Breast cancer is the most common cancer in women. Bisphenol A (BPA), as a known endocrine disrupter, is closely related to the development of breast cancer. Curcumin has been clinically used in chemoprevention and treatment of cancer; however, it remains unknown whether microRNAs are involved in curcumin-mediated protection from BPA-associated promotive effects on breast cancer. In the present study, we showed that BPA exhibited estrogentic activity by increasing the proliferation of estrogen-receptor-positive MCF-7 human breast cancer cells and triggering transition of the cells from G1 to S phase. Curcumin inhibited the proliferative effects of BPA on MCF-7 cells. Meanwhile, BPA-induced upregulation of oncogenic miR-19a and miR-19b, and the dysregulated expression of miR-19-related downstream proteins, including PTEN, p-AKT, p-MDM2, p53, and proliferating cell nuclear antigen, were reversed by curcumin. Furthermore, the important role of miR-19 in BPA-mediated MCF-7 cell proliferation was also illustrated. These results suggest for the first time that curcumin modulates miR-19/PTEN/AKT/p53 axis to exhibit its protective effects against BPA-associated breast cancer promotion. Findings from this study could provide new insights into the molecular mechanisms by which BPA exerts its breast-cancer-promoting effect as well as its target intervention. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: bisphenol A; curcumin; MCF-7 breast cancer cells; miR-19; proliferation; inhibition.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women. Lifestyles, genetic, and environmental factors affect the risk of breast cancer. Exposure to endocrine-disrupting compounds is suspected of contributing to increased breast cancer incidence. It is reported that low-dose exposure of endocrine disruptors increases the susceptibility to breast cancer, particularly during critical periods of breast development when breast tissue is less differentiated (Birnbaum and Fenton, 2003; Fenton, 2006; Tsuda et al., 2003).

Bisphenol A (BPA), as a known endocrine disrupter, has a close relationship with the development of breast cancer. Bisphenol A is one of the highest volume chemicals produced worldwide. It is presented in the manufacture of plastics and epoxy resins that are universal in the environment and daily life of humans. Numerous evidences have demonstrated that BPA can release from food or beverage containers, and diet is the principal human exposure route to BPA (Kang et al., 2006; Richter et al., 2007). Because of the extensive usage of plastic products, BPA exposure is ubiquitous in general population (Kang et al., 2006). Besides its adverse effects in immune system, developmental function, and reproductive function, much attention has been attracted to the association between BPA and hormone-related cancers such as breast cancer. In vitro study has shown that BPA is able to induce neoplastic transformation in human breast epithelial cells (Fernandez and Russo, 2010). Bisphenol A induces MCF-7 breast cancer cell proliferation through upregulation of genes that promote cell cycle and downregulation of anti-proliferative genes (Keri et al., 2007; Lee et al., 2012). Evidence from animal models has demonstrated that maternal oral exposure to low concentrations of BPA during lactation increases mammary carcinogenesis (Jenkins et al., 2009). Unlike mutation-inducing chemicals, the effects of BPA occur rapidly, compromising molecular signaling pathways involved in cellular proliferation and apoptosis probably through epigenetic modifications. Alterations in signaling pathways will ultimately lead to changes in gene expression (Goodson et al., 2011; Keri et al., 2007; Lee et al., 2012). Recent studies have demonstrated microRNAs (miRNAs) regulation as an important mechanism in gene expression and cell signaling. To date, however, little information is available regarding the involvement of miRNAs in BPA-mediated breast cancer development.

MicroRNAs constitute a class of highly conserved, single-stranded non-coding small RNAs that function as post-transcriptional regulators of gene expression.
Materials and methods

Materials. Bisphenol A, 17β-estradiol (E2), and curcumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). MCF-7, an estrogen receptor (ER)-positive human breast cancer cell line, and MDA-MB-231, an ER-negative human breast cancer cell line, were purchased from Chinese Academy of Typical Culture Collection Cell Bank. Dulbecco’s modified Eagle’s medium (DMEM) and L-15 medium were purchased from Gibco (New York, NY, USA). Minimum essential medium (MEM) and 3-(4,5)-dimethylthiazol-(2-yl)-2,5-di-phenyltetrazoliumromide (MTT) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was obtained from PAA Laboratories (Pasching, Austria). Charcoal-striped FBS (CS-FBS) was obtained from Biological Industries (Kibbutz Be’er Haemek, Israel). miR-19a/miR-19b mimic and miR-19b/miR-19b inhibitor were purchased from RIBOBIO (Guangzhou, China). The primary antibodies of anti-PTEN, anti-α-AKT, anti-α-MDM2, anti-p53, and anti-proliferating cell nuclear antigen (PCNA) were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin antibody was purchased from Bioworld (Shanghai, China).

Cell culture. MCF-7 cells were grown in DMEM-containing antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), 1 nM E2, and 10% (v/v) FBS in a 37 °C humidified incubator with 5% CO2. MDA-MB-231 cells were grown in L-15 medium containing antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and 10% (v/v) FBS in a 37 °C humidified incubator without CO2. The long-term estrogen-deprived MCF-7 cells were cloned from wild-type MCF-7 cells following long-term culture in phenol-red-free MEM (without E2) supplemented with 5% charcoal-stripped FBS (CS-FBS), 0.29 g/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.11 g/L sodium pyruvate.

Cell proliferation assay. Experiments were accomplished in 96-well plates containing phenol-red-free MEM supplemented with 5% CS-FBS. MCF-7 cells and MDA-MB-231 cells were seeded at a plating density of 2 × 10^3 cells/well in 100 μL of medium. Two days later, the medium was replaced, and the cells were treated with 0.1% DMSO, E2 (1 nM, 10 nM), BPA (10^–7–10^–9 M), curcumin (1 μM), curcumin (1 μM) + E2 (10 nM), or curcumin (1 μM) + BPA (10^–5 M) for 4 days. Cell viability was then assessed using MTT assay. Ten microlitre of MTT solution (5 mg/mL) was added to each well, and the plates were further incubated for 4 h at 37 °C. Medium containing MTT was removed, and precipitants were solubilized in DMSO. Absorbance was measured at 490 nm using a microplate reader. All measurements were performed in triplicate.

Cell cycle analysis. The distribution of MCF-7 cells at different stages in the cell cycle was determined by flow cytometric analysis. Briefly, cells (5 × 10^5) were incubated at 37 °C overnight in triplicate of 60 cm plastic dishes in phenol-red-free MEM supplemented with 5% CS-FBS and cultured with MEM without FBS for 6 h. Cells were then treated with E2 (10 nM), BPA (10^–5 M), curcumin (1 μM), curcumin (1 μM) + E2 (10 nM), or curcumin (1 μM) + BPA (10^–5 M) for 4 days. Cells were trypsinized, washed twice with cold PBS, and fixed overnight with 70% ethanol at 4 °C followed by resuspension in 500 μL of PBS. After addition of 10 μL RNAse (10 mg/mL), cells were left for 30 min at 37 °C in the dark and stained with 10 μL propidium iodide (1 mg/mL). Cell cycle analysis was then performed by flow cytometry. The percentages of cells in each cell cycle phase (G0/G1, S, or G2/M) were calculated using MULTICYCLE v.3.0 software, with a minimum of 1 × 10^4 cells/sample being evaluated.

Transient transfection. MCF-7 cells were plated onto 96-well plates with a density of approximately 1.0 × 10^4 cells in the phenol-red-free MEM containing 5% CS-FBS without antibodies. Following 12 h of incubation, cells were then transfected with miR-19a/miR-19b mimic (50 nM) or miR-19a/miR-19b inhibitor or negative vector control by lip 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. Cells were treated with E2 (10 nM), BPA (10^–5 M), curcumin (1 μM), curcumin (1 μM) + E2 (10 nM), or curcumin (1 μM) + BPA (10^–5 M) for 4 days. Cell viability was assessed using MTT assay. For western blot analysis, 5 × 10^5 cells/well of MCF-7 were plated onto 60-mm plates, and 12 h later, cells were transfected with miR-
19a and 19b mimic (50 nM) or negative vector control by lip 2000, as described previously. After 48 h, cells were collected for western blot analysis.

**Statistical analysis.** All data are presented as the means ± standard deviation. All experiments were repeated three times independently. Statistical analyses were performed by one-way analysis of variance, using SPSS version 11.0 software (SSPS, Inc., Chicago, IL, USA). A p-value of <0.05 was considered statistically significant.

**RESULTS**

**BPA-induced MCF-7 breast cancer cell proliferation**

We first evaluated the effect of BPA on the proliferation of MCF-7 and MDA-MB-231 human breast cancer cells by MTT assay, using E2 as a positive control. Fig. 1A shows that E2 promoted the proliferation of ER-positive MCF-7 human breast cancer cells. Meanwhile, treatment of MCF-7 cells with 10⁻² or 10⁻³ M BPA for 4 days also significantly increased MCF-7 cell viability in comparison with vehicle control (p < 0.05), an effect similar to that of E2 on MCF-7. On the other hand, both E2 and BPA did not induce the proliferation of ER-negative MDA-MB-231 breast cancer cells (Fig. 1B). These data revealed that BPA exerted its estrogenic activity and induced MCF-7 breast cancer cell proliferation.

**Curcumin prevented BPA-triggered MCF-7 cell proliferation and cell cycle progression**

To determine whether curcumin could suppress BPA-triggered MCF-7 cell proliferation, cells were treated with 10 nM E2, 10⁻⁵ M BPA, 1 μM curcumin, curcumin (1 μM) + E2 (10 nM), or curcumin (1 μM) + BPA (10⁻⁵ M) for 4 days. Results revealed that curcumin effectively suppressed BPA-mediated cell proliferation, an inhibitory effect similar to that of curcumin on E2 (Fig. 2A). On the basis of the growth inhibition results, further studies were performed to explore the possible mechanisms by which curcumin exhibits its inhibitory effects in BPA-treated MCF-7 cells. To examine whether the anti-proliferative effect of curcumin on BPA-treated MCF-7 cells was mediated via cell cycle modulation, we investigated the cell cycle distribution in curcumin-treated cells by flow cytometric analysis. Treatment of MCF-7 cells with 10⁻³ M BPA accelerated...
G1 to S phase progression, with 21.98% of cells entering into the S phase, significantly higher than that of the control value (8.11%). In contrast, combination treatment of curcumin and BPA reversed BPA-elicited increase in the percentage of cells in S phase (Fig. 2B and 2C), indicating that curcumin prevented BPA-triggered cell cycle progression.

**Curcumin suppressed BPA- upregulated expression of miR-19a/miR-19b**

MicroRNAs function as important post-transcriptional regulators of gene expression. The well-characterized oncogenic miR-17-92 cluster plays crucial role in the regulation of cell proliferation, apoptosis, and carcinogenesis. As a key oncogenic component of the miR-17-92 cluster, miR-19 overexpression is implicated in carcinogenic processes. In the present study, we examined whether ectopic expression of miR-19 can cause MCF-7 breast cancer cell proliferation. Cells were transfected with miR-19a or miR-19b mimics, and cell viability was measured. As shown in Fig. 3, miR-19a and miR-19b increased the proliferation of MCF-7 cells. To determine if miR-19 is involved in BPA-induced proliferation of MCF-7 cells, we examined the expression of miR-19a and miR-19b in cells exposed to BPA. As expected, marked increases in the levels of miR-19a and miR-19b expression were observed following BPA treatment. Moreover, curcumin treatment significantly decreased BPA-mediated elevation of miR-19a and miR-19b expression (Fig. 4).

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**Figure 2.** Curcumin prevented Bisphenol A (BPA)-triggered MCF-7 cell proliferation and cell cycle progression. (A) MCF-7 cells were treated with 10^{-5} M BPA with or without 1 μM curcumin for 4 days, and cell viability was measured by MTT. Data are expressed as mean±standard deviation (SD). *p < 0.05 compared with the control group. (B) and (C): MCF-7 cells were treated with 10^{-5} M BPA in the absence or presence of 1 μM curcumin for 4 days, cells were fixed in 70% ethanol, and cell cycle analysis was performed by flow cytometry. E2 was used as a positive control. Data are expressed as mean±SD. *p < 0.05, compared with the control group. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

**Figure 3.** MicroRNA (miRNA)-19a/miR-19b mimic promoted MCF-7 cell proliferation. MCF-7 cells were transfected with miR-19a/miR-19b mimic (50 nM) or negative vector control. miRNA-19a (A) or miR-19b (B) transfection efficiency was measured by quantitative reverse transcription PCR assays 48 h post-transfection. U6 snRNA was served as an internal control. Data are expressed as mean±standard deviation (SD). *p < 0.05 compared with the control group. (C) MCF-7 cells were transfected with miR-19a/miR-19b mimic (50 nM) or negative vector control. After 48 h incubation, cell viability was measured by MTT. Data are expressed as mean±SD. *p < 0.05, compared with the control group.
Curcumin abrogated BPA-mediated alterations in miR-19-targeted PTEN-AKT-p53 pathway

miR-19 exerts its oncogenic function by targeting critical cancer-related genes such as PTEN—a tumor suppressor gene that inhibits the activity of the PI3K-Akt signaling pathway and subsequently leading to activation of MDM2, inactivation of p53, and cell growth. In the present study, we indeed observed that ectopic expression of miR-19a and miR-19b decreased the expression of PTEN and p53 and increased the expression of p-AKT (Fig. 5A). We then examined the alterations in the expression of miR-19-associated cell proliferation proteins, including PTEN, AKT, MDM2, p53, and PCNA, in MCF-7 cells treated with BPA as well as the combination of curcumin and BPA. Our results indicated that, consistent with the induction of miR-19, BPA downregulated the expression of PTEN, along with increased levels of phosphorylated (p)-AKT and p-DMDM2, decreased p53, and increased PCNA. On the contrary, curcumin abrogated the spectrum of effects associated with BPA-mediated changes in the expression of PTEN, p-AKT, p-DMDM2, p53, and PCNA (Fig. 5B). These data suggested the role of miR-19/PTEN/AKT/p53 axis in the modulation of the breast-cancer-promoting effects of BPA and the anti-proliferative effects of curcumin.

DISCUSSION

The notion that BPA is closely related to the development of breast cancer is supported by studies with rodents, non-human primates, and human cancer cell lines (Jenkins et al., 2009; Keri et al., 2007; Lee et al., 2010).
Many miRNAs functionally participate in cancer development, involving the regulation of cell proliferation, apoptosis, carcinogenesis, invasion, and metastasis. The miR-17-92 cluster has been identified as a polycistronic and potent oncogenic miRNA (O’Donnell et al., 2005; Fontana et al., 2008; Kim et al., 2012; Wang et al., 2008). Downregulation of miR-17-92 cluster results in inhibition of cancer cell proliferation and invasion (Kim et al., 2012). Among miR-17-92, miR-19 is a key oncogenic miRNA of the cluster, which is vital for promoting tumorigenesis (Mu et al., 2009; Olive et al., 2009). miR-19 exerts its oncogenic function by targeting critical tumor suppressor genes such as PTEN.

PTEN is a major tumor suppressor gene that is frequently mutated or deleted in multiple tumor types (Di Cristofano and Pandolfo, 2000; Knobbe et al., 2008). It acts to repress the intracellular level of phosphatidylinositol 3'-kinase (PI3K), thus negatively regulating the PI3K pathway and its downstream effector AKT (Comer and Parent, 2002; Rossi and Weissman, 2006). It has been demonstrated that PTEN is a key target gene of miR-19. There are two miR-19-binding sites in 3'UTR of PTEN; direct binding exists between miR-19 and PTEN 3'UTR, and mutations of the miR-19-binding sites in PTEN 3'UTR hampers mir-19 mediated PTEN repression; upregulation of miR-19 suppresses PTEN expression and activates AKT to promote cancer cell growth (Olive et al., 2009). Because of the central role of PTEN/PI3K/AKT signaling pathway for cancer growth and development, aberrant activation of this pathway has been verified in many human cancers including breast cancer (Chandarlapaty et al., 2012; Lopez-Knowles et al., 2010; Ptak and Gregoraszczuk, 2012). Activation of AKT downregulates p53 abundance through the phosphorylation of MDM2—a negative regulator of p53—thereby stabilizing p53 and promoting cancer cell growth.
In colorectal cancer cells, curcumin inhibited the cancer cells (Zhang et al., 2013; Li and Lozano, 2013; Wade et al., 2012). In the present study, we showed that ectopic expression of miR-19a or miR-19b increased MCF-7 cell proliferation, and decreased the expression of PTEN and p53, and increased the expression of p-AKT. In contrast, inhibition of miR-19a or miR-19b decreased MCF-7 cell proliferation and prevented BPA-induced MCF-7 cell growth. These data illustrated the important role of miR-19 in BPA-mediated MCF-7 cell proliferation. Furthermore, in line with the ability of miR-19 to repress PTEN expression and function, we observed in our study that BPA-triggered miR-19a and miR-19b induction was associated with PTEN downregulation, AKT and MDM2 activation, and p53 inactivation. These data suggest that BPA modulates miR-19/PTEN/AKT/p53 pathway in its breast-cancer-promoting effects. It is noteworthy that the interactive mechanism between BPA and miR-19 is unclear at the present time, and further studies are warranted to investigate the mechanisms implicated in BPA regulation of miR-19.

Phytochemicals have been gaining increasing attention as anticancer agents. Curcumin, a natural polyphenol, exhibits potent chemopreventive and therapeutic potential against a variety of cancers. This potent anticancer property of curcumin is attributable to its ability to regulate numerous proteins and cellular signal pathways, altering the expression and activities of genes involved in cell proliferation, apoptosis, and metastasis. However, the precise molecular mechanisms for the anti-carcinogenic activity of curcumin are not fully understood. Recently, curcumin has been demonstrated to possess a novel mechanism of regulation, that is, by modulating miRNA expression in various human cancers. In human pancreatic cancer cells, curcumin upregulated miR-22 and downregulated oncosgenic miR-196 (Sun et al., 2008). Curcumin decreased miR-186 and miR-21 to promote apoptosis of A549 lung cancer cells (Zhang et al., 2010; Zhang and Bai, 2013). In colorectal cancer cells, curcumin inhibited the expression of miR-21 to suppress cell proliferation, tumor growth, invasion, and metastasis (Mudduluru et al., 2011). While several groups depicted the effects of curcumin on miRNA expression in various cancers, so far, only one study illustrated curcumin-mediated miRNA regulation in breast cancer. Yang et al. showed that curcumin upregulated miR-15a and miR-16 expression, reduced Bcl-2 expression, and induced apoptosis in breast cancer cells (Yang et al., 2010). Currently, however, no information is available regarding the role of miRNAs in the anticancer activity of curcumin against BPA-associated carcinogenic effects on breast cancer cells. In the present study, we revealed that curcumin suppressed BPA-mediated upregulation of oncosgenic miR-19a and miR-19b in MCF-7 cells and that the dysregulated expression of miR-19-related downstream proteins, including PTEN, p-AKT, p-MDM2, p53, and PCNA, were reversed by curcumin treatment. These data suggest that curcumin modulates miR-19 and its downstream targets to exhibit its protective effects against BPA-elicited breast cancer promotion.

In conclusion, the present study demonstrates for the first time that modulation of miR-19/PTEN/AKT/p53 axis is critically involved in the proliferation-stimulating effects of BPA on MCF-7 breast cancer cells and the anti-proliferative effects of curcumin. Findings from this study could provide new insights into the molecular mechanisms by which BPA exerts its breast-cancer-promoting effects as well as its target intervention.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81373005, No. 81072330, No. 81202194) and by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Conflict of Interest

The authors declare no conflict of interest.

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