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GPER/Hippo-YAP signal is involved in Bisphenol S induced migration of triple negative breast cancer (TNBC) cells

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ABSTRACT

Nowadays, risk factors of triple-negative breast cancer (TNBC) metastasis are not well identified. Our present study reveals that an industrial chemical, bisphenol S (BPS), can promote the migration, but not the proliferation, of TNBC cells *in vitro*. BPS activates YAP, a key effector of Hippo pathway, by inhibiting its phosphorylation, which promotes YAP nuclear accumulation and up-regulates its downstream genes such as *CTGF and ANKRD1*. Inhibition of YAP blocks the BPS-triggered cell migration and up-regulation of fibronectin (FN) and vimentin (Vim). BPS rapidly decreases the phosphorylation levels of LATS1 (Ser909) in TNBC cells, which regulates the activation and functions of YAP. Silencing LATS1/2 by siRNA increases BPS-induced dephosphorylation of YAP and extended the half-life of YAP protein. Inhibition of G protein-coupled estrogen receptor 1 (GPER) and its downstream PLCβ/PKC signals attenuate the effects of BPS-induced YAP dephosphorylation and CTGF up-regulation. Targeted inhibition of GPER/YAP inhibits BPS-induced migration of TNBC cells. Collectively, we reveal that GPER/Hippo-YAP signal is involved in BPS-induced migration of TNBC cells.

1. INTRODUCTION

Bisphenol A (BPA) is an endocrine disrupting chemical which has been widely used in the production of plastic containers for food and beverages, adhesives, paints, and dental sealants [1]. Since increasing evidences showed the negative effects of BPA on human health, it has been prompted to remove from consumer products [2]. Bisphenol S (BPS) is considered as the substitution for BPA in the plastic industry due to its excellent stability [2]. Up to date, BPS has become world widely used in industrial products, including washing agents, phenolic resin, electro plating solvent, and thermal paper [3]. As reported, the estimated daily intake of BPS is 9.55 ng/kg body weight/day for men and 9.56 ng/kg body weight/day for women [4]. Epidemiological studies reveal that BPS can be widely detected in human urine and blood [5–7]. For example, 81% urine samples from the United States and Asia contain detectable BPS [7], suggesting that biological safety of BPS is an urgent issue for public health [2].

Increasing evidences show that BPS has strong estrogenic responses *in vitro* and *in vivo*, resulting in the disruption of endocrine functions. Combining fluorescence polarization system and E-screen test, studies revealed that BPS had a comparable estrogenic activity as BPA [8]. This comparable effect was further confirmed in both MELN cells and BG-1Luc 4E2 cells [9]. Meanwhile, BPS can bind to membrane estrogen receptors (ERs) and disrupt the non-genomic 17β-estradiol (E2) signaling in rat pituitary cell line [10], and the estrogenic potency of BPS is equivalent to or even greater than E2 [11–13]. In addition, BPS exposure can increase the plasma estrogen level, while decrease the testosterone level, in hypothalamus [14,15].

Progression of human breast cancer can be regulated by estrogenic signals [16]. Emerging reports revealed that BPA triggers the proliferation, migration and invasion of breast cancer cells [17,18]. Furthermore, few studies showed that BPS modulates the growth and epithelial mesenchymal transition of breast cancer cells [19]. Triple-negative breast cancer (TNBC) are characterized by testing negative for the estrogen receptor (ER), progesterone receptor (PGR) and human epidermal growth factor receptor 2 (HER-2) [20]. Compared to other

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breast cancer subtypes, TNBC cells are more biologically aggressive and easily metastatic [21]. It has been reported that estrogenic signals can also modulate the progression of TNBC via G-protein coupled estrogen receptor (GPER) and other pathways [22]. However, effects of BPS on progression of TNBC cells have not been investigated yet. Our present study revealed that BPS can promote *in vitro* cell migration, but not proliferation, of human TNBC cells. The GPER-regulated Hippo-YAP pathway is involved in BPS-induced migration of TNBC cells.

2. MATERIALS AND METHODS

2.1. Cell culture, treatment, and transfection

Human TNBC MDA-MB-231 and BT-549 cells were cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (Invitrogen). For BPS treatments, all plastic items, reagents and water used for the experiments were pretreated by enhanced sonochemical degradation to reduce potential background according to our previous study [23]. Chemicals or inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Less than 0.1% DMSO was contained in the working solution. Steroid-free medium containing DMSO was used as control. The siRNA transfection was operated by lipofectamine RNAiMAX (Invitrogen; Carlsbad, CA) according to the instructions with the working concentration of 50 nM.

2.2. Cell proliferation assays

Effects of BPS on cell proliferation were evaluated by CCK-8 kit (Dojindo Molecular Technologies, Gaithers burg, MD, USA) according to the manufactures' instruction and our previous study [23]. Briefly, 1×10^4 cells per well were seeded into 96-well plates before treatments. Indicated concentrations of BPS were incubated with cells for different time points as mentioned in text. At the end of experiment, 10 μL of CCK-8 solution was added to each well and incubated for 2 h, followed by measurement of absorbance at 450 nm using microplate reader. The control cells were set as 100% for normalization.

2.3. Cell migration assays

Wound healing and transwell assay were used to study the effects of BPS on cell migration according to previous studies with slight modifications [24,25]. For wound healing assay, about 90% confluent monolayers of cells were scratched and treated with BPS in medium containing no FBS. The migration distance of the cells into the scratched area was measured in 10 randomly chosen fields. The polycarbonate filters (8 μm pore size, Corning) was used for transwell assay. The upper chamber was added 200 μl medium (containing 0.1% FBS) containing 1×10^5 cells treated with or without BPS. The lower chamber was added 600 μl medium with 10% FBS to serve as a chemotactic agent. After incubation for 48 h, cells migrated onto the lower chamber were fixed, stained, and counted under upright microscope (5 fields per chamber). Each transwell assay was repeated in five independent experiments.

2.4. Western blot analysis

Western blot analysis was performed as previously described [24]. Briefly, cells were lysed in cell lysis buffer, and then lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Aliquats of protein were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Following blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with the primary antibody at 1:1000 dilution overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 2 h at room temperature, and detected with the Western Lightning Chemiluminescent detection reagent (Perkin-Elmer Life Sciences, Wellesley, MA). GAPDH was used as the loading control for all western blot analysis. Densitometry analysis was performed using the ImageJ Gel Analysis tool, where gel background was also removed individually for each band.

2.5. Quantitative real-time PCR (qRT-PCR)

The RNA extraction, reverse transcription, and real time PCR were performed according to our previous study [24] with the following primers: GAPDH, forward 5′-GCA CCG TCA AGG CTG AGA AC-3' and reverse 5′-TGG TGA AGA CGC CAG TGG A-3′; CTGF, forward 5′- ACC GAC TGG AAG ACA CGT TTG-3' and reverse 5′- CCA GGT CAG CTT CGC AAG G-3′; ANKRD1, forward 5′- CGA CTC CTG ATT ATG TAT GGC GC-3' and reverse 5′- GCT TTG GTT CCA TTC TGC CAG TG-3′; YAP, forward 5′- CGC TCT TCA ACG CCG TCA -3' and reverse 5′- AGT ACT GGC CTG TCG GGA GT -3′. GAPDH was used as a control for normalization. The relative levels of gene expression were represented as $\Delta Ct = Ct^{gene} - Ct^{reference}$, and the fold change of gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Experiments were repeated in triplicate.

2.6. Immunofluorescence

Immunofluorescent staining was carried out as stated previously [22]. Briefly, 50% confluent cells were cultured on confocal dishes and then exposed to BPS or G-1 for the indicated times. Cells were washed three times with phosphate-buffered saline solution (PBS), fixed in 4% paraformaldehyde for 20 min, and permeabilized with 0.3% Triton X-100 for 10 min. After blocking with goat serum for 2 h, cells were incubated for 1 h with antibody against YAP. Then, dishes were washed three times with PBS and incubated with Alexa Fluor 488 conjugated secondary antibody (1:1000dilution) for 1 h at room temperature. Nuclei were stained with DAPI (10 mg/ml) for 10 min. Samples were examined with Confocal Laser Scanning Microscopy (Zeiss) to analyze expression and nuclear translocation of YAP.

2.7. Statistical analyses

The data were reported as mean ± SD from at least three independent experiments and analyzed by SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Data were analyzed by two-tailed unpaired Student's t-test between two groups. For comparisons among three or more groups, ANOVA was performed and groups had significant differences compared to the control group were marked. A p-value of < 0.05 was considered to be statistically significant.

3. RESULTS

3.1. BPS triggers the migration, but not proliferation, of TNBC cells

Previous study suggested that BPS can regulate the proliferation of human cancer cells [9]. Our data showed that concentrations of BPS from nanomole (10^{-9} M) to milimole (10^{-3} M) had no significant effect on proliferation of either MDA-MB-231 (Fig. 1A) or BT-549 (Fig. S1A) cells. Next, we investigated the effect of BPS on *in vitro* migration of TNBC cells. Wound healing assays showed that both 10^{-8} M and 10^{-6} M BPS can significantly trigger the migration of MDA-MB-231 cells after treatment for 48 h (Fig. 1 B). Transwell analysis confirmed this promotive effect of BPS on cell migration (Fig. 1C). Similar effects of BPS were observed on *in vitro* migration of BT549 cells (Fig. S1 B). We then detected the expression of migration related proteins in BPS-treated cells. Western blot results showed that BPS treatment can markedly