Original Article

Sirtuin 1 promotes the growth and cisplatin resistance of endometrial carcinoma cells: a novel therapeutic target

Running title: SIRT1 in endometrial carcinoma

Authors: Ryoichi Asaka, Tsutomu Miyamoto, Yasushi Yamada, Hirofumi Ando, David Hamisi Mvunta, Hisanori Kobara and Tanri Shiozawa

Affiliation: Department of Obstetrics and Gynecology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

This work was supported by JSPS KAKENHI Grant Number 24791696.

Address correspondence to: Miyamoto T: Department of Obstetrics and Gynecology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

Tel.: 81-263-37-2719 Fax: 81-263-39-3160 e-mail: tmiya@shinshu-u.ac.jp

Key words: Sirtuin1, endometrial carcinoma, cisplatin resistance, cell survival, SIRT1 inhibitor

Abstract

Sirtuin 1 (SIRT1), originally identified as a longevity gene, is induced by caloric restriction, and regulates various cellular functions including DNA repair, cell survival and metabolism via the deacetylation of target proteins such as histone and p53. These functions are considered to act dualistic as preventing or facilitating cancer. This study aimed to clarify the expression and role of SIRT1 in endometrial carcinoma. Because high calorie diet was a well-known risk factor for endometrial carcinoma, we first hypothesized that SIRT1 might be down-regulated in normal endometrial glandular cells of obese women. However, no correlation was observed between the expression of SIRT1 and body mass index (BMI). In contrast, regardless of BMI, the immunohistochemical expression of SIRT1 was significantly higher in endometrial carcinoma (108 cases) than in normal endometria (60 cases) ($p<0.05$), and its overexpression was associated with a shorter survival ($p<0.05$). Our experiments *in vivo* revealed that SIRT1 accelerated the proliferation of endometrial carcinoma cell lines (HHUA, HEC151 and HEC1B). SIRT1 overexpression significantly enhanced the resistance for cisplatin and paclitaxel in HHUA cells. Although p53 is an important target protein for SIRT1, the selective SIRT1 inhibitor (EX527) significantly suppressed the proliferation and cisplatin resistance of three endometrial carcinoma cell lines regardless of the p53 mutation status. In addition, SIRT1 overexpression in HHUA cells accelerated tumor growth and cisplatin resistance in nude mice, and EX527 significantly suppressed the growth of tumors of HHUA and HEC1B cells. No adverse effect of EX527 was observed in these mice. In conclusion, SIRT1 is involved in the acquisition of the aggressive behavior associated with endometrial carcinoma, and the SIRT1 inhibitor, EX527, may be a useful agent for the treatment of this malignancy.

47

48 **List of abbreviations**

49 AMPK: 5' adenosine monophosphate-activated protein kinase

50 BMI: body mass index

51 CDDP: cisplatin

52 CPα: cyclic pifithrin-α

53 ER: estrogen receptor

54 ERK: extracellular signal-regulated kinase

55 FOXO: forkhead box protein O

56 IARC: the International Agency for Research on Cancer

57 MAPK: mitogen-activated protein kinase

58 MEK1/2: MAPK/ERK kinase 1/2

59 NAD: nicotinamide adenine dinucleotide

60 NADH: dihydronicotinamide adenine dinucleotide

61 PI: Positivity Index

62 PI3K: phosphoinositide 3-kinase

63 PIP3: Phosphatidylinositol (3,4,5)-triphosphate

64 PR: progesterone receptor

65 PTEN: phosphatase and tensin homolog

66 PTX: paclitaxel

67 RT-PCR: reverse transcriptase polymerase chain reaction

68 SIRT1: sirtuin 1

69 Skp2: S-phase kinase associated protein 2

The incidence of endometrial carcinoma has been correlated with an elevated body mass index (BMI) [1]. And several nutritional factors, such as obesity, a high calorie diet, and low physical activity, have been identified as risk factors for this malignancy[2]. The reasons for these factors have been explained in terms of peripheral estrogen production[3], stimulation of the insulin-like growth factor-1 receptor by insulin[4], and a decrease in the adiponectin / leptin ratio[5]. However, the mechanisms underlying how obesity or a high calorie diet leads to endometrial carcinoma are not fully understood.

Caloric restriction was previously demonstrated to prolong lifespan in various organisms[6, 7]. Sir (Silent information regulator) family genes were reported to be involved in longevity and the Sir2 mutant decreased the lifespan of yeast by 50%[8]. Sirtuins (SIRT1 ~ SIRT7) have been identified as the human homologues of Sir2 and may act as longevity genes. Sirtuin 1 (SIRT1) was found to be very similar to Sir2 and encoded one of the nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases[9]. Several stresses such as caloric restriction are known to enhance the expression of SIRT1 in human cells. In addition, the increase in the NAD / dihydronicotinamide adenine dinucleotide (NADH) ratio induced by a decrease in energy production was shown to enhance the activity of SIRT1[10]. SIRT1 can suppress the transcription of target genes by the deacetylation of histone. Furthermore, SIRT1 can directly deacetylate target proteins and suppress their functions by inducing their degradation[11]. Several molecules including p53 have been identified as the target genes or proteins of SIRT1. SIRT1 is known to be involved in various cellular functions, such as glucose metabolism, the regulation of cell proliferation, DNA repair, the suppression of cell death, and anti-oxidative stress effects, which suppress tumorigenesis and prolong cell survival in non-neoplastic cells. Accordingly, SIRT1 may be involved in the

protection against age-related pathogenesis, such as various types of cancers, diabetes, hepatic steatosis, and cardiovascular disease[12]. Therefore, our first hypothesis is that SIRT1 may be down-regulated in normal endometrial glandular cells of obese women, and that down-regulation of SIRT1 may be involved in facilitating endometrial carcinogenesis.

On the other hand, the oncogenic function of SIRT1 has also been reported in cancer. SIRT1 was found to be overexpressed in many cancers including colon and prostate cancers[13, 14]. The findings of these studies suggested that once cancer cells acquired the ability to produce SIRT1, the presumed function of SIRT1 may promote the survival of carcinoma cells. Therefore, our next hypothesis is that SIRT1 may be overexpressed in endometrial carcinoma cells and facilitate the survival of these cells.

In the present study, we immunohistochemically examined the expression of SIRT1 in normal endometria and endometrial carcinoma, and analyzed the function of SIRT1 in endometrial carcinoma cells *in vitro* and *in vivo*. We also investigated the efficacy of a SIRT1 inhibitor, EX527, in the treatment of endometrial carcinoma.

Materials and Methods

Samples for immunohistochemistry

A total of 108 formalin-fixed and paraffin-embedded tissue specimens of endometrial carcinoma, 24 endometrial hyperplasia, and 60 normal endometria obtained by hysterectomy or biopsy were selected from the pathology files of Shinshu University Hospital, and used for immunohistochemistry. Histological diagnoses were made by two or more pathologists in the Department of Laboratory Medicine of Shinshu University Hospital. Endometrial carcinomas were treated between 1996 and 2007 and body mass

indices and follow-up survival data were known. According to the International Federation of Gynecology and Obstetrics classification (2008) [15], 87 patients had stage I and II, while 21 had stage III and IV. Histologically, all of the 108 carcinomas were endometrioid adenocarcinoma (68 were grade 1, 21 were grade 2, and 19 were grade 3). The endometrial hyperplasia samples included 8 simple, 8 complex, and 8 atypical hyperplasia. Normal endometria were classified into 16 proliferative phase, 21 secretory phase, and 23 post-menopausal atrophic endometria according to endometrial dating[16]. Each tissue sample was used with the approval of the Ethics Committee of Shinshu University, after obtaining written consent from the patients.

Immunohistochemistry and evaluation

Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase complex method according to the manufacturer's instructions (Histofine MAX-PO kit; Nichirei, Tokyo, Japan). The primary antibody was rabbit monoclonal anti-SIRT1 (1:50; Epitomics, Inc., Burlingame, CA). Regarding SIRT1 staining, 4-μm-thick sections were deparaffinized and boiled in 0.01mol/L citrate buffer (pH 6.0) for 20 minutes in a 500W microwave oven. They were then treated with 0.3% hydrogen peroxide and incubated with the primary antibody at 4°C overnight. After the sections had been washed in phosphate-buffered saline (PBS), they were incubated with biotinylated anti-rabbit immunoglobulin G, treated with peroxidase-conjugated streptavidin, and stained with diaminobenzidine and 0.15% hydrogen peroxidase. Counterstaining was performed with hematoxylin. Staining with a rabbit IgG-Isotype control antibody (Abcam, Cambridge, UK) was used as a negative control. Renal cell carcinoma was used as a positive control (Supplementary figure 2A). Immunoreactivity was evaluated according to the percentage of positive cells observed, which was defined as similar or stronger

staining than endothelial cells as an internal control, among 500 cells in 5 high power fields by three independent reviewers (R.A., T.M. and Y.Y.), and these results were described as a positivity index (PI) with a maximal score of 100. The PIs of normal endometria were counted in the glandular epithelium, except for the surface epithelium. The significance of differences in PI among the histological grades was examined using Scheffe's test. The significance of differences in PI between other clinicopathological parameters was examined using the Mann-Whitney U test. A P value of less than 0.05 was considered significant. Cumulative survival was also analyzed using the Kaplan-Meier method. Because the 75 percentile of PI in carcinoma was 52.75, we tentatively defined PI=50 as the reference point of SIRT1 overexpression. Cases were classified based on the expression of SIRT1 (PI≥50 vs. PI<50), and differences in survival were then evaluated using the log-rank test.

Cell lines, transfection of SIRT1 siRNA, or cDNA

Normal endometrial glandular cells were extracted from surgical specimens with a previously described protocol[17]. The endometrial carcinoma cell line, Ishikawa was kindly provided by Dr. Nishida (Kasumigaura Medical Center, Ibaraki, Japan), HHUA was provided by the RIKEN BioResource Center (Ibaraki, Japan), HEC108, HEC151, and HEC265 were provided by the JCRB Cell Bank (Osaka, Japan), HEC1A, HEC1B, ECC1, AN3CA, KLE, and RL95-2 were purchased from the ATCC (Manassas, VA). The expression of the SIRT1 protein in these endometrial carcinoma cell lines was examined by western blot analysis (Supplementary figure 1A). All of the cell lines apparently expressed the SIRT1 protein. SIRT1-specific siRNA and scramble siRNA (as control), the plasmids containing SIRT1-cDNA, and an empty plasmid (as the control) were purchased from Origene (Rockville, MD), and each was transfected into the cell

lines using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. SIRT1-specific siRNAs were used for HEC151, HEC1B, HHUA, ECC1 and Ishikawa. SIRT1-cDNAs were used for HEC151, HEC1B, ECC1 and Ishikawa. The stable SIRT1-overexpressing HHUA (HHUA-SIRT1) was established by the transfection of SIRT1-cDNA and the isolation of colonies using neomycin. Control HHUA was established by the same techniques using empty vector. The effects of silencing and overexpressing of SIRT1 in all cell lines were confirmed by Western blot and real time RT-PCR (Supplementary figure 1B, C).

Cell cycle analysis (flow cytometry)

Cell cycle was analyzed by quantitation of DNA content using flow cytometry. In brief, cells were removed using tripsin-EDTA solution from the culture dishes. Then, the cells were adjusted to 10^6 cells/ml using PBS containing 0.2% triton X-100 and ribonuclease (RNase), stained with 1μg/ml of propidium iodide (PI), and detected the fluorescence in flow cytometry (BD FACS CANTO Becton, Dickinson and Company)(Measurement condition was 585/42 nm of fluorescence wavelength, and 488nm of excitation wavelength).

Ultraviolet (UV) irradiation

Cells were cultured on 60mm-dish to 60~70% confluency. Medium was replaced to PBS, and the culture-dishes without lid were irradiated for 5 minutes by UV-C (around 254 nm wavelength) using a UV germicidal lamp. Cells were harvested 8 hours after UV irradiation.

Western blotting

Proteins extracted from cultured cells were subjected to Western blot analysis, as described previously[18], using antibodies against human SIRT1 (rabbit-polyclonal; Cell

Signaling, Danvers, MA), p53 (mouse-monoclonal; Cell Signaling, Danvers, MA), acetylated-p53 (which recognized acetylation at Lys382; rabbit polyclonal; Cell Signaling), p21 (mouse monoclonal; Cell Signaling), phosphorylated Akt (pAkt) (rabbit polyclonal; Cell Signaling), phosphorylated MAPK (pMAPK) (rabbit monoclonal; Cell Signaling), FOXO3A (rabbit monoclonal; abcam, Cambridge, UK) and β-actin (mouse monoclonal; BioMakor, Rehovot, Israel) as the primary antibody. The membranes were blotted with a primary antibody at 4°C overnight and then incubated with a peroxidase-conjugated secondary antibody. Bound antibodies were visualized using the ECL Western blot detection reagent (Amersham, Piscataway, NJ).

Assay of SIRT1 activity

Protein was extracted from the cells cultured on 60mm dishes, and the deacetylase activity of SIRT1 was measured using SIRT1 Activity Assay Kit (Fluorometric) (abcam, Cambridge, UK) according to the manufacturer's instructions.

Assay for cell proliferation and anticancer drug resistance (WST-1 assay)

The PI3K inhibitor, wortmannin (Sigma-Aldrich, St. Lois, MO), was used to inhibit the PI3K pathway. The MEK 1/2 inhibitor, U0126 (Sigma-Aldrich, St. Lois, MO), was used to inhibit the MAPK pathway. The inhibitory effect of wortmannin (1μM) and U0126 (10μM) was confirmed by Western blotting (Supplementary figure 1D). The SIRT1 selective inhibitor, EX527 (Merck Millipore, Billerica, MA) was used at functional concentrations as described previously[19]. The anticancer drugs, cisplatin (CDDP) (Sigma-Aldrich, St. Lois, MO) and paclitaxel (PTX) (Wako, Osaka, Japan), diluted with 5% dimethylformamide (DMFA) and saline were added to the culture medium at various concentrations, and cell viability was measured after 72 hours.

Cell viability in the assay for proliferation or anticancer drug resistance was

214 evaluated using the WST-1 reagent (Roche Diagnostics, Basel, Switzerland) according
215 to the manufacturer's instructions. Briefly, cells were seeded onto 96-well plates. After
216 culturing the cells under various conditions, the WST-1 reagent was added to the
217 medium. After 2.5 hours, A450 wavelength was measured using the microplate reader,
218 SYNERGY HT (BioTek, Winooski, VT). Each result was obtained from 3 independent
219 experiments with 16 replicates.

220 ***Soft-agar colony formation assay***

221 Each 60mm plate was prepared by adding 2 mL of agar medium (0.8 mL of 1.25% agar
222 and 1.2 mL of F12 medium with 15% FBS. Five hundred cells of control-HHUA or
223 HHUA-SIRT1were suspended in 1 mL of F12 medium with 15% FBS and 0.33% agar
224 and then layered on top of the hardened agar medium in each 60mm plate. Plates were
225 maintained at 37°C for 4 weeks. Colonies were stained with 0.04% crystal violet-2%
226 ethanol in PBS. The total number of stained colony in each 60mm plate was counted by
227 three independent reviewers (R.A., T.M. and Y.Y.). Each result was obtained from 3
228 independent experiments with 3 replicates.

229 ***Real time reverse transcriptase polymerase chain reaction (real time RT-PCR)***

230 Total RNA was extracted using the TRIzol reagent (Life Technologies) according to the
231 manufacturer's instructions, and reverse-transcribed to cDNA for PCR using the
232 PrimeScript RT-PCR Kit (Takara Bio, Shiga, Japan). Sequences of the SIRT1-specific
233 primer sets were 5'-TCAGTGTCATGGTTCCTTTGC-3' for the forward primer and
234 5'-AATCTGCTCCTTTGCCACTCT-3' for the reverse primer (annealing 57°C). Real
235 time quantitative PCR was performed using the Light Cycler 480 DNA SYBR Green I
236 Master (Roche Diagnostics) in Light Cycler 480 system II (Roche Diagnostics)
237 according to the manufacturer's instructions. The amplified efficiency of the SIRT1

primers used for real-time RT-PCR was 1.970. The expression of SIRT1 mRNA was quantitated using β-actin as an internal control gene. The sequences of the β-actin specific primer sets were 5'-GACAGGATGCAGAAGGAGATTACT-3' for the forward primer and 5' -TGATCCACATCTGCTGGAAGGT-3' for the reverse primer (annealing 56°C). Each experiment for real-time RT-PCR was independently repeated 5 times with 3 replicates.

Apoptosis assay

Immunofluorescent staining with propidium iodide (PI) and annexin-V (Annexin-V-FLUOS Staining Kit, Roche Applied Science) to detect apoptosis was performed according to the manufacturer's instructions. Pictures of cells were taken busing the Floid cell imaging station (Life Technologies). The cells stained with green color are indicating apoptotic cells. The number of green-colored cells was counted in 3 different fields under 40x magnification.

p53 sequencing

The database for the p53 gene status was obtained from the International Agency for Research on Cancer (IARC) TP53 Database (http://p53.iarc.fr). No data was available for ECC1 or RL95-2 in the Database. These two cell lines were examined using direct sequencing with the protocol and primer sets of IARC. Sequencing analysis was performed by an Applied Biosystems 130 genetic analyzer (Life Technologies)

Assay of tumor growth in nude mouse

The backs of six-week-old nude mice (BALB/c-nu, Charles River Laboratories Japan, Kanagawa, Japan) were subcutaneously injected with $1x10^7$ resuspended cells under anesthesia. The care and use of experimental animals was in accordance with the institutional guidelines. CDDP (5mg/kg) and EX527 (10mg/kg) were diluted with 5%

DMFA and saline, and were injected intraperitoneally every week. Mice in DMFA group (as control) were injected intraperitoneally with 400μl of 5% DMFA and saline every week. The tumor size and body weight of each mouse were measured twice a week.

Statistical analysis

Statistical analysis was conducted using the SPSS Statistics system (IBM, Armonk, NY).

Results

The immunohistochemical expression of SIRT1 in normal and neoplastic endometria

The results of immunohistochemical staining for the SIRT1 protein were shown in Figure 1. Staining for SIRT1 was predominantly observed in glandular cells, but not in stromal cells (Fig. 1A). The positive SIRT1 staining was predominantly localized in the cytoplasm. In normal endometria, the expression of the SIRT1 protein was significantly higher in the secretory phase than in the proliferative phase and postmenopausal endometria (Fig. 1B). Among the three secretory phases, the expression of SIRT1 was only higher in the late secretory phase, which showed pre-decidual changes in the stroma (Supplementary figure 2B) [16]. Contrary to our expectations, no correlation was observed between body mass index (BMI) and expression of the SIRT1 protein in normal endometrial glandular cells (Fig. 1B). SIRT1 protein expression was significantly higher in endometrial carcinomas than in normal endometria (31.3 vs 8.5; median Positivity Index (PI)) (Fig. 1A, B, Supplementary figure 2C). However, no correlation was observed between the expression of SIRT1 in endometrial carcinomas and the BMI of patients (data not shown). The expression of SIRT1 was significantly

elevated in patients with higher histological grades (54.5 as grade 3 vs. 26.5 as grade 1; median PI) and lymph-vascular space invasion (34.0 as positive vs. 28.0 as negative; median PI) (Fig. 1C). In addition, the overexpression of SIRT1 (PI≥50) aggravated overall survival (p=0.040) and disease-free survival (p=0.035) in endometrial carcinoma patients (Fig. 1D). These results suggested that the expression of SIRT1 was associated with the aggressive behavior of endometrial carcinoma.

Effects of SIRT1 on cell proliferation

The effect of SIRT1 on the proliferation of endometrial carcinoma cell lines was examined using the WST-1 assay. SIRT1 knockdown by SIRT1-specific siRNA significantly reduced the proliferative activities of HHUA, ECC1, Ishikawa, HEC1B and HEC151 cells ($p<0.05$) (Fig. 2A). In contrast, the proliferative activity of these cells with SIRT1-overexpression was stronger than that of control cells ($p<0.05$) (Fig. 2B). The result of flow cytometry indicated that S-phase fraction was increased by SIRT1-overexpression in HHUA cells (13.7% of HHUA-SIRT1 versus 10.8% of HHUA-Control) and decreased by SIRT1-knockdown in Ishikawa cells (7.7% of Ishikawa-siRNA versus 11.2% of Ishikawa-Control) (Supplementary figure 4A, B). However, other changes in G1, G2 and sub-G1 fractions by alteration of SIRT1 expression were not observed in HHUA, ECC1 and Ishikawa cells. This effect SIRT1-overexpression on proliferation was cancelled by the addition of a selective SIRT1 inhibitor (EX527), phosphoinositide 3-kinase (PI3K) inhibitor (Wortmannin), or mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK 1/2) inhibitor (U0126) (Fig. 2C). EX527 also decreased the viability of control HHUA cells ($p<0.05$), whereas Wortmannin and U0126 could not decrease it. In addition, the colony formation assay using HHUA revealed that the number of

colonies were increased by the overexpression of SIRT1 (Fig. 2D). These results indicated that SIRT1 stimulated the proliferative activity of endometrial carcinoma cells. The PI3K and MAPK pathways might be involved in the part of SIRT1-induced cell proliferation.

The expression of SIRT1 under stresses

Next, we focused on the effect of SIRT1 on cell survival. The expression of SIRT1 mRNA and protein in HHUA cells with cytotoxic treatment, such as ultraviolet (UV) and cisplatin (CDDP), was examined by real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) and Western blotting. The expression of SIRT1 mRNA was increased by UV exposure (150% increase, $p<0.05$) (Fig. 3A) and CDDP treatment (400% increase, $p<0.05$) (Fig. 3B). The expression of SIRT1 protein was also increased by UV (Fig. 3A) and CDDP (Fig. 3B). In addition, the CDDP treatment increased the deacetylase activity of SIRT1 in endometrial carcinoma cells (Supplementary figure 4C).

Effects of SIRT1 on anticancer drug resistance

Because the immunoreactivity of recurrent tumors was higher for SIRT1 after platinum-based chemotherapy (Supplementary figure 5A), the effects of SIRT1 on cell viability were examined in cells treated with an anticancer agent using the WST-1 assay. The results obtained revealed that the overexpression of SIRT1 significantly increased the viability of HHUA cells against CDDP (31% increase compared with control under 20μM CDDP treatment, $p<0.05$) and paclitaxel (PTX) (37% and 96% increase compared with control under 2.5 and 5μM PTX treatment, respectively, $p<0.05$) (Fig. 3C). This effect of SIRT1 on the CDDP resistance was cancelled by the SIRT1 inhibitor (EX527) (Fig. 3D). EX527 additionally decreased the viability of both HHUA cells

($p<0.05$). On the other hand, immunofluorescence for annexin-V revealed that EX527 markedly increased apoptosis in both control HHUA and HHUA-SIRT1 with the CDDP treatment (Fig. 4A, Supplementary figure 5B). However, the PI3K inhibitor (Wortmannin) did not affect CDDP resistance or apoptosis (Fig. 3D, 4A). U0126 also did not affect resistance or apoptosis (data not shown).

CDDP resistance by SIRT1 and p53

A previous study demonstrated that p53 was one of the target proteins of deacetylation by SIRT1[20]; therefore, SIRT1-induced CDDP resistance may be mediated via the inhibition of p53. The p53 inhibitor, cyclic pifithrin-α (CPα), was shown to decrease apoptosis in HHUA (Fig. 4A), which confirmed the involvement of p53. The p53 gene mutation has frequently been reported in endometrial carcinomas[21, 22] and may influence the effects of SIRT1. Therefore, we examined the p53 mutational status of 11 endometrial carcinoma cell lines by the International Agency for Research on Cancer (IARC) database or direct sequencing (Fig. 4B, Supplementary table 1). Of these, three cell lines had wild type p53 and 8 cell lines had p53 mutations. In the present study, we used 5 cell lines; HEC151 with wild-type p53 and strong expression of p21, HHUA with a partially functional p53 mutation and detectable expression of p21, Ishikawa, ECC1 and HEC1B with a non-functional p53 mutation and undetectable expression of p21. In HEC151, Ishikawa and ECC1 cells, the addition of the SIRT inhibitor (EX527) and CDDP increased the expression of p53 acetylated at Lys382 (Fig. 4C). These results identified p53 as the substantial target protein of SIRT1 in endometrial carcinoma cells.

The WST-1 assay revealed that EX527 significantly decreased the survival of HEC151 cells (with wild-type p53) with the CDDP treatment (45% decrease from CDDP, $p<0.05$), and the addition of CPα partially recovered survival (18% recovery

from CDDP + EX527, $p<0.05$) (Fig. 4D). EX527 significantly reduced CDDP resistance in HEC1B cells with mutated and non-functional p53 (18% decrease from CDDP, $p<0.05$). However, the addition of CPα did not recover CDDP resistance (8% decrease compared with CDDP + EX527 without significant difference). These results indicated that SIRT1 may act by enhancing CDDP resistance via p53-dependent and p53-independent pathways.

Effects of SIRT1 and the SIRT1 inhibitor on tumor growth in nude mice

The effects of SIRT1 on tumor formation were examined *in vivo* using a mouse xenograft model of endometrial carcinoma cell lines. Four weeks after grafting, the size of HHUA-SIRT1 tumors was approximately 40% larger than that of control HHUA ($p<0.05$) (Fig. 5A, B). Hematoxylin-eosin (HE) staining revealed that HHUA-SIRT1 tumors were less necrotic than those of control-HHUA (Fig. 5C). In addition, immunofluorescent staining of the cleaved-caspase3 protein showed apoptosis was less in HHUA-SIRT1 than in control HHUA (Fig. 5C).

We then examined the effects of EX527 and CDDP *in vivo* using mouse xenografts. HHUA-SIRT1 tumors exhibited stronger resistance against the CDDP treatment than HHUA control tumors (Fig. 6A). However, the treatment with EX527 markedly inhibited the tumor growth of HHUA-SIRT1 to the same level as that of control HHUA (Fig. 6A). On the other hand, body weight gain was not impaired in mice, and no other adverse events were observed (Fig. 6B) with the EX527 treatment. In addition, we confirmed the effects of EX527 on tumor growth in the p53 non-functional endometrial carcinoma cell line, HEC1B. The intraperitoneal infusion of EX527 significantly reduced the tumor growth of HEC1B (Fig. 6C, D). These results indicated that EX527 could suppress the tumor growth of endometrial carcinoma regardless of the

382 p53 mutational status.

383

384 **Discussion**

385 Obesity is a risk factor for endometrial carcinoma[23]. SIRT1 was reported to be involved
386 in the longevity induced by caloric restriction[10], and its function in DNA repair and
387 control of the cell cycle[24] was found to be similar to that of a tumor suppressor gene.
388 Therefore, we first hypothesized that SIRT1 may be down-regulated in the normal or
389 cancerous tissues of women with a high BMI. Contrary to this expectation, a correlation
390 was not found between BMI and SIRT1 in the present study.

391 We also revealed that the expression of SIRT1 did not change in a normal
392 cyclic endometrium or menopause, except for the late secretory phase. The effects of
393 estrogen and progesterone are known to be evoked via their specific receptors, the
394 estrogen receptor (ER) and progesterone receptor (PR). We previously reported that the
395 expression of ER and PR was significantly diminished in the late secretory phase of
396 normal endometrial glandular cells[25]. Therefore, the expression of SIRT1 in a normal
397 endometrium does not appear to be controlled by sex steroids. However, the expression
398 of SIRT1 was found to be up-regulated by estrogen in a breast cancer cell line[26].

399 The present study showed that the expression of SIRT1 was significantly
400 higher in endometrial carcinomas than in normal endometrial glands. Its expression was
401 also elevated in grade 3 tumors, and was associated with a shorter survival rate in
402 endometrial carcinoma patients. Furthermore, this study revealed that SIRT1 stimulated
403 the proliferation of endometrial carcinoma cells. These results suggest the oncogenic
404 nature of SIRT1 in this malignancy. Although the overexpression of SIRT1 has already
405 been reported in breast, lung, pancreas, colon, and prostate cancers[27-31] and has been

406 associated with a poor prognosis[27-29], this is the first study to demonstrate the expression
407 of SIRT1 in endometrial carcinoma.
408 We showed that SIRT1-induced cell proliferation was cancelled by the SIRT1
409 inhibitor (EX527), PI3K inhibitor (Wortmannin), and MEK inhibitor (U0126). The
410 growth inhibition by Wortmannin and U0126 was smaller than that by EX527. These
411 results suggested that the PI3K and MAPK pathways might be involved in the part of
412 SIRT1-induced cell proliferation. Regarding the target molecule of SIRT1, PTEN
413 (phosphatase and tensin homolog) and Akt were reported to be the subject of
414 deacetylation by SIRT1[32]. The deacetylation of the Lys14 and Lys20 of Akt were
415 necessary for the binding of Akt to PIP3 (Phosphatidylinositol (3,4,5)-triphosphate) and
416 for its membrane localization and activation[33]. However, our results could not
417 demonstrate the relation between the expression of pAkt or pMAPK and the expression
418 of SIRT1.
419 We also revealed that SIRT1 increased resistance against CDDP or PTX in 3
420 endometrial carcinoma cell lines, which was consistent with the findings of a previous
421 study on the effects of SIRT1 on anticancer drug resistance in other carcinomas[34]. The
422 p53 protein is known to be an important target of deacetylation by SIRT1[11, 20]. The DNA
423 binding capacity of deacetylated p53 was previously shown to be reduced[35], which
424 promoted MDM2-mediated ubiquitination and degradation[36]. Therefore, p53 is
425 considered to be a key factor in SIRT1-mediated survival in tumor cells with wild type
426 p53 such as HEC151. In addition, we showed that SIRT1-mediated CDDP resistance
427 may be independent of the PI3K and MAPK pathways.
428 We also showed that EX527 significantly suppressed the CDDP resistance of
429 endometrial carcinoma cells not only with wild-type p53, but also those harboring

mutated p53. Apart from the p53-dependent pathway, the mechanism underlying SIRT1-mediated CDDP resistance is not well understood. However, the FOXO (forkhead box protein O) family may be involved in SIRT1-mediated CDDP resistance. In tamoxifen-resistant breast cancer cells, SIRT1 boosted anticancer drug resistance by regulating the nuclear localization of the transcriptional factor, FOXO1, which induced the expression of multidrug resistance protein 2[37]. The elevated expression of FOXO1 in HEC1B cells has been reported previously[38]. Wang et al. demonstrated that SIRT1 deacetylated FOXO3, which, in turn, triggered apoptosis by upregulating the genes necessary for cell death, and facilitated its degradation via poly-ubiquitination by the ubiquitin-ligase, Skp2 (S-phase kinase associated protein 2) [39]. The expression of FOXO3A protein was not changed by knockdown or overexpression of SIRT1 in our study. However, acetylation status of FOXO3A was not confirmed.

To date, no agent has demonstrated efficacy in molecular-targeted therapy against endometrial carcinoma. Our results indicated that SIRT1 may be a therapeutic target candidate. Several studies previously reported the anticancer effects of SIRT1 inhibitors. Ueno et al. showed that tenovin-6, an inhibitor of SIRT1 and 2, induced apoptosis in five colon carcinoma cell lines regardless of the p53 mutation status, and the synergistic antitumor effects of tenovin-6 were also observed in combination with either 5-FU or oxaliplatin[40]. In contrast, Kabra et al. reported that EX527 (2μM), which is highly specific to SIRT1, but not SIRT2, enhanced the proliferation of the colon cancer cell line, HCT116[41]. Peck et al. reported that 25μM or higher of Sirtinol and Salermide (inhibitors of SIRT1 and 2) significantly inhibited the proliferation of the breast cancer cell line, MCF-7, whereas 100μM or higher of EX527 repressed proliferation[42]. These findings suggested that the inhibitors of SIRT1 and 2 had stronger

anti-cancer effects than the selective SIRT1 inhibitor, EX527. However, the present study revealed that the lower dose (1μM) of EX527 significantly inhibited proliferation and had a synergic anticancer effect on CDDP chemotherapy in 3 endometrial carcinoma cell lines regardless of the p53 mutation status. Zhang et al. also recently reported that EX527 (1μM) had significant anti-cancer effects in PANC-1 and ASPC-1 cell lines[43]. In addition, our *in vivo* experiments demonstrated that EX527 significantly reduced the tumor growth of endometrial carcinoma in nude mice as a single agent. Therefore, EX527 appeared to be a more effective agent of molecular targeted therapy for SIRT1, especially in endometrial carcinoma.

In contrast to the CDDP treatment, an intraperitoneal injection of EX527 (10mg/kg/weekly for 4 weeks) did not promote adverse events or affect body weight gain in nude mice. There were a few studies on the systemic administration of EX527 to mice[44, 45], and no adverse effect of EX527 was noted. Among the other inhibitors of SIRT1 and 2, an intravenous injection (twice a week for 4 weeks) of sirtinol (10mg/kg) and a daily intraperitoneal injection of cambinol (100mg/kg) did not induce weight loss or adverse effects[46, 47]. However, serum glucose levels were not examined in this study. SIRT1 was previously reported to be involved in glucose metabolism due to the activation of AMPK (5' adenosine monophosphate-activated protein kinase) [48], and also contributed to the regulation of serum glucose levels in type 2 diabetes mellitus[49]. Therefore, further studies are needed to clarify the adverse effects associated with the administration of EX527, especially regarding metabolic disorders.

Our results showed that SIRT1 contributed tumor progression and chemoresistance of endometrial carcinoma. In addition, SIRT1 increased colony formation in soft agar. These findings suggest SIRT1 to provide stem cell nature to the

478 cells. In breast cancer, CD44+/CD24- cancer stem cell had high level of SIRT1[50].
479 However, there is no stem cell marker established in endometrial carcinoma.
480 In summary, the present study revealed that SIRT1 played important roles in
481 tumor progression, growth, and anticancer drug resistance in endometrial carcinoma.
482 The SIRT1 inhibitor effectively cancelled these functions of SIRT1 regardless of the
483 p53 mutation status. These results suggest that SIRT1 is a novel and promising
484 therapeutic target candidate, and SIRT1 inhibitors especially EX527 may be useful
485 agents for the treatment of endometrial carcinoma.
486

487 **Acknowledgments**

488 The authors are grateful to Fumi Tsunoda and Eiji Uchida (Research Assistants;
489 Department of Obstetrics and Gynecology, Shinshu University School of Medicine) for
490 their excellent technical assistance.
491 This work was supported by JSPS KAKENHI Grant Number 24791696.
492

493 **Disclosure of Interest**

494 The authors declare that there are no conflicts of interest.

495 **References**

496 1. Renehan AG, Tyson M, Egger M, et al. Body-mass index and incidence of cancer: a
497 systematic review and meta-analysis of prospective observational studies. Lancet.
498 2008; 371: 569-578.
499 2. Goodman MT, Hankin JH, Wilkens LR, et al. Diet, body size, physical activity, and
500 the risk of endometrial cancer. Cancer Res. 1997; 57: 5077-5085.
501 3. Berstein LM, Tchernobrovkina AE, Gamajunova VB, et al. Tumor estrogen content
502 and clinico-morphological and endocrine features of endometrial cancer. J Cancer
503 Res Clin Oncol. 2003; 129: 245-249.
504 4. Hirano S, Ito N, Takahashi S, et al. Clinical implications of insulin-like growth
505 factors through the presence of their binding proteins and receptors expressed in
506 gynecological cancers. Eur J Gynaecol Oncol. 2004; 25: 187-191.
507 5. Petridou E, Mantzoros C, Dessypris N, et al. Plasma adiponectin concentrations in
508 relation to endometrial cancer: a case-control study in Greece. J Clin Endocrinol
509 Metab. 2003; 88: 993-997.
510 6. Viswanathan M, Guarente L. Regulation of Caenorhabditis elegans lifespan by
511 sir-2.1 transgenes. Nature. 2011; 477: E1-2.
512 7. Weindruch R. The retardation of aging by caloric restriction: studies in rodents and
513 primates. Toxicol Pathol. 1996; 24: 742-745.
514 8. Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone
515 promote longevity in Saccharomyces cerevisiae by two different mechanisms.
516 Genes Dev. 1999; 13: 2570-2580.
517 9. North BJ, Verdin E. Sirtuins: Sir2-related NAD-dependent protein deacetylases.
518 Genome Biol. 2004; 5: 224.

519 10. Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span
520 extension by calorie restriction in Saccharomyces cerevisiae. Science. 2000; 289:
521 2126-2128.
522 11. Vaziri H, Dessain SK, Ng Eaton E, et al. hSIR2(SIRT1) functions as an
523 NAD-dependent p53 deacetylase. Cell. 2001; 107: 149-159.
524 12. Herranz D, Serrano M. SIRT1: recent lessons from mouse models. Nat Rev Cancer.
525 2010; 12: 819-823.
526 13. Stünkel W, Peh BK, Tan YC, et al. Function of the SIRT1 protein deacetylase in
527 cancer. Biotechnol J. 2007; 2: 1360-1368.
528 14. Lee H, Kim KR, Noh SJ, et al. Expression of DBC1 and SIRT1 is associated with
529 poor prognosis for breast carcinoma. Hum Pathol. 2011; 42: 204-213.
530 15. Pecorelli S. Revised FIGO staging for carcinoma of vulva, cervix, and
531 endometrium. Int J Gynaecol Obstet. 2009; 105: 103-104.
532 16. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol.
533 1975; 122: 262-263.
534 17. Shiozawa T, Miyamoto T, Kashima H, et al. Estrogen-induced proliferation of
535 normal endometrial glandular cells is initiated by transcriptional activation of cyclin
536 D1 via binding of c-Jun to an AP-1 sequence. Oncogene. 2004; 23: 8603-8610.
537 18. Miyamoto T, Kashima H, Suzuki A, et al. Laser-captured
538 microdissection-microarray analysis of the genes involved in endometrial
539 carcinogenesis: stepwise up-regulation of lipocalin2 expression in normal and
540 neoplastic endometria and its functional relevance. Hum Pathol. 2011; 42:
541 1265-1274.
542 19. Napper AD, Hixon J, McDonagh T, et al. Discovery of indoles as potent and

543 selective inhibitors of the deacetylase SIRT1. J Med Chem. 2005; 48: 8045-8054.
544 20. Luo J, Nikolaev AY, Imai S, et al. Negative control of p53 by Sir2alpha promotes
545 cell survival under stress. Cell. 2001; 107: 137-148.
546 21. Risinger JI, Dent GA, Ignar-Trowbridge D, et al. p53 gene mutations in human
547 endometrial carcinoma. Mol Carcinog. 1992; 5: 250-3.
548 22. Feng YZ, Shiozawa T, Horiuchi A, et al. Intratumoral heterogeneous expression of
549 p53 correlates with p53 mutation, Ki-67, and cyclin A expression in
550 endometrioid-type endometrial adenocarcinomas. Virchows Arch. 2005; 447:
551 816-822.
552 23. Wynder EL, Escher GC, Mantel N. An epidemiological investigation of cancer of
553 the endometrium. Cancer. 1966; 19: 489-520.
554 24. Brachmann CB, Sherman JM, Devine SE, et al. The SIR2 gene family, conserved
555 from bacteria to humans, functions in silencing, cell cycle progression, and
556 chromosome stability. Genes Dev. 1995; 9: 2888-2902.
557 25. Shiozawa T, Li SF, Nakayama K, et al. Relationship between the expression of
558 cyclins/cyclin-dependent kinases and sex-steroid receptors/Ki67 in normal human
559 endometrial glands and stroma during the menstrual cycle. Mol Hum Reprod. 1996;
560 2: 745-752.
561 26. Elangovan S, Ramachandran S, Venkatesan N, et al. SIRT1 is essential for
562 oncogenic signaling by estrogen/estrogen receptor α in breast cancer. Cancer Res.
563 2011; 71: 6654-6664.
564 27. Wu M, Wei W, Xiao X, et al. Expression of SIRT1 is associated with lymph node
565 metastasis and poor prognosis in both operable triple-negative and
566 non-triple-negative breast cancer. Med Oncol. 2012; 29: 3240-3249.

567 28. Noh SJ, Baek HA, Park HS, et al. Expression of SIRT1 and cortactin is associated
568 with progression of non-small cell lung cancer. Pathol Res Pract. 2013; 209:
569 365-370.
570 29. Stenzinger A, Endris V, Klauschen F, et al. High SIRT1 expression is a negative
571 prognosticator in pancreatic ductal adenocarcinoma. BMC Cancer. 2013; 13: 450.
572 30. Kriegl L, Vieth M, Kirchner T, et al. Up-regulation of c-MYC and SIRT1
573 expression correlates with malignant transformation in the serrated route to
574 colorectal cancer. Oncotarget. 2012; 3: 1182-1193.
575 31. Huffman DM, Grizzle WE, Bamman MM, et al. SIRT1 is significantly elevated in
576 mouse and human prostate cancer. Cancer Res. 2007; 67: 6612-6618.
577 32. Ikenoue T, Inoki K, Zhao B, et al. PTEN acetylation modulates its interaction with
578 PDZ domain. Cancer Res. 2008; 68: 6908-6912.
579 33. Sundaresan NR, Pillai VB, Wolfgeher D, et al. The deacetylase SIRT1 promotes
580 membrane localization and activation of Akt and PDK1 during tumorigenesis and
581 cardiac hypertrophy. Sci Signal. 2011; 4: ra46.
582 34. Wang Z, Chen W. Emerging Roles of SIRT1 in Cancer Drug Resistance. Genes
583 Cancer. 2013; 4: 82-90.
584 35. Kim EJ, Kho JH, Kang MR, et al. Active regulator of SIRT1 cooperates with SIRT1
585 and facilitates suppression of p53 activity. Mol Cell. 2007; 28: 277-290.
586 36. Li M, Luo J, Brooks CL, et al. Acetylation of p53 inhibits its ubiquitination by
587 Mdm2. J Biol Chem. 2002; 277: 50607-50611.
588 37. Choi HK, Cho KB, Phuong NT, et al. SIRT1-mediated FoxO1 deacetylation is
589 essential for multidrug resistance-associated protein 2 expression in
590 tamoxifen-resistant breast cancer cells. Mol Pharm. 2013; 10: 2517-2527.

591 38. Goto T, Takano M, Albergaria A, et al. Mechanism and functional consequences of
592 loss of FOXO1 expression in endometrioid endometrial cancer cells. Oncogene.
593 2008; 27: 9-19.
594 39. Wang F, Chan C-H, Chen K, et al. Deacetylation of FOXO3 by SIRT1 or SIRT2
595 leads to Skp2-mediated FOXO3 ubiquitination and degradation. Oncogene. 2012;
596 31: 1546-1557.
597 40. Ueno T, Endo S, Saito R, et al. The sirtuin inhibitor tenovin-6 upregulates death
598 receptor 5 and enhances cytotoxic effects of 5-Fluorouracil and oxaliplatin in colon
599 cancer cells. Oncol Res. 2014; 21: 155-164.
600 41. Kabra N, Li Z, Chen L, et al. SirT1 is an inhibitor of proliferation and tumor
601 formation in colon cancer. J Biol Chem. 2009; 284: 18210-18217.
602 42. Peck B, Chen CY, Ho KK, et al. SIRT inhibitors induce cell death and p53
603 acetylation through targeting both SIRT1 and SIRT2. Mol Cancer Ther. 2010; 9:
604 844-855.
605 43. Zhang JG, Hong DF, Zhang CW, et al. Sirtuin 1 facilitates chemoresistance of
606 pancreatic cancer cells by regulating adaptive response to chemotherapy-induced
607 stress. Cancer Sci. 2014; 105: 445-454.
608 44. Oon CE, Strell C, Yeong KY, et al. SIRT1 inhibition in pancreatic cancer models:
609 contrasting effects in vitro and in vivo. Eur J Pharmacol. 2015; 757: 59-67.
610 45. Yang Y, Jiang S, Dong Y, et al. Melatonin prevents cell death and mitochondrial
611 dysfunction via a SIRT1-dependent mechanism during ischemic-stroke in mice. J
612 Pineal Res. 2015; 58: 61-70.
613 46. Gong DJ, Zhang JM, Yu M, et al. Inhibition of SIRT1 combined with gemcitabine
614 therapy for pancreatic carcinoma. Clin Interv Aging. 2013; 8: 889-897.

615 47. Laemmle A, Lechleiter A, Roh V, et al. Inhibition of SIRT1 impairs the
616 accumulation and transcriptional activity of HIF-1α protein under hypoxic
617 conditions. PLoS One. 2012; 7: e33433.
618 48. Suchankova G, Nelson LE, Gerhart-Hines Z, et al. Concurrent regulation of
619 AMP-activated protein kinase and SIRT1 in mammalian cells. Biochem Biophys
620 Res Commun. 2009; 378: 836-841.
621 49. Milne JC, Lambert PD, Schenk S, et al. Small molecule activators of SIRT1 as
622 therapeutics for the treatment of type 2 diabetes. Nature. 2007; 450: 712-716.
623 50. Ma W, Xiao GG, Mao J, et al. Dysregulation of the miR-34a-SIRT1 axis inhibits
624 breast cancer stemness. Oncotarget. 2015; 6: 10432-10444.
625

626 **Titles and legends to figures**

627 **Fig. 1**

628 **A;** Immunohistochemical staining for the SIRT1 protein in a normal endometrium
629 (upper lane) and endometrial carcinoma (lower lane). PP: Proliferative phase, SP:
630 Secretory phase, PM: Post-menopause, G1-3: Endometrioid adenocarcinoma Grade1-3.
631 **B;** Positivity indexes (PI, maximum score=100) in the groups classified by body mass
632 index (BMI, low: BMI<25 and high: BMI≥25), menstrual cycle, and histology (N:
633 Normal, HP: Hyperplasia, CA: Carcinoma). Box plot: Box describes 25-75 percentiles.
634 Lines in boxes show the median. Lines above/under the box show the
635 maximum/minimum. Dots by circles are scores over 1.5xIQR (interquartile range). *:
636 P<0.05, NS: No significant difference. **C;** PIs in the clinicopathological status. MI:
637 Myometrial invasion (- negative, + positive in under half of the myometrium, ++
638 positive in over half of the myometrium), LVSI: Lymph-vascular space invasion, LNM:
639 Lymph node metastasis. *: P<0.05. **D;** Overall survival and disease-free survival of
640 patients with PI<50 and PI≥50 using the Kaplan-Meier method.

641

642 **Fig. 2**

643 **A;** Relative cell viabilities of endometrial carcinoma cell lines (HEC151, HEC1B,
644 HHUA, ECC1 and Ishikawa) with SIRT1 silencing by SIRT1 specific siRNA
645 transfection (siRNA) or their control cells by scramble siRNA transfection (Control). **B;**
646 Relative cell viabilities of endometrial carcinoma cell lines (HEC151, HEC1B, HHUA,
647 ECC1 and Ishikawa) with SIRT1 overexpression by SIRT1 cDNA transfection (cDNA)
648 or their control cells by empty vector transfection (Control). **C;** Relative cell viabilities
649 of HHUA-SIRT1 and control cells when treated with the vehicle (Veh, 5% DMFA as the

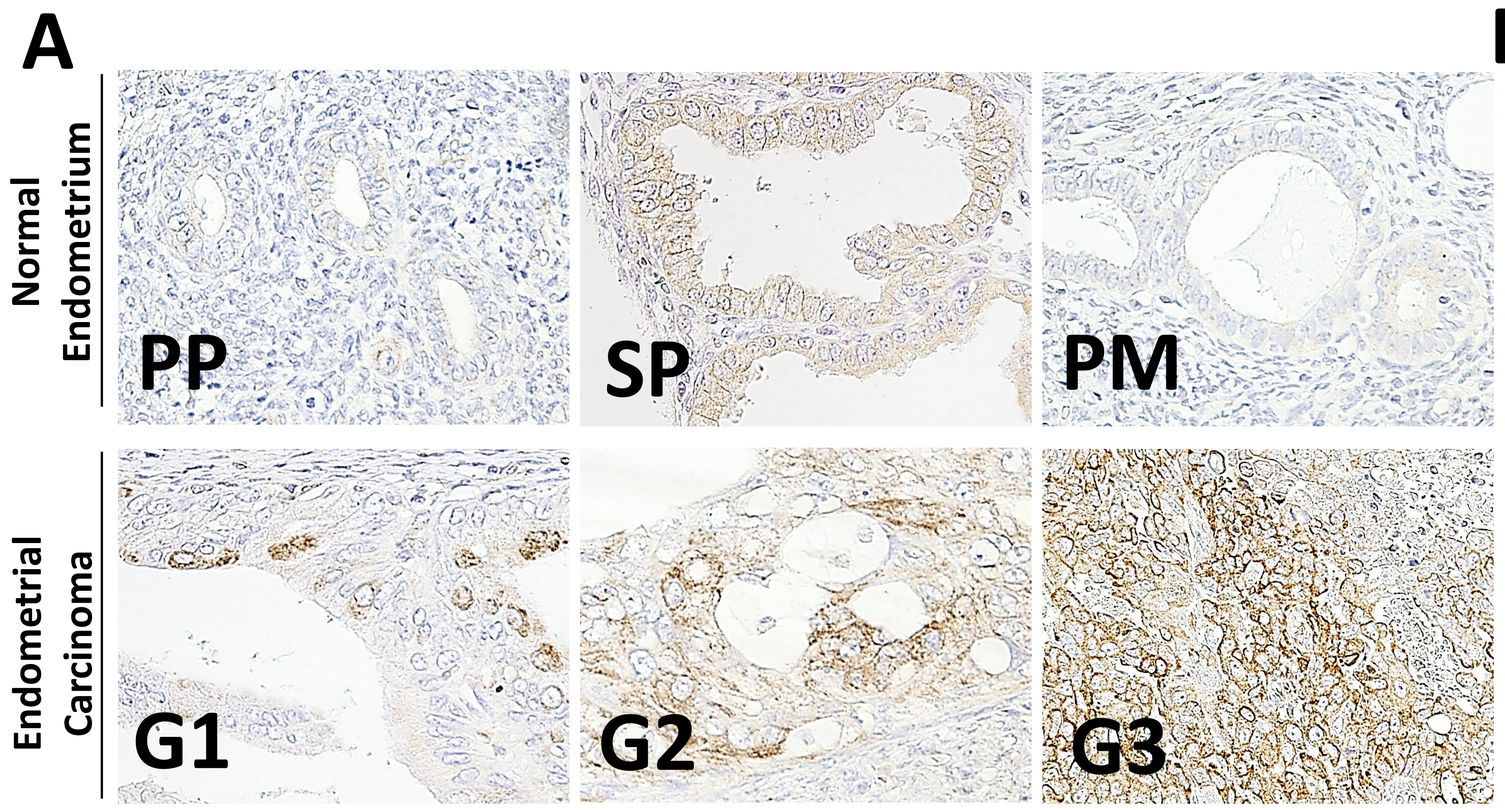
650 solvent), EX527 (SIRT1 inhibitor: 1μM), wortmannin (Wort, PI3K inhibitor: 1μM), or
651 U0126 (MEK inhibitor: 10μM) for 24 hours. **D;** The number of colonies formed by
652 control HHUA and HHUA-SIRT1 were counted.
653 *1: significantly different (P<0.05) from control cells, *2: significantly different
654 (p<0.05) from control treated with Veh, *3: significantly different (p<0.05) from
655 HHUA-SIRT1 treated with Veh, but not different from control treated with the same
656 agent. The error bars show the standard deviation.
657
658 **Fig. 3**
659 **A;** Relative expression of SIRT1 mRNA (real-time RT-PCR) and protein (Western
660 blotting) in HHUA cells with (+) or without (-) ultraviolet (UV) exposure. **B;** Relative
661 expression of SIRT1 mRNA (real-time RT-PCR) and protein (Western blotting) in
662 HHUA cells treated by 72 hours with CDDP. **C;** Relative cell viabilities of
663 HHUA-SIRT1 and control cells treated with CDDP (0-40μM) and paclitaxel (PTX:
664 0-10nM) for 72 hours. **D;** Relative cell viabilities with the CDDP (20μM) treatment in
665 HHUA-SIRT1 and control cells with the vehicle (Veh; 5% DMFA as the solvent),
666 EX527 (1μM), or wortmannin (Wort; 1μM) for 72 hours.
667 *1: significantly different (P<0.05) from no treatment, *2: significantly different
668 (P<0.05) from control cells, *3: significantly different (p<0.05) from control treated
669 with Veh, *4: significantly different (p<0.05) from HHUA-SIRT1 treated with Veh, *5:
670 significantly different (p<0.05) from control treated with the same agent. The error bars
671 show the standard deviation.
672
673 **Fig. 4**

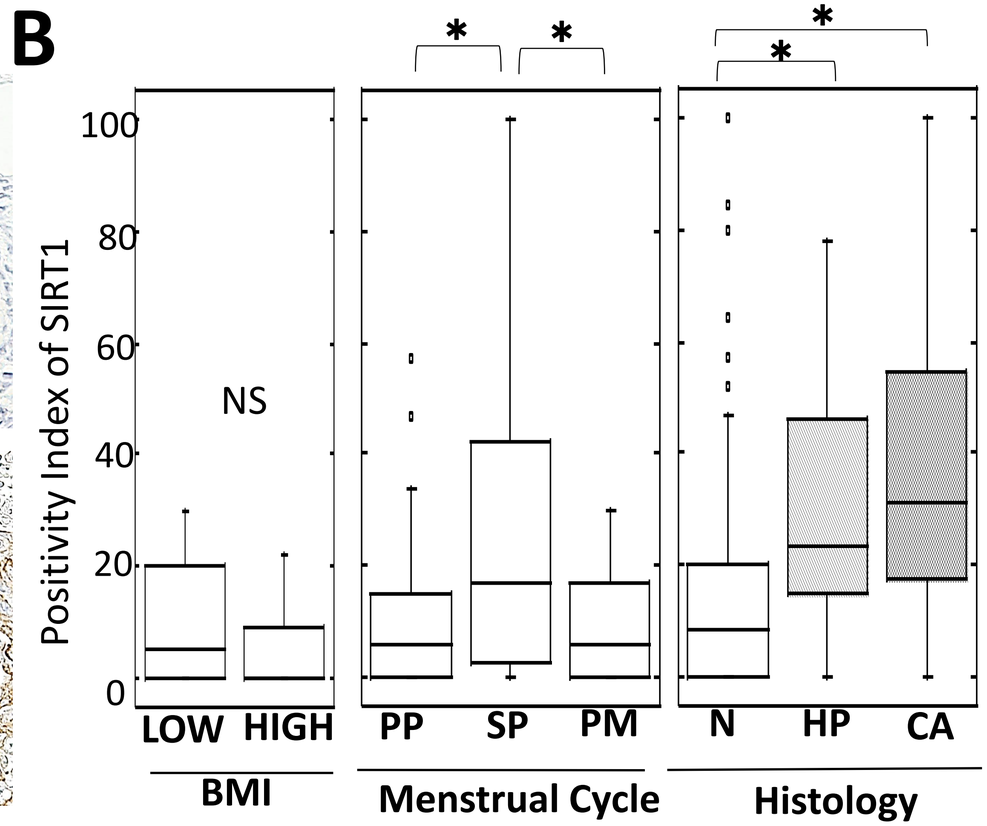
674 **A;** Results of immunofluorescent staining for annexin-V and propidium iodide.
675 HHUA-SIRT1 and control cells were treated with CDDP (20μM) and Veh (5% DMFA
676 as the solvent), EX527 (1μM), Wort (1μM), or Cyclic pifithrin-α (CPα: p53 inhibitor,
677 10μM) for 24 hours. Graph indicates the percentage of apoptotic cells evaluated by
678 counting the green-colored cells **B;** Description of the p53 mutation and the expression
679 of p53, p21, and β-actin (internal control) proteins in endometrial carcinoma cell lines
680 and normal endometrial glandular cells (Western blotting). **C;** Western blot analysis for
681 SIRT1, Acetyl-p53 (Lys 382), and β-actin (internal control) in HEC151, Ishikawa and
682 ECC1 cells treated with/without EX527 (1μM) and CDDP (20μM for HEC151 and
683 Ishikawa, and 5μM for ECC1) for 24 hours. **D;** Relative cell viabilities of HEC1B and
684 HEC151 cells treated with/without CDDP (20μM), EX527 (1μM), and CPα (10μM).
685 *: $P<0.05$, NS: No significant difference. The error bars show the standard deviation.
686
687 **Fig. 5**
688 **A;** HHUA-SIRT1 and control cells (10^7 cells respectively) were subcutaneously grafted
689 in nude mice (left dorsal: control, right dorsal: HHUA-SIRT1). **B;** Estimated tumor
690 volume of control and HHUA-SIRT1 4 weeks after grafting. Estimated tumor volume =
691 (long axis) x (short axis)^2 x 1/2 (cm^3). **C;** Control (left) and HHUA-SIRT1 (right)
692 tumors were extracted from grafted mice. Upper lane: HE staining (x5). Middle lane:
693 HE staining (x40). Lower lane: immunofluorescent staining for cleaved-caspase3 (red)
694 and DAPI (blue) (x200).
695 *: $P<0.05$, NS: No significant difference. The error bars show the standard deviation.
696

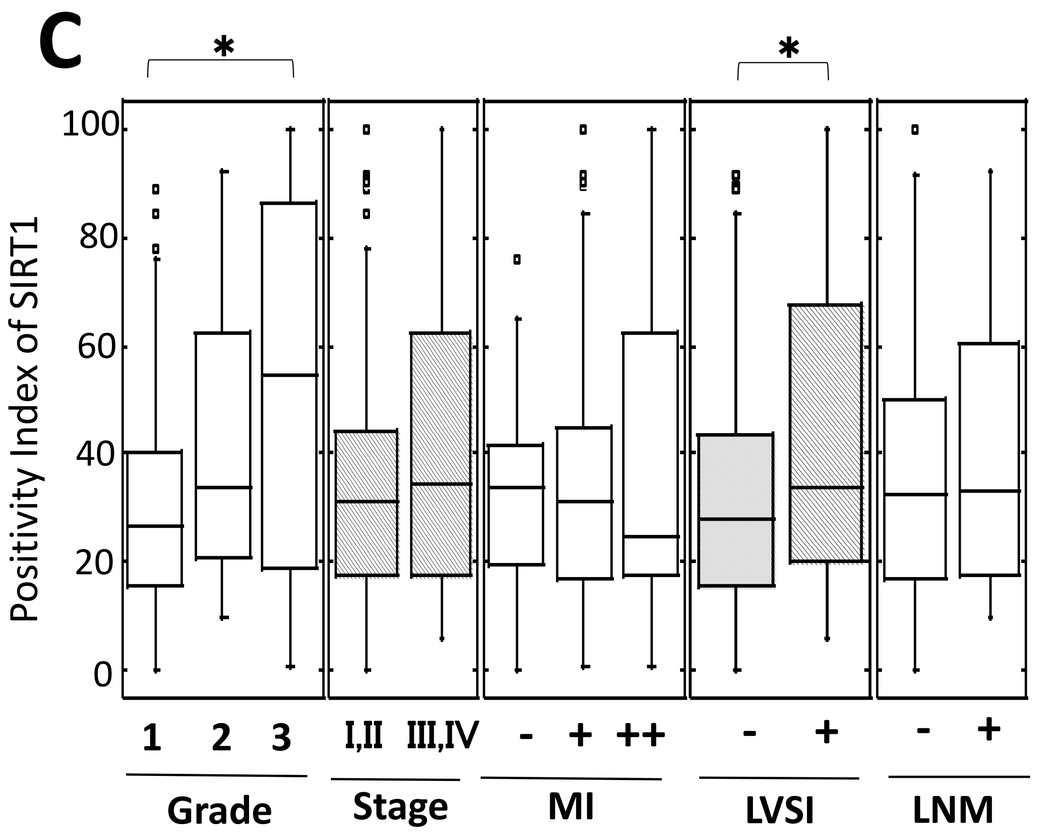
697 **Fig. 6**

698 **A;** Time-dependent changes in relative tumor volumes in nude mice treated with 5%
699 DMFA (as control), CDDP (5mg/kg/weekly) or EX527 (10mg/kg/weekly). **B;** Body
700 weight of each treatment group of mice. **C;** HEC1B cells were subcutaneously grafted
701 in nude mice. DMFA (left, as control) and EX527 (right, 10mg/kg/weekly) were
702 administrated. **D;** Relative tumor volume of HEC1B treated with DMFA (black) and
703 EX527 (blue).
704 *: $P<0.05$, NS: No significant difference. The error bars show the standard deviation.

Figure 1


A
Normal Endometrium
PP
SP
PM
Endometrial Carcinoma
G1
G2
G3


B
Positivity Index of SIRT1
100
80
60
40
20
0
NS
*
*
*
*
LOW
HIGH
BMI
PP
SP
PM
Menstrual Cycle
N
HP
CA
Histology


C
*
*
Positivity Index of SIRT1
100
80
60
40
20
0
1
2
3
Grade
I,II
III,IV
Stage
-
+
++
MI
-
+
LVSI
-
+
LNM

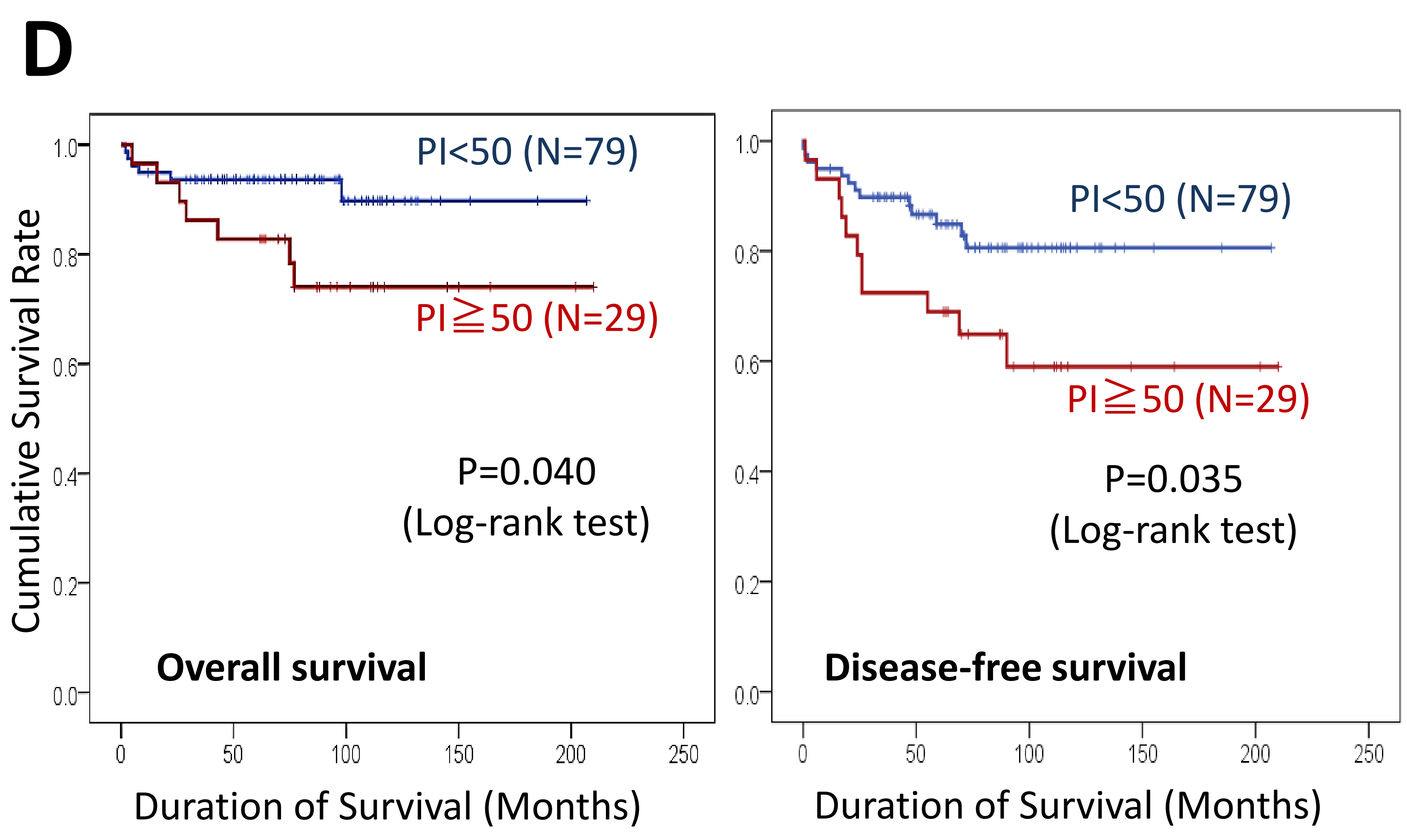
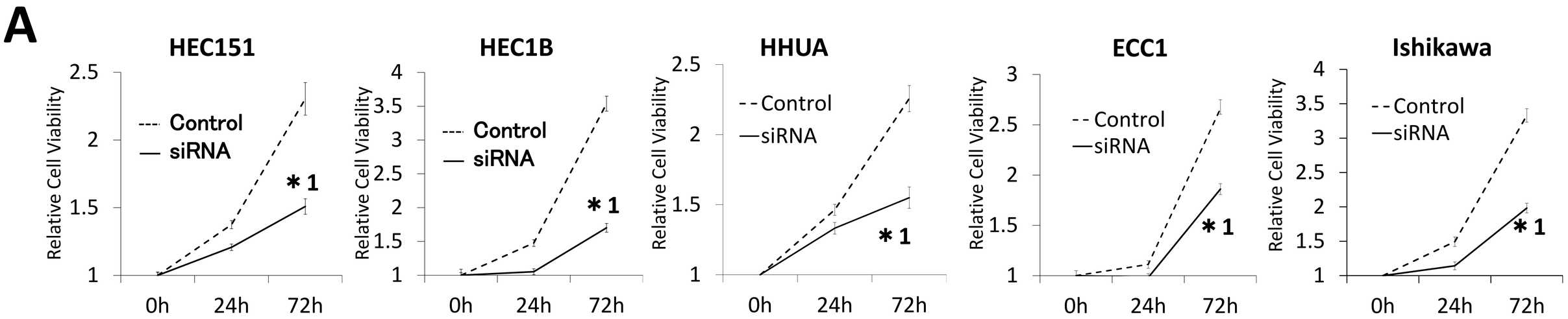
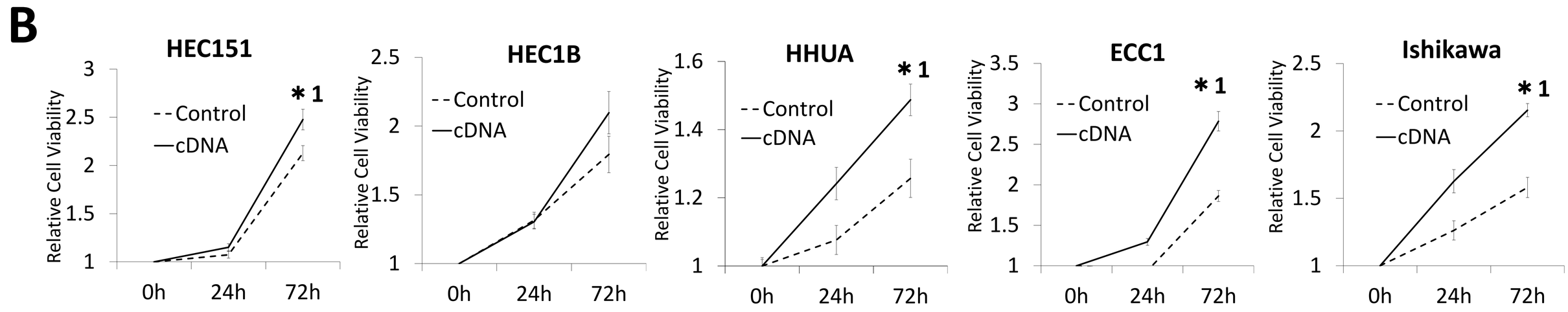
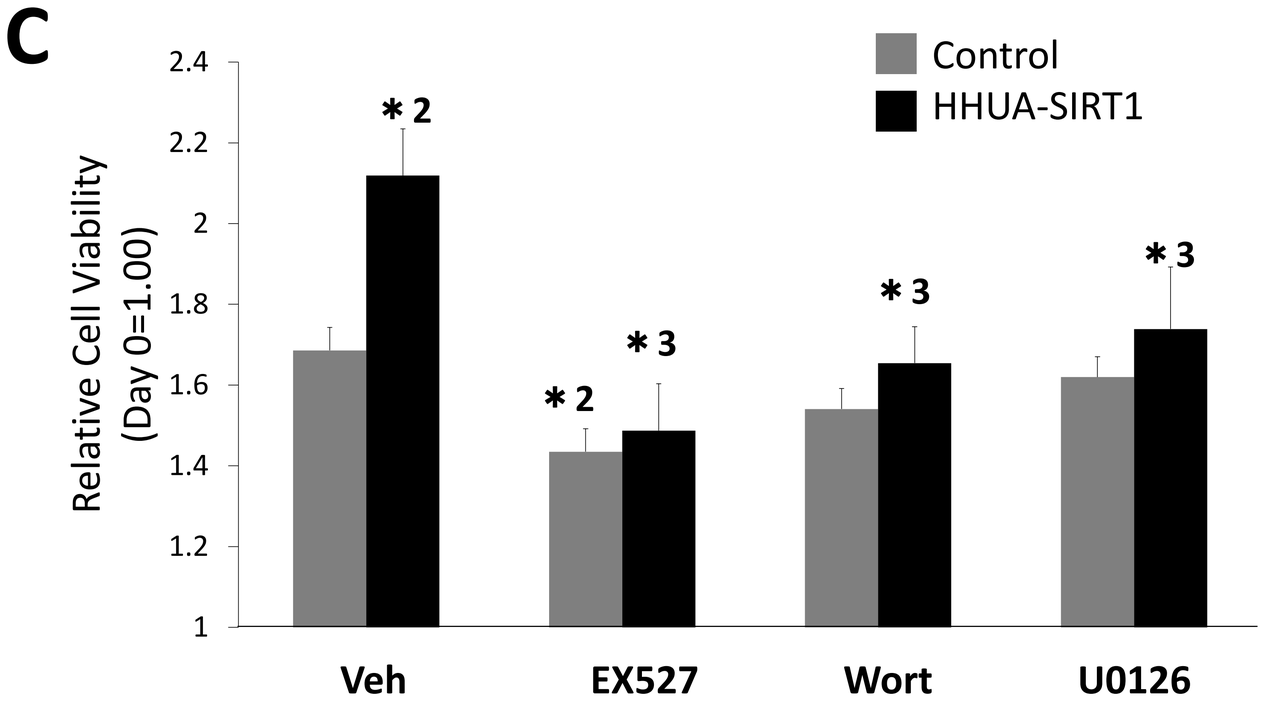

D
Cumulative Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
PI<50 (N=79)
PI≧50 (N=29)
P=0.040
(Log-rank test)
Overall survival
0
50
100
150
200
250
Duration of Survival (Months)
PI<50 (N=79)
PI≧50 (N=29)
P=0.035
(Log-rank test)
Disease-free survival
Duration of Survival (Months)

Figure 2


A
HEC151
HEC1B
HHUA
ECC1
Ishikawa
Relative Cell Viability
Control
siRNA
*1
0h
24h
72h


B
HEC151
HEC1B
HHUA
ECC1
Ishikawa
Relative Cell Viability
Control
cDNA
*1
0h
24h
72h


C
Relative Cell Viability
(Day 0=1.00)
Control
HHUA-SIRT1
*2
*3
Veh
EX527
Wort
U0126

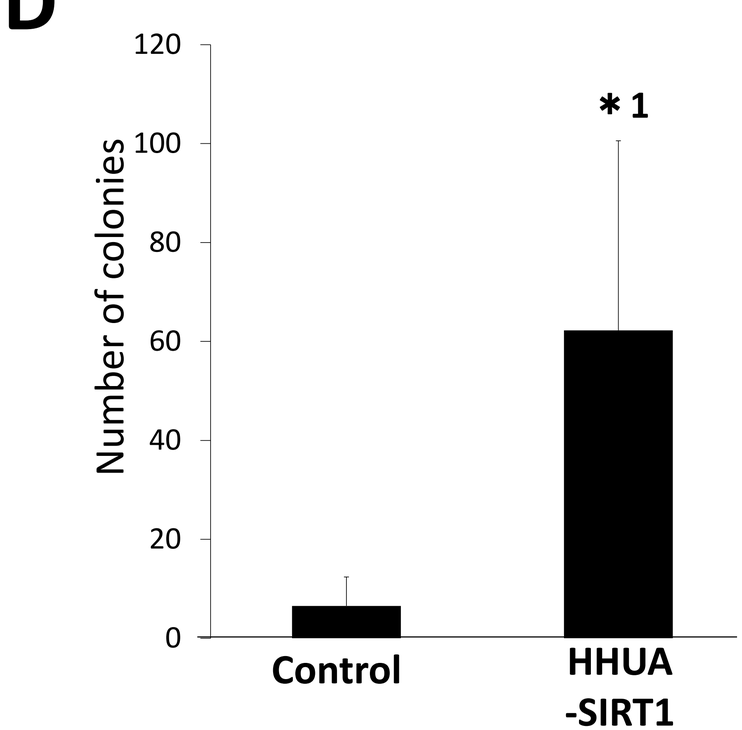
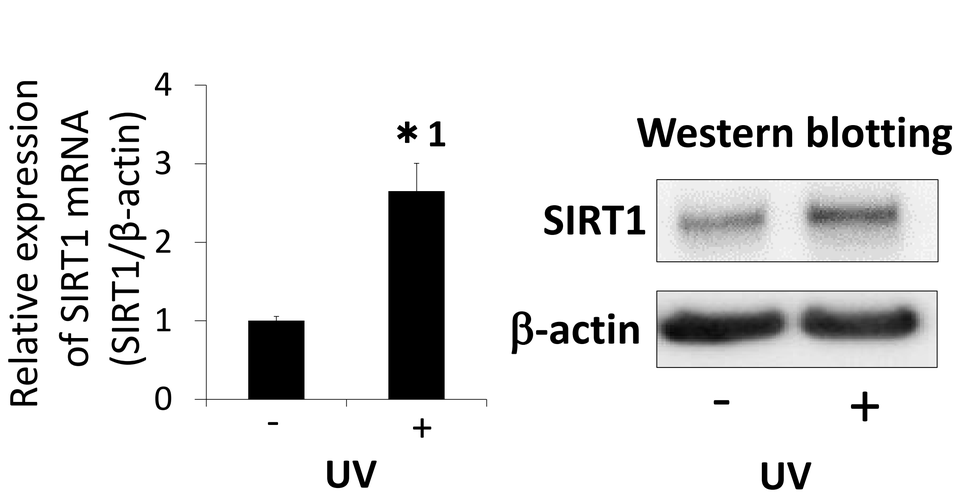
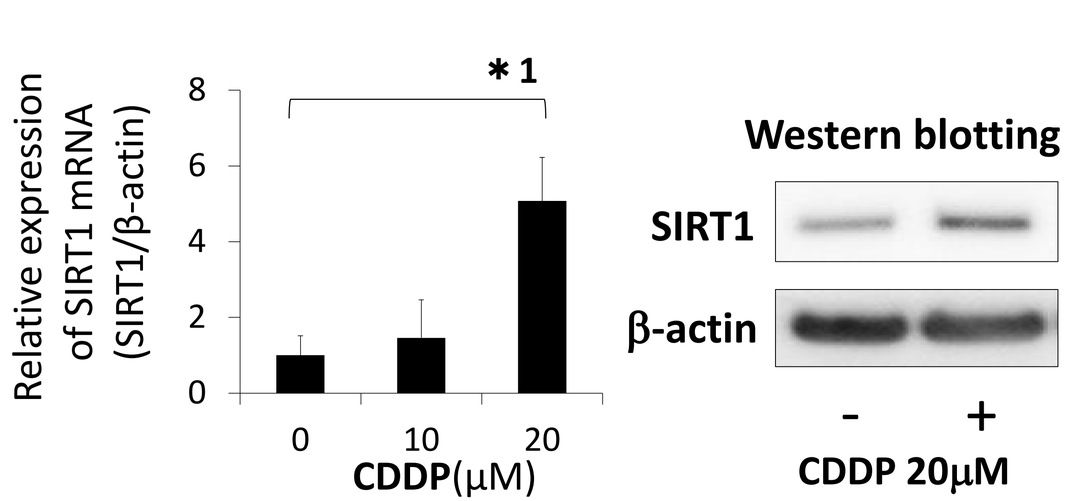
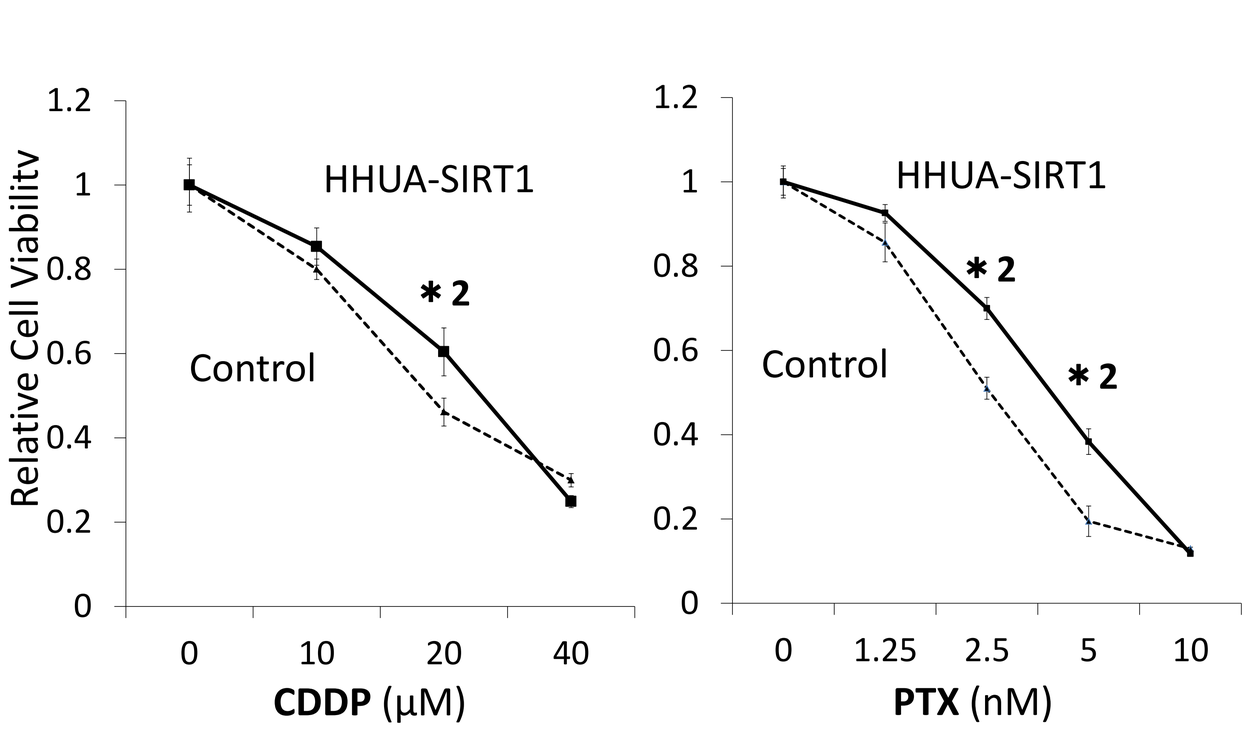

D
Number of colonies
*1
Control
HHUA
-SIRT1

Figure 3


A
Relative expression of SIRT1 mRNA (SIRT1/β-actin)
4
3
2
1
0
＊1
-
+
UV
Western blotting
SIRT1
β-actin
-
+
UV


B
Relative expression of SIRT1 mRNA (SIRT1/β-actin)
8
6
4
2
0
＊1
0
10
20
CDDP(μM)
Western blotting
SIRT1
β-actin
-
+
CDDP 20μM


C
Relative Cell Viability
1.2
1
0.8
0.6
0.4
0.2
0
HHUA-SIRT1
Control
＊2
0
10
20
40
CDDP (μM)
HHUA-SIRT1
Control
＊2
＊2
0
1.25
2.5
5
10
PTX (nM)

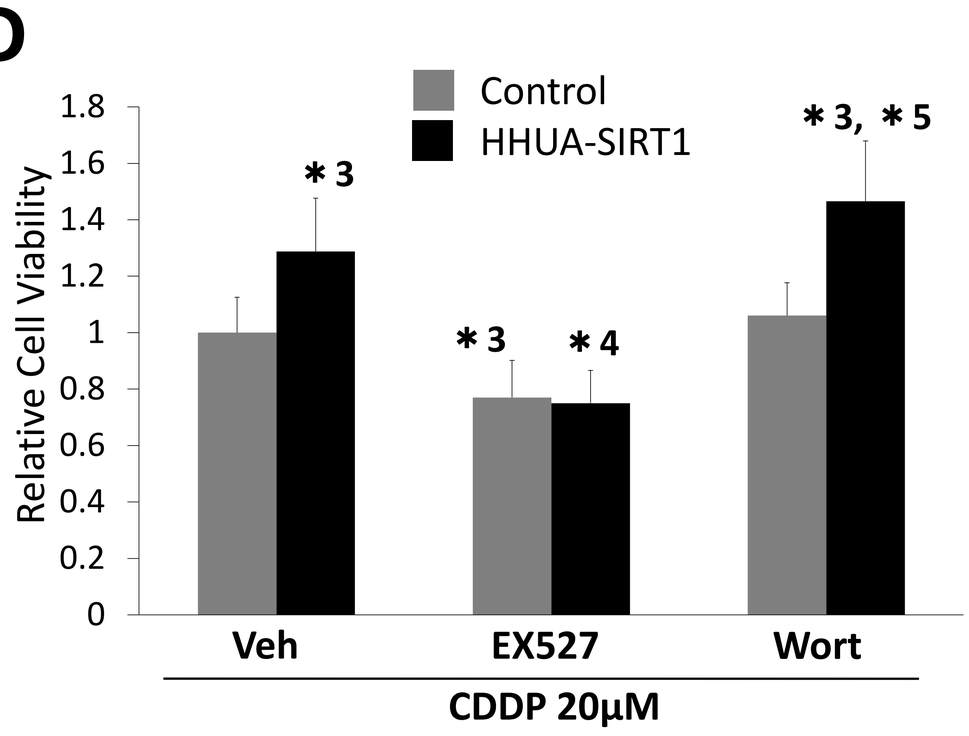
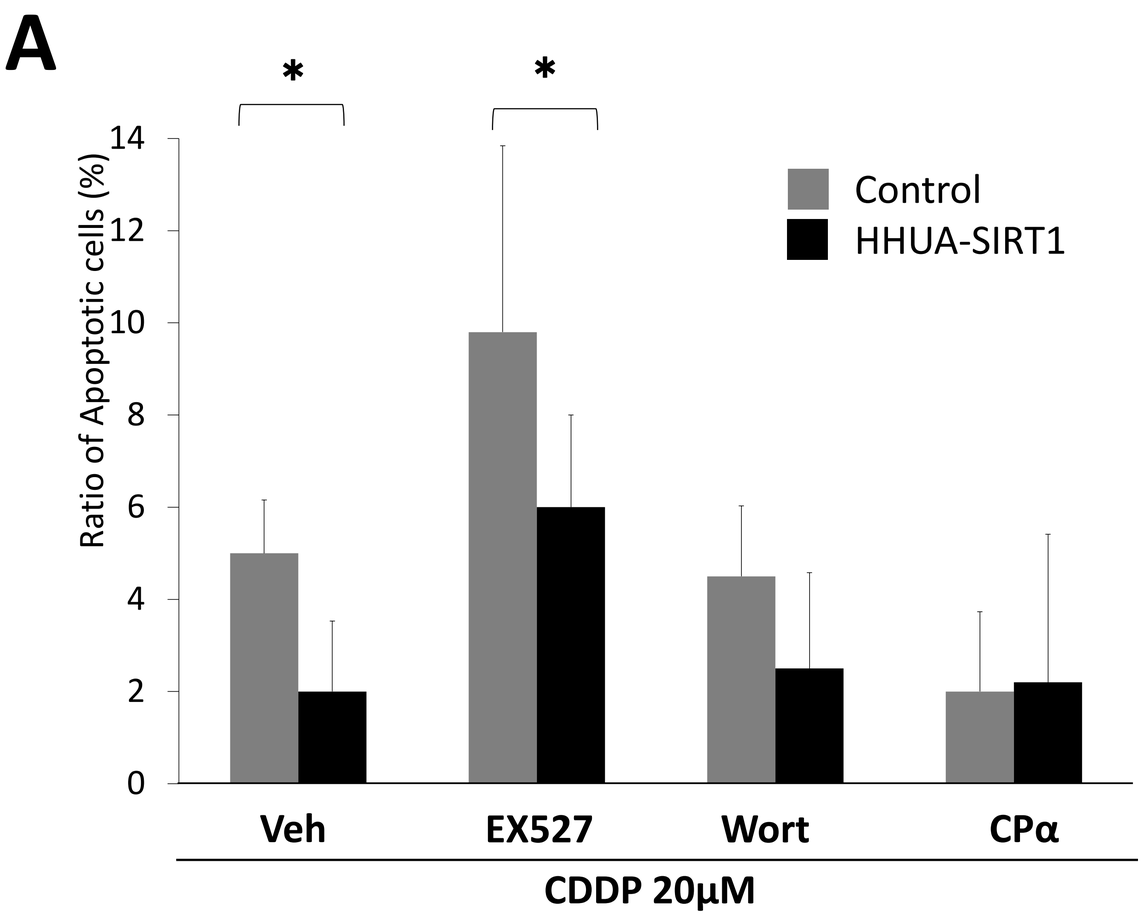
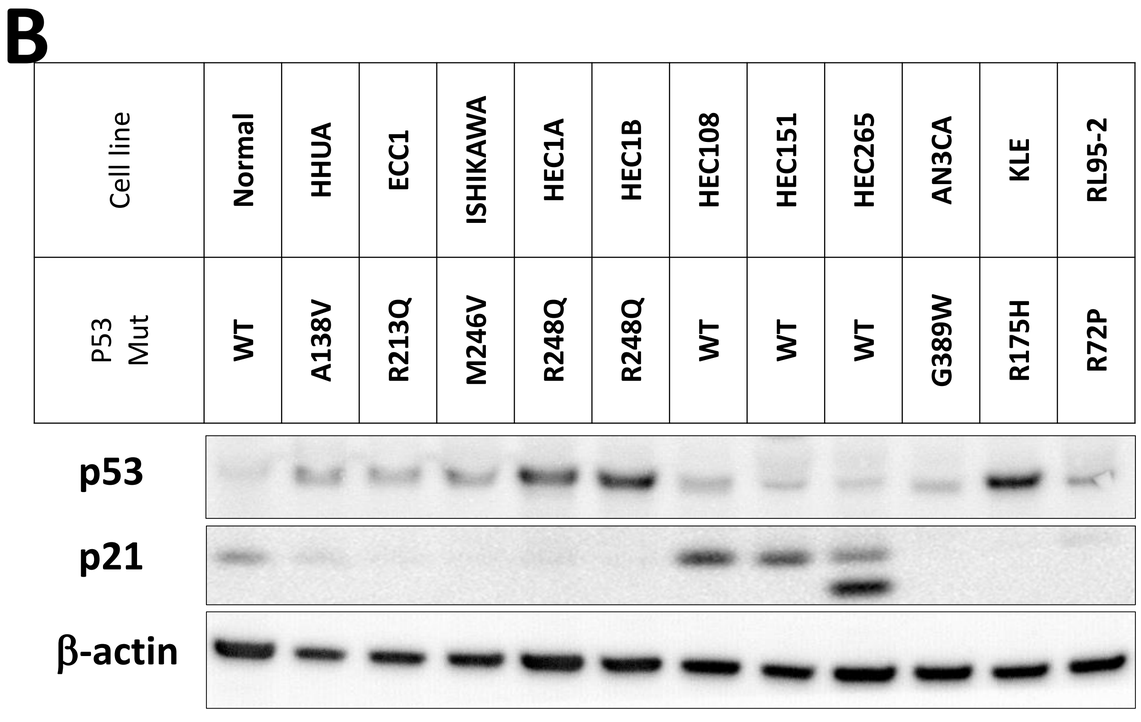
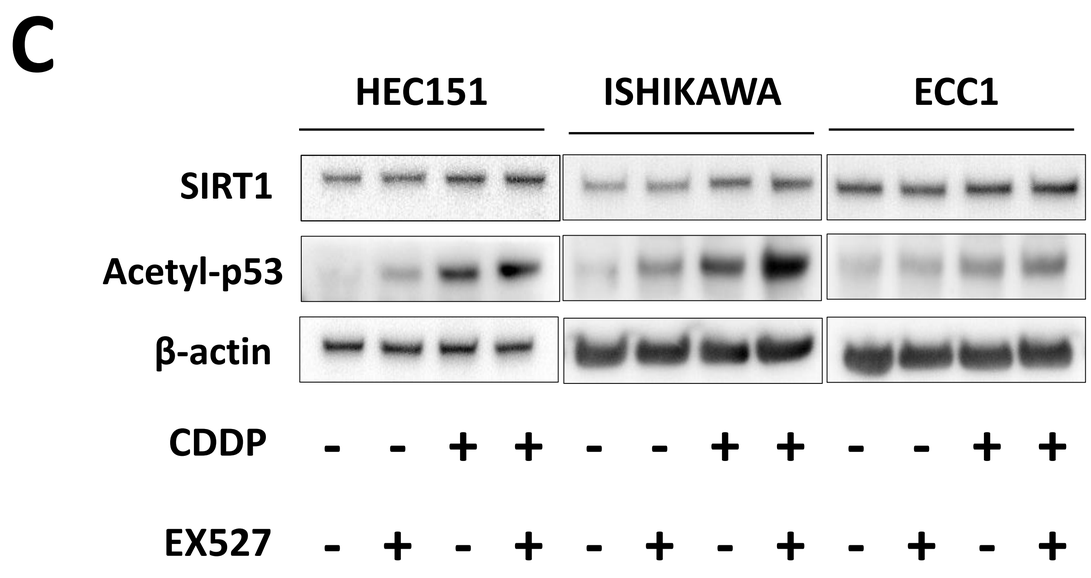

D
Control
HHUA-SIRT1
Relative Cell Viability
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
＊3
＊3
＊4
＊3, ＊5
Veh
EX527
Wort
CDDP 20μM

Figure 4


A
*
*
Ratio of Apoptotic cells (%)
14
12
10
8
6
4
2
0
Control
HHUA-SIRT1
Veh
EX527
Wort
CPα
CDDP 20μM


B
Cell line
Normal
HHUA
ECC1
ISHIKAWA
HEC1A
HEC1B
HEC108
HEC151
HEC265
AN3CA
KLE
RL95-2
P53 Mut
WT
A138V
R213Q
M246V
R248Q
R248Q
WT
WT
WT
G389W
R175H
R72P
p53
p21
β-actin


C
HEC151
ISHIKAWA
ECC1
SIRT1
Acetyl-p53
β-actin
CDDP
EX527

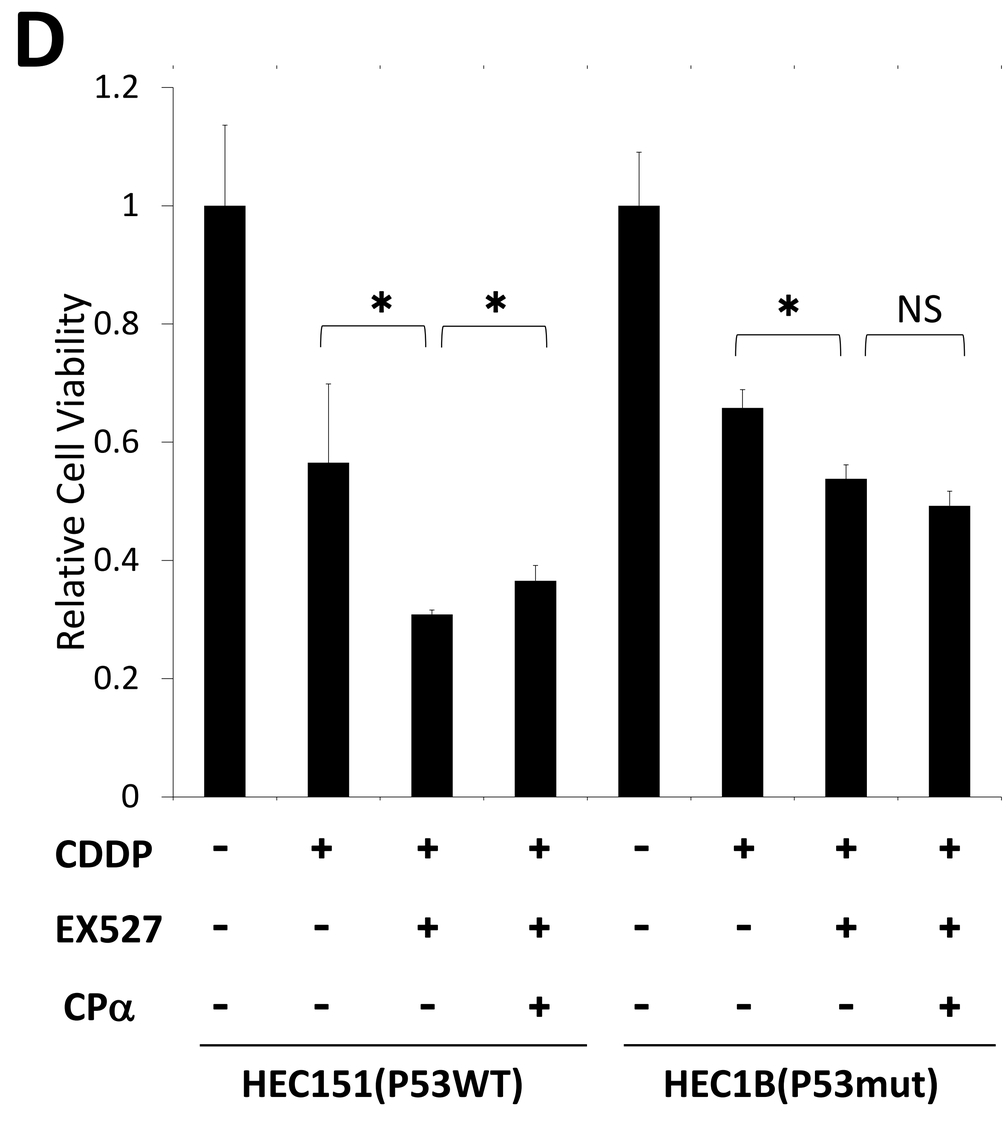
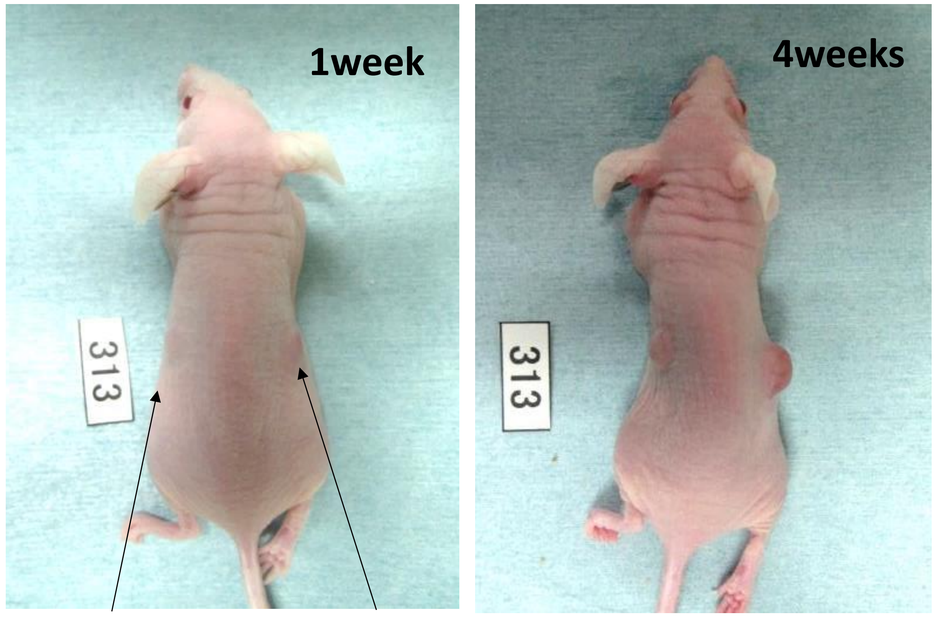
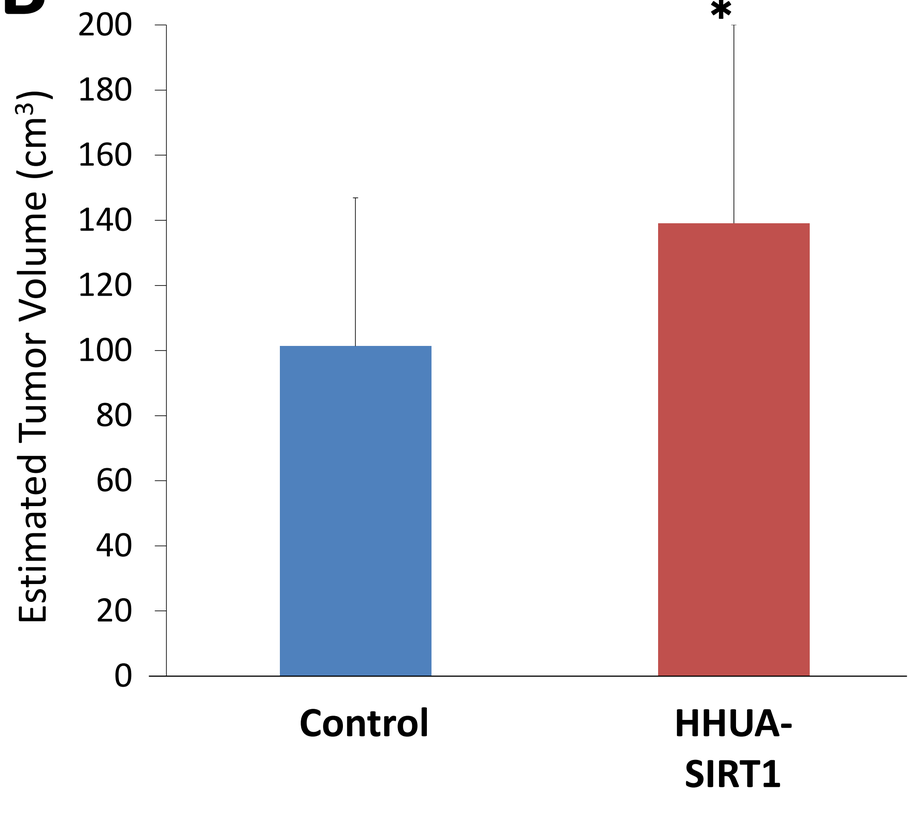

D
1.2
1
0.8
0.6
0.4
0.2
0
Relative Cell Viability
*
*
*
NS
CDDP
EX527
CPα
HEC151(P53WT)
HEC1B(P53mut)

Figure 5

A


1week
4weeks
313
313
Control
HHUA-SIRT1

B


*
200
180
160
140
120
100
80
60
40
20
0
Estimated Tumor Volume (cm³)
Control
HHUA-SIRT1

C

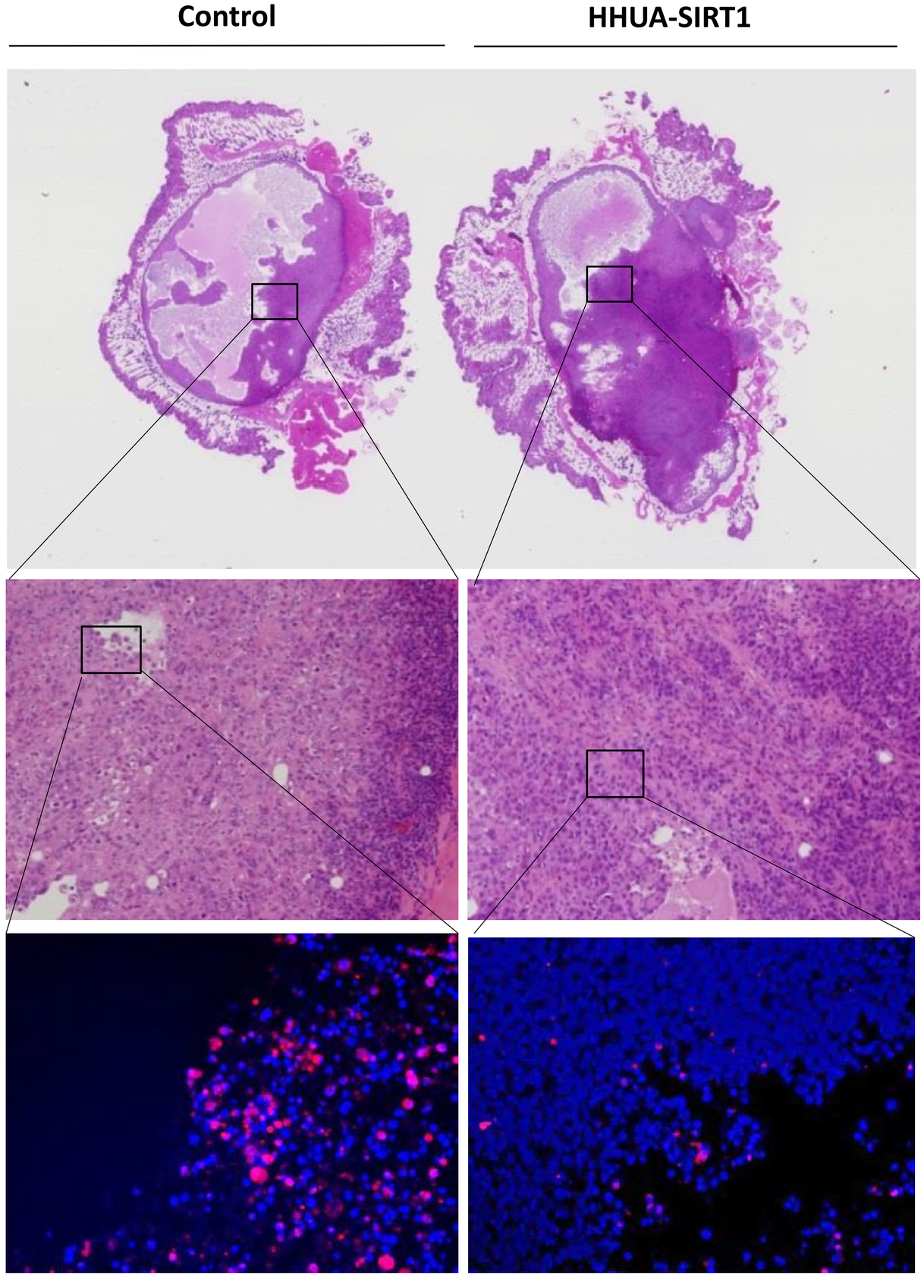
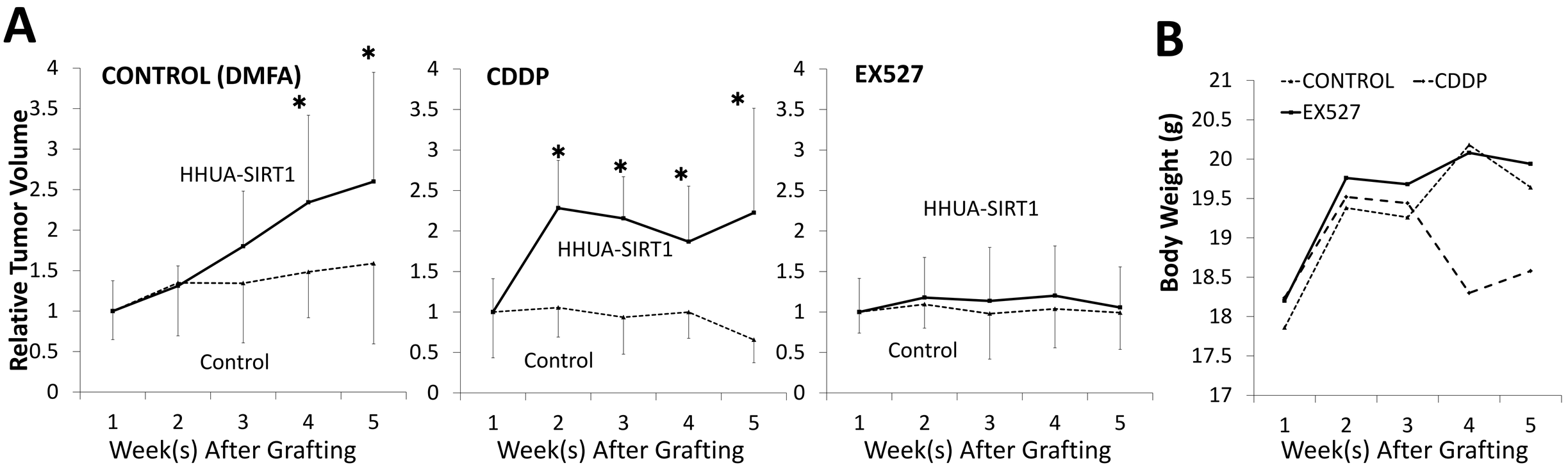
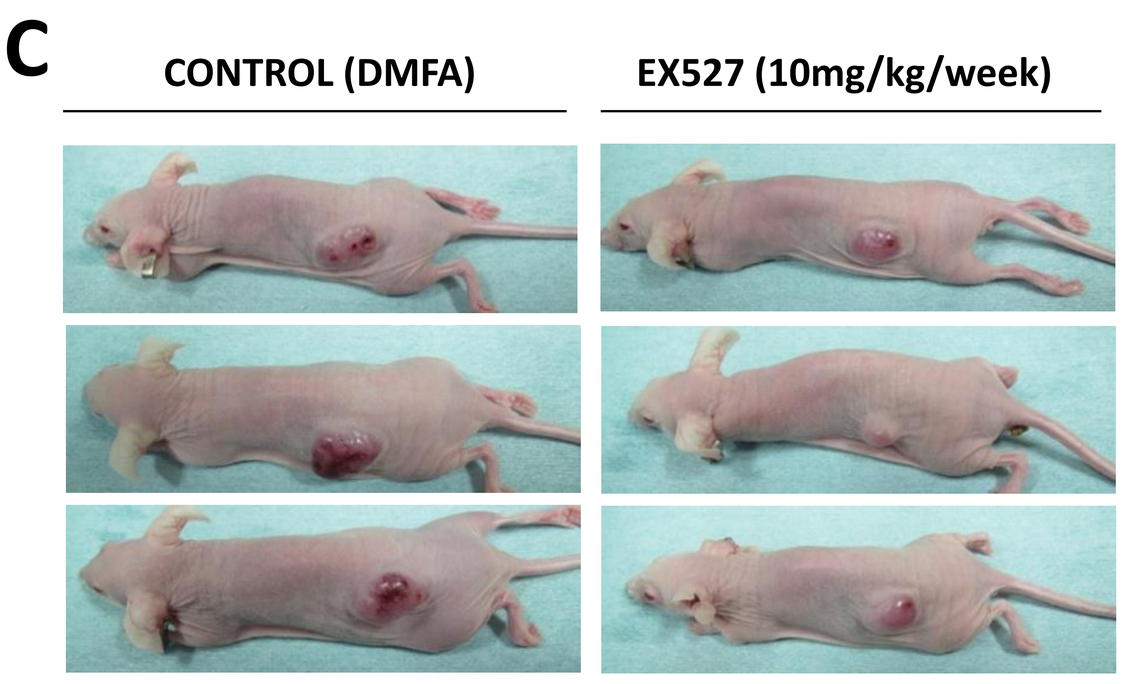
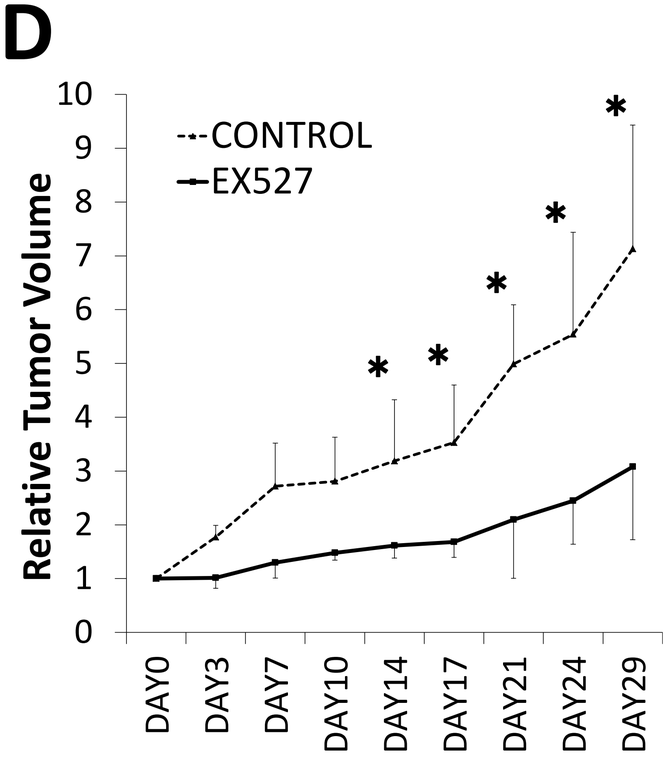

Control
HHUA-SIRT1
HE
Cleaved-Caspase3

Figure 6


A
CONTROL (DMFA)
CDDP
EX527
HHUA-SIRT1
Control
Relative Tumor Volume
Week(s) After Grafting
B
CONTROL
CDDP
EX527
Body Weight (g)
Week(s) After Grafting


C
CONTROL (DMFA)
EX527 (10mg/kg/week)


D
CONTROL
EX527
Relative Tumor Volume
DAY0
DAY3
DAY7
DAY10
DAY14
DAY17
DAY21
DAY24
DAY29