Apoptosis Repressor with Caspase Recruitment Domain Contributes to Chemotherapy Resistance by Abolishing Mitochondrial Fission Mediated by Dynamin-Related Protein-1

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Abstract

One obstacle of cancer therapy is the development of cancer resistance to chemotherapy. The molecular mechanisms by which the resistance is developed remain to be fully understood. Apoptosis repressor with caspase recruitment domain (ARC) is an endogenous antiapoptotic protein. Here, we report that ARC contributes to chemotherapy resistance by abolishing mitochondrial fission mediated by dynamin-related protein-1 (Drp1). Our results show that both HeLa and human gastric cancer (SGC-7901) cells have a high expression level of ARC. Doxorubicin at a low dose can slightly induce apoptosis in HeLa and SGC-7901 cells. In contrast, knockdown of ARC by its RNA interference enables the same low dose of doxorubicin to significantly induce apoptosis in HeLa and SGC-7901 cells. These data indicate that ARC is responsible for the cell resistance to doxorubicin treatment. Mitochondrial fission has recently been shown to be involved in triggering apoptosis. In exploring the molecular mechanism by which ARC participates in antagonizing doxorubicin-induced apoptosis, we observed that doxorubicin is able to induce mitochondrial fission that can be inhibited by ARC. Our results further show that Drp1 accumulates in mitochondria and mediates the signal of doxorubicin to induce mitochondrial fission. ARC is able to prevent Drp1 accumulations in mitochondria. Finally, we identified that PUMA is required for Drp1 accumulations in mitochondria. ARC inhibits Drp1 accumulations in mitochondria by directly binding to PUMA. Taken together, our results reveal a chemotherapy-resistant model in which ARC inhibits PUMA-mediated Drp1 accumulations in mitochondria and the consequent mitochondrial fission. [Cancer Res 2009;69(2):492–500]

Introduction

One obstacle of cancer therapy is the development of cancer resistance to chemotherapy. The molecular mechanisms by which the resistance is developed remain to be fully understood. Apoptosis repressor with caspase recruitment domain (ARC) is an endogenous inhibitor of apoptosis. Previous studies reveal that ARC is specifically expressed in heart and skeletal muscle (1, 2). However, a growing body of evidence shows that ARC is also expressed in a variety of human cancer cell lines and primary human cancers (3, 4). In particular, overexpression of ARC in cancer cell lines may inhibit chemical- and radiation-induced apoptosis (5, 6). Given the important role of chemotherapy for treating cancers, it is necessary to elucidate the molecular mechanism by which ARC influences chemotherapy.

Doxorubicin is the most widely used chemotherapeutic agent in the treatment of human tumors. It has been well-documented that doxorubicin exerts its death effects by inducing apoptosis. Although doxorubicin may initiate apoptotic program by using molecules such as p53, reactive oxygen species, and caspases (7, 8), the detailed mechanism by which doxorubicin induces apoptosis has not been fully clarified.

Apoptosis can be initiated through the extrinsic and/or the intrinsic pathways. The extrinsic pathway is initiated through the death receptors, whereas the intrinsic pathway is initiated through mitochondria. A death signal can initiate apoptotic program by inducing the release of mitochondrial proapoptotic proteins such as cytochrome c (9), apoptosis-inducing factor (10), and Smac/Diablo (11, 12).

It has been recently shown that the mitochondrial morphology is an important determinant of mitochondrial function (13). Mitochondrial fusion and fission participate in the regulation of apoptosis. Mitochondrial fusion is able to inhibit apoptosis, whereas mitochondrial fission is involved in the initiation of apoptosis (14, 15). It remains largely unknown as to whether doxorubicin triggers apoptosis through inducing mitochondrial fission.

ARC is originally identified to be a caspase-inhibiting protein and can specifically inhibit the activation of caspase-2 and caspase-8, thereby blocking apoptosis induced by a variety of stimuli requiring the engagement of these caspases (1). Further studies reveal that ARC may also elicit its antiapoptotic function by other means. It can interact with Bax (16, 17), inhibit cytochrome c release (18), and maintain mitochondrial membrane potential (19, 20). Furthermore, ARC seems to be a calcium-binding protein and can suppress the intracellular Ca^{2+} increase thereby blocking Ca^{2+}-mediated apoptosis (21). In addition, our previous work shows that ARC is regulated by protein kinase CK2. CK2 can phosphorylate ARC at threonine-149 enabling ARC translocation from cytoplasm to mitochondria. ARC requires T149 phosphorylation to protect cells against oxidative stress–induced apoptosis (22). Strikingly, ARC phosphorylation by CK2 is constitutive, indicating the importance of phosphorylation for ARC function (22, 23). Despite of these observations, it is not yet clear whether ARC is able to regulate mitochondrial fission.

Mitochondrial fission requires the activity of a dynamin-related protein-1 (Drp1; ref. 24). Drp1 is a GTPase that causes scission of the mitochondrial outer membrane, resulting in fission of mitochondrial tubules into fragments. Drp1 is responsible for cytochrome c release and caspase activation (14). The introduction

Figure 1. Doxorubicin induces mitochondrial fission inhibited by ARC. *A,* analysis of ARC expression levels in cancer cells including HeLa and SGC-7901 cells. HEK-293 cells served as a negative control. ARC was detected by immunoblot using the anti-ARC antibody. Actin served as a loading control. A representative result of three independent experiments is shown. *B,* doxorubicin induces mitochondrial fission and a reduction in ARC expression levels in HeLa cells. HeLa cells were treated with 2 μmol/L doxorubicin (*DOX*). Representative photos show mitochondrial fission (*left*). Photos were taken 6 h after doxorubicin treatment. Bar, 10 μm. ARC expression levels were analyzed by immunoblot using the anti-ARC antibody (*right*). The percentage of cells undergoing mitochondrial fission was counted and summarized (*right*). *C,* ARC prevents doxorubicin-induced mitochondrial fission. HeLa cells were infected with Adβ-gal or AdARC at a moi of 100. Twenty-four hours after infection they were treated with 2 μmol/L doxorubicin. Twelve hours after treatment, cells were collected for the detection of mitochondrial fission. *, $P < 0.05$ versus doxorubicin alone. *D,* doxorubicin induces mitochondrial fission in SGC-7901 cells inhibited by ARC. SGC-7901 cells were treated and analyzed as described for *A, B,* and *C.* *, $P < 0.05$ versus doxorubicin alone. *Columns,* mean of three independent experiments; *bars,* SE.
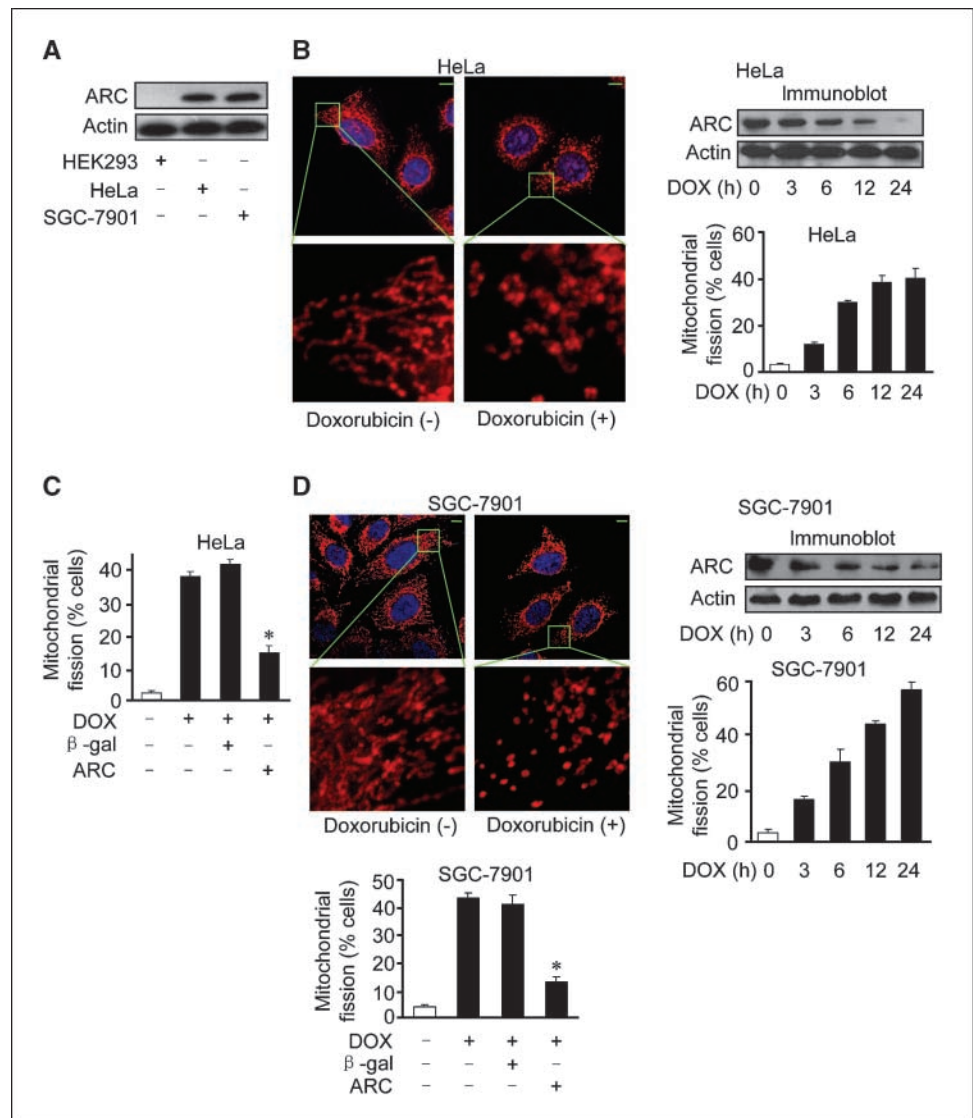
of mutation in its catalytic GTPase active site has been shown to block mitochondrial fission and cytochrome *c* release during apoptosis (24, 25). It has been reported that Bax/Bak promotes sumoylation of Drp1 and its stable association with mitochondria during apoptosis (26). PUMA is a member of Bcl-2 family, and exclusively located to mitochondria. It can bind to Bcl-2 and Bcl-X(L), thereby inducing cytochrome *c* release and the consequent activation of caspase-9 and caspase-3 (27, 28). It remains unknown as to whether PUMA can facilitate Drp1 accumulations in mitochondria.

The present study was designed to elucidate whether doxorubicin induces apoptosis by triggering mitochondrial fission, and if so, whether it is regulated by ARC. Our results show that the susceptibility of doxorubicin to inducing mitochondrial fission and apoptosis in cancer cells is dependent on the expression levels of ARC. ARC is able to inhibit mitochondrial fission induced by doxorubicin. Strikingly, ARC prevents Drp1 accumulations in mitochondria through directly associating with PUMA, the latter facilitates Drp1 to accumulate in mitochondria. Our data suggest that ARC contributes to cell resistance to chemotherapy by targeting the mitochondrial fission machinery.

Materials and Methods

Reagents, cell cultures, doxorubicin treatment, and cell viability assay. Doxorubicin was purchased from Sigma. Anti-ARC antibody was from Chemicon. Anti-Drp1 antibody was from BD Biosciences. Anti-PUMA antibody and anti-cyclooxygenase IV antibody were from Abcam. Anti-actin antibody was from Santa Cruz Biotechnology. HeLa cells were as we described (29). Human gastric cancer cell line SGC-7901 was as described elsewhere (30). The cells were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. The treatment with doxorubicin was performed as we described (31). Cell death was determined by Trypan Blue exclusion, and the numbers of Trypan Blue–positive and Trypan Blue–negative cells were counted on a hemocytometer.

Preparations of siRNA constructs of ARC, PUMA, and Drp1. The siRNA constructs were designed using the siRNA Design Tools from Ambion. The functional siRNA constructs used in the present work include the following: ARC-siRNA-A sense sequence is 5′-AGGGACGAGTCCGAA-GATT-3′; ARC-siRNA-A antisense sequence is 5′-AATCTTCGGACTCG-TCCCT-3′. The scramble ARC-siRNA-A sense sequence is 5′-TGCGGA-GAAGTGCGAATCA-3′; the scramble ARC-siRNA-A antisense sequence is 5′-TGATTCGCACTTCTCCGCA-3′. ARC-siRNA-B sense sequence is

5′-GCTGAGGCCTCTAAAGAGG-3′; ARC-siRNA-B antisense sequence is 5′-CCTCTTTAGAGGCCTCAGC-3′. The scramble ARC-siRNA-B sense sequence is 5′-GGCTCAAGCGAGAGCGTAT-3′; the scramble ARC-siRNA-B antisense sequence is 5′-ATACGCTCTCGCTTGAGCC-3′. PUMA-siRNA-A sense sequence is 5′-TCTCATCATGGGACTCCTG-3′; PUMA-siRNA-A antisense sequence is 5′-CAGGAGTCCCATGATGAGA-3′. The scramble PUMA-siRNA-A sense sequence is 5′-CTTACGTCATGCGTCGTAC-3′; the scramble PUMA-siRNA-A antisense sequence is 5′-GTACGACGCATGACGTAAG-3′. PUMA-siRNA-B sense sequence is 5′-CAGTGGGCCCGGGAGATCG-3′; PUMA-siRNA-B antisense sequence is 5′-CGATCTCCCGGGCCCACTG-3′. The scramble PUMA-siRNA-B sense sequence is 5′-TCGGCATGGGCGGCAGCGA-3′; the scramble PUMA-siRNA-B antisense sequence is 5′-TCGCTGCCGCCCATGCCGA-3′. Drp1-siRNA-A sense sequence is 5′-CTGGAGAGGAATGCTGAAA-3′; Drp1-siRNA-A antisense sequence is 5′-TTTCAGCATTCCTCTCCAG-3′. The scramble Drp1-siRNA-A sense sequence is 5′-CTGGAAATGGAGGGAACTA-3′; the scramble Drp1-siRNA-A antisense sequence is 5′-TAGTTCCCTCCATTTCCAG-3′. Drp1-siRNA-B sense sequence is 5′-GGATATTGAGCTTCAAATC-3′; Drp1-siRNA-B antisense sequence is 5′-GATTTGAAGCTCAATATCC-3′. The scramble Drp1-siRNA-B sense sequence is 5′-GTGAACGATTATGAATCTC-3′; the scramble Drp1-siRNA-B antisense sequence is 5′-GAGATTCATAATCGTTCAC-3′. They were cloned into p*Silencer* adeno 1.0-CMV vector (Ambion) according to the manufacturer's instructions. The specificity of the oligonucleotides was confirmed by comparison with all other sequences in Genbank using Nucleotide BLAST. There was no homology to other known human DNA sequences.

Adenovirus construction and infection. Adenovirus harboring the cDNA of ARCT149A (AdARCT149A) was constructed using the Adeno-X Expression System (Clontech) according to the manufacturer's instructions. Adenovirus ARC (AdARC) and adenovirus β-galactosidase (Adβ-gal) were as we described (31). Viruses were amplified in 293 cells. Cells were infected with the virus at the indicated multiplicity of infection (moi). After washing with PBS, culture medium was added and cells were cultured until the indicated time.

Immunoprecipitation. Immunoprecipitation was carried out as we described (29). In brief, cells were lysed for 1 h at 4°C in a lysis buffer. To perform immunoprecipitation, the cell lysates were precleared with 10% (vol/vol) protein A-agarose (Roche) for 1 h on a rocking platform. Specific antibodies were added and rocked for 1 h. Immunoprecipitates were captured with 10% (vol/vol) protein A-agarose for another hour. The agarose beads were spun down and washed thrice with NET buffer. The antigens were released and denatured by adding SDS sample buffer.

Immunoblotting. Immunoblotting was performed as we reported earlier (29). In brief, cells were lysed for 1 h at 4°C in a lysis buffer [20 mmol/L Tris (pH 7.5), 2 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 and a protease inhibitor cocktail]. Samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Equal-protein loading was controlled by Ponceau red staining of membranes. Blots were probed using primary antibodies. Blots were then probed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruse). Antigen-antibody complexes were detected by enhanced chemiluminescence (Cell Signal Biosciences).

Preparations of subcellular fractions. Subcellular fractions were prepared as we described (23). In brief, cells were washed twice with PBS and the pellet was suspended in 0.2 mL of buffer A [20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 1 mmol/L EGTA, 1 mol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, 250 mmol/L sucrose] containing a protease inhibitor cocktail. The cells were homogenized by 12 strokes in a Dounce homogenizer. The homogenates were centrifuged twice at 750 g for 5 min at 4°C to collect nuclei and debris. The supernatants were centrifuged at 10,000 g for 15 min at 4°C to collect mitochondria-enriched heavy membrane pellet (HM). The resulting supernatants were centrifuged to yield cytosolic fractions.
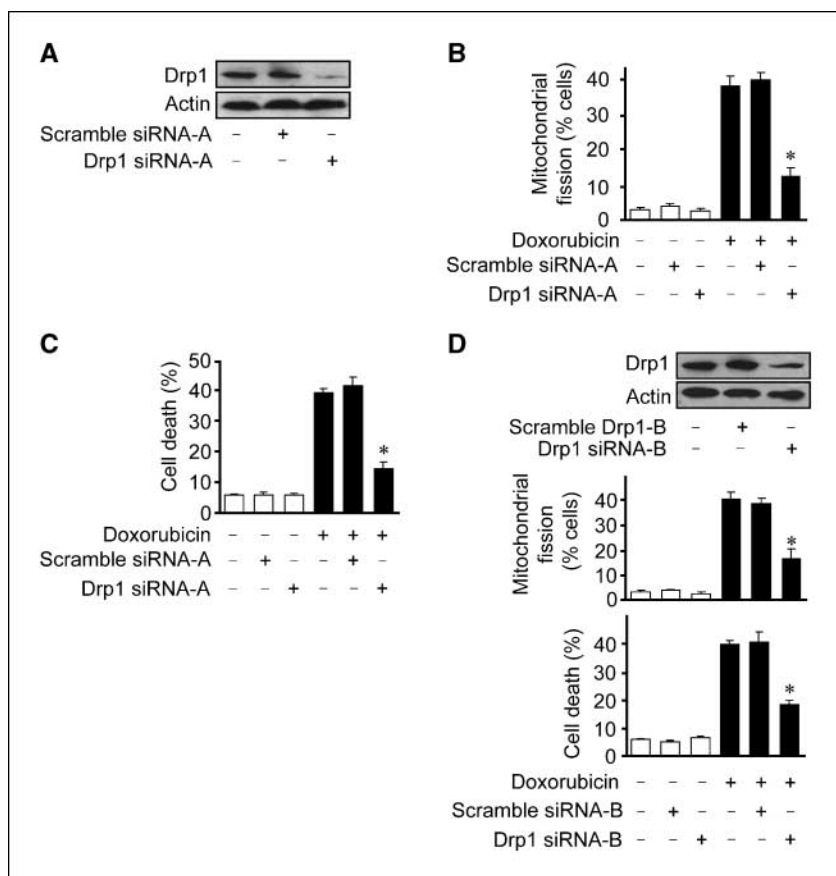
Figure 2. Drp1 is required for doxorubicin to induce mitochondrial fission. *A,* Drp1 siRNA is able to reduce the expression levels of Drp1. HeLa cells were infected with adenoviruses harboring Drp1 siRNA or its scramble form at a moi of 200. Cells were harvested 48 h after infection for the analysis of Drp1 levels by immunoblot. A representative blot of three independent experiments is shown. *B,* knockdown of Drp1 attenuates doxorubicin-induced mitochondrial fission. HeLa cells were infected with adenoviruses harboring Drp1 siRNA or its scramble form at a moi of 200. Twenty-four hours after infection, cells were treated with 2 μmol/L doxorubicin. Mitochondrial fission was analyzed 12 h after doxorubicin treatment. *, $P < 0.05$ versus doxorubicin alone. *C,* knockdown of Drp1 attenuates doxorubicin-induced cell death. HeLa cells were treated as described for *B.* Cell death was analyzed 36 h after doxorubicin treatment. *, $P < 0.05$ versus doxorubicin alone. *D,* a second siRNA of Drp1 could inhibit mitochondrial fission and cell death induced by doxorubicin. *, $P < 0.05$ versus doxorubicin alone. *Columns,* mean of three independent experiments; *bars,* SE.

Figure 3. ARC prevents Drp1 accumulations in mitochondria upon treatment with doxorubicin. *A,* doxorubicin induces Drp1 accumulations in mitochondria. HeLa cells were treated with 2 μmol/L doxorubicin. Cells were harvested at the indicated time after treatment for the immunoblot analysis of Drp1 levels in the cytosol and mitochondria-enriched HM. A representative blot of three independent experiments is shown. *B,* immunofluorescent analysis of the localizations of Drp1 before and after doxorubicin treatment. HeLa cells were treated with 2 μmol/L doxorubicin. Six hours after treatment cells were labeled with MitoTracker (*red*), stained with anti-Drp1 antibody, and monitored by FITC-labeled secondary antibody (*green*). The overlay of red and green yields orange and/or yellow. *Bar,* 10 μm. *C,* ARC prevents Drp1 accumulations in mitochondria upon treatment with doxorubicin. HeLa cells were infected with Adβ-gal or AdARC at a moi of 100. Twenty-four hours after infection, they were treated with 2 μmol/L doxorubicin. Six hours after treatment, cells were harvested for the detection of Drp1 in HM and cytosol. A representative blot of three independent experiments is shown.
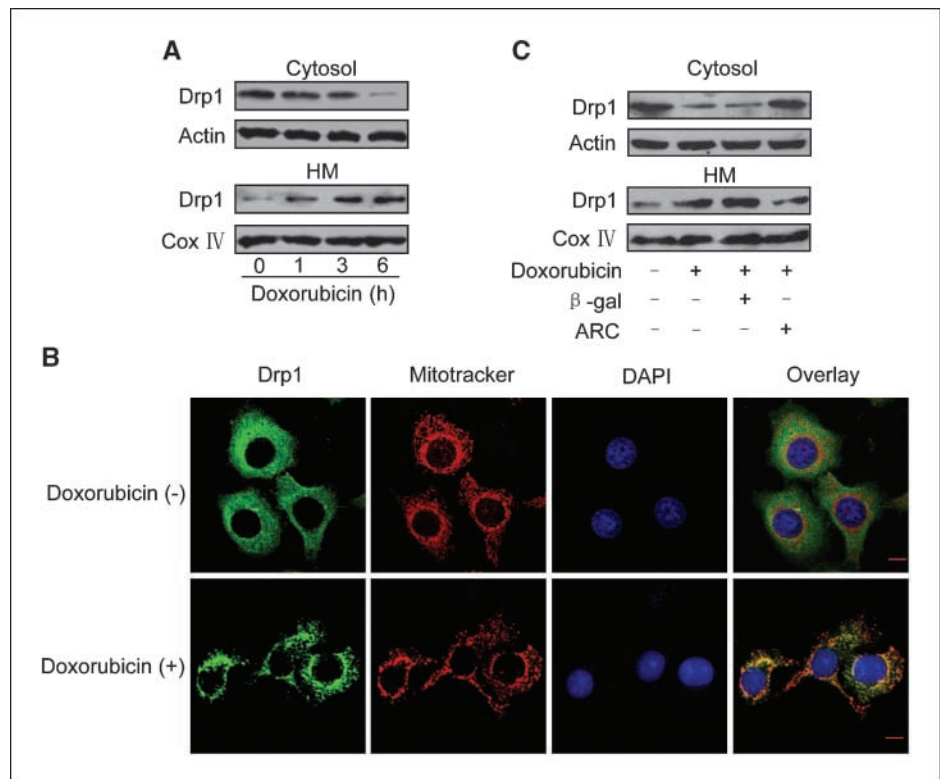
Mitochondrial staining and immunofluorescence. Cells were plated onto the cover-slips coated with 0.01% poly-L-lysine. After treatment they were stained for 20 min with 0.02 μmol/L MitoTracker Red CMXRos (Molecular Probes). Immunofluorescence was performed as we described (23). The samples were imaged using a laser scanning confocal microscope (Zeiss LSM 510 META).

Statistical analysis. The results are expressed as means ± SE. The statistical comparison among different groups was performed by one-way ANOVA. *P* values of <0.05 was considered statistically significant.

Results

Doxorubicin induces mitochondrial fission inhibited by ARC. It has been previously found that ARC is highly expressed in the cardiac and skeletal muscle (1, 2). Recent studies have shown that ARC also is expressed in some types of cancer cells (5, 6). However, the role of ARC in the chemotherapeutic resistance remains largely unknown. Mitochondrial fission is related to the initiation of apoptosis. We tested whether ARC is related to mitochondrial fission in doxorubicin-induced cell death. ARC could be detectable in cancer cells including HeLa and SGC-7901 but not in the normal HEK-293 cells (Fig. 1*A*). Administration of doxorubicin at a high dose (2 μmol/L) could induce mitochondrial fission in HeLa cells as revealed by the morphologic alterations (Fig. 1*B*). We counted the cells undergoing mitochondrial fission upon doxorubicin treatment. A time-dependent increase in mitochondrial fission could be observed (Fig. 1*B, right*). We analyzed the expression levels of ARC in response to doxorubicin treatment. Doxorubicin led to a decrease in ARC expression levels in HeLa cells (Fig. 1*B, right*). These data suggest that ARC can be a target of doxorubicin in its apoptotic program. The reduction in ARC expression levels upon treatment with doxorubicin led us to consider whether ARC can influence mitochondrial fission induced

by doxorubicin. Enforced expression of ARC could attenuate mitochondrial fission upon doxorubicin treatment (Fig. 1*C*).

To understand whether the effect between ARC and doxorubicin-induced mitochondrial fission exists in other types of cancer cells, SGC-7901 cells were used. We observed a similar result in SGC-7901 cells (Fig. 1*D*). Taken together, it seems that ARC is able to inhibit doxorubicin-induced mitochondrial fission.

Drp1 is required for doxorubicin to induce mitochondrial fission. Drp1 is involved in mitochondrial fission (14, 26). We asked whether Drp1 is necessary for doxorubicin to induce mitochondrial fission. To this end, we prepared the siRNA construct of Drp1. Drp1 levels could be reduced by its siRNA but not its scramble form (Fig. 2*A*). Mitochondrial fission could be induced by doxorubicin in the absence but not presence of Drp1 siRNA (Fig. 2*B*). Concomitantly, doxorubicin-induced cell death could be attenuated by Drp1 siRNA (Fig. 2*C*). To further confirm the role of Drp1 in doxorubicin-induced mitochondrial fission, we produced another Drp1 siRNA construct. This construct could reduce mitochondrial fission and cell death induced by doxorubicin (Fig. 2*D*). These results suggest that Drp1 is a prerequisite for doxorubicin to induce mitochondrial fission and cell death.

ARC prevents Drp1 accumulations in mitochondria. Drp1 participates in regulating mitochondrial fission by translocating from the cytoplasm to mitochondria (14, 32). We detected the subcellular locations of Drp1 upon doxorubicin treatment. The levels of Drp1 in HM were increased upon doxorubicin treatment in HeLa cells (Fig. 3*A*). Immunofluorescence was used to further detect the localizations of Drp1 before and after doxorubicin treatment. In the control untreated cells, Drp1 was distributed throughout the cytoplasm and showed a distinct pattern from the mitoTracker. Upon doxorubicin treatment, Drp1 accumulated around the perinuclear region, and its pattern coincided very

closely with that of the mitoTracker (Fig. 3B). These results suggest the accumulations of Drp1 in mitochondria in response to doxorubicin treatment.

Because ARC is able to prevent mitochondrial fission as shown in Fig. 1, we asked whether ARC can influence Drp1 distributions. Enforced expression of ARC could attenuate Drp1 accumulations in mitochondria in HeLa cells (Fig. 3C). ARC also could inhibit Drp1 accumulations in mitochondria in SGC-7901 cells upon treatment with doxorubicin (data not shown). Thus, these data suggest that ARC can prevent Drp1 accumulations in mitochondria.

PUMA is required for Drp1 to accumulate in mitochondria. In the following experiments, we explored the molecular mechanism by which ARC inhibits Drp1 accumulations in mitochondria. PUMA has been shown to be a prerequisite for doxorubicin to initiate apoptosis (27, 28, 33). Our previous work has shown that ARC is able to target PUMA, thereby preventing PUMA-mediated apoptosis (31). This encouraged us to test whether PUMA is related to Drp1 accumulations in mitochondria in the apoptotic pathway of doxorubicin. To this end, we produced the siRNA construct of PUMA. Doxorubicin treatment led to an elevated level of PUMA. PUMA siRNA but not its scramble form could attenuate PUMA elevation induced by doxorubicin (Fig. 4A). Knockdown of PUMA resulted in the

abolishment of Drp1 accumulations in mitochondria induced by doxorubicin (Fig. 4B). These data suggest that PUMA is necessary for Drp1 accumulations in mitochondria.

To understand whether the regulation of PUMA on Drp1 accumulations plays a functional role in mitochondrial fission, we detected whether PUMA knockdown can influence mitochondrial fission. Doxorubicin-induced mitochondrial fission could be attenuated by PUMA knockdown (Fig. 4C). Concomitantly, knockdown of PUMA resulted in a reduction in cell death upon doxorubicin treatment (Fig. 4D). A second siRNA construct of PUMA exerted a similar effect on mitochondrial fission induced by doxorubicin (Fig. 4E). Thus, it seems that PUMA is required for Drp1 accumulations in mitochondria and the consequent fission of mitochondria.

ARC prevents Drp1 accumulations in mitochondria by directly binding to PUMA. The regulation of PUMA on Drp1 accumulations in mitochondria led us to consider whether it can be interrupted by ARC. ARC could inhibit Drp1 accumulations in mitochondria in response to PUMA stimulation (Fig. 5A). PUMA has been shown to be exclusively located in mitochondria (27, 28). Our previous work has shown that the phosphorylated ARC at threonine-149 is located in mitochondria, whereas the nonphosphorylatable ARC with threonine-149 mutated to an
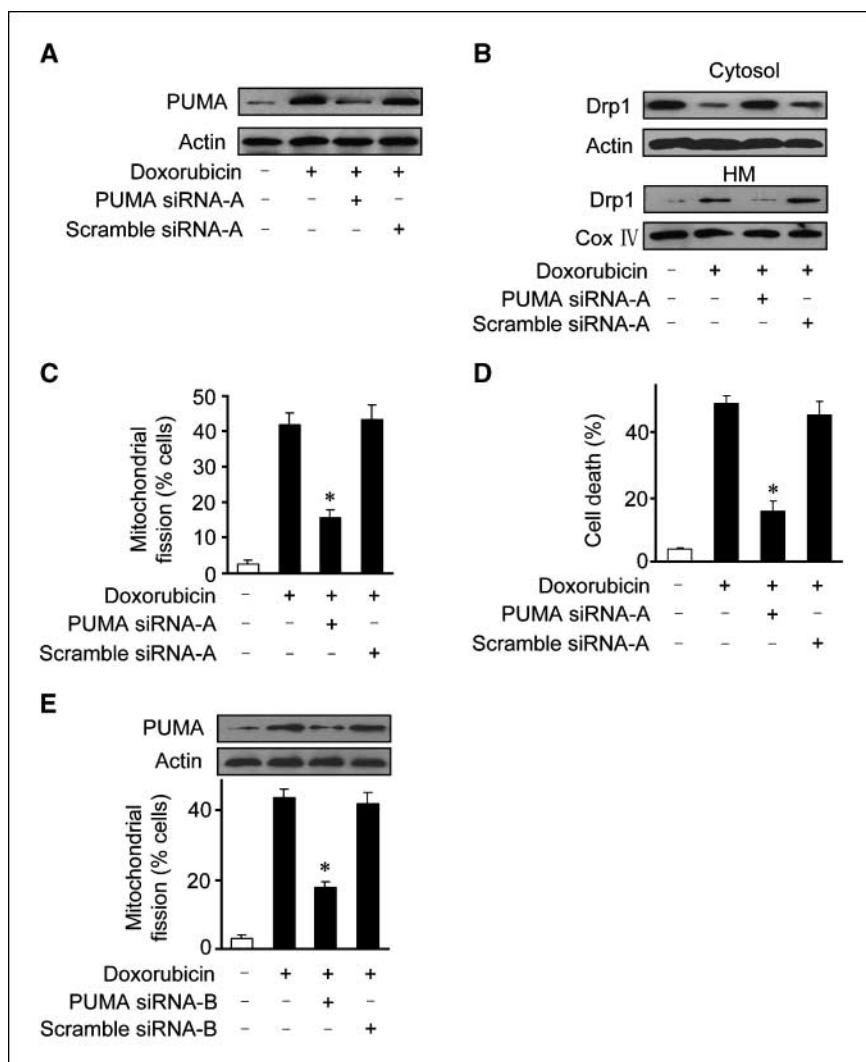
Figure 4. PUMA is required for Drp1 to translocate to mitochondria. A, PUMA siRNA attenuates PUMA levels upon doxorubicin treatment. HeLa cells were infected with adenoviruses harboring PUMA siRNA or its scramble form at a moi of 150. Twenty-four hours after infection, cells were treated with 2 μmol/L doxorubicin. Cells were harvested 6 h after doxorubicin treatment for the analysis of PUMA levels by immunoblot. A representative blot of three independent experiments is shown. B, knockdown of PUMA attenuates Drp1 accumulations in mitochondria. HeLa cells were treated as described for (A). Six hours after treatment, cells were harvested for the detection of Drp1 in the cytosol and mitochondria-enriched HM. A representative blot of three independent experiments is shown. C and D, knockdown of PUMA abolishes mitochondrial fission and cell death upon doxorubicin treatment. HeLa cells were treated as described for A. Mitochondrial fission was analyzed 12 h after doxorubicin treatment (C). Cell death was analyzed 36 h after doxorubicin treatment (D). E, a second siRNA of PUMA could inhibit mitochondrial fission induced by doxorubicin. *, P < 0.05 versus doxorubicin alone. Columns, mean of three independent experiments; bars, SE.

Figure 5. ARC prevents PUMA-induced mitochondrial fission by directly binding to PUMA. *A,* PUMA-induced Drp1 accumulations in mitochondria are abolished by ARC. HeLa cells were coinfected with AdPUMA and Adβ-gal, AdARC, or AdARCT149A at a moi of 100. Twenty-four hours after infection, cells were harvested for the immunoblot analysis of PUMA and ARC expression levels (*top*) as well as Drp1 distributions in the cytosol (*middle*) and HM (*lower*). A representative blot of three independent experiments is shown. *B* and *C,* ARC prevents PUMA-induced mitochondrial fission and cell death. HeLa and SGC-7901 cells were treated as described for *A.* Mitochondrial fission was analyzed 24 h after infection (*B*). Cell death was analyzed 48 h after infection (*C*). *, *P* < 0.05 versus PUMA alone. *D,* ARC binds to endogenous PUMA. HeLa cells were infected with AdARC or AdARCT149A at a moi of 100. Twenty-four hours after infection, cells were treated with 2 μmol/L doxorubicin. The association between PUMA and ARC or ARC149A was analyzed by immunoprecipitation (*IP*) followed by immunoblot (*IB*). *Columns,* mean of three independent experiments; *bars,* SE.
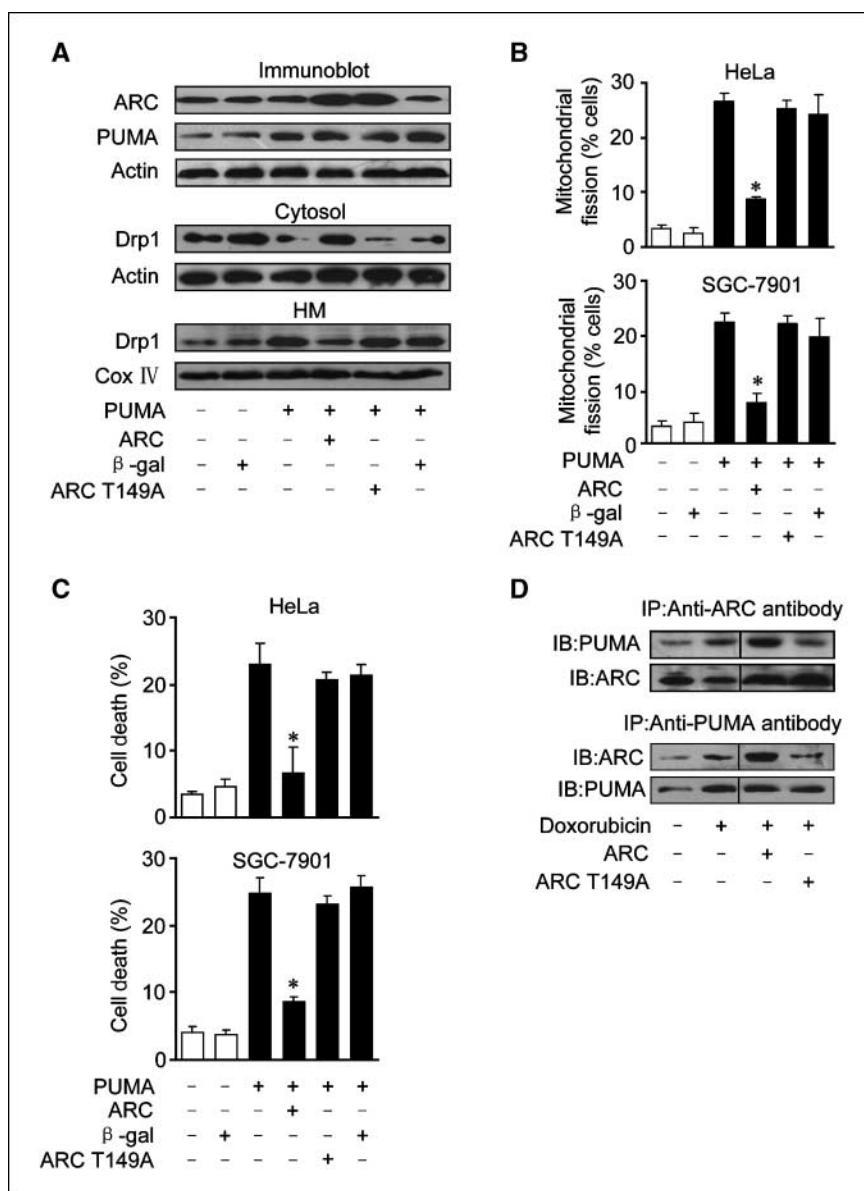
alanine residue (ARCT149A) is localized in the cytoplasm. Furthermore, the phosphorylated ARC but not ARCT149A is able to inhibit apoptosis (23). To confirm the role of ARC in controlling Drp1 accumulations induced by PUMA, we tested whether ARCT149A is able to influence Drp1 accumulations. The accumulations of Drp1 in mitochondria could not be significantly influenced by ARCT149A (Fig. 5*A, lane 5, middle* and *bottom*), although it had a comparable expression level as ARC (Fig. 5*A, lane 5, top*). These data suggest that ARC can control PUMA-induced Drp1 accumulations in mitochondria.

Subsequently, we detected whether ARC can influence mitochondrial fission and cell death in response to PUMA stimulation. PUMA was able to induce mitochondrial fission that was inhibited by ARC but not ARCT149A (Fig. 5*B*). Also, cell death induced by PUMA could be attenuated by ARC but not ARCT149A (Fig. 5*C*). These data indicate that ARC plays a functional role in controlling PUMA-induced mitochondrial fission and cell death.

We tested whether ARC inhibits PUMA-mediated Drp1 accumulations through directly interacting with PUMA or not. A weak

association between ARC and PUMA could be observed in response to doxorubicin treatment. A strong association between ARC and PUMA could be observed in cells expressing exogenous ARC. Enforced expression of ARCT149A led to no significant alterations in ARC and PUMA association levels (Fig. 5*D*). Taken together, these data indicate that there is a cross-talk between ARC and PUMA in regulating mitochondrial fission.

Knockdown of endogenous ARC sensitizes doxorubicin to inducing Drp1 accumulations in mitochondria. We carried out experiments to test whether endogenous ARC participates in the regulation of mitochondrial fission. First, we used RNAi technology to knockdown ARC. Doxorubicin at a low dose was able to slightly induce Drp1 accumulations in mitochondria. In contrast, doxorubicin at the same low dose induced a significant elevation of Drp1 levels in mitochondria upon ARC knockdown. The scramble form of ARC-siRNA had no obvious effects on doxorubicin-induced Drp1 accumulations (Fig. 6*A*). These data suggest that endogenous ARC participates in controlling Drp1 accumulations in mitochondria.

Subsequently, we detected mitochondrial fission and cell death. As shown in Fig. 6*B*, 0.2 μmol/L doxorubicin alone induced a limited amount of cells undergoing mitochondrial fission and death. Strikingly, an elevated amount of cells underwent mitochondrial fission and death in response to the same dose of doxorubicin treatment upon ARC knockdown. A second siRNA construct of ARC could exert a similar effect on Drp1 localization (Fig. 6*C*), mitochondrial fission, and cell death (Fig. 6*D*). These data suggest that the abrogation of endogenous ARC can reduce the resistance of cancer cells to doxorubicin treatment.

Discussion

Chemotherapy plays an important role for the treatment of a variety of cancers. However, there is a distinct problem that the cancer cells are resistant to a variety of therapeutic drugs such as doxorubicin. In exploring the mechanism by which the cancer cells are resistant to doxorubicin, our present study reveals that doxorubicin triggers apoptosis by inducing mitochondrial fission. However, this effect of doxorubicin can be inhibited by the antiapoptotic protein ARC in the cancer cells. Our results further revealed that doxorubicin activates PUMA, the latter facilitates Drp1 accumulations in mitochondria. Strikingly, ARC inhibits mitochondrial fission by directly targeting PUMA. Our data may provide a new clue in understanding the molecular mechanism of chemotherapy resistance.

Doxorubicin is the most widely used chemotherapeutic agent in the treatment of human tumors. However, the mechanism of apoptosis induced by doxorubicin has not been fully clarified. It has

been shown that the anticancer action of doxorubicin is elicited through DNA damage and/or reactive oxygen species generation by redox reaction (7, 34). Doxorubicin also can activate caspases (8). Our present study shows that doxorubicin is able to activate the mitochondrial fission machinery. It would be interesting to elucidate the relationship between mitochondrial fission and other events such as reactive oxygen species generation in the apoptotic cascades of doxorubicin.

The molecular mechanism by which Drp1 is recruited from the cytosol to the mitochondrial surface is not fully understood. It has been shown that Drp1 accumulation in mitochondria is likely mediated by membrane-associated receptors (35, 36). hFis1 is an outer membrane protein that is proposed to mediate Drp1 accumulation in mitochondria in mammalian cells (37). On the contrary, a recent study shows that the accumulation of Drp1 in mitochondria results from its irreversibly locking on the membrane in an hFis1-independent but Bax/Bak-dependent manner (26). However, there is also opposite evidence showing that the fission of mitochondria is a dispensable event in Bax/Bak-dependent apoptosis (38). PUMA is a member of Bcl-2 family, and can be up-regulated by p53. It can bind to Bcl-2 and Bcl-X(L) thereby inducing cytochrome *c* release and the consequent activation of caspase-9 and caspase-3 (27, 28). We have previously showed that PUMA can be activated by daunomycin in a p53-dependent manner (31). Our present work reveals that PUMA is a prerequisite for doxorubicin to induce mitochondrial fission. In contrast to Bax that is predominantly distributed in the cytoplasm (39, 40), PUMA is exclusively located to mitochondria (27, 28). If the recruitment of
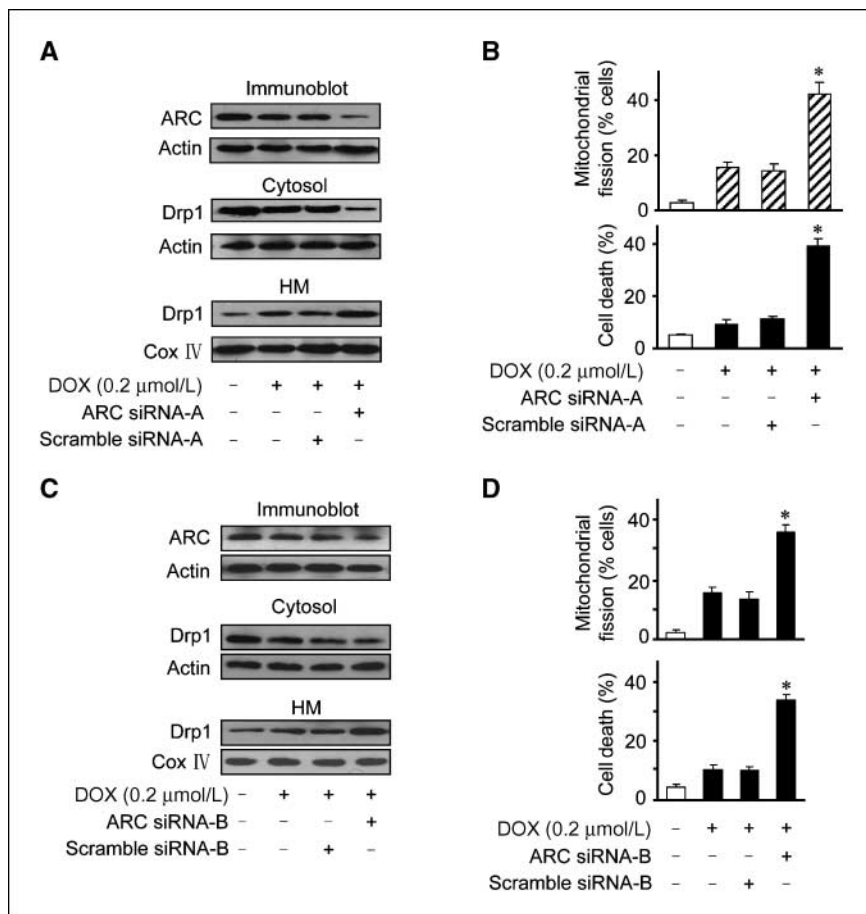
Figure 6. Knockdown of endogenous ARC sensitizes doxorubicin to inducing Drp1 accumulations in mitochondria and cell death. *A,* knockdown of ARC sensitizes doxorubicin to inducing Drp1 accumulations in mitochondria. HeLa cells were infected with adenoviruses harboring ARC siRNA or its scramble form at a moi of 150. Twenty-four hours after infection, cells were treated with doxorubicin. Cells were harvested 6 h after doxorubicin treatment for the immunoblot analysis of ARC levels or Drp1 levels. A representative blot of three independent experiments is shown. *B,* knockdown of ARC sensitizes doxorubicin to inducing mitochondrial fission and cell death. HeLa cells were infected with adenoviruses and then treated with doxorubicin as described for *A.* Mitochondrial fission was analyzed 12 h after doxorubicin treatment. Cell death was analyzed 36 h after treatment. *, $P < 0.05$ versus doxorubicin alone. *C* and *D,* a second siRNA of ARC could influence Drp1 accumulations, mitochondrial fission, and cell death induced by doxorubicin. *Columns,* mean of three independent experiments; *bars,* SE.

Drp1 in mitochondria is due to its irreversibly locking on the membrane (26), it can be speculated that PUMA may directly or indirectly facilitate the locking of Drp1 in mitochondrial membrane.

Our present study reveals that ARC can inhibit doxorubicin-induced mitochondrial fission. We and others have previously shown that ARC plays a role in maintaining mitochondrial integrity in multiple ways. These include the prevention of cytochrome *c* release from mitochondria into cytosol (18, 23), and the maintenance of mitochondrial permeability transition (18). The consequent events of mitochondrial fission include the collapse of mitochondrial membrane potential and cytochrome *c* release (24, 25, 38). It is possible that ARC elicits its effects against the collapse of mitochondrial membrane potential and cytochrome *c* release through inhibiting mitochondrial fission. This hypothesis needs to be tested in future studies.

The subcellular localization of ARC in cancer cells is cell type dependent. For example, ARC is predominantly distributed in the nuclei of HCT116 and A549 cells (4, 6). However, in melanoma cell lines ARC is predominantly distributed in the mitochondria (3). Our present study reveals that ARC is localized in the mitochondria in HeLa and SGC-7901 cells. PUMA is exclusively localized in the mitochondria (27, 28). Our results show that ARC can directly bind to PUMA. This further suggests the functional site of ARC is in mitochondria. Bax has been show to be involved in Drp1 accumulations in mitochondria (26), whereas ARC is able to bind to Bax thereby inhibiting Bax-mediated apoptosis (16, 17). Bax in the healthy cells is predominantly localized in the cytoplasm. In response to apoptotic stimulation, it translocates to mitochondria where it triggers cytochrome *c* release (41–43). It remains to be determined as to whether the association between ARC and Bax occurs in the cytoplasm and/or mitochondria.

It is of note that the expression levels of ARC are decreased in response to doxorubicin treatment. Our recent work has shown that p53 can negatively regulate ARC expression (31). p53 is known to play an important role in mediating DNA damage–induced apoptosis (44). The chemotherapeutic agents such as doxorubicin require p53 to initiate apoptosis (33). It would be interesting to understand the role of p53 in mitochondrial fission.

The induction of apoptosis is beneficial for the treatment of those diseases that are related to abnormal cell proliferation such as cancers. In contrast, the heart is an organ composed of terminally differentiated postmitotic cardiac myocytes. Induction of apoptosis in cardiomyocytes may lead to pathophysiologic disorders. In fact, doxorubicin has been shown to induce cardiomyocyte apoptosis, thereby leading to heart failure (45, 46). ARC is the first anti-apoptotic protein thus far identified to be highly expressed in the heart. Our present study reveals that doxorubicin needs to be in a high dose to induce apoptosis in cancer cells expressing ARC. The cardiotoxicity induce by doxorubicin at a high dose limits its usefulness in chemotherapy. In light of the key role of ARC in controlling doxorubicin-induced apoptosis, it is necessary to find out the molecular approaches that can down-regulate ARC expression in cancer cells but up-regulate ARC in cardiomyocytes. Our present work warrants further studies to explore ARC as a potential target for cancer diagnosis and therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Apoptosis Repressor with Caspase Recruitment Domain Contributes to Chemotherapy Resistance by Abolishing Mitochondrial Fission Mediated by Dynamin-Related Protein-1

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