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p53 modulates the AMPK inhibitor compound C induced apoptosis in human skin cancer cells

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ABSTRACT

Compound C, a well-known inhibitor of the intracellular energy sensor AMP-activated protein kinase (AMPK), has been reported to cause apoptotic cell death in myeloma, breast cancer cells and glioma cells. In this study, we have demonstrated that compound C not only induced autophagy in all tested skin cancer cell lines but also caused more apoptosis in p53 wildtype skin cancer cells than in p53-mutant skin cancer cells. Compound C can induce upregulation, phosphorylation and nuclear translocalization of the p53 protein and upregulate expression of p53 target genes in wildtype p53-expressing skin basal cell carcinoma (BCC) cells. The changes of p53 status were dependent on DNA damage which was caused by compound C induced reactive oxygen species (ROS) generation and associated with activated ataxia-telangiectasia mutated (ATM) protein. Using the wildtype p53-expressing BCC cells versus stable p53-knockdown BCC sublines, we present evidence that p53-knockdown cancer cells were much less sensitive to compound C treatment with significant G2/M cell cycle arrest and attenuated the compound C-induced apoptosis but not autophagy. The compound C induced G2/M arrest in p53-knockdown BCC cells was associated with the sustained inactive Tyr15 phosphor-Cdc2 expression. Overall, our results established that compound C-induced apoptosis in skin cancer cells was dependent on the cell's p53 status.

Introduction

Apoptosis and autophagy, two self-destructive processes, are physiologically regulated and evolutionally conserved in eukaryotic organisms (Eisenberg-Lerner et al., 2009). Apoptosis is the best-described programmed cell death; it is characterized by membrane-blebbing, DNA fragmentation, and the formation of apoptotic bodies in the cells (Adams, 2003). Central to the apoptotic processes are "initiator caspases", which start the apoptotic caspase cascade, and "effector caspases", which can disassemble the cellular structures in an orderly fashion. The therapeutic effects of various anticancer reagents in cancer cells are mainly mediated through apoptosis (Reed, 2003). However, autophagy involves both the adaption for survival or the induction of another type of programmed cell death that is independent of caspase cascade activation (Gozuacik and Kimchi, 2007). The process of autophagy involves the sequestration of cell structures into autophagosomes, which then fuse with lysosomes and degrade the cytosolic components through the formation of acidic autophagolysosomes (Mizushima, 2007). Other studies have reported that the induction of autophagic cell death can coordinate and collaborate with apoptosis to cause efficient cell death in various types of cancer cells in response to specific anti-cancer therapies. Conversely, the

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activation of autophagy could rescue cancer cells from anti-cancer reagent-induced apoptosis under certain conditions (Eisenberg-Lerner et al., 2009). Therefore, the regulation of autophagy has been suggested to be a potential anti-cancer therapeutic strategy (Kondo et al., 2005).

AMP-activated protein kinase (AMPK) is a principal intracellular energy sensor, which switches on catabolic pathways that generate ATP and switches off anabolic pathways that consume ATP when the cellular AMP/ATP ratio is increased (Luo et al., 2010). AMPK regulates these effects through the direct phosphorylation of target proteins or via transcriptional control of target genes. AMPK has recently been implicated in the regulation of apoptosis and autophagy, although the results obtained thus far appear to be conflicting (Baumann et al., 2007; Okoshi et al., 2008). Compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)] 3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine) is a cell-permeable and selective ATP-competitive inhibitor of AMPK that efficiently blocks the metabolic actions of AMPK (Zhou et al., 2001). The application of compound C has been reported to attenuate apoptosis (Bae et al., 2007). In contrast, it has also been suggested that compound C treatment directly causes apoptosis in breast cancer cells and glioma cells (Jin et al., 2009; Vucicevic et al., 2009). Compound C has been described as a blocker of AMPK-dependent autophagy in yeast, hepatocytes and Hela cells (Law et al., 2010; Meley et al., 2006; Yan et al., 2010). Controversially, compound C could also induce protective autophagy against compound C-induced apoptosis in glioma cancer cells (Vucicevic et al., 2011). These observations indicate that the effect of compound C on apoptosis and autophagy may be dose-, cell type- and/or context-dependent. However, the effects of compound C on human skin cancers have never been assessed.

The p53 tumor suppressor is a vital genome guardian and controller of cell growth; thus, p53 has a critical function in preventing cancer development and is the most frequently disrupted tumor suppressor gene in human cancers (Bargonetti and Manfredi, 2002; Pietsch et al., 2008). Activation of p53 leads to the inhibition of cell cycle progression and results in apoptosis, autophagy or senescence (Tasdemir et al., 2008a; Vousden and Lu, 2002). The loss of p53 functions contributes to chemotherapy resistance via apoptosis inhibition and autophagy activation in some cancer cells (Hollstein et al., 1999; Tasdemir et al., 2008b). The reactivation of p53 functions in cancer cells has been demonstrated, which could defend against oncogenesis by apoptosis and senescence (Martins et al., 2006). Therefore, the status of p53 could be a potential marker for the efficacy of cancer therapy. However, the role of p53 in the compound C-induced effects remains unknown.

In the present study, we demonstrate that compound C can induce apoptosis and autophagy in several human skin cancer cell lines. Additionally, compound C also induced upregulation and activation of the p53 protein via ROS induced DNA damage and associated with activated ATM. Using the wildtype p53-expressing BCC cells versus stable p53-knockdown BCC sublines, we present evidence that compound C-induced apoptosis but not autophagy was dependent on p53 as p53 knockdown cancer cells were much more resistant to compound C-induced apoptosis and with significant G2/M cell cycle arrest.

Materials and methods

Reagent and antibodies. Compound C was purchased from Calbiochem (Darmstadt, Germany). N-acetylcysteine (NAC), bafilomycin A1, Pan caspase inhibitor (zVAD-FMK) and caffeine were obtained from Sigma (St. Louis, MO, U.S.A.). The antibody specific for LC3 was purchased from Novus (Littleton, CO, U.S.A.). The antibodies specific for cleaved-caspase 3, caspase 9 and PARP were contained in the Apoptosis Sampler Kit from Cell Signaling Technology (Danvers, MA, U.S.A.). The antibodies specific for p53, phosphorylated p53, AMPKα, phosphor-AMPKα(Thr172), p21, phosphor-γ-H2AX, phosphor-ATM, ATM, phosphor-ATR, ATR, phosphor-Chk1, Chk1, phosphor-Chk2, Chk2, phosphor-Cdc2, Cdc2, cyclinB1 and Bax were also purchased from Cell Signaling Technology. The antibody specific for β-actin was purchased from Santa Cruz (Santa Cruz, CA, U.S.A.).

Cells and culture conditions. Primary human keratinocytes from freshly excised neonatal foreskins were purchased from GIBCO (Carlsbad, CA, USA). Keratinocytes were maintained in flasks coated with type IV collagen and cultured in a serum-free keratinocyte growth medium (Clonetics, San Diego, CA, USA). Human basal cell carcinoma (BCC/KMC1) cells which carry functional p53 proteins were established as described previously (Huang et al., 2010; Jee et al., 1998). Melanoma cell lines A375 and C32 contain wild-type *p53* gene. MeWo melanoma cell line contains mutant *p53* gene (Forbes et al., 2006). Skin squamous cell carcinoma cell line, SCC12, contains a mutation CTG→GCG (valine→glycine) at condon 216 of p53 gene (Burns et al., 1993). BCC cells were cultured in RPMI 1640 medium. A375, C32 and MeWo cells were cultured in MEM medium. SCC12 cells were cultured in DMEM/F12 medium. The p53-wildtype and p53-null HCT116 cells were cultured in McCoy's 5a medium. All media were supplemented with 10% fetal calf serum.

Cell proliferation assay. The effects of compound C on cell viability were evaluated in vitro using the XTT assay (Roche, Madison, WI, U.S.A.). In this experiment, different cell lines were maintained in 96-well plates and treated with increasing doses (0–40 μM) of compound C. After 24 and 48 h, the XTT assay was performed according to the manufacturer's instructions. Results were quantified using an ELISA plate reader at 450 nm and compared with untreated controls.

Cell cycle analysis. Skin cancer cells treated with compound C were harvested at the indicated time points and fixed in 70% ethanol at 4 °C. After centrifugation (300×*g*), the cell pellets were resuspended in a buffer containing phosphate-buffered saline (PBS), 0.05% RNase A and 40 μg/ml propidium iodide (PI) at 37 °C for 30 min. After staining, the cells were collected and resuspended in PBS. The fluorescence emitted from the PI-DNA complexes following laser excitation of the fluorescent dye was quantified using the Cytomics™ FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA, U.S.A.).

Mitochondrial membrane potential measurement. Cells were incubated in a medium containing 1 μg/ml JC-1 (Molecular Probe, Carlsbad, CA, U.S.A.) for 30 min at 37 °C, washed with PBS, and then measured with a fluorescence microplate reader (BioTek, Winooski, VT, U.S.A.) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm as well as with an excitation wavelength of 560 nm and an emission wavelength of 595 nm. The mitochondrial membrane potential was expressed as the ratio of emission at 595 nm to that at 535 nm.

TUNEL assay. TUNEL assay was performed by using the ApopTaq Fluorescein Direct in Situ Apoptosis Detection kit (Chemicon, Billerica, MA, USA). Briefly, cells were plated at a density of 5×10^4 cells/35-mm dish. The next day, cells were exposed to compound C in complete culture medium. After incubation, cells were washed with PBS, and fixed in a PBS-buffered paraformaldehyde solution (1%, pH 7.4) on ice for 30 min. The cells were washed with PBS and then maintained in 70% (v/v) ice-cold ethanol and stored at −20 °C until the assay. To perform this assay, cells were washed with PBS, labeled with the TUNEL reaction mixture and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. The reaction was stopped by adding 500 μl of PBS and washed. The apoptotic cells were then monitored by confocal microscopy (Olympus, FV1000D, Tokyo, Japan).

EGFP-LC3 puncta detection. Cells were plated at a density of 1×10^5 cells/well in six-well plates and cultured to 70% confluence. Cells were transfected with pEGFP-LC3 plasmid DNA (Addgene plasmid 11,546) for 24 h and then treated with 40 μM compound C.

Transfection was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Photomicrographs of EGFP-LC3 for autophagy detection were obtained by confocal microscopy (Olympus, FV1000D, Tokyo, Japan).

Detection of autophagic vacuoles by acridine orange. Cells were seeded in 60-mm petri dishes. At 70% confluence, cells were treated with 40 μM compound C or control DMSO. At the appropriate time points, cells were incubated with 1.0 μM acridine orange (Molecular Probes) in serum-free medium for 30 min at 37 °C. The acridine orange was then removed, the cells were washed, and fluorescent micrographs were obtained using an inverted fluorescence microscope. The cytoplasm and nuclei of the stained cells fluoresced bright green, whereas the acidic autophagic vacuoles fluoresced bright red. To quantify the development of acidic vesicular organelles, compound C-treated cells were stained with 1.0 μM acridine orange for 15 min and removed from the plate with trypsin–EDTA. The stained cells were then analyzed using a Cytomics™ FC500 Flow Cytometer.

Immunocytochemistry. Cells (5×10^4 cells per chamber) were cultured on two-chamber slides (Nalgene Nunc International, Naperville, IL, U.S.A.) and then treated with compound C. The treated cells were fixed in 3.7% formaldehyde in PBS. For intracellular staining, the cells were blocked with 2% normal horse serum, incubated with antibodies against p53 or phosphor γ-H2AX in PBS containing 0.2% Triton X-100 (PBST), and then incubated with goat FITC-conjugated anti-rabbit IgG (Sigma). After washing with PBST, the cells were mounted using an anti-fade, DAPI contained and water-based mounting medium (Vector Lab, Burlingame, CA, U.S.A.) and analyzed under a confocal microscopy (Olympus, FV1000D, Tokyo, Japan).

Construction of RNA interference (RNAi) vectors and introduction of RNAi vectors into BCC cells. To construct the p53 RNAi vector, the DNA fragment containing a human U6 promoter followed by p53 shRNA sequence (5′-GACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGG AGTCT TTTT-3′) was retrieved from the CMV-/Hu6-RNAi plasmid by HindIII and EcoRI double digestion. This DNA fragment was then inserted into the mammalian expression vector pcDNA3, which resulted in a plasmid carrying a U6 promoter that drives the expression of p53 shRNA and allows for the selection of stably transfected clones. An EGFP RNAi vector was employed for the negative control of p53 knockdown. The p53 RNAi vector and the EGFP RNAi vector were subsequently transfected into BCC cells using jetPEI™ (Polyplus, Illkirch, France) followed by G418 (500 μg/ml, Invitrogen) treatment for 48 h posttransfection to select for stably transfected clones. The degree of p53 knockdown in these stable clones was verified by immunoblotting analysis on the level of endogenous p53 protein using anti-human p53 antibodies.

Protein immunoblotting. Cells were harvested, and whole-cell extracts were prepared with the PRO-PREP protein extraction reagent (iNtRON, Taipei, Taiwan). Protein samples (30 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane by electroblotting. After blocking, the blots were incubated overnight at 4 °C in PBS containing 0.05% Tween-20 (TBST) containing primary antibodies. After washing with TBST, the blots were incubated with the corresponding horseradish peroxidase-coupled anti-rabbit or anti-mouse secondary antibody (Pierce Biotechnology, Rockford, IL, U.S.A.). Proteins were visualized with SuperSignal West Pico ECL reagents (Pierce Biotechnology), and chemiluminescence was detected by exposing the membranes to Kodak-X-Omat Films. The levels of β-actin signal were used to verify equal protein loading in each lane.

ROS detection. The generation of ROS for the cells was evaluated by a fluorometry assay using intracellular oxidation of dichlorofluorescin diacetate (DCFDA) (Molecular Probes). The cells in logarithmic growth phase were incubated in a 6-well plate for overnight, and then the medium was replaced with medium containing 40 μM of compound C for 24 h. After exposure, the cells were washed with PBS, then they were resuspended at a concentration of 1×10^6 cells/ml and were stained by the staining solution for 30 min at 37 °C, the cells were detected and analyzed by the Cytomics™ FC500 Flow Cytometer.

Statistical analyses. Three independent experiments were conducted in all studies, and all assay conditions were performed in duplicate or triplicate. Data were analyzed using the Student's *t* test, and significant differences were set at a p value of 0.05.

Results

p53-wildtype skin cancer cells are sensitive to compound C-induced apoptosis

Compound C has been reported to directly induce apoptosis in myeloma and breast cancer cell lines in the absence of any stress (Baumann et al., 2007; Jin et al., 2009). Additionally, compound C also causes G2/M cell cycle arrest, which is accompanied by apoptosis in glioma cell lines (Vucicevic et al., 2009). To investigate the same potential of compound C in skin cancer cells, we examined the effect of compound C on cell growth in primary human keratinocytes, one human basal cell carcinoma (BCC) cell line, one human skin squamous cell carcinoma cell line (SCC12) and three human melanoma cell lines (A375, C32 and MeWo) using the XTT test. Compound C significantly decreased the cell viability in a concentration-dependent manner in p53-wild-type normal keratinocytes, BCC, A375 and C32 cells more than in p53 mutant MeWo and SCC12 cells (Fig. 1A). To examine the mechanism responsible for compound C-mediated growth inhibition, we choose wild-type-p53 BCC and mutant-p53 SCC12 to evaluate the cell cycle distribution using flow cytometric analysis. The results showed that treating cells with high dosage of compound C caused significant apoptosis in BCC cells at 48 h (Fig. 1B), which resulted in a clear increase in the percentage of cells in the sub-G1 phase. Compound C efficiently caused G2/M cell cycle delay in SCC12 cells, but less response to compound C induced sub-G1 increasing as compared to BCC cells (Figs. 1B and C). We found that the mitochondrial membrane potential was significantly decreased in BCC cells but not in SCC12 cells within 4 h of compound C treatment (Fig. 1D). We also found that compound C treatment led to significantly more cells with TUNEL labeling of DNA strand breaks in BCC as compare to SCC12 cells (Fig. 1E). Additionally, the pan caspase inhibitor zVAD-fmk could significantly inhibit the compound C-induced sub-G1 population increase in BCC cells (Fig. 1F). Therefore, compound C-mediated apoptosis in skin cancer cells is due to caspase-dependent apoptosis, and p53 may modulate this process.

Compound C induces autophagy in skin cancer cells

The conversion of microtubule-associated protein light chain 3 (LC3)-I (18-kDa) to LC3-II (16-kDa) and its translocalization from the cytosol to the autophagosome are one reliable marker of autophagy (Klionsky et al., 2008). Immunoblotting analyses indicate that the accumulation of lipid-conjugated LC3-II protein in compound C-treated BCC cells occurs in a time- and dose-dependent manner (Figs. 2A and B). The increase in LC3-II may due to a consequence of reduced autophaglysosomal proteolysis. Thus, we examined the compound C induced LC3 conversion in the presence and absence of the lysosomal inhibitor, Bafilomycine A1, in BCC cells. Bafilomycin A1 significantly enhanced the compound C induced LC3 conversion (Fig. 2C). This finding indicates that compound C increased autophagic flux, rather than inhibition of autophagolysosomal function in BCC cells. The conversion of LC3-I to LC3-II was also detected in all studied skin cancer cells after compound C treatment (Fig. 2D).

The recruitment of LC3-II to autophagic vesicles was further tested by the punctate expression of EGFP-LC3 in response to compound C treatment. In pEGFP-LC3-transfected BCC and SCC12 cells, the puncta accumulation of EGFP-LC3-II was detected 4 h after compound C treatment (Fig. 2E). Additionally, compound C significantly enhanced acidic autophagolysosome formation in BCC, SCC12, A375 and MeWo cells after staining with acridine orange (Fig. 2F). Taken together, we concluded that compound C could directly induce autophagy in skin cancer cells.

Compound C treatment causes p53 activation

To determine whether compound C activated a p53-dependent pathway, we evaluated whether compound C increased the expression level of p53 protein. BCC cells containing wildtype p53 were treated with 40 μM compound C, cell extracts were prepared at various time intervals from 0 to 24 h and p53 levels were assessed by immunoblotting. Compound C treatment was found to not only reduce phosphor-AMPK but also significantly induce the expression of p53

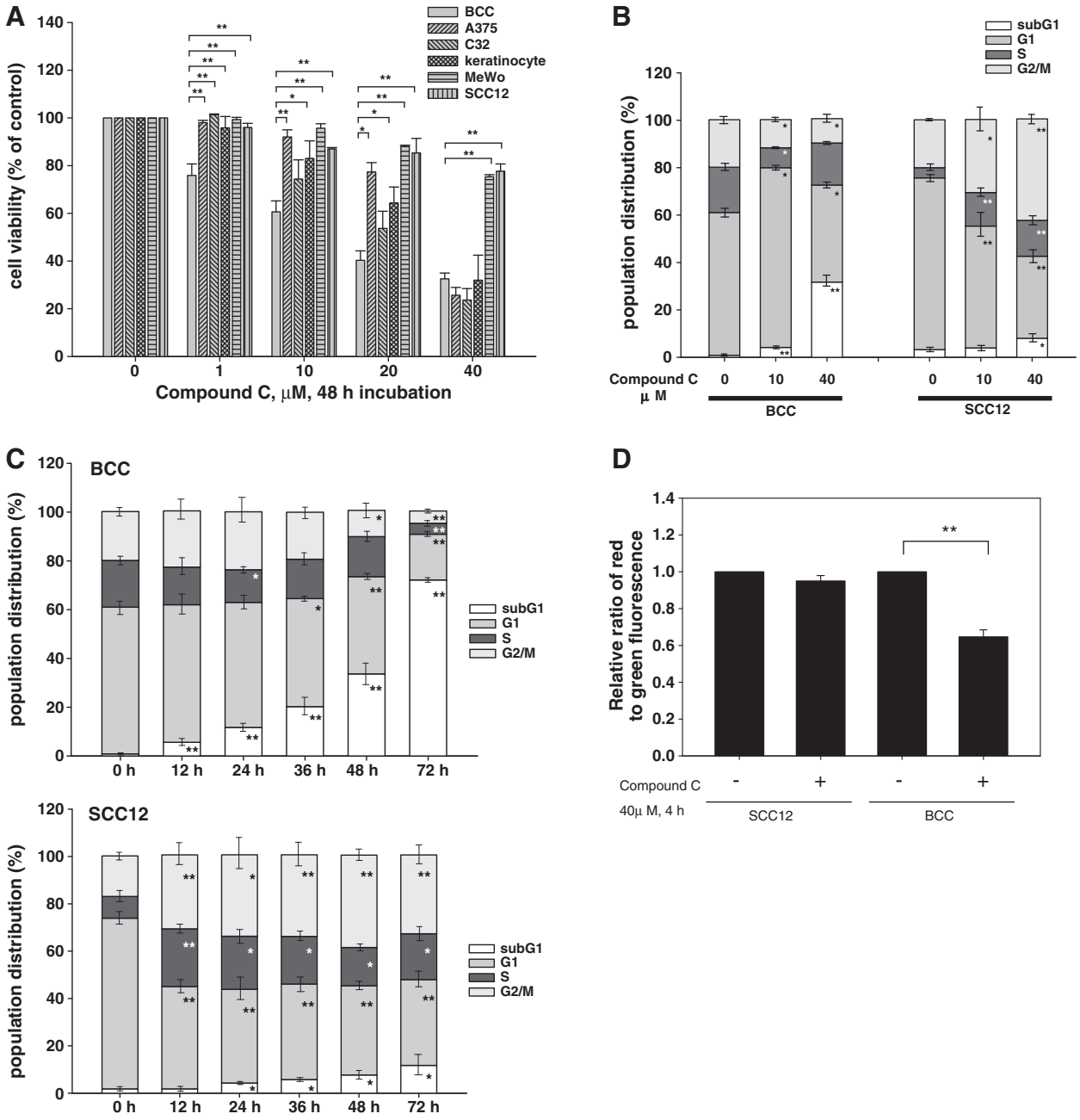


Fig. 1. Compound C induced intrinsic apoptotic cell death in p53-wildtype skin cancer cell lines. (A) Primary keratinocytes, BCC, SCC12, A375, C32 and MeWo skin cancer cells were treated with indicated concentrations of compound C for 48 h. Cell viability was determined by the XTT assay. (B and C) Compound C treatment caused an increase in the percentage of sub-G1 phase cells and G2/M phase cells in p53-wildtype BCC and p53 mutant SCC12, respectively. BCC and SCC12 were treated with compound C (0, 10 and 40 μM) for 48 h (B) or treated with 40 μM compound C for different time interval (C). The DNA content was analyzed. (D) Disruption of the mitochondrial potential in BCC cells but not in SCC12 cells when treated with compound C. Cells were left untreated or treated with 40 μM compound C for 4 h. The mitochondrial potential was determined. (E) Compound C-induced apoptotic DNA fragmentation. Cells were treated for 36 h with 40 μM compound C. The appearance of apoptotic DNA strand breaks in a representative field was detected by the TUNEL method. Scale bars, 20 μm. (F) Compound C-induced apoptosis is caspase-dependent. BCC cells were treated with 10 μM or 40 μM compound C alone or in combination with 20 μM zVAD-fmk for 24 h. The subG1 population of treated cells was analyzed by flow cytometry. Data are expressed as the mean ± S.E.M. of three independent experiments. (* $p<0.05$; ** $p<0.001$).

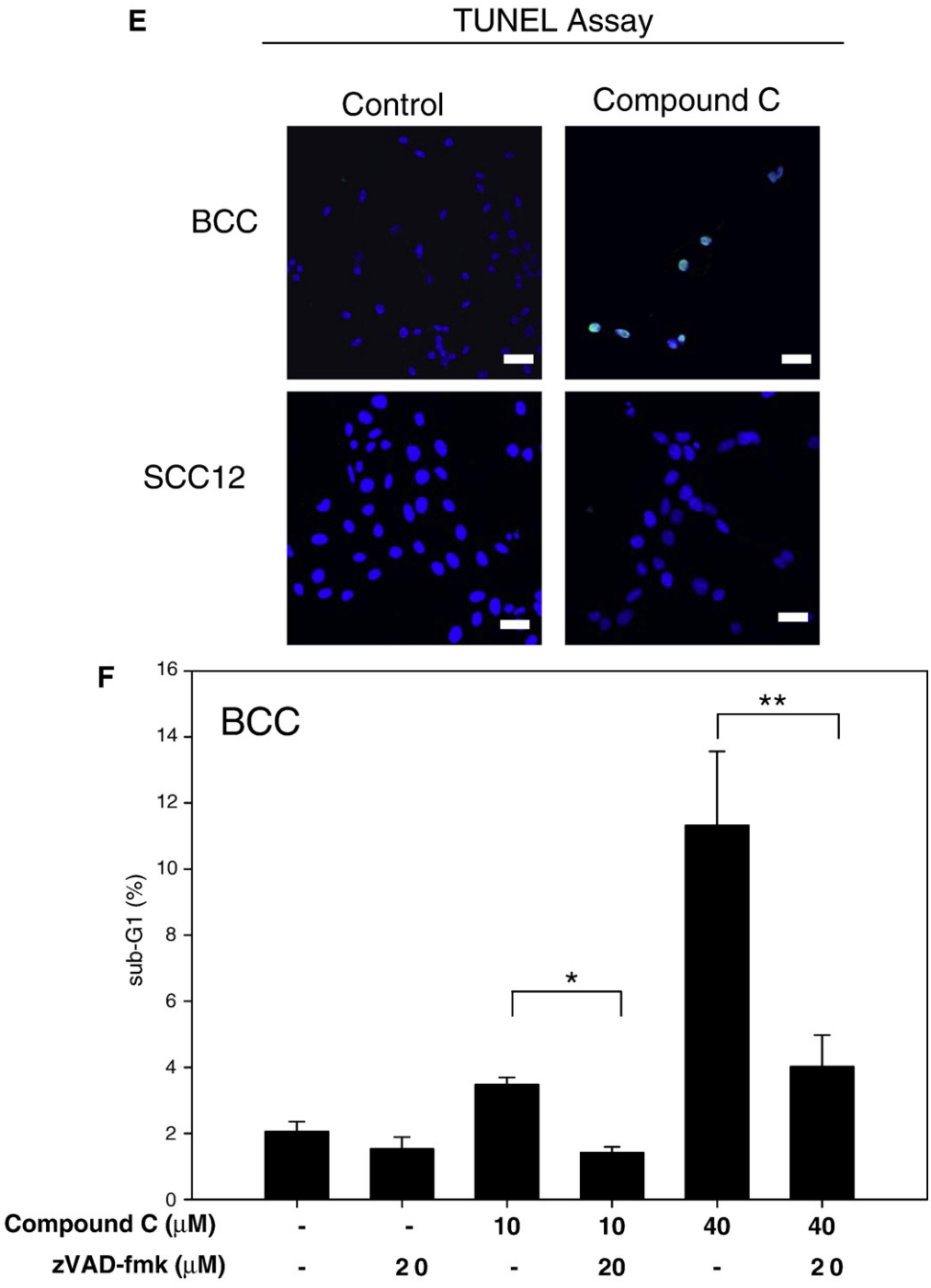


Fig. 1 (*continued*).

protein and also induce the expression of its downstream target genes, such as p21 and Bax, in BCC cells (Fig. 3A). To examine the phosphorylation of p53, we conducted immunoblotting using anti-phospho-p53 antibodies. The signal of anti-phospho-p53 at Ser15 was remarkably increased when BCC cells were exposed to compound C (Fig. 3B). In contrast, there was no remarkable change in other phospho-p53 signals detected in compound C-treated BCC cells. To examine the localization of the p53 protein after treatment with compound C, BCC cells were subjected to immunofluorescence staining and confocal microscopy. The p53 protein was concentrated and specifically located in the nuclei of the compound C-treated cells (Fig. 3C). Therefore, compound C could upregulate p53 expression and activate p53 by inducing its phosphorylation and translocalization from the cytoplasm to the nucleus to regulate the expression of p53 target genes.

Compound C-induced p53 activation is associated with DNA damage caused by compound C induced ROS generation

DNA damage induces phosphorylation of p53 at Ser15 (Shieh et al., 1997). Reactive oxygen species (ROS) is a causal risk factor to induce oxidation damage of DNA (Cooke et al., 2003). Compound C has been reported to increase ROS production in glioma cells (Vucicevic et al., 2009). Thus, we presumed that compound C-induced p53 activation may depend on DNA damage response which was caused by compound C induced ROS production. First, we investigated whether

compound C could cause ROS production in BCC cells using DCFDA staining and flow-cytometry. We had observed an increasing of ROS generation after compound C treatment and this effect was diminished after antioxidant N-Acetyl Cysteine (NAC) addition (Fig. S1). We next treated the BCC cells with or without 40 μM compound C for 12 h and an immunofluorescent assay were performed to examine DNA damage foci by using anti-phosphorylated γ-H2AX antibody. As shown in Fig. 4A, DNA damage foci were clearly observed in the compound C-treated cells, but it was not the case in control, NAC treatment only and the cells co-treated with compound C and NAC. This result indicated that compound C could cause DNA damage via compound C induced ROS. Compound C treatment not only increased phosphorylated γ-H2AX, but also increased phosphorylated ATM, a DNA damage activated kinase which phosphorylated Ser15 of p53 (Canman et al., 1998), and phosphorylated Chk2, an ATM downstream target (Chaturvedi et al., 1999). The NAC co-treatment could efficiently diminish the protein levels of phosphor-γ-H2AX, phospho-ATM, phosphor-Chk2, phosphor-p53 and total p53 proteins (Fig. 4B). To verify the possible role of ATM in compound C induced p53 activation, BCC cells were pre-treated for 1 h with specific inhibitor for ATM, caffeine. Subsequently, the caffeine-treated cells were exposed to compound C. As shown in Fig. 4C, the compound C-induced ATM activation was efficiently inhibited by caffeine. In addition, pretreatment of cells with caffeine also decreased the compound C mediated p53 upregulation, Ser15 phosphorylation of p53, p21 upregulation and phosphor-Chk2. Interestingly, the compound C cannot significantly induce the phosphorylation of ATR and Chk1 protein (Fig. S2). Thus, the compound C may specifically activate ATM/Chk2 pathway, but not ATR/Chk1 pathway. Taken together, these data suggested that the ROS may play a role in compound C induced DNA damage. The DNA damage induced ATM activation is essential for compound C induced p53 activation.

Compound C-induced apoptosis is attenuated in p53-knockdown BCC cells

As p53 is activated by compound C, we were eager to elucidate the role of p53 in compound C-induced apoptosis and autophagy. To answer this question, we examined the effect of compound C on BCC cells deficient in p53 function. This deficiency was achieved by means of RNA interference to reduce the level of endogenous p53, which is sufficient to abolish p53 function. A p53 shRNA vector carrying a human U6 promoter was transfected into BCC cells, and the stably transfected clones were retrieved after G418 selection. BCC cells stably expressing EGFP shRNA were used in this study as a negative control of p53 knockdown. The degree of p53 knockdown in these cells was validated by p53 immunoblotting. In contrast to control cells, the endogenous p53 and p21 level in BCC/p53 shRNA stable clones was dramatically reduced to a level barely detectable after compound C treatment, which indicated efficient knockdown of p53 (Fig. 5A). As expected, 40 μM compound C triggered a significant reduction in the cell number in cells transfected with control vector (BCC control). However, compound C caused a small decline in the cell number of BCC/p53-shRNA clone-C16 and clone-C18 cells (Fig. 5B), suggesting that the compound C-induced decrease in cell number was partially prevented or attenuated by p53 knockdown. Sequentially, the apoptosis was characterized by a DNA content assay, and it was found that approximately 32% of the apoptosis caused by compound C (40 μM for 48 h treatment) was due to BCC control but less than 7% of apoptotic cells were observed in BCC/p53-shRNA clone-C16 and clone-C18 cells (Fig. 5C). Interestingly, the percentage of G2/M phase was significantly increased in BCC/p53-shRNA clone-C16 and clone-C18 cells as compared to BCC control at 48 h of compound C treatment. Consistently, cleaved caspase 3 and PARP were significantly decreased in BCC/p53-shRNA clone-C16 and clone-C18 cells after compound C treatment as compared to BCC control cells (Fig. 5A). Furthermore, there are more sustained cyclin B1 and inactive phosphor-Cdc2 (Tyr15) in BCC/p53-shRNA clones after compound C treatment as compared to BCC control cells, which may one of the reasons to cause G2/M delay in BCC/p53-shRNA clones (Fig. 5D). However, there was no difference in the amount of LC3-II protein and acridine orange-stained acidic vesicular organelles between the BCC control cells and BCC/p53-shRNA cells (Figs. 5A and E). Taken together, the collected data revealed that p53 could modulate compound C-induced cell apoptosis but not autophagy in skin cancer cells.

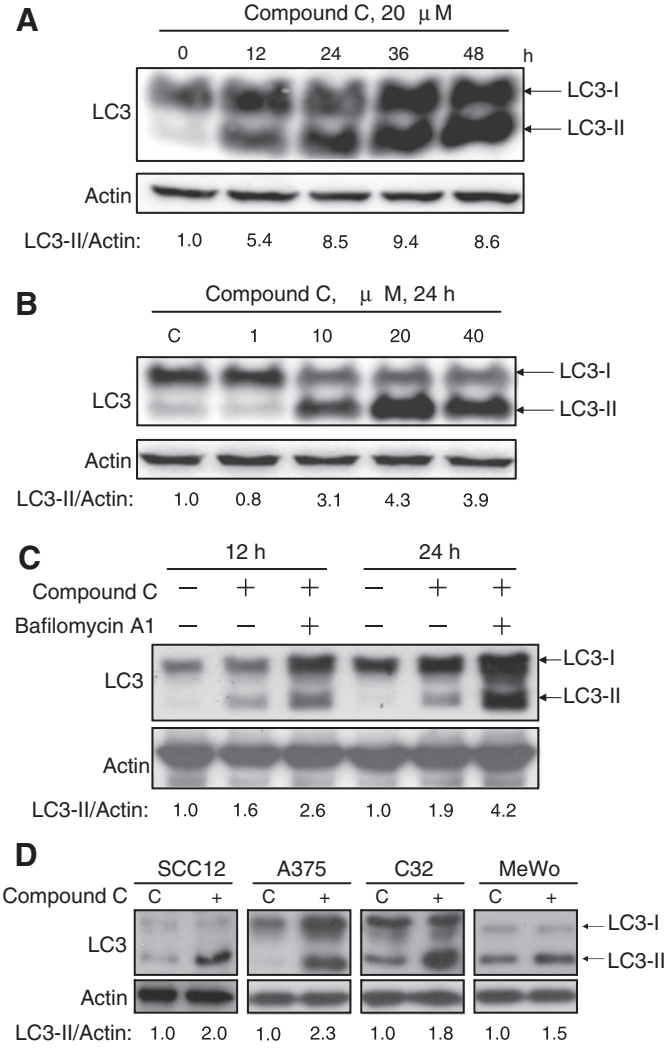


Fig. 2. Compound C induces cell autophagy in different skin cancer cells. (A and B) Induction of the conversion of LC3-I to LC3-II in response to compound C treatment was time- and dose-dependent. BCC cells were treated with 20 μM compound C for various amounts of time or treated for 24 h with different compound C concentrations as indicated and analyzed by immunoblotting. Control (C) cells were treated with DMSO only. (C) Bafilomycin A1 increases the compound C induced LC3-II conversion in BCC cells. BCC cells were treated with 20 μM compound C or co-treated with 2 μM bafilomycin A1 for 12 and 24 h and analyzed by immunoblotting. (D) Induction of the conversion of LC3-I to LC3-II by compound C in different skin cancer cell lines. A375, C32, SCC12 and MeWo cells were treated for 4 h with 40 μM compound C or DMSO control (C) and analyzed by immunoblotting. Densitometric LC3-II/actin ratios are shown underneath the blot. (E) Induction of EGFP-LC3 puncta in response to compound C treatment. The EGFP-LC3 transfected BCC and SCC12 cells were treated with 40 μM compound C for 4 h, fixed with 1% paraformaldehyde and observed using a confocal microscope. Scale bars, 20 μm. (F) Detection of compound C-induced acidic autophagic vacuoles in skin cancer cells. Skin cancer cells were treated with 40 μM compound C for 12 h. The acidic autophagic vacuoles were analyzed by acridine orange staining. Scale bars, 50 μm.

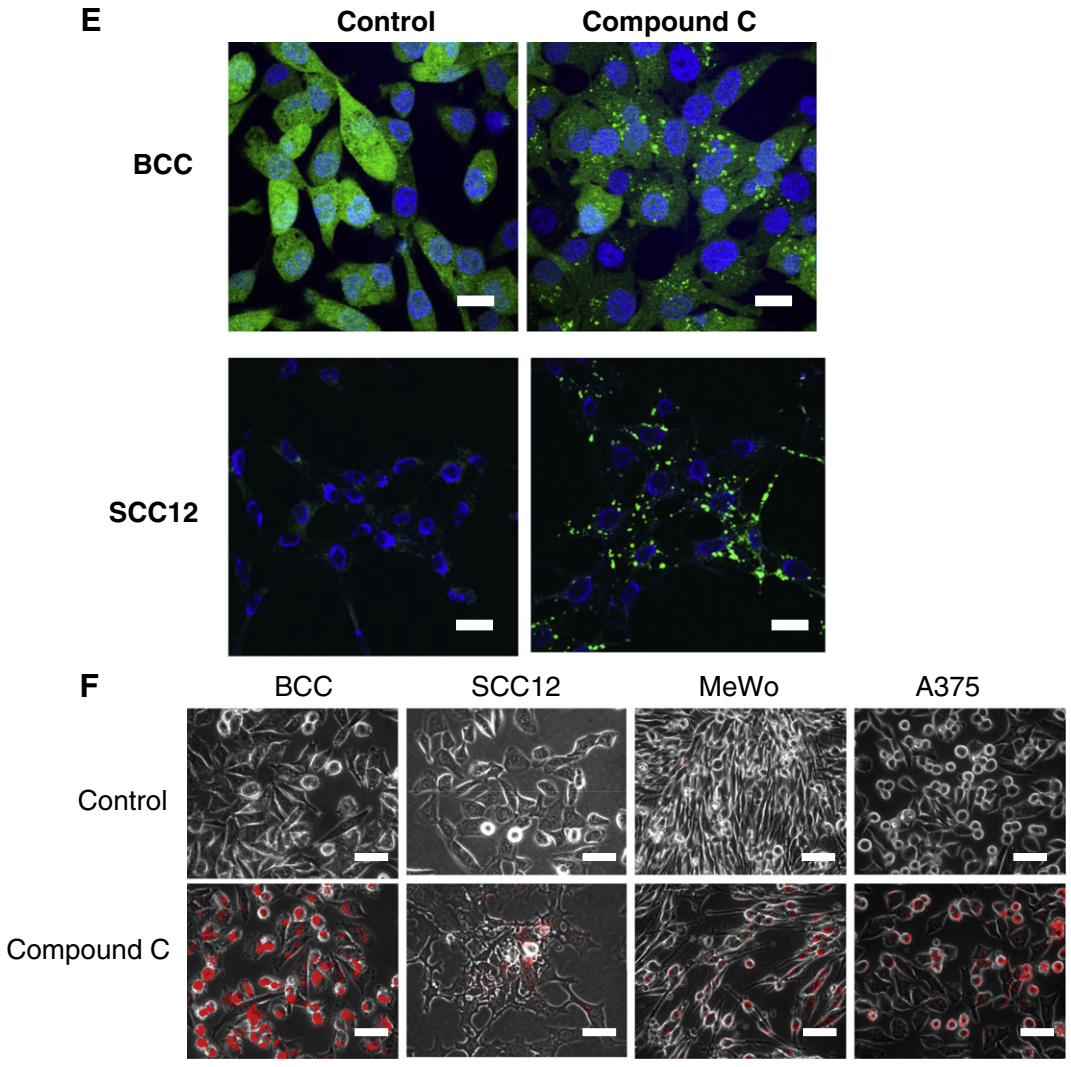


Fig. 2 (*continued*).

Discussion

The present study demonstrates that the AMPK inhibitor compound C not only induces autophagy but also selectively causes caspase-dependent and intrinsic apoptosis on some skin cancer cells in vitro. The p53-mutant skin cancer cells were less sensitive to compound C-induced apoptosis when compared with p53-wildtype cells. In addition to the upregulation of p53 and its target gene expression, compound C also induced p53 phosphorylation and nuclear translocalization. The compound C induced p53 activation was caused by DNA damage response which resulted from compound C induced ROS production and mediated by activated ATM. Finally, we demonstrated that compound C-induced apoptosis but not autophagy was much less sensitive in p53-knockdown cells when compared with their isogenic counterparts with wildtype p53 expression. Our data indicate that p53 may modulate compound C-induced apoptosis in skin cancer cells.

Earlier studies have demonstrated that the inhibition of AMPK activity by compound C could rescue cells from the apoptosis caused by glucose depletion or AMPK activator stimulation (such as AICAR and metformin) (Bae et al., 2007; Misumi et al., 2009). On the other hand, some evidence suggested that compound C could indirectly enhance the apoptosis induced by some cell death stimulation (Shaw et al., 2007). Our data support the hypothesis that compound C could directly induce apoptosis in multiple human skin cancer cell lines. Interestingly, different skin cancer cell lines have different sensitivities to compound C-induced apoptosis. In general, p53-wildtype cancer cells were more sensitive to compound C-induced apoptosis than p53-mutant cells. Compound C-induced apoptosis was reversed when the cells were pretreated with the pan caspase inhibitor zVAD-fmk. Additionally, the observation of mitochondrial membrane potential reduction, caspase 3 activation, and PARP cleavage in cells after compound C treatment indicated that this cell death is mediated by mitochondrial-mediated and caspase-dependent apoptosis. In accordance with our findings, compound C was recently found to directly induce apoptosis in myeloma cell lines via the downregulation of Mcl-1 and Bcl-xL (Baumann et al., 2007). Compound C has also been shown to directly induce apoptosis in breast carcinoma cells and glioma cells by enhancing cermide production and through an AMPK-independent mechanism, respectively (Jin et al., 2009; Vucicevic et al., 2009). These results indicate that compound C may have anti-tumor activity in different tumor lineages.

Under energy stress conditions, AMPK maintains energy homeostasis by inducing autophagy and inhibiting protein translation and cell growth mainly through the inhibition of its downstream target mTOR (Meijer and Codogno, 2007). Recent studies also indicated that mammalian autophagy-initiating kinase Ulk1 could be phosphorylated and activated by AMPK during glucose starvation (Egan et al., 2011; Kim et al., 2011). AMPK is thus a positive regulator of autophagy induction.

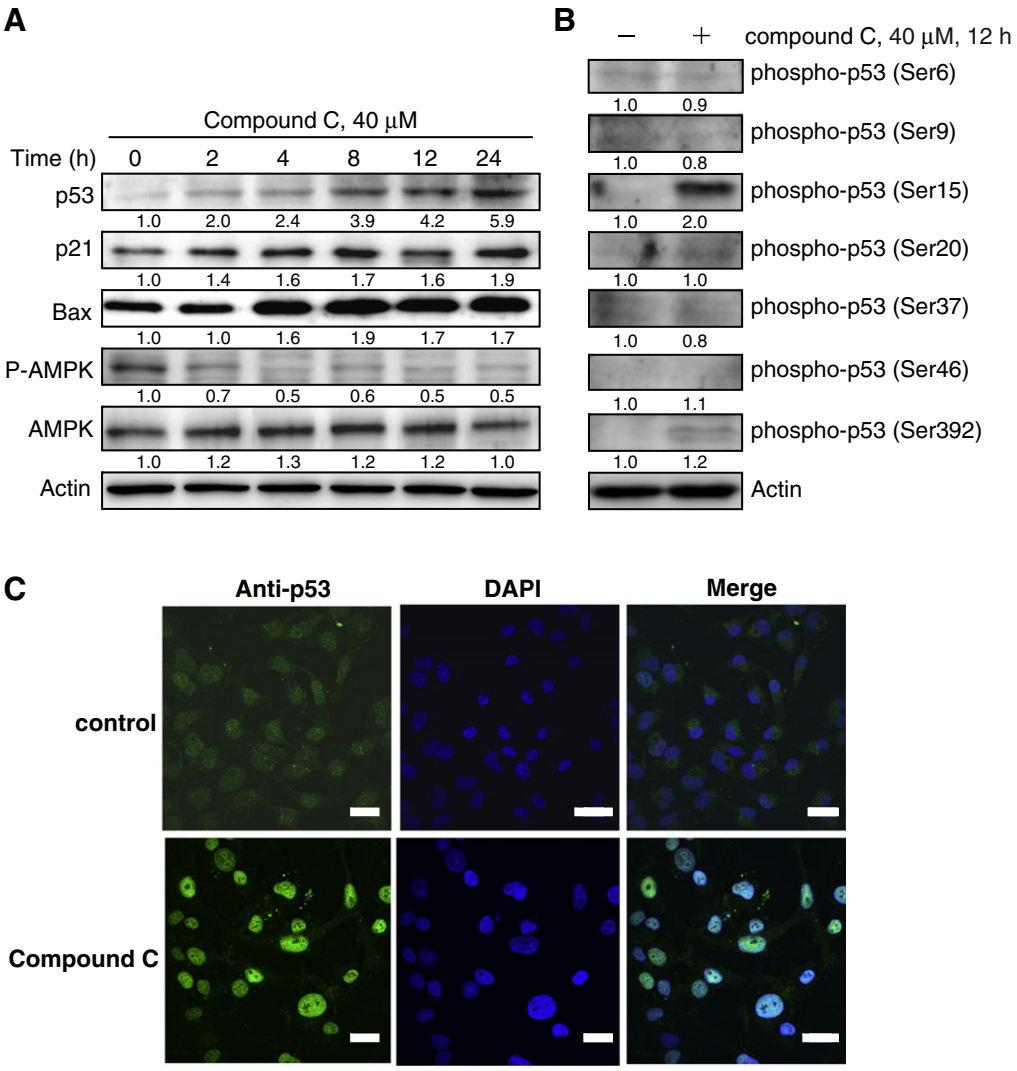


Fig. 3. Compound C increases the expression of p53 and induces p53 activation in BCC cells. (A) Compound C increased of p53 and its target genes. BCC cells were treated with 40 μM compound C for the time indicated. The p53, p21, and Bax proteins were analyzed by immunoblotting. (B) Induction of p53 phosphorylation by compound C in BCC cells. BCC cells were incubated with DMSO control (−) or 40 μM compound C for 12 h. Immunoblotting was performed using various anti-phospho-p53 antibodies. (C) The nuclear localization of p53 proteins following treatment with compound C in BCC cells. BCC cells were treated with DMSO control or with 40 μM compound C for 12 h. After fixation, the cells were incubated with rabbit anti-p53 antibodies and then incubated with goat anti-rabbit FITC. Scale bars, 20 μm.

Furthermore, the inhibition of AMPK by compound C has been said to occur by blocking AMPK-dependent autophagy in yeast, hepatocytes and Hela cells (Law et al., 2010; Meley et al., 2006; Yan et al., 2010). However, our data surprisingly demonstrated that compound C at the experimental concentration (10–40 μM) could directly and strongly induce autophagy and enhance the autophagic flux in skin cancer cells. Consistent with our results, the compound C-induced autophagy in the human glioma cell line has been reported to occur via downregulation of the Akt/mTOR pathway independent of AMPK inhibition (Vucicevic et al., 2011). Therefore, compound C-induced autophagy may not be restricted to several specific cell lines and may not be dependent on AMPK activity. For this reason, caution should be taken when using compound C to study the AMPK-dependent response, especially for autophagy.

The induction of cell cycle arrest and apoptosis has been considered to be the most important function of p53 when cells respond to various cellular stresses (Bargonetti and Manfredi, 2002). The AMPK-dependent phosphorylation of p53 at Ser-15 and Ser-46 had been reported to mediate the effects of activated AMPK on p53-dependent cell cycle arrest and apoptosis during glucose depletion (Okoshi et al., 2008; Vousden and Lu, 2002). In the current study, we suggest that compound C-induced apoptosis is AMPK independent but at least partly mediated by the phosphorylation and subsequent activation of p53 for the following reasons. First, compound C can efficiently inhibit the phosphorylation of AMPK and ACC (acetyl CoA carboxylase), a downstream target of AMPK, which indicates that the compound C-induced p53 phosphorylation and nuclear translocalization and the subsequent apoptosis are AMPK-independent. Consistent with our results, it has been found that the compound C-mediated inhibition of AMPK is not sufficient for compound C-induced apoptosis in glioma cells (Vucicevic et al., 2009). Compound C may thus bypass the AMPK pathway to induce cell apoptosis. Second, compound C induced both the phosphorylation of p53 at Ser-15 and the nuclear accumulation of p53, which are recognized as p53 activation signals (Lambert et al., 1998). Additionally, the inhibition of p53 by shRNA attenuated the compound C-trigged apoptosis in BCC cells. Similarly, we also found higher levels of compound C-induced apoptosis in HCT116 cells than in isogenic cells with the p53 gene deleted, which further supports the finding that compound C-induced p53 activation is important for compound

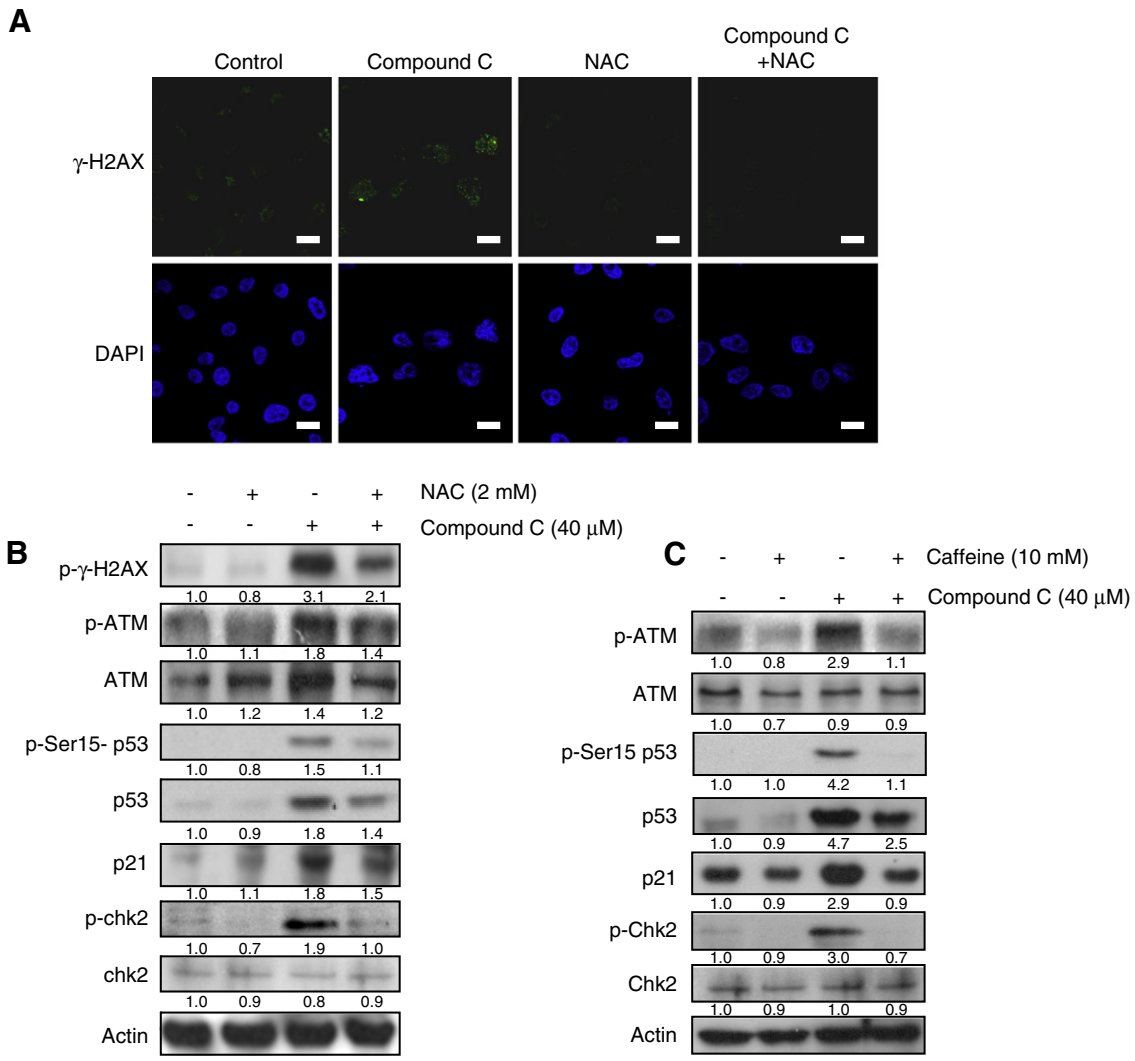


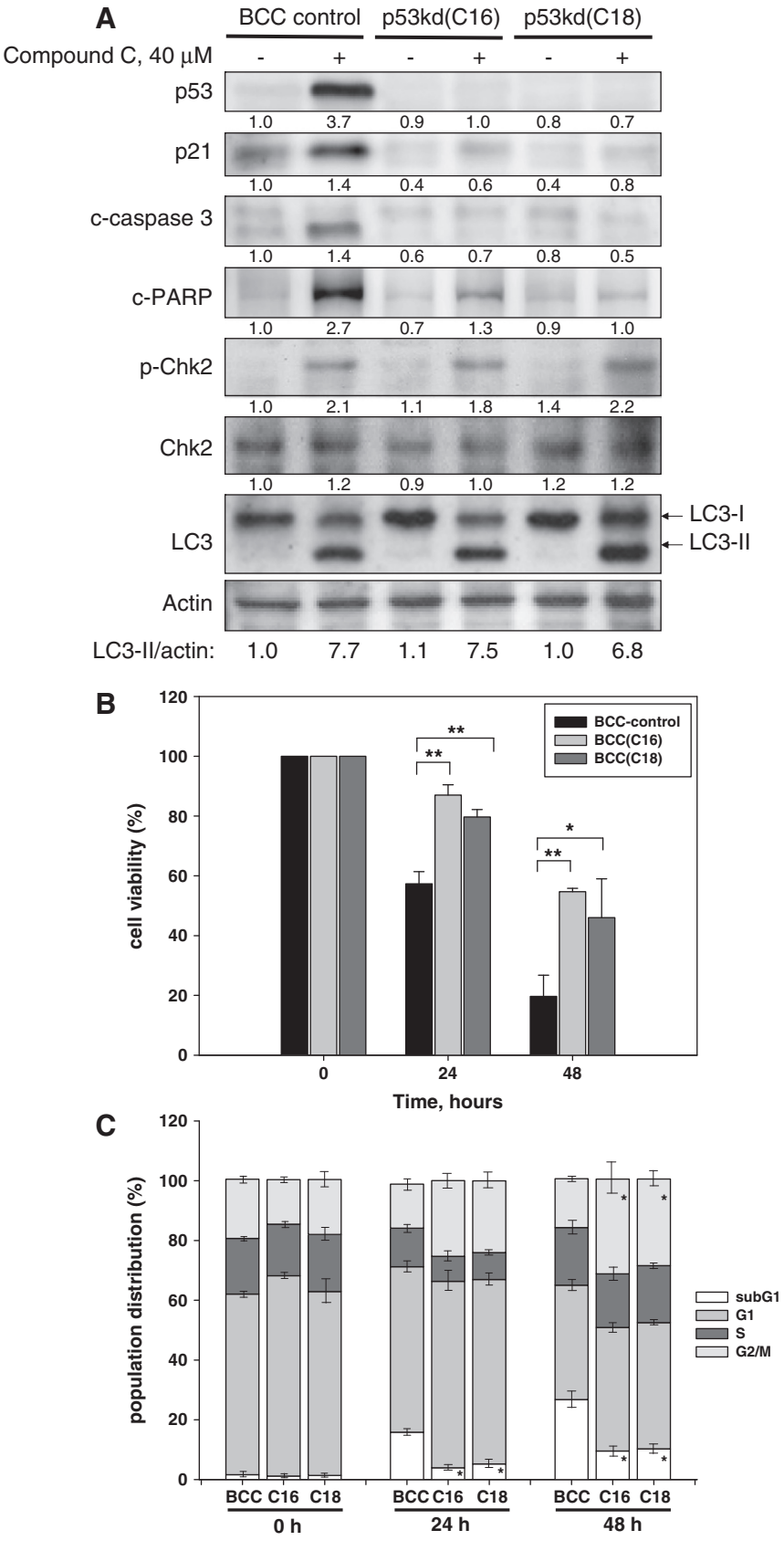
Fig. 4. Compound C induced ROS dependent DNA damage and activated ATM to phosphorylate p53. (A) Compound C induced DNA damage is dependent on ROS production. BCC cells were treated with 40 μM of compound C, 2 mM of NAC, co-treatment with 40 μM of compound C and 2 mM of NAC or vehicle for 12 h, then immunofluorescent assays were conducted using antibody against the phosphorylated γ-H2AX to display DNA damage foci. Scale bars, 20 μm. (B) Antioxidant NAC can diminish the compound C induced activation of DNA damage markers and phosphorylation of ATM. BCC cells were treated with 40 μM of compound C, 2 mM of NAC, co-treatment with 40 μM of compound C and 2 mM of NAC or vehicle for 8 h, then the cell lysates were prepared for immunoblotting. (C) ATM inhibitors blocked compound C-mediated phosphorylation of p53. Cells were incubated for 1 h in the presence or absence of 10 mM caffeine, then 40 μM compound C was added and incubated for 8 h for p53 phosphorylation by immunoblot analyses.

C-induced apoptosis (Fig. S3). Third, compound C induced apoptosis has been reported to involve induction of oxidative stress (Vucicevic et al., 2009). In addition to compound C induced DNA damage response, we demonstrated that depletion of compound C induced ROS production by antioxidant partially inhibited compound C induced apoptosis and autophagy (Fig. S4). Furthermore, the ATM, a DNA damage activated kinase which had been reported to phosphorylate Ser15 of p53 and induce cell apoptosis (Canman et al., 1998), involved in the compound C induced p53 activation. This suggests that p53 induced apoptosis may be one of the mechanisms underlying the pro-apoptotic action of compound C which is shared with compound C induced apoptosis mediated by ROS induced DNA damage. Collectively, different lines of evidence argue that p53 may play a critical role in compound C-induced apoptosis, and compound C can activate p53 through a mechanism that is AMPK independent and associate with compound C induced DNA damage response.

Both ATM and ATR can be activated by DNA damage. However, ATM is activated by double-stranded breaks (DSBs) resulting from conditions such as ionizing irradiation (IR). On the other hand, ATR mainly responds to single-stranded DNA lesions and is crucial for resolving replication stress from conditions such as stalled replication forks and ultraviolet (UV) light (Yang et al., 2003). We demonstrated that compound C specifically induces the phosphorylation of ATM and Chk2 protein, but not ATR and Chk1 protein. We also observed that the phosphorylated γ-H2AX, a marker for DNA DSBs, increased after compound C treatment. Thus, the possibility that compound C specifically induced DSBs to activate ATM/Chk2 pathway should not be ruled out and need to be further characterized in future.

In p53-deficient cells, G2/M arrest is primary determined by the ATM which response to DNA damage (Reinhardt et al., 2007). The Chk1 and Chk2 are downstream targets of ATM, which induce G2/M arrest by inactivating Cdc25 tyrosine phosphatases (Bartek and Lukas, 2003). The critical regulatory step in the activation of Cdc2 during progression from the G2 phase into mitosis is removal of inhibitory phosphotyrosines (Tyr15) on Cdc2 of cyclin B/Cdc2 complex by active Cdc25. In this study, we demonstrated that compound C-induced G2/M cell cycle arrest correlates with sustained Cdc2 phosphorylation at Tyr15 in p53 knockdown cells (Fig. 5D), indicating the

possibility that compound C induced ROS-dependent DNA damage activates the ATM signaling pathway, and thus activate Chk2 (Figs. 4B and C), to accumulate inactivated Cdc2 and resulting in cells that were arrest in G2 phase and unable to move into the mitosis phase. The dominant G2 cell cycle arrest was evident in intact nuclei without disassembly of the nuclear envelope and absence of



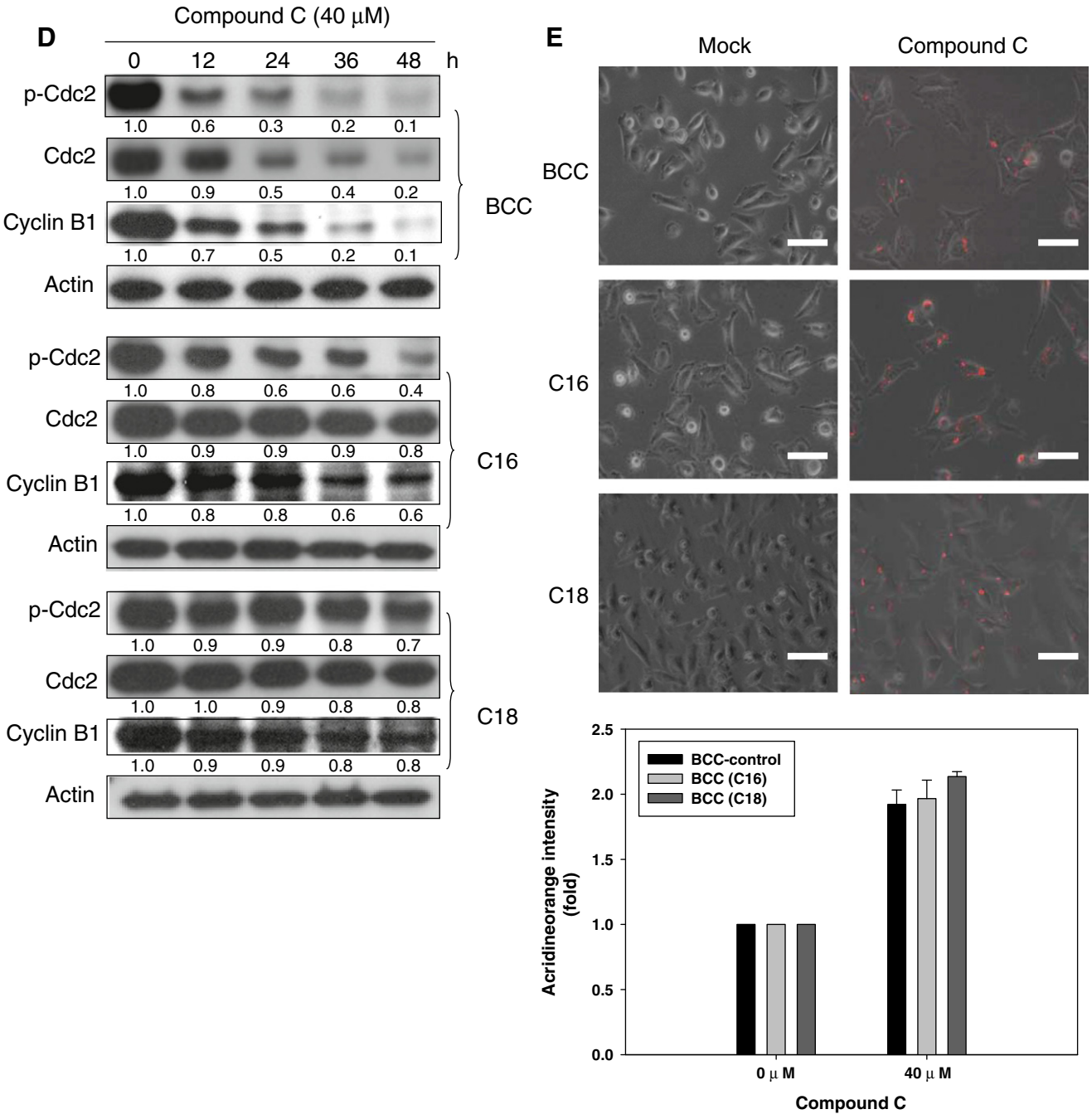


Fig. 5. Compound C-induced apoptosis is attenuated in p53 knockdown BCC cells. (A) Knockdown of p53 levels in BCC cells by RNA interference. Cell extracts were prepared from BCC/EGFP RNAi cells (BCC control) and two individual BCC/p53 RNAi stable clones (p53kdC16 and C18) treated with DMSO (−) or with 40 μM of compound C for 12 h and then analyzed by immunoblotting. (B) Effect of compound C on the cell viability of p53-wildtype and p53-knockdown BCC cells. The BCC control and BCC/p53 RNAi stable clones (C16 and C18 cells) were treated with 40 μM compound C for 24 and 48 h. The cytotoxicity of compound C was determined using the XTT assay. (C) The effect of compound C on the cell cycle of p53-wildtype and p53-knockdown BCC cells. The BCC control and BCC/p53 RNAi stable clones were treated with 40 μM compound C for 24 and 48 h. Compound C-induced cell cycle population change between p53 wild type BCC and p53 knock-down BCC cells (C16 and C18) in each group with different time intervals of compound C treatment was determined by DNA content assay. (D) The effect of compound C on cell cycle regulatory proteins of BCC control and BCC/p53 RNAi stable clones. The Tyr^{15} phosphor-Cdc2, Cdc2 and cyclin B1 levels in BCC control and two BCC/p53 RNAi stable clones with 40 μM of compound C treatment for 0, 12, 24, 36 and 48 h were determined by immunoblotting. (E) The effect of compound C on acridine orange stained autophagolysosome of BCC control and BCC/p53 RNAi stable clones. Cells were treated with 40 μM compound C for 12 h and then the acridine orange staining was examined on fluorescence microscopy (upper panel) and quantitated using flow cytometry (lower panel). Scale bars, 50 μm. The data are expressed as the mean ± S.E.M. of three independent experiments. (* $p<0.05$; ** $p<0.001$).

chromosome condensation in compound C treated C16 and C18 cells (Fig. S5). These evidences may explain the mechanism of the compound C induced G2/M arrest in p53 deficient cells.

In conclusion, this study shows that compound C can induce both autophagy and apoptosis in skin cancer cell lines. Additionally, p53 is upregulated, phosphorylated and undergoes nuclear translocalization during treatment with compound C which is independent on AMPK activity but associated with ROS induced DNA damage response. The shRNA-based inhibitory experiments in BCC cells with different p53 statuses provide compelling evidence for a role for p53 in compound C-induced apoptosis. However, p53 is not necessary for compound C-induced autophagy. Understanding the mechanisms through which

p53 pathways regulate apoptosis following treatment with compound C may contribute to basic research for exploring the AMPK-dependent cellular response or to develop novel therapeutic strategies in skin cancers.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2012.12.016.

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