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Bisphenol-A-induced inactivation of the p53 axis underlying deregulation of proliferation kinetics, and cell death in non-malignant human breast epithelial cells

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Widespread distribution of bisphenol-A (BPA) complicates epidemiological studies of possible carcinogenic effects on the breast because there are few unexposed controls. To address this challenge, we previously developed non-cancerous human high-risk donor breast epithelial cell (HRBEC) cultures, wherein BPA exposure could be controlled experimentally. BPA consistently induced activation of the mammalian target of rapamycin (mTOR) pathway—accompanied by dose-dependent evasion of apoptosis and increased proliferation—in HRBECs from multiple donors. Here, we demonstrate key molecular changes underlying BPA-induced cellular reprogramming. In 3/3 BPA-exposed HRBEC cell lines, and in T47D breast cancer cells, proapoptotic negative regulators of the cell cycle (p53, p21^{WAF1} and BAX) were markedly reduced, with concomitant increases in proliferation-initiating gene products (proliferating cell nuclear antigen, cyclins, CDKs and phosphorylated pRb). However, simultaneous exposure to BPA and the polyphenol, curcumin, partially or fully reduced the spectrum of effects associated with BPA alone, including mTOR pathway proteins (AKT1, RPS6, pRPS6 and 4EBP1). BPA exposure induced an increase in the ER α (Estrogen Receptor): ER β ratio—an effect also reversed by curcumin (analysis of variance, $P < 0.02$ for all test proteins). At the functional level, concurrent curcumin exposure reduced BPA-induced apoptosis evasion and rapid growth kinetics in all cell lines to varying degrees. Moreover, BPA extended the proliferation potential of 6/6 primary finite-life HRBEC cultures—another effect reduced by curcumin. Even after removal of BPA, 1/6 samples maintained continuous growth—a hallmark of cancer. We show that BPA exposure induces aberrant expression of multiple checkpoints that regulate cell survival, proliferation and apoptosis and that such changes can be effectively ameliorated.

Introduction

The chemical bisphenol-A (BPA) is so widely used that over 90% of North Americans test positive for recent exposure (1,2). The estrogenic properties of this compound are well known since the 1930s, and continuing studies by us, and others suggest that as a xenoestrogen, BPA might contribute to breast cancer. Unlike mutation-inducing chemicals, such as polycyclic hydrocarbons, and alkylating agents, the effects of BPA occur rapidly, compromising molecular signaling

Abbreviations: ANOVA, analysis of variance; BPA, bisphenol-A; BrdU, bromodeoxyuridine; E2, 17 β -estradiol; FACS, fluorescence-activated cell sorting; HRBEC, high-risk donor breast epithelial cell; IMM, immortalized; MCM2, minichromosome maintenance 2; mTOR, mammalian target of rapamycin; 4-OHT, 4-hydroxytamoxifen; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; RPFNA, random periareolar fine needle aspirate.

aimed at the targeted removal of cells through apoptotic cell death (3), probably through epigenetic modifications. This is analogous to the carcinogenic effects of natural estrogens that do not cause mutations *per se*, but when combined with progestins, are known to increase breast cancer incidence (4). Such cancer-promoting effects of estrogen are reversible when its action is blocked with aromatase inhibitors (5) and SERMs (6), leading to a decline in breast cancer incidence. Concerns regarding estrogenic mimicry by BPA are supported by studies with rodents, non-human primates and human cancer cell lines (7–9). However, knowledge of alterations in signaling pathways underlying BPA's deleterious effects is incomplete.

Based on the premise that promotion of carcinogenesis is best studied in cells that are not already malignant, we have evaluated shifts in key regulatory proteins induced by BPA *in vitro* using benign high-risk donor breast epithelial cells (HRBECs). Unlike the general population, BPA exposure of these cells can be experimentally controlled. HRBECs meet multiple criteria for normalcy, such as non-malignant cytology, moderate expression of both ER α and ER β , polarized growth in three-dimensional cultures and a finite lifespan. Non-malignant HRBECs exposed to BPA concentrations, similar to those measured in humans, demonstrate significant alterations in the transcriptional profiles of genesets, including activation of the mammalian target of rapamycin (mTOR) pathway (10). In an independent HRBEC sample set, we demonstrated quantitative shifts in mTOR pathway transcripts and gene products. These effects were associated with BPA-induced pro-survival changes conferring resistance to the estrogen receptor antagonist—tamoxifen and the mTOR inhibitor—rapamycin (3). The failure of rapamycin to override BPA exposure suggests additional downstream perturbations in the mTOR pathway by BPA, such as inhibition of apoptosis by down regulation of reactive oxygen species as described earlier (3). To investigate if another type of mTOR pathway inhibitor might abrogate the effects of BPA, we compared BPA alone to concurrent BPA and curcumin exposure. Unlike rapamycin, which largely acts through inhibition of the mTORC1 complex (and thus is not universally proapoptotic; reviewed in ref. 11), curcumin is thought to inhibit both mTORC1 and mTORC2 complexes (12) and has other consistent downstream effects such as induction of reactive oxygen species, thus triggering apoptotic cell death (13,14). By comparing exposure to BPA alone, and in combination with curcumin, we now define a range of molecular consequences of BPA-induced cell signaling that encompasses p53 suppression and inactivation of G₀/G₁ cell cycle checkpoints. These changes evident as evasion of apoptotic cell death, and increased rate of proliferation, contribute to the inappropriate extension of the lifespan of non-malignant HRBECs. Our data add molecular and functional insights into the action of a widespread environmental chemical. Most importantly, we provide proof of principle that if molecular changes underlying the mechanism of action of an environmental chemical are identified, steps can be designed to ameliorate potential adverse effects.

Materials and methods

Random periareolar fine-needle aspirate sample isolation and cell culture

With written informed consent approved by the California Pacific Medical Center Institutional Review Board, we collected HRBECs from random periareolar fine-needle aspirates (RPFNA) of the unaffected contralateral breast of patients undergoing surgical procedures for benign or malignant disease. RPFNA-derived primary HRBEC cultures and spontaneously immortalized HRBEC cell lines were propagated in MCDB170 medium supplemented with 2% fetal bovine serum at 37°C as before (3). To meet the requirements of both live cells and cell lysates for the range of assays and endpoints examined, we supplemented primary finite-life HRBECs with three novel, spontaneously immortalized (designated IMM) HRBEC lines derived from among 130 independent RPFNA samples attempted and reported earlier (3). Live cell assays for determining 24h growth kinetics

and effects on long-term growth maintenance included three and six finite-life HRBEC specimens, respectively. T47D breast cancer cells were adapted to the same growth medium as HRBECs. To minimize variables in assay endpoints described below, control versus treated HRBECs were uniformly harvested from subconfluent populations at passages 25–27. Under test conditions, all cells were maintained in phenol red-free medium, supplemented with 0.2% charcoal/dextran-treated serum, and other components as indicated. 17 β -Estradiol (E2), BPA, 4-hydroxytamoxifen (4-OHT) and curcumin were purchased from Sigma–Aldrich (St Louis, MO). Cells were plated at a density of 100 000 cells per well in 6-well plates and exposed to BPA for 7 days with or without curcumin as noted.

Protein isolation and western blot analysis

Cells were lysed with NP40 buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.1% NP40, 2 mM dithiothreitol, 5% glycerol) containing protease and phosphatase inhibitors. For DNA-bound proteins, total cell lysates were fractionated after NP40 lysis and the remaining pellet lysed with a urea-based buffer (8 M urea, 50 mM Tris–HCl pH 7.4, 100 mM NaCl). Total proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes. After incubation with appropriate primary and secondary immunoreagents, and enhanced chemiluminescence detection, immunolocalized protein signals were recorded on X-ray film (Amersham, Piscataway, NJ). Actin served as a loading control. Primary antibodies, directed at 17 different proteins, and their commercial source is as follows: BAX, RPS6 total and phosphorylated p70S6K (Thr389/Ser235/Ser236) and ER β from Genetex (Irvine, CA); cyclin D3, CDK6 and pRb from Cell Signaling Technology (Danvers, MA); AKT1, 4EBP1, CDK2, cyclin A, minichromosome maintenance 2 (MCM2), p21, ER α , proliferating cell nuclear antigen (PCNA), p53 and actin from Santa Cruz Biotechnology (Santa Cruz, CA). Densitometric analysis of western blots was done using AlphaEase FC software (Alpha Innotech, Santa Clara, CA).

Cell cycle analysis and determination of growth rate by fluorescence-activated cell sorting

DNA synthesis was quantitated by incorporation of 10 μ M bromodeoxyuridine (BrdU) (Sigma–Aldrich) for defined time periods in test cells subsequently fixed with 70% ethanol. Fixed cells were incubated with anti-BrdU (Santa Cruz Biotechnology), followed by fluorescein isothiocyanate-conjugated secondary antibody (Life Technologies, Grand Island, NY), counterstained with propidium iodide (PI) and analyzed by FACScan (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences).

Quantitation of apoptotic cell death

Baseline and 4-OHT-induced apoptosis was measured in unfixed cells with Annexin V-fluorescein isothiocyanate (BD Biosciences) following manufacturer's instructions. Data acquisition and analysis were performed by FACScan.

Statistics

All quantitative data were summarized as mean \pm standard error. Each data point was composed of least two and up to nine replicates in some cases. The results from apoptosis evasion, proliferation, cell cycle and protein expression assays were used to test the effects of BPA and curcumin, both individually and concurrently by analysis of variance (ANOVA). The statistics were analyzed using STATA v12, with $P < 0.05$ considered to be statistically significant.

Results

We used non-malignant, human, high-risk breast epithelial cells as a model to seek possible carcinogenic effects of BPA in cells that were not yet malignant. The finite lifespan of primary HRBECs limits the number of cells available from a single donor sample for analysis and thus limits the number of repetitions of any assay. However, the ability to assay non-immortalized cell populations allows studies on a wider sample of the human population of interest. In this case, therefore, the central conclusions of the study are based on multiple assay endpoints to test the effects of BPA with and without curcumin on three novel, spontaneously immortalized HRBEC lines. These effects were then confirmed on fresh, donor-derived primary HRBEC samples.

A molecular basis for BPA-disrupted apoptotic signaling

We asked whether suppression of p53 expression or its regulatory activity was an underlying factor in BPA-induced apoptosis evasion in HRBECs, previously observed (3). Lysates prepared from immortalized

HRBEC lines (PA024, PA025 and PA115) and the ER-positive breast cancer cell line, T47D, were analyzed for baseline p53 expression and after pretreatment with BPA (100 nM) or curcumin (100 nM) or simultaneously with both compounds. Baseline p53 levels were unequivocally downregulated by BPA ($P = 0.0001$) (Figure 1A, Supplementary Figure 1, available at *Carcinogenesis* Online). In contrast, exposure to curcumin increased p53 levels ($P = 0.0000$), as did a combined exposure to BPA and curcumin for the same time period. This effect was consistent on a cell line-by-cell line basis (P value, PA024: 0.0002, PA025: 0.0074, PA115: 0.0006, T47D: 0.0016).

Downregulation of p53 promoted by BPA exposure was also reflected in the simultaneous reduction in the downstream proapoptotic BAX gene product. Conversely, curcumin alone induced the BAX protein. Synergistic with p53 induction, higher BAX levels were maintained in BPA + curcumin-treated cells (Figure 1A, Supplementary Figure 1, available at *Carcinogenesis* Online). These findings provide a clear molecular rationale for BPA-induced evasion of apoptotic cell death.

Using functional endpoints to test the impact of p53 and BAX modulation by BPA and curcumin, HRBECs were treated with the ER α antagonist, 4-OHT, for 24 h to induce apoptotic cell death quantified by Annexin V-based fluorescence-activated cell sorting (FACS) analysis. Percent cell death under various pretreatment conditions was determined for each test cell line relative to untreated control (Figure 1B). BPA pretreatment resulted in apoptosis reduction in all test cell lines to varying degrees. In contrast, curcumin alone increased apoptotic cell death. Experimentally induced cell death in cells concurrently exposed to curcumin during BPA pretreatment was increased compared with BPA alone over a range of 11.5–74%. Although, the magnitude of the difference varied between cell lines, each comparison showed a significant effect of adding curcumin to BPA. For example, in PA024, BPA alone reduced the apoptotic fraction to 65% of control; the addition of curcumin restored it to 78% of control ($t = 4.3$, $P = 0.004$). Together, our data provide proof of principle that treatment with curcumin as a drug, whose effects are antithetical to that of the toxicant BPA, can reduce the breadth of consequences that arise due to a BPA-disabled p53 circuitry by restoring normal p53 function.

BPA-triggered changes in the repertoire of cell cycle proteins

Next, we assessed consequences of BPA exposure on proteins that regulate cell cycle progression (Figure 1A, Supplementary Figure 1, available at *Carcinogenesis* Online). Consistent with BPA-induced p53 down regulation, the negatively regulating p21^{WAF1} protein remained significantly suppressed in the presence of BPA ($P = 0.0106$). In contrast, an increase in p21^{WAF1} observed in HRBECs in curcumin alone ($P = 0.0006$) was maintained in BPA + curcumin in 2/4 test cell lines (PA024: $P = 0.04$, PA115: $P = 0.017$).

As expected of fast-growing cancer cells, the baseline expression of positively regulating cell cycle proteins was consistently higher in T47D, whereas it was at the lower limit of detection in HRBECs. BPA-induced pRb phosphorylation ($P = 0.0002$) detected at two sites: Ser765 (Figure 1A) and Ser608 (data not shown), resulted in its functional inactivation, which enables cell cycle progression into S phase. Similarly, BPA exposure increased components of the CDK complexes, cyclin D3–CDK4/6, which promote exit from the G₁ phase ($P = 0.0005$, 0.0001, respectively) and cyclin A–CDK2, which drive progression through S phase ($P = 0.0001$, 0.0010, respectively). Congruent with BPA-induced increase in positively associated cell cycle proteins, significant accumulation of the fork protein—PCNA ($P = 0.0000$) and the replication origin helicase—MCM2 ($P = 0.0000$) was observed within DNA-bound fractions of BPA-exposed HRBEC lysates. Curcumin consistently decreased phosphorylated pRb, cyclin D3, CDK6, cyclin A and CDK2 ($P < 0.01$ for each) with mixed results on other gene products tested (Figure 1A, Supplementary Figure 1, available at *Carcinogenesis* Online). However, in cells exposed to BPA + curcumin, protein levels of all proliferation promoting test genes were decreased (Figure 2B; ANOVA, $P < 0.02$ for all proteins).

Protein quantitation data derived by densitometric scanning of western blots for 16 test gene products and evaluated by ANOVA of

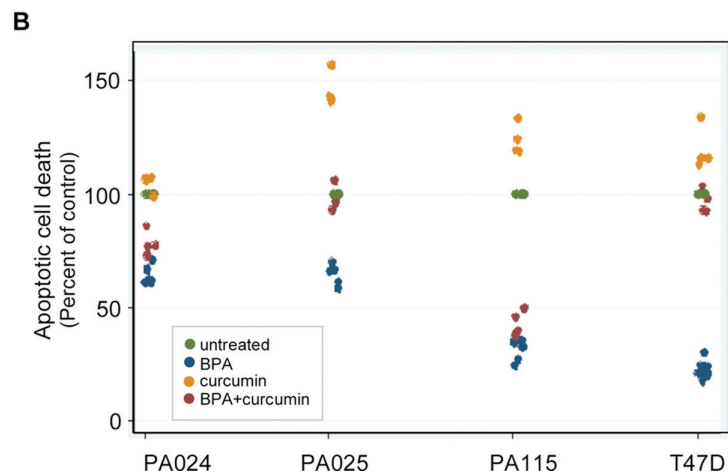
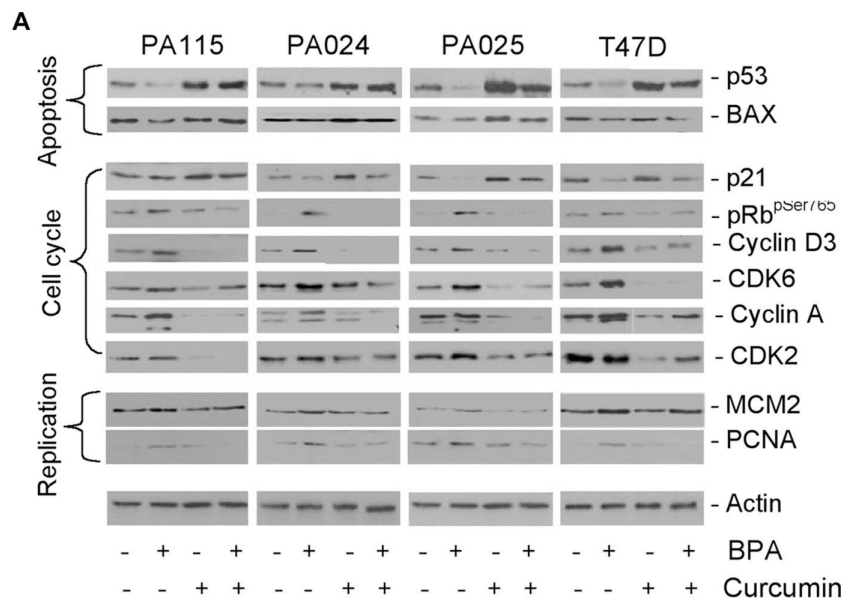


Fig. 1. BPA-induced pro-survival changes in proapoptotic and cell cycle proteins; effects of curcumin. **(A)** Representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis of cell lysates of HRBECs (PA115, PA024 and PA025), and T47D after 7 day exposure to 100 nM BPA and/or curcumin to evaluate alterations in proteins related to apoptosis regulation and cell cycle progression. Note downregulation of p53 by BPA alone and its induction by curcumin exposure in all cell lines. In parallel to p53, these chemicals also modulate levels of the proapoptotic protein—BAX. Similarly, BPA exposure results in the suppression of the p53-regulated CDK inhibitor, p21^{WAF1}, and the induction and activation of CDKs and cyclins followed by pRb phosphorylation in all cell lines to varying degrees. Accumulation of proteins associated with DNA replication, MCM2 and PCNA, is evident in the DNA-bound fraction of test cell lysates. Curcumin alone had the opposite effects for p53, p21^{WAF1}, cyclin D3, cyclin A, pRb, CDK6 and CDK2, with mixed effects for MCM2, BAX and PCNA. Curcumin reduces BPA-mediated pro-survival changes in all cell lines. Plots derived from the densitometric scans of blots generated from two independent runs are included as [Supplementary Figure 1](#), available at [Carcinogenesis Online](#). **(B)** Annexin V-based FACS analysis of tamoxifen (4-OHT) induced apoptosis in BPA-treated cells, with or without curcumin supplementation in four test cell lines. Scatter plot of induced apoptotic cell death plotted as a percentage of untreated control. Data represent two to three replicates from two independent experiments on each cell line; each dot represents one or more replicates in the event of overlapping values. There was a minimum of four replicates per cell line for each treatment. BPA alone treatment (blue spheres) significantly reduced the apoptotic fraction. The addition of curcumin to BPA (maroon spheres) increased apoptotic cell death to varying degrees. Two-sample *t*-tests were used to compare percent apoptosis induction in BPA + curcumin versus BPA alone for each cell line. Estimated improvement in apoptosis induction by curcumin in the presence of BPA is as follows: PA024: 13% ($t = 4.3$, $P = 0.004$); PA025: 34% ($t = 10.2$, $P = 0.0001$); PA115: 11.5% ($t = 3.6$, $P = 0.007$); T47D: 74% ($t = 29.5$, $P = 0.0001$).

mean replicate values normalized to actin controls demonstrate that the differences induced by pretreatment of test cell lines with BPA alone or BPA + curcumin do not merely occur by chance. Moreover, the pretreatment effects are consistent across the four cell lines for each of the cellular proteins tested ([Figure 2B](#)). Taken together, these results provide the required molecular framework for reversing BPA-induced HRBEC proliferation. They demonstrate that (i) BPA exposure not only induces exit from G₀/G₁ (quiescence) by pRb phosphorylation but also actively stimulates DNA replication and

cell cycling and suppresses apoptosis through suppression of p53 and BAX, (ii) the addition of curcumin to BPA restores p53 and reduces BPA modulation of major proliferation-associated proteins, i.e. cyclin D3, CDK6, cyclin A, CDK2, MCM2 and PCNA. Overall, close similarities in BPA-activated proliferation endpoints in HRBECs and in T47D breast cancer cells support a role for BPA in the promotion of characteristic hallmark cancerous traits of continuous proliferation and apoptosis evasion in high-risk cells that have not yet become malignant.

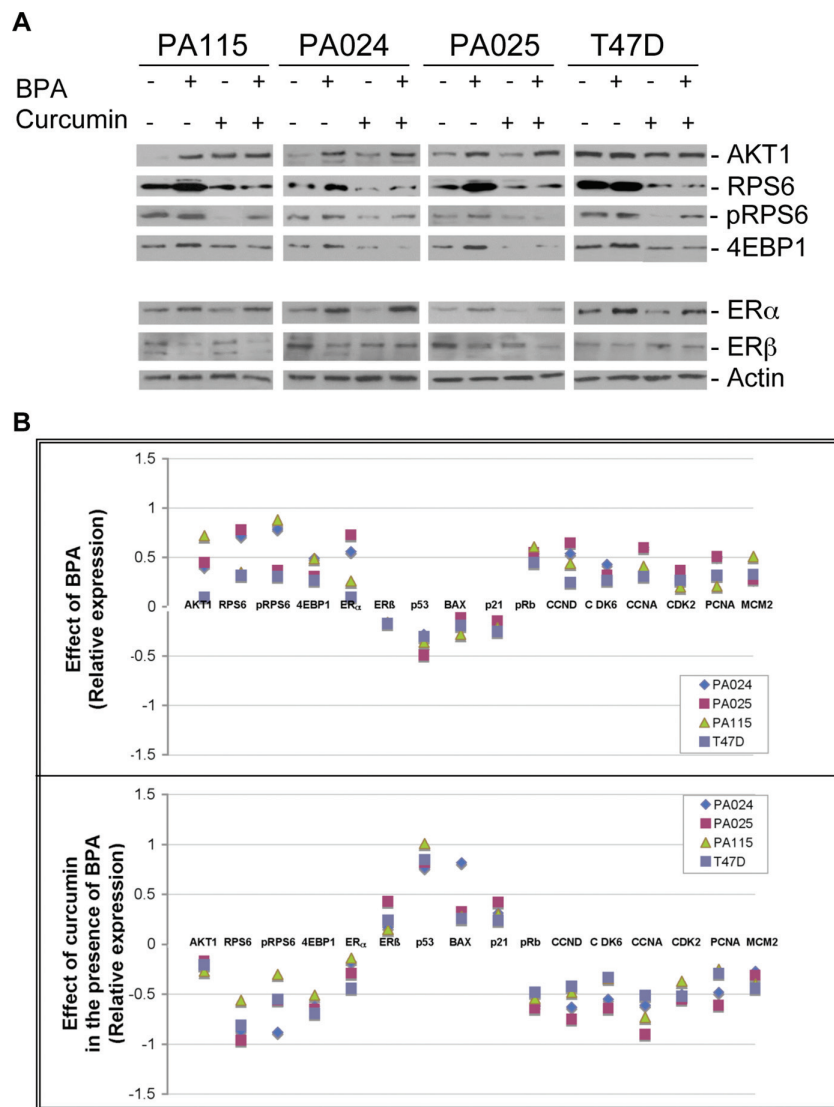


Fig. 2. BPA and curcumin modulation of mTOR pathway proteins and ER subtypes. (A) Representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis of cell lysates of spontaneously immortalized non-malignant HRBEC lines (PA115, PA024 and PA025), and T47D breast cancer cells exposed to 100 nM BPA and/or curcumin for 7 days to determine effect on mTOR pathway proteins. AKT1, RPS6 (total and phosphorylated) and 4EBP1 proteins increased with BPA alone. Curcumin induced an opposite effect on RPS6, pRPS6 and 4EBP1, but not AKT expression. BPA + curcumin reduced the effect of BPA alone for all four mTOR proteins. BPA alone induced ER α and reduced ER β , thereby raising the ER α :ER β ratio. Adding curcumin to BPA reduced induction of ER α protein and increased ER β protein resulting in a relative decrease in the ER α :ER β ratio compared with BPA exposure alone. Plots derived from the densitometric scans of the blots generated from two independent runs are included as [Supplementary Figure 2](#), available at *Carcinogenesis Online*. (B) Summary representation of 64 two-way ANOVA tests for change in the expression of 16 gene products in four test cell lines partially illustrated in [Figure 1A](#) and part (A) of this figure. All measurements shown represent duplicate values from two independent gel runs. A minimum of eight data points was included for ANOVA of each gene product under each test conditions (HRBECs: $n = 6$, T47D: $n = 2$). The directional effect on each gene (positive or negative) was the same across all four cell lines. Top panel—effect of BPA alone. Bottom panel—effect of curcumin in the presence of BPA. The two treatment conditions display opposite effects consistent across all test cell lines. (ANOVA, $P < 0.02$ for all gene products.)

Contrasting effects of BPA and curcumin on mTOR pathway signaling

To demonstrate continuity with our previous work on BPA-induced mTOR pathway activation, lysates prepared from HRBEC lines (PA024, PA025, PA115) and the ER-positive breast cancer cell line, T47D, exposed to BPA (100 nM) or curcumin (100 nM) or simultaneously to both compounds were analyzed for expression of the key mediators of this pathway—AKT1, RPS6 and 4EBP1 ([Figure 2A](#), [Supplementary Figure 2](#), available at *Carcinogenesis Online*). Consistent with our previous data, BPA alone led to an increase in these proteins (AKT: $P = 0.007$, RPS6: $P = 0.0004$, pRPS6: $P = 0.0072$, 4EBP1: $P = 0.0002$). Curcumin treatment with or without BPA visibly reduced RPS6, pRPS6 and 4EBP1 in all test cell lines

($P < 0.005$). In some cell lines, AKT levels were relatively high in the presence of curcumin alone as noted previously (15). Taken together, the data demonstrate contrasting effects of BPA and BPA + curcumin for critical downstream events in the pro-survival PI3K/AKT/mTOR pathway as summarized by ANOVA ([Figure 2B](#)).

Altered ER α :ER β ratio induced by BPA exposure

In clinical studies, the ER α :ER β ratio has predictive value for progression of breast hyperplasia to malignancy, higher baseline ER α being characteristic of breast tissue in which cancer develops (16). We therefore determined the effect of BPA on this ratio in the HRBEC model. BPA exposure significantly induced ER α protein in 3/3 HRBEC lines and in T47D cells ($P = 0.0008$), whereas ER β protein

levels were consistently reduced by BPA ($P = 0.0038$) (Figure 2A and B, Supplementary Figure 2, available at *Carcinogenesis* Online). The effect of curcumin alone on ER α was inconsistent, however ER β was consistent, though slightly elevated (Figure 2A). In all cases, ER α levels were lower (and ER β higher) in BPA + curcumin exposure compared with BPA alone. Due to an overall increase in ER β and a decline in ER α , the ER α :ER β ratio was reduced upon the addition of curcumin to BPA (Figure 2), an effect opposite of BPA alone.

BPA-enhanced proliferation rate is reduced by curcumin

We used BrdU labeling and FACS analysis of three immortalized HRBEC lines (IMM-HRBECs) and three finite-life HRBEC samples (PA126, PA127 and PA128) in comparison with T47D cancer cells to ask whether cell proliferation occurred as predicted by treatment-induced changes in checkpoint proteins. Primary finite-life HRBEC cultures showed the lowest average number of proliferating cells ($S=22 \pm 1.4\%$, $G_1=72 \pm 1.5\%$), characteristic of adult non-malignant breast tissue relative to cancer (17). At the other end of the spectrum, T47D displayed high S phase ($53 \pm 2.0\%$) and low G_1 phase ($35 \pm 1.0\%$) fractions. IMM-HRBECs were moderately proliferative ($S: 31 \pm 0.7\%$, $G_1: 58 \pm 0.6\%$). As expected of an estrogenic stimulus, BPA exposure led to a marked increase in replicating cells with the greatest proportional increase in S phase, accompanied by a decline in the G_1 fraction, occurring in cells whose baseline proliferation rate was significantly slower, i.e. primary finite-life HRBECs. Interestingly, IMM-HRBECs displayed greater similarity to their non-malignant counterparts than to cancer cells (Figure 3A). Curcumin exposure alone resulted in a partial G_1 arrest in all three groups of cells. In cells concurrently exposed to curcumin and BPA, a reversal to baseline cell cycle distribution was observed for all cell lines. ANOVA-based data demonstrating the magnitude and significance of the effect of curcumin in the presence of BPA for all cell cycle phases in each of the seven test samples are summarized in Supplementary Table 1, available at *Carcinogenesis* Online. In 6/7 samples, treatment-induced changes in cell cycling were statistically significant ($P < 0.05$).

To develop insights regarding the changes induced in the growth dynamics of BPA-exposed cells, BrdU incorporation was also measured at multiple intervals up to 24 h (18 h for finite-life HRBECs). A higher percentage of BrdU-positive cells relative to control was consistently observed in the presence of BPA at all time points assessed (1, 3, 6 and 24 h). Conversely, the proliferation rate in curcumin alone or in combination with BPA was significantly lower. Despite fewer replicating cells in untreated control populations of non-malignant cells compared with T47D, the effect of BPA on accelerating the growth rate was evident by an increased accumulation of the S-phase fraction within 1 h of BrdU labeling. The initial replication rate calculated by the slope of the linear regression of the growth curves between 0 and 1 h showed that BPA increased the replication rate by 53.3% in IMM-HRBECs, 30.6% in primary HRBECs and 13.7% in T47D, thereby shortening the duration of the cell cycle of non-malignant cell lines to a range characteristic of fast-growing malignant cells. Remarkably, concurrent exposure to BPA and curcumin at a concentration as low as 1 nM decreased the growth rate below control by 19.8% in IMM-HRBECs, 10.8% in primary HRBECs and 13.6% in T47D (Figure 3B).

In addition to the contrasting effects of BPA and curcumin on short-term cell growth measured over a 24 h period, HRBECs exposed continuously to these compounds for 7 days, provided further confirmation (Figure 3C) of these effects. Of note, unlike IMM-PA025, finite-life HRBECs (PA124) were relatively non-responsive to the mitogenic effects of E2. Both types of HRBECs, however, were responsive to BPA, and in both, curcumin reduced cell numbers back to essentially 100% of control values (ANOVA estimate compared with BPA alone PA025: -7088 , 95% CI -7339 , -6837 , $P < 0.0001$; PA124: -6391 , 95% CI -7075 , -5707 , $P < 0.0001$). Curcumin alone had little effect on cells not exposed to BPA. These findings demonstrate that (i) BPA induction of the S-phase fraction observed by cell cycle analysis does result in the accumulation of more cells at

a faster rate, (ii) percent increase in S phase is abolished in the presence of curcumin and (iii) despite the slow baseline replication rate of primary finite-life HRBECs, overall responsiveness of these cells is remarkably similar to immortalized non-malignant and malignant cell lines.

Perturbation of the finite lifespan of primary HRBECs by BPA exposure; normalization with curcumin

To estimate the growth promoting effects of BPA on live human breast epithelial cells as close as possible to *in vivo* exposure, six consecutive HRBEC specimens were treated with BPA immediately after RPFNA collection from volunteers. The cellular content of such patient-derived samples is severely limited, on average 15 000 total cells/case, and RPFNA samples are generally not amenable to passaging more than three times at 1:2 splits of the culture. After this stage, the cultures attain quiescence, remain growth arrested and eventually undergo apoptosis. In contrast, 6/6 consecutive primary HRBEC samples maintained in BPA-supplemented growth medium for a 40 day period exhibited more robust growth than unexposed control cultures (Figure 4A). Finite-life primary HRBECs exposed to BPA + curcumin were less robust (Figure 4B). Curcumin only treated cells did not survive. BPA-induced checkpoint perturbations documented above yield long-term vigorous proliferation of non-malignant human breast cells that are not yet malignant or immortalized. Such changes are reduced by curcumin supplementation.

To evaluate whether cessation of BPA treatment in prior exposed primary HRBEC populations would reverse the activated cell proliferation program of finite-life cells, a primary HRBEC culture (PA151) exposed to BPA, or BPA + curcumin for 40 days, was maintained BPA free and curcumin free for an additional 30 days, labeled with BrdU and harvested for cell cycle analysis. The culture from prior BPA-alone exposure continued to display vigorous growth during this period as confirmed by cell cycle analysis despite a 30 day interval without BPA and without E2 (in charcoal-stripped serum). Sizeable cycling S phase and G_2/M phase populations were sustained (Figure 5). However, the prior BPA + curcumin culture displayed cells mostly in the G_1 or sub G_1 phase of the cycle. This demonstrates that growth-enhancing effects of BPA can linger even after its withdrawal and provides an example of the continued proliferation of benign cells that it is feared may be induced in humans by BPA. Such a perturbation *per se* could potentially culminate in cell immortalization.

Discussion

Since the discovery that BPA leaches into cell culture media from polycarbonate containers, binds to the estrogen receptor and stimulates DNA synthesis in MCF7 breast cancer cells (18), a role for BPA in breast carcinogenesis has been considered possible. This concern begs two broad questions: (i) What effects of BPA at the cellular level might initiate, promote or sustain carcinogenesis? (ii) Can such effects be reversed? Here, we show that BPA disrupts checkpoints that normally signal apoptotic cell death and limit cell proliferation, and we provide proof of principle that such molecular consequences can be reduced in non-malignant cell cultures isolated from human volunteers.

A wide spectrum of biological perturbations was observed in BPA-exposed HRBECs. BPA-induced apoptosis evasion has been reported previously in HRBECs (3) and in the dimethylbenz[*a*]anthracene model of rodent mammary carcinogenesis (19). By evaluating critical checkpoints for apoptosis and cell proliferation, we show that BPA suppresses p53, the 'guardian of the genome', and downstream signaling mediated by BAX, a major effector of p53-induced proapoptotic activity (20). This parallels p53 loss of function that occurs in many types of solid tumors and in individuals with the Li-Fraumeni syndrome who are predisposed to breast and other cancer because of inherited loss of p53 (21,22). Our HRBEC-derived data provide a molecular basis for histopathological observations of BPA-induced increase in cell proliferation within estrogen-sensitive tissues of rodent models (23). Although levels of negative regulators of the cell

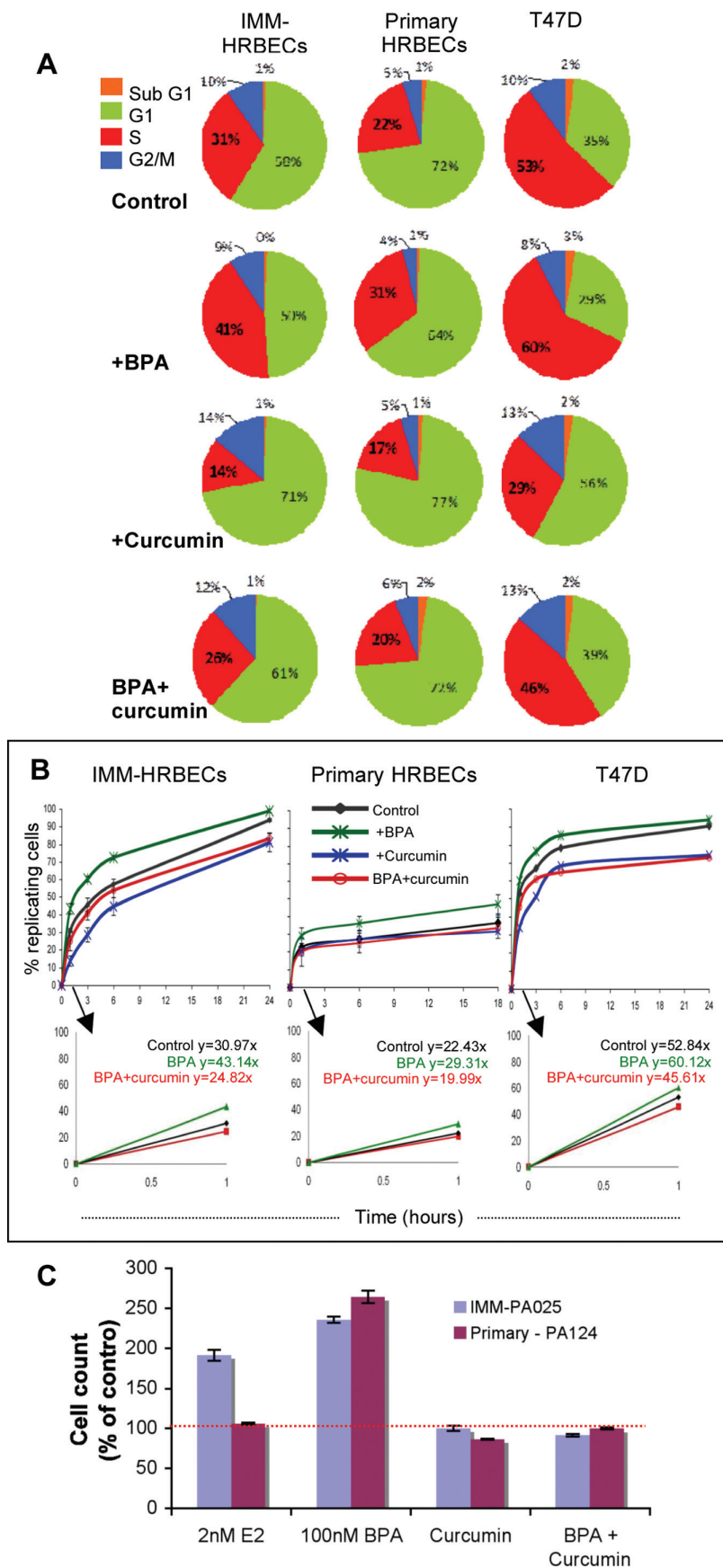


Fig. 3. BPA-induced cell cycle progression and increased rate of cell proliferation; effects of curcumin. (A) Distribution of cell cycle phases in cultures pretreated with BPA with or without concurrent curcumin exposure for 7 days. Seven cell samples were evaluated—three IMM-HRBECS lines (PA024, PA025 and PA115), three primary finite-life HRBECS cultures (PA126, PA127 and PA128) and T47D breast cancer cells. Representative pie charts derived from FACS

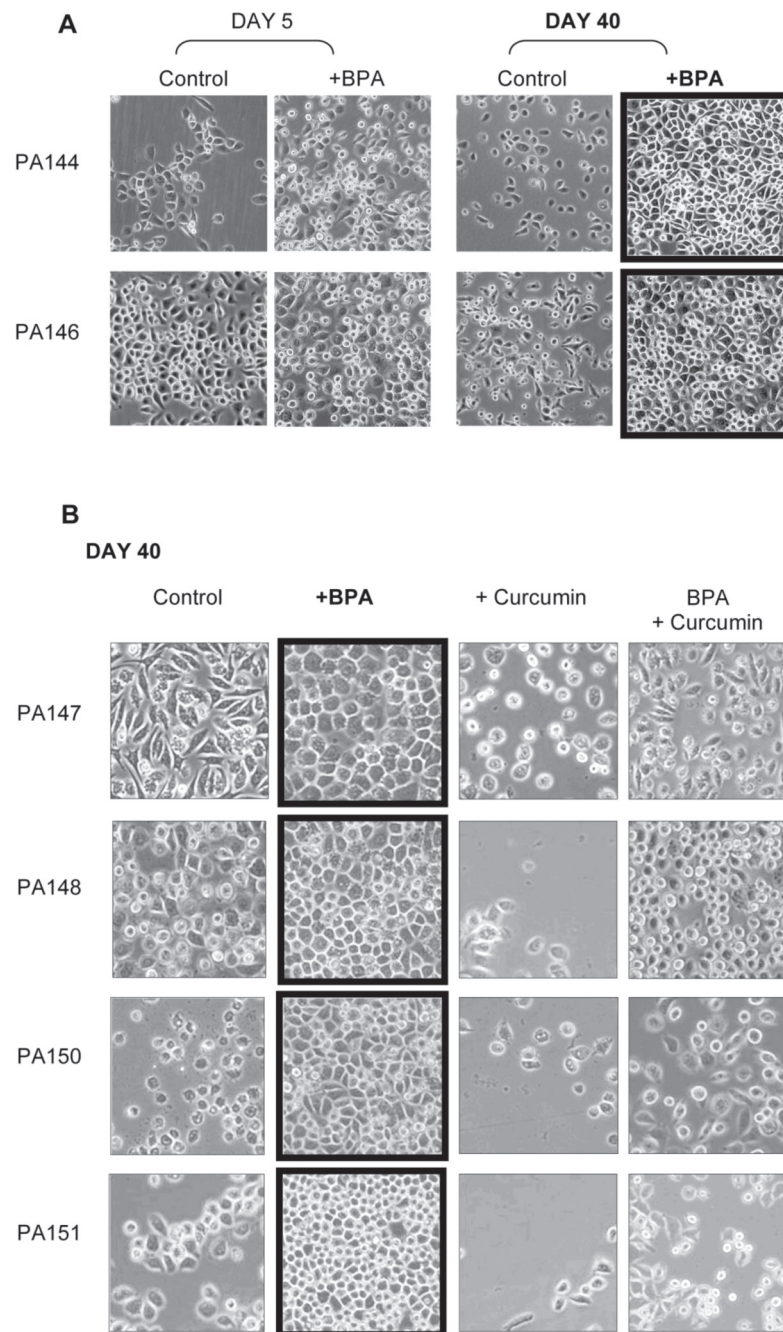


Fig. 4. Prolonged survival and abrogation of quiescence induced by BPA in finite-life primary HRBECs. Brightfield micrographs of six independent cases of finite-life primary HRBECs (PA144, PA146, PA147, PA148, PA150 and PA151) demonstrate vigorous growth stimulated by BPA within a 40 day period (darkened squares). A minimal amount of sample precludes multiple replicates of each treatment condition to be set up directly after acquisition from volunteer donors, but six of six consecutive specimens were consistent in the results shown. **(A)** The growth differential induced by BPA is visible as early as day 5 after initial plating (PA145 and PA146) and later as well. **(B)** Forty-day cultures in the presence of curcumin show growth reduction compared with controls, and those exposed to BPA + curcumin display growth attenuation in comparison with the stimulation by BPA alone (PA147, PA148, PA150 and PA151).

← analysis of BrdU and PI-labeled cells demonstrate altered distribution of cell cycle phases to varying degrees in the presence of BPA and its reversal by curcumin supplementation evident as an increase in the percentage of cells in G₁ and G₂/M and a reduction in percent S phase. Statistical analysis of the data by ANOVA is summarized in [Supplementary Table 1](#), available at *Carcinogenesis Online*. **(B)** Changes in proliferation kinetics of cultures pretreated with BPA with or without concurrent curcumin exposure analyzed as the percentage of BrdU-labeled cells over a 1–24 h period. Note that the proliferation rate is highest in the presence of BPA but reverts to control or lower rates with curcumin. Arrows from each plot point to the initial replication rate of exposed cells calculated by measuring the slope of the linear regression of the curves between 0 and 1 h. BPA consistently increased the replication rate, whereas concomitant exposure to BPA and curcumin reset the replication rate to values similar to or lower than control. **(C)** Cell counts of IMM-PA025 and primary HRBEC (PA124) at the end of a 7 day period of exposure to 5 nM E2 or 100 nM BPA, with or without curcumin, confirm the FACS data shown in panels A and B. The effect of curcumin in reducing cell number in the presence of BPA compared with BPA alone was statistically significant for both PA025 and PA124, as estimated by ANOVA ($P = 0.0001$). Control cell counts represented an average of 4500 ± 500 . In (A–C), each data set represents the averaged values from two independent experiments performed in triplicate on each immortalized cell line and in duplicate for each finite lifespan primary HRBEC culture exposed to all test conditions.

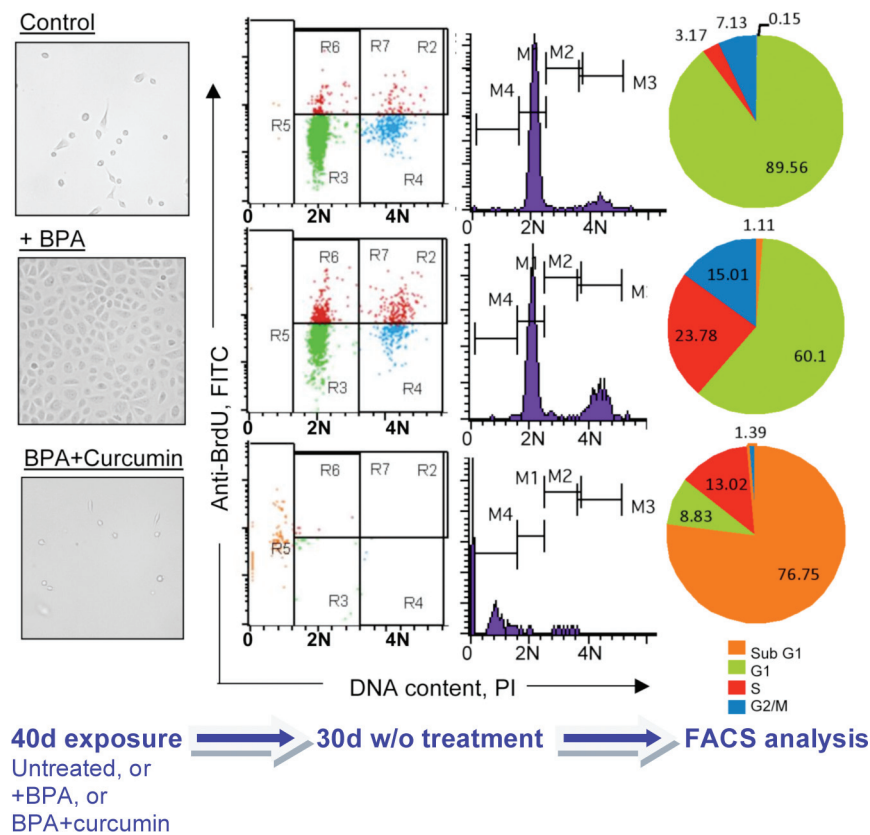


Fig. 5. Long-term effects of prior BPA exposure on the cell cycle. Primary HRBECs (PA151) exposed to BPA for 40 days, followed by growth in BPA-free and E2-free medium for an additional 30 days display S-phase characteristics of early passage finite-life primary HRBEC control cultures (see Figure 3A). In contrast, parallel controls without BPA or in BPA + curcumin cease proliferation and are represented largely by cells in G₁ and subG₁, respectively. Cells exposed for the first 30 days to curcumin alone did not survive. The differences in percent BrdU-positive cells in various treatment groups were significant (curcumin-induced reduction compared with BPA alone for G₁ phase was 51%, $P = 0.0007$; S phase—10.7%, $P = 0.009$; G₂/M—13.7%, $P = 0.006$; subG₁—75.5%, $P = 0.0001$).

cycle—p21^{WAF1} and pRb—are repressed by BPA, gene products supporting enhancement in growth rate are consistently overexpressed. Such induction encompasses higher levels of (i) MCM2, a replicative helicase that unwinds double-strand DNA to the single strands required for replication (24), (ii) PCNA, a protein that increases the processivity of leading strand synthesis during DNA replication (25), (iii) RPS6 and pRPS6, which enhance growth by increasing transcript translation (26), (iv) 4EBP1 and p4EBP1, the ‘funnel factor’ mediating mTOR pathway signaling to multiple cell cycle control genes (27) and (v) cyclin–CDK complexes that drive cell cycle progression (28). This data complement and provide continuity with our previous demonstration of BPA-induced mTOR pathway activation by upregulation of PI3K, AKT, pAKT and 4EBP1 with concurrent suppression of PTEN (3). The contrasting effects of BPA and curcumin in HRBECs are also congruent with previously reported effects of decreased cell proliferation and increased apoptosis by curcumin treatment of the human mammary epithelial cell line, MCF10A (29) and of breast cancer cell lines stimulated by the environmental estrogens, 4-nonylphenol, 4-octylphenol and *o,p'*-DDT (30). Together, this spectrum of molecular changes could expedite the initial rate of cell proliferation, thereby generating a greater number of cells that can evade apoptosis. As summarized in Figure 6, we hypothesize that in non-malignant finite-life human breast epithelial cells, such changes circumvent barriers to extend replicative potential and cellular life span that normally serve to protect from the induction of further carcinogenic changes.

Additionally, we measured the ER α :ER β ratio as an endpoint relevant to the malignant phenotype of the human breast. Unlike widely used breast cancer cell lines, benign breast cells generally express both ER α and ER β (16). ER α is known to suppress p53 and enhance cell survival (39). However, since ER β limits ER α signaling (40), a

decline in ER β might fail to regulate ER α -induced cell growth. Our observed BPA-induced shift in the ER α :ER β ratio to favor ER α parallels the clinical phenotype where relatively more ER α favors progression of hyperplasia to breast cancer (16).

In functional assays of live HRBECs, we have previously reported BPA-mediated resistance to both tamoxifen and rapamycin-induced apoptosis (3). Here, we confirm BPA-induced evasion of tamoxifen-induced apoptosis, and further demonstrate that addition of curcumin reduced its occurrence in all test populations, albeit to a limited extent in some cell lines. Similarly, BPA-induced increases in the S-phase fraction and subsequent growth rate were generally reversed by concurrent curcumin exposure in all test cells. We further validated these observations in an independent set of test cells freshly obtained from human donors. BPA enhanced the growth potential of 6/6 consecutive specimens, whereas in combination with curcumin, its effects on S phase and apoptosis evasion sufficed to reduce overall growth. Since the cell yield of primary HRBEC samples is limited, we substituted repetitive sampling of different individuals from the population of interest ($n = 6$) for conventional experimental replicates of each sample. From a broader perspective, this illustrates the strength of using HRBECs. In the course of this and two previous studies, we have assayed samples from 40 individuals to demonstrate that BPA reproducibly induces pro-survival changes in high-risk human breast cells that are not malignant at the outset. Persistence of BPA-induced proliferation for 30 days after withdrawal of BPA in even one of these cases constitutes confirmation that BPA can indeed induce ongoing change. Knowing that the effects of BPA exposure can linger after it is discontinued forces reconsideration of the assertion that BPA might not be biologically relevant in humans because it is rapidly metabolized (41). Further research is needed to determine the minimal length

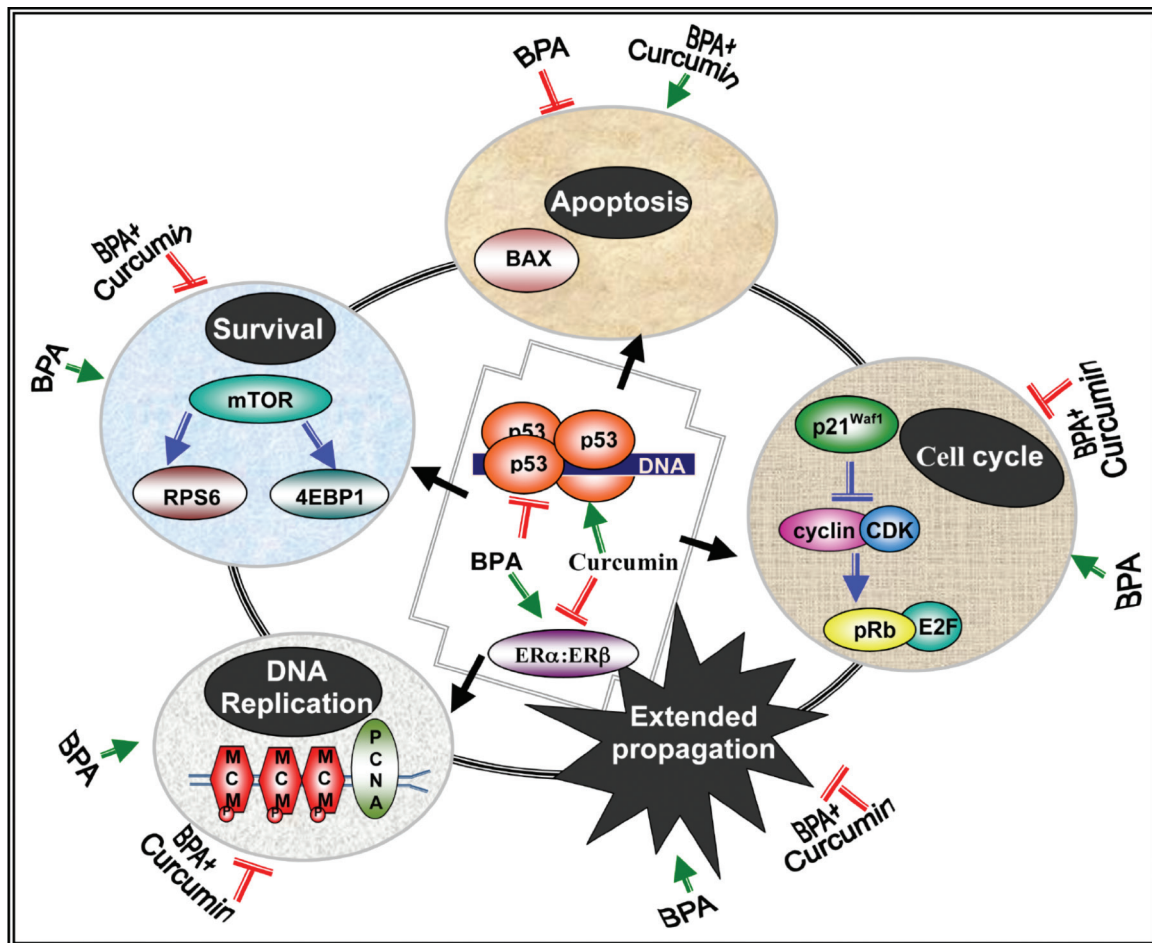


Fig. 6. Schematic model of BPA-induced cellular reprogramming and its reversal. We postulate that BPA exposure perturbs multiple regulatory hubs within target cells, thereby promoting the functional manifestation of well-accepted ‘hallmarks of cancer’ and culminating in the sustained propagation of finite-life non-malignant HRBECs. Green arrow represents activation; red ‘T’ denotes inhibition. Our findings support the antagonist effects of ER and p53 on cellular programs altered by BPA (depicted by large circles), each of which is associated with clinical breast cancer outcome. For example (i) cell survival—mediated by activation of the PI3K/AKT/mTOR pathway is known to result from estrogenic stimuli among others (31). Activation of this pathway confers a poor prognosis (32), as we demonstrated previously using a BPA-response gene signature (10). (ii) Apoptosis evasion—a role for BPA in the inhibition of p53, and downstream effects on BAX expression is consistent with its estrogenic action. TP53 loss of function is a hallmark of all cancer in general. Specifically in breast cancer, p53 status identifies women at a high risk of disease-specific mortality among African-Americans (33) and other ethnicities. (iii) Cell cycle—analogue to estrogen-stimulated early G_1 changes (34), BPA exposure induces cyclin D, inactivates p21^{Waf1}, facilitating cyclin E–Cdk2 activation and pRb inactivation in mid-to-late G_1 , allowing S phase entry to HRBECs. Overexpression of cell cycle progression genes is prognostic in breast cancer (35). (iv) DNA replication—ER α interacts with PCNA to initiate DNA replication and repair (36). Similarly, the MCM gene family required for DNA replication is frequently upregulated in various cancers, including breast tumors (37). (v) Extended propagation potential—is often a prelude to *in vitro* cell immortalization and is a characteristic feature of tumor cells derived from aggressive high-grade breast cancer (38). Our hypothesis predicts that BPA-induced aberrant gene expression alters the normal regulation of ER-responsive genes that influence pathways underlying tumorigenesis. As such, this model provides a molecular basis for BPA-promoted breast carcinogenesis and a mechanistically defined role for curcumin or curcumin-like agents in its prevention.

of exposure that initiates lasting cellular changes. However, since BPA exposure is an almost daily occurrence for the North American population, the brevity of any single exposure might be irrelevant in the context of repeated exposure.

Based on this study and our previous data, BPA neutralizes the effects of tamoxifen on normal breast cell proliferation, undermining a widely used breast cancer preventive measure, and as we have shown previously, BPA blocks the proapoptotic effects of rapamycin (3), a possible obstacle for effective single agent mTOR-targeted therapy. However, as demonstrated here, BPA does not override curcumin-induced downstream effects that reduce cell proliferation and, to a degree, increase apoptosis. In this context, our results support the use of downstream mTOR pathway inhibitors, i.e. curcumin or curcumin-like drugs, to reduce the emergence of resistant cell clones in treatment plans such as those targeting hormone-refractory tumors with aromatase inhibitors and rapalogs (42).

Supplementary material

Supplementary Table 1 and Figures 1 and 2 can be found at <http://carcin.oxfordjournals.org/>

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resources. All authors contributed to data analysis and manuscript preparation and approved the final version.

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