Activator Protein 2α Status Determines the Chemosensitivity of Cancer Cells: Implications in Cancer Chemotherapy

Narendra Wajapeyee,[1] Chandrashekhar Ganpat Raut,[2] and Kumaravel Somasundaram[1]

[1]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India and [2]National Institute of Virology, Pune, India

Abstract

Cancer chemotherapeutic drugs induce apoptosis by several pathways. Inactivation of proapoptotic genes, or activation of survival signaling, leads to chemoresistance. Activator protein 2α (AP-2α), a developmentally regulated sequence-specific DNA-binding transcription factor, has been shown to function like a tumor suppressor. Here, we show that controlled expression of AP-2α, using tetracycline-inducible system, increased the chemosensitivity of cancer cells by severalfold by sensitizing cells to undergo apoptosis upon chemotherapy. Under these conditions, neither AP-2α expression nor drug treatment resulted in apoptosis induction, whereas in combination the cancer cells underwent massive apoptosis. We found that endogenous AP-2α protein is induced posttranscriptionally by various chemotherapeutic drugs. Blocking the endogenous AP-2α by small interfering RNA in human cancer cells lead to decreased apoptosis, increased colony formation, and chemoresistance irrespective of their p53 status upon chemotherapy. We further show that 5-aza-2′-deoxycytidine induced reexpression of AP-2α in MDA-MB-231 breast cancer cells (wherein AP-2α expression is silenced by hypermethylation), resulted in massive apoptosis induction, increased chemosensitivity, decreased colony formation, and loss of tumorigenesis upon chemotherapy. However, in MDA-MB-231 cells transfected with AP-2α small interfering RNA, 5-aza-2′-deoxycytidine treatment failed to increase apoptosis and chemosensitivity. The treatment also resulted in increased colony formation and efficient tumor formation upon chemotherapy. These results establish an important role for AP-2α in cancer cell chemosensitivity and provide new insights for modifying the chemosensitivity of cancer cells by activating apoptotic pathways. (Cancer Res 2005; 65(19): 8628-34)

Introduction

Activator protein 2α (AP-2α) is a sequence-specific DNA-binding transcription factor that is required for normal growth and morphogenesis (1). *AP-2α* gene is the archetypal member of the AP-2 family of transcription factors, which in mouse and human also includes AP-2β, AP-2γ, AP-2δ, and AP-2ε (2). AP-2α has been shown to regulate many genes related to variety of biologically important functions (3). Several lines of evidence indicate that *AP-2α* may act as a tumor suppressor gene. *AP-2α* gene is located in chromosome position 6p22, a region of frequent loss of heterozygosity in breast and other cancers (4). Diminished AP-2α function has been correlated with N-*ras* oncogene-mediated transformation (5). The functions of AP-2α are regulated by SV40 T antigen and adenovirus E1A oncoproteins (6, 7). In addition, reduced, or loss of, AP-2α expression has been reported in human cancers of breast, ovary, colon, skin, brain, and prostate (8–13). In good correlation, expression of dominant-negative mutant AP-2α resulted in increased invasiveness and tumorigenicity (13).

Based on the following reports, we hypothesized that AP-2α may have a role to play in determination of cancer cell chemosensitivity: Overexpression of AP-2α in cancer cells is highly cytotoxic by inducing cell cycle arrest and apoptosis (14, 15); Targeted overexpression of AP-2α in mouse mammary gland using mouse mammary tumor virus promoter resulted in reduced cellular proliferation and increased cell death (16); AP-2α expression is lost in many human cancers (8–13). In the present study, we show that cancer cells expressing AP-2α are highly sensitive to many chemotherapeutic drugs and loss of AP-2α leads to chemoresistance. Further, we show that chemotherapy induces endogenous AP-2α posttranscriptionally, which contributes to chemosensitivity.

Materials and Methods

Plasmids, adenoviruses, and cell lines. pSG5/AP-2, 3X-AP2-CAT, pUHD15.1, and adenovirus-expressing tetracycline-responsive transcriptional activator (Ad-tTA) are described before (15, 17). AP-2α was cloned in pUHD15.1/AP-2α as an *Eco*R1 fragment. Human cancer cell lines SW480, MDA-MD-231, H460, HCT116, H1299, and SK-N-MC were described previously (8, 15).

Western blot analysis, flow cytometry, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and clonogenic assay. Western blot analysis was done as described previously (15) with mouse anti-human poly(ADP-ribose) polymerase (PARP) monoclonal antibody (Ab-2; Oncogene), rabbit anti-human AP-2 polyclonal (sc-184; Santa Cruz), and goat anti-human actin polyclonal antibody (sc-1616, Santa Cruz; Ab-1, Oncogene). For fluorescence-activated cell sorting (FACS) analysis, the cells were harvested at indicated time points after the appropriate treatment and carried out as described (15). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assays were done as described previously (15, 18).

Small interfering RNA and reverse transcription-PCR. AP-2α specific small interfering (siRNA) was obtained from Dharmacon as si*GENOME SMART* pool reagent, which contains a pool of four different double-stranded RNA oligonucleotides (siRNA) directed against AP-2α. lamin siRNA was purchased as si*GLO* lamin A/C siRNA (human/mouse/rat) from Dharmacon. The cells were transfected using OligofectAMINE (Invitrogen, Inc.) at 100 nmol/L concentration of siRNA. Total RNA was extracted from tissue culture cells by TRIzol method (Invitrogen) according to the instructions of the manufacturer. Reverse transcription-PCR (RT-PCR) was carried out using a two-step strategy as per instructions of the manufacturer: cDNA was generated using Reverse Transcription kit (Promega) in the first step, and then using gene-specific primer sets, PCR

Note: K. Somasundaram is a Wellcome Trust International Senior Research Fellow.

Requests for reprints: Kumaravel Somasundaram, Department of Microbiology and Cell Biology, Indian Institute of Science, Sir C.V. Raman Road, Bangalore, Karnataka 560-012, India. Phone: 91-80-22932973; Fax: 91-80-23602697; E-mail: skumar@mcbl.iisc.ernet.in.

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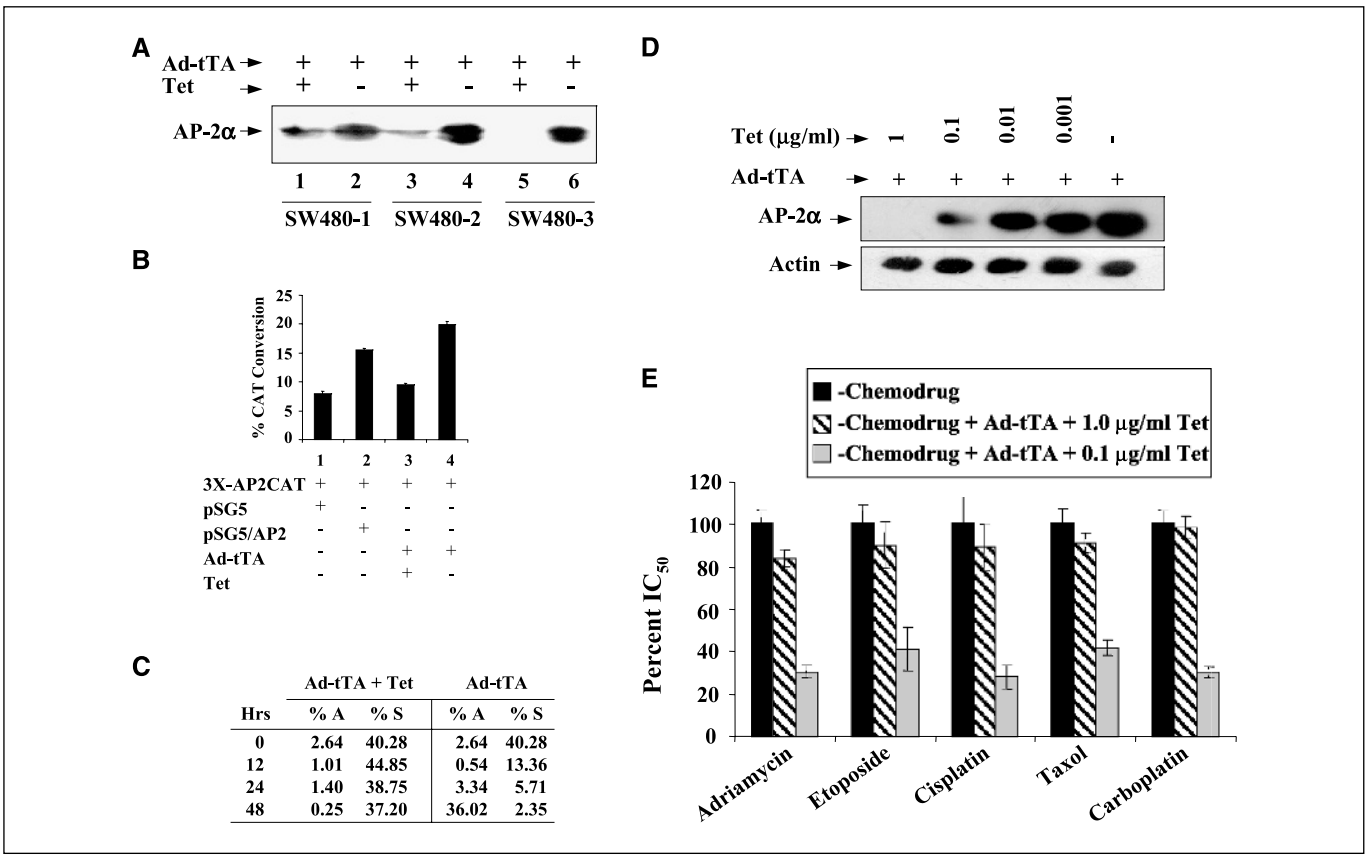

Figure 1. Conditionally expressed AP-2α using Tet-Off transcription system increases the chemosensitivity of cancer cells. AP-2α expression from the tetracycline-responsive promoter in stable SW480 cell clones (see Materials and Methods) was induced by infecting them with Ad-tTA in the absence of tetracycline. *A,* conditional expression of AP-2: The Tet-Off stable clones of AP-2α (three independent clones: SW480-1, SW480-2, SW480-3) were infected with Ad-tTA in the presence (1 μg/mL) or absence of tetracycline. Total cell extracts were made after 24 hours and subjected to Western blot analysis for AP-2α protein. *B,* conditionally expressed AP-2α activates transcription from AP-2α–specific reporter 3X-AP2-CAT: SW480-3 cells (this clone was used for subsequent experiments as it had very minimal uninduced levels of AP-2α) was transfected with 5 μg of 3X-AP2-CAT (*lanes 1-4*) and control vector pSG5 (5 μg; *lane 1*), or pSG5/AP-2α (5 μg; *lane 2*). For samples in lanes 3 and 4, cells were infected with Ad-tTA in the presence or absence of tetracycline at 20 multiplicity of infection (MOI) after 6 hours of transfection. The total amount of DNA per transfection was kept constant at 10 μg. Lysates were prepared and analyzed for chloramphenicol acetyltransferase reporter activity 24 hours posttransfection as described in Materials and Methods. All the samples were done in duplicate. *C,* SW480-3 cells were infected with Ad-tTA (20 MOI) in the presence and absence of tetracycline. The cells were allowed to incorporate BrdUrd during the last 4 hours of time points at which they were collected. The cells were harvested at 0, 12, 24, and 48 hours after infection and subjected to flow cytometry analysis as mentioned in Materials and Methods. %S, which indicates the percentage of cells undergoing DNA synthesis (S phase), and %A, which indicates the percentage of cells containing <2*N* amount of DNA (apoptotic cells), were calculated and shown. *D,* SW480-3 cells were infected with Ad-tTA at 20 MOI in the absence or presence of the tetracycline (indicated amounts). Total cell extract were made after 24 hours of virus infection and subjected to Western blot analysis for AP-2α and actin (sc1616; Santa Cruz) proteins. *E,* SW480-3 cells were mock infected or infected with Ad-tTA (20 MOI) in the presence either 1 or 0.1 μg/mL tetracycline. Varying concentrations of indicated drugs (Adriamycin: 0.1, 0.2, 0.4, and 0.8 μg/mL; etoposide: 0.5, 1, 2, and 4 μmol/L; cisplatin: 0.25, 0.5, 1.0, 2.0 μg/mL; Taxol: 2, 4, 8, and 16 μmol/L; carboplatin: 2.5, 5, 10, and 20 μg/mL) were added 6 hours postinfection. At 48 hours postdrug addition, the proportion of live cells was quantified by MTT assay as described in Materials and Methods. The IC_{50} levels were calculated for each drug at different conditions and converted to percentage, keeping IC_{50} of drug alone as 100% and shown.

was carried out with cDNA as templates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used for internal normalization. The sequences of the sense and antisense primers used for RT-PCR are available upon request.

Activator protein 2α Tet-Off inducible system. SW480 cells were transfected with 5 μg of pUHD15.1/AP-2α plasmid using LipofectAMINE (Invitrogen) and selected at 800 μg/mL G418 (Calbiochem). The G418-resistant colonies were cloned and screened for AP-2α expression after Ad-tTA infection. Positive clones expressing AP-2α only in the absence of tetracycline were used for further experiments.

Tumorigenicity experiment. NIH Swiss *nu/nu* strain of mice was used for the animal experiments (National Institute of Virology, Pune, India). Mice were divided into six experimental groups. MDA-MB-231 viable cells (1×10^7, as scored by trypan blue exclusion test), which underwent different treatments as indicated in the figure legends (Fig. 4*F*), were injected into the right flank of nude mice. The tumors were measured from the day 5 postinjection at every 3-day interval. Tumor volume was calculated using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2.

Results

Activator protein-2α overexpression increases the chemosensitivity of cancer cells. We found Ad-AP2 (AP-2α–expressing adenovirus) infected cancer cells of different tissue types [(SW480 (mutant p53), H460 (wild-type p53), HCT116 (wild-type p53), H1299 (deleted for p53), and SK-N-CH (mutant p53)] are manyfold more sensitive to commonly used chemotherapeutic drugs (Adriamycin, etoposide, cisplatin, Taxol, and carboplatin; ref. 15; data not shown). To better understand the mechanism of increased chemosensitivity in AP-2α–overexpressing cells, we generated SW480 cell clones in which AP-2α expression is regulated by the Tet-Off transcription system. In three independently isolated clones, AP-2α is induced severalfold in Ad-tTA (17) infected cells only in the absence of tetracycline (Fig. 1*A*, compare *lanes 2, 4*, and *6* with *1, 3*, and *5*) and is functional as it activated transcription from AP-2–specific reporter, 3X-AP2-CAT (Fig. 1*B*,

compare *column 4* with *3*), inhibited cellular DNA synthesis and induced apoptosis as measured by FACS analysis (Fig. 1*C*). Because the overexpression of AP-2α by itself induces apoptosis (15), we fine tuned the expression of AP-2α by using decreasing amounts of tetracycline and found that the addition of 0.1 μg/mL tetracycline induced AP-2α levels by ~2-fold (Fig. 1*D*). Under these conditions, 2-fold induction of AP-2α did not inhibit the growth of cells significantly (data not shown) and failed to induce apoptosis (Fig. 2*A*). Next, we analyzed the sensitivity of SW480-3 cells infected with Ad-tTA and treated with 0.1 μg/mL tetracycline to chemotherapy. Under these conditions, the IC_{50} levels reduced ~2- to 3-fold for all drugs used (Fig. 1*E*). These results suggest that controlled expression of AP-2α, which by itself is not cytotoxic, indeed increases chemosensitivity of cancer cells.

To understand the mechanism of AP-2α–mediated increase in chemosensitivity, we examined cells expressing AP-2α to undergo apoptosis upon chemotherapy. SW480-3 cells were infected with Ad-tTA in the presence of 0.1 μg/mL tetracycline, treated with chemotherapeutic drugs, and subjected to FACS analysis. Whereas the induction of AP-2α by the addition of 0.1 μg/mL tetracycline resulted in inhibition of cellular DNA synthesis, no apoptosis induction was detected (Fig. 2*A*). Similarly, Adriamycin addition (both IC_{25} and IC_{50}) in the absence of AP-2α induction resulted in efficient inhibition of DNA synthesis, leading to accumulation of cells in G_2-M phase; however, no apoptosis was detected (Fig. 2*A*). In contrast to this, Adriamycin induced massive apoptosis upon induction of AP-2α by the addition of 0.1 μg/mL tetracycline (Fig. 2*A*). Similarly, other chemotherapeutic drugs, e.g., cisplatin and Taxol, induced high levels of apoptosis under AP-2α–induced condition (Fig. 2*B*). Etoposide also induced apoptosis under AP-2α–overexpressed condition, although to a lesser extent (Fig. 2*B*). These experiments suggest that controlled expression of AP-2α does not induce apoptosis by itself, but it sensitizes the cancer cells to undergo very high levels of apoptosis upon chemotherapy.

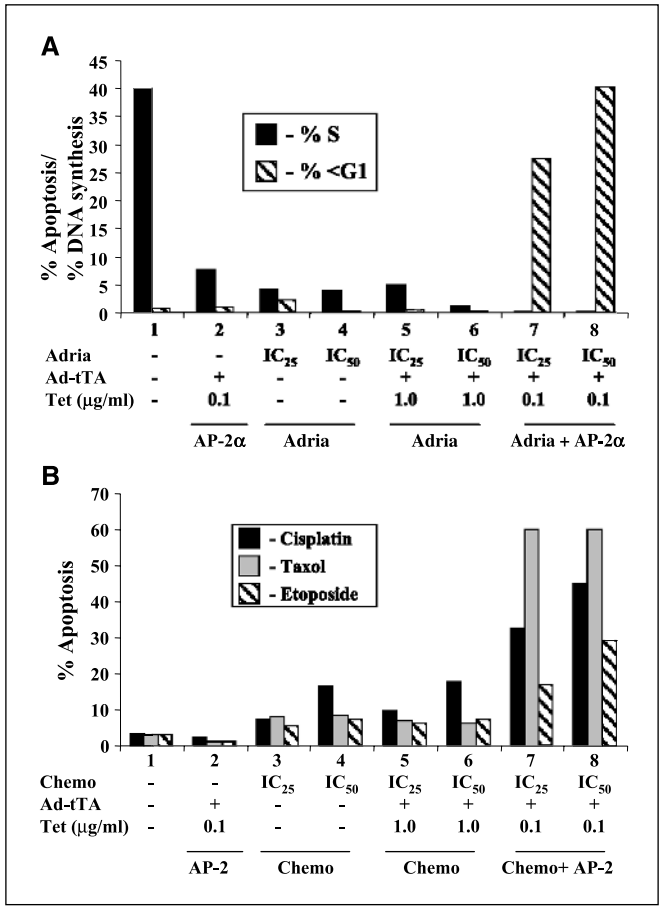

Figure 2. Regulated AP-2α expression sensitizes cancer cells to undergo apoptosis upon chemotherapy. *A,* SW480-3 cells were infected with Ad-tTA (20 MOI) in the presence (either 1 or 0.1 μg/mL as indicated) or absence of tetracycline. In some samples, Adriamycin was added as indicated (IC_{25}: 0.15 μg/mL; IC_{50}: 0.30 μg/mL) after 6 hours of virus infection. The cells were allowed to incorporate BrdUrd during the last 4 hours of time points at which they were collected. The cells were harvested at 48 hours after drug addition and subjected to flow cytometry analysis as mentioned in Materials and Methods. %S, which indicates the percentage of cells undergoing DNA synthesis (S phase), and %A, which indicates the percentage of cells containing <2*N* amount of DNA (apoptotic cells), were calculated and shown. *B,* SW480-3 cells were subjected to similar experiments mentioned in (*A*), but the following drugs were added: cisplatin (IC_{25}: 0.475 μg/mL; IC_{50}: 0.95 μg/mL), Taxol (IC_{25}: 4.35 μmol/L; IC_{50}: 8.7 μmol/L), and etoposide (IC_{25}: 0.96 μmol/L; IC_{50}: 1.92 μmol/L). %A, which indicates the percentage of cells containing <2*N* amount of DNA (apoptotic cells), were calculated and shown.

Chemotherapy induces posttranscriptionally endogenous activator protein-2α, which contributes to chemosensitivity. To ask whether endogenous AP-2α has any role in chemosensitivity determination, we examined the AP-2α transcript and protein levels in SW480 cells treated with chemotherapeutic drugs. Adriamycin, cisplatin, and Taxol, but not etoposide, induced AP-2α protein levels in time-dependent manner (Fig. 3*A*). We found that AP-2α induction upon chemotherapy was revealed only after careful experiments that examined the influence of drug dose and duration. Chemotherapy-mediated induction of AP-2α protein is posttranscriptional, as no significant change was observed in AP-2α transcripts levels under similar conditions (Fig. 3*B*). Further, to find out the role of chemotherapy-induced endogenous AP-2α in chemosensitivity, we resorted to siRNA approach. Transfection of SW480 cells with AP-2α siRNA but not lamin siRNA lead to complete loss of AP-2α transcript (Fig. 3*C*). Neither AP-2α siRNA nor lamin siRNA transfection affected the levels of GAPDH transcripts (Fig. 3*C*). Similarly, AP-2α siRNA, but not lamin siRNA, inhibited the chemotherapy-induced accumulation of endogenous AP-2α (Fig. 3*D*) and conferred protection against Adriamycin, cisplatin, and Taxol (Fig. 3*E*). Notably, AP-2α siRNA protected cells treated with etoposide much less significantly (Fig. 3*E*), which correlates with inability of etoposide to induce endogenous AP-2α (Fig. 3*A*). Because long-term assays reflect more accurately chemosensitivity of a tumor, we did clonogenic assays using SW480 cells transfected with AP-2α siRNA. Adriamycin-treated cells transfected with AP-2α siRNA formed more colonies than cells transfected with lamin siRNA or buffer alone (mock; data not shown). Neither AP-2α nor lamin siRNA affected colony formation by untreated cells (data not shown). As expected, the increased viability of chemotherapy-treated cells transfected with AP-2α siRNA was found to be due to decreased apoptosis. PARP cleavage was significantly decreased in AP-2α siRNA-transfected cells compared with lamin siRNA or buffer (mock)-transfected cells upon chemotherapy (except etoposide; Fig. 3*F*). Taken together, these experiments suggest that chemotherapy posttranscriptionally induces accumulation of endogenous AP-2α, which contributes to chemosensitivity.

Hypermethylation-induced silencing of activator protein-2α in breast cancer contributes to chemoresistance *in vitro* and *in vivo*. Whereas no genetic alterations in *AP-2α* gene in cancers have been reported, progressive loss of AP-2α expression with tumor progression has been reported in cancers (8–13). The loss of AP-2α expression in invasive breast cancer has been correlated

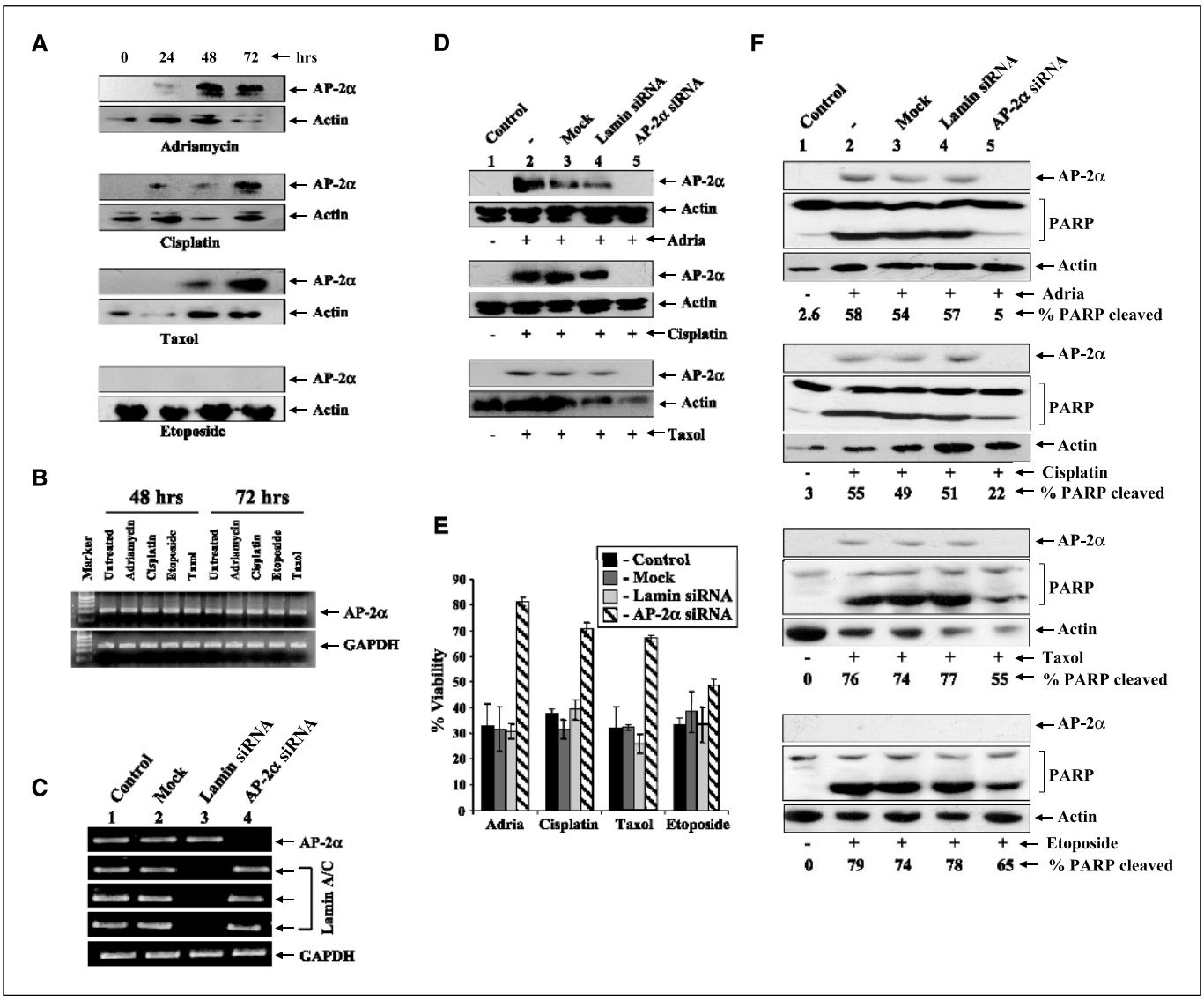

Figure 3. Posttranscriptional induction of endogenous levels of AP-2α by chemotherapy and its role in chemosensitivity. *A* and *B,* SW480 cells were treated with Adriamycin (0.1 μg/mL), cisplatin (2 μg/mL), Taxol (2.5 μmol/L), and etoposide (1 μmol/L). The cells were harvested at 0, 24, 48, and 72 hours after drug treatment and the total cell lysates were subjected to Western blot analysis for AP-2α and actin proteins in (*A*). Total RNA was isolated after 48 and 72 hours of the treatment and subjected to the RT-PCR analysis for AP-2α and GAPDH in (*B*). *C,* SW480 cells were left untransfected (*Control*) or mock transfected or transfected with lamin siRNA or AP-2α siRNA, and total RNA was prepared after 96 hours of the transfection and subjected to RT-PCR analysis for AP-2α, lamin A/C (three different transcripts), or GAPDH. *D,* SW480 cells were left untreated (*Control*), untransfected (−), or mock transfected or transfected with 100 nmol/L of lamin siRNA or AP-2α siRNA. At 48 hours posttransfection, the cells were treated as indicated with Adriamycin (0.1 μg/mL), cisplatin (2 μg/mL), or Taxol (2.5 μmol/L) for another 48 hours and, after which, the cells were harvested and subjected for Western blot analysis of AP-2α and actin proteins. *E,* SW480 cells were either left untransfected (*Control*), mock transfected, or transfected with lamin siRNA or AP-2α siRNA. At 48 hours posttransfection, the cells were treated with different concentrations (Adriamycin: 0.1, 0.2, 0.4, and 0.8 μg/mL; etoposide: 0.5, 1, 2, and 4 μmol/L; cisplatin: 0.25, 0.5, 1.0, 2.0 μg/mL; Taxol: 2, 4, 8, and 16 μmol/L) of indicated drugs. After 48 hours of drug treatment, proportion of live cells was quantified by MTT assay. The absorbance of control cells was considered as 100%. The proportion of viable cells at 0.4 μg/mL Adriamycin, 1 μg/mL cisplatin, 2 μmol/L etoposide, and 8 μmol/L Taxol were calculated and shown. *F,* SW480 cells were either left untreated (*Control*), untransfected (−), mock transfected, or transfected with lamin siRNA or AP-2α siRNA, and after 48 hours of transfection the cells were treated with 0.8 μg/mL Adriamycin, 2 μg/mL cisplatin, 4 μmol/L etoposide, and 16 μmol/L Taxol. After 48 hours of the drug treatment, cells were harvested and then subjected to Western blot analysis for AP-2α, PARP (Ab-2; Oncogene), and actin proteins. Percentage PARP cleavage in each of the sample is quantitated and shown below.

with hypermethylation of CpG island in the exon 1 of *AP-2α* gene and consequent silencing (8). We hypothesized that methylation-mediated (epigenetic) silencing of AP-2α expression may contribute to chemoresistance in breast cancer. To prove this hypothesis, we used a breast cancer line MDA-MB-231, where AP-2α expression is silenced by hypermethylation of its exon 1 (8). Treatment of MDA-MB-231 cells with the methylation inhibitor 5-aza-2′ deoxycytidine (5aza2dC) resulted in reexpression of AP-2α transcript (Fig. 4*A*, compare *lane 2* with *1*) and protein (Fig. 4*B* compare *lane 2* with *1*). In addition, 5aza2dC-induced reexpression of AP-2α resulted in increased chemosensitivity to Adriamycin and cisplatin (Fig. 4*C*). To ask whether reexpressed AP-2α (upon 5aza2dC treatment) is indeed responsible for increased chemosensitivity, we again resorted to siRNA approach. In MDA-MB-231 cells transfected with AP-2α siRNA, but not lamin siRNA or buffer alone (mock), 5aza2dC treatment failed to reexpress AP-2α (Fig. 4*B*), and the cells remained resistant to Adriamycin and cisplatin (Fig. 4*C*). In MDA-MB-231 cells treated with 5aza2dC,

Adriamycin inhibited colony formation much more efficiently compared with untreated cells (data not shown). However, in 5aza2dC-treated MDA-MB-231 cells transfected with AP-2α siRNA, but not lamin siRNA or buffer alone (mock), Adriamycin failed to inhibit colony formation (data not shown). As expected, the chemoresistance seen in MDA-MB-231 cells transfected with AP-2α siRNA was due to decreased apoptosis as seen by flow cytometry (Fig. 4*D*) and PARP cleavage (Fig. 4*E*). These results suggest that reexpression of epigenetically silenced AP-2α in breast cancer cells increases the chemosensitivity by sensitizing them to undergo apoptosis upon chemotherapy.

To test whether the above results would correlate with tumor growth *in vivo*, MDA-MB-231 cells either untreated or treated with 5aza2dC or Adriamycin or both drugs for 24 hours were injected

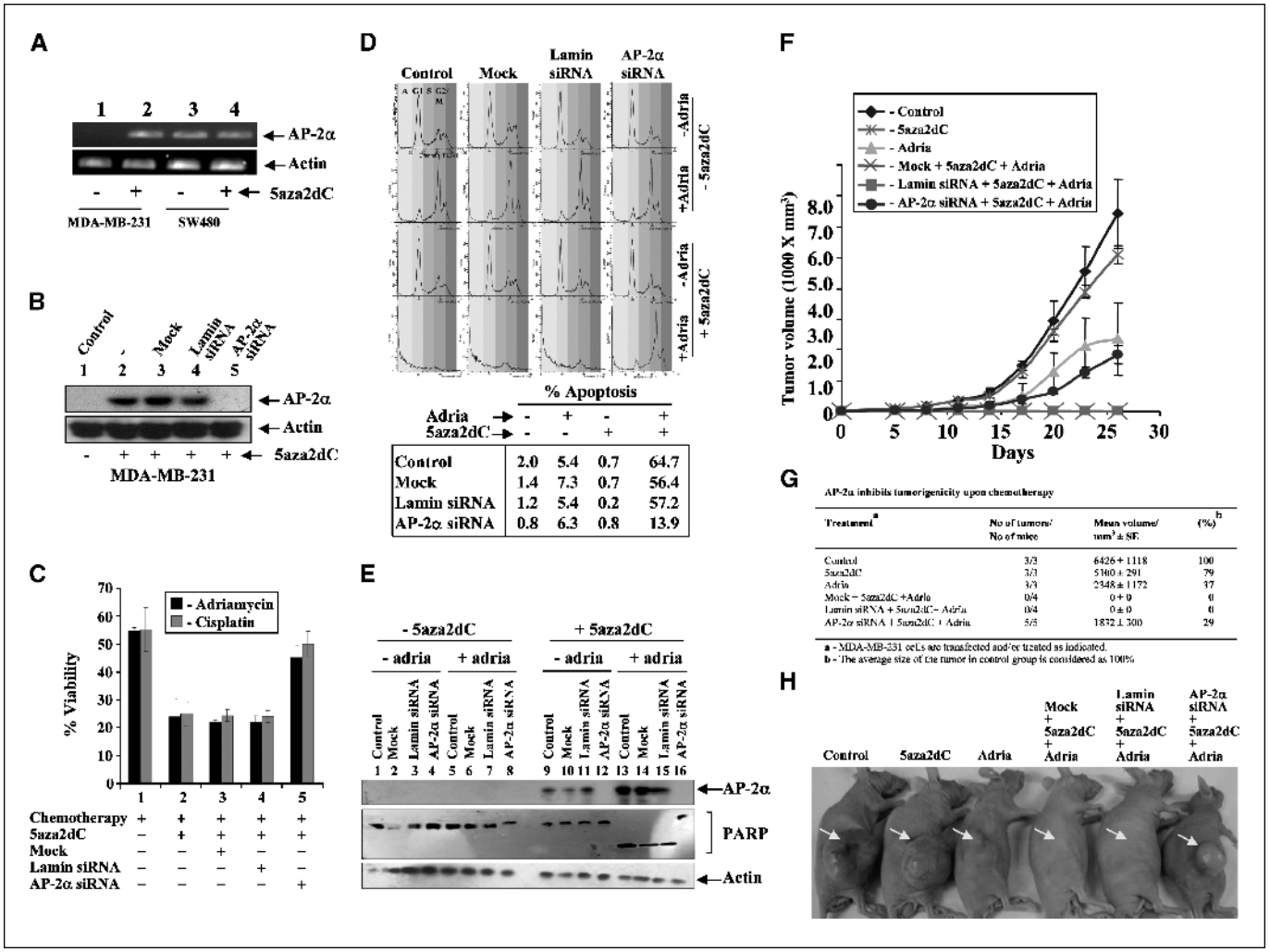

Figure 4. Reexpression of epigenetically silenced AP-2α sensitizes MDA-MB-231 for chemotherapeutic drugs and inhibits tumorigenesis in an AP-2α–dependent manner. *A,* MDA-MB-231 or SW480 cells were treated (as indicated) with 0.5 μmol/L 5aza2dC. Total RNA was made 48 hours after treatment and RT-PCR analysis was carried for AP-2α and GAPDH transcripts. *B,* MDA-MB-231cells were left untreated (*Control*), untransfected (−), mock (buffer) transfected, or transfected with lamin siRNA or AP-2α siRNA. After 48 hours of transfection, the cells were treated with 0.5 μmol/L 5aza2dC (as indicated) for another 48 hours and, after which, the various samples were subjected to Western blot analysis for AP-2α or actin proteins. *C,* MDA-MB-231 cells were either left untransfected, mock (buffer) transfected, or transfected with lamin siRNA or AP-2α siRNA. At 48 hours posttransfection, the cells were either untreated or treated (as indicated) with 5aza2dC (0.5 μmol/L). After 24 hours of 5aza2dC addition, the cells were treated (as indicated) with various amounts of Adriamycin or cisplatin (Adriamycin: 0.1, 0.2, 0.4, and 0.8 μg/mL; cisplatin: 0.25, 0.5, 1.0, 2.0 μg/mL). After 48 hours of the drug treatment, proportion of live cells was quantified by MTT assay. The absorbance of control cells was considered as 100%. The proportion of viable cells from the above experiment at 0.8 μg/mL Adriamycin and 2 μg/mL cisplatin were calculated and shown. *D,* MDA-MB-231 cells were left untransfected, mock (buffer) transfected, or transfected with lamin siRNA or AP-2α siRNA. At 48 hours posttransfection, the cells were either untreated or treated (as indicated) with 5aza2dC (0.5 μmol/L). After 24 hours of 5aza2dC addition, the cells were either untreated or treated (as indicated) with Adriamycin (0.1 μg/mL). The cells were harvested at 48 hours after Adriamycin addition and subjected to flow cytometry analysis. "S" indicates cells undergoing DNA synthesis (S phase); "A" indicates cells containing <2*N* DNA (apoptotic cells); "G_1" indicates cells containing 2*N* DNA; and "G_2/M" indicates cells containing 4*N* DNA. Percentage apoptosis under different conditions were calculated and shown below. *E,* MDA-MB-231 cells were left untransfected, mock (buffer) transfected, or transfected with lamin siRNA or AP-2α siRNA. At 48 hours posttransfection, the cells were either untreated or treated (as indicated) with 5aza2dC (0.5 μmol/L). After 24 hours of 5aza2dC addition, the cells were either untreated or treated (as indicated) with Adriamycin (0.1 μg/mL). The cells were harvested at 48 hours after Adriamycin addition and subjected to Western blot analysis for AP-2α, PARP, and actin proteins. *F,* MDA-MB-231 cells that were untransfected or mock transfected, lamin A/C siRNA or AP-2α siRNA transfected, were treated (as indicated) with 5aza2dC (0.5 μmol/L) or Adriamycin (0.1 μg/mL) for 24 hours or first treated with 5aza2dC for 24 hours and followed by Adriamycin treatment for the next 24 hours. The cells were then harvested, tested for viability using trypan blue exclusion test, and 1×10^7 viable cells (per animal) were injected s.c. under the right flank of nude mice. Tumor volumes were measured and plotted. *G,* the details of the experiment described above (*F*) are shown in the form of a table. *H,* representative animals from each group of the experiment described in (*F*) at the end of the experiment were photographed and shown. *Arrows,* location of tumor or the site of injection of cells. Note that mock- or lamin siRNA–transfected, but not AP-2α– siRNA-transfected MDA-MB-231 cells, which underwent combined treatment with Adriamycin and 5aza2dC, failed to form tumors.

into the flank of nude mice and the tumor growth was monitored over time. 5aza2dC treatment inhibited tumor growth marginally compared with control cells (Fig. 4*F-H*). Although the tumor formation was delayed in Adriamycin-treated cells, they ultimately grow to an average size of ~40% of control cells derived tumor (Fig. 4*F-H*). However, combined 5aza2dC and Adriamycin treatment completely abolished the tumor growth (Fig. 4*F-H*). Next, to find out the role of AP-2α reexpressed upon 5aza2dC treatment on tumorigenicity inhibition, we monitored tumorigenic potential of AP-2α siRNA-transfected cells after treatment with 5aza2dC and Adriamycin. 5aza2dC and Adriamycin combined treatment of MDA-MB-231 cells transfected with AP-2α siRNA failed to inhibit tumor formation with the average size of the tumor in the similar range that of cells treated with Adriamycin alone (Fig. 4*F-H*). However, 5aza2dC and Adriamycin combined treatment of MDA-MB-231 cells transfected with lamin siRNA inhibited the tumor formation very efficiently (Fig. 4*F-H*). The tumor sample derived from 5aza2dC-treated group showed reexpression of AP-2α transcript and protein (data not shown), which are lost upon prior AP-2α siRNA transfection (data not shown). These results suggest that increased chemosensitivity as a result of AP-2α reexpression upon 5aza2dC treatment indeed results in inhibition of tumor formation in an AP-2α–dependent manner.

Discussion

Many chemotherapeutic drugs induce apoptosis and, hence, defects in apoptotic pathways in tumors promote chemoresistance. Although p53 has been shown to play a major role in DNA damage–induced apoptotic response, the cytotoxicity of DNA-damaging agents is not restricted to cells containing wild-type p53. This is suggestive of presence of other markers, which, either alone or together, could determine the chemosensitivity. Our findings suggest that AP-2α is an important factor in determining chemosensitivity of cancer cells by sensitizing them to undergo apoptosis upon chemotherapy. The growth inhibitory activity of AP-2α is mediated through direct interaction with p53 (19). Contrary to this, AP-2α also has been shown to inhibit cancer cell growth independent of p53 (14, 15). Because majority of our experiments were carried out in SW480, MDA-MB-231 cells, and other cell lines, which carry mutated p53 or deleted for p53, the ability of AP-2α to enhance the apoptotic potential of cancer cells and, therefore, chemosensitivity is independent of p53. Thus, results from the current study suggest that AP-2α status, independent of p53, has a major role in determining the chemosensitivity of cancer cells.

Our results also suggest that AP-2α is induced posttranscriptionally in response to chemotherapy. AP-2 activity has previously been shown to be stimulated by 12-*O*-tetradecanoylphorbol-13-acetate or okadaic acid treatment via posttranscriptional mechanism (3). Similarly, protein kinase A–induced phosphorylation of AP-2α has been shown to increase the ability of AP-2α to transactivate APO-E promoter (20). We show for the first time that chemotherapy induces the AP-2α levels involving a posttranscriptional mechanism. At present, we do not know the mechanism of AP-2α induction by chemotherapy. However, based on our siRNA experiments, it is clear that chemotherapy-induced accumulation of AP-2α indeed contributes to chemosensitivity.

We also show that the ability of AP-2α to sensitize cancer cells to chemotherapy correlates with its ability to induce apoptosis. Although we do not know at present the mechanism of apoptosis induction by AP-2α, preliminary evidence from a separate study under progress in our laboratory suggests an important role for mitochondria-dependent intrinsic pathway of apoptosis in Ad-AP2–infected cells. To identify target genes regulated by AP-2α that could affect apoptosis, we carried out cDNA microarray analysis using RNA derived from AP-2α–overexpressing cells (details will be published elsewhere). We found that insulin growth factor binding protein 3 (IGFBP3) is induced by AP-2α consistently (2.2 ± 0.2-fold), which is known to induce apoptosis by several mechanisms. We also found that AP-2α down-regulated BCL2-related protein A1 transcript (BCL2A1; 2.68-fold), an antiapoptotic member of Bcl-2 family. Thus, it is clear from these results that perhaps transcriptional activation of IGFBP3 and repression of BCL2A1.

In addition, our study has important implications for considering combination therapy. By showing that 5aza2dC treatment of MDA-MB-231 breast cancer cells can sensitize them to chemotherapy, we show that by activating an apoptotic pathway one can restore chemosensitivity *in vivo*. Considering the fact that 75% of invasive breast tumors have epigenetically silenced AP-2α, this approach provides excellent opportunity to modify the chemosensitivity of breast cancer. Taken together, our data provides both *in vitro* and *in vivo* validation for a strategy to reverse chemoresistance in human cancers, in particular breast cancer, and underscores the value of tailoring cancer therapy on the basis of tumor genotype.

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Narendra Wajapeyee, Chandrashekhar Ganpat Raut and Kumaravel Somasundaram

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