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Aspirin targets SIRT1 and AMPK to induce senescence of colorectal carcinoma cells

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Abbreviation

AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMPK	AMP-activated protein kinase
CRC	colorectal carcinoma cells
GI	gastrointestinal
HCoEpiCs	human colonic epithelial cells
IR	Ionizing radiation
LKB	liver kinase B1
NSAIDs	nonsteroidal anti-inflammatory drugs
NAD ⁺	nicotinamide adenine dinucleotide
PI	Propidium iodide
SA- β gal	senescence-associated β -galactosidase
SASP	senescence-associated secretory phenotype
SIRT1	sirtuin 1
TBST	Tris-buffered saline with 0.1% Tween-20
TIS	therapy-induced senescence
TSC2	tuberous sclerosis complex

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Abstract

Cancer therapies attempt to destroy the entire tumor, but this tends to require toxic compounds and high doses of radiation. Recently, considerable attention has focused on therapy-induced senescence (TIS), which can be induced in cancer cells by low doses of therapeutic drugs or radiation, and provides a barrier to tumor development. However, the molecular mechanisms governing TIS remain elusive. Special attention has been paid to the potential chemopreventive effect of aspirin against human colorectal cancer. In this study, we investigated the effects of aspirin on TIS of human colorectal carcinoma (CRC) cells, and show that it occurs via sirtuin 1 (SIRT1) and AMP-activated protein kinase (AMPK), two key regulators of cellular metabolism. Aspirin increased the senescence of CRC cells, increased the protein levels of SIRT1, phospho-AMPK (T172), and phospho-acetyl CoA carboxylase (S79), and reduced the cellular level of ATP. SiRNA-mediated down-regulation or pharmacological inhibition of SIRT1 or AMPK significantly attenuated the aspirin-induced cellular senescence in CRC cells. In contrast, treatment with a SIRT1 agonist or an AMP analog induced cellular senescence. Remarkably, SIRT1 knockdown abrogated the aspirin-induced activation of AMPK, and vice versa. During the progression of aspirin-induced cellular senescence in CRC cells, SIRT1 showed increased deacetylase activity at a relatively early time point, but was characterized by decreased activity with increased cytoplasmic localization at a later time point. Collectively, these novel findings suggest that aspirin could provide anticancer effects by inducing senescence in human CRC cells through the reciprocal regulation of SIRT1-AMPK pathways.

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Introduction

Cellular senescence, which is a well-known physiological or pathological response induced by many factors, is a key component of permanent cell cycle arrest, normal tissue repair, and tumor suppression (Rodier and Campisi, 2011). Recently, senescence was shown to bar the initiation and development of cancer (Rodier and Campisi, 2011). Accumulating data indicate that certain therapeutic compounds or radiotherapies can induce senescence; this effect has been called therapy-induced senescence (TIS) (Ewald et al., 2010; Lee and Lee, 2014; Suzuki and Boothman, 2008). Since much lower total doses of drugs or radiation are required to induce senescence compared to cancer cell death, TIS-based strategies may trigger fewer toxicity-related side effects while stimulating tumor-specific immune activity (Kang et al., 2011; Sagiv and Krizhanovsky, 2013).

Cancer is among the metabolic diseases that are accompanied by disturbances in the energy balance (Mendonca et al., 2015). Previous studies suggest that there is a positive relationship between type 2 diabetes/obesity and cancer risk/cancer-related mortality (O'Neill and O'Driscoll, 2015; Vucenik and Stains, 2012). Compared to normal cells, tumor cells show distinct metabolic features, such as enhanced aerobic glycolysis, glutaminolysis, and lipid synthesis (Currie et al., 2013; Daye and Wellen, 2012; Vander Heiden et al., 2009). The master regulators of cellular metabolic homeostasis include sirtuin 1 (SIRT1; the mammalian homolog of silent information regulation 2 of the yeast, *Saccharomyces cerevisiae*), which is a member of the highly conserved family of nicotinamide adenine dinucleotide (NAD⁺)-dependent protein lysine modifying enzymes. In recent years, SIRT1 has emerged as a potent protector against aging-related pathologies, such as diabetes, cardiovascular disease, hepatic steatosis, neurodegeneration, and numerous types of cancer (Sebastian et al., 2012). Another key regulator of metabolism, AMP-activated protein kinase (AMPK), is predominantly activated in response to decreases in the ATP/ADP ratio; it plays central roles in regulating energy homeostasis, tumorigenesis, and aging (Hardie, 2014). Crosstalk between SIRT1 and AMPK governs major metabolic activities and is believed to regulate senescence in mammalian cells (Wang et al., 2011). SIRT1 stimulates oxidative energy production by activating AMPK and several regulators of mitochondrial biogenesis (Price et al., 2012). Conversely, AMPK enhances SIRT1 activity by

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increasing cellular NAD⁺ levels, leading to the up-regulation of the downstream targets of SIRT1 (Canto et al., 2009). However, the mechanisms underlying these metabolic phenomena in cancer have not been fully elucidated, and we do not yet know whether treatment with therapeutic activators of these enzymes could be an effective strategy for curing cancer via the induction of senescence.

Multiple reports have shown that nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, are promising chemopreventive agents (Alfonso et al., 2014; Rao and Reddy, 2004; Thun et al., 2002). Although aspirin or other NSAIDs may confer adverse effects, their daily use can reduce the risk of colorectal cancer (CRC) (Chan et al., 2007; Chan et al., 2009; Huls et al., 2003). Improving our understanding of how NSAIDs influence cancer metabolism could provide substantial insights into the mechanisms by which these unique agents control CRC cell growth, potentially directing the development of better prevention and treatment strategies. To date, aspirin has been shown to reduce the development of CRC through the direct activation of AMPK (Din et al., 2012; Lissa et al., 2014).

In this study, we investigated the therapeutic effect of aspirin, focusing on the interplay of the SIRT1-AMPK pathway in the TIS of human CRC cells. Our results revealed that aspirin treatment increased the senescence of both SW620 and HCT116 human CRC cells, but had more minimal effects on normal human colonic epithelial cells. Aspirin treatment increased the protein levels of SIRT1, phospho-AMPK, and phospho-ACC, while decreasing the level of ATP. In aspirin-treated CRC cells, the deacetylase activity of SIRT1 was increased at an early time point (3 h after treatment) but decreased at 72 h post-treatment. SiRNA mediated down regulation of SIRT1 or AMPK or their pharmacological inhibitions with EX527 or Compound C, respectively, significantly attenuated the aspirin-induced senescence in CRC cells. In contrast, treatment with a SIRT1 agonist (resveratrol) or an AMP analog (AICAR) induced cellular senescence in these cells. The senescence-induced activation of AMPK was abrogated by inhibition of SIRT1 expression, whereas AMPK knockdown decreased the senescence-induced activation of SIRT1. Interestingly, aspirin-induced senescence increased the cytoplasmic localization of SIRT1 at a later time point, when deacetylase activity was reduced (72 h). Taken together, these results suggest that aspirin could potentially act as a TIS-inducing therapeutic drug in human CRC cells via the potentially interdependent regulation of the

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SIRT1-AMPK pathways.

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Materials and Methods

Reagents. Aspirin (acetylsalicylic acid) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Resveratrol was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). AICAR (ab120358), Compound C (ab120843), EX527 (ab141506) and celecoxib (ab141988) were purchased from Abcam (Cambridge, MA, USA). Antibodies against AMPK α , phospho-AMPK α (T172), phospho-acetyl CoA carboxylase (ACC; S79) and ACC were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against SIRT1, p21CIP, DcR2, and β -actin were purchased from Santa Cruz Biotechnology.

Cell culture, irradiation, aspirin treatment and transfection. The SW620 and HCT116 human CRC cell lines were obtained from the American Type Culture Collection (Philadelphia, PA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM). Human colonic epithelial cells (HCoEpiC) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA), cultured in colonic epithelial cell medium, and passaged three to four times for use. Cells were grown in a 37°C incubator with a 5% CO₂ atmosphere in culture media supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics. For γ -irradiation, cells were exposed to a ¹³⁷Cs γ -ray source (Atomic Energy of Canada, Ltd., Ontario, Canada) at a dose rate of 3.2 Gy/min. Cells were treated with aspirin added to the culture medium for the indicated durations (0, 3, 6, 12, 24, 48 and 72 h) and at various concentrations (10, 50, 200, 500 and 1000 μ M). SiRNAs specific for AMPK α 1 and SIRT1 were purchased from Bioneer (Seoul, Republic of Korea). Cells were transfected with si-AMPK α 1 and si-SIRT1 (final concentration, 50 nM) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA).

MTT assay. Cells were seeded (1×10^4 cells per well) to 96-well plates in culture medium containing 10% (v/v) FBS, allowed to attach for 24 h, and then treated with different concentrations of aspirin (1 to 10,000 μ M) for 24 h. The cells were washed, treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (20 μ L of 5 mg/mL) at 37 °C for 1 h, and then lysed with DMSO. The absorbance in each well was measured at 540 nm using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). The results were generated from

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three independent experiments, each performed in triplicate.

Protein preparation and immunoblot analysis. Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin). Protein samples were denatured, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and blocked with 5% nonfat dry milk in TBST (Tris-buffered saline with 0.1% Tween-20). The membrane was incubated with the primary antibody overnight at 4°C, and then with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Immuno-reactive proteins were visualized using enhanced chemiluminescence.

Senescence-associated β -galactosidase (SA- β -gal) staining. Cells were washed with PBS, fixed for 10 min at room temperature in 3% formaldehyde, washed, and incubated for 16 h at 37°C in a CO₂-free atmosphere with a senescence-associated (SA- β -gal) staining solution that contained 1 mg of 5-bromo-4-chloro-3-indolyl P β -D-galactoside (X-gal) per ml (stock = 20 mg of dimethylformamide per ml), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 150 mM NaCl₂. The percentage of blue cells was calculated from 200 cells observed under a bright-field microscope (Debacq-Chainiaux et al., 2009).

Propidium iodide (PI) staining. Cells were trypsinized, washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.4), and collected by centrifugation at 200 \times g for 10 min. The supernatant was discarded, and the pelleted cells were carefully suspended in 500 μ L PBS. PI was added to a final concentration of 40 μ g/mL, and cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA).

SIRT1 activity assay. NAD-dependent deacetylase activity was measured using a SIRT1 Assay Kit (ab156915, Abcam) according to the manufacturer's instructions. The absorbance in each well was measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA)

Measurement of intracellular ATP levels. Intracellular ATP levels were measured by luminescence using an ATP Determination Kit (Invitrogen). Briefly, 10,000 sub-confluent cells were loaded to a 96-

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well plate in triplicate. Luciferin/luciferase reagents (100 μ l) were added, and relative luminescence units were measured using a MicroLumat Plus LB96V (Berthold Technologies, Germany)

Immunofluorescence and confocal microscopy. Cells were seeded onto coverslips, fixed with 4% formaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100, and then stained with anti-SIRT1 (Santa Cruz, CA, USA) followed by Alexa 488-conjugated immunoglobulins (Invitrogen, Carlsbad, CA, USA). Immunostained cells were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA). The results were visualized using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Le Pecq, France),

Statistical analysis. Data are expressed as means \pm standard deviations (SDs) of at least three experiments. Statistical significance was determined using the Student's t-test for comparisons between two means, and differences were accepted at *p*-values less than 0.05 (*), 0.01 (**) or 0.001 (***).

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Results

Aspirin induces cellular senescence in HCT116 and SW620 human CRC cells. Aspirin has been strongly suggested to possess chemopreventive activities in human CRC, perhaps via the anti-proliferative influence of cellular senescence. Here, we investigated whether aspirin can induce cellular senescence in human CRC cells. First, we performed a cell viability analysis to determine the range of aspirin concentrations over which cell death was not induced. We found that aspirin concentration-dependently reduced cell viability in both SW620 human metastatic colorectal carcinoma cells (p53 mutant-type) and HCT116 human primary colorectal carcinoma cells (p53 wild-type), and that it highly inhibited cell proliferation at concentrations greater than 1 mM (Fig. 1A). Next, we performed staining for senescence-associated β galactosidase (SA- β -gal) (Debacq-Chainiaux et al., 2009), a marker of senescent cells, and examined the ability of aspirin to induce senescence in CRC cells. Indeed, we found that aspirin induced cellular senescence, with maximum senescence-inducing effects observed at a concentration of 500 μ M in SW620 cells (Fig. 1B and 1C).

Further experiments showed that aspirin (500 μ M) significantly induced cellular senescence in both SW620 (about 91%) and HCT116 (about 67%) cells after 72 h of treatment (Fig. 1D-F). PI staining and FACS analysis of CRC cells treated with 500 μ M of aspirin for 72 h showed that cell death was induced in less than 1% of the cells (Fig. 1G). As cellular senescence is well known to occur in response to IR (Suzuki and Boothman, 2008), we compared the senescent phenotypes between aspirin-treated and irradiated CRC cells. As expected, IR increased the SA- β -gal positivity in irradiated CRC cells (Fig. 1D-F). Therapy-induced senescence (TIS) is an effective therapeutic strategy for malignant tumors (Ewald et al., 2010), but its clinical induction may injure the surrounding normal tissue. Since aspirin is associated with upper and lower gastrointestinal (GI) damage (Lanas and Gargallo, 2015; Sostres et al., 2013), we questioned whether such adverse effects would be present in the normal colonic epithelium following senescence-inducing aspirin treatment. Accordingly, we examined the effect of aspirin (500 μ M for 72 h) on normal colonic epithelial cells (HCoEpiC). Aspirin did not trigger cell death or SA- β -gal-positive staining under our experimental

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conditions, whereas IR enlarged the cell shape and increase the SA- β -gal-positive staining (Fig. 1D-F). Thus, the concentration of aspirin capable of inducing senescence in CRC cells (500 μ M) may have fewer effects in normal colonic epithelial cells than in CRC cells. The p53 protein and the cyclin dependent kinase inhibitor, p21, which are both required for cellular senescence (Deng et al., 2008), were both up-regulated in HCT116 cells following aspirin treatment. Contradictory, the p53-independent induction of senescence and up-regulation of p21 were observed in aspirin-treated SW620 cells (Fig. 1H). We additionally validated the aspirin-induced cellular senescence by monitoring the senescence marker, DcR2 (Ewald et al., 2010). We observed that the protein level DcR2 was increased in aspirin-treated CRC cells (Fig. 1H and 1I). This finding was confirmed by densitometric quantification of DcR2 (Fig. 1J). Taken together, these results indicate that aspirin highly induces cellular senescence in SW620 and HCT116 human CRC cells.

Aspirin activates SIRT1 and AMPK in HCT116 and SW620 cells. It has been suggested that aspirin may significantly impact cellular metabolism (Kamble et al., 2014; Steinberg et al., 2013). Furthermore, studies have suggested that SIRT1 or AMPK may contribute to the anti-cancer activity of aspirin mediating its ability to inhibit cell growth (Kumazaki et al., 2013; Liu et al., 2009; Motoshima et al., 2006). However, the tumor suppressive activities of metabolic sensors such as SIRT1 or AMPK have not been well studied in CRC in view of cellular senescence. Thus, we examined the potential involvement of SIRT1 and AMPK in the aspirin-induced cellular senescence of CRC cells. We examined the protein expression and deacetylase activity of SIRT1, and tested the activity of AMPK by monitoring increases in phospho-AMPK (p-AMPK T172) or phospho-acetyl CoA carboxylase (p-ACC S79; an AMPK substrate known to be a reliable indicator of its activity) (Hardie, 2014). Aspirin treatment was found to dose- and time-dependently increase the protein levels of SIRT1, phospho-AMPK (p-AMPK T172), and p-ACC S79 (Fig. 2A and 2B). Interestingly, the increase in SIRT1 protein expression was followed by AMPK activation (Fig 2B). Next, we examined the enzyme activity of SIRT1 by measuring SIRT1 deacetylase activity. The enzymatic activity of SIRT1 in CRC cells was significantly increased following treatment with 500 μ M of aspirin for 3 h (Fig. 2C). As the activities of SIRT1 and AMPK mainly depend on the cellular energy level (Canto

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and Auwerx, 2009), we determined the intracellular ATP level in aspirin-treated HCT116 and SW620 cells. As shown in Figure 2D, the ATP level was dramatically and dose-dependently reduced in aspirin-treated CRC cells. We then compared the effects of IR and aspirin on SIRT1 and AMPK activity in SW620 and HCT116 cells. Similar to the responses induced by aspirin treatment, the protein level and activity of SIRT1 were increased by IR, as were the protein levels of p-AMPK (T172) and p-ACC (S79) (Fig. 2E and 2F). AMPK is a heterotrimeric protein comprising a catalytic subunit (α) and two regulatory subunits (β and γ) (Hardie, 2014). Interestingly, whereas the protein level of AMPK α was unaltered following aspirin treatment (Fig. 2A and 2B), this catalytic subunit was specifically upregulated by IR (Fig. 2E). Similar to our observations in aspirin-treated cells, however, the ATP level was considerably reduced in IR-exposed CRC cells (Fig. 2G). These results collectively indicate that aspirin activates SIRT1 and AMPK activity through an imbalance in the energy status of CRC cells.

Knockdown of SIRT1 or the AMPK α 1 catalytic subunit attenuates the aspirin-induced senescence of CRC cells. Given that the activities of SIRT1 and AMPK were increased in aspirin-treated CRC cells, we tested whether aspirin-induced cellular senescence was dependent on these activities. We down-regulated SIRT1 using siRNA to block its expression or EX527 to inhibit its deacetylase activity (Peck et al., 2010), and examined the aspirin-induced senescence phenotype using SA- β -gal staining. SIRT1 knockdown or EX527 treatment clearly decreased the activity of SIRT1, as confirmed by a SIRT1 activity assay (data not shown). Our results revealed that aspirin increased SA- β -gal-positive staining in control SW620 and HCT116 cells, but this staining was significantly decreased by pretreatment with si-SIRT1 or EX527. IR-induced senescence was also diminished by the inhibition of SIRT1 (Fig. 3A-D). Previously, we demonstrated that the AMPK α 1 catalytic subunit is expressed in various CRC cells (Park et al., 2012). To determine whether aspirin-induced senescence requires AMPK activation, we depleted AMPK α 1 using si-AMPK α 1 or Compound C (a well-known AMPK inhibitor) (Zhou et al., 2001) and examined aspirin-induced senescence. Our results revealed that knockdown or inhibition of AMPK (as confirmed by decreases in the level of p-ACC S79; data not shown) dramatically reduced the aspirin-induced senescence of CRC cells, as

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evidenced by decreased SA- β -gal-positive staining. Moreover, IR-induced senescence was diminished by AMPK inhibition (Fig. 4A-D). These findings indicate that the activations of both SIRT1 and AMPK are required for the aspirin-induced senescence of CRC cells.

SIRT1 and AMPK are interdependent in the process of aspirin-induced CRC senescence. To confirm that the activities of SIRT1 and AMPK are necessary for cellular senescence in CRC cells, we tested whether other pharmacological activators of SIRT1 or AMPK could induce this effect. Resveratrol (a well-known SIRT1 activator) (Baur, 2010; Price et al., 2012) and AICAR (an AMP analog that induces allosteric activation of AMPK) (Gaidhu et al., 2006) were found to increase SA- β -gal positivity (Fig. 5A and 5B), SIRT1 activity (Fig. 5C), and the levels of p-AMPK (T172) and p-ACC (S79) (Fig. 5D). Meanwhile, the protein expression of SIRT1 was increased by resveratrol, but not AICAR (Fig. 5D). These results confirmed that the cellular senescence of CRC cells was highly dependent on the activities of both SIRT1 and AMPK. To examine whether crosstalk between SIRT1 and AMPK governs cellular senescence in mammalian cells, we investigated the correlation between the activities of SIRT1 and AMPK during aspirin-induced cellular senescence. Since aspirin activated SIRT1 earlier than AMPK (Fig. 2B), we tested whether the depletion of SIRT1 could affect AMPK activity. Compared with si-control-treated cells, knockdown of SIRT1 diminished the aspirin-induced increases in the levels of p-AMPK (T172) and p-ACC (S79) (Fig. 5E). To examine whether AMPK acts upstream of SIRT1 in aspirin-induced cellular senescence, we tested the effect of AMPK α 1 knockdown on SIRT1, and found that depletion of AMPK α 1 attenuated the aspirin-induced increases in SIRT1 protein expression and enzymatic activity (Fig. 5F and 5G). Collectively, our results show that aspirin induces senescence in human CRC cells via a process that requires both SIRT1 and AMPK, and involves crosstalk between these two metabolic regulators.

At a later time point, aspirin reduces the deacetylase activity and increases the cytoplasmic localization of SIRT1. Although SIRT1 deacetylase activity was increased at an earlier time point following aspirin treatment (3 h) (Fig. 2C), such activity was decreased at a later time point (72 h) (Fig. 6A). AICAR and IR, but not resveratrol, also reduced SIRT1 enzymatic activity at the later stage of treatment (Fig. 6B and 6C). To further examine this late-stage activity decrease, we tested whether

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a SIRT1 inhibitor could influence the latter stages of aspirin-induced cellular senescence. SW620 cells were treated with aspirin for 24 or 48 h, further treated with EX527 (10 μ M), and incubated for an additional 48 or 24 h, respectively (for a total of 72 h in both cases). SA- β -gal staining revealed that whereas pretreatment with EX527 resulted in nearly complete inhibition of aspirin-induced cellular senescence (Fig. 3B and 3D), the addition of this inhibitor after 48 h of aspirin treatment only partially blocked senescence (Fig. 6D and 6E). This suggests that SIRT1 deacetylase activity may be important for the early stage of aspirin-induced cellular senescence. However, this led us to question our observation that the protein level of SIRT1 continued increasing at a time when its deacetylase activity was diminishing. The subcellular localization of SIRT1 differs in various tissues and cells. The protein is generally nuclear (Michishita et al., 2005), but its shuttling between the nucleus and cytosol may change in response to diverse pathophysiological stimuli (Byles et al., 2010; Jin et al., 2007; Tanno et al., 2007). Therefore, we tested whether aspirin treatment could alter the localization of SIRT1 in CRC cells, using Lamin A/C and GAPDH as nuclear and cytoplasmic markers, respectively. We first performed immunoblotting using whole cell extracts, and found that aspirin treatment induced the protein levels of SIRT1 in CRC cells (Fig. 6F). Next, we used a subcellular fractionation analysis to examine the localization of SIRT1 in CRC cells. As shown in Figure 6G, SIRT1 was mainly localized in the cytosol of HCT116 and SW620 cells. The protein levels were confirmed to be increased after 72 h of aspirin treatment in CRC cells. Moreover, immunofluorescence analysis revealed that aspirin-treated SW620 (Fig. 6H, panel e) and HCT116 (Fig. 6H, panel k) cells showed profound induction of cytosolic SIRT1. Therefore, with the deacetylase activity of nuclear SIRT1 at early phase of senescence way, cytoplasmic SIRT1 may have important role in the process of cellular senescence in aspirin-treated CRC cells.

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Discussion

Recently, researchers have paid increasing attention to therapy-induced senescence (TIS), a novel therapeutic approach in which low doses of therapeutic compounds or radiation are used to induce senescence, which has been shown to contribute to successful anti-cancer therapy (Ewald et al., 2010). This avoids the severe side effects caused by injury to normal tissues and decreases the possibility of multidrug resistance. Therefore, TIS is suggestive for therapeutic approaches by evading initiation of oncogenes and limiting the proliferation of cancer cells (Cairney et al., 2012; Ewald et al., 2010; Tchkonja et al., 2013). However, cellular senescence is not equivalent to cell death. Indeed, senescence may play contradictory roles in tumorigenesis: although senescent cells themselves cannot become neoplastic, they stimulate the growth of neighboring nascent cells and tissues by secreting a specific profile of cytokines, growth factors and proteases that are collectively called the senescence-associated secretory phenotype (SASP) (Coppe et al., 2010; Davalos et al., 2010). On the other hand, the components of the SASP have anti-cancer functions that contribute to the removal of tumor cells by the immune system (Kang et al., 2011; Sagiv and Krizhanovsky, 2013). Thus, the cytostatic effects of TIS may need to be assessed on a case-by-case basis.

One promising preventative strategy is the use of aspirin, which has been shown to notably reduce the incidence and mortality of CRC (Chan et al., 2007; Chan et al., 2009; Huls et al., 2003; Li et al., 2014; Ye et al., 2014). These specific protective actions against CRC suggest that aspirin might target distinct pathways in this disease, but the precise molecular basis of this effect is largely unknown.

Although the regular use of aspirin for more than 5 years was found to significantly reduce cancer mortality independent of the aspirin dose (Cuzick et al., 2015), the applied dosage of aspirin does appear to be important. For example, high-dose aspirin (5 mM) was found to have very different effects on tumor cells compared to two lower concentrations (50 and 500 μ M) (Hardwick et al., 2004). Similarly, the clinical evidence suggests that high- and low-dose aspirin has different effects on patients (Campbell et al., 2007). Therefore, we hypothesized that there may be different mechanisms underlying the anticancer effects of low- and high-dose aspirin in CRC cells. The mean IC_{50} value of

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aspirin for the growth reduction of CRC cells was reported to be 2.38mM (Din et al., 2004), and we found that aspirin at concentrations greater than 1mM reduced cell viability, effectively inhibiting proliferation (Fig. 1A). However, treatment of CRC cells with 500 μ M aspirin for 72 h induced less than 1% cell death, but was associated with an extreme level of cellular senescence (fig 1D-1G). Therefore, we examined the senescence-inducing effects of 500 μ M of aspirin, which is a 10- to 20-fold lower dose than that (5-10 mM) reported to be deadly against CRC cells (Din et al., 2012; Lissa et al., 2014). Clinically, the regular use of aspirin (375 mg) has been associated with a 20% (statistically significant) reduction in the risk of CRC (Usman et al., 2015). However, other studies have suggested that the treatment benefits of aspirin are increased at higher doses. Typically, the plasma salicylate concentrations in humans treated with high-doses of aspirin (30-90 mg/kg) are 1 to 3 mM, which is moderately relevant to the pharmacological levels obtained in clinical practice (Day et al., 1989; Hawley et al., 2012; Hundal et al., 2002). However, it is difficult to equate the concentrations of aspirin used *in vitro* with those obtained *in vivo*, because we are not able to precisely mimic the *in vivo* drug metabolism and intestinal tissue concentrations in the lab. Thus, it will be challenging to further determine a more accurate dose of aspirin that can confer clinically meaningful cellular senescence while diminishing the risk for potentially harmful effects, such as gastrointestinal hemorrhage. In reverence to dose-related toxicity, aspirin administration has been associated with upper GI bleeding and lower GI injury (Sostres and Gargallo, 2012; Sostres et al., 2013). While our data suggest that aspirin (500 μ M) may have decreased effects on normal colonic epithelial cells under conditions that induce senescence in CRC cells (Fig. 1D-1F), additional *in vivo* studies are needed to examine the toxicity of aspirin dose in the GI tract.

Cancer may be seen as metabolic disease that is accompanied by deregulation of the energy balance (Mendonca et al., 2015). Other metabolic diseases, including obesity and type 2 diabetes, confer increased risks for CRC (O'Neill and O'Driscoll, 2015; Vucenik and Stains, 2012). Thus, it is logical to speculate that metabolic regulators could contribute to the pathophysiology of CRC. The anti-tumor activity of aspirin has predominantly been attributed to the inhibition of cyclooxygenase-2 (COX-2) (Chan et al., 2007; Hennekens and Dalen, 2013). However, accumulating evidence indicates that

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COX-2 inhibition is not the only basis for the anti-cancer effects of aspirin; other targets have been identified, and researchers have begun to recognize that this old drug has a startling variety of effects (Alfonso et al., 2014). We herein show that aspirin induces cellular senescence in SW620 and HCT116 cells, which are known to lack COX-2 expression (Liu et al., 2003; Sade et al., 2012). We further tested whether aspirin or celecoxib, two known COX-1/2 inhibitors (Seedher and Bhatia, 2003), could induce senescence in COX-2-expressing HT-29 cells. Our results revealed that aspirin increased SA- β gal staining in HT-29 cells, whereas celecoxib did not (data not shown). This suggests that aspirin induces senescence in CRC cells via a COX-1/2 independent mechanism. Recently, aspirin was shown to affect type 2 diabetes in a manner similar to that of the accepted therapeutic agents, metformin and insulin (Hardie, 2013). Likewise, aspirin has been shown to influence the cellular energy status (Hardie, 2014; Kamble et al., 2014). Based on these findings, we suggest that aspirin may specifically induce senescence in CRC cells via a mechanism other than the classical ability of aspirin to inhibit COX proteins.

Here, we investigated aspirin-induced cellular senescence, focusing on the key regulators of cellular metabolism, SIRT1 and AMPK. SIRT1 confers anti-aging activity in normal physiology by limiting calories and regulating cellular energy metabolism (Canto and Auwerx, 2012). Recent studies have demonstrated that SIRT1 antagonizes senescence in various cell types, including cancer cells (Huang et al., 2008; Jung-Hynes and Ahmad, 2009; Ota et al., 2007; Ota et al., 2006). In contrast to these observations, however, our data indicated that inhibition of SIRT1 by si-SIRT1 or a SIRT1 inhibitor (EX527) substantially decreased the senescence-inducing effects of aspirin. Although the deacetylase activity of SIRT1 functions solely in the nucleus, nucleo-cytoplasmic translocation of SIRT1 has been recognized in various mammalian cells. For example, in a cell culture system in which cellular senescence was induced by growth factor starvation, SIRT1 was transported to the cytosol with a concomitant reduction of SIRT1 activity (Meng et al., 2011). Aberrant cytoplasmic localization of SIRT1 was previously noted in cancer, and found to be associated with SIRT1 protein stabilization (Byles et al., 2010). Furthermore, cell death has been shown to be enhanced by cytosol-localized SIRT1 in a deacetylase activity-independent manner (Jin et al., 2007). Therefore, the cytosolic SIRT1

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present in CRC cells is likely to play a very significant function in aspirin-induced cellular senescence. Although additional work is needed to elucidate the mechanism through which cytosolic SIRT1 triggers the deacetylase activity-independent induction of senescence, our data suggest that SIRT1 is essentially responsible for aspirin-induced cellular senescence in CRC cells. There is some debate regarding the role of SIRT1 in CRC, however. Several reports have described that loss of SIRT1 activity is associated with progression of CRC, and SIRT1 expression in HCT116 cells (primary CRC cells) was shown to inhibit the growth of tumor xenografts (Kabra et al., 2009). In contrast, high levels of SIRT1 were shown to enhance the tumorigenesis of CRC, and SIRT1 deficiency in SW620 (metastatic CRC cells) was associated with low CRC tumorigenesis in tumor xenografts (Chen et al., 2014). Because the initiation and progression of cancer differs from the development of metastasis (Kroemer and Pouyssegur, 2008), SIRT1 is likely to play different roles in primary and metastatic CRC. In the future, given the function of SIRT1 as a metabolic regulator in cancer, it will be useful to define other systemic alterations in key metabolic pathways that occur during CRC progression and metastasis.

AMPK may also play essential roles in the aspirin-induced senescence of CRC cells. The ancient protein, AMPK, is a central regulator of energy balance that is known to play many roles in human cancer (Hardie, 2014). Tumor suppressors, such as liver kinase B1 (LKB1), tuberous sclerosis complex 2 (TSC2) and p53, are highly involved in AMPK signaling and thus contribute to the network connecting cancer metabolism with tumor development (Hardie and Alessi, 2013; Inoki et al., 2006; Zhou et al., 2014). The results of a previous study strongly suggest that aspirin, metformin or other pharmaceuticals can inhibit colon carcinogenesis through the activation of AMPK (Hardie, 2013). Thus, our observation of a novel connection between aspirin-induced senescence and metabolic alterations in CRC cells, particularly in SIRT1-AMPK signaling, suggests a molecular basis for the particular sensitivity of CRC to aspirin.

Notably, the reciprocal regulations of the SIRT1 and AMPK signaling pathways in cellular senescence have been observed in different tissues and cell types (Wang et al., 2011). For example, AMPK augments SIRT1 activity by increasing cellular NAD⁺ levels in skeletal muscle (Canto et al., 2009),

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and this amplification of SIRT1 signaling is lessened under AMPK-deficient conditions (Canto et al., 2010). In contrast, resveratrol was found to stimulate AMPK in a SIRT1-independent manner in neuron (Dasgupta and Milbrandt, 2007). Moreover, resveratrol has been shown to activate AMPK signaling in both SIRT1-dependent and -independent manners in hepatocellular carcinoma cells (Hou et al., 2008; Shin et al., 2009). The molecular mechanism through which SIRT1 modulates AMPK activity is not yet clear. AMPK is activated by phosphorylation at Thr172 in the catalytic subunit by the Ca²⁺-dependent kinase, CaMKK β , or the tumor suppressor, liver kinase B1 (LKB1) (Hardie, 2008). Therefore, the SIRT1/CaMKK β -AMPK or SIRT1/LKB1-AMPK pathways may be involved in the induction of senescence. Notably, LKB1 has been identified as a substrate of SIRT1 deacetylase (Hou et al., 2008). Together, the previous observations and our present findings suggest that SIRT1/LKB1-mediated AMPK activation may be implicated in the aspirin-induced senescence of CRC cells.

As the regulations of energy metabolism differ widely across tissues and cells, it is difficult to envision how SIRT1 and AMPK might be linked in a coordinated network governing cellular senescence in CRC. It will thus be important to investigate the mechanisms by which they interact and the consequences of their cross-regulations under senescence-inducing conditions in CRC.

In sum, based on the novel findings described herein, we propose that aspirin may confer a dominant anti-cancer effect in CRC by inducing cellular senescence, and that this occurs through creation of an energy imbalance and subsequent activation of metabolic regulators, SIRT1 and AMPK (Fig. 6I). Our findings may help explain why aspirin has specific therapeutic effects or chemopreventive activities in human CRC.

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Conflict of interest

The authors declare that they have no competing interests.

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Authorship Contributions

Participated in research design: Y.R.J., H.S.K., M.L.

Conducted experiments: Y.R.J

Contributed new reagents or analytical tools: J.-J.P., H.J.C.

Performed data analysis: Y.R.J., Y.J.L., M.-J.P

Wrote or contributed to the writing of the manuscript: E.J.K., M.L.

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Footnote

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Figure legends

Figure 1. Aspirin induces cellular senescence in human colorectal carcinoma (CRC) cells. (A) SW620 and HCT116 cells were treated with the indicated doses of aspirin, and cell viability was analyzed by MTT assay. (B) SW620 cells were treated with 200 μ M, 500 μ M, or 1 mM of aspirin, and cellular senescence was examined by staining of senescence-associated β -galactosidase (SA- β gal). Senescent cells were (blue-stained) were observed by bright-field microscopy. (C) Quantitative analysis of SA- β gal-positive cells. (D) SW620 and HCT116 cells were exposed to 500 μ M of aspirin for 72 h or 6 Gy (SW620) or 8 Gy (HCT116) of IR for 48 h, and cellular senescence was examined by SA- β gal staining. Additionally, SA- β gal staining was performed in human colonic epithelial cells (HCoEpiCs) treated with aspirin (500 μ M) or IR (6 Gy). (E and F) The percentage of SA- β Gal-positive cells was quantified. (G) SW620 and HCT116 cells were treated with aspirin (500 μ M) for 72 h, and then stained with PI (propidium iodide) and analyzed by flow cytometry. The percentage of PI-positive cells was quantified. (H) HCT116 and SW620 cells were treated with 500 μ M of aspirin for different durations and the protein levels of p53, p21, DcR2, and β -actin were analyzed by immunoblotting. HCT116 and SW620 cells were treated with 500 μ M of aspirin for 72 h, the protein levels of DcR2 and β -actin were analyzed by immunoblotting (I), and the three-independent results were scanned and analyzed by densitometry (J). The values are presented as the mean \pm SEM; * p < 0.05, ** p < 0.01 and *** p < 0.001 versus the control.

Figure 2. Aspirin increases the protein levels of SIRT1, phospho-AMPK (p-AMPK T172), and phospho-acetyl CoA carboxylase (p-ACC S79) in human CRC cells. (A and B) HCT116 and SW620 cells were treated with different concentrations of aspirin (10, 50, 200, 500 and 1000 μ M) (A) for different durations (0, 3, 6, 12, 24, 48 and 72 h). (B) Cell lysates were harvested, and SIRT1, AMPK α , p-AMPK α (Thr172), p-ACC (S79), ACC and β -actin were detected by immunoblotting. (C) HCT116 and SW620 cells were treated with aspirin (500 μ M) for 3 h, and intracellular SIRT1 activity was measured using a SIRT1 assay kit. (D) HCT116 and SW620 cells were treated with different

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concentrations (10, 50, 200, 500 and 1000 μM) of aspirin for 72 h, and the intracellular ATP content was measured using an ATP Determination Kit. (E) SW620 and HCT116 cells were exposed to 6 Gy or 8 Gy of IR for different durations (0, 3, 6, 12, 24, 36 and 48 h), and SIRT1, AMPK α , p-AMPK α (Thr172), p-ACC (S79), ACC, and β -actin were determined by immunoblotting. (F) SW620 and HCT116 cells were irradiated for 3 h (6 and 8 Gy, respectively), and SIRT1 activity was measured based on an enzymatic reaction. (G) HCT116 and SW620 cells were irradiated for 72 h, and the intracellular ATP content was measured using an ATP Determination Kit. The values are presented as the mean \pm SEM; * p < 0.05, ** p < 0.01, *** p < 0.001 versus the control.

Figure 3. Aspirin-induced cellular senescence is abrogated by inhibition of SIRT1 in human CRC cells. (A and B) HCT116 cells were transfected with si-control or si-SIRT1 and incubated for 24 h. The cells were then treated with aspirin (500 μM) or exposed to IR (8 Gy) for another 72 or 48 h, respectively. SA- β gal staining was performed and SA- β gal-positive cells were quantified. (C and D) SW620 cells were pretreated with the SIRT1 inhibitor, EX527 (2 μM), for 1 h and then treated with aspirin (500 μM) or IR (6 Gy) for another 72 or 48 h, respectively. SA- β gal staining was performed and SA- β gal-positive cells were quantified. The values are presented as the mean \pm SEM; * p < 0.05, ** p < 0.01 and *** p < 0.001 versus the control.

Figure 4. Aspirin-induced cellular senescence was diminished by inhibition of AMPK in human CRC cells. (A and B) HCT116 cells were transfected with si-control or si-AMPK α 1, incubated for 24 h, and then treated with aspirin (500 μM) or IR (8 Gy) for another 72 or 48 h, respectively. SA- β gal staining was performed, and the SA- β gal-positive cells were quantified. (C and D) SW620 cells were pretreated with the AMPK inhibitor, Compound C (10 μM) for 1 h, and then treated with aspirin (500 μM) or IR (6 Gy) for another 72 or 48 h, respectively. SA- β gal staining was performed, and the SA- β Gal-positive cells were quantified. The values are presented as the mean \pm SEM; * p < 0.05, ** p < 0.01 and *** p < 0.001 versus the control.

Figure 5. The aspirin-induced cellular senescence of CRC cells involves the potentially reciprocal of SIRT1 and AMPK. (A and B) HCT116 cells were treated with 10 μM of resveratrol or 500 μM of AICAR for 72 h, SA- β gal staining was performed, and the SA- β gal-positive cells were quantified.

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(C) HCT116 cells were treated with resveratrol or AICAR for 6 h, and SIRT1 enzyme activity was measured. (D) HCT116 cells were treated with resveratrol (10 μ M) or AICAR (500 μ M) for 72 h, and levels of SIRT1, AMPK α , p-AMPK α (Thr172), p-ACC (S79), ACC and β -actin were determined by immunoblotting. (E) HCT116 cells were transfected with si-SIRT1, incubated for 24 h, and then treated with 500 μ M of aspirin for another 72 h. Cell lysates were prepared, and the protein levels of SIRT1, AMPK α , p-AMPK α (Thr172), p-ACC (S79), ACC and β -actin were detected by immunoblotting. (F) HCT116 cells were transfected with si-AMPK, incubated for 24 h, and further treated with aspirin (500 μ M) for another 72 h. Cell lysates were prepared, and the protein levels of SIRT1, AMPK α , p-ACC (S79), ACC and β -actin were detected by Western blotting. (G) HCT116 cells were transfected with si-AMPK, incubated for 24 h, and then treated with aspirin for 3 hr. Intracellular SIRT1 activity was measured using a SIRT1 assay kit. The values are presented as the mean \pm SEM; * p < 0.05, ** p < 0.01 and *** p < 0.001 versus the control.

Figure 6. Aspirin decreases the deacetylase activity of SIRT1 at a later time point, followed by enhancement of cytoplasmic localization. HCT116 and SW620 cells were treated with aspirin (A), or irradiation (B), and SIRT1 enzyme activity was measured. (C) HCT116 cells were treated with resveratrol or AICAR, and SIRT1 enzyme activity was measured. SW620 cells were treated with aspirin for 24 or 48 h, EX527 (10 μ M) was added to the culture medium, and the cells were incubated for an additional 48 or 24 h, respectively (for a total of 72 hours of aspirin treatment). SA- β gal staining was performed (D), and the SA- β gal-positive cells were quantified (E). (F) SW620 cells and HCT116 cells were treated with 500 μ M of aspirin for 72 h, and the protein levels of SIRT1 and β -actin were analyzed by immunoblotting. (G) SW620 cells and HCT116 cells were treated with aspirin for 72 h, nuclear and cytoplasmic proteins were prepared, and SIRT1, GAPDH, Lamin A/C, and β -actin were detected by immunoblotting. (H) SW620 cells and HCT116 cells were treated with 500 μ M of aspirin for 72h. Cells were fixed, and stained with SIRT1 for immunostaining. Nuclei were stained with DAPI. Bar, 10 μ M. (I) Proposed scheme for the coordinated activations of SIRT1 and AMPK in the aspirin-induced cellular senescence of CRC cells. The values are presented as the mean \pm SEM; * p < 0.05, ** p < 0.01 and *** p < 0.001 versus the control.

Figure 1

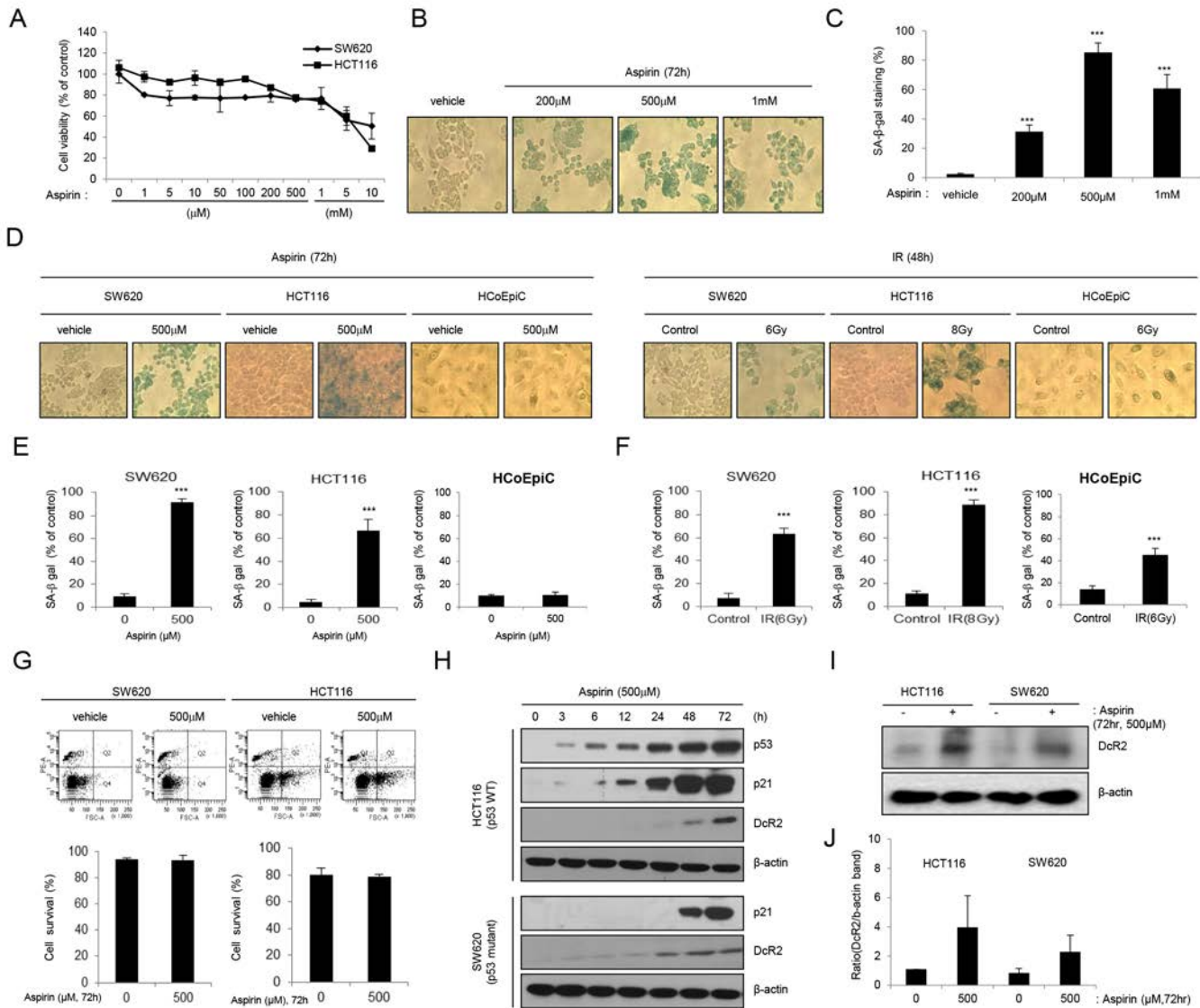
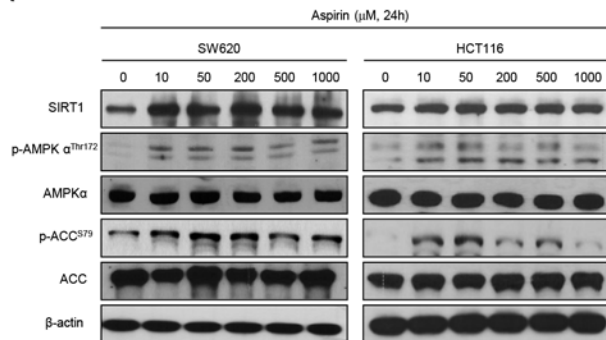
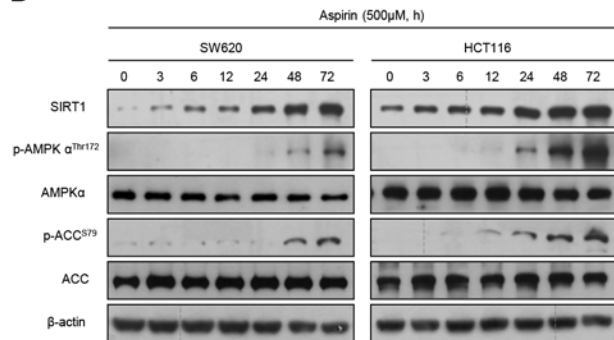


Figure 2

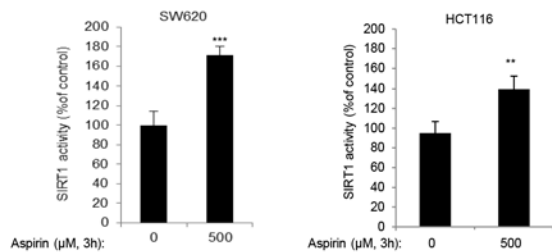
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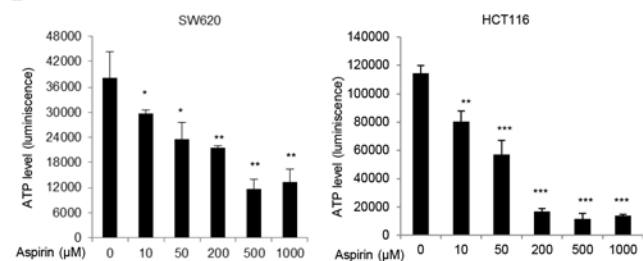
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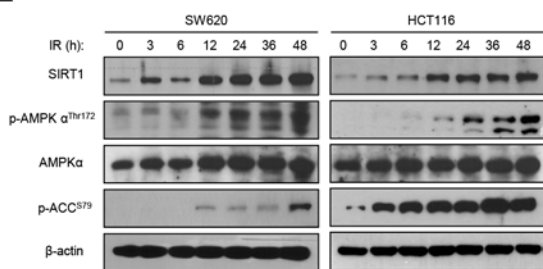
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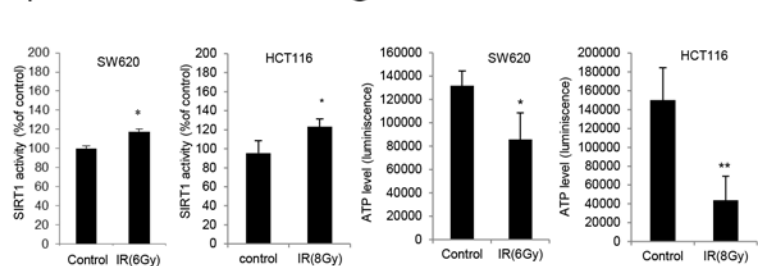
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E



F



G

Figure 3

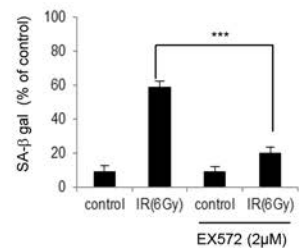
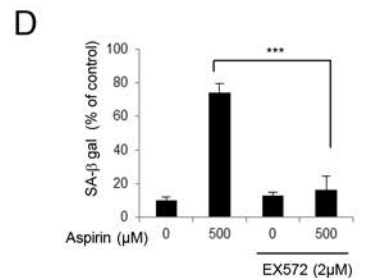
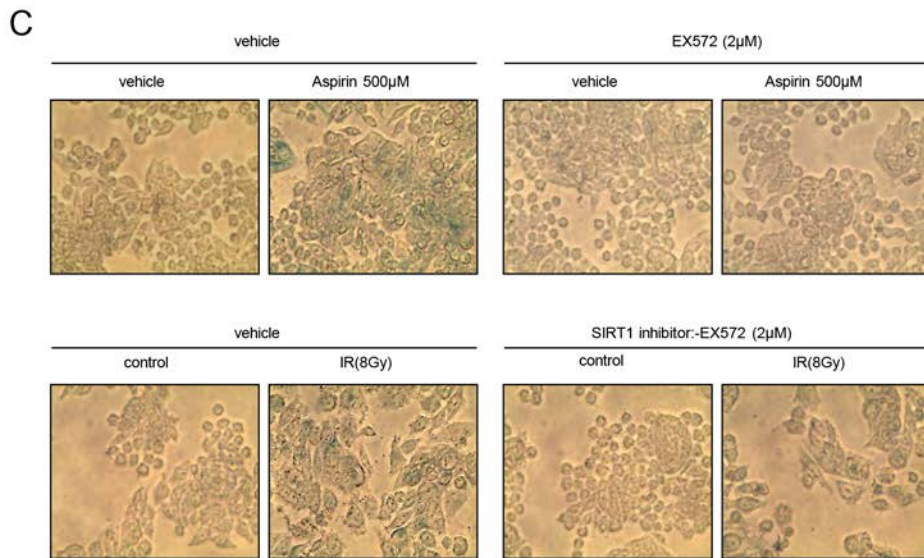
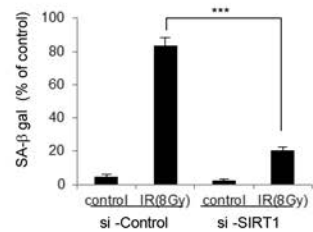
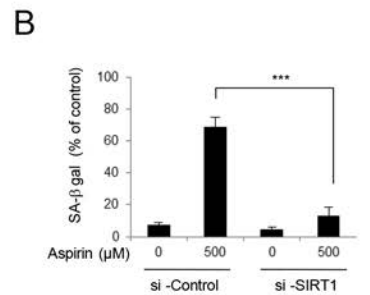
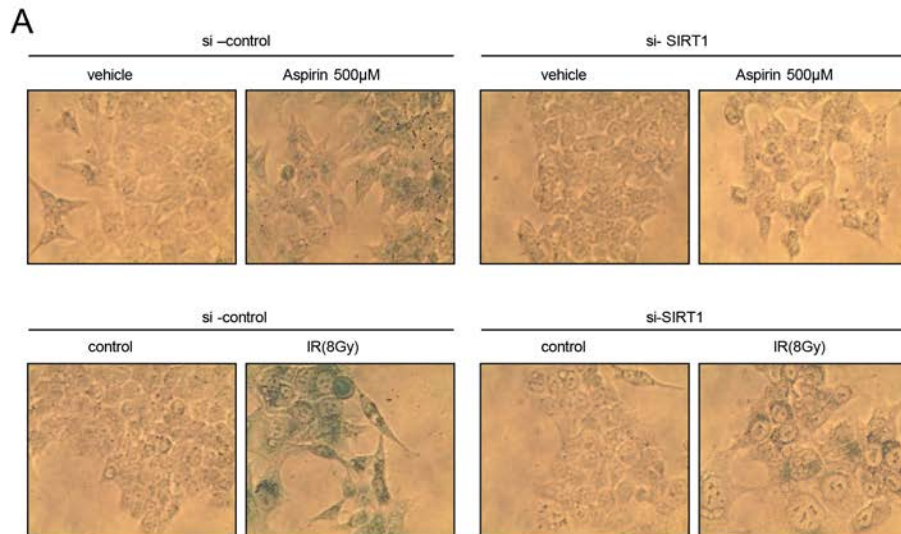
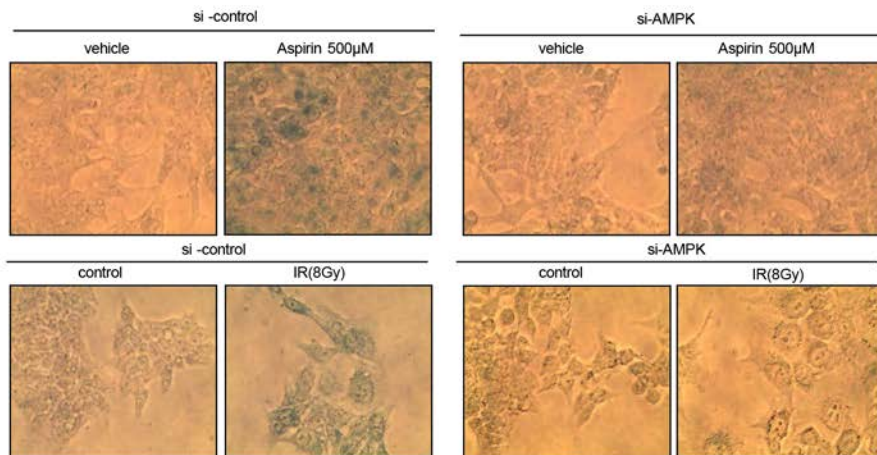
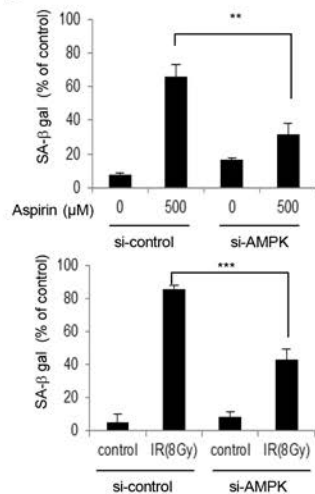


Figure 4

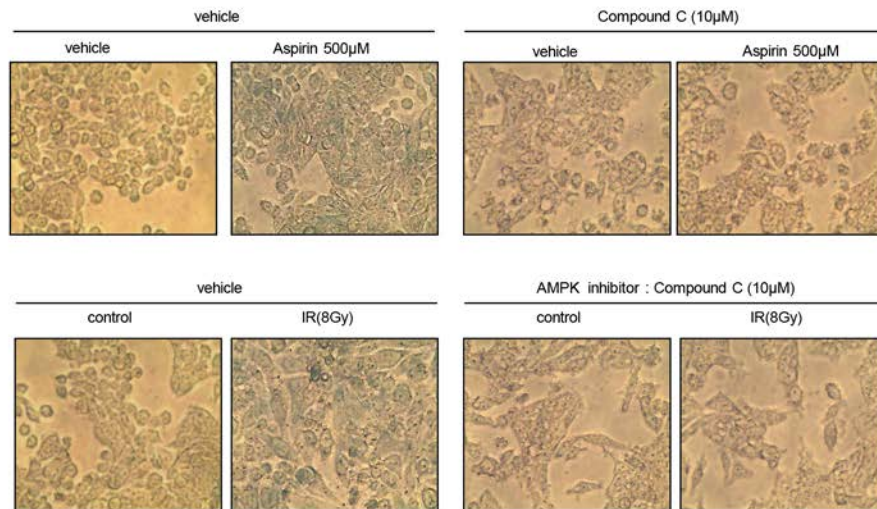
A



B



C



D

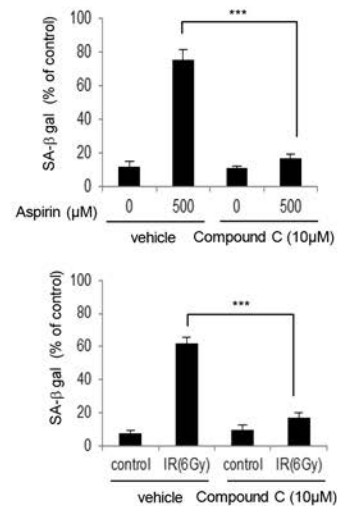


Figure 5

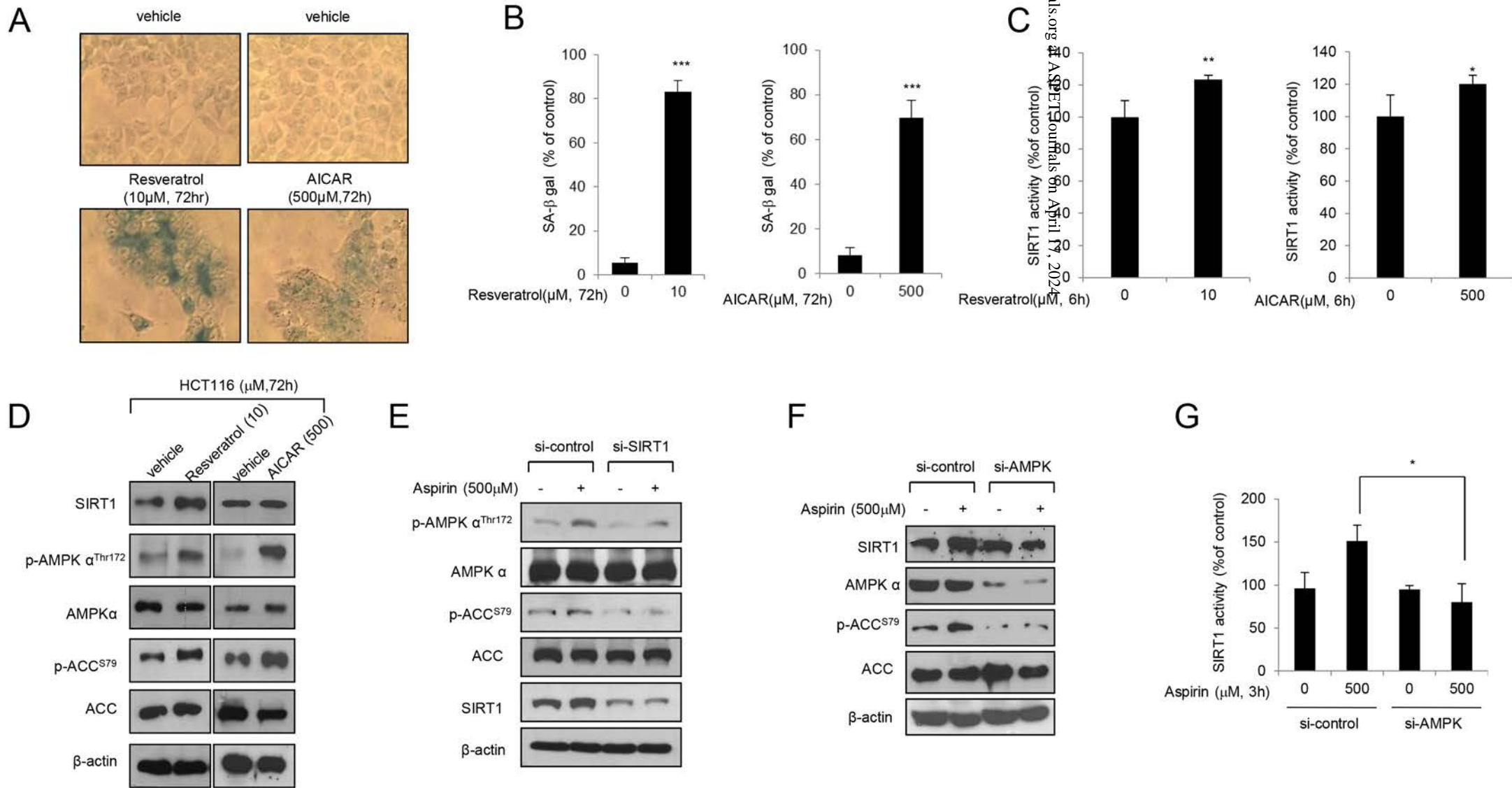


Figure 6

