented by SIRT1 restoration, which was attenuated by AMPKα knock-down. In addition, the mitochondrial parameters were reduced under normoxia by SIRT1 knock-down, which was reversed by AMPKα expression. To evaluate mitochondrial function, ATP levels were measured in NSCLC cells (Fig. 5B). The normoxic ATP levels were reduced by SIRT1 knock-down, which was recovered by AMPKα expression. ATP levels decreased during hypoxia were recovered by SIRT1 expression, which was attenuated by AMPKα knock-down. These results suggest that the inactivation of the SIRT1-AMPK pathway leads to mitochondrial suppression under hypoxia. These findings strongly indicate that the hypoxic inactivation of the SIRT1-AMPK pathway blocks mitochondriogenic apoptosis through mitochondrial suppression and subsequently desensitizes cancer cells to anticancer drugs.

The anticancer effect of cisplatin is augmented in H1299 tumors by activation of the SIRT1-AMPK pathway.

We examined if the anticancer effect of cisplatin is modulated through the SIRT1-AMPK pathway in H1299 xenografted tumors. Cisplatin reduced tumor volume and weight by 23% and 50%, respectively, 21 days after the first drug injection. Cisplatin in combination with SRT1720 retarded tumor growth more efficiently, but the combination effect was abolished by an
additional treatment with compound C (Figs. 6A and 6B). Body weights of mice on day 21 were not significantly different among the six experimental groups (Fig. 6C). Representative photographs of excised tumors are shown in Supplementary Fig. 10. To evaluate AMPK activity and apoptosis in xenografts, Western blotting of tumor tissue homogenates was carried out. AMPKα in grafted tumors was activated by SRT1720 injection but was inactivated by compound C injection. The combined treatment with SRT1720 and cisplatin increased the levels of cleaved caspase-9 and caspase-3. However, the addition of compound C attenuated the combination effect (Supplementary Figs. 11A and 11B). The tumor levels of activated AMPKα and caspases were quantified using the ImageJ program and compared among the groups (Fig. 6D). Apoptotic death of cancer cells in H1299 xenografts was examined using TUNEL staining. SRT1720 sensitized tumors to cisplatin but compound C desensitized tumors (Supplementary Fig. 11C). The in vivo results supported the notion that the SIRT1-AMPK pathway can be a target for sensitizing NSCLC cells to cisplatin.

Discussion

Lung cancer is the most common cancer worldwide in terms of incidence and mortality, and NSCLC constitutes approximately 80% of all primary lung cancers (30, 31). Chemotherapy for NSCLC is based on the combined treatment of cisplatin and another anticancer drug including gemcitabine, irinotecan, taxanes, and vinorelbine (32). Although these combination regimens have produced better therapeutic results than cisplatin alone, the best regimens can only give an overall response rate of less than 50% (33). Strategies to further improve the survival of NSCLC patients involve discovery or development of more effective drugs, or the sensitization
of NSCLC cells to pre-existing drugs. For the latter strategy, the mechanisms underlying chemoresistance need to be clearly understood. Yet, this goal is difficult to achieve because resistance is multifactorial, involving pharmacokinetic resistance, cellular resistance, and the microenvironment. The present study focused on the mechanism underlying hypoxia-induced chemoresistance, and implicated hypoxic repression of SIRT1 in the resistance of NSCLC cells to cisplatin and doxorubicin.

In the present study, we first investigated whether SIRT1 participates in the hypoxia-induced chemoresistance in NSCLC cells. As SIRT1 was down-regulated during hypoxia, we tested the possibility that the hypoxia-induced chemoresistance could be attributed to SIRT1 repression. SIRT1 was required for the cytotoxic actions of cisplatin and doxorubicin, which was shown in the experiments controlling SIRT1 expression and activity. SIRT1 activated AMPK by deacetylating and activating the AMPK activator LKB1. The involvement of the SIRT1-AMPK pathway in the tumor-killing actions of cisplatin and doxorubicin was also demonstrated by controlling the expressions and activities of AMPKα and SIRT1. Furthermore, the SIRT1-AMPK pathway promoted mitochondrial biogenesis and by doing so ensured mitochondriogenic apoptosis in the presence of cisplatin or doxorubicin. Under hypoxia, however, the drug-mediated apoptosis was attenuated due to mitochondrial suppression induced by the SIRT1-AMPK inactivation. The graphical summary of this mechanism is presented in Fig. 7.

Although the relation between SIRT1 and cancer has been extensively investigated in the past decade, the roles of SIRT1 in tumorigenesis and tumor development are still controversial. The oncogenic role of SIRT1 has been supported by clinical and pharmacological reports showing that SIRT1 is highly expressed in some human cancers and that tumor growth can be retarded by SIRT1 inhibition (34). For example, Li et al. found that SIRT1 was highly expressed in stem cells of chronic myelogenous leukemia (CML). They also demonstrated that SIRT1
inhibition could suppress the growth of CML both in vitro and in vivo due to p53 activation (35). In contrast, many studies using mice with genetically modified SIRT1 levels have consistently demonstrated that SIRT1 serves as a tumor suppressor (36). Since it targets a variety of key factors determining cell fate, SIRT1 could act as a tumor suppressor or promoter depending on cell context. In the present study, we treated H1299 tumor-bearing mice with SRT1720 (SIRT1 activator) for three weeks, but observed no significant change in tumor growth (Fig. 6A). This result suggests that SIRT1 does not play a critical role in H1299 tumor growth. Nonetheless, SIRT1 could be a promising target for cancer therapy because it participates in the apoptotic process induced by anticancer drugs.

SIRT1 regulation under hypoxia is dependent on the cell context. As shown in Fig. 1B, the hypoxic repression of SIRT1 was not observed in about 50% of tested cell-lines. In particular, RCC4, MKN28, and HeLa cells induce SIRT1 expression during hypoxia. Indeed, the hypoxic regulation of SIRT1 expression remains as a conflicting phenomenon. Chen et al. demonstrated that SIRT1 in Hep3B and HT1080 cell-lines is induced under hypoxia at the transcriptional level by both HIF-1α and HIF-2α (37). In contrast, Zhang et al. showed that the SIRT1 transcription in fibroblasts is noticeably repressed under hypoxia in a CtBP-dependent fashion (21). Presently, SIRT1 was constantly down-regulated in the tested NSCLC cell lines. To rule out the possible involvement of HIF-1/2α in SIRT1 regulation, we overexpressed or knocked-down both HIFs in NSCLC cells, but found no differences in SIRT1 levels under either normoxia or hypoxia. More defined experiments should be done to elucidate why SIRT1 under hypoxic conditions is differentially regulated among cell-lines.

AMPK is activated in response to poor energy states, such as nutrient deficiency, severe exercise, and other cellular stresses, due to increased levels of AMP, a product of ATP breakdown. AMP binds to the γ-subunit of the AMPK complex, which allosterically promotes the
LKB1-mediated phosphorylation and activation of the catalytic α-subunit (26). AMPK restores energy homeostasis by facilitating the catabolic processes of glucose and fatty acid. Many recent studies have supported the existence of bi-directional cross-talk between AMPK and SIRT1. In terms of the AMPK regulation of SIRT1, Canto et al. demonstrated that AMPK stimulates SIRT1 activity in myoblasts by increasing the ratio of NAD⁺/NADH (38). In contrast, Lan et al. demonstrated that SIRT1 activates AMPK signaling in human embryonic kidney cells by deacetylating LKB1 at Lys48 (39). Briefly, after being deacetylated by SIRT1, LKB1 is translocated from the nucleus to the cytoplasm, where it forms an active complex with STE20-related adaptor protein (STRAD) and mouse embryo scaffold protein (MO25), and then activates the α-subunit of AMPK. Of the two regulatory routes, the SIRT1-to-AMPK pathway might be involved in NSCLC cell responses to cisplatin and doxorubicin because the SIRT1 action on drug resistance could be reversed by inhibiting AMPK. Moreover, the acetylated (inactive) LKB1 level increased during hypoxia due to SIRT1 suppression. However, we did not check if the AMPK-to-SIRT1 pathway determines anticancer effects.

The metabolic sensors SIRT1 and AMPK have been reported to promote mitochondrial biogenesis through the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) signaling pathway. PGC-1α binds and activates nuclear respiratory factor 1 and 2, mitochondrial transcription factor A, and estrogen-related receptor gamma transcription factors that express the nuclear genes essential for mitochondrial replication and respiratory function (40). Under energy-deficient conditions, the intracellular levels of AMP and NAD⁺ increase, leading to activation of both AMPK and SIRT1. Then, PGC-1α is phosphorylated and deacetylated cooperatively by the two enzymes, and activates the genes required for mitochondrial biogenesis in the nucleus (38). Hypoxia may drive cell metabolism toward the adaptation to energy deprivation because the energy level also drops during hypoxia. However,
mitochondrial biogenesis should be blocked during hypoxia because the mitochondria become useless for ATP generation due to lack of oxygen, and instead produce free electron-driven toxic metabolites like reactive oxygen species. Fig. 7 depicts a proposed mechanism underlying hypoxia-induced mitochondrial suppression.

Based on the present results, we can suggest a new potential strategy for overcoming the hypoxia-induced chemoresistance in NSCLC. The combination of a SIRT1 activator or/and an AMPK activator with conventional anticancer drugs like cisplatin and doxorubicin. Considering that drug-induced apoptosis is triggered in hypoxia by activating the SIRT1-AMPK pathway, this strategy may be useful for augmenting the anticancer effects of molecularly targeted drugs. In this case, however, the outcome of the SIRT1-AMPK activation might depend on the target signaling of the drugs because SIRT1 and AMPK can cross-talk with multiple signaling pathways that confer a survival benefit on the cells. The advantages and disadvantages of SIRT1-AMPK activation in cancer therapy should be carefully evaluated before its clinical application.

Acknowledgments

We thank professors Yang-Sook Chun and Dr. Chung-Hyun Cho in Seoul National University College of Medicine for their kind discussions and comments.

Authors’ Contributions
Conception and design: D.H. Shin, J.W. Park

Acquisition of data: D.H. Shin, Y.J. Choi

Analysis and interpretation of data: D.H. Shin

Writing, review and/or revision of the manuscript: D.H. Shin, J.W. Park

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Figure legends

Figure 1. Hypoxia-induced resistances against cisplatin and doxorubicin are attributed to SIRT1 repression in NSCLC cells. **A**, Cell viabilities with increasing concentrations of cisplatin (CP) and doxorubicin (DOXO) under normoxic and hypoxic condition for 48 hours were determined by MTT assay. IC50 values are presented as the means ± SDs (n=4) and * denotes p<0.05. **B**, SIRT1 protein levels in the indicated cancer cells subjected to 48-hour hypoxia were determined by Western blotting. The density of each blot was quantified using the ImageJ program (NIH, U.S.A), and β-actin was a loading control. Gray columns present the results from NSCLC cells. **C**, RT-qPCR was done to check the SIRT1 mRNA levels in NSCLCs subjected to 48-hour hypoxia. Each bar represents the mean ± SD (n=3) and * denotes p<0.05. **D**, H1299 and A549 cells, which had been transfected with SIRT1 plasmid (2 μg) or siRNA (80 nM), were treated with CP under normoxic or hypoxic conditions for 48 hours. Cell viabilities (the means ± SD, n=4) were presented as percentages of the values in untreated groups, and average IC50 values are written with boxes. * and #: p<0.05 versus normoxic control and versus hypoxic control, respectively. **E**, Cells within 0.4% top agar were stained with crystal violet and counted. Each bar represents the mean ± SD (n=4; * p<0.05).

Figure 2. AMPKα mediates SIRT1-dependent, hypoxia-induced chemoresistance. **A**, AMPKα and its phosphorylated form were analyzed by Western blotting in the cell lysates used in Figure 1B, and quatified using ImageJ. Gray columns represent the results from NSCLCs. **B**, H1299 and A549 cells, which had been transfected as indicated, were incubated under normoxic or hypoxic conditions for 48 hours. Proteins were analyzed using Western blotting. **C**, Transfected cells were incubated for 48 hours as indicated. Cell lysates were added to a plate pre-coated with insulin receptor substrate-1, and S789 phosphorylation of the substrate was analyzed.
spectrophotometrically at 450 nm. Each bar represents the mean + SD (n=4). D, Transfected H1299 cells were incubated for 48 hours as indicated, cell lysates were subjected to immunoprecipitation with anti-LKB1, and then to immunoblotting with the indicated antibodies. E, Transfected cells were treated with CP for 48 hours, and cell viabilities were measured using MTT. Results (means ± SDs, n=4) were expressed as percentages of the values of untreated groups, and average IC50 values are written with boxes. * and #: p<0.05 versus normoxic control and versus hypoxic control, respectively.

Figure 3. The SIRT1-AMPKα pathway determines cellular sensitivities to cisplatin and doxorubicin in NSCLC. A, Cells were treated with CP, DOXO, SRT1720 (100 nM), EX527 (100 nM), A769662 (10 μM) and Compound C (1 μM) under normoxic or hypoxic conditions for 48 hours, and then their viabilities were measured by MTT. B, Transfected cells were treated with CP or DOXO for 48 hours, and their viabilities were determined by MTT. C, Cells, which had been transfected with the indicated plasmids or siRNAs, were treated with SRT1720 or EX527 in combination with CP or DOXO, and then incubated under normoxic or hypoxic conditions for 48 hours. Cell viabilities were determined by MTT. D, Cells were transfected with pSIRT1_H363Y and/or pAMPKα, and then treated with CP or DOXO for 48 hours. Each bar represents the mean + SD (n=4) and * denotes p<0.05.

Figure 4. The SIRT1-AMPKα pathway mediates apoptosis in NSCLC. Transfected H1299 and A549 cells were treated with CP for 48 hours. Apoptosis was analyzed by measuring the indicated markers: A, TUNEL staining; B, caspase-9 cleavage; C, caspase-3 and PARP cleavages. D, The transfected cells were treated with CP and/or Z-LEHD-FMK (100 μM) for 48 hours. Cell viabilities were measured by MTT assay and each bar represents the mean + SD (n=4, * p<0.05).
Figure 5. The hypoxic inhibition of SIRT1-AMPKα pathway decreases mitochondrial biogenesis in NSCLC. A, Cells, which had been transfected as indicated in the box, were incubated under normoxic or hypoxic conditions for 48 hours. Mitochondrial number and morphology were analyzed based on electron microscopic images (scale bar: 1 µm) (left panel). Area (µm²) and perimeter (µm) of mitochondria were measured using ImageJ. Bars present the mean + SD (n=4) and * denotes p<0.05 (right panel). B, ATP levels were analyzed using the EnzyLight kit under the same conditions to “A”. Results are presented as the relative values to “a” level. Bars present the means + SD (n=4) and * denotes p<0.05.

Figure 6. The activation of SIRT1-AMPKα pathway overcomes the tumor resistance to cisplatin in NSCLC tumor xenografts. H1299 cells were implanted in the flank of nude mice. After tumor volume reached about 100 mm³, tumor-bearing mice were treated three times a week with cisplatin (3 mg/kg, i.p.), SRT1720 (0.2 mg/kg, i.v.) and/or compound C (1 mg/kg, i.p.). A, Tumor volumes were measured once per 3 days for 21 days. The results of tumor volumes are expressed as the means ± SEM (n=10) and * denotes p<0.05. B, Tumors were removed from mice and weighed (means + SEM, n=10). C, Body weights (means ± SEM, n=10) of mice were not significantly different among all groups. D, The expressions of pAMPKα (T172), PGC-1α, cleaved caspase-9, and cleaved caspase-3 were detected in four xenografts per each group by Western blotting, and the blot intensities were analyzed using ImageJ. Each bar represents the mean + SEM (n=4) and * denotes p<0.05.

Figure 7. A proposed mechanism underlying hypoxia-induced resistances to cisplatin and doxorubicin in NSCLC cells.
**A**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cisplatin</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>H</td>
</tr>
<tr>
<td>H1299</td>
<td>5.1±0.9</td>
<td>10.8±3*</td>
</tr>
<tr>
<td>A549</td>
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<td>20.2±0.7*</td>
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<td>HT1080</td>
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</tr>
<tr>
<td>HCT116</td>
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</tr>
<tr>
<td>MCF7</td>
<td>5.9±0.4</td>
<td>4.0±0.4</td>
</tr>
</tbody>
</table>

**B**

Hypoxic levels of SIRT1 (% versus normoxic levels)

**C**

Hypoxic levels of SIRT1 mRNA (versus normoxic levels)

**D**

Cell viability (%)

**E**

Colony number/190 mm²

*Significant differences.
Figure 7

**Normoxia**

- SIRT1
- LKB1
- AMPKα
- Active mitochondria
- Cyt-c
- Caspase 9
- Caspase 3
- Apoptosis
- Sensitive to drugs

**Hypoxia**

- SIRT1
- LKB1
- AMPKα
- Inactive mitochondria
- Cyt-c
- Caspase 9
- Caspase 3
- Apoptosis
- Resistant to drugs
SIRT1 and AMPK mediate hypoxia-induced resistance of non-small cell lung cancers to cisplatin and doxorubicin

Dong Hoon Shin, Yong-Joon Choi and Jong-Wan Park

*Published OnlineFirst November 15, 2013.*

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