p28^{GANK} knockdown-derived reactive oxygen species induces apoptosis through mitochondrial dysfunction mediated by p38 in HepG2 cells

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Abstract. Oncoprotein *p28^{GANK}* knockdown by RNA interference (RNAi) can induce hepatoma cells apoptosis. However, the mechanisms have not been well defined yet. In the present study the *p28^{GANK}* knockdown-induced apoptosis in HepG2 cells was prevented by caspase-9 inhibitor (Z-LEHD-FMK). During the knockdown of *p28^{GANK}*, mitochondrial translocation of Bax, loss of mitochondrial transmembrane potential (ΔΨm) and release of cytochrome c were observed. In this study, the activation of p38 was found to be critical for the *p28^{GANK}* knockdown-induced apoptosis, as suggested by the finding that pharmacological inhibition of p38 with SB203580 suppressed the redistribution of Bax, the loss of ΔΨm and the apoptosis. Moreover, generation of reactive oxygen species (ROS) contributed to the cell death because N-acetyl-L-cystenine (NAC), a ROS scavenger, suppressed the phosphorylation of p38 and the apoptosis. Our studies established the signaling pathway of *p28^{GANK}* knockdown-induced apoptosis in HepG2 cells, namely, mitochondrial dysfunction mediated by p38 downstream of intracellular ROS generation.

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Abbreviations: Adsi, adenovirus small interfering; Apaf-1, apoptotic protease-activating factor-1; BA, bongkrekic acid; Cox4, cytochrome oxidase 4; DCFH-DA, 2,7-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulating kinase; FADD, Fas-associated death domain; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethy-benzimidazolylcarbo-cyanine iodice; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cystenine; PARP, poly (ADP) ribose polymerase; ROS, reactive oxygen species; TNFR, tumor necrosis factor receptor; ΔΨm, mitochondrial membrane potential

Key words: apoptosis, *p28^{GANK}* knockdown, p38, ΔΨm, reactive oxygen species

Introduction

The ability to use double-stranded RNA to inhibit gene expression sequence-specifically (RNA interference, or RNAi) is currently revolutionizing science and medicine alike. Numerous pre-clinical studies are evaluating RNAi as a novel therapeutic modality in the battle against gain-of-function autosomal dominant diseases, cancer, and viral infections. *p28^{GANK}* is a novel oncogene especially over-expressed in human hepatocellular carcinoma (1). Down-regulation of *p28^{GANK}* by RNAi in several human hepatoma cell lines induces apoptosis (2,3). Our previous study showed that oncogene *p28^{GANK}* knockdown by adenovirus small interfering *p28^{GANK}* (Adsi*p28^{GANK}*) infection induced caspase-8 and -9 activation in hepatoma cells (2).

Mammalian cells possess two major apoptotic signal pathways known as the extrinsic pathway and intrinsic pathway (4). The extrinsic pathway is activated by tumor necrosis factor receptor (TNFR), which requires Fas-associated death domain (FADD) and caspase-8, while the intrinsic pathway is initiated by stress-induced signals inside the cell, which requires apoptotic protease-activating factor-1 (Apaf-1) and caspase-9 (5). Mitochondria play a central role in the integration of the intrinsic cell death pathway. Mitochondrial alterations such as the release of sequestered apoptogenic proteins, the loss of mitochondrial transmembrane potential (ΔΨm) and the production of reactive oxygen species (ROS) have been shown to be involved in the apoptosis (6). This process is mainly regulated by the proteins of Bcl-2 family (7,8). The antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, prevent the release of mitochondrial apoptogenic factors, such as cytochrome c, whereas the pro-apoptotic members of this family, such as Bax, Bak and Bid,

trigger this event (9). Accumulated evidence suggest that the mitogen-activated protein kinase (MAPK) superfamily which includes extracellular signal-regulating kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 are important regulators of apoptosis (10-14). More importantly, the roles of JNK and p38 involving in the intrinsic apoptotic pathway have also been observed (11,12,15,16).

This study aimed at the elucidation of the precise mechanisms in the apoptosis induced by *p28^{GANK}* knockdown in hepatoma cells. Therefore, the roles of the Bcl-2 family, mitochondria, MAPK signal pathways and ROS in this apoptotic process were investigated.

Materials and methods

Cell culture, reagents and siRNA transfection. HepG2 cell lines were obtained from American Type Culture Collection (Manassas, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, MD, USA) contained 10% fetal bovine serum (Gibco) and cultured in a humidified 37˚C incubator with 5% CO_2. Z-IETD-FMK, Z-LEHD-FMK, bongkrekic acid (BA), SB203580, SP600125, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethy-benzimidazolyl carbocyanine iodice (JC-1), 2,7-dichloro-fluorescein diacetate (DCFH-DA), and N-acetyl-L-cystenine (NAC) were purchased from Merck (La Jolla, CA). The method of transfection of Adsi*p28^{GANK}* or Adsi-Green fluorescence protein (Adsi*GFP*) was performed as described (2).

Western blotting. Cell lysates were made, and protein extracts were separated by SDS-PAGE according to standard methods. Antibodies used were anti-Bax, anti-Bid, anti-Bak, anti-Bcl-XL, anti-cytochrome c, anti-ERK, anti-JNK, anti-p38, anti-phospho-ERK (Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p38 (Thr180/Tyr182), anti-cleaved poly (ADP) ribose polymerase (PARP), anti-H3-histone (Cell Signaling Technologies, Beverly, USA), anti-glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (Kangchen, Shanghai, P.R. China) and anti-cytochrome c oxidase subunit 4 (Cox4) (Molecular Probes, Eugene, USA).

Preparation of subcellular fractions. The mitochondrial fractions of cells were prepared as following. The cells were washed with ice-cold PBS, resuspended in ice-cold HMKEE buffer (20 mM HEPES-KOH, pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DDT, 0.1 mM PMSF, 10 ng/ml pepstatin A and 10 ng/ml leupeptin) containing 250 mM sucrose. The cells were homogenized by passages through a 26-gauge needle, and then centrifuged at 1,000 g to remove cell debris and discard nuclei. The postnuclear supernatants were centrifuged then centrifuged at 14,000 g for 20 min at 4˚C. The supernatants obtained were designated as the cytosolic fraction, and the pellets containing the mitochondria were resuspended in lysis buffer. Nuclear fractions were prepared using a nuclear isolation kit (Pierce, Rockford, USA) according to the manufacturer's instructions.

Analysis of apoptosis. Apoptosis was assayed as described previously (2). Briefly, the cells were infected with AdSi*p28^{GANK}* or AdSi*GFP* virus for 72 h. Adherent cells were then collected by trypsinization and fixed with 70% ethanol overnight at 4˚C. After washing with PBS, the cells were treated with 100 μg/ml RNase A (Roche Diagnostics), 50 μg/ml of propidium iodide (PI) (Sigma), and 0.05% (v/v) Triton X-100, and incubated for 45 min at room temperature (RT). The samples were analyzed using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA). The cell cycle distribution was established by plotting the intensity of PI signal, which reflects the cellular DNA content. Apoptotic cells were identified as a hypodiploid DNA peak representing cells (sub-G1).

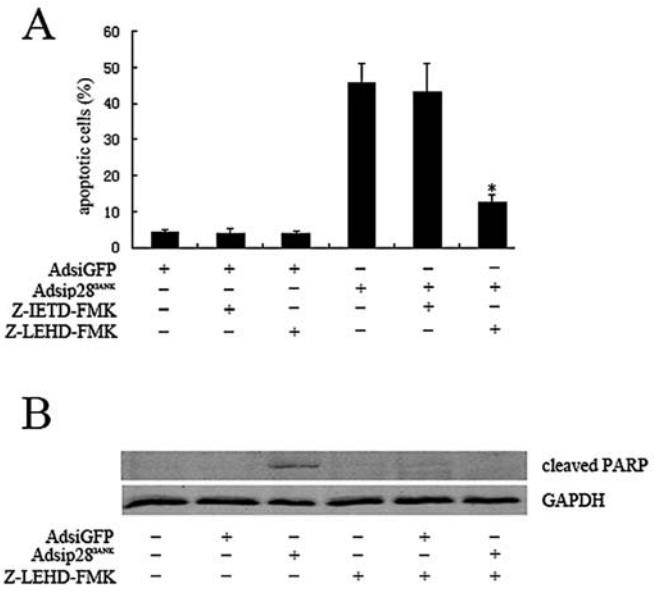

Figure 1. *p28^{GANK}* knockdown-induced apoptosis is caspase-9 dependent. (A) HepG2 cells infected with adenovirus small interfering green fluorescence protein (Adsi*GFP*, 15 MOI) or Adsi*p28^{GANK}* (15 MOI) were treated with (+) or without (-) 40 μM Z-IETD-FMK (caspase-8 inhibitor) or 50 μM Z-LEHD-FMK (caspase-9 inhibitor). After incubation for 72 h, apoptotic cells were estimated by flow cytometry. Adsi*GFP* infection served as a control. Data were presented as means ± SD of three independent experiments. $^{*}p<0.05$ vs. the Adsi*p28^{GANK}*-infected group. (B) Cells were transfected with Adsi*GFP* or Adsi*p28^{GANK}* in the presence (+) or absence (-) of Z-LEHD-FMK (50 μM) for 48 h. Western blotting was performed to analyze cleaved poly (ADP) ribose polymerase (PARP). Equal protein loading was confirmed by Western blotting for glyceraldehydes 3-phosphate dehydrogenase (GAPDH). The figure was obtained from three separate experiments with similar patterns.

Measurement of ΔΨm. Changes of ΔΨm were assessed as described (17). In brief, cells were stained with JC-1 (3 μM) for 15 min at 37˚C in the dark. After washing with PBS, the cells were observed with a laser scanning confocal microscope (Olympus Fluoview, Tokyo, Japan). The green fluorescence from JC-1 monomer (with a 515-nm barrier filter) and the red fluorescence from the aggregated form of JC-1 (with a 590-nm barrier filter) were visualized simultaneously.

Measurement of ROS. Generation of intracellular ROS was assessed as described (18). In brief, cells incubated with DCFH-DA (20 μM) for 30 min at 37˚C in the dark. After washing with PBS, the cells were observed with a laser scanning confocal microscope (Olympus Fluoview).

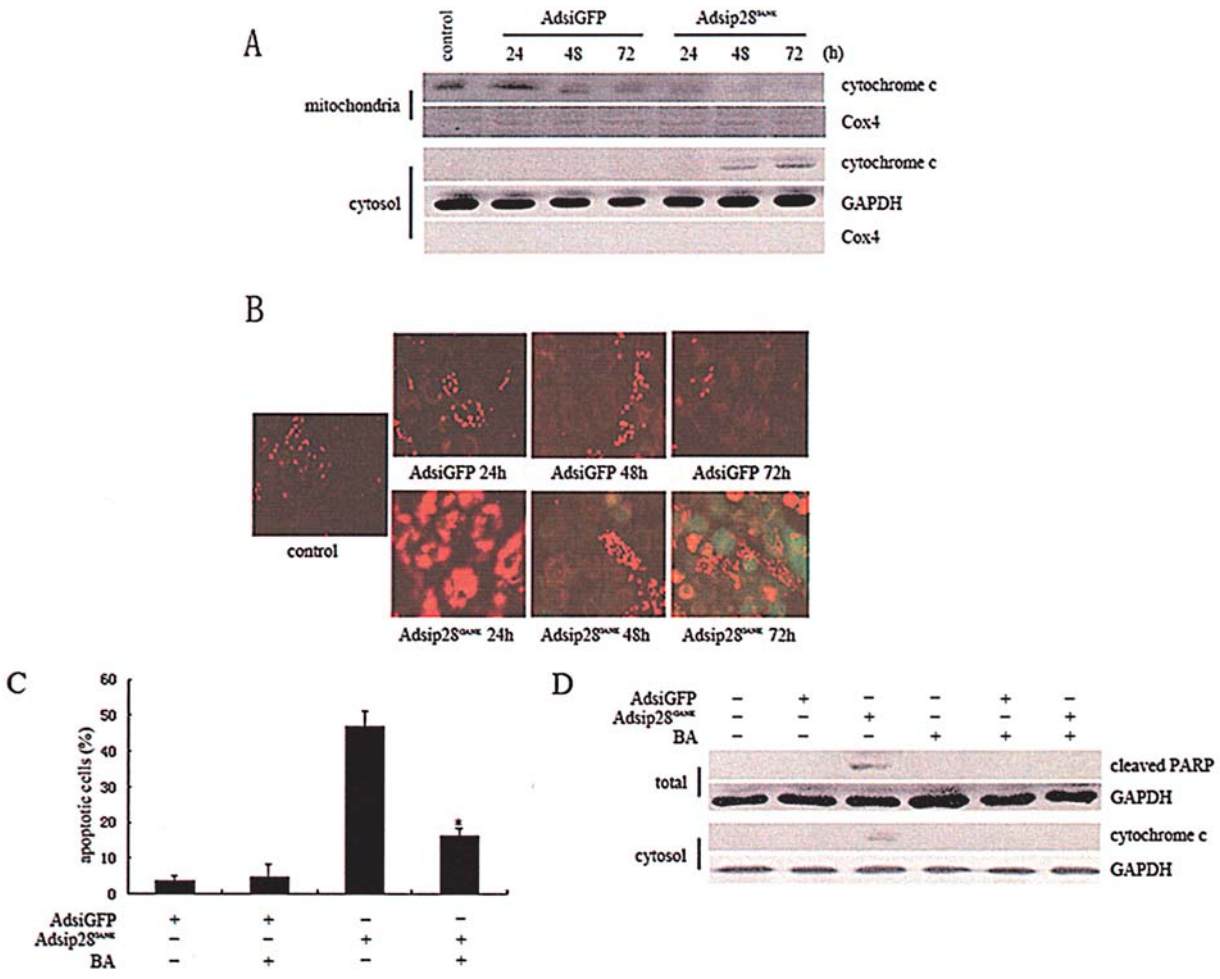

Figure 2. Downregulation of *p28*^{GANK} induces cytochrome c release from the mitochondria into the cytosol and change of mitochondrial membrane potential (ΔΨm). (A) HepG2 cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} for indicated time-points. Mitochondrial and cytosolic fractions were separated and cytochrome c content was analyzed by Western blotting. Equal proteins loading in the mitochondrial fractions and cytosolic fractions were confirmed by Western blotting for cytochrome oxidase 4 (Cox4) and GAPDH, respectively. (B) Changes of ΔΨm of HepG2 cells were measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethy-benzimidazolyl-carbocyanineiodice (JC-1) staining. Cells infected with Adsi*GFP* or Adsi*p28*^{GANK} for indicated time-points were incubated with JC-1 (3μM), then observed with a fluorescence microscope (magnification x200). Bright red and bright green fluorescence indicated high and low mitochondrial membrane potential respectively. (C) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} in the presence (+) or absence (-) of bongkrekic acid (BA, a potent inhibitor of mitochondrial megachannel, 50 μM) incubation for 72 h. Apoptotic cells were analyzed using flow cytometry. Data are presented as means ± SD of three independent experiments. *p<0.05 vs. the Adsi*p28*^{GANK}-infected group. (D) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} in the presence (+) or absence (-) of BA (50 μM) incubation for 48 h. Western blotting was performed to analyze cleaved PARP in whole-cell lystate and cytochrome c in cytosolic fraction without mitochondria. GAPDH levels are shown as controls for equal loading. (A), (B) and (D) are representatives of three independent experiments. Similar results were obtained for each of the three experiments.

Statistical analysis. Statistical analysis was performed using SPSS 10.1.4 software (SPSS Inc., Chicago, IL, USA). The results were expressed as mean value ± standard deviation (SD). The data from different treated groups were compared by using the unpaired, two-tailed Student's t-test. A p<0.05 was considered statistically significant.

Results

p28^{GANK} *knockdown in hepatoma cells induces apoptosis in a caspase-9-dependent manner*. Caspases play a pivotal role in apoptosis induced by diverse stimuli in most situations (19). To clarify the roles of specific caspases in the apoptotic process induced by *p28*^{GANK} knockdown, we treated HepG2 cells with inhibitors of caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK). Addition of 40 μM Z-IETD-FMK and 50 μM Z-LEHD-FMK significantly inhibited activity of caspase-8 and -9 induced by *p28*^{GANK} depletion respectively (data not shown). Z-LEHD-FMK drastically blocked the apoptosis induced by *p28*^{GANK} depletion. However, Z-IETD-FMK had almost no effect on the cell death (Fig. 1A). PARP is a substrate of caspase-3 yielding a product of 89 kDa and is considered a late marker for apoptosis. We observed that *p28*^{GANK} knockdown in HepG2 cells induced PARP cleavage which was markedly inhibited by Z-LEHD-FMK (Fig. 1B).

p28^{GANK} *depletion causes alterations of ΔΨm and cytochrome c release into cytosol*. The release of cytochrome c from mitochondria into cytosol is a key step in the mitochondrial apoptosis pathway (20), which interacts with Apaf-1, ATP/dATP and caspase-9 to form the apoptosome (4). In the present experiment, the release of cytochrome c from mitochondria into cytosol was observed at 48 h, continuing through 72 h after HepG2 cells were infected with Adsi*p28*^{GANK} (Fig. 2A).

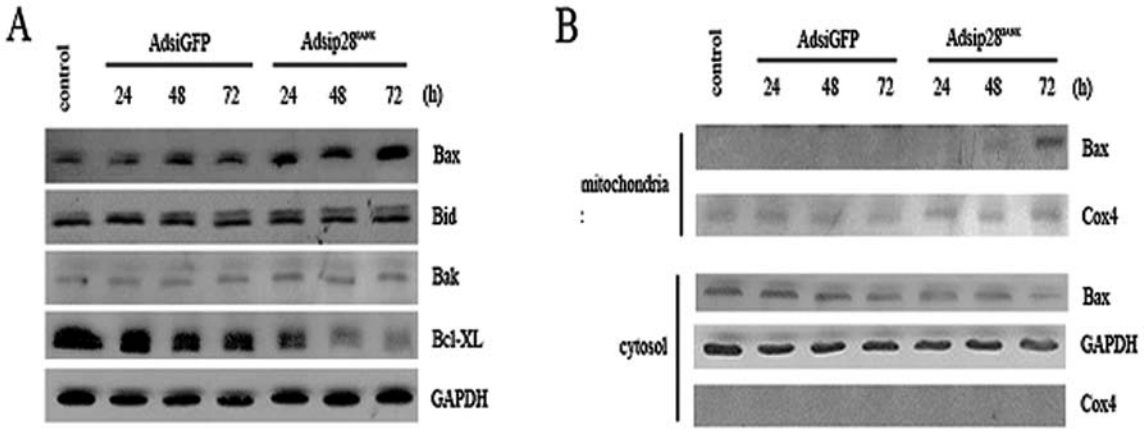

Figure 3. Downregulation of *p28*^{GANK} in HepG2 cells altered the ratio of Bax/Bcl-XL, caused the translocation of Bax into mitochondria. (A) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} for indicated time-points. Whole-cell lysates were separated on SDS-PAGE, followed by immunoblotting. Each target protein was detected with the specific antibody against Bax, Bid, Bak and Bcl-XL. GAPDH levels were shown as controls for equal loading. (B) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} for indicated time-points. Mitochondrial fractions and cytosolic fractions were separated and analyzed by Western blotting for Bax. Equal protein loading in the mitochondrial fractions and cytosolic fractions were confirmed by Western blotting for Cox4 and GAPDH, respectively. (A) and (B) were obtained from three separate experiments with similar patterns.

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis, and during this process, the ΔΨm collapses, inducing the release of cytochrome c (21,22). We used JC-1 (3 μM) to measure the effect of *p28*^{GANK} knockdown on the ΔΨm. With JC-1 staining, red and green fluorescence demonstrate high and low ΔΨm respectively. As shown in Fig. 2B, the ΔΨm increased within 24 h, then descended after Adsi*p28*^{GANK} infection in HepG2 cells. Furthermore, a potent inhibitor of mitochondrial megachannel, BA (50 μM) suppressed cytochrome c release, PARP cleavage and cell death induced by *p28*^{GANK} knockdown (Fig. 2C and D).

p28^{GANK} *knockdown alters antiapoptotic and proapoptotic proteins of Bcl-2 family expression and causes Bax translocation into mitochondria in HepG2 cells*. Because the ratio of proapoptotic and antiapoptotic proteins of Bcl-2 family is considered as a critical factor for initiating mitochondria-mediated apoptosis (9), we examined the expression of several proteins of Bcl-2 family, including the anti-apoptotic protein Bcl-XL and the proapoptotic proteins Bax, Bid and Bak, during *p28*^{GANK} knockdown-induced apoptosis in HepG2 cells. As shown in Fig. 3A, time-dependently Bax upregulation and B cl-XL downregulation were observed in Adsi*p28*^{GANK}-infected HepG2 cells. However, no considerable changes in Bid and Bak were detected. Cleavage of Bid was not observed.

Recent studies have suggested that some apoptosis-promoting conditions induce Bax translocation into mitochondria and conformational changes in Bax that involve releasing cytochrome c (23,24). In this study, translocation of Bax into mitochondria was observed at 48 h, continuing through 72 h after that HepG2 cells were infected with Adsi*p28*^{GANK} (Fig. 3B).

p38 plays critical roles in p28^{GANK} *knockdown-induced cell death*. To investigate the role of MAPK signal transduction pathways in *p28*^{GANK} knockdown-induced apoptosis, we measured the level of MAPK phosphorylation by Western blot analysis. We found that *p28*^{GANK} knockdown induced phosphorylation of p38 and JNK rather than phosphorylation of ERK in HepG2 cells. No significant changes were observed in total-ERK, total-p38 or total-JNK (Fig. 4A). When HepG2 cells were treated with inhibitors of p38 (SB203580, 10 μM) or JNK (SP600125, 10 μM), phosphorylations of these two proteins induced by *p28*^{GANK} depletion was inhibited (data not shown). However, only SB203580 blocked apoptosis promoted by *p28*^{GANK} depletion (Fig. 4B). Moreover, SB203580 suppressed PARP cleavage induced by *p28* knockdown (Fig. 4C).

We further investigated the role of p38 in changes of ΔΨm, Bcl-2 family proteins induced by *p28*^{GANK} knockdown. As shown in Fig. 5A, SB203580 suppressed the loss of ΔΨm, but had no effect on the elevation of ΔΨm during early period after Adsi*p28*^{GANK} infection in HepG2 cells. In addition, SB203580 suppressed Bax upregulation, Bcl-XL down-regulation, the translocation of Bax into mitochondria (Fig. 5B).

ROS are involved in p28^{GANK} *knockdown-induced apoptosis and p38 phosphorylation*. Recent studies showed that ROS were involved in diverse aspects of stress response, including the induction of cell death (25,26). To determine whether *p28*^{GANK} knockdown in HepG2 cells induced ROS generation, we used DCFH-DA as an indicator to measure intracellular ROS generation. As shown in Fig. 6A, a time-dependent increase of ROS generation was observed in HepG2 cells infected with Adsi*p28*^{GANK}. We next examined the effect of ROS on *p28*^{GANK} knockdown-induced cell death using the ROS scavenge NAC. As shown in Fig. 6B and C, addition of 50 μM NAC suppressed PARP cleavage and markedly inhibited apoptosis. Furthermore, we investigated the relationship between ROS generation and p38 phosphorylation. As shown in Fig. 6C, NAC abolished *p28*^{GANK} knockdown-induced p38 phosphorylation.

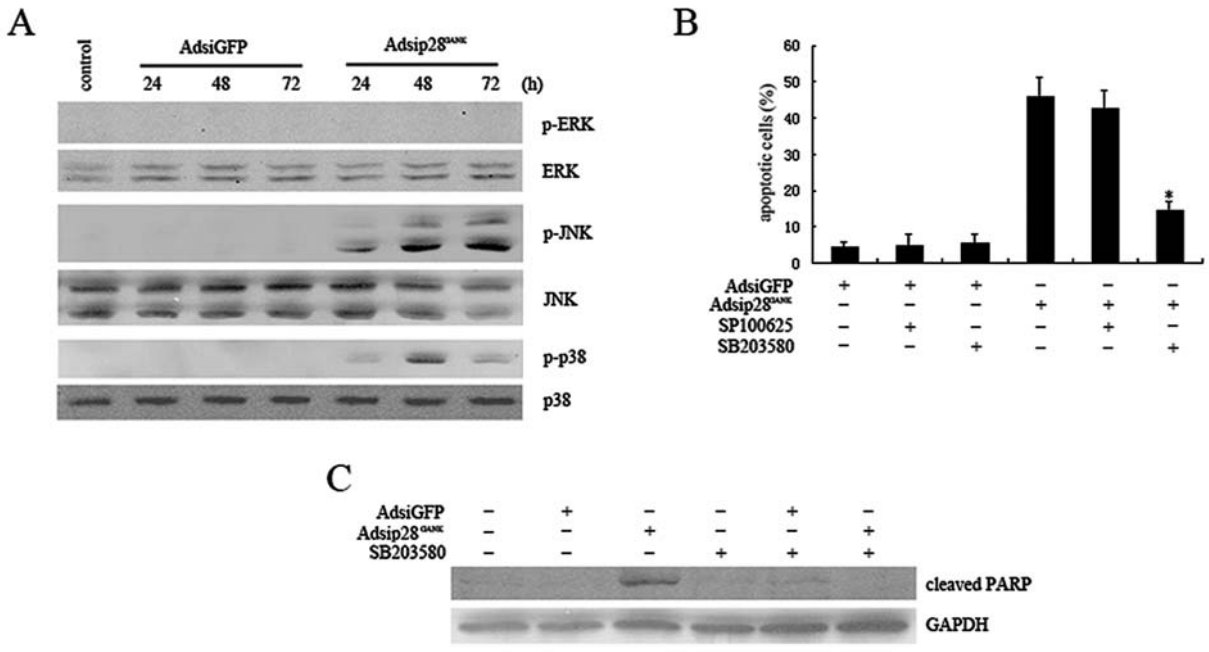

Figure 4. Involvement of p38 in *p28*^{GANK} knockdown-induced apoptosis in HepG2 cells. (A) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} for indicated time-points. Analysis of phosphor-ERK, phosphor-JNK and phosphor-p38 were performed with Western blotting. (B) Cells infected with Adsi*GFP* or Adsi*p28*^{GANK} were treated with JNK inhibitor, SP600125 (10 μM), or p38 inhibitor, SB203580 (10 μM). After incubation for 72 h, apoptotic cells were estimated by flow cytometry. Data are presented as means ± SD of three independent experiments. $^{*}p<0.05$ vs. the Adsi*p28*^{GANK}-infected group. (C) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} in the presence (+) or absence (-) of SB203580 (10 μM) incubation for 48 h. Western blotting was performed to analyze cleaved PARP. GAPDH levels were shown as controls for equal loading. (A) and (C) were representatives of three independent experiments with similar results.

Discussion

Caspases, a family of intracellular proteins which play an important role in the initiation and execution of apoptosis, are classified into initiator caspases (e.g. caspase-8 and -9) and effector caspases (e.g. caspase-3, caspase-6 and -7) (19). Caspase-8 and -9 are key initiator caspases of death receptor and mitochondrial apoptosis pathways respectively (5). Since our previous study showed that *p28*^{GANK} knockdown in hepatoma cells activated both caspase-8 and -9 (2), we examined the roles of these two caspases in *p28*^{GANK} knockdown-induced apoptosis using their inhibitors. Caspase-9 inhibitor, but not caspase-8 inhibitor, markedly protected cells from death. To further confirm that caspase-8 activation was not involved in the *p28*^{GANK} knockdown-induced cell death, we determined the proapoptotic protein expression status of Bid. Following certain types of stimuli, Bid, a BH3-domain-only molecule is cleaved by caspase-8 to generate a tBid that translocates to mitochondria, subsequently induces cytochrome c release and caspase-9 activity (9). In our study, tBid was not observed after the *p28*^{GANK} was downregulated by RNAi, indicating that the apoptosis induced by *p28*^{GANK} knockdown was caused by caspase-9 rather than -8. The ratios of antiapoptotic and proapoptotic proteins of Bcl-2 family are pivotal for controlling mitochondria-mediated cell death in several cell types (9). Our data showed that *p28*^{GANK} knockdown upregulated the expression of proapoptotic protein, Bax, and downregulated the expression of anti-apoptotic protein, Bcl-XL. These results suggested that *p28*^{GANK} depletion-induced apoptosis is due to the activation of caspase-9 which was induced by the mitochondrial dysfunction. The reason why caspase-8 had no effect on the apoptotic process induced by *p28*^{GANK} depletion is unclear.

Change of ΔΨm is one of the characteristics of mitochondrial dysfunction. Appropriate regulation of ΔΨm is essential for cellular homeostasis, for it is indispensable to generate ATP. Following certain types of apoptotic stimuli, change of ΔΨm correlated with the release of cytochrome c (27,28). Korean mistletoe lectin caused loss of ΔΨm, then triggered cytochrome c release and apoptosis in human hepatoma Hep3B cells (28). However, the elevation of ΔΨm was observed in apoptosis triggered by growth factor withdrawal, and Bcl-XL was shown to prevent the increase of ΔΨm, thereby inhibiting apoptosis (29). Our study showed that *p28*^{GANK} knockdown in HepG2 induced decrease of ΔΨm and cytochrome c release. Furthermore, a potent inhibitor of mitochondrial megachannel, BA, prevented breakdown of ΔΨm and reduced cell death induced by *p28*^{GANK} knockdown, indicating that loss of ΔΨm is involved in the apoptotic process. However, there is a transient elevation of ΔΨm before its collapse. Whether the elevation of ΔΨm contributes to the *p28*^{GANK} knockdown-induced apoptosis remains to be elucidated. The collapse of ΔΨm is thought to occur through formation of pores in the mitochondria by dimerized Bax or activated Bid (20,21). In viable cells, a substantial portion of Bax is monomeric and found either in the cytosol or loosely attached to membranes. Following a death stimulus, cytosolic and monomeric Bax translocates into the mitochondria where it becomes an integral membrane protein and cross-linkable as a homodimer, then results in the release of cytochrome c

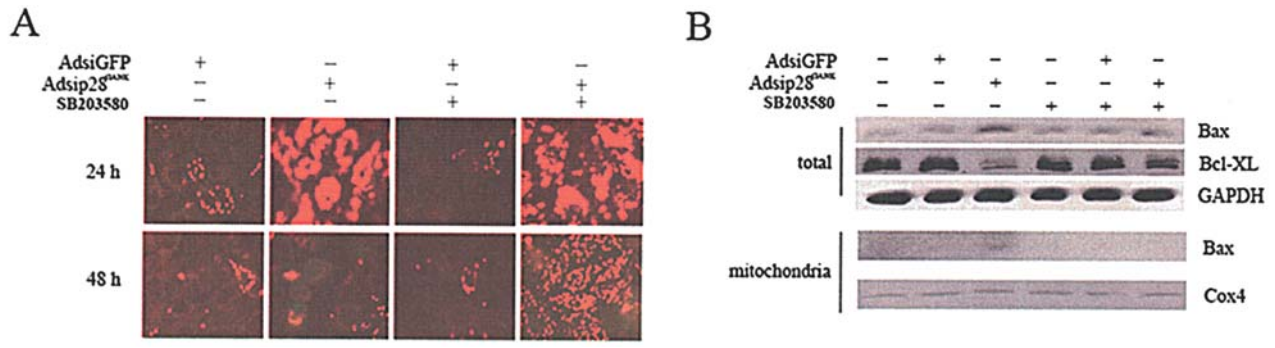

Figure 5. (A) HepG2 cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} in the presence (+) or absence (-) of SB203580 (10 μM) incubation for 24 or 48 h. After incubation with JC-1 (3 μM), the cells were observed with a fluorescence microscope (magnification x200). (B) HepG2 cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} in the presence (+) or absence (-) of SB203580 (10 μM) incubation for 48 h. Whole-cell lysates and mitochondrial fractions were prepared. Western blotting was performed to analyze the expression of Bax and Bcl-XL in whole-cell lysates as well as the expression of Bax in mitochondrial fractions. GAPDH and Cox4 are shown as controls for equal loading of whole-cell lysates and mitochondrial fractions, respectively. (A) and (B) are representatives of three independent experiments with similar results.

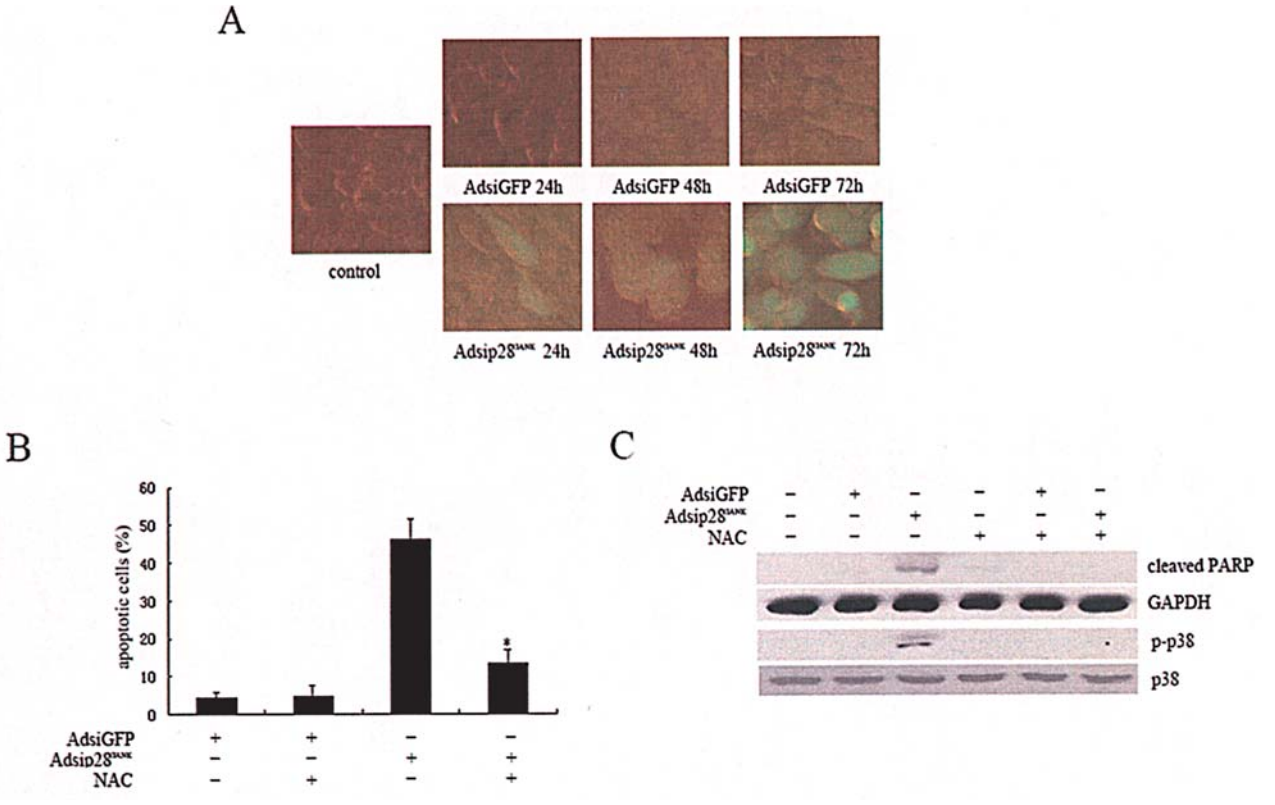

Figure 6. Involvement of reactive oxygen species (ROS) in *p28*^{GANK} knockdown-induced apoptosis in HepG2 cells. (A) Intracellular ROS were measured using 2,7-dichlorofluorescein diacetate (DCFH-DA). Cells infected with Adsi*GFP* or Adsi*p28*^{GANK} for indicated time-points were incubated with DCFH-DA (5 mM), then observed with a fluorescence microscope (magnification x200). (B) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} in the presence (+) or absence (-) of N-acetyl-L-cystenine (NAC, a ROS scavenger, 50 μM) incubation for 72 h. Apoptotic cells were analyzed by flow cytometry. Data are presented as means ± SD of three independent experiments. *p<0.05 vs. the Adsi*p28*^{GANK}-infected group. (C) Cells were infected with Adsi*GFP* or Adsi*p28*^{GANK} in the presence (+) or absence (-) of NAC (50 μM) incubation for 48 h. Western blotting was performed to analyze cleaved PARP and phopho-p38. GAPDH levels are shown as controls for equal loading. (A) and (C) were representatives of three independent experiments with similar results.

(30-33). In the present study, Bax translocation into mitochondria was observed in HepG2 cells after Adsi*p28*^{GANK} infection. However, loss of ΔΨm preceded the translocation of Bax in HepG2 cells, indicating that ΔΨm loss is only partly mediated by Bax and there are other mediators, such as p53. Following certain cytotoxic signals, a small fraction of p53 which trans-locate into mitochondria (34), directly induces permeabilization of the outer mitochondrial membrane by forming complex with the protective Bcl-XL and Bcl-2 proteins, resulting in cytochrome c release (35). In the present study, the trans-location of p53 into mitochondria was also observed after Adsi*p28*^{GANK} infection in HepG2 cells, being consistent with loss of ΔΨm (data not shown).

Growing evidence shows that MAPK signaling plays an important role in the response to mitogen or stress to

determine cell proliferation or apoptosis. The major MAPKs are ERK, JNK and p38. ERK is generally associated with cell proliferation and growth (36), while JNK and p38 are stress-inducible and involved in cytokine-mediated differentiation and cell death (12,14). Here, we observed that *p28^{GANK}* knockdown in HepG2 cells induced phosphorylation of p38 and JNK, but not ERK. It seemed that p38 contributed to *p28^{GANK}* knockdown-induced apoptosis, as suggested by the finding that this apoptotic process was inhibited by p38 specific inhibitor, SB203580. Furthermore, we demonstrated that SB203580 suppressed the decrease of ΔΨm, but not the increase of ΔΨm during early period of the apoptosis, indicating that ΔΨm elevation and ΔΨm decrease were two independent events. Moreover, SB203580 inhibited Bax upregulation, Bcl-XL downregulation. These data indicated that p38 played a key role in the apoptosis induced by *p28^{GANK}* knockdown. Although *p28^{GANK}* knockdown induced phosphorylation of JNK, the JNK inhibitor, SP600125, had no effect on the apoptosis induced by *p28^{GANK}* depletion. The role of JNK in the apoptotic process remains to be clarified.

ROS, shown to regulate activation of MAPK (15,37-39), are ubiquitously generated mainly by oxidative phosphorylation at mitochondria and are released during ATP synthesis (40). Recent studies have shown that ROS were important signal molecules modulating protein phosphorylation, and regulating cell growth, differentiation and apoptosis (41). Low levels of ROS can modify cell-signal proteins for cell growth and survival (42), however, increased generation of ROS can induce cell death (15,28,37). In the study, we observed that *p28^{GANK}* depletion in HepG2 cells induced excess generation of ROS. Moreover, a ROS scavenger, NAC, completely inhibited phosphorylation of p38 and markedly suppressed PARP cleavage and apoptosis. These data suggested that excess generation of ROS was an early event in the *p28^{GANK}* knockdown-induced apoptosis, which mediated the apoptotic process via p38 phosphorylation. However, the mechanism of *p28^{GANK}* knockdown inducing ROS generation remains to be elucidated.

In conclusion, our results indicated that *p28^{GANK}* knockdown in HepG2 cells induced ROS generation, which in turn activated p38. p38 activation then altered the ratio of Bax/Bcl-XL, caused translocation of Bax into mitochondria, resulted in the loss of ΔΨm and triggered the release of cytochrome c from mitochondria to cytosol which led to caspase-9 activation and cell death. The results provided a rationale for using *p28^{GANK}* as a therapeutic target in human hepatic cancers.

Acknowledgements

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