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A Pathway From JNK Through Decreased ERK and Akt Activities for FOXO3a Nuclear Translocation in Response to UV Irradiation

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Forkhead box O (FOXO) transcription factors play an important role in physiological and pathological processes. Extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) can phosphorylate FOXO and cause its degradation or cytoplasmic retention, respectively, leading to tumorigenesis. In addition, C-Jun N-terminal protein kinase (JNK) can promote FOXO nuclear localization, leading to apoptosis. Using confocal imaging of cells transfected with GFP-FOXO3a, we visualized the dynamic translocation of GFP-FOXO3a from the cytoplasm to the nucleus after UV irradiation in a time- and dose-dependent manner. We also found that UV irradiation caused activation of JNK, which in turn inactivated ERK and Akt, leading to FOXO3a translocation and Bim expression. Our results indicate that nuclear translocation of FOXO3a can be regulated by UV irradiation through the JNK-ERK/Akt pathway.

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Members of the class O of forkhead box transcription factors (FOXO) contain four homologous mammalian proteins: FOXO1 (FKHR), FOXO3 (FKHR-L1), FOXO4 (AFX), and FOXO6 (Greer and Brunet, 2005). FOXO factors are involved in multiple signaling pathways and physiological and pathological processes, including apoptosis, aging, proliferation, metabolism, immunity, and tumorigenesis (Burgering and Kops, 2002; Tran et al., 2003; Accili and Arden, 2004; Brunet et al., 2004; Arden, 2007; Van der Horst and Burgering, 2007; Fu and Tindall, 2008). These factors are often inactivated in a variety of tumors. For example, FOXO3a was found mainly in the cytoplasm of human breast tumor cells, closely correlated with poor prognosis (Nicholson and Anderson, 2002; Brunet et al., 2004; Hu et al., 2004). In addition, the activation of JNK-regulated FOXO factors was shown to enhance resistance to oxidative stress and longevity in animals (An and Blackwell, 2003; Essers et al., 2004; Chong et al., 2005). Furthermore, stimulation by cell death signals or growth factor deprivation causes FOXO factors reside in the nucleus and are active as transcription factors, thereby resulting in pro-apoptotic signaling via induction of TRAIL, FasL, or Bim (Brunet et al., 1999; Dijkers et al., 2000; Urbich et al., 2005; Griswold et al., 2008; Wang et al., 2009).

Subcellular localization of FOXO factors plays a major role in the regulation of their activities and functions (Zanella et al., 2008). These activities are tightly controlled by multiple signaling pathways, including PI3K/Akt, RAS/ERK, and stress/JNK. FOXO factors are negatively regulated by a number of survival signals, such as extracellular signal-regulated kinase (ERK), Akt, and IKK (Nicholson and Anderson, 2002; Hu et al., 2004). Higher basal activation of ERK and Akt signaling causes the retention of FOXO factors in the cytoplasm or degradation through the proteasome pathway. Akt has been shown to modulate the activities of FOXO factors, such as FOXO1, FOXO3a, and FOXO4, through phosphorylation at three conserved serine/threonine residues (Thr32, Ser253, and Ser315 of FOXO3a; Burgering and Kops, 2002; Tran et al., 2003). This modulation leads to the release of FOXO factors from the DNA and translocation to the cytoplasm, where 14-3-3 protein binds to the phosphorylated FOXO factors and retains them in the cytoplasm by masking their nuclear localization signals and preventing their reentry into the nucleus (Sunayama et al., 2005; Rinner et al., 2007; Nielsen et al., 2008). ERK have recently been shown to down-regulate FOXO3a by directly phosphorylating FOXO3a at Ser 294, Ser 344, and Ser 425, and to cause degradation of FOXO3a via the murine double minute-2 (MDM2) pathway, which consequently promotes cell survival and anti-apoptosis (Yang et al., 2008).

Abbreviations: FOXO3a, Forkhead boxO3; UV, ultraviolet; LCSM, laser confocal scanning microscopy; FRAP, flourescence recovery after photobleaching; MDM2, murine double minute-2; ERK, extracellular-regulated kinase; JNK, C-jun N-terminal kinase; Akt, protein kinase B; shRNA, short hairpin RNA

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Thus, it seems plausible that ERK and Akt signals may be involved in the regulation of FOXO factors.

Recent studies reported that JNK activation could respond to a variety of environmental insults, including UV irradiation and oxidative stress (Davis, 2000; Kockel et al., 2001). The roles of JNK in the regulation of FOXO activity at multiple levels and modes have been identified in mammalian cells, *Caenorhabditis elegans* and *Drosophila* (Essers et al., 2004; Wang et al., 2005b). JNK has been shown to phosphorylate 14-3-3, releasing FOXO to enter the nucleus (Sunayama et al., 2005; Nielsen et al., 2008). Phosphorylation of FOXO4 by JNK at threonine 447 and threonine 451 induces FOXO4 activation and retains FOXO4 in the nucleus (Essers et al., 2004). JNK phosphorylation sites in FOXO4 are found in a region with a low degree of sequence similarity to other members of the FOXO4 family (Anderson et al., 1998; Biggs et al., 2001; Van Der Heide et al., 2004; Sunters et al., 2006). Additional mechanisms of FOXO regulation by JNK may be related to the JNK-dependent nuclear localization of FOXO factors. Under certain conditions, JNK may also regulate FOXO activities by effecting MST1 activation (Bi et al., 2009). Recently, stress/JNK signaling was reported to inhibit ERK activation, promote apoptosis, and limit cell growth by antagonizing survival/growth signaling (Wang et al., 2005b; Friedman and Perrimon, 2006; Luo et al., 2007; Karpac and Jasper, 2009). Typically, FOXO factors become phosphorylated and localized in the cytoplasm in response to survival and growth factors. However, the presence of stress stimuli overrides the sequestration of FOXO by growth factors in mammalian cells during stress events, FOXO remains in the nucleus despite the presence of survival and growth factors (Brunet et al., 2001; Wang et al., 2005a,b). These results allow us to speculate that JNK could mediate FOXO activities by counterbalancing ERK and Akt signals.

Based on previous studies (Brunet et al., 2001; Wang et al., 2005a,b; Luo et al., 2007; Karpac and Jasper, 2009), we investigated interactions among JNK, ERK/Akt and FOXO3a to illustrate how JNK initiates FOXO3a nuclear translocation. Using UV irradiation as stimulation, we focused on the following areas: (i) the subcellular localization of FOXO3a, (ii) the effect of JNK activation on ERK/Akt activities, (iii) the effect of ERK/Akt activities on FOXO3a nuclear translocation and Bim expression, and (iv) the mechanism of FOXO3a nuclear translocation under UV irradiation.

Materials and Methods

Reagents, plasmids, and antibodies

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY). LipofectamineTM 2000 Reagent was purchased from Invitrogen (Carlsbad, CA). DNA Extraction kits were purchased from Qiagen (Valencia, CA). Epidermal growth factor (EGF, diluted in DMSO) was purchased from PeproTech (Rocky Hill, NJ). Wortmannin was purchased from BIOMOL Research Laboratories, Inc. (Plymouth, PA). SP600125 was purchased from Sigma (St. Louis, MO). U0126 was purchased from Biosource (Camarillo, CA). Plasmids for GFP-FOXO3a were a gift from Prof. Wolfgang Link (Zanella et al., 2008). The oligonucleotides for shRNA-JNK and shRNA-FOXO3a were purchased from GenePharma (Shanghai, China). Nuclear and cytoplasmic protein extraction kits were purchased from Calbiochem (ProteinExtract™, Darmstadt, Germany). Protease inhibitor cocktail set I was purchased from Calbiochem (cat.No.539131; San Diego, CA). Antibodies to Akt, pAkt (Thr308), ERK1/2, pERK1/2 (Thr202/Tyr204), JNK, pJNK (Thr183/Tyr185), Bim, Caspase-3, FOXO3a, and pFOXO3a (Ser253) were purchased from Cell Signaling (Hitchin, UK). Anti-β-actin was purchased from Santa Cruz (La Jolla, CA). IRDye RDye® 800CW anti-rabbit IgG and Alexa Fluor 680® goat anti-Mouse IgG were purchased from Molecular Probes (Eugene, OR).

Cell culture and treatments

Cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. For fluorescence experiments, cells were transiently transfected with plasmids using lipofectamine 2000 (Invitrogen) in a 35-mm dish. The medium was replaced with fresh culture medium after 5 h. The efficiency of transfection was approximately 60% as determined by fluorescent protein, and maximal levels of protein expression were observed between 24 and 48 h.

During UV irradiation treatment (Wang et al., 2009), medium was removed and saved, cells were rinsed with PBS and irradiated, and medium was restored after treatment. Unless otherwise specified, cells were exposed to UV irradiation and observed at the time indicated. For experiments with the inhibitors or growth factors, cells were pre-incubated with either Wortmannin (100 nM), SP600125 (20 μM), U0126 (10 mg/ml), or EGF (50 ng/ml) 30 min before UV irradiation. The inhibitors or growth factors were kept in the medium throughout the experimental process. After irradiation, cells were observed using LCSM or allowed to recover at 37°C for the indicated periods of time before extraction.

Nuclear translocation assays

To observe nuclear translocation of FOXO3a, ASTC-a-1 cells were transfected with GFP-FOXO3a before specific treatments. Subcellular GFP localization was assessed by using a 488 nm excitation light from an argon laser and a 500–550 nm band-pass filter. Cells showing nuclear translocation of GFP-FOXO3a were usually scored under an inverted fluorescence microscope using a 20× objective.

Fluorescence recovery after photobleaching (FRAP, Wasiak et al., 2007; Sheridan et al., 2008) was carried out to further assess nuclear translocation of GFP-FOXO3a in single cells. During the experiment, the probe (GFP) was selectively bleached by repeated scanning of the nucleus with the 488 nm laser line. The GFP in this region received high intensity illumination, which causes their fluorescence lifetime to quickly decrease. The image in the microscope is that of a uniformly fluorescent field with a noticeable dark spot due to the loss of fluorescence. If FOXO3a is able to migrate into the nucleus, the still-fluorescing probes in the cytoplasm will diffuse into the nucleus and replace the non-fluorescent probes in the bleached region such that the dark area will gradually increase in brightness.

Western blot assays

Cells were extracted in lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% TritonX-100, 100 μg/ml PMSF] supplemented with protease inhibitor cocktail set I on ice for 60 min (Wang et al., 2009). After centrifugation, cell lysates (about 30–50 μg protein extract was loaded on each lane) were resolved by 10–15% SDS–PAGE gel (10 × 10 cm^2), and then transferred onto BioTraceTM NT nitrocellulose membranes (Pall). The resulting membrane was blocked with 5% skim milk and incubated with designated primary and secondary antibodies. Fluorescent signals were then detected with an ODYSSEY1 Infrared Imaging System (LI-COR, Lincoln, NE). To extract cytoplasm proteins, the pellet was resuspended by cytoplasm extraction buffer [10 mM Hepes or Tris (pH 7.5), 40 mM KCl, 2 mM $MgCl_2$, 10% glycerol, 1 mM NaPPi, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM NaVO4, 1 mM NaF, 1 mM PMSF]. The solution was put into a 15-ml glass pestle, crushed 25–30 times on ice, and spun down at 2000 rpm for 5 min at 4°C. The supernatant was removed and transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was used as the cytoplasmic protein solution.

Three milliliter of 0.35 M sucrose buffer [1.2 g sucrose, 1 M $MgCl_2$, 1 M Hepes (pH 7.5)] was added to 14 ml round bottom centrifuge tube. Pellet in 3 ml 0.25 M sucrose buffer [0.855 g sucrose, 1 M $MgCl_2$, 1 M Hepes (pH 7.5)] was resuspended.

Resuspended pellet was carefully laid over the 0.35 M sucrose buffer. Supernatant was collected after centrifugation at 4°C for 5 min at 1,430 × *g*. Then the pellet was resuspended in nuclear lysis buffer [10 mM Hepes or Tris (pH 7.5), 500 mM NaCl, 1% Triton-X100, 10% glycerol, 1 mM NaPPi, 1 (g/ml pepstatin, 1 (g/ml aprotinin, 1 (g/ml leupeptin, 1 mM $NaVO_4$, 1 mM NaF, 1 mM PMSF). The pellet was then sonicated for 30 sec with a 1-min rest five to eight times at power setting 5. The pellet was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was used as nuclear protein solution. The cytosolic and nuclear protein solutions were analyzed by Western blot analysis.

RNA interference (RNAi)

JNK1/2 suppression was accomplished using JNK1/2 short hairpin RNA (shRNA-JNK) constructs. The target sequence for the down-regulation of both JNK isoforms was 5′-GAAAGAATGTCCTACCTTC-3′ (Chen et al., 2010). FOXO3a suppression was accomplished using FOXO3a short hairpin RNA (shRNA-FOXO3a) constructs. The oligonucleotides for shRNA were synthesized as follows (Barreyro et al., 2007): shRNA-FOXO3a:5'-GACTCCGGGTCCAGCTCCACTTCAAGAGA-GTGGAGCCGGAGTTT-3', shRNA-NC: 5'-GTTCTCCGAAC-GTGTCACGTCAAGAGATTACGTGACACGTTCGGAG-AATT-3'. The shRNA sequences were transfected into cells using TurboFect siRNA Transfection Reagent (Fermentas, Vilnius, Lithuania) in a 35-mm dish and incubated for 36 h.

Statistical analysis

All data represent at least three independent experiments and are expressed as mean ± SEM. Differences between groups were compared by Student's *t*-test using SPSS software, and significance was defined as $P < 0.005$.

Results

Translocation of FOXO3a from cytoplasm to nuclei in ASTC-a-1 cells under UV irradiation

To monitor the nuclear translocation of FOXO3a upon UV irradiation, cells were transfected by GFP-FOXO3a expression plasmid. In healthy cells, GFP-FOXO3a was distributed diffusely in the cytoplasm only (control group, Fig. 1A). However, UV irradiation resulted in dynamic nuclear translocation of GFP-FOXO3a from the cytoplasm. FRAP was performed in order to further confirm these findings. As shown in Fig. 1B,C, rapid detectable fluorescence recovery of GFP in the nucleus was observed 2 h after UV treatment, suggesting that FOXO3a undergoes translocation from the cytoplasm to the nucleus after UV irradiation.

FOXO3a activation by UV irradiation was dose- and time-dependent: as shown in Fig. 1D, the percentage of cells exhibiting GFP-FOXO3a nuclear translocation increased with UV irradiation dose and time. When cells were treated with a low dose of UV irradiation (40 mJ/cm^2), translocated FOXO3a returned to the cytoplasm (Fig. 1E). However, nuclear translocation was irreversible at a higher dose of UV irradiation (80 mJ/cm^2, Fig. 1A,D). As shown in Figure 1F, quantitative analysis of nuclear areas A and B in Figure 1E further confirmed this finding.

JNK plays an important role in FOXO3a translocation under UV irradiation

Western blot assays were performed to test the effect of JNK activation on the subcellular localization of FOXO3a in response to UV irradiation. As shown in Figure 2A, an increase in pJNK (Thr183/Tyr185) (activated JNK) and nuclear-FOXO3a, as well as a decrease in pFOXO3a (Ser253) (inactivated FOXO3a), was seen in the samples after UV irradiation (Fig. 2A, lanes 2 and 6, Fig. 2B–G). Knockdown of endogenous JNK by shRNA-JNK (Fig. 2A, lanes 3 and 7) or treatment with JNK inhibitor SP600125 (Fig. 2A, lanes 4 and 8) before UV irradiation resulted in increased phosphorylation of FOXO3a at the Ser-253 site and blocked FOXO3a nuclear movement (Fig. 2B–G).

Real-time single-cell analysis was applied to further confirm our findings. As shown in Fig. 2H, the nuclear translocation of FOXO3a was delayed either by suppressing the expression of endogenous JNK or by preincubating with JNK inhibitor. Quantitative analysis of nuclear-positive cells (GFP fluorescence from nuclei, Fig. 2I) and comparison of FOXO protein levels in nuclei and cytoplasm (relative fluorescence of GFP in nuclei and cytoplasm, Fig. 2J) showed consistent results. Together, these data suggest that JNK activation plays an important role in FOXO3a phosphorylation and sub-cellular localization in response to UV irradiation.

ERK and Akt activities affect nuclear translocation of FOXO3a under UV irradiation

To determine the effect of ERK and Akt on FOXO3a movement, we first detected the activities of ERK and Akt under UV irradiation. As shown in Figure 3A, UV irradiation resulted in decreased levels of both ERK and Akt phosphorylation (lane 2), similar to those of cells treated by U0126 (lane 3) or Wortmannin (lane 4), respectively. There was no apparent difference in the levels of pAkt (Thr308) (activated Akt) and pERK1/2 (Thr202/Tyr204) (activated ERK) between the co-treatment group (lane 6) and the UV irradiation treatment alone group (lane 2). We also found that inactivation of ERK and/or Akt resulted in decreased levels of pFOXO3a (Ser253), as shown in Fig. 3B, and promoted FOXO3a nuclear translocation, as shown in Fig. 3C.

To further determine whether inactivation of ERK and Akt could cause nuclear localization of FOXO3a, real-time single-cell and FRAP analyses were performed. As shown in Figure 3D, GFP-FOXO3a was translocated from the cytoplasm to the nucleus in cells treated with Wortmannin and/or U0126. As shown in Figure 3E, fluorescence recovery of GFP was observed in nuclei after cells were incubated with either Wortmannin and/or U0126. Moreover, quantitative analysis of nuclear-positive cells (green fluorescence from nuclei) further confirmed these findings (Fig. 3F). Combining these results with our findings of diminished pERK1/2 (Thr202/Tyr204) and pAkt (Thr308) under UV irradiation (Fig. 3A, lane 2 and Fig. 3B), we hypothesize that FOXO3a translocation triggered by UV irradiation is caused by ERK and Akt activities.

Downregulation of ERK and Akt via JNK to affect nuclear translocation of FOXO3a under UV irradiation

Two assays were performed to study the mechanism of FOXO3a nuclear translocation. First, we detected the activities of JNK, ERK, Akt, and FOXO3a after UV irradiation using Western blot assays. As shown in Figure 4A–C, the levels of total JNK, ERK, Akt, and FOXO3a proteins remained the same after UV irradiation. However, the level of pJNK (Thr183/Tyr185) was significantly increased while the levels of pAkt (Thr308), pERK1/2 (Thr202/Tyr204), and pFOXO3a (Ser253) were significantly decreased.

Second, we used shRNA-JNK and SP600125 to determine whether inactivation of ERK and Akt is regulated by JNK in response to UV irradiation. As shown in Figure 2A, UV irradiation resulted in lower levels of both pAkt (Thr308) and pERK1/2 (Thr202/Tyr204) (lanes 2 and 6), which could be blocked by adding shRNA-JNK (lanes 3 and 7) or SP600125 (lanes 4 and 8). Moreover, we observed the inhibition of both ERK and Akt, as well as activation of JNK and FOXO3a, in cells that underwent UV irradiation (Fig. 4D,H). Combining UV irradiation with EGF (lane 4), we found decreased levels of pAkt

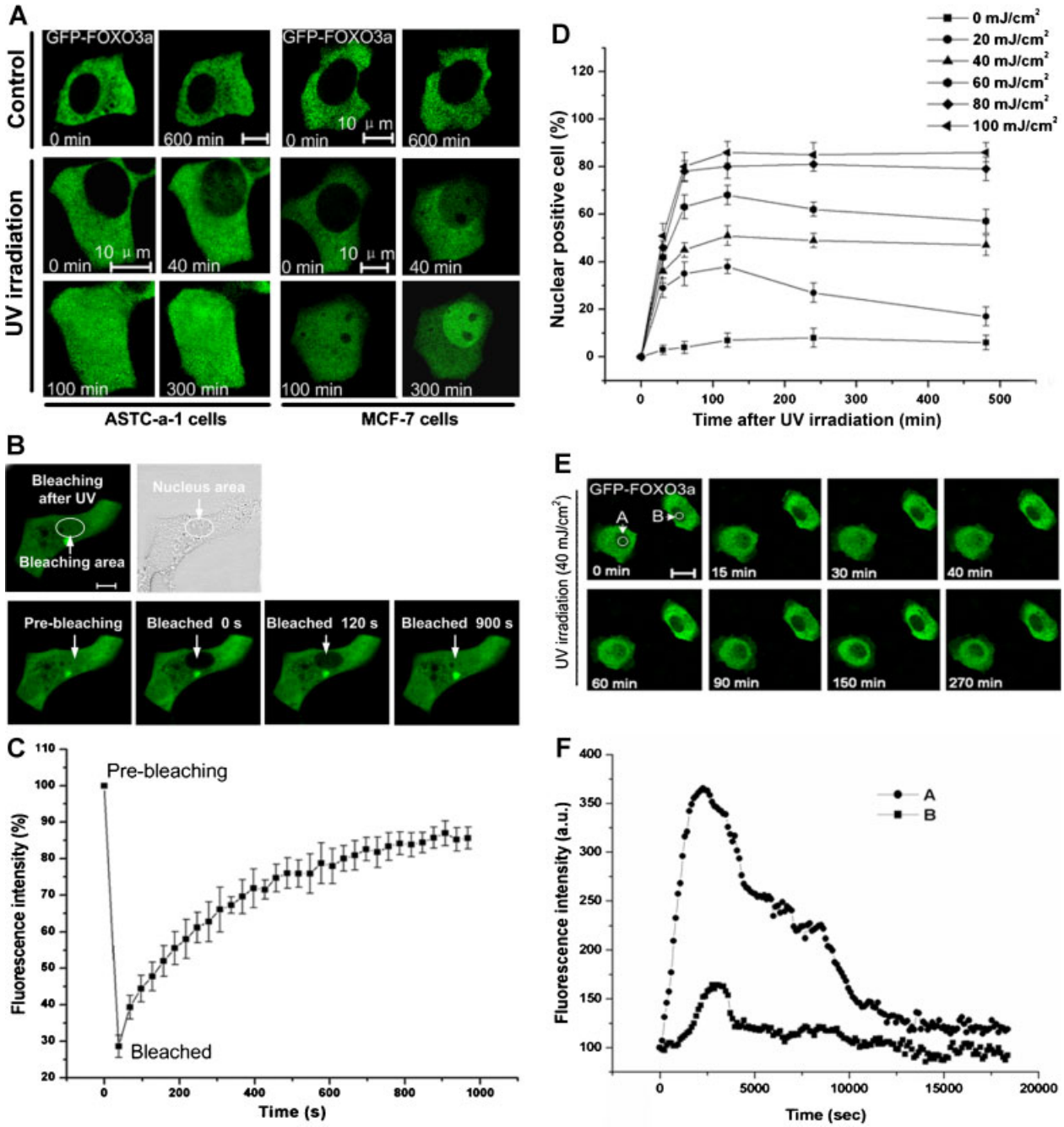

Fig. 1. Translocation of GFP-FOXO3a from cytoplasm to nuclei in response to UV irradiation. A: Cells were transfected with GFP-FOXO3a and subjected to UV irradiation (80 mJ/cm^2). The translocation of GFP fluorescence was monitored by laser confocal scanning microscopy (LCSM). Bar, 10 μm. B: ASTC-a-1 cells were transfected with GFP-FOXO3a and subjected to UV irradiation. About 2 h after UV irradiation (80 mJ/cm^2), the marked regions containing GFP-FOXO3a in the nucleus were photobleached (0 sec), and fluorescence recovery was monitored over time (900 sec). Bar, 5 μm. C: Relative fluorescence intensity of GFP-FOXO3a, indicated in (B) and recorded during photobleaching, was plotted as a function of time (n = 5 cells). D: The percentage of ASTC-a-1 cells undergoing GFP-FOXO3a nuclear translocation after the indicated time and dose of UV irradiation. The number of nuclear GFP cells (nuclear positive cell) was determined according to the majority of GFP fluorescence in the nucleus. Data represented mean ± SEM from the average of 10 fields from 100 cells for each treatment. E: ASTC-a-1 cells were transfected with GFP-FOXO3a and subjected to UV irradiation (40 mJ/cm^2). The translocation of GFP fluorescence was monitored by confocal laser scanning microscopy. Bar, 20 μm. F: Relative GFP fluorescence intensities in nuclear areas A and B of cells indicated in (E) were recorded after UV irradiation. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

(Thr308), pERK1/2 (Thr202/Tyr204), and pFOXO3a (Ser253) proteins, compared to EGF treatment alone (lane 3). When cells were pretreated with SP600125 before UV irradiation (lane 6), we found inhibition of JNK activation, higher basal activation of ERK and Akt, and reduction of FOXO3a activity, compared to UV treatment alone (lane 2). Statistical analysis further confirmed that UV irradiation increased the levels of pJNK (Thr183/Tyr184) (Fig. 4E,I) and decreased the levels

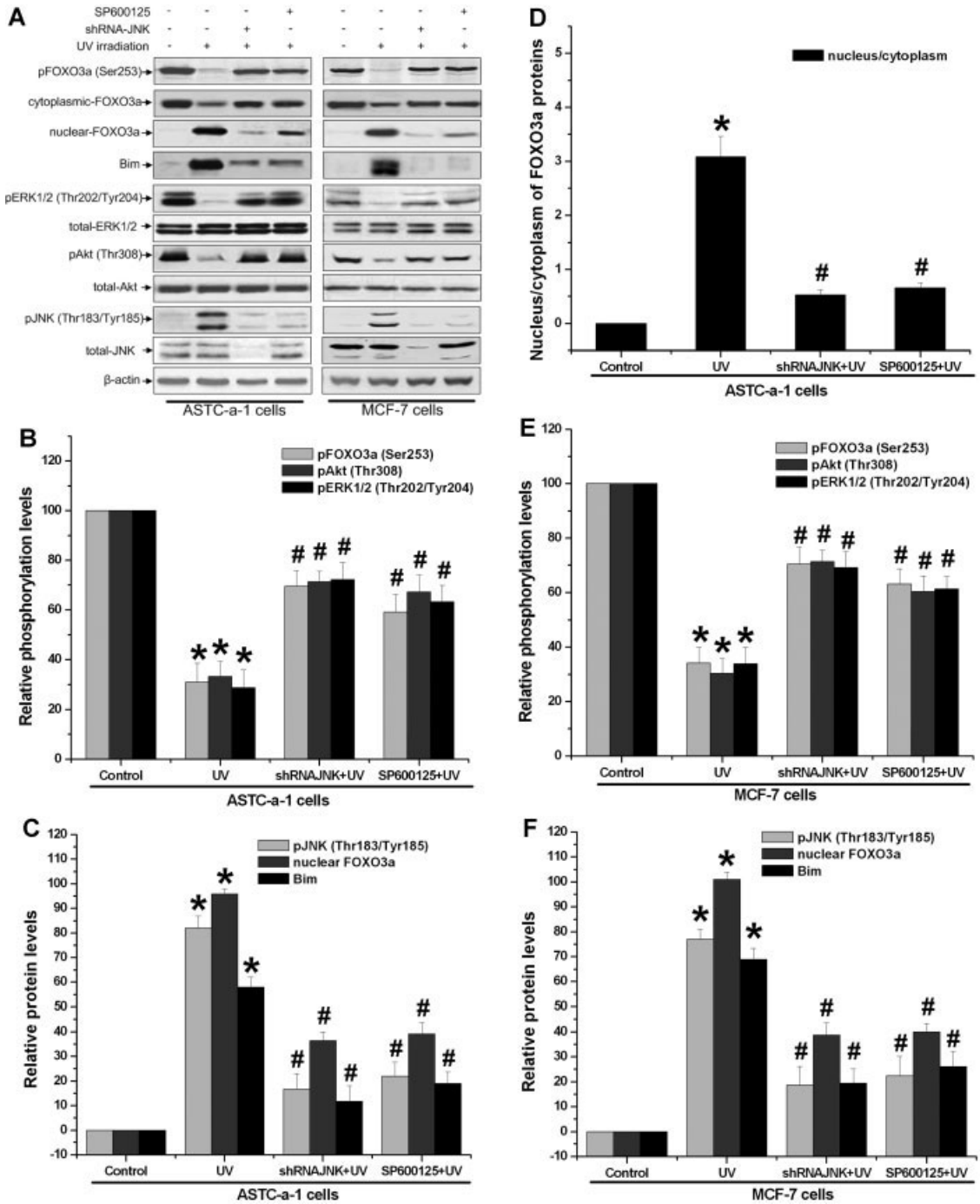

Fig. 2. Delay of nuclear translocation of FOXO3a and Bim expression due to suppression of endogenous JNK and inhibition of JNK activation under UV irradiation. A: ASTC-a-1 and MCF-7 cells were treated with UV irradiation (80 mJ/cm^2), or pretreated with shRNA-JNK or SP600125 before UV irradiation, or untreated (Control). About 2 h after UV irradiation, Western blot analysis was performed to detect the levels of JNK, pJNK (Thr183/Tyr185), total-Akt, pAkt (Thr308), total-ERK1/2, pERK1/2 (Thr202/Tyr204), nuclear-FOXO3a, cytoplasmic-FOXO3a, pFOXO3a (Ser253), and Bim. β-actin was used as a control. B-D: Quantitative analysis of the levels of pFOXO3a (Ser253), pAkt (Thr308), pERK1/2 (Thr202/Tyr204), pJNK (Thr183/Tyr185), nuclear-FOXO3a, Bim, and nucleus-cytoplasm ratio (N/C) of FOXO3a in ASTC-a-1 cells after different treatments. Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. control, #$P < 0.005$ vs. UV treatment). E–G: Quantitative analysis of the levels of pFOXO3a (Ser253), pAkt (Thr308), pERK1/2 (Thr202/Tyr204), pJNK (Thr183/Tyr185), nuclear-FOXO3a, Bim, and nucleus–cytoplasm ratio (N/C) of FOXO3a in MCF-7 cells after different treatments. Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. control, #$P < 0.005$ vs. UV treatment). H: ASTC-a-1 cells were transfected with GFP-FOXO3a and preincubated with shRNA-JNK or SP600125 (20 μM) before UV irradiation (80 mJ/cm^2), the translocation of GFP fluorescence was monitored by LCSM. Bar, 10 μm. I: The percentage of ASTC-a-1 cells undergoing GFP-FOXO3a nuclear translocation in response to the indicated treatments. The number of nuclear GFP cells (nuclear positive cells) was determined according to the majority of GFP fluorescence in the nucleus. Data represent mean ± SEM of three independent experiments with 300 cells per condition (*$P < 0.005$ vs. control, #$P < 0.005$ vs. UV treatment). J: Quantitative analysis of comparison of GFP fluorescence in nuclear and cytoplasmic compartments in response to the indicated treatments. Data represent mean ± SEM of three independent experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

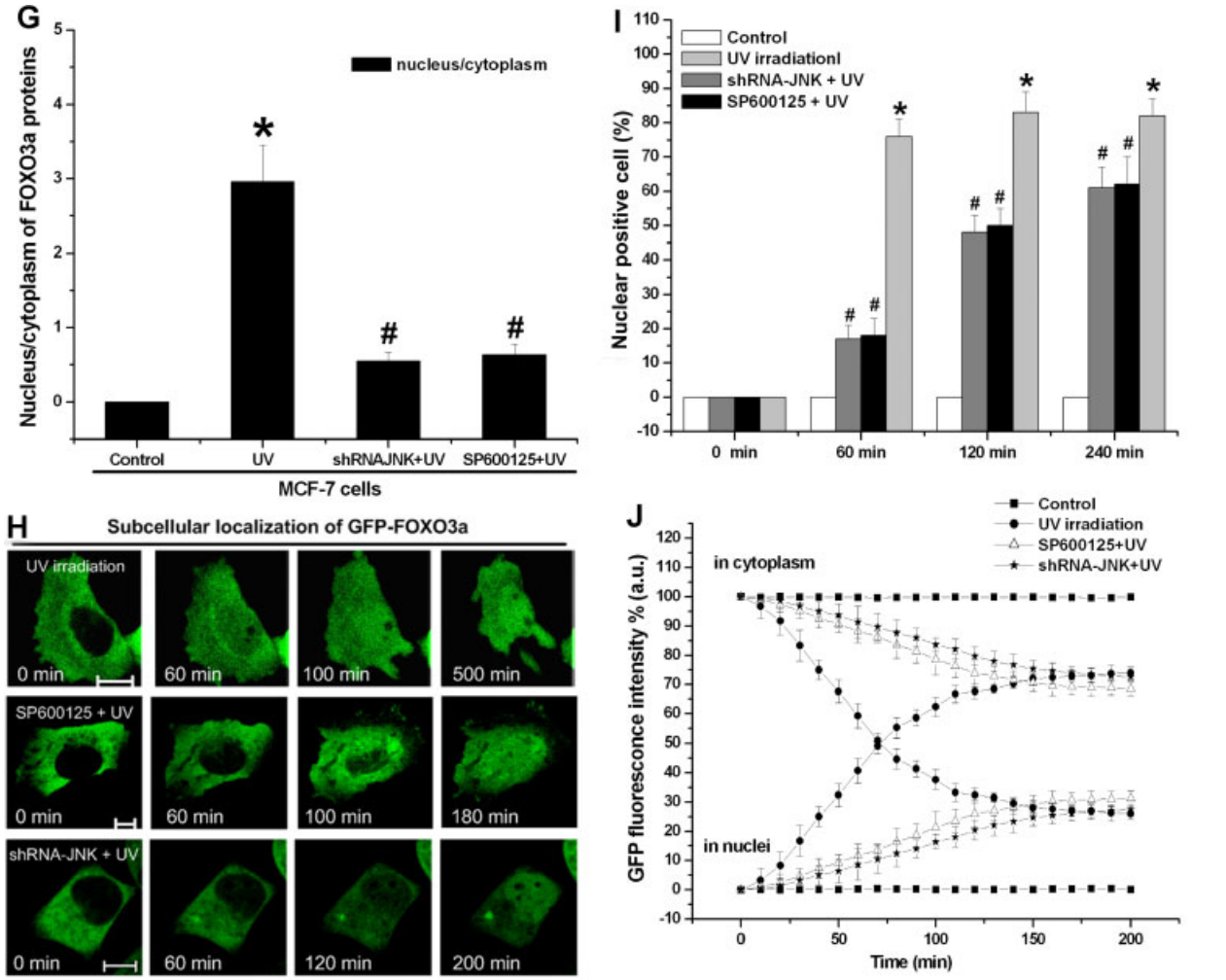

Fig. 2. (*Continued*)

of pAkt (Thr308), pERK1/2 (Thr202/Tyr204), and pFOXO3a (Ser253) (Fig. 4F,J), leading to nuclear translocation of FOXO3a (Fig. 4G,K). These data suggest that UV irradiation leads to JNK activation that in turn inactivates basal ERK and Akt activities, which results in FOXO3a nuclear translocation.

FOXO3a promotes UV irradiation-mediated apoptosis

To determine the effect of FOXO3a on apoptosis, we analyzed the expression of Bim and the activity of Caspase-3 under UV irradiation. We found that UV irradiation decreased the levels of pFOXO3a (Ser253), promoted FOXO3a nuclear movement and resulted in Bim expression, as shown in Fig. 2A,C,F. Moreover, knockdown of endogenous FOXO3a in cells by RNAi (Fig. 5C, lanes 2 and 5D) reduced Bim expression. Furthermore, Caspase-3 was cleaved under UV irradiation (Fig. 5A,B); however, this cleavage was not detected in cells pre-treated with shRNA-JNK. These data suggest that UV irradiation induces FOXO3a nuclear translocation and promotes cell apoptosis.

Discussion

We used laser scanning microscopy and biochemical assays to study the mechanisms that modulate FOXO3a subcellular localization. Our data demonstrate that FOXO3a translocation from the cytoplasm to the nucleus in response to UV irradiation is correlated with the activity of JNK and is inversely related to the activation of ERK and Akt. Under normal culture conditions (with 10% serum), GFP-FOXO3a was distributed exclusively in the cytoplasm, whereas FOXO3a showed a dynamic translocation from the cytoplasm to the nucleus in response to UV irradiation. Interestingly, we observed that GFP-FOXO3a could return to the cytoplasm after low-dose UV irradiation (below 40 mJ/cm^2, Fig. 1D–F), but not after high-dose UV irradiation (above 80 mJ/cm^2, Fig. 1A,D). It is therefore possible that there is a mechanism at low-dose UV irradiation that allows reversal of FOXO3a translocation. However, under high doses of UV stimuli, translocation is irreversible.

FOXO is negatively regulated by a number of survival signaling pathways, such as ERK, Akt, and IKK (Nicholson and Anderson, 2002; Hu et al., 2004; Sunters et al., 2006; Yang et al., 2008). Phosphorylation by Akt and ERK inhibit FOXO activity by promoting its nuclear export or proteasome-mediated degradation. High levels of pAkt (Thr308)/pERK1/2 (Thr202/Tyr204) cause inactivation and nuclear export of GFP-FOXO3a in healthy cells (Fig. 1A). With the inhibition of ERK and Akt, the GFP-FOXO3a fusion protein is rapidly localized in the nucleus (Fig. 3). Moreover, knockdown of JNK by RNAi or inhibition of

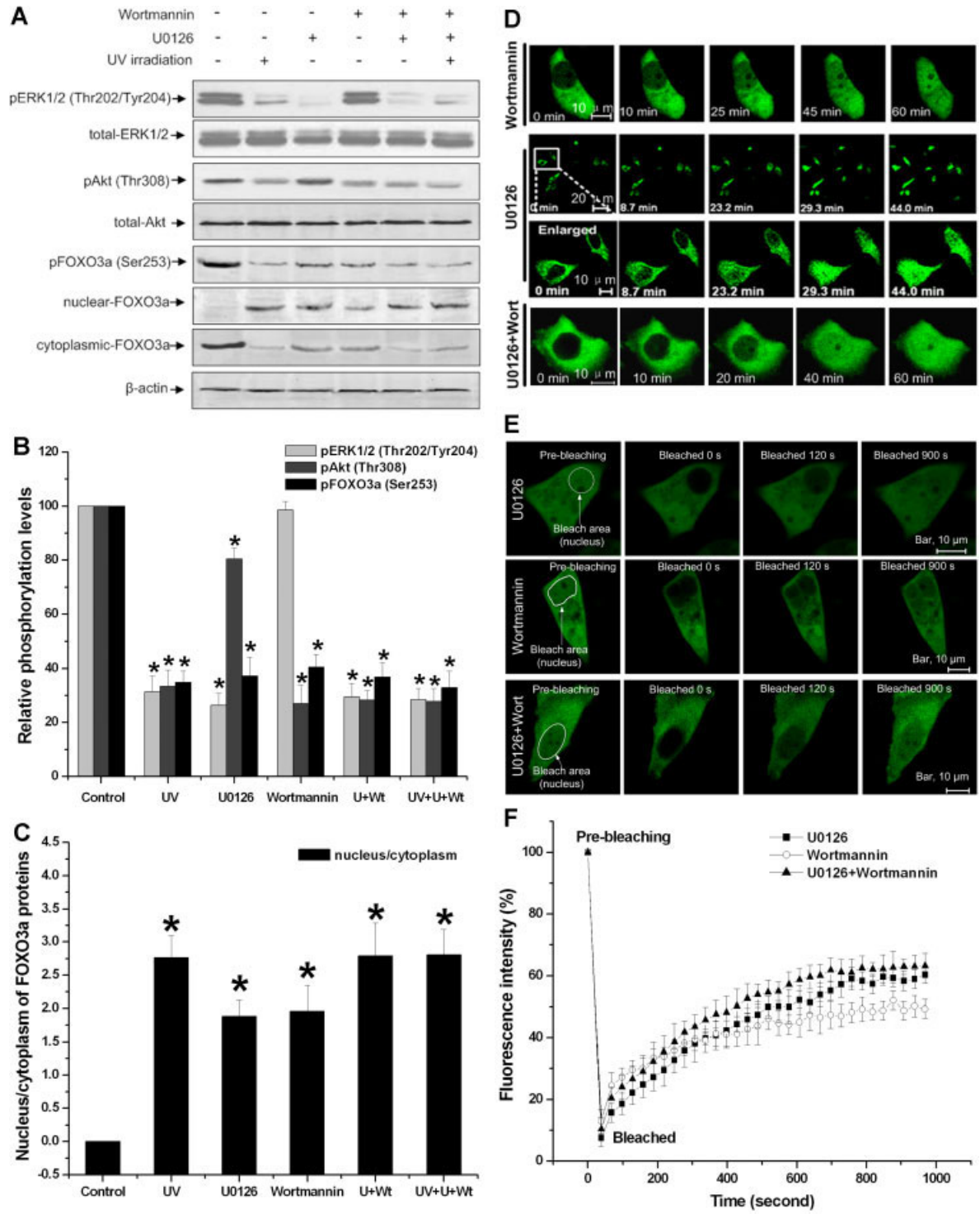

Fig. 3. FOXO3a nuclear translocation due to inactivation of ERK and Akt after UV irradiation. A: The lysates from ASTC-a-1 cells treated with UV irradiation (2 h after 80 mJ/cm^2 UV light, lane 2), U0126 (10 mg/ml, lane 3), Wortmannin (100 nM, lane 4), combination with U0126 and Wortmannin (lane 5), combination with two inhibitors (U0126 and Wortmannin) and UV irradiation (lane 6), or untreated (lane 1) were subjected to Western blot analysis with the antibodies of total-ERK1/2, pERK1/2 (Thr202/Tyr204), total-Akt, pAkt (Thr308), total-FOXO3a nuclear-FOXO3a, cytoplasmic-FOXO3a, and pFOXO3a (Ser253). β-actin was used as a control. B,C: Quantitative analysis of levels of pFOXO3a (Ser253), pAkt (Thr308), pERK1/2 (Thr202/Tyr204), and nucleus–cytoplasm ratio (N/C) of FOXO3a in ASTC-a-1 cells after different treatments. Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. control, #$P < 0.005$ vs. UV treatment). D: ASTC-a-1 cells were transfected with GFP-FOXO3a and pretreated with the inhibitor of PI3K/Akt (Wortmannin, 100 nM) and/or with the inhibitor MEK1/2 (U0126, 10 mg/ml); the translocation of GFP fluorescence was monitored by LCSM. Bar, 10 and 20 μm (middle, pre-enlarged). E: ASTC-a-1 cells were transfected with GFP-FOXO3a and pretreated with Wortmannin (Wort, 100 nM) and/or U0126 (10 mg/ml) for about 30 min. The marked regions containing GFP-FOXO3a in the nuclei were photobleached (0 sec), and fluorescence recovery was monitored over time (900 sec). Bar, 10 μm. F: Relative fluorescence intensity of GFP-FOXO3a, as indicated in (E) and recorded during photobleaching, was plotted as a function of time (n = 5 cells). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

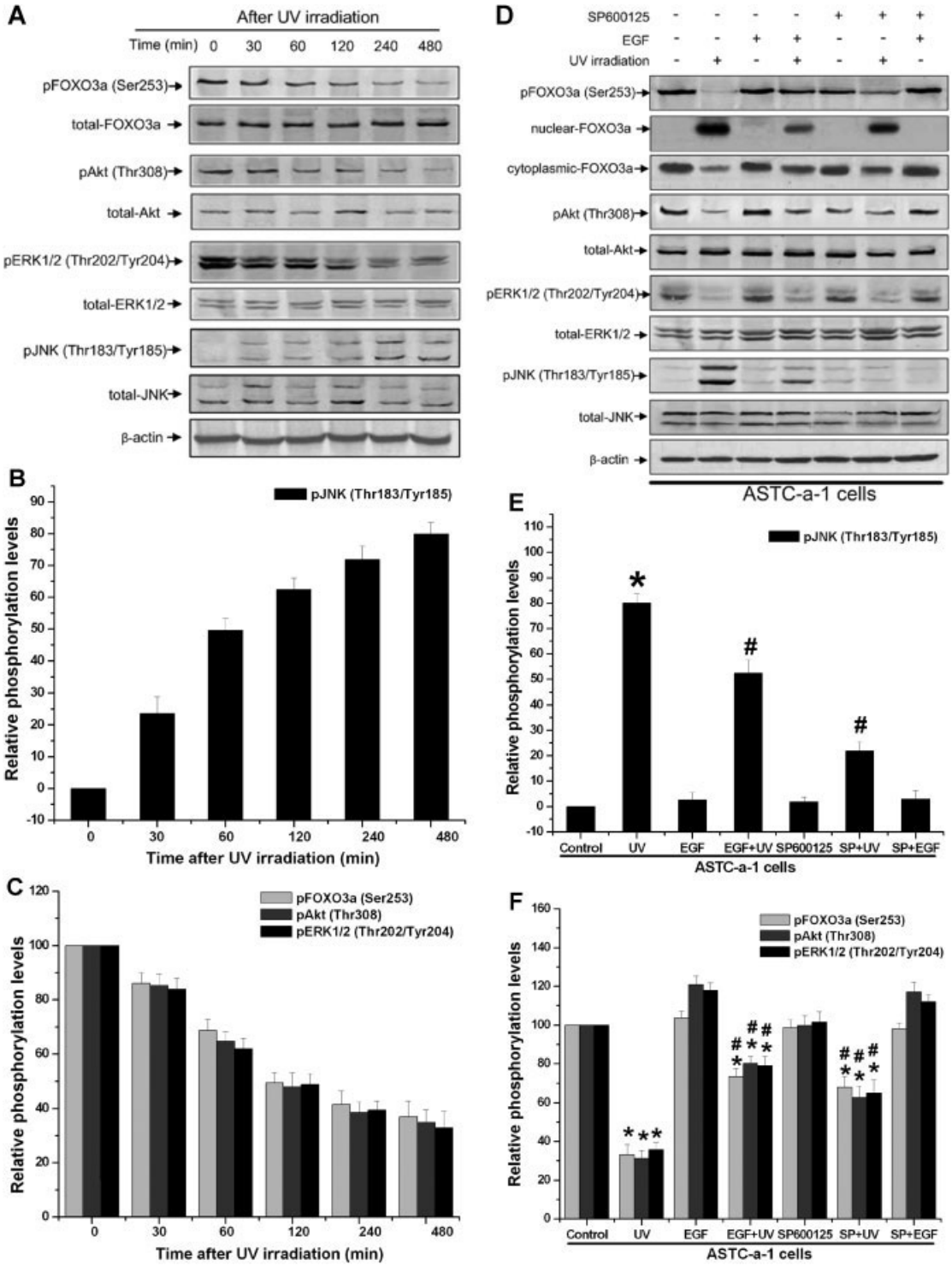

Fig. 4. Nuclear translocation of FOXO3a due to decreased ERK and Akt activities through JNK under UV irradiation. A: The lysates from ASTC-a-1 cells treated after UV irradiation (80 mJ/cm^2) or untreated (Control) were subjected to Western blot analysis with the antibodies of total-JNK, pJNK (Thr183/Tyr185), total-ERK1/2, pERK1/2 (Thr202/Tyr204), total-Akt, pAkt (Thr308), total-FOXO3a, and pFOXO3a (Ser253). β-actin was used as a control. B,C: Quantitative analyses of the levels of pJNK (Thr183/Tyr185), pFOXO3a (Ser253), pAkt (Thr308), and pERK1/2 (Thr202/Tyr204) in ASTC-a-1 cells after UV treatments. Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. control, #$P < 0.005$ vs. UV treatment). D: ASTC-a-1 cells were treated with UV irradiation (80 mJ/cm^2, lane 2), EGF (50 ng/ml, lane 3), combination with EGF and UV (lane 4), SP600125 (20 μM, lane 5), combination with SP600125 and UV (lane 6), combination with EGF and SP600125 (lane 7), or untreated. Cell lysates were subjected to Western blot analysis with the antibodies of total-JNK, pJNK (Thr183/Tyr185), total-ERK1/2, pERK1/2 (Thr202/Tyr204), total-Akt, pAkt (Thr308), total-FOXO3a, nuclear-FOXO3a, cytoplasmic-FOXO3a and pFOXO3a (Ser253). β-actin was used as a control. E–G: Quantitative analysis of levels of pJNK (Thr183/Tyr185), pFOXO3a (Ser253), pAkt (Thr308), pERK1/2 (Thr202/Tyr204) and nucleus–cytoplasm ratio (N/C) of FOXO3a in ASTC-a-1 cells after different treatments. Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. control, #$P < 0.005$ vs. UV treatment). H–K: Repeated the experiments from 4D to 4G in MCF-7 cells.

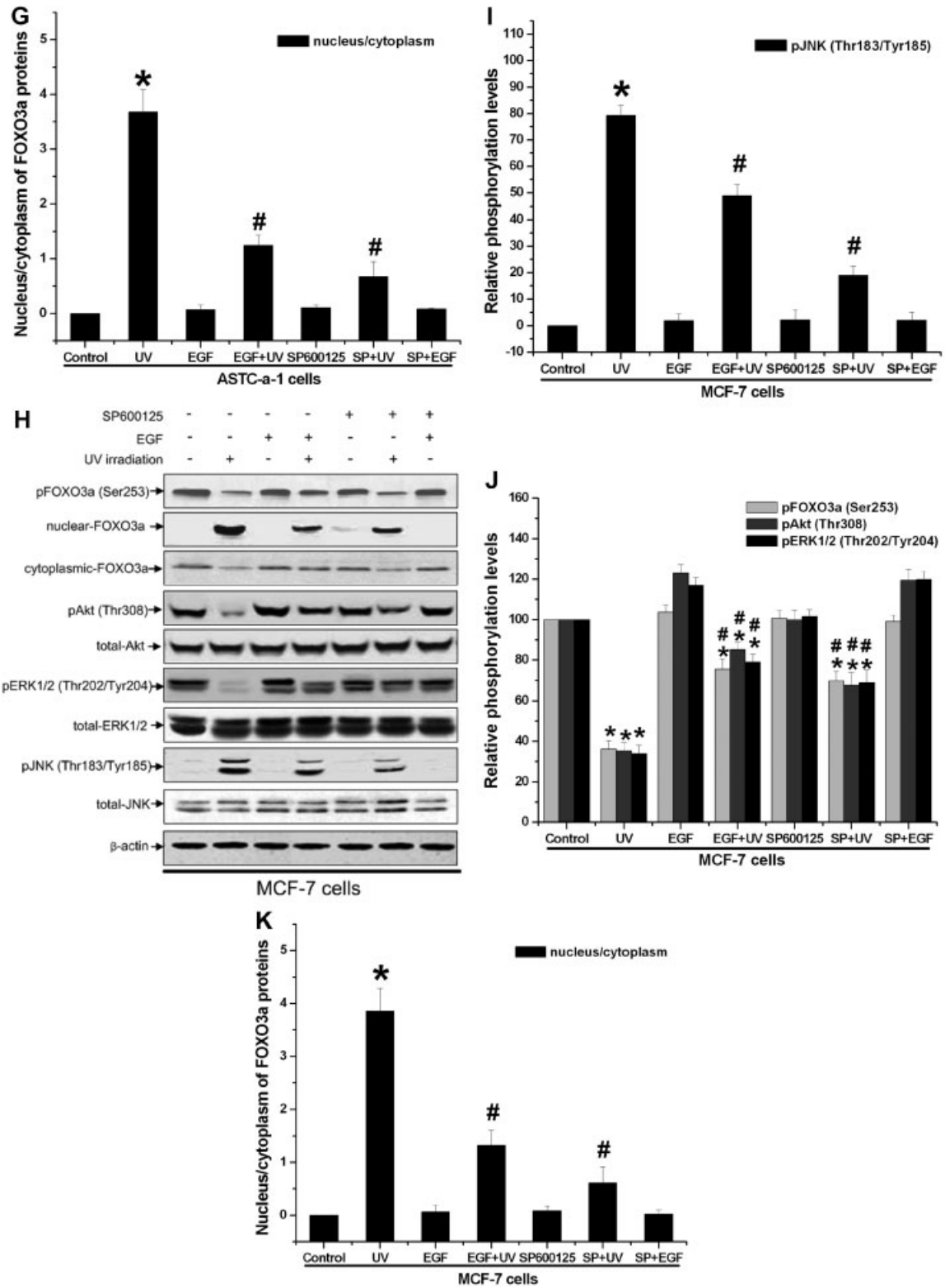

Fig. 4. (*Continued*)

early JNK activation by SP600125 is correlated with attenuated FOXO3a nuclear translocation (Fig. 2A,C,D,F,G). These results suggest that UV irradiation-activated JNK and growth factor-activated ERK and Akt have opposing effects on FOXO3a subcellular localization. Based on these findings and previous studies (Kockel et al., 2001; Wang et al., 2005b; Karpac and Jasper, 2009), it appears that cancer cells that receive abundant JNK signals are resistant to survival signal-mediated FOXO

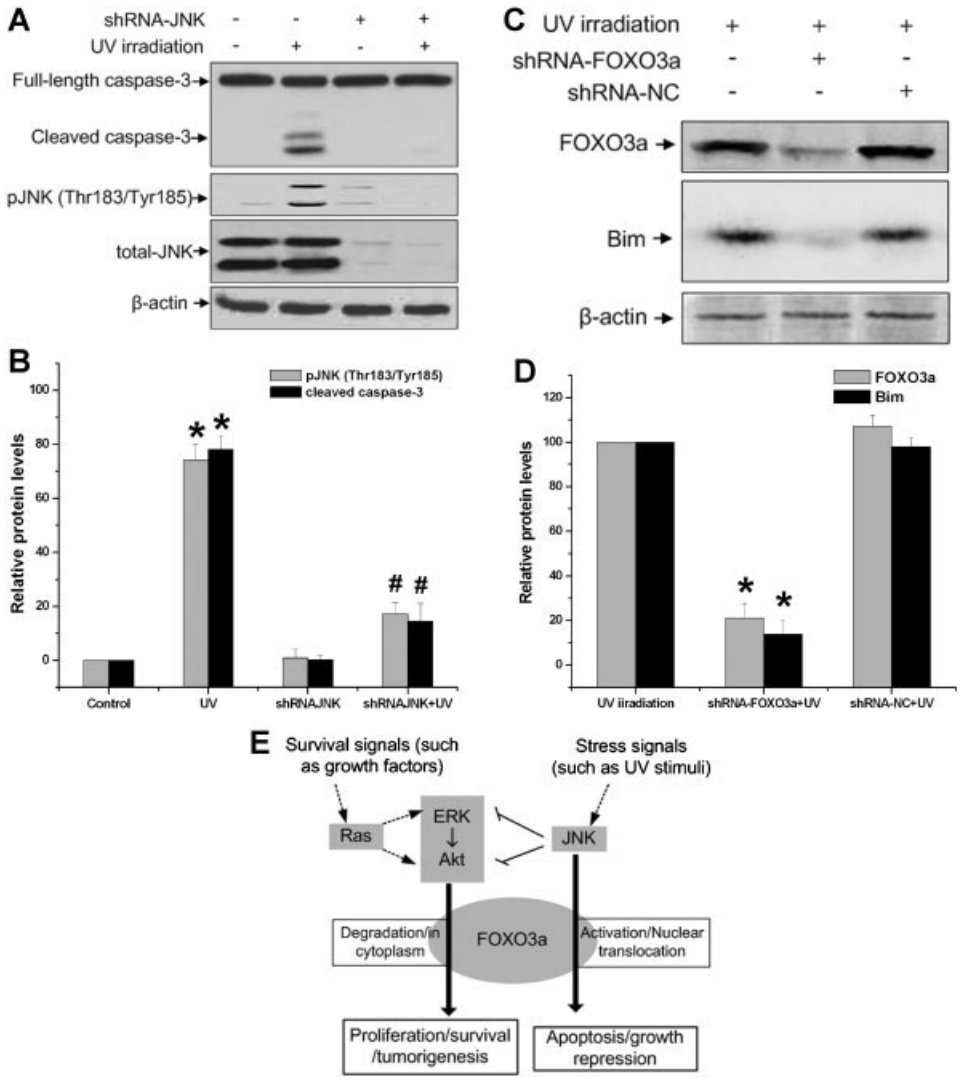

Fig. 5. UV irradiation-mediated apoptosis promoted by FOXO3a. A: ASTC-a-1 cells were treated with UV irradiation (80 mJ/cm^2), or pretreated with shRNA-JNK, or combined with shRNA-JNK and UV treatments, or untreated (control). Western blot analysis was performed to detect the levels of JNK, pJNK (Thr183/Tyr185) and the activity of Caspase-3. β-actin was used as a control. B: Quantitative analysis of the levels of pJNK (Thr183/Tyr185) and cleaved-Caspase-3 in ASTC-a-1 cells after different treatments. Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. control, #$P < 0.005$ vs. UV treatment). C: ASTC-a-1 cells were treated with UV irradiation (80 mJ/cm^2), or pretreated with shRNA-FOXO3a before UV treatment (lane 2), or pretreated with shRNA-NC before UV treatment (lane 3, a negative control for shRNA). Western blot analysis was performed to detect the levels of total-FOXO3a and Bim expression. β-actin was used as a control. D: Quantitative analysis of the levels of total-FOXO3a and Bim in ASTC-a-1 cells after different treatments. Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. UV treatment). E: Model for FOXO3a nuclear translocation regulated through the pathway of JNK-ERK/Akt under UV irradiation. Survival signals (e.g., growth factors) initiate a signaling cascade that results in the activation of ERK and Akt. ERK and Akt phosphorylate FOXO3a, causing its degradation or cytoplasmic retention, respectively, leading to cell survival, proliferation, and tumorigenesis. Stress signals, such as UV irradiation, cause activation of JNK, which in turn inactivates ERK and Akt, resulting in an irreversible nuclear translocation of FOXO3a and causing apoptosis, growth repression or longevity.

nuclear export and tumorgenesis. Recent studies suggest that PI3K/Akt inhibition leads to increased JNK phosphorylation and islet cell death, which can be reversed by the specific JNK inhibitor SP600125 (Brunet et al., 2001; Karpac and Jasper, 2009). We speculate that the balance between JNK and survival signals which regulates subcellular localization of FOXO3a is destroyed when cells are exposed to high-dose UV irradiation. Our results show that UV irradiation induces JNK activation that in turn inactivates basal ERK and Akt activities, leading to FOXO3a nuclear translocation (Figs. 2–4). UV irradiation-activated JNK appears to override the sequestration of FOXO by ERK and Akt. FOXO factors are involved in multiple signaling pathways and are closely correlated with apoptosis. In previous studies (Wang et al., 2009), we found that Bim protein played important roles in UV irradiation-induced apoptosis. Here, we find that nuclear translocation of FOXO3a can be regulated through the pathway of JNK-ERK/Akt, resulting in the expression of Bim and apoptosis (Fig. 5).

FOXO factors are regulated by diverse signals and a wide range of other upstream factors. Protein phosphatase 2A (PP2A) was also reported to regulate the activities of FOXO1 under certain conditions (Yan et al., 2008). In mammals, FOXO contains four homologous proteins, and in response to diverse upstream signals, FOXO may be activated by several different

mechanisms. Recent studies (Wang et al., 2005a; Gottlieb et al., 2002) showed that UV treatment involves JNK signalling/p53 pathway activation and in turn promotes Akt degradation, suggesting that p53 pathway activation benefits FOXO nuclear translocation. However, the works of Wang et al. (2008) and Miyaguchi et al. (2009) provided strong evidence that p53 could interact with FOXO3a and negatively regulate the transcriptional activity of FOXO3a. As shown in Figure 1E,F, nuclear FOXO3a can also return to the cytoplasm after low doses of UV irradiation. Therefore, it is possible that nuclear translocation of FOXO does not represent transcriptional activity. Many other unknown factors may affect the transcriptional activities of FOXO proteins. Moreover, studies by Kugler (Kugler et al., 2005) showed that UV could induce apoptosis by direct activation of Caspase-3. Our data in this study show that cleaved Caspase-3 becomes less significant after adding shRNA-JNK during UV irradiation (Fig. 5A,B), suggesting that suppression of endogenous JNK downregulates the activation of Caspase-3. The detailed mechanism awaits further investigation.

In summary, we demonstrate that JNK plays an important role in FOXO3a translocation and Bim expression. More importantly, our findings provide new insight into the mechanism of FOXO3a nuclear translocation: a pathway from JNK through decreased ERK and Akt activities in response to UV irradiation. Our findings contribute to the understanding of molecular mechanisms involving FOXO factors during cell apoptosis.

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