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ORIGINAL ARTICLE

Akt2, but not Akt1, is required for cell survival by inhibiting activation of JNK and p38 after UV irradiation

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The serine/threonine protein kinase, Akt/PKB, has an essential function in cell survival during response to various stresses. Recent studies have demonstrated that Akt isoforms exhibit some distinct physiological functions, but the isotype-specific functions for Akt in the stress response have not been fully identified. In this study, we analysed the cellular response to genotoxic stress using isogenic wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ mouse embryonic fibroblasts (MEFs). Marked hypersensitivity of Akt2$^{-/-}$ MEFs was observed to UV irradiation, whereas wild-type and Akt1$^{-/-}$ MEFs showed comparable levels of resistance. Akt2$^{-/-}$ mouse aortic endothelial cells also showed hypersensitivity to UV and the reconstitution of Akt2 expression in the Akt2$^{-/-}$ MEFs restored the UV resistance of the cells. Interestingly, upon UV irradiation, JNK and p38 were significantly upregulated in Akt2$^{-/-}$ MEFs, compared to wild-type and Akt1$^{-/-}$ MEFs. Additionally, inhibition of JNK and p38 activation reduced UV-induced cell death. Furthermore, both the hyperactivation of JNK and p38 and the UV-induced cell death in Akt2$^{-/-}$ MEFs were completely inhibited by restoring Akt2 expression. These results indicate that Akt2, but not Akt1, is essential for cell survival upon UV irradiation, and that Akt2 prevents UV-induced cell death by inhibiting activation of JNK and p38.
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Keywords: Akt1; Akt2; isotype-specific function; UV; JNK; p38

Introduction

Akt, also called PKB or RAC kinase, is a serine/threonine kinase known as a major signal transducer for cell proliferation and survival mediated by extracellular stimuli. In mammalian cells, three isoforms of Akt, Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ, have been identified, where all isoforms are activated in a phosphatidylinositol 3-kinase (PI3-K)-dependent manner (Datta *et al*., 1999). Once activated, Akt controls critical cellular processes such as proliferation, cell growth and cell survival by regulating the function of target proteins through phosphorylation (Brazil *et al*., 2004). Recent studies have demonstrated that PI3-K and Akt are activated by numerous types of stresses (Chan *et al*., 1999; Ronai, 1999). Activated Akt suppresses apoptosis and enhances cell survival through phosphorylation of various target proteins, such as GSK3β, Bad, MDM2, caspase-9, FOXO and ASK1 (Kim *et al*., 2001; Brazil *et al*., 2004), indicating that Akt has an essential function in the cellular response to genotoxic stress. Given the essential function of Akt in cell survival, it is not surprising that upregulation of Akt activity is frequently observed in human cancers (reviewed in Vivanco and Sawyers, 2002).

Although Akt has been the subject of extensive studies, the specific functions of Akt isoforms have been underscored until recently. Akt1, Akt2 and Akt3 are encoded by three separate genes with more than 80% sequence homology (Brazil *et al*., 2004). Akt1 and Akt2 are ubiquitously expressed in all tissues, whereas Akt3 is expressed in testis and neuronal tissue. Because of a similar tissue distribution and activation pattern, it has been assumed that the Akt1 and Akt2 isoforms have redundant and overlapping functions. In addition, because all Akt isoforms share a similar protein structure, containing N-terminal pleckstrin homology domain, a central catalytic domain and C-terminal regulatory domain, all Akt isoforms have been previously considered to possess identical or similar substrate specificity (Alessi and Cohen, 1998; Coffer *et al*., 1998). However, recent *in vivo* studies using knockout mouse models have provided evidences for isotype-specific functions of Akt. For example, Akt1 knockout mice are growth retarded and undergo increased apoptosis (Cho *et al*., 2001b). Akt2 knockout mice show diabetes-like symptoms due to an impaired insulin response (Cho *et al*., 2001a; George *et al*., 2004). Akt3 knockout mice show reduced brain size (Easton *et al*., 2005). These results indicate that Akt isoforms proteins have both distinct and overlapping functions in physiological conditions. Therefore, an investigation into isotype-specific functions of Akt is important

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for understanding the oncogenic function of Akt. Furthermore, because Akt is increasingly thought to be a potential target for anticancer drugs (Cheng *et al*., 2005), the characterization of specific Akt isotype function is a critical step for cancer treatment.

In this report, we describe our finding that Akt2, but not Akt1, has an important function in cellular survival during the response to UV irradiation. Additionally, our data suggest that Akt2 renders UV resistance of the cells by inhibiting the activation of JNK and p38 in response to UV treatment.

Results

Akt2-deficient MEFs are hypersensitive to UV irradiation

To address the isotype-specific functions of Akt in the cellular response to genotoxic stresses, we first examined cell survival upon γ-ray (IR) and UV irradiation using previously generated isogenic wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Bae *et al*., 2003). The absence of Akt1 or Akt2 isoform protein in the respective Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs was confirmed by western blot analysis (Figure 1a), indicating that our isogenic MEFs provide an ideal system for examining the functions of Akt isoforms.

Cell survival after treatment with various doses of IR and UV irradiation was measured by colony-forming assay. As shown in Figure 1b, both Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs were more sensitive to IR than wild-type MEFs (Figure 1b). In contrast, wild-type and Akt1$^{-/-}$ MEFs showed considerable resistance to UV irradiation up to 4 J/m^2, suggesting that Akt1 is not critical in cellular resistance to UV. However, interestingly, Akt2$^{-/-}$ MEFs showed marked hypersensitivity to UV irradiation compared to wild-type and Akt1$^{-/-}$ MEFs. With 4 J/m^2 of UV irradiation, Akt2$^{-/-}$ MEFs were about 10-fold more sensitive than wild-type and Akt1$^{-/-}$ MEFs, suggesting Akt2, but not Akt1, is critical for proper cell survival after UV irradiation.

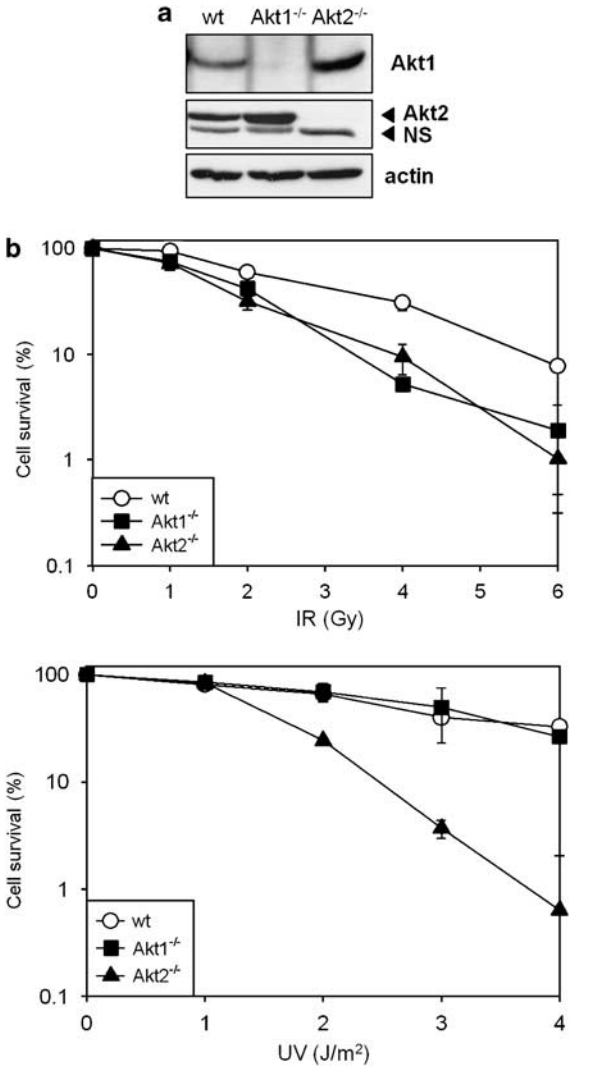

Figure 1 Akt2-deficient mouse embryonic fibroblasts (MEFs) are hypersensitive to UV irradiation. (**a**) Cell lysates from wild-type (wt), Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs were analysed by western blotting using anti-Akt1, anti-Akt2 and anti-actin antibodies. The non-specific band (NS) is indicated. (**b**) Clonogenic survival of wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs was examined with various doses of γ-ray (IR) and UV irradiation, as described in the Materials and methods section. Error bars indicate the standard deviation.

Akt2-deficient MEFs undergo extensive apoptotic cell death upon UV irradiation

To understand the molecular basis for the hypersensitive phenotype of Akt2$^{-/-}$ MEFs, we examined the cell-cycle response of UV-irradiated MEFs by flow cytometry (fluorescence-activated cell sorting, FACS) after propidium iodide (PI) staining. Akt1$^{-/-}$ MEFs were arrested in G_1 and G_2/M 24 h after UV irradiation, and this arrest was maintained until 36 h (Figure 2a). However, unlike the Akt1$^{-/-}$ MEFs, Akt2$^{-/-}$ MEFs were not arrested in G_2/M and a large proportion of sub-G_1 fraction, indicative of cell death, emerged after 24 h and increased in the later time course (Figures 2a and b). To examine whether Akt1$^{-/-}$ MEFs were undergoing cell death, we performed FACS analysis until 96 h after UV irradiation, but there was no sub-G_1 fraction observed (data not shown).

In addition to the sub-G_1 fraction, caspase-3 and PARP cleavage, which are well-known markers of apoptosis, were only detected in the Akt2$^{-/-}$ MEFs after UV irradiation (Figure 2c). Furthermore, as a typical feature of the apoptotic process, DNA fragmentation was observed in the Akt2$^{-/-}$ MEFs after 24 h UV irradiation (Figure 2d). These results suggest that Akt2$^{-/-}$ MEFs are deficient in proper response to UV irradiation and thus are hypersensitive to UV-induced apoptosis.

Akt2 is required for cell survival upon UV irradiation

To confirm that the absence of Akt2 results in hypersensitivity to UV irradiation, we examined cell survival in a different cell system, using previously generated isogenic wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ mouse aortic endothelial cells (AECs) (Cho *et al*., 2001a). Consistent with previous data in the MEFs

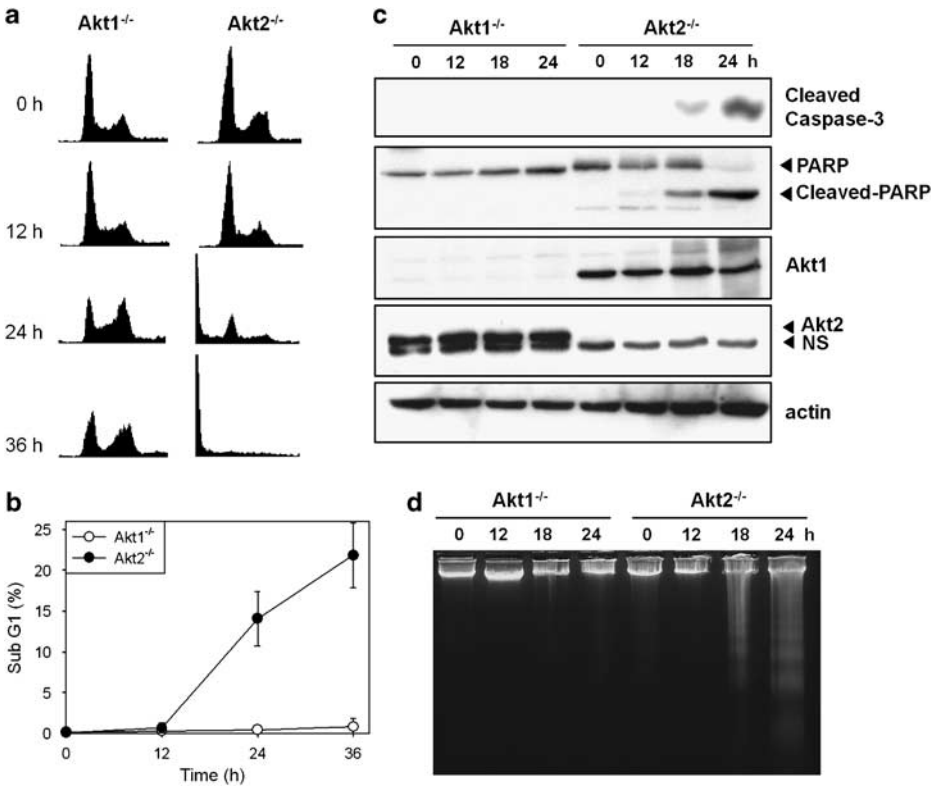

Figure 2 UV irradiation induces extensive apoptosis of Akt2-deficient mouse embryonic fibroblasts (MEFs). (**a**) Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs were treated with 10 J/m^2 of UV and harvested at the indicated time points. Cells were fixed, stained with propidium iodide (PI) and then analysed by flow cytometry as described in the Materials and methods section. (**b**) The percentage of cells in the sub-G_1 phase was determined with CellQuest software and plotted. (**c**) The cell lysates of Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs were prepared at the indicated time points after UV irradiation (10 J/m^2) and subjected to western blotting using anti-caspase-3, anti-PARP, anti-Akt1 and anti-Akt2 antibodies. Actin levels were examined as a loading control using anti-actin antibodies. (**d**) Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs were harvested at the indicated time points after UV irradiation (10 J/m^2), and DNA ladder formation was examined as described in the Materials and methods section, using a 2% agarose gel electrophoresis. The data shown are the representative of three independent experiments.

(Figure 1), Akt2$^{-/-}$ AECs were also hypersensitive to UV irradiation compared to wild-type and Akt1$^{-/-}$ AECs (Figure 3).

To further substantiate the critical function of Akt2 in mediating UV resistance, we reintroduced Akt1 and Akt2 into Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs, respectively, as described in the Materials and methods section. Western blotting results confirmed that Akt1- and Akt2-reconstituted cell lines (Akt1M1 and Akt2M2) expressed high level of Akt1 and Akt2, respectively (Figure 4a). In colony-forming assays, Akt2-reconstituted MEFs (Akt2M2) were indeed resistant to UV irradiation, at a degree comparable with wild-type and Akt1$^{-/-}$ MEFs (Figure 4b). These results confirm that the hypersensitivity of Akt2$^{-/-}$ MEFs to UV is solely due to the absence of Akt2 function. Additionally, these data indicate that the function of Akt2, but not Akt1, is essential for cell survival after UV irradiation.

The activation of p38 and JNK are dramatically upregulated in Akt2-deficient MEFs

To understand the underlying molecular mechanism for how Akt2 could increase resistance to UV irradiation, we first examined whether both Akt1 and Akt2 were activated upon UV treatment, using anti-phospho-Thr308 and anti-phosho-Ser473 Akt-specific antibodies. As shown in Figure 5a, both Akt1 and Akt2 were activated upon UV irradiation. Although Akt1 was activated a little more strongly and inactivated earlier in some experiments, a significant difference was not observed in repeated experiments (data not shown). In addition, the phosphorylation pattern of GSK3β and MDM2, representative target proteins of Akt, were similar in wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs, suggesting that the hypersensitivity of Akt2$^{-/-}$ MEFs is not because Akt1 and Akt2 are not differentially activated upon UV treatment.

Both JNK and p38 are activated after various stress signals including UV irradiation and both kinases have an important function in the induction of cell death (Ichijo, 1999). To determine whether JNK and p38 are involved in Akt2-mediated cell survival after UV irradiation, we examined JNK and p38 activation using an anti-phospho-specific antibody for JNK and p38 in wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs. Strikingly, we observed that JNK and p38 were activated much earlier and more intensely in Akt2$^{-/-}$ MEFs, compared to wild-type and Akt1$^{-/-}$ MEFs (Figure 5b). These results suggest that Akt1 and Akt2 may differentially regulate activation of JNK and p38 upon UV irradiation.

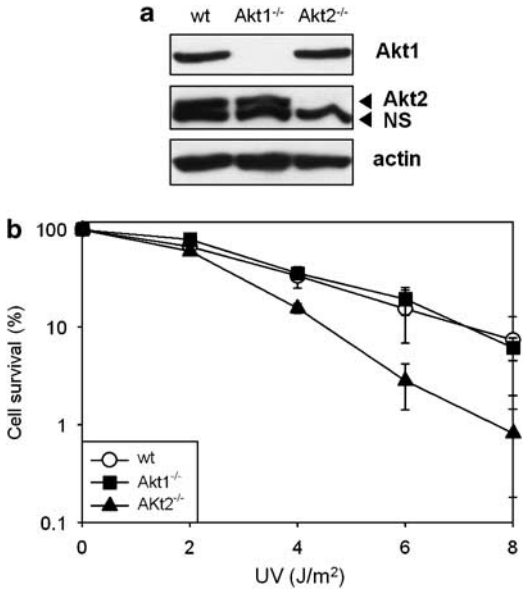

Figure 3 Akt-deficient aortic endothelial cells (AECs) are also hypersensitive to UV irradiation. (**a**) Cell lysates from wild-type (wt), Akt1$^{-/-}$ and Akt2$^{-/-}$ AECs were analysed by western blotting using anti-Akt1, anti-Akt2 and anti-actin antibodies. The non-specific band (NS) is indicated. (**b**) Clonogenic survival of wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs was examined with various doses of UV, as described in the Materials and methods section. Error bars indicate the standard deviation.

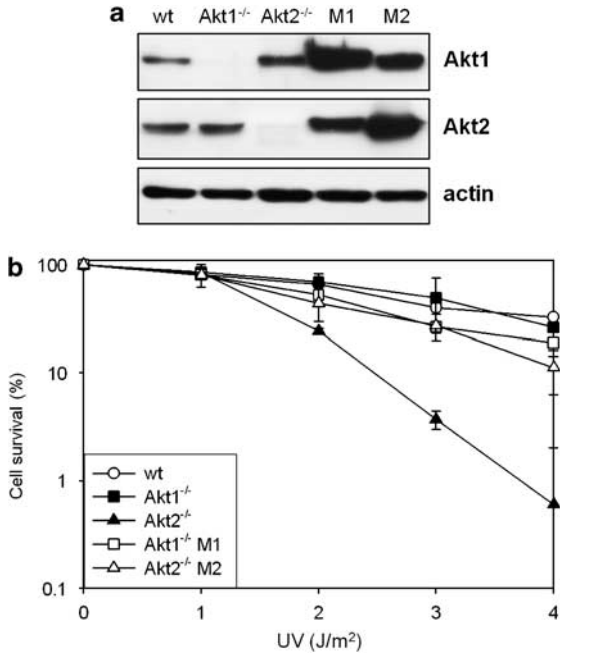

Figure 4 UV resistance is restored by Akt2 reconstitution. (**a**) Cell lysates from wild-type (wt), Akt1$^{-/-}$, Akt2$^{-/-}$ mouse embryonic fibroblasts (MEFs), Akt1-reconstituted MEFs (M1) and Akt2-reconstituted MEFs (M2) were subjected to western blotting using anti-Akt1, anti-Akt2 and anti-actin antibodies. (**b**) Clonogenic survival of wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs was examined with various doses of UV, as described in Materials and methods section. Error bars indicate the standard deviation.

Akt2 enhance cell survival by inhibiting the activation of p38 and JNK upon UV irradiation

Because JNK and p38 are key stress kinases for UV-induced cell death, it could be hypothesized that inhibiting JNK and p38 activity would reduce cell death of Akt2$^{-/-}$ MEFs upon UV irradiation. To test this possibility, we examined the effect of SP600125 and SB202190, specific inhibitors of JNK and p38, respectively, on UV-induced cell death of Akt2$^{-/-}$ MEFs by monitoring the sub-G_1 fraction, as described above. As expected, the proportion of Akt2$^{-/-}$ MEFs that were dead after UV irradiation was significantly reduced after treatment with SP600125 (62%) and with SB202190 (52.5%) (Figure 6a), indicating that the activation of JNK and p38 is an important step for UV-induced cell death of Akt2$^{-/-}$ MEFs.

To confirm the function of Akt2 in regulating JNK and p38 activation, we then examined levels of active JNK and p38 after UV irradiation in Akt2$^{-/-}$- and Akt2-reconstituted MEFs (M2). As shown in Figure 6b, UV-induced activation of both JNK and p38 was significantly decreased by reexpressing Akt2, suggesting that Akt2 has an essential function in suppressing JNK and p38 activation in response to UV. Accordingly, it was expected that restoring Akt2 expression would reduce the apoptosis in response to UV. Indeed, we found that UV-induced apoptotic cell death was also significantly reduced in Akt2-reconstituted MEFs, compared to Akt2-deficient MEFs (Figure 6c). Taken together, these results suggest that Akt2 is the isoform of Akt that is responsible for cell survival in response to UV, and specifically, Akt2 increases cell survival by inhibiting hyperactivation of JNK and p38.

Discussion

In this study, we examined whether Akt1 and Akt2, two major Akt isoforms, have specific functions in response to genotoxic stress. Although both Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs showed a similar increased sensitivity to IR, they demonstrated unique sensitivities to UV irradiation. Interestingly, Akt1$^{-/-}$ MEFs were resistant to UV, which was similar to wild-type MEFs. However, Akt2$^{-/-}$ MEFs exhibited marked hypersensitivity to UV irradiation (Figure 1b). To rule out possible artifacts due to the use of established specific MEF cell lines, we examined UV sensitivity in isogenic AECs and observed the same pattern of results (Figure 3). In addition, restoring Akt2 expression in Akt2$^{-/-}$ MEFs enhanced the resistance to UV (Figure 4), confirming that Akt2 has an essential function in cell survival after UV irradiation, whereas Akt1 function is not critical. Therefore, our results described here indicate that different Akt isoforms have unique physiological functions depending on the type of genotoxic stress.

In the detailed analysis, we found that the hypersensitivity of Akt2$^{-/-}$ MEFs to UV is mainly due to

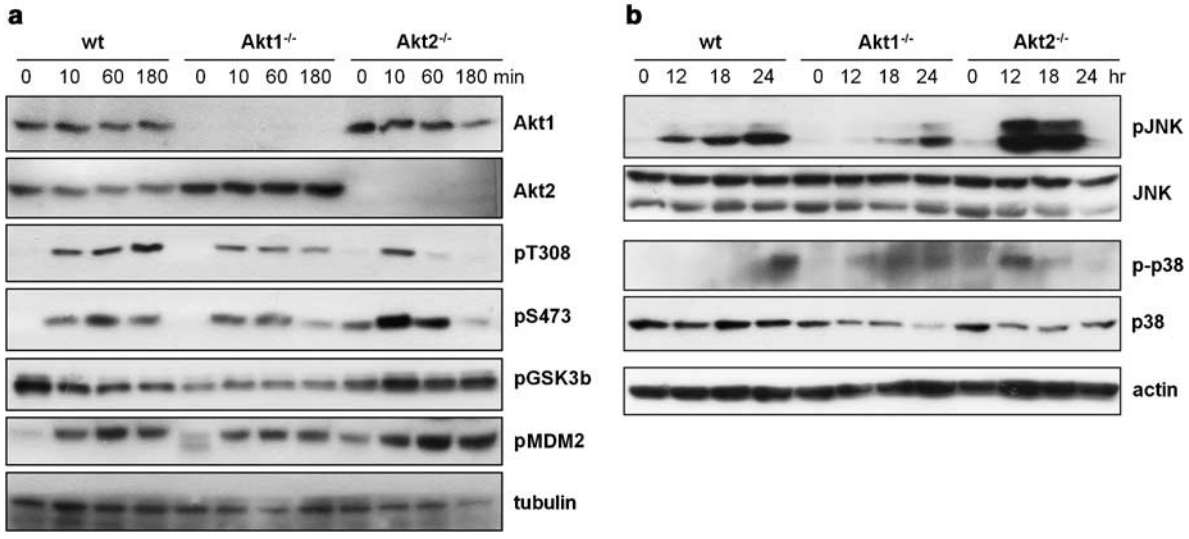

Figure 5 Activation of JNK and p38 is upregulated in Akt2-deficient mouse embryonic fibroblasts (MEFs). (**a**) Wild-type (wt), Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs were treated with 10 J/m^2 of UV and harvested at the indicated time points. Cell lysates were subjected to western blotting using indicated antibodies. The western blot analysis was repeated three times. (**b**) Cell lysates were prepared as in (**a**) and were subjected to western blotting using indicated antibodies. The western blot analysis was repeated three times.

extensive apoptosis. First, the sub-G_1 fraction was sharply increased after UV irradiation (Figures 2a and b). Second, activation of caspase-3 and the cleavage of PARP were detected (Figure 2c). Finally, the DNA fragmentation formation, a notable feature of apoptosis, was also observed in response to UV irradiation, only in Akt2$^{-/-}$ MEFs (Figure 2d). These results indicate that Akt2$^{-/-}$ MEFs are more susceptible to UV-induced apoptosis, and Akt2 has anti-apoptotic function in response to UV treatment.

Importantly, in this study, we found that JNK and p38 were activated much more strongly in Akt2$^{-/-}$ MEFs upon UV treatment, compared to wild-type and Akt1$^{-/-}$ MEFs (Figure 5b). JNK and p38 are mitogen-activated protein kinases that are stimulated by a variety of stresses, such as growth factor withdrawal, hydrogen peroxide, X-rays and UV (Ichijo, 1999). Interestingly, recent studies showed that the PI3-K–Akt pathway can cross-talk with the JNK/p38 pathway under condition of cellular stress. Rane *et al*. (2001) reported that p38 regulates Akt activation in neutrophils. Liao and Hung (2003) showed that adenovirus E1A sensitizes cells to stress-induced apoptosis by inactivating Akt and activating p38. More interestingly, it has been recently demonstrated that activation of Akt2 causes the inhibition of UV/tumor necrosis factor-α-induced activation of JNK and p38 (Yuan *et al*., 2002). These studies suggest that the survival factor Akt and the pro-apoptotic JNK–p38 pathways can negatively regulate each other, and the balance between these factors is important in determining cellular fate. Consistent with this notion, the specific inhibitors of JNK and p38 decreased UV-induced cell death (Figure 6A), confirming that the activation of JNK and p38 is a critical step for UV-induced cell death. Moreover, restoring Akt2 expression reduced the activation of JNK and p38, as well as UV-induced apoptosis. These results suggest that Akt2 promotes cell survival, at least partially through inhibiting the activation of JNK and p38 after UV irradiation.

One important issue that arises from this work is how Akt2 suppresses JNK and p38 activation upon UV treatment, whereas Akt1 does not. Akt2 may regulate JNK and p38 activation through a direct interaction. It is reported that Akt and p38 could exist in same signal complex (Rane *et al*., 2001). Alternatively, Akt2 could inhibit JNK and p38 function indirectly by controlling part of the upstream pathway. The activation of JNK and p38 is regulated by several upstream kinases including SEK1, MEKK3/6 and ASK1 (Ichijo, 1999). It has been shown that Akt directly phosphorylates Ser83 of ASK1 and in turn inhibits ASK1-mediated JNK activation (Kim *et al*., 2001). Interestingly, Yuan *et al*. (2003) showed that Akt2 promotes resistance to cisplatin-induced cell death through ASK1 phosphorylation. Therefore, it is possible that Akt2 inhibits JNK and p38 activation through the phosphorylation of ASK1 upon UV irradiation. Whether Akt2 specifically suppresses JNK and p38 activation through direct phosphorylation of ASK1 upon UV irradiation has yet be determined. Any future study on this topic would provide a better understanding for how Akt2 renders resistance to UV irradiation.

Although we report here Akt2 promotes cell survival through inhibition of UV-induced activation of JNK and p38, it is still possible that Akt2 also specifically regulates other target proteins after UV irradiation. Akt enhances cell survival in response to cellular stresses by inhibiting various pro-apoptotic target proteins such as Bad, MDM2, p21, FOXO1 and caspase-9 (Brazil *et al*., 2004). Interestingly, several recent studies demonstrate that some target proteins are differentially regulated by

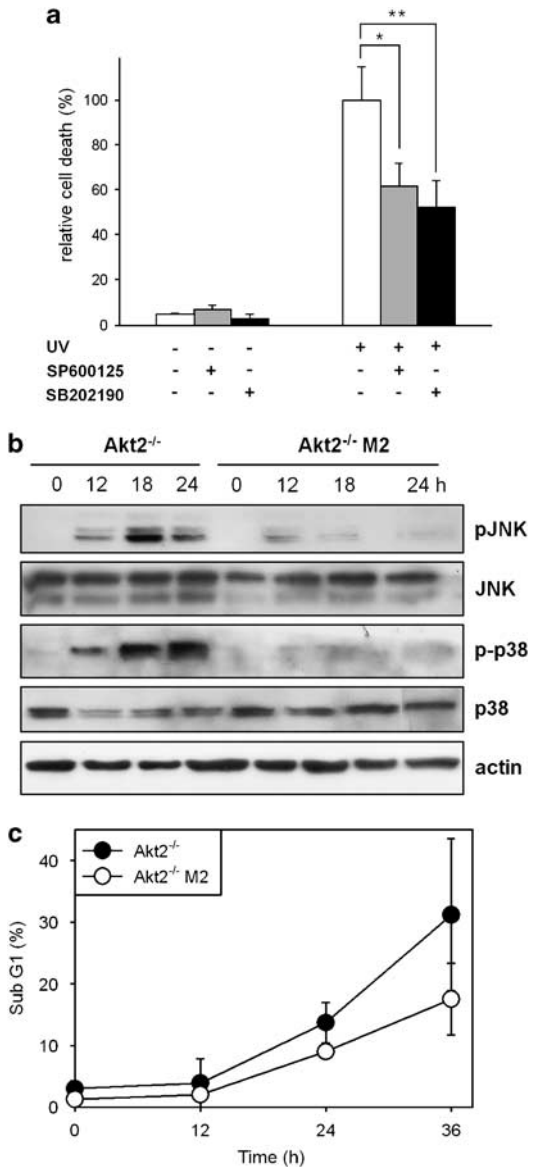

Figure 6 JNK and p38 activation are required for UV-induced cell death. (**a**) Akt2$^{-/-}$ MEFs were treated with 10 J/m^2 of UV irradiation and incubated with either SP600125 (10 μM) or SB202190 (10 μM) for 18 h. Cells were harvested, and the percentage of cells in the sub-G_1 phase was determined as in Figure 2(a). (**b**) Akt2$^{-/-}$- and Akt2-reconstituted MEFs (M2) were treated with 10 J/m^2 of UV irradiation and harvested at the indicated time points. Cell lysates were subjected to western blotting using indicated antibodies. The western blot analysis was repeated three times. (**c**) Akt2$^{-/-}$- and Akt2-reconstituted MEFs (M2) were treated with 10 J/m^2 of UV irradiation and the percentage of cells in the sub-G_1 phase was determined as in Figure 2(a). $*P<0.05$; $**P<0.01$ by Student's *t*-test.

Akt isoforms. It has been reported that the Tcl1 oncoprotein interacts with Akt1, but not with Akt2 (Pekarsky *et al*., 2000). More recently, Heron-Milhavet *et al*. (2006) demonstrated that Akt isoforms regulate p21 function in opposite manners. Whereas Akt1 phosphorylates p21, inhibiting its function and thereby promoting cellular proliferation, Akt2 binds to p21 and prevents phosphorylation by Akt1. Therefore, although we did not observe a difference in phosphorylation of GSK3β and MDM2 among wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs, Akt1 and Akt2 might differently regulate other target proteins after UV irradiation, which could contribute to an Akt2-specific function in UV resistance.

In conclusion, this study revealed that Akt isoforms have different functions in the cellular response to UV irradiation. The results presented in this report provide a mechanistic explanation for how Akt2 specifically promotes cell survival upon UV irradiation. Although the exact mechanism for how Akt2 renders the cell resistant to UV irradiation remains to be elucidated, further studies will provide important information for understanding the distinct functions of Akt isoforms, offering more efficient strategies for specific cancer treatments targeting Akt family proteins.

Materials and methods

Cell lines and treatments

Wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ MEFs, along with wild-type, Akt1$^{-/-}$ and Akt2$^{-/-}$ AECs, were generated, as previously described (Bae *et al*., 2003). Akt1- and Akt2-reconstituted cell lines were generated using retrovirus-mediated gene transfer, as described previously (Yun *et al*., 2008). All MEF and AEC cell lines were cultured in Dulbecco's modified Eagle's medium—high glucose media containing 10% fetal bovine serum.

For genotoxic stresses, cells were treated with γ-irradiation (IR) using a 131 Cs source or with UV using a germicidal UV lamp and UV radiometer (UVX-25; Ultra-Violet Products Ltd) with the indicated dose.

Western blot analysis and antibodies

Cells harvested after treatment were lysed in radioimmuno precipitation assay buffer and subjected to western blot analysis, as described previously (Kim *et al*., 2007). Antibodies for Akt1, Akt2, caspase-3, PARP, phospho-Akt (T308), phospho-Akt (S473), phospho-GSK3β, phospho-MDM2, phospho-JNK, JNK, phospho-p38 and p38 were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Levels of actin or tubulin proteins were monitored as an internal loading control using anti-actin (Sigma, St Louis, MO, USA) or anti-tubulin (Santa Cruz Inc., Santa Cruz, CA, USA) antibodies.

Clonogenic survival assay

To measure cell survival after genotoxic stress, a clonogenic survival assay was performed as described previously (Yun *et al*., 2005). Cells (1×10^3) were seeded into a 100 mm dish and exposed to the indicated dose of IR or UV. After 10–12 days, the colonies were fixed and stained with methylene blue, and the number of colonies was counted. Survival experiments were repeated at least three times, and the data were presented as a mean value with standard deviation.

Cell-cycle analysis

To determine the cell-cycle distribution, 1×10^6 cells were seeded into a 100 mm dish. After UV treatment, cells were trypsinized at indicated time points and fixed with 70%

ethanol. Subsequently, cells were stained with PI, and flow cytometric analysis was performed using EPICS XL cytometer (Beckman Coulter Inc.).

DNA fragmentation assay

To detect UV-induced DNA fragmentation, 4×10^6 of cells were treated with 10 J/m^2 of UV and harvested at the indicated time points. Cells were lysed with 0.5 ml of lysis buffer (10 mM Tris (pH 8.0), 25 mM EDTA (pH 8.0), 150 mM NaCl, 0.5% SDS, 200 μg/ml proteinase K). The lysates were extracted twice with phenol/chloroform and DNA was precipitated with ethanol. DNA was dissolved in TE buffer and treated with RNase A for 1 h. DNA was separated on a 2% agarose gel and visualized by staining with ethidium bromide.

Acknowledgements

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