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Sirt1 Promotes DNA Damage Repair and Cellular Survival

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Abstract

Sirt1, a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase, is known to deacetylate a number of proteins that are involved in various cellular pathways such as the stress response, apoptosis and cell growth. Modulation of the stress response by Sirtuin 1 (Sirt1) is achieved by the deacetylation of key proteins in a cellular pathway, and leads to a delay in the onset of cancer or aging. In particular, Sirt1 is known to play an important role in maintaining genomic stability, which may be strongly associated with a protective effect during tumorigenesis and during the onset of aging. In these studies, Sirt1 was generated in stably expressing cells and during the stimulation of DNA damage to examine whether it promotes survival. Sirt1 expressing cells facilitated the repair of DNA damage induced by either ionizing radiation (IR) or bleomycin (BLM) treatment. Fastened damaged DNA repair in Sirt1 expressing cells corresponded to prompt activation of Chk2 and γ-H2AX foci formation and promoted survival. Inhibition of Sirt1 enzymatic activity by a chemical inhibitor, nicotinamide (NIC), delayed DNA damage repair, indicating that promoted DNA damage repair by Sirt1 functions to induce survival when DNA damage occurs.

Key Words: Sirt1, DNA damage, Ionizing radiation, DNA repair, Radio-chemo sensitizer, Bleomycin

INTRODUCTION

Silencing information regulator 2 (Sir2) in yeast is involved in transcriptional regulation of the DNA damage response, senescence and longevity by caloric restriction (Guarente and Picard, 2005). Sirtuin 1 (Sirt1), a mammalian homolog of Sir2, belongs to the nicotinamide adenine dinucleotide (NAD) dependent histone deacetylase family and has been implicated as a regulator of a variety of important biological processes such as aging, metabolism and stress resistance (Michan and Sinclair, 2007).

As a deacetylase, Sirt1 regulates such biological responses through the deacetylation of various target proteins by modulating or promoting their activity. For example, deacetylation of the p53 tumor suppressor protein by Sirt1 attenuates transcriptional activity toward its downstream targets, which are mostly involved in stress responses (Vaziri *et al.*, 2001; Kim *et al.*, 2007). In a similar fashion, members of the FOXO family, important transcription factors that trans-activate a number of stress genes such as p27Kip1, Gadd45, and Bim (Sedding, 2008), are deacetylated by an interaction with Sirt1, leading to the repression of transcriptional activity (Motta *et al.*, 2004). Under DNA damage stress conditions, Sirt1 deacetylates Ku-70 (Cohen *et al.*, 2004), the Nijmegen breakage syndrome-1 (NBS1) protein, which promotes survival (Yuan *et al.*, 2007). Therefore, modulation of the stress response by Sirt1 under a variety of conditions - DNA damage, oxidative stress, and metabolic stress - is closely associated with its role in controlling senescence, longevity and survival (Brooks and Gu, 2009). Given that Sirt1 is known to express in a relatively high level in many types of cancers (Bradbury *et al.*, 2005; Stunkel *et al.*, 2007), expression of Sirt1 in cancers favors the survival and growth of tumors (Deng, 2009). In contrast, Sirt1 has also demonstrated the augmentation of apoptosis in response to TNFα by inhibiting NF-κB-dependent, anti-apoptotic gene expression (Yeung *et al.*, 2004), as well as playing a role in the prevention of tumor development and in the maintenance of genomic stability (Wang *et al.*, 2008).

Cancer stem cells (CSCs) have shown a high chemo/radio resistance (Dean *et al.*, 2005) as a result of increased recognition of the DNA damage signal and enhanced activation of the DNA damage repair system (Bao *et al.*, 2006). Therefore, the molecular mechanism of an enhanced DNA damage repair system in cancer stem cells is regarded as an urgent factor in enhancing the efficiency of radiotherapy.

In the present study, Sirt1 promoted DNA damage repair

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and survival. Facilitated DNA damage repair occurs in parallel with the enhanced DNA damage stress signaling through rapid Chk2 activation. Thus, inhibition of Sirt1 enzymatic activity by a chemical inhibitor or depletion of Sirt1 significantly delayed DNA damage repair. These results suggest that a pharmacological inhibitor of Sirt1 may be a potential therapeutic reagent to lower the chemo/radio-resistance of cancer cells.

MATERIALS AND METHODS

Reagents and cell culture

The Sirt1 (cat#:SC-15404), ERK2 (cat#:SC-154), anti-phospho-Chk2 (Threonine 68, cat#:CS-16297), and PARP-1/2 (cat#:SC-7150) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The phospho-Histone H2AX (Ser139, cat#:9718S), acetyl-p53 (cat#2525) and 53BP1 (cat#:4937) antibodies were obtained from Cell Signaling Technology (Danvers, MA). HEK 293T and Sirt1 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum with gentamycin (50 μg/ml).

Gene delivery

Sirt1-expressing HEK293T cells were generated using a viral gene delivery system, as previously described (Lee *et al.*, 2009). In brief, each viral plasmid (20 μg; MFG-Sirt1 (Hong *et al.*, 2010)) was transfected into Phoenix Amphor viral packaging cells (5×10^6) using lipofectamine 2000 (11668-027; Invitrogen, Carlsbad, CA). After 48 h, culture media containing the viruses were collected from the transfected Phoenix cells and filtered (0.45-lm filter; Millipore, Billerica, MA).

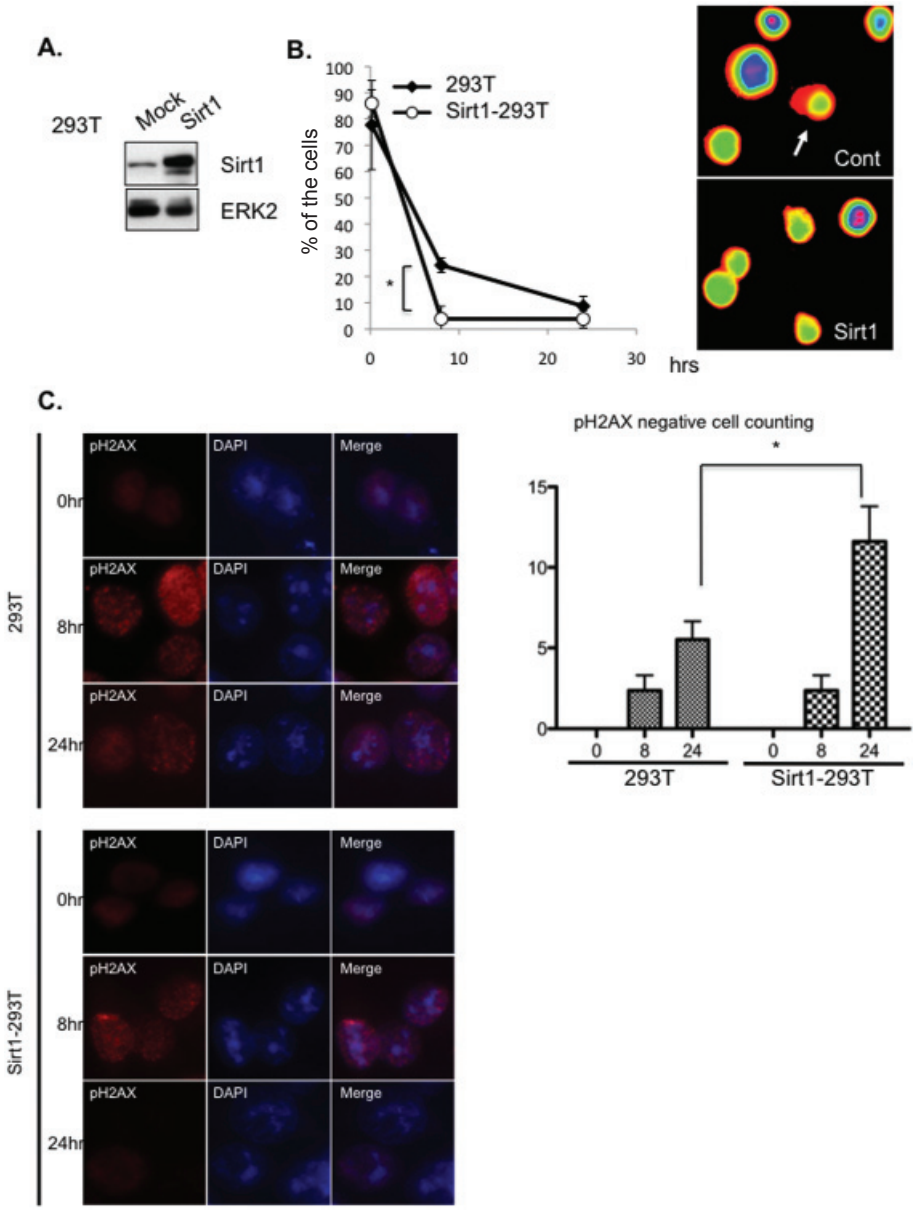

Fig. 1. Sirt1 enhanced the repair of damaged DNA. (A) Sirt1 was stably expressed to HEK 293T, generating Sirt1-293T cells. Stable expression of Sirt1 in Sirt1-293T cells was confirmed by immunoblotting analysis (top panel). ERK2 was used as loading control (bottom panel). (B) HEK 293T cells and Sirt1-293T were subjected to single cell gel DNA electrophoresis (comet assay). Both types of cells were exposed by 5 Gy of γ ray. (C) Immunofluorescence of γ-H2AX foci (red) staining in HEK 293T and Sirt1-293T cells and DAPI (blue) counterstaining for nuclei was shown (left panel). Quantification of γ-H2AX foci negative cell was graphically presented (right panel). At each condition, around 200 cells were counted. Statistical analysis (Student T-test) was performed as described in the Materials and Methods (*p<0.05).

Induction of DNA damage

50 μg/ml of bleomycin (BLM, cat#203408, calbiochem) was treated for varying time periods (0, 4, 8, 16, and 24 h). For some experiments, cells were exposed to γ-Rays using a model IBL 437C 95-495 (dose rate 2.93 Gy/min, CIS BIO International, Bagnols France).

Immunoblotting

Immunoblotting analysis was performed as standard protocol. In brief, cells were washed twice with ice-cold phosphate buffered saline (PBS), lysed with tissue lysis buffer (20 mM Tris-base, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine), and centrifuged at 14,000 rpm for 10 min (to clarify lysates). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF (Perkin Elmer Life Sciences; Boston, MA), blocked for 1-2 hr with 5% nonfat dry milk in Tris-buffered saline tween (TBS-T: 50 mM Tris-base, pH 7.4, 0.15 M NaCl, and 0.1% Tween-20) and incubated with the appropriate primary antibodies in TBS-T containing 1% BSA solution for 1-16 h. The primary antibodies were Sirt1, acetyl p53, Parp-1, γ-H2AX, and pchk2 (Santa Cruz biotechnology, Cell signaling Technology). Membranes were washed several times in TBS-T solution and incubated with anti mouse or rabbit secondary antibodies with 5% nonfat dry milk in TBS-T for 1 h at room temperature. Immunoreactivity was detected using an ECL Advance Western Blotting Detection kit (Amersham Biosciences, Piscataway, NJ, http://www.gelifesciences.com).

Immunofluorescence

HEK 293T cells on cover glasses were fixed for 30 min with −20°C methanol. Subsequently, the cells were permeabilized and blocked with PBS-BT (1×PBS, 3% BSA, and 0.1% Triton X-100) for 30 min at room temperature. Coverslips were subsequently incubated in primary and secondary antibodies diluted in PBS-BT. Images were acquired with an LSM image examiner (Carl Zeiss) under a Zeiss LSM510 confocal microscope using a 63 x oil immersion lens.

Comet assay

DNA double-stranded breaks were analyzed using a commercial comet assay (Trevigen, Inc., Gaithersburg, MD) following the manufacturer's protocol. For quantification, comet positive cells were scored in random fields of cells. More than 200 cells from each sample were scored. The analysis of the comet tail length was performed using Cometscore software version 1.5 (TriTek Corporation, http://www.autocomet.com).

Crystal violet staining

Crystal violet stock solution consisted of 0.1% crystal violet and 25% methanol. Cells were fixed with with ice-cold methanol for 10 min. Subsequently, the crystal violet stock solution was applied at room temperature for additional 10 min. After repeated rinsing with PBS, the plates were air dried and scanned with a Bio-Rad Gel-Doc ™ XR+System.

Statistical analysis

The graphical data were presented as means ± S.D. Statistical significance among three groups and between groups were determined using Student t-test. Significance was assumed for $p<0.05$ (*) and $p<0.01$ (**). Statistical analysis was performed using SAS statistical package, v.9.13 (SAS Inc., Cary, NC; http://www.sas.com/).

RESULTS AND DISCUSSION

Sirt1 promotes DNA damage repair

In order to examine the role of Sirt1 in the DNA damage response, Sirt1 was stably expressed in HEK 293T cells by a retroviral gene delivery system, as described previously (Lee *et al.*, 2009). The stable expression of Sirt1 in the Sirt1-293T cells was revealed by immunoblotting analysis (Fig. 1A). Upon 5 Gy of IR, which is sufficient to induce a double-strand break (dsb) of DNA, DNA repair kinetics between HEK293T cells and Sirt1-293T cells were determined using a comet assay, which is a typical method used to measure damaged DNA (Cha *et al.*, 2010) (Fig. 1B). As shown in Fig. 1B, Sirt1-293T cells appeared to facilitate the DNA repair that was indicated by prompt removal of the comet positive cell population (Fig. 1B, left panel). Consistently, γ-H2AX foci - a typical marker for a dsb event upon DNA damage stimuli (Fig. 1C, left panel) - more rapidly disappeared in Sirt1-293T cells when γ-H2AX foci negative cells were counted between HEK293T cells and Sirt1-293T cells (Fig. 1C).

Rapid sensing of DNA damage by Sirt1

Because Sirt1 expression in HEK293T cells promoted the repair of damaged DNA, DNA damage stress signaling was examined next. Since facilitated dsb repair in glioma stem cells was caused by rapid activation of stress signaling (Bao *et al.*, 2006) and CD133 positive brain cancer stem cells expressed high levels of Sirt1 (Chang *et al.*, 2009), the hypothesis was that efficient DNA damage repair in Sirt1 expressing

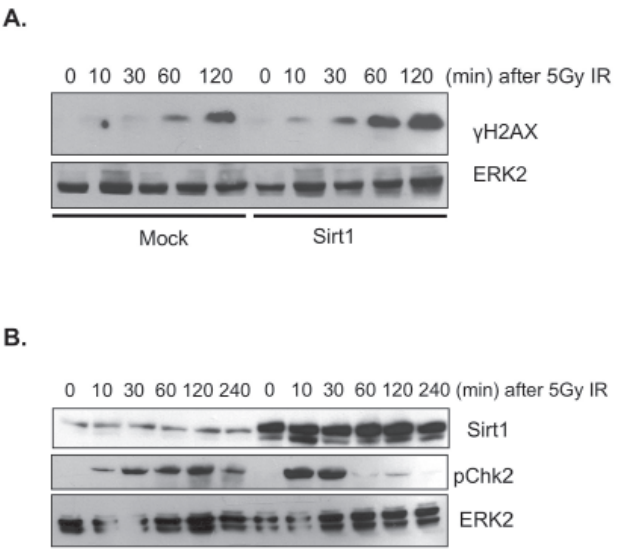

Fig. 2. Facilitated DNA damage signaling in Sirt1-293T cells. HEK 293T and Sirt1-293T were treated 5 Gy of γ ray and incubated for the indicated period of time. Immunoblotting results of γ-H2AX (A) and phosphorylated chk2 (B) were shown in respectively. ERK2 was used as loading control.

cells may be associated with rapid activation of DNA damage stress signaling. As predicted, Sirt1-293T cells (Fig. 2A top, panel), showed facilitated Chk2 phosphorylation upon 5 Gy of IR (Fig. 2A, bottom panel), indicating that ATM, a specific upstream kinase of Chk2, which is a major sensor protein for a dsb event, was activated more promptly in Sirt1 expressing cells. Thus, γ-H2AX foci generation that is also mediated by ATM activation (Burma *et al.*, 2001), also appeared to be more rapidly induced in Sirt1-293T cells than in control cells (Fig. 2B). These results are consistent with a previous report that glioma stem cells, which may express a high level of Sirt1 (Chang *et al.*, 2009), showed radioresistance by preferential activation of DNA damage stress signaling (Bao *et al.*, 2006).

Sirt1 inhibition delayed DNA damage repair

NIC (IC_{50} was lower than 50 μM) is known to be equal to or better than the most effective known synthetic inhibitors at blocking NAD^+ hydrolysis (Bitterman *et al.*, 2002; Lee *et al.*, 2010). The present study examined whether Sirt1 inhibition by NIC would affect DNA damage repair kinetics. As shown in Fig. 3A, cells treated by bleomycin (BLM) showed gradual induction of γ-H2AX, indicating an increase in the occurrence of a dsb. Interestingly in the pretreatment of NIC, the level of γ-H2AX failed to be lowered even after 24 h of BLM treatment, suggesting that dsb repair, subsequently lowering γ-H2AX (Keogh *et al.*, 2006), was significantly delayed by inhibition of Sirt1 enzymatic activity (Fig. 3A). Given that p53 is a known target protein for Sirt1 (Vaziri *et al.*, 2001), increased levels of p53 acetylation by BLM in the pretreatment of NIC strongly indicated that inhibition of Sirt1 enzymatic activity by NIC showed a clear correlation to DNA damage repair efficiency. Consistently, 53BP1 foci, another well-established dsb indica-

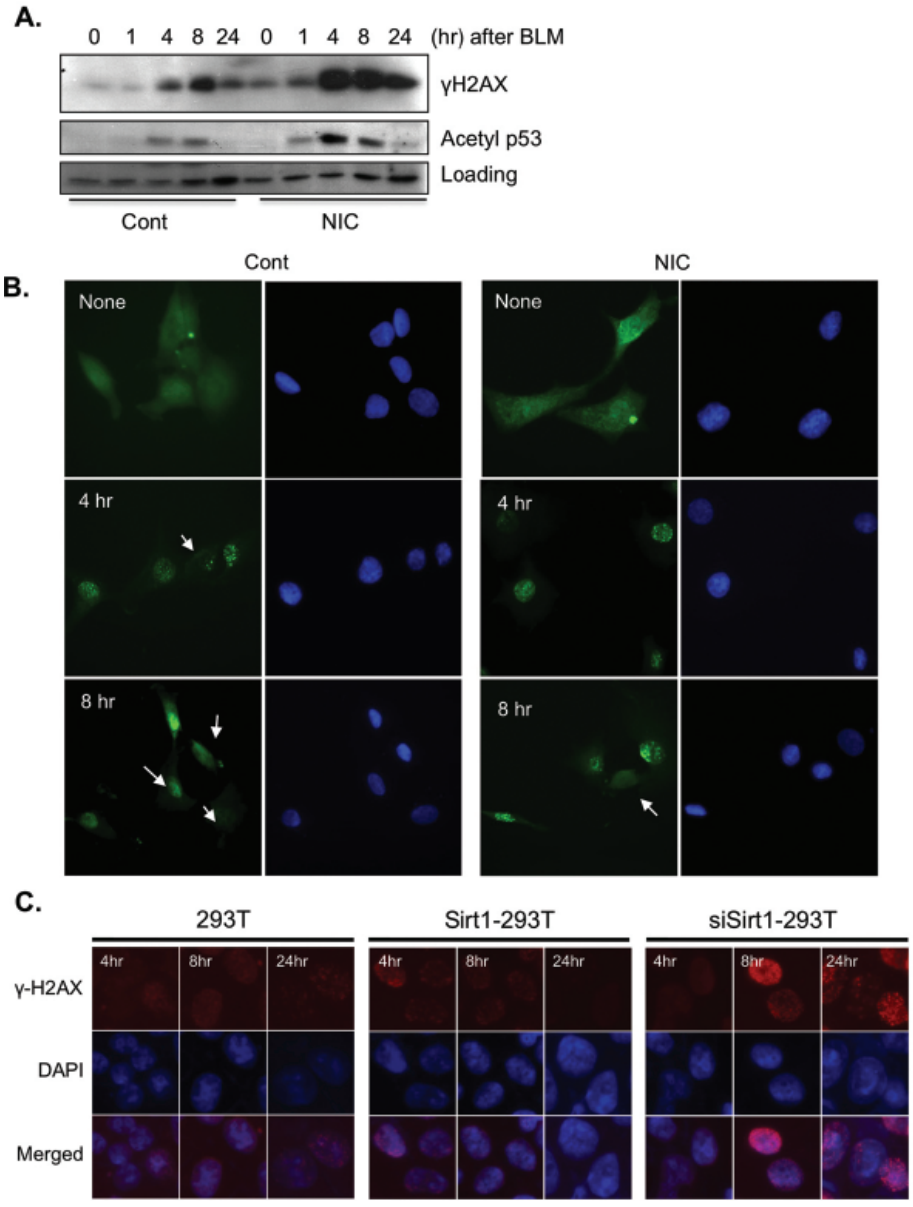

Fig. 3. Inhibition of Sirt1 delays DNA damage repair. DNA damage was induced by 50 μg/ml of bleomycin (BLM) and cells were further incubated with or without 10 mM of Nicotinamide (NIC). (A) γ-H2AX and acetylated p53 level were determined by immunoblotting analysis. α-tubulin for equal loading control. (B) 53BP1 foci (green) was determined by immunofluorescence and Nculei was counterstained with DAPI (blue). (C) γ-H2AX foci (red) indicating DNA damage were determined by immnuofluroscence analysis in 293T, Sirt1 expressing 293T (Sirt1-293T) and Sirt1 depleted 293T cells (siSirt1-293T) at indicative time after 5 Gy of γ-ray.

tor (Mochan *et al.*, 2003), remained longer in the pretreatment of NIC (Fig. 3B, white arrows), also confirming that Sirt1 inhibition impaired the DNA damage repair process. As inhibition of Sirt1 by NIC delays removal of DNA damage foci (determined by 53BP1 foci), next we further confirmed the positive role of Sirt1 in DNA damage repair in Sirt1 depleted cells. Sirt1 was depleted by siRNA (siSirt1-293T cells) and DNA damage repair rate was determined by γ-H2AX foci. As consistent, γ-H2AX foci disappeared 24 h after IR in Sirt1 expressing cells, whereas significant number of foci remained positive in Sirt1 depleted cells (Fig. 3C). These results clearly indicate that Sirt1 is closely associated with DNA damage repair process.

Sirt1 promotes cellular survival under the DNA damage stress

Since the DNA damage repair process is important for protecting cells from apoptosis, preferential DNA damage repair promotes the survival of cells. Thus, it is readily surmised that Sirt1 expressing cells survived DNA damage stress, inflicted by IR. To test this idea, 293T and Sirt1-293T cells were both irradiated at a variety of dose and survival of resultant cells was determined by the clonogenic assay, a typical survival assay as described previously (Franken *et al.*, 2006). As predicted, cells expressing high level of Sirt1 (Sirt1-293T) showed promoted survival rate under relatively high dose of IR (5 Gy of IR) (Fig. 4B). This result demonstrated that Sirt1 promoted survival under DNA damage stress.

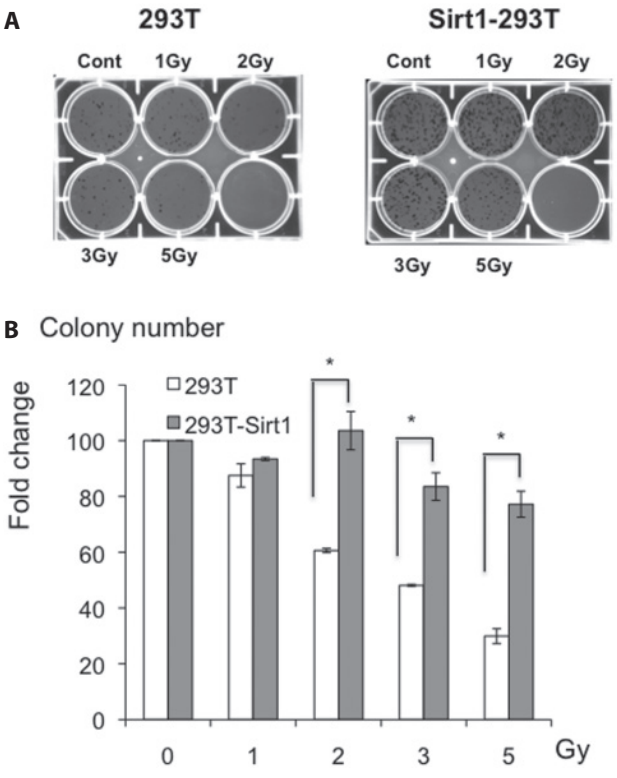

Fig. 4. Sirt1 promotes cellular survival under DNA damage. (A) 293T and Sirt1-293T cells were irradiated with a variety of dose of IR and 500 cells of each cell condition were plated and allowed them to growth additional 10 days. Numbers of colony from surviving cells were counted after crystal violet staining (A). Fold ratio of surviving colony versus control was graphically presented (B). Statistical analysis (Student T-test) was performed as described in the Materials and Methods (*p<0.05).

Sirt1 has been demonstrated to be involved in DNA damage repair and survival. However the molecular mechanism to govern the event of Sirt1 in DNA damage repair remains unclear although a number of DNA repair factors such as Kuy-70 or Nbs1 were studied as a Sirt1 substrate (Jeong *et al.*, 2007; Yuan *et al.*, 2007). We have provided the evidence that ATM activation and subsequent Chk2 activation, which are critical for triggering DNA damage signaling, become prompt in the presence of Sirt1 expression (Fig. 2). The facilitated DNA damage sensing that is mostly governed by prompt activation of ATM has been demonstrated to be an important process for high DNA damage repair and consequent high resistance to radio/chemo therapy in cancer stem cells (Bao *et al.*, 2006). Thus, we hypothesized that high expression of Sirt1 may be responsible for chemo/radio resistance of a number of cancer cells. We also showed the evidence that DNA damage repair process was delayed by either chemical inhibitor of Sirt1 (NIC) or siRNA of Sirt1, indicating that Sirt1 plays role in facilitated DNA damage repair process.

Increased resistance to conventional cancer therapies such as chemo or radiotherapy that induce DNA damage is a major reason for the failure of cancer therapy and the frequent recurrence of cancer. Therefore, development of a chemo/radio-sensitizer to increase sensitivity to developed chemo/radio-therapeutic agents would be as important as developing a new type of cancer drug. Considering that high Sirt1 expression is frequently found in a number of cancer cells (Bradbury *et al.*, 2005; Huffman *et al.*, 2007; Stunkel *et al.*, 2007) and CD133 positive stem-like cancer cells (Chang *et al.*, 2009), inhibiting Sirt1 enzymatic activity and subsequently impairing its role in the DNA damage repair process by a pharmacological inhibitor may increase sensitivity to chemo/radio-therapeutic reagents. Thus, these results suggest that a specific inhibitor targeting Sirt1 may be utilized as a chemo/radio-sensitizer to lower the chemo/radio-resistance of cancer cells and cancer stem cells.

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