



UV light induces premature senescence in Akt1-null mouse embryonic fibroblasts by increasing intracellular levels of ROS

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ARTICLE INFO

Article history:

Received 30 March 2009

Available online 11 April 2009

Keywords:

UV-irradiation

Senescence

Akt1

Isotype-specific function

ROS

ABSTRACT

Akt/PKB plays a pivotal role in cell survival and proliferation. Previously, we reported that UV-irradiation induces extensive cell death in Akt2^{-/-} mouse embryonic fibroblasts (MEFs) while Akt1^{-/-} MEFs show cell cycle arrest. Here, we find that Akt1^{-/-} MEFs exhibit phenotypic changes characteristics of senescence upon UV-irradiation. An enlarged and flattened morphology, a reduced cell proliferation and an increased senescence-associated β -galactosidase (SA β -gal) staining indicate that Akt1^{-/-} MEFs undergo premature senescence after UV-irradiation. Restoring Akt1 expression in Akt1^{-/-} MEFs suppressed SA β -gal activity, indicating that UV-induced senescence is due to the absence of Akt1 function. Notably, levels of ROS were rapidly increased upon UV-irradiation and the ROS scavenger NAC inhibits UV-induced senescence of Akt1^{-/-} MEFs, suggesting that UV light induces premature senescence in Akt1^{-/-} MEFs by modulating intracellular levels of ROS. In conjunction with our previous work, this indicates that different isoforms of Akt have distinct function in response to UV-irradiation.

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Introduction

Growth arrest and cell death are the most important cellular responses upon genotoxic stress. Severely damaged cells are actively eliminated either by apoptotic cell death or irreversible growth arrest, known as cellular senescence [1]. Recently, a number of studies demonstrated that cellular senescence acts as an important barrier to genomic instability and tumorigenesis [1,2]. The acquisition of genetic defects that impair mechanisms of senescence and apoptosis is therefore an essential prerequisite for cancer development.

Akt/PKB is a serine/threonine kinase and is known to play a central role in the regulation of both cell proliferation and cell survival [3]. The hyperactivation of Akt is frequently observed in various human cancers and is thought to convey antiapoptotic protection and uncontrolled cell growth to the tumor cell [4]. In mammalian cells, three isoforms of Akt; Akt1, Akt2 and Akt3, have been identified [3]. Akt isoforms are activated in a phosphatidylinositol 3-kinase (PI3K) dependent manner upon a variety of extracellular stimuli including growth factor treatment and genotoxic stress [3]. Activated Akt suppresses cell death and enhances cell proliferation through phosphorylation of various target proteins [5]. Mounting evidence indicates that Akt isoforms have distinct physiological functions despite a high degree of structure and sequence homology [6–9]. Given the importance of Akt function in cell pro-

liferation and survival and the frequent upregulation of Akt activity in various human cancers, Akt is increasingly thought to be a potential target for anticancer treatment [10]. Thus, the characterization of Akt isotype specific functions is an important step in the development of effective strategies for cancer treatment.

Recently, we have shown that Akt1 and Akt2, two major Akt isoforms, play distinct role in response to UV-irradiation using isogenic Akt1^{-/-} and Akt2^{-/-} mouse embryonic fibroblasts (MEFs) [11]. While Akt2^{-/-} MEFs underwent extensive apoptotic cell death through hyperactivation of JNK and p38, Akt1^{-/-} MEFs showed cell cycle arrest in the G1 and G2/M phases. In this report, we show that Akt1^{-/-} MEFs undergo senescence-like phenotypic changes upon UV-irradiation and that the increase of intracellular levels of ROS is required for this process.

Materials and methods

Cell lines and UV treatments. Akt1^{-/-} and Akt1-reconstituted MEFs were generated as previously described [12,13]. All MEF cell lines were cultured in DMEM-high glucose media containing 10% FBS. UV-irradiation was administered at 10 J/m² using a germicidal UV lamp and UV radiometer (UVX-25, Ultra-Violet Products Ltd.).

Cell proliferation assay. Akt1^{-/-} MEFs were seeded at a density of 1×10^4 of cells in a 12-well plate and treated with or without 10 J/m² of UV. Cells were trypsinized and counted using a hemacytometer after Trypan blue staining at the indicated time points.

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Experiments were repeated three times, and data were presented as mean values with standard deviations.

Western blot analysis and antibodies. Cells harvested after treatment were lysed in RIPA buffer and subjected to Western blot analysis as described previously [14]. Antibodies for Akt1 were obtained from Cell Signaling Technology Inc. (Danvers, MA). Antibodies for p53 and p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Levels of actin were monitored as an internal loading control using anti-actin (Sigma) antibodies.

Cell cycle analysis. To determine the cell cycle distribution, 1×10^6 cells were seeded onto a 100 mm dish. After UV treatment, cells were trypsinized at indicated time points and fixed with 70% ethanol. Subsequently, cells were stained with propidium iodide (PI), and flow cytometric analysis was performed using an EPICS XL cytometer (Beckman Coulter Inc.).

Senescence associated (SA)- β -galactosidase staining. SA β -galactosidase staining was performed at pH 6.0 as described previously [15]. Briefly, cells were fixed with 0.25% glutaraldehyde and incubated with freshly prepared staining solution overnight. After incubation, at least 300 cells in several fields were examined and SA β -gal positive cells were counted. These experiments were repeated three times and the results were presented as mean values with standard deviations.

Measurement of intracellular ROS level. In order to measure intracellular levels of ROS, Akt1^{-/-} MEFs were stained with 50 μ M of dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma–Aldrich Co., Ltd.) for 30 min and then harvested. The fluorescent intensities were quantified using an EPICS XL cytometer (Beckman Coulter Inc.). To examine the effect of N-acetylcystein (NAC) (Sigma–Aldrich Co., Ltd.), Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and incubated with 10 mM of NAC for 2 days. Experiments were performed in triplicate and the results were presented as mean values with standard deviations.

Results

Akt1-null MEFs show persistent growth arrest after UV-irradiation

To investigate the physiological consequence of UV-irradiation in Akt1^{-/-} MEFs, we first examined cell proliferation after UV-irradiation. Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and cell proliferation was measured at various time points by cell counting after Trypan blue staining. We found that the growth of Akt1^{-/-} MEFs was completely inhibited after UV-irradiation while untreated Akt1^{-/-} MEFs proliferated normally (Fig. 1A).

In a previous study, we observed that Akt1^{-/-} MEFs arrested in G1 and G2/M phase upon UV treatment [11]. Thus, the growth inhibition after UV-irradiation may be due to prolonged growth arrest. Alternatively, Akt1^{-/-} MEFs may undergo cell death, but at later time points. Cell cycle analysis revealed that the G1 and G2/M phase arrest induced by UV-irradiation was maintained for six days. Additionally, a sub-G1 fraction, indicative of cell death, was never observed. (Fig. 1B). Thus, these data suggest that the growth retardation of Akt1^{-/-} MEFs upon UV-irradiation is due to persistent growth arrest.

UV treatment induces premature senescence in Akt1-null MEFs

Interestingly, morphological analyses indicate that Akt1^{-/-} MEFs become enlarged and flattened after UV-irradiation (Fig. 2A). Previous studies have documented that cells entering senescence show distinct features including reduced proliferation and dramatic changes in morphology [1]. Since UV-irradiated Akt1^{-/-} MEFs show a phenotype similar to that of cells undergoing senescence, we examined whether Akt1^{-/-} MEFs exhibit senescence associated β -galactosidase (SA β -gal) activity, a reliable marker for cellular senescence [15], after UV-irradiation. Indeed, Akt1^{-/-} MEFs clearly show SA β -gal activity on day 6 after UV-irradiation (Fig. 2B).

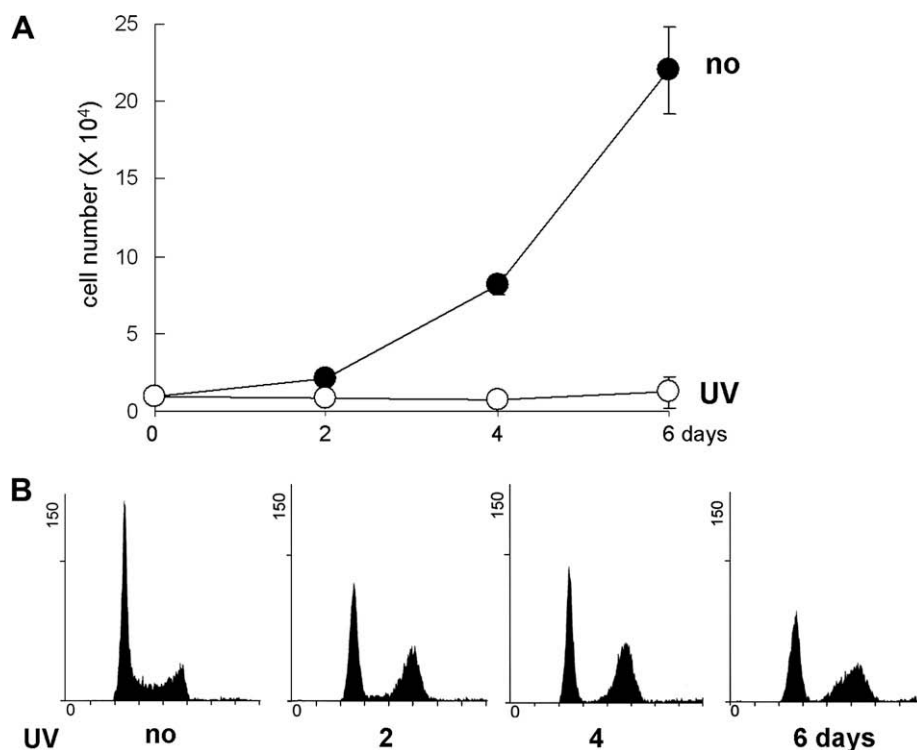


Fig. 1. UV light induces persistent growth arrest in Akt1-null MEFs. (A) Akt1^{-/-} MEFs were seeded at a density of 1×10^4 cells in a 12-well plate and treated with or without 10 J/m² of UV. Cell proliferation was measured at the indicated time points as described in Materials and methods. Error bars indicate standard deviation. (B) Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and harvested at the indicated time points. Cells were fixed, stained with PI and then analyzed by flow cytometry as described in Materials and methods. The data shown represent three independent experiments.

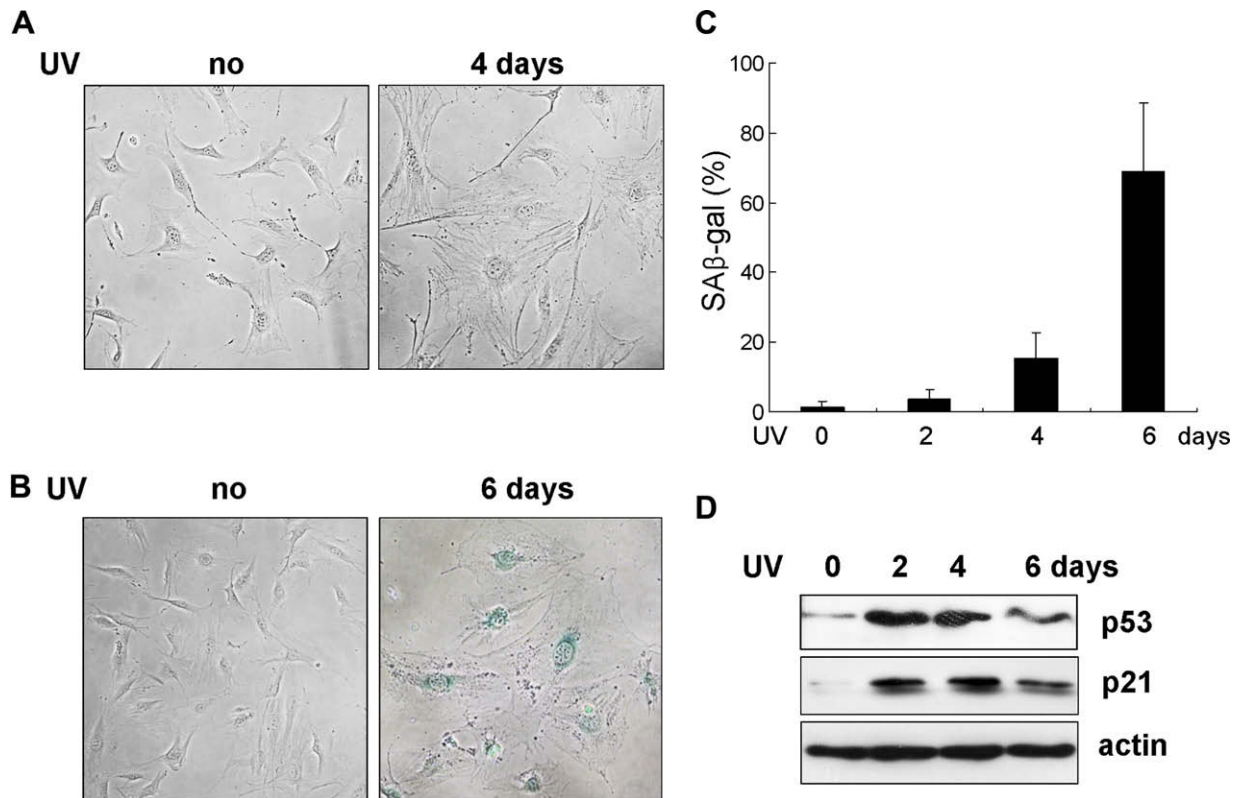


Fig. 2. UV-irradiation induces senescence-associated phenotypic changes in Akt1-null MEFs. (A) Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and cell images were captured 4 days later under a microscope. (B) Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and stained with freshly prepared SA β-gal staining solution 6 days later. (C) Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and stained with freshly prepared SA β-gal staining solution at the indicated time points. SA β-gal positive cells were counted in three independent experiments and the results were presented as mean values with standard deviations. (D) The cell lysates of Akt1^{-/-} MEFs were prepared at the indicated time points after UV-irradiation and subjected to Western blotting using anti-p53 and anti-actin antibodies.

Time-dependent kinetic analyses reveal that about 20% of cells were positive for SA β-gal on day 4 and that more than 70% of cells were positive for SA β-gal on day 6 after UV-irradiation (Fig. 2C). Moreover, upregulation of p53 and p21, tumor suppressors important in senescence [16], were observed from 2 days after UV-irradiation (Fig. 2D). Together, these results indicate that UV-irradiation induces cellular senescence in Akt1^{-/-} MEFs.

Akt1 reconstitution inhibits the induction of senescence in Akt1-null MEFs after UV treatment

To confirm that the absence of Akt1 is responsible for the UV-induced senescence in Akt1^{-/-} MEFs, we examined whether reintroduction of Akt1 expression is able to inhibit UV-induced senescence. Akt1 expression was reconstituted in Akt1^{-/-} MEFs via retrovirus-mediated gene transfer as described previously [13]. Western blotting results confirmed that Akt1-reconstituted cell lines (Akt1M1) expressed high levels of Akt1 while vector-only reconstituted cell lines (Akt1M) did not (Fig. 3A). SA β-gal assay results show that the reconstitution of Akt1 indeed caused a significant reduction of SA β-gal positive cells upon UV-irradiation while Akt1M showed levels of SA β-gal comparable with Akt1^{-/-} MEFs (Fig. 3B). These results confirm that the UV-induced senescence of Akt1^{-/-} MEFs is due solely to the absence of Akt1 function.

An increase in intracellular levels of ROS is required for UV-induced senescence in Akt1^{-/-} MEFs

Previous studies showed that reactive oxygen species (ROS) play an important role in the induction of premature senescence upon various types of genotoxic stresses [16]. To examine whether

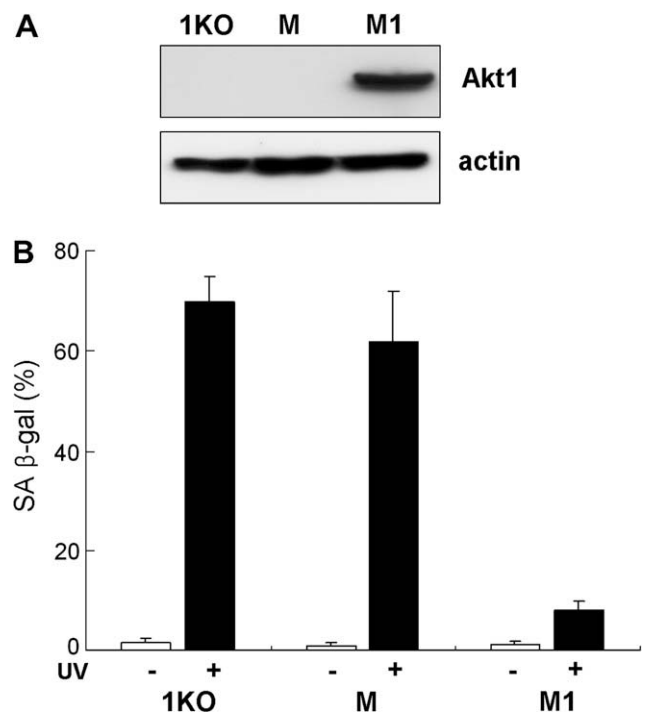


Fig. 3. Akt1 reconstitution inhibits UV-induced senescence induction in Akt1^{-/-} MEFs. (A) Cell lysates from Akt1^{-/-} MEFs, control retrovirus infected MEFs (M), and Akt1-reconstituted MEFs (M1) were subjected to Western blotting using anti-Akt1 and anti-actin antibodies. (B) SA β-gal staining was performed at 6 days later after UV-irradiation as described in Materials and methods. The experiments were repeated three times and the results were presented as a mean value with standard deviation.

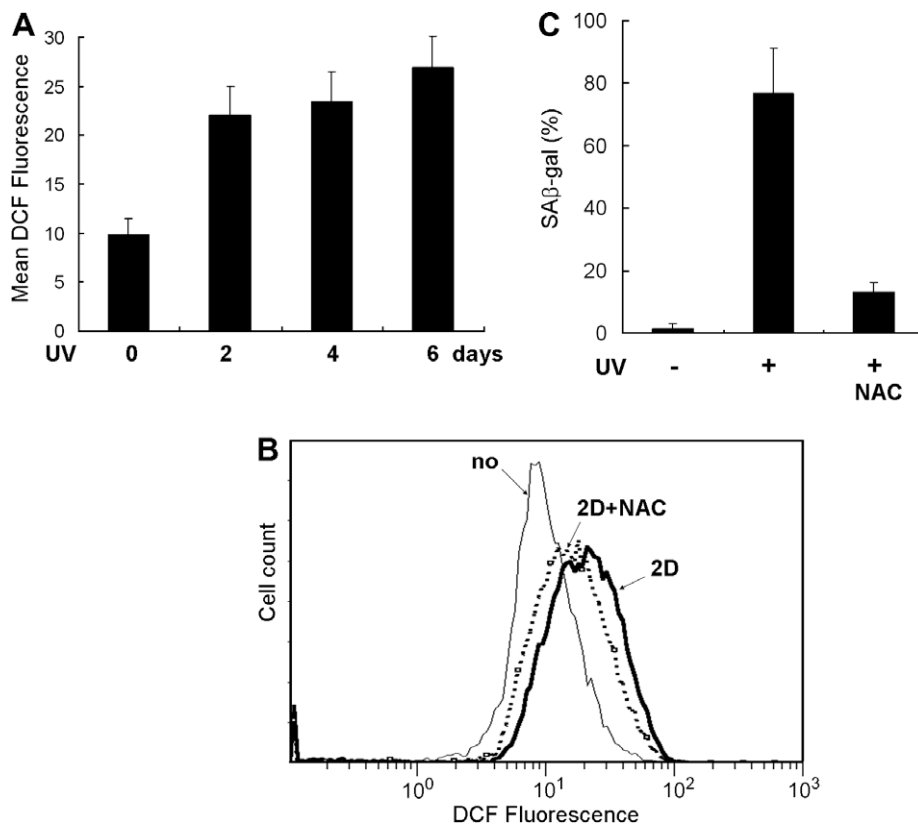


Fig. 4. An increase in intracellular levels of ROS is essential for UV-induced senescence. (A) Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and intracellular levels of ROS were determined as described in Materials and methods. Error bars indicate standard deviation. (B) Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and incubated with or without 10 mM of NAC for 2 days. Intracellular levels of ROS were measured as described in Materials and methods. (C) Akt1^{-/-} MEFs were treated with 10 J/m² of UV light and incubated with or without 10 mM of NAC for 2 days. SA β-gal staining was performed 6 days after UV-irradiation as described in Materials and methods. Experiments were repeated three times and the results are presented as mean values with standard deviations.

ROS are involved in UV-induced senescence in Akt1^{-/-} MEFs, we measured intracellular levels of ROS after UV-irradiation using the redox-sensitive fluorophore 2',7'-dichlorofluorescein diacetate (DCF). As shown in Fig. 4A, levels of ROS increased about two fold on day 2 after UV-irradiation and were maintained until day 6.

To address whether the increase in levels of ROS is important for the induction of premature senescence in Akt1^{-/-} MEFs, we employed N-acetylcystein (NAC), a potent ROS scavenger. Treatment with NAC (10 mM) for 2 days after UV-irradiation inhibited UV-induced increase in the levels of ROS by about 40% (Fig. 4B). Although NAC treatment did not completely inhibit the induction of ROS after UV-irradiation, NAC treatment for 2 days after UV-irradiation dramatically reduced the number of SA β-gal positive cells on day 6 (from 70% to 13%) (Fig. 4C). Thus, these data suggest that an increase in intracellular levels of ROS is essential for senescence-induction after UV-irradiation in Akt1^{-/-} MEFs.

Discussion

In this report, we describe our findings that UV light induces premature senescence in Akt1^{-/-} MEFs. First, cell growth was irreversibly inhibited upon UV-irradiation (Fig. 1A). Flow cytometric analyses indicate that cells were arrested in G1 and G2/M cell cycle phases in the absence of cell death until 6 days after UV-irradiation (Fig. 1B). Secondly, Akt1^{-/-} MEFs become enlarged and flattened in response to UV, a morphological change characteristic of senescence (Fig. 2A), a morphological change characteristic of senescence (Fig. 2A). Finally, SA β-gal activity is dramatically increased on day 4 after UV-irradiation (Fig. 2B and C). Upregulation of p53 and p21 are also observed (Fig. 2D). These results indicate that Akt1^{-/-} MEFs undergo premature senescence upon UV-irradiation.

These results in combination with our previous results [11] indicate that Akt1 and Akt2 dysfunctions result in different cellular responses to UV treatment. Akt2^{-/-} MEFs are more susceptible to UV-induced apoptosis indicating that Akt2 plays an anti-apoptotic role in response to UV-irradiation. However, Akt1^{-/-} MEFs show premature senescence upon UV-irradiation suggesting that Akt1 function is more important in cell proliferation. Consistent with this, it has been previously shown that mice lacking Akt1 are small in size compared to wild type mice and demonstrate slow growth, while Akt2 knockout mice develop normally [7]. In addition, we have recently shown that Akt1 function is important for the G1/S cell cycle transition and cell proliferation [17]. These results suggest that the Akt1 and Akt2 isoforms have distinct functions on cell proliferation and survival, respectively.

Cellular senescence is an irreversible growth arrest that occurs when a cells has exhausted its capacity to divide [2]. Initially, it was assumed that only normal cells were able to enter the senescence stage called "replicative senescence" after a limited number of cell divisions. However, a number of recent studies demonstrate that cancer cells also undergo cellular senescence after various treatments such as oxidative stress, DNA damage and restoration of tumor suppressor function [16]. This phenomenon, called "premature senescence", is now considered to be a potential mechanism by which to control human cancer. Consistent with this notion, two groups recently showed that the restoration p53 function in tumors lacking functional p53 induces tumor regression by premature senescence [18,19]. Thus, identification of the key regulatory proteins required for premature senescence of cancer cells is critical not only to understand mechanisms of senescence but also important to develop anti-cancer treatments. Since our results in

this report indicate that the absence of Akt1 function sensitizes cells to UV-induced premature senescence, targeting Akt1 function may be a useful strategy to sensitize cancer cells to premature senescence by various genotoxic treatments including drugs that induce DNA damage and ionizing irradiation. Also, since our results suggest that the absence of Akt1 or Akt2 function makes cells more susceptible to apoptotic cell death or premature senescence upon genotoxic treatment, Akt isoforms could be important targets for overcoming the resistance of tumor cells to chemotherapy and gamma irradiation. Although the exact molecular mechanisms of Akt1 and Akt2 function after genotoxic stress remain to be determined, further studies will help to unveil distinct roles of Akt isoforms on tumorigenesis and to develop efficient strategies for cancer treatment targeting these proteins.

Acknowledgment

This work was supported by Dong-A university research fund in 2006.

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