Letter to the Editor

ARC, an apoptosis suppressor limited to terminally differentiated cells, is induced in human breast cancer and confers chemo- and radiation-resistance

Dear Editor,

Carcinogenesis often involves defects in apoptosis that are mediated by endogenous inhibitors of cell death, including Bcl-2, IAPs, and FLIP.[1,2] ARC (Apoptosis Repressor with a CARD (caspase recruitment domain)) is an endogenous inhibitor of apoptosis that is expressed primarily in terminally differentiated cells such as cardiac and skeletal myocytes and neurons.[3,4] In contrast to most apoptosis inhibitors, which interfere with circumscribed portions of either the extrinsic (death receptor) or intrinsic (mitochondrial/ER) death pathways, ARC antagonizes both central death cascades.[4,5] We have recently demonstrated that this inhibition is mediated by ARC's direct binding to components of each pathway via nonhomotypic death-fold interactions.[5] Thus, ARC's binding to Fas, FADD, and procaspase-8 precludes formation of the death inducing signaling complex (DISC), disabling the extrinsic pathway. ARC's interaction with Bax prevents Bax conformational activation and translocation to the mitochondria, antagonizing the intrinsic pathway. In this way, ARC potently inhibits a wide array of death signals. As inhibition of apoptosis has been implicated in carcinogenesis, we hypothesized that ARC is induced in cancer and plays a functionally important role.

To begin to test this hypothesis, lysates from 48 cancer cell lines from the NCI-60 panel were screened for ARC expression by immunoblot (not shown). ARC was present in cancer cell lines derived from breast (e.g. MCF-7), lung (non-small-cell, e.g. A-549), colon (e.g. HT-29), prostate (e.g. PC-3), kidney (e.g. A-498), and others. ARC levels were highly variable, however, even among different cell lines derived from the same tissue. For example, in contrast to HT-29 and PC-3 cells, SW-60 colon cancer cells and DU-145 prostate cancer cells exhibited low ARC levels. Within breast cancer lines, ARC was expressed at high levels in MCF-7 and T-47D cells, at intermediate levels in BT-549 and ADR-RES cells, and to a much lesser extent, in Hs578T, MDA-MB-231, MDA-MB-435, and HCC1419 cells. Significant ARC was present in both estrogen receptor positive (e.g. MCF-7) and negative (e.g. BT-549) cell lines. Notably, MCF-10A and MCF-12A, immortalized epithelial cell lines derived from benign breast tissue, exhibited very low ARC levels. These data demonstrate that ARC is expressed at high levels in some, but not all, cancer cell lines.

Owing to limitations inherent to immortalized cell lines, we next examined ARC expression in breast tissue from 36 women with invasive ductal breast carcinoma and 10 women without breast cancer who underwent reduction mammoplasty (Figure 1). Immunohistochemistry was performed using an antiserum against a peptide from the unique C-terminus of ARC. This reagent was used to avoid potential ambiguity from antibodies that recognize the N-terminus of the protein, which contains a CARD, a motif found in other proteins. This antiserum identifies a single band on SDS-PAGE of molecular mass consistent with ARC (not shown). As demonstrated in representative sections, ARC was present in the cytoplasm and nuclei of epithelial cells in invasive ductal carcinoma (Figure 1b, e). In contrast, ARC in reduction mammoplasty specimens was limited almost exclusively to nuclei (Figure 1a, d). ARC staining in invasive ductal carcinoma was specific, as it was extinguished by a 50-fold molar excess of peptide corresponding to the epitope (Figure 1c) as well as by omission of the primary antiserum (not shown).

In aggregate (Table 1), invasive ductal carcinomas from 32/36 (89%) women exhibited cytoplasmic ARC staining compared with benign breast tissue from 6/29 (21%) of the same women ($P<0.0001$) or reduction mammoplasty specimens from 1/10 (10%) women ($P<0.0001$). Moreover, on a 0–2 scale, the mean intensities of cytoplasmic ARC staining in invasive ductal carcinomas, benign breast tissue from the same patients, and mammoplasty specimens were 1.31, 0.21, and 0.1 respectively ($P<0.0001$ for invasive ductal carcinoma *versus* each control group). Within invasive ductal carcinomas, cytoplasmic ARC staining did not differ significantly among tumor grades (not shown). These data demonstrate that ARC is present at high levels in the cytoplasm of most invasive ductal carcinomas, but not benign breast tissue.

ARC has been previously localized to the cytosol and mitochondria,[6] and its antiapoptotic actions have been be ascribed to pools in those locations.[5] Thus, our finding that ARC is also present in nuclei of epithelial cells of benign and malignant breast tissues is unexpected. To further examine whether endogenous ARC is present in the nucleus, we carried out immunostaining and cellular fractionation experiments on MCF-7 breast cancer cells and primary cultures of cardiac myocytes, benign nonepithelial cells. While most ARC was cytoplasmic (including cytosol and cytoplasmic membranes), a significant portion resided in the nucleus of both cell
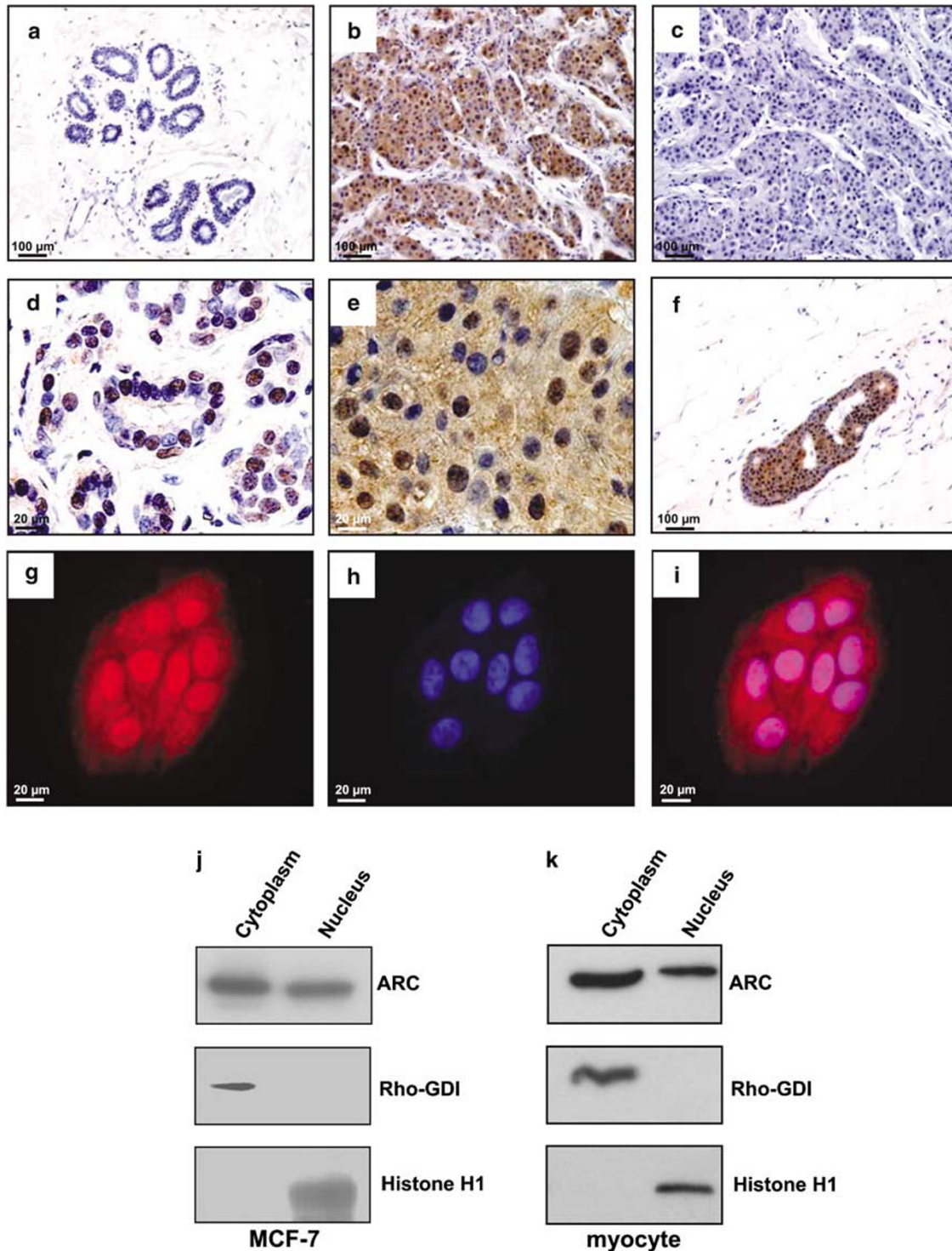
Figure 1 ARC in human breast cancer tissue. (**a–f**) ARC immunohistochemistry with antiserum against residues 176–195 (Neuromics, Northfield, MN, USA). Similar results obtained with antiserum against residues 191–208 (Neomarkers, Fremont, CA, USA). Tissues counterstained with hematoxylin. Mammoplasty specimen under low and high power respectively (**a**, **d**). Invasive ductal carcinoma under low and high power respectively (**b**, **e**). Signal in panel (**b**) ablated by competing peptide (**c**). DCIS (**f**). (**g–i**) MCF-7 cells stained for ARC (Cayman antiserum against residues 191–208; similar results with Neuromics antiserum) (**g**), Hoechst 33258 (**h**), and merged images (**i**). (**j**, **k**) ARC immunoblots (Cayman antisera) of cytoplasmic and nuclear fractions from MCF-7 cells and primary cardiac myocytes. Replica immunoblots for Rho-GDI (cytoplasmic marker) and Histone-H1 (nuclear marker)

types (Figure 1g–k and immunostaining of cardiac myocytes (not shown)). The use of antisera against the ARC C-terminus excludes the possibility that our signal was due to Nop30, a hypothetical nucleolar protein that may be generated through alternative splicing of the ARC locus.[7] Thus, ARC resides in nuclei of both epithelial and nonepithelial cells.

Table 1 ARC in human breast cancer[a]

Patients[c]	Cytoplasmic ARC immunostaining[b]			Nuclear ARC immunostaining[b]		
	Invasive ductal CA	Benign tissue[d]	DCIS[d]	Invasive ductal CA	Benign tissue[d]	DCIS[d]
Cancer						
%Positive	32/36 (89%)[e]	6/29 (21%)	20/23 (87%)[e]	23/36 (64%)[g]	12/29 (41%)	14/23 (61%)[g]
Mean intensity	1.31[f]	0.21	1.30[f]	N/A[h]	N/A	N/A
Controls						
%Positive	N/A	1/10 (10%)	N/A	N/A	6/10 (60%)	N/A
Mean intensity	N/A	0.1	N/A	N/A	N/A	N/A

[a]This study was approved by the Institutional Review Board of Montefiore Medical Center. [b]Cytoplasmic staining scored 0–2: 0 (absent signal even at 40 × or faint signal present in <10% of the tissue); 1 (signal barely perceptible at 4 × and clearly seen at 40 ×); 2 (strong signal at 4 ×). Nuclear staining scored detectable or not detectable. [c]Breast cancer – patients with invasive ductal carcinoma. Controls – patients with benign breast tissue who underwent reduction mammoplasty. [d]Benign tissue from either the ipsilateral or contralateral breast of patients with invasive ductal carcinoma. DCIS from the ipsilateral breast. [e]Percent patients with cytoplasmic ARC staining: $P < 0.0001$ for invasive ductal carcinoma *versus* mammoplasty control (Fisher exact test); DCIS *versus* mammoplasty control (Fisher exact test); invasive ductal carcinoma *versus* same patient benign tissue (McNemars's test); DCIS *versus* same patient benign tissue (McNemar's test). [f]Mean cytoplasmic ARC staining intensity: $P < 0.0001$ for invasive ductal carcinoma *versus* mammoplasty control (Wilcoxon Rank Sum test); invasive ductal carcinoma *versus* same patient benign tissue (Wilcoxon Signed Rank test); DCIS *versus* same patient benign tissue (Wilcoxon Signed Rank test). $P < 0.0005$ for DCIS *versus* mammoplasty control (Wilcoxon Rank Sum test). [g]Percent patients with nuclear ARC staining: P NS for invasive ductal carcinoma vs mammoplasty control (Fisher exact test); DCIS *versus* mammoplasty control (Fisher exact test); invasive ductal carcinoma *versus* same patient benign tissue (McNemar's test); DCIS *versus* same patient benign tissue (McNemar's test). [h]N/A - not applicable

We next assessed whether the presence of nuclear ARC differs between cancerous and benign breast tissues. ARC was present in nuclei of similar percentages of invasive ductal carcinomas (64%), benign breast tissue in the same patients (41%), and reduction mammoplasty specimens (60%) (*P*, NS). Thus, approximately half of both benign and cancerous breast specimens exhibit ARC in nuclei of epithelial cells. The function of this nuclear pool is not known.

Some tissues from patients with invasive ductal carcinoma also contain DCIS (Figure 1f). While the frequency of cytoplasmic ARC staining in DCIS (20/23 patients (87%)) and its intensity (1.30) were similar to that observed in invasive ductal carcinoma (*P*, NS), both parameters differed markedly from that of benign breast tissue from the same patients (*P* < 0.0001) and mammoplasty specimens (*P* < 0.0001 for frequency and *P* < 0.0005 for intensity). In contrast, the frequency of nuclear ARC staining (14/23 patients (61%)) in DCIS lesions was similar to that found in benign and other cancerous breast tissues (*P*, NS).

In summary, these data demonstrate that high levels of cytoplasmic ARC are found in most invasive ductal carcinomas and DCIS, but not in benign breast tissues. In contrast, ARC is present in the nuclei of benign and cancerous breast tissue.

Since ARC is known to inhibit apoptosis through cytoplasmic protein–protein interactions[5] and since ARC is present at high levels in the cytoplasm of most invasive ductal carcinomas, we hypothesized that this protein may contribute to chemo- and radiation-resistance in breast cancer. To test this hypothesis, we exploited the fact that some breast cancer cell lines, such as Hs578T cells, contain low levels of endogenous ARC. Accordingly, we generated pools of Hs578T cells that were stably transfected with ARC driven by a constitutive promoter (Hs578T-ARC) or with vector (Hs578T-vector). Hs578T-ARC cells, but not Hs578T-vector cells, expressed high levels of ARC, and these levels did not change following treatment with doxorubicin or γ-radiation (not shown). The number of surviving cells was higher in Hs578T-

ARC cells, compared with Hs578T-vector cells, following treatment with 1 μM, but not 5 μM, doxorubicin (Figure 2a, left). This effect could not be attributed to ARC-induced proliferation because basal cell numbers following 36 h in culture did not differ between the two cell types (CTL in Figure 2a, left). Moreover, cell death induced by 1 μM doxorubicin was inhibited by ARC, as evidenced by the lower percentage of apoptotic nuclei in Hs578T-ARC cells compared with Hs578T-vector cells (Figure 2a, right). Similarly, ARC inhibited basal levels of cell death as well as cell death induced by 2.1–12.9 Gy of γ-radiation (Figure 2b). These data indicate that ARC partially protects against doxorubicin and γ-radiation, and suggests that this apoptosis inhibitor may contribute to chemo- and radiation-resistance in breast cancer cells.

This study demonstrates that ARC, an apoptosis inhibitor restricted to terminally differentiated cells, is expressed at high levels in multiple cancer cell lines. Moreover, abundant ARC is present in the epithelial cytoplasm of most invasive ductal carcinomas and DCIS, but not benign breast epithelium. In contrast, nuclear ARC is present in both benign and cancerous breast epithelial cells as well as in terminally differentiated heart muscle cells. Finally, ARC overexpression in a breast cancer line inhibits doxorubicin- and γ-radiation-induced apoptosis.

The presence of abundant ARC in cancer cells is consistent with the notion that these cells co-opt diverse mechanisms to promote their survival.[1] Often these mechanisms involve increases in the levels of ubiquitously expressed endogenous inhibitors of apoptosis.[2] In contrast, the expression of ARC is normally restricted to terminally differentiated cells,[3,4] which often possess limited regenerative potential. Moreover, endogenous ARC appears to function as an obligate survival factor in some such cells.[5] Thus, the ectopic expression of ARC may provide cancer cells with a survival mechanism that normally serves to protect poorly renewable cell populations.

The mechanisms that lead to increased cytoplasmic ARC in breast cancer are not known. The lack of grossly visible
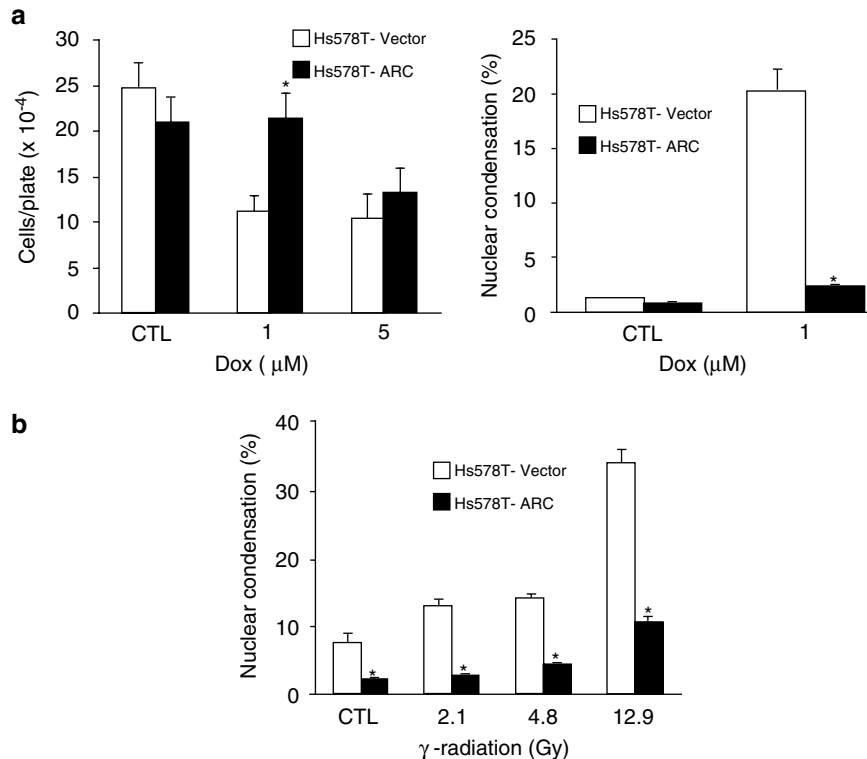
Figure 2 Inhibition of doxorubicin- and γ-radiation-induced apoptosis in breast cancer cells. (**a**) Pools of Hs578T cells stably transfected with ARC (Hs578T-ARC) or vector (Hs578T-vector) were treated with doxorubicin for 2 h and then cultured for an additional 22 h. Mean ± S.E.M. number of adherent cells (left) and percentage of condensed nuclei as assessed by Hoechst 33258-staining of adherent and floating cells (right). (**b**) The same transfectants in serum-free media were subjected to γ-radiation from a self-contained Cesium-137 irradiator and then cultured in serum-containing media for 7 days. Mean ± S.E.M. percentage of condensed nuclei. Five fields scored per condition. Experiments performed in duplicate with 2–4 independent replicates. *$P < 0.05$ by two-tailed t-test

differences in the amount of nuclear ARC in benign *versus* cancerous breast epithelial cells suggests that increases in cytoplasmic ARC reflect augmentation of total cellular ARC levels and not simply a redistribution from the nuclear compartment. As nuclear ARC levels were not assessed quantitatively in this study, however, this possibility requires formal testing. Assuming that total cellular ARC levels are increased in breast cancer cells, it will be of interest to determine the mechanisms that regulate these changes and the associated pathways.

Although a mechanistic role for ARC in breast carcinogenesis remains to be proven, the marked induction of this protein in both invasive ductal carcinoma and DCIS suggests that ARC may be involved in early pathogenic events. An expected role for ARC would be to inhibit apoptosis, which is consistent with increases of ARC in the cytoplasm, the location in which its antiapoptotic functions are believed to take place.[5] Indeed, inhibition of apoptosis has been shown to play important roles in several aspects of breast cancer pathogenesis. For example, the loss of the intraluminal space requires inhibition of cell death as well as stimulation of cell proliferation.[8] Moreover, suppression of apoptosis by ligation of α6β4 integrin is critical for survival of tumorigenic breast epithelial cells either when maintained by basement membrane as polarized structures or when growing in an anchorage-independent manner.[9,10] ARC's unusual ability to inhibit both central death pathways[4,5] may be ideally suited to the task of inhibiting apoptosis during carcinogenesis. For

example, ARC interacts with and inhibits Bax,[5] an activator of the intrinsic pathway, that mediates anoikis of breast epithelial cells.[11] Similarly, by inhibiting DISC formation in the extrinsic pathway[4,5] and by blocking Bax-dependent amplification through the intrinsic pathway,[12–14] ARC would be predicted to inhibit TRAIL-mediated killing, a process important in host defences against cancer cells.[15,16] In this regard, it would be interesting to determine whether BAR, another repressor of both central pathways,[17] whose expression is usually neuron-restricted,[18] is also induced in cancer.

Apart from its potential role in breast cancer pathogenesis, ARC may be an important factor in determining the response of breast cancers to a variety of therapies. Our data suggest that ARC contributes to radiation- and chemoresistance in breast cancer cells. This finding is not unexpected given ARC's potent inhibition of the intrinsic pathway.[5] As discussed above, ARC may also interfere with TRAIL-based therapies.

In summary, the apoptosis repressor ARC is ectopically induced in the cytoplasm of most invasive ductal carcinomas and DCIS lesions and may contribute to the resistance of these tumors to chemotherapy and radiation.

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