SPANDIDOS PUBLICATIONS

Environmental endocrine disruptors promote invasion and metastasis of SK-N-SH human neuroblastoma cells

HAITAO ZHU[1], JICUI ZHENG[1], XIANMIN XIAO[1], SHAN ZHENG[1], KUIRAN DONG[1], JIANGBIN LIU[1] and YANG WANG[2]

[1]Department of Surgery of Children's Hospital, [2]Anatomy and Histology and Embryology of Shanghai Medical School, Fudan University, Shanghai, P.R. China

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Abstract. Neuroblastoma (NB) is the most common pediatric extracranial cancer. Metastasis is the main cause of mortality in NB patients. Currently, little is known about the risk factors and their mechanisms that cause metastasis. Environmental endocrine disruptors (EED) are recently identified risk factors associated with various human diseases including malignant tumors. Our previous studies have implicated the role of di(2-ethylhexyl) phthalate (DEHP) and bisphenol A (BPA), two of the most common EED, in neuroblastoma cell proliferation. Here, we further investigated the effects of DEHP, BPA as well as 17ß-estradiol (E_2) on the invasion and metastasis of human neuroblastoma SK-N-SH cells *in vitro*. SK-N-SH cells expressed estrogen receptor (ER)-ß, matrix metalloproteinases-2 (MMP-2), MMP-9 and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) at readily detectable levels. 50 μM DEHP, 0.1 μM BPA and 10 μM E_2 exposure all resulted in enhanced motility and invasiveness of SK-N-SH cells ($P<0.001$), elevated expression of MMP-2 and MMP-9, and decreased expression of TIMP-2 ($P<0.01$). Furthermore, phosphorylation of Akt (Ser473) was also induced following the exposure ($P<0.01$). Importantly, both ER antagonist ICI182,780 and phosphoinositide 3-kinase (PI3K) specific inhibitor LY294002 significantly inhibited the DEHP, BPA, or E_2-induced cell migration and invasion, as well as the disregulation of MMP-2, MMP-9 and TIMP-2 expression. ICI182,780 may have worked through abolishing Akt (Ser473) phosphorylation. In conclusion, DEHP, BPA, and E_2 potently promote invasion and metastasis of neuroblastoma cells through overexpression of MMP-2 and MMP-9 as well as downregulation of TIMP-2. ER-dependent pathway and PI3K/Akt pathway are involved, which may become potential therapeutic targets for neuroblastoma treatment.

Correspondence to: Dr Xianmin Xiao, Department of Surgery, Children's Hospital, Fudan University, 399 Wanyuan Road, Shanghai 201102, P.R. China
E-mail: xmxiao@shmu.edu.cn

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Introduction

Neuroblastoma (NB) is one of the most frequent solid malignant tumors in children (1). NB affects around 10.5/10 million children younger than 15 years of age per year (2). It accounts for 7-10% of all malignacies of childhood and about 15% of all pediatric cancer deaths (2-5). However, little is known about the risk factors and their mechanisms involved in NB's etiopathogenesis. The prognosis for patients, particularly those with advanced stage disease, has remained poor despite great advances in clinical therapies occurred during the past few years (2,3). Therefore, NB continues to be a great challenge to physicians as well as basic scientists.

Metastasis is the leading cause of death in neuroblastoma. It occurs in near 70% of NB patients at the time of primary diagnosis (6). Most patients with metastatic disease are currently classified into the high-risk group which has an overall survival rate less than 40% (3,4). Currently, the mechanisms of metastasis in NB are largely unknown. We believe exploring the risk factors involved in NB cell metastasis and understanding their mechanisms will contribute to improved preventive strategies and more effective treatments for this cancer.

Environmental endocrine disruptors (EED) are a large number of either natural or man-made exogenous compounds that have the potential to interfere with the normal endocrine system of the animals including human beings (7,8). These chemicals are also called environmental estrogens or xenoestrogens since most of them mainly mimick the action of natural hormone estrogens such as 17ß-estradiol in the body. During past decades, with aggravation of global environmental problems, there have been growing concerns on relationships between daily exposure to environmental chemical contaminants including EED and human health. Accumulating evidence suggested that EED are causative factors for multiple human diseases including birth defects,

developmental and metabolic disorders, reproductive disturbances, and malignant tumors (9-14). Children are especially susceptible to adverse effects of EED exposure due to their physiological characteristics such as small body size and limited capacity to metabolize EED (9,10).

Di(2-ethylhexyl) phthalate (DEHP) and bisphenol A (BPA) are most common compounds in EED. They are widely used as plasticizers in most of plastic products including floor and wall coverings, food containers, blood storage bags, medical devices, baby bottles and children's toys (7,15). Both animal experiments and epidemiological studies have shown harmful effects of exposure to DEHP or BPA on human health. Prenatal exposure to BPA induces neurobehavioral developmental disorders in mouse model (16). Morphological and functional alterations of both mouse and human reproductive system often occur after prenatal or adult exposure to DEHP or BPA (17-20). Higher BPA exposure is also associated with abnormal liver functions, cardiovascular diseases, diabetes and other metabolic disorders in human beings (10,21). Furthermore, developmental exposure to DEHP or BPA also influence the promotion or progression of most common adult cancers, like breast cancer, prostate cancer and colon cancer (13,22-23). It is of great interest to further investigate the relationship between DEHP or BPA exposure and pediatric solid malignant tumors including neuroblastoma due to children's susceptibility to these chemicals.

There are few reports concerning the relationship between environmental endocrine disruptors and neuroblastoma or other children solid malignant tumors. In our previous studies, we demonstrated that both DEHP and BPA had growth-promoting effect on neuroblastoma (24-26). In the present study, we further investigated whether these environmental endocrine chemicals as well as 17ß-estradiol (E_2) were also involved in SK-N-SH human neuroblastoma cell invasion and metastasis. We have also attempted to understand their mechanisms.

Materials and methods

Cell culture and treatment. SK-N-SH, a neuroblastoma cell line purchased from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom, Berlin, Germany), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.1 M L-glutamine. Cells were grown in a humidified atmosphere with 5% CO_2 in air at 37˚C. For steroid-depleted conditions, phenol red-free RPMI-1640 medium (Sigma-Aldrich) and 10% charcoal-dextran-stripped FBS (cd-FBS; Biological Industries Ltd., Kibbutz Beit Haemek, Israel) were utilized for 48 h before experiments (25). MCF-7 human breast cancer cells, a gift from Dr Tiewei Chen of Cancer Hospital, Fudan University, were cultured in RPMI-1640 medium (Sigma-Aldrich) with 10% FBS (Biochrom).

Chemicals. DEHP, BPA, and E_2 were obtained from Sigma-Aldrich. Estrogen receptor (ER) antagonist ICI182,780 was purchased from Tocris (Bristol, UK) and phosphoinositide 3-kinase (PI3K) inhibitor LY294002 was obtained from Cell Signaling Technology (Beverly, MA). All reagents were dissolved in DMSO, and then diluted with the cell culture medium at the indicated concentrations. The final solvent concentration in the culture did not exceed 0.1%.

Cell migration and invasion assay. Cell migration assay was performed using transwell cell culture chambers (Millipore, Bedford, MA) with 6.5-mm diameter polycarbonate filters (8 μm pore size). Each upper chamber was filled with 100 μl cell suspension ($1x10^5$/chamber) that was prepared with 50 μM DEHP, 0.1 μM BPA, 10 μM E_2 or solvent only with or without 1-h pretreatment of 10 μM ICI182,780 or 20 μM LY294002 in serum-free and phenol red-free RPMI-1640 medium. Each lower chamber was filled with 600 μl phenol red-free RPMI-1640 medium supplemented with 10% cd-FBS. After 16 h, cells that had invaded the lower side of the filters were fixed with 4% paraformaldehyde followed by hematoxylin and eosin staining. The number of migrating cells was counted under a light microscope at a magnification of x200, and five random visual fields were selected for each examination.

For invasion assays, 50 μl diluted 1:3 Matrigel (BD Biosciences, Bedford, MA) in serum-free cell culture medium was added to each upper chamber and incubated at 37ºC for 2 h for gelling. Then the invasion assay procedure was the same as for the migration assay described above except that the incubation time of the experiment was 24 h. Both experiments were repeated three times.

Reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR. The neuroblastoma cells were seeded in 60-mm dishes with phenol red-free RPMI-1640 medium containing 10% cd-FBS. After 24 h, cells were cultured in phenol red-free RPMI-1640 with 5% cd-FBS overnight. Cells were further incubated for another 24 h in 5% cd-FBS-supplemented medium with DEHP (50 μM), BPA (0.1 μM), E_2 (10 μM), or solvent (control cells), with or without 1-h pretreatment of ICI182,780 (10 μM) or LY294002 (20 μM). Total cellular RNA was then extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the protocol recommended by the manufacturer. Total RNA concentrations were measured by absorbance reading at 260 nm on a SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA). The purity of total RNA specimen was examined by determing the A_{260}/A_{280} ratio. cDNA templates for use in PCR were synthesized from 0.5 μg of total RNA by *in vitro* transcription in 10 μl reaction containing 1 mM dNTPs, 0.125 μM oligo(dT), and 0.5 μl of Avian Myeloblastosis Virus (AMV) reverse transcriptase (Takara Biotechnology, Dalian, China) at 42˚C for 30 min. RT-PCR reaction mixture including 2 μl of cDNA templates from RT, 0.1 μM of each primer, 5 μl 5X PCR buffer, 2 μl Takara Ex Taq HS (Takara Biotechnology), and sterile water in a reaction volume of 25 μl. Conditions for RT-PCR followed the manufacturer's protocol. The PCR products were then electrophoresed on a 1% agarose gel. For quantification, real-time PCR was performed and the reaction mixture including 1 μl of cDNA, 0.2 μM of each primer, 12.5 μl SYBR® Premix Ex Taq™ (Takara Biotechnology), and sterile water

Oligonucleotide sequences of primer pairs used for RT-PCR or quantitative real-time PCR.

Gene	Sense (5'-3')	Antisense (5'-3')	Size (bp)	Annealing temperature (˚C)
ESR1	CCC ACG GCC AGC AGG TGC CCT ACT AC	TCA CCC CTG CCC TCC CCA TCA TCT CT	469	69
ESR2	AAT TGA CCA CCC CGG CAA GCT CAT CTT T	TGC CCT TGT TAC TCG CAT GCC TGA CG	377	68
MMP-2	GCA AGC CCA AGT GGG ACA AGA A	AAA ACC GCA GTG GGG TCA CAT C	135	60
MMP-9	GGA CGG GCT CCT GGC ACA C	GAG CGG CCC TCG AAG ATG AAG	168	69
TIMP-2	AGG GCC AAA GCG GTC AGT GA	AGG AGG GGG CCG TGT AGA TAA ACT	148	59
GAPDH	GAA GGT GAA GGT CGG AGT C	GAA GGT GAA GGT CGG AGT C	226	50

in a reaction volume of 25 μl. The PCR conditions were 5 min at 94˚C followed by 40 cycles at 94˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 20 sec. Experiments were carried out using the Rotor-Gene 3000™ system and rotor gene 6.1.90 detection software (Corbett Life Science, Sydney, Australia). The primer pairs for target genes including human estrogen receptor 1 (ESR1), estrogen receptor 2 (ESR2), matrix metallopeptidase (MMP)-2, MMP-9, and tissue inhibitor of metalloproteinase 2 (TIMP-2) were designed using DNAStar software (DNAStar, Inc., Madison, WI), and manufactured by Shanghai Invitrogen Biotechnology. All primer pairs were first tested by regular RT-PCR to be highly effective and specific for amplification. Primer sequences and annealing temperature are listed in Table I. GAPDH was used as the standard housekeeping gene. Ratios of target gene expression levels were calculated by subtracting the threshold cycle number (Ct) of the target gene from the Ct of GAPDH. Ct values were defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. Target gene expression was expressed relative to GAPDH gene expression. Then ratios of target gene expression between the treatment groups and the untreated group were also calculated. As a positive control, ESR1 and ESR2 gene expression in both ERα and ß positive MCF-7 cells were also detected with the same RT-PCR protocol described above (27). The whole experiments were repeated four times.

Immunocytochemistry. SK-N-SH cells were initially seeded at a density of $5x10^4$ in 12-well plate containing 0.01% poly-L-lysine-coated glass slides. Growth medium was then removed, and the plate was rinsed with 0.01 M phosphate-buffer solution (PBS, pH 7.2). Slides containing the attached cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were permeable for 15 min using 0.1% Triton X-100 in PBS, washed in PBS and then incubated in 5% normal goat serum (Boster, Wuhan, China) for 30 min at room temperature. After rinsing with PBS, cells were incubated with 5% normal goat serum (negative control), anti-ERα (1:100 dilution, Thermo Fisher Scientific Inc., Fremont, CA), anti-ERß, anti-MMP-2, anti-MMP-9, and anti-TIMP-2 primary antibody (all 1:500 dilutions, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4˚C. After being rinsed, cells were incubated with goat anti-rabbit/rabbit anti-mouse IgG (H+L) conjugated to Oregon green (FITC) (Invitrogen) at a 1:1000 dilution and 1 μg/ml DAPI (Invitrogen) for 2 h at room temperature. Stained slides were coverslipped and visualized with an Olympus IX71 fluorescence microscope (Olympus Corp., Tokyo, Japan). As a positive control, the ER expression in MCF-7 cells was also detected with the same immuocytochemistry protocol described above.

Western blot analysis. Cells were treated with DEHP, BPA, E_2, ICI182,780, or LY294002 as described for experimental conditions. After 24 h, cells were collected in an appropriate volume of RIPA lysis buffer containing 50 mM Tris (pH 8.0), 1.0 mM EDTA, 150 nM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 2 mM Na_3VO_4, 5 mM NaF and a protease inhibitor cocktail and incubated on ice for 30 min with occasional agitation. The suspension was then cleared by centrifugation at 20,000 g for 30 min at 4˚C to yield supernatants comprising the total cell extract. Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific Inc.). Protein extracts (50-100 μg) were resolved by 6-8% SDS-polyacrylamide gels (SDS-PAGE) according to target protein sizes. After SDS-PAGE, the protein was then transferred onto polyvinylidene difluoride membranes (Millipore) at 300 mA for 2.5 h. Membranes were blocked for 1 h in TBST [20 mM Tris-HCl (pH 7.6), 150 Mm NaCl, 0.05% Tween] containing 5% non-fat dried milk and then incubated overnight at 4˚C with the appropriate primary antibody: anti-MMP-2, anti-MMP-9, anti-TIMP-2 (all 1:1000 dilutions; Santa Cruz Biotechnology Inc.); anti-Akt, anti-phospho-Akt (Ser473) (all 1:1000 dilutions; Cell Signaling Technology Inc.). After incubation for 3 h with goat anti-rabbit immunoglobulin G secondary antibodies (1:2000 dilution; Cell Signaling Technology Inc.) at room temperature, bands were visualized using an ECL chemiluminescence system (Millipore). Corresponding membranes were reprobed with anti-GAPDH antibody (1:2000 dilution; Abcam Inc., Cambridge, MA) to ensure equal loading of protein. The experiments were repeated three times.

Statistical analysis. Statistical analyses were performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) in conjunction with least significant

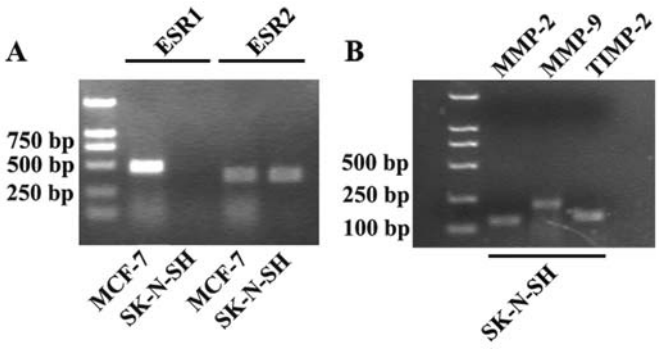

Figure 1. Estrogen receptor, MMP-2, MMP-9 and TIMP-2 gene mRNA expression in SK-N-SH cells detected by RT-PCR. (A) Expression of ESR1 and ESR2 which encode estrogen receptor (ER) α and ß, respectively. Human breast cancer MCF-7 cells were used as the positive control. (B) MMP-2, MMP-9 and TIMP-2 mRNA expression in SK-N-SH cells.

difference test was performed to analyze experiments. The level of significance was set at $P<0.05$.

Results

Estrogen receptors, MMP-2, MMP-9 and TIMP-2 expression in SK-N-SH cells. We examined the mRNA expression levels of MMP-2, MMP-9, TIMP-2 genes as well as estrogen receptor (ER) genes in SK-N-SH cells by regular RT-PCR. There was detectable expression of MMP-2, MMP-9, TIMP-2 genes as well as ESR2 gene encoding ERß protein in this cell line (Fig. 1). However, the expression of ESR1 gene encoding ERα protein was negative in these cells (Fig. 1A). ERß, MMP-2, MMP-9 and TIMP-2 expression were further confirmed at protein level by immuocytochemistry (Figs. 2 and 3).

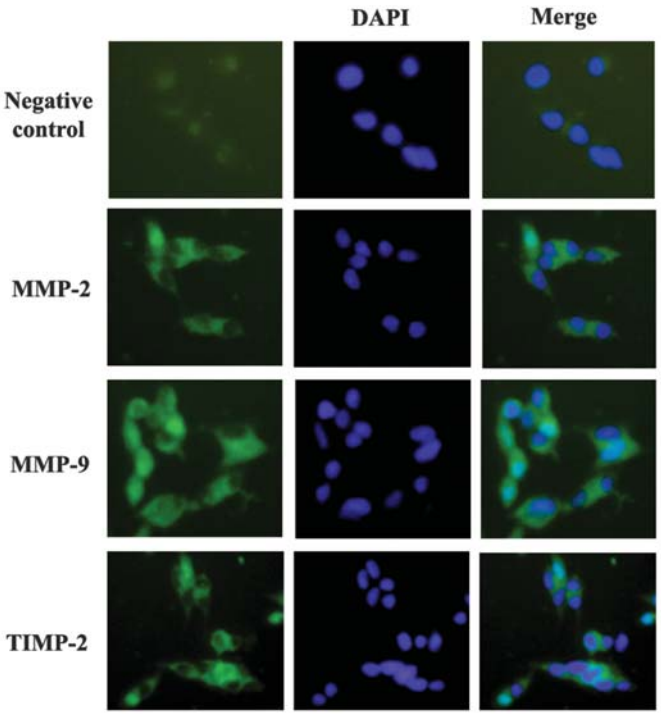

Figure 3. MMP-2, MMP-9 and TIMP-2 protein expression in SK-N-SH human neuroblastoma cells (original magnification, x200). MMP-2, MMP-9, or TIMP-2 protein (green), DAPI (blue).

DEHP, BPA or E_2 exposure enhances SK-N-SH cell migration and invasion. Based on a dose response experiment and our previous studies (24-26), the concentration of chemicals were determined: 50, 0.1, 10, 10 and 20 μM was the concentration of DEHP, BPA, E_2, ICI182,780 and LY294002 respectively used in the experiments.

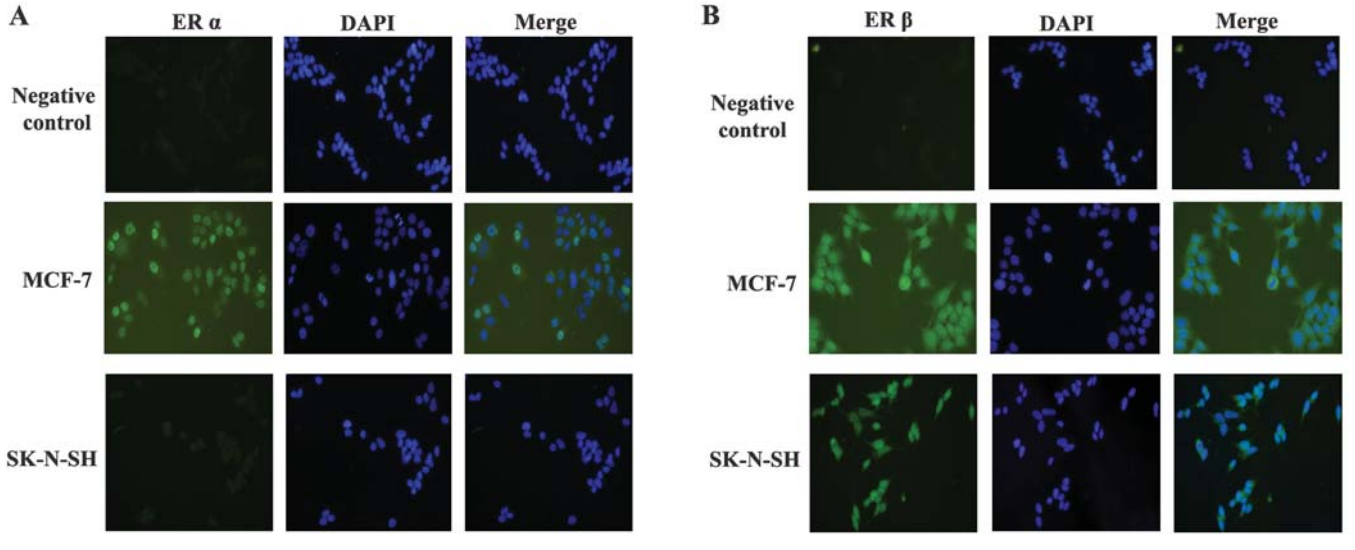

Figure 2. The detection of estrogen receptor (ER) α and ß protein expression in SK-N-SH cells. The positive control in the experiment was human breast cancer MCF-7 cells which were previously identified to express both ERα and ß protein. (A) Detection of ERα protein expression in SK-N-SH cells (original magnification, x200). ERα (green), DAPI (blue). (B) Detection of ERß protein expression in SK-N-SH cells (original magnification, x200). ERß (green), DAPI (blue).

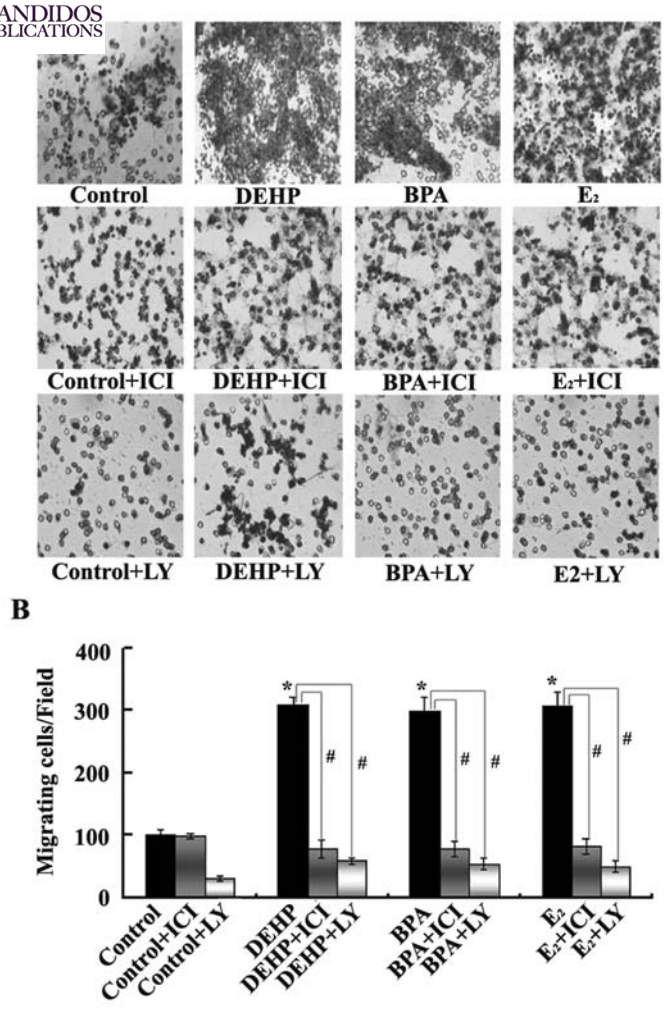

Figure 4. Migration assay in SK-N-SH cells after 16-h treatment. Cells were treated with DEHP (50 μM), BPA (0.1 μM), E_2 (10 μM) or solvent only with or without pretreatment of estrogen receptor antagonist ICI182,780 (ICI, 10 μM) or PI3K specific inhibitor LY294002 (LY, 20 μM). (A) Representative images of SK-N-SH cells on the lower surface of the transwell membranes after 16-h treatment (original magnification, x200). (B) Quantitative comparison of migrating cells of different treatment groups. The number of migrated cells for each group was counted from 5 randomly selected microscopic fields (original magnification, x200). Quantitative data are presented as mean ± SEM. (*P<0.001, vs. the control group; #P<0.001, compared with the DEHP, BPA, or E_2 group).

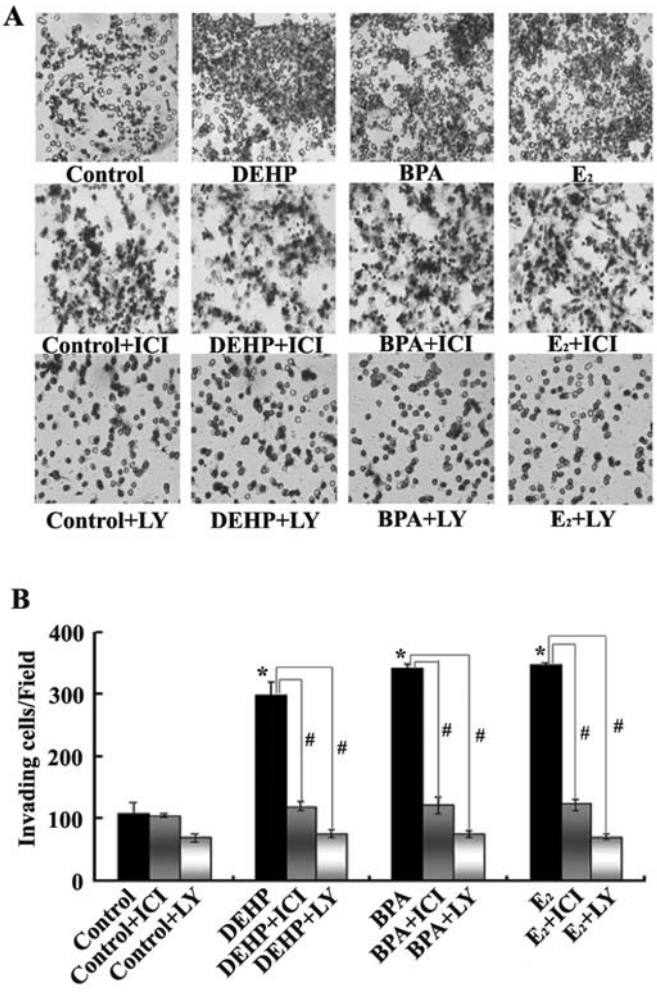

Figure 5. SK-N-SH cell invasion assay after 24-h treatment. Cells were treated with DEHP (50 μM), BPA (0.1 μM), E_2 (10 μM) or solvent only with or without pretreatment of estrogen receptor antagonist ICI182,780 (ICI, 10 μM) or PI3K specific inhibitor LY294002 (LY, 20 μM). (A) Representative images of changes in SK-N-SH cell invasion capability after 24-h treatments (original magnification, x200). (B) Quantitative evaluation of invading cells of different treatment groups. The number of invaded cells for each group was counted from 5 randomly selected microscopic fields (original magnification, x200). Quantitative data are presented as mean ± SEM. (*P<0.001, vs. the control group; #P<0.001, compared with the DEHP, BPA, or E_2 group).

The cell migration and invasion assay were performed to investigate effects of DEHP, BPA, or E_2 exposure on SK-N-SH human neuroblastoma cell migration and invasion *in vitro*. The changes in motility and invasiveness of SK-N-SH cells were observed 16 and 24 h post-exposure, respectively. The hematoxylin and eosin staining of migrating or invading cells through transwell filters suggested that the capacity of motility and invasiveness of cells in the DEHP, BPA or E_2 group were much higher than those of control group (Figs. 4A and 5A). Compared to untreated cells (100.80±8.93), the capacity of cell migration was significantly elevated by exposure to DEHP, BPA or E_2 (308.80±12.28, 298.60±22.50, and 306.80±21.51, respectively; P<0.001; Fig. 4B, black columns). Moreover, the ability of cell invasion also significantly increased in the DEHP, BPA or E_2 group (298.60±18.40, 342.40±6.70, and 347.60±3.90, respectively) when compared with the control group (107.20±18.40, P<0.001; Fig. 5B, black columns).

Exposure to DEHP, BPA or E_2 results in increased expression of MMP-2 and MMP-9 but a decrease in TIMP-2 expression. To further understand the effect of DEHP, BPA or E_2 on SK-N-SH cell migration and invasion, quantitative real-time PCR and Western blotting were performed to detect mRNA and protein expression of MMP-2, MMP-9 and TIMP-2, which have been implicated in cell invasion and metastasis in neuroblastoma and other cancers (28,29). Exposure to DEHP, BPA, or E_2 for 24 h resulted in a significant increase in mRNA levels of both MMP-2 and MMP-9. Compared with the untreated group, the DEHP, BPA and E_2 groups had (113.74±6.20), (105.06±15.76) and (94.21±21.03)

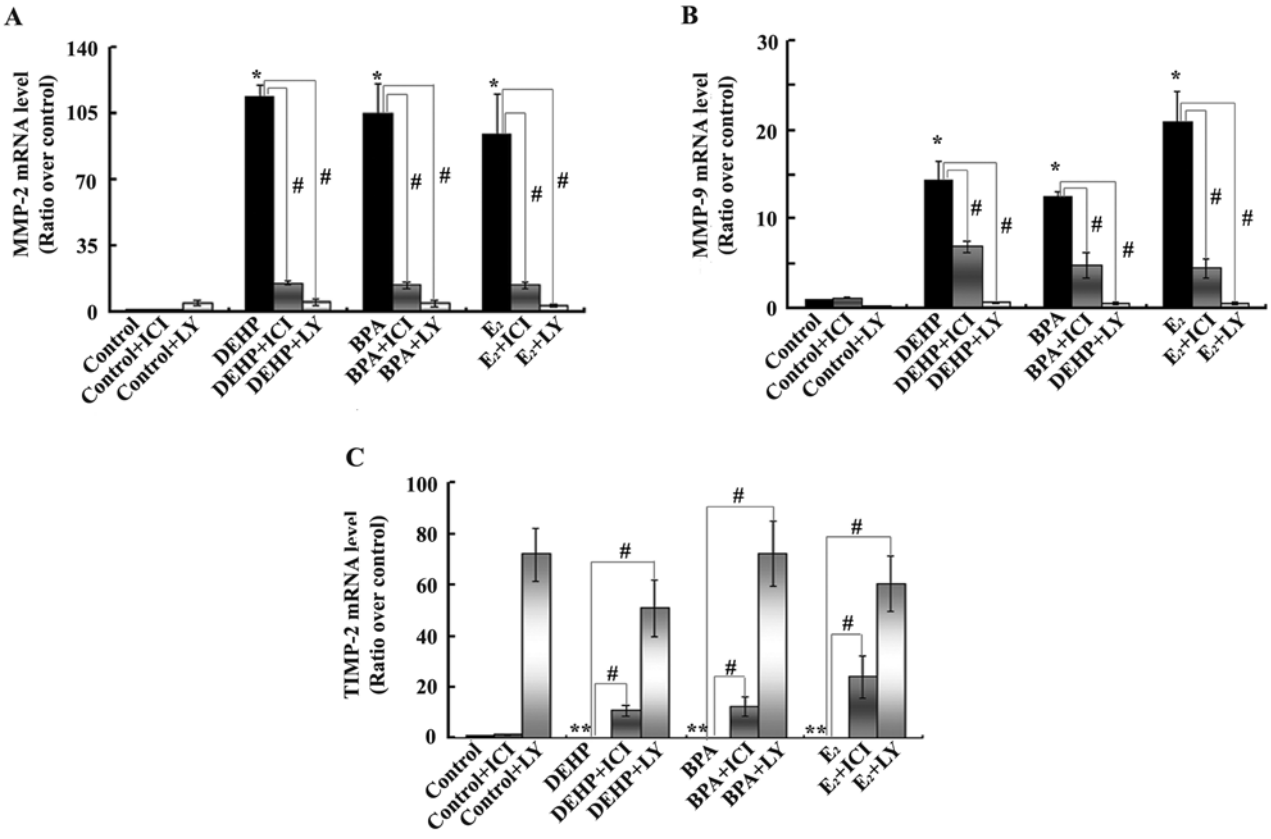

Figure 6. Quantification of MMP-2, MMP-9 and TIMP-2 gene mRNA expression in SK-N-SH cells after 24 drug treatments. Cells were treated with the indicated concentrations of DEHP, BPA or E_2 with or without pretreatment of ICI182,780 (IC, 10 μM) or LY294002 (LY, 20 μM). Cells treated with solvent only were used as control. After 24 h, the mRNA levels of MMP-2, MMP-9 and TIMP-2 mRNA in all groups were determined by quantitative real-time PCR. The mean mRNA expression in each group (normalized to GAPDH mRNA expression) was calculated. Then ratios of target gene expression between the drug treatment groups and the control group were also calculated. Quantitative data are presented as mean ± SEM from four independent experiments. (A) MMP-2 mRNA expression in SK-N-SH cells with different treatments. (B) MMP-9 mRNA expression in SK-N-SH cells with different treatments. (C) TIMP-2 mRNA expression in SK-N-SH cells with different treatments. (*P<0.001 compared to the control group; **P<0.01 compared with the control group; $^\#$P<0.001 compared with the DEHP, BPA, or E_2 group, respectively).

times higher MMP-2 mRNA expression respectively (P<0.001; Fig. 6A, black columns). mRNA levels of MMP-9 in the DEHP, BPA and E_2 groups were also respectively (14.34±2.10), (12.44±0.73) and (20.91±3.39) times higher than that in the control group (P<0.001; Fig. 6B, black columns). On the contrary, a decrease in TIMP-2 mRNA expression was induced by DEHP, BPA, or E_2 treatment. The DEHP, BPA and E_2 groups respectively had 74, 65 and 88% lower TIMP-2 mRNA expression compared with the control group (P<0.01; Fig. 6C, black columns). In addition, Western blot analysis showed MMP-2 protein expression as well as MMP-9 protein obviously increased in the DEHP, BPA and E_2 groups (1.348±0.035 and 1.104±0.002, 1.301±0.050 and 1.223±0.010, 1.485±0.040 and 1.042±0.034, respectively) compared with the untreated group (0.545±0.014 and 0.345±0.014; P<0.01, Fig. 7B and C, first 4 lanes and columns). But TIMP-2 protein expression significantly decreased in cells treated with DEHP, BPA or E_2 (0.133±0.005, 0.110±0.013, and 0.174±0.005, respectively) compared to untreated cells (0.33±0.027; P<0.05; Fig. 7C, first 4 lanes and columns).

DEHP, BPA and E_2 induce phosphorylation of Akt (Ser473) in neuroblastoma cells. Western blot analyses of both Akt and phosphorylation of Akt (Ser473) protein were examined to further observe whether activation of PI3K/Akt signaling pathway was involved in promoting effects of DEHP, BPA or E_2 on SK-N-SH cell migration and invasion. The basal level of phosphorylation of Akt (Ser473) apparently increased by treatment with DEHP, BPA or E_2 (Fig. 8A and C). Densitometry analysis showed that phosphorylation levels of Akt (Ser473) in the DEHP and BPA groups were significantly higher than that in the control group (2.176±0.065 vs. 0.717±0.006, and 2.337±0.037 vs. 0.605±0.006, respectively, P<0.01; Fig. 8B and D). Phosphorylations of Akt (Ser473) was increased by 145 and 173% in DEHP and BPA-treated cells, respectively. A similar trend also occurred in the E_2 group (P<0.01, Fig. 8B and D). However, there were no significant change in Akt protein expression in any of the experimental groups (P>0.05, Fig. 8).

ICI182,780 and LY294002 treatments both abolished the effect of DEHP, BPA and E_2 on the migration and invasion of SK-N-SH cells. Estrogen receptor (ER) dependent pathway is known to be involved in the estrogenic activities of environmental endocrine disruptors and estrogen (30-32). Therefore, ER antagonist ICI182,780 was applied to determine whether ER-dependent pathway was also involved in

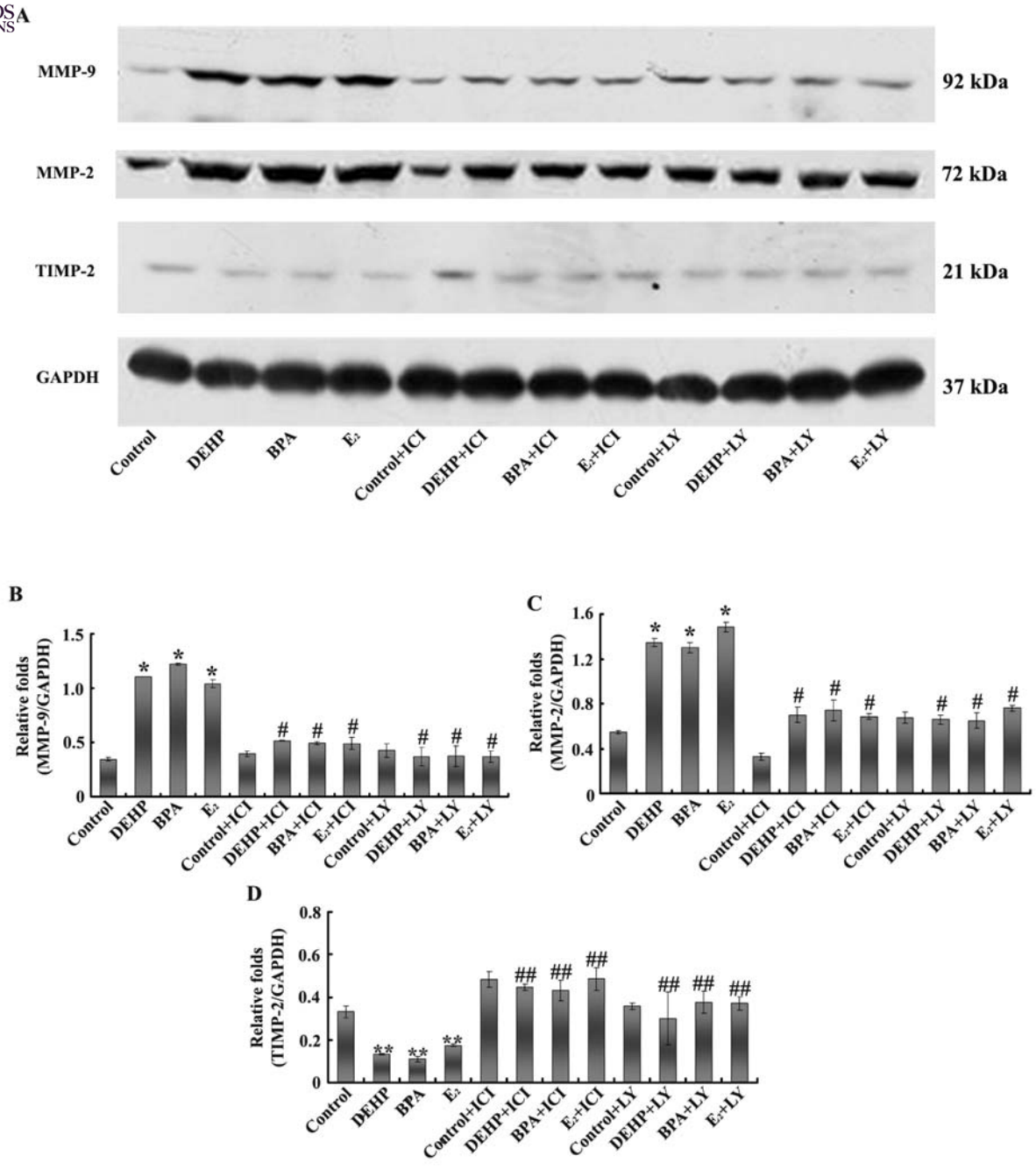

Figure 7. MMP-2, MMP-9, and TIMP-2 protein expression in human neuroblastoma SK-N-SH cells treated with DEHP, BPA, or E_2 with or without ICI182,780 (ICI, 10 μM) or LY294002 (LY, 20 μM) pretreatments. After 24 h drug treatments, Western blot analysis was performed to detect the change in expression of MMP-2, MMP-9, and TIMP-2 protein. Equal loading was confirmed by Western blotting probed with an anti-GAPDH antibody. The mean protein expression (normalized to GAPDH protein expression) in each group was calculated from three independent experiments. (A) Representative images of MMP-2, MMP-9 and TIMP-2 protein expression in different treatment groups. (B) Quantitative evaluation histograms of MMP-9 protein expression. (C) Quantitative evaluation histogram of MMP-2 protein expression. (D) Quantitative evaluation histogram of TIMP-2 protein expression. Data are presented as mean ± SEM. (*P<0.01, vs. the control group; $^{\#}$P<0.01, vs. the DEHP, BPA, or E_2 group, respectively; **P<0.05, vs. the control group; $^{\#\#}$P<0.05, vs. the DEHP, BPA, or E_2 group, respectively).

the process of DEHP, BPA or E_2 regulating cellular motility or invasiveness. PI3K specific inhibitor LY294002 was also used to investigate whether downregulation of PI3K pathway activity affects DEHP, BPA or E2-induced SK-N-SH cell migration or invasion.

In vitro cell migration assay showed that the capacity of cell migration in the groups pretreated with either ICI182,780 or LY294002 did not significantly increase when exposed to DEHP, BPA, or E_2 (77.60±14.94 and 58.80±4.93, 79.00±11.85 and 54.20±8.85, 81.60±12.00 and 50.00±8.04, respectively) compared to those in the group treated with DEHP, BPA, or E_2 only (308.80±12.28, 298.60±22.50, and 306.80±21.51, respectively; P<0.001; Fig. 4A and B). Moreover, cells pretreated with ICI182,780 or LY294002 still

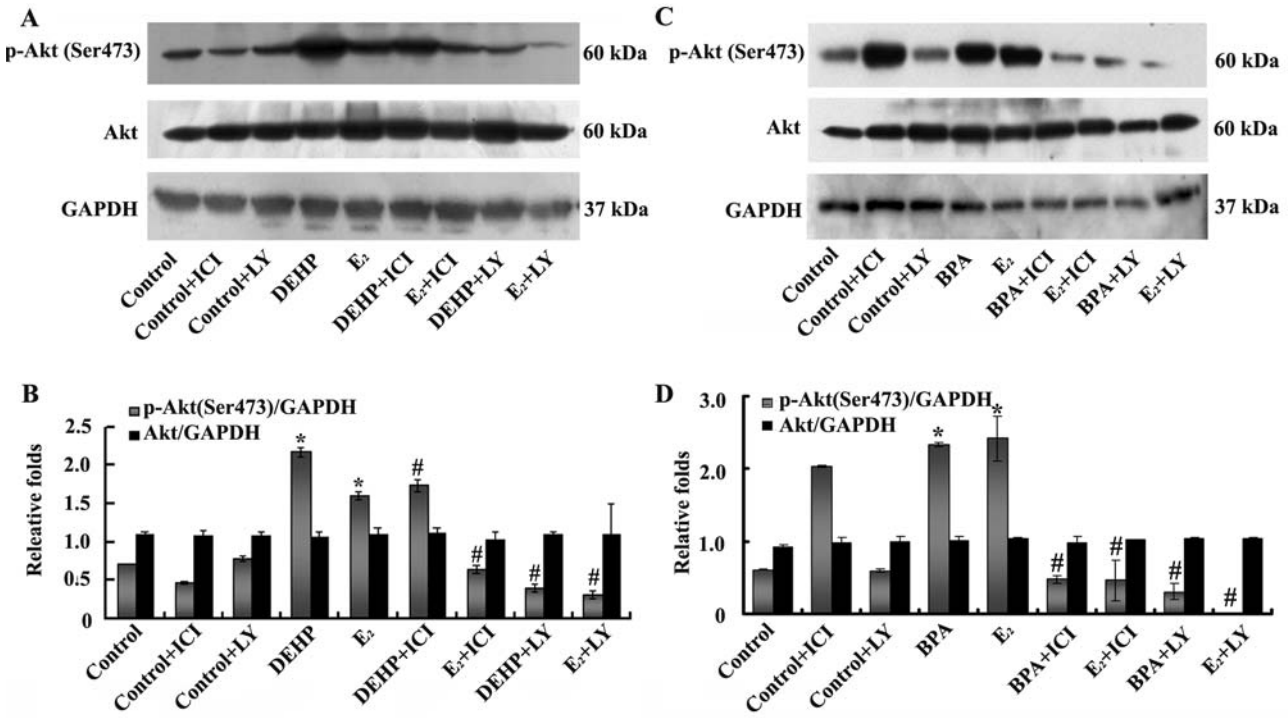

Figure 8. phospho-Akt-Ser473 (p-AktSer473) and Akt protein expression in human neuroblastoma SK-N-SH cells after 24-h treatments. Cells were treated with DEHP, BPA, or E_2 with or without ICI182,780 (ICI, 10 μM) or LY294002 (LY, 20 μM). p-Akt (Ser473) and Akt protein expression were detected by Western blot analysis. Equal loading was confirmed by Western blotting probed with an anti-GAPDH antibody. The mean protein expression (normalized to GAPDH protein expression) in each group was calculated from three dependent experiments. (A) Representative images of p-Akt (Ser473) and Akt protein expression in groups treated with DEHP, E_2 or solvent only with or without ICI or LY. (B) The quantitative evaluation histogram of p-Akt (Ser473) and Akt protein expression in groups treated with DEHP, E_2 or solvent only with or without ICI or LY. (C) Representative images of p-Akt (Ser473) and Akt protein expression in groups treated with BPA, E_2 or solvent only with or without ICI or LY. (D) The quantitative evaluation histogram of p-Akt (Ser473) and Akt protein expression in groups treated with DEHP, E_2 or solvent only with or without ICI or LY. All quatitative data are presented as mean ± SEM. (*P<0.01, vs. the control group; $^\#$P<0.01, vs. the DEHP, BPA, or E_2 group, respectively).

had lower capacity of invasiveness after exposure to DEHP, BPA, or E_2 (119.40±7.50 and 75.60±6.60, 121.80±13.50 and 74.40±6.00, 122.80±8.70 and 70.40±4.40, respectively) than cells treated with DEHP, BPA, or E_2 only (298.60±18.40, 342.40±6.70, and 347.60±3.90, respectively; P<0.001; Fig. 5A and B).

Furthermore, DEHP, BPA or E_2 exposure was not able to enhance MMP-2 and MMP-9 mRNA as well as protein expression in groups pretreated with ICI182,780 or LY294002. Compared with the DEHP, BPA or E_2-only treatment group, the groups treated with both DEHP, BPA, or E_2 and ICI182,780 or LY294002 had (7.53±1.18 and 22.48±1.82), (7.52±1.69 and 23.56±1.63) and (6.73±1.68 and 29.72±0.81) times lower MMP-2 mRNA expression, respectively (P<0.001; Fig. 6A). Lower MMP-2 protein expression was observed in the groups treated with both DEHP, BPA, or E_2 and ICI182,780 or LY294002 as well (0.702±0.068 and 0.661±0.040, 0.744±0.095 and 0.652±0.071, 0.688±0.024 and 0.762±0.027, respectively) compared with the groups treated with DEHP, BPA or E_2 only (1.348±0.035, 1.301±0.050, and 1.485±0.040, respectively; P<0.01; Fig. 7A and C). MMP-9 mRNA expression in the DEHP, BPA and E_2 groups pretreated with ICI182,780 or LY294002 were also (2.08±0.66 and 23.13±0.11), (2.58±1.44 and 23.04±0.10) and (4.66±1.05 and 38.72±0.13) times lower than those in the groups treated with DEHP, BPA or E_2 only, respectively (P<0.001; Fig. 6B). Lower levels of MMP-9 protein were still observed in the groups with both DEHP, BPA or E_2 and ICI182,780 or LY294002 treatments (0.515±0.005 and 0.317±0.086, 0.494±0.014 and 0.374±0.095, 0.488±0.057 and 0.371±0.052, respectively) compared with the DEHP, BPA or E_2-only treatment group (1.104±0.002, 1.223±0.010, and 1.042±0.034, respectively; P<0.01, Fig. 7A and B). On the contrary, TIMP-2 mRNA levels in the groups treated with both DEHP, BPA, or E_2 and ICI182,780 or LY294002 were (41.85±2.34 and 195.12±10.99), (35.66±3.63 and 206.34±12.83) and (200.67±8.14 and 504.83±10.91) times higher than the DEHP, BPA, or E_2 group without the IC182,780 or LY294002 treatment, respectively (P<0.001; Fig. 6C). TIMP-2 protein levels were also significantly higher in both DEHP, BPA or E_2 and ICI182,780 or LY294002-treated groups than those in the DEHP, BPA or E_2-only treatment group (0.446±0.015 and 0.300±0.124 vs. 0.133±0.005, 0.432±0.049 and 0.377±0.052 vs. 0.110±0.013, 0.485±0.055 and 0.371±0.032 vs. 0.174±0.005, respectively; P<0.05; Fig. 7A and D).

Moreover, the elevated phosphorylation level of Akt (Ser473) could not be induced by DEHP, BPA or E_2 exposure in the groups pretreated with either ICI182,780 or LY294002 (Fig. 8A and C). Densitometry analysis showed that phos-

on of Akt (Ser473) in both DEHP or BPA and 80 or LY294002 treated groups were obviously lower than those in the only DEHP or BPA-treated group (1.745±0.079 and 0.402±0.061 vs. 2.176±0.065, 0.478±0.056 and 0.306±0.109 vs. 2.337±0.037, respectively; $P<0.01$; Fig. 8B and D). The similar trend was also observed in both E_2 and ICI182,780 or LY294002 treated groups ($P<0.01$, Fig. 8B and D). However, there was no significant change in Akt protein expression in the experimental groups ($P>0.05$, Fig. 8).

Discussion

Neuroblastoma is one of the most common pediatric cancers. It arises from neural crest tissue and can occur anywhere along the sympathetic chain. Mechanisms of cell metastasis which is the most important cause of death in neuroblastoma are still poorly understood. Some studies suggested that genetic variations may contribute to the cell meatstasis in NB (6). Recently, it is reported that the incidence of neuroblastoma is increasing while environmental pollutions are becoming worse, and our previous studies also revealed the relationship between EED and neuroblastoma cell proliferation (24-26,33). Therefore, we further hypothesize that EED are risk factors for NB cell metastasis and they may also be involved with genetic risk factors that are associated with NB metastasis.

The SK-N-SH cell line was established from a 4-year-old female patient with a bone marrow metastasis and SK-N-SH cells show multiple biological properties of neural crest cells *in vitro* culture (34,35). This cell line is a better *in vitro* model for studies of the biology of neuroblastoma than other neuroblastoma cell lines (34) and we used this cell line as an *in vitro* model in our previous studies (24-26). In this study, we first detected neurobastoma metastasis related gene (MMP-2, MMP-9, and TIMP-2) expression in SK-N-SH cells. Both RT-PCR and immunochemistry analysis suggested that MMP-2, MMP-9 and TIMP-2 expression at mRNA and protein levels are detectable. Therefore, this cell line is also useful for neuroblastoma metastasis related studies *in vitro*. Furthermore, we confirmed SK-N-SH cells were estrogen receptor (ER)-ß positive but ERα negative cells, which is consistent with results of previous studies (36).

In the current study, we examined effects of DEHP and BPA, two of the most important EED, as well as estrogen (E_2) on SK-N-SH neuroblastoma cell migration and invasion. In the cell migration and invasion assay, DEHP, BPA or E_2 exposure resulted in higher capacity of the motility or invasiveness in SK-N-SH cells, which implicated that neuro-blastoma cells would be more susceptible to invasion and metastasis. MMPs are a group of zinc-dependent endopeptidases that are responsible for degrading extracelluar matrix components and play key roles in cancer cell proliferation, invasion, metastasis and angiogenesis (37). MMPs are also involved in neuroblastoma metastasis. Cheng *et al* found higher levels of MMP-2 and MMP-9 expression, but a lower level of TIMP-2 expression in metastatic neuroblastoma than those in neuroblastoma without metastasis (29). Moreover, an increased production of MMP-2 and MMP-9 induced angiogenesis in neuroblastoma and enhanced tumor dissemination (38). Therefore, we further investigated whether exposure to DEHP, BPA or E_2 affect the MMP-2, MMP-9 or TIMP-2 expression. As revealed by real-time PCR and Western blot analysis, all these drugs could upregulate the expression of MMP-2 and MMP-9, and downregulate the expression of TIMP-2. The results demonstrated that DEHP, BPA and E_2 promote neuroblastoma cell invasion and metastasis *in vitro*.

It has been reported that EED as well as E_2 mainly exert their estrogenic effects through classic ER-dependent pathway (30-32). ERα and ERß, encoded by ESR1 and ESR2 gene, respectively, are the main ER subtypes (39). ICI182,780 is a specific ER antagonist and has an ability to block ER-dependent biologic effects (40). In the current study, ICI182,780 treatment effectively abolished DEHP, BPA, and E_2-induced cell migration and invasion, and significantly blocked DEHP, BPA, or E_2-induced upregulation of MMP-2 and MMP-2 expression as well as downregulation of TIMP-2 expression. It suggested that EED and E_2 promote neuroblastoma cell invasion and metastasis through ER-dependent pathway and ICI182,780 may be a new potential drug for improved neuroblastoma therapies. In addition, we demonstrated that EED and E_2 could still exert their biologic effects through ER-dependent pathway in ERß positive, ERα negative cells although substantial evidence indicates that ERα played a more important role than ERß in cancer promotion and progression (31,41-43). Therefore, ERß is also strongly associated with EED or E_2 mediated carcinogenesis.

Phosphatidylinositide 3-kinases (PI3Ks) are a group of intracellular lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphonositides (44). PI3K and its signaling pathway regulate various processes including cell metabolism, cell cycle and survival, cell apoptosis, and protein synthesis in the body (44). The protein serine/threonine kinase (Akt) is the cellular homolog of v-Akt oncogene and a member of the AGC family of kinases (45). It acts as a principal downstream component of PI3K pathway and the level of its phosphorylation on Ser473 represents the activity of PI3K/Akt signaling pathway. The activity of PI3K pathway can be modified by growth factors, vitamins as well as hormones (45-47). PI3K/Akt pathway deregulation is strongly related to metabolism disorders, mental disease including schizophrenia, and tumorigenesis as well (46-49). Furthermore, PI3K/Akt pathway involved in EED and estrogen biological effects has also been reported (32,50). In our study, DEHP, BPA and E_2 also induced Akt (Ser473) phosphorylation and activate the PI3K pathway. Additive LY294002 treatment could block DEHP, BPA and E_2 induced SK-N-SH cell migration and invasion as well as MMP-2, MMP-9 and TIMP-2 deregulation. The above evidence indicates that PI3K/Akt pathway is involved in promoting effects of EED and estrogen on neuroblastoma cell invasion and metastasis. Moreover, like LY294002, additive ICI182,780 also resulted in marked reduction in the activity of PI3K/Akt pathway, which demonstrates that inhibition of ICI182,780 on EED and estrogen-mediated cell invasion and metastasis also involves the PI3K/Akt pathway.

In summary, DEHP, BPA as well as E_2 potently promote invasion and metastasis of neuroblastoma cell *in vitro*. These effects are mediated by ERß and involve the PI3K/Akt pathway. EED including DEHP and BPA or estrogen are novel risk factors for neuroblastoma metastasis. Estrogen receptor and downstream kinases in PI3K pathway will be potential targets for new target therapies for neuroblastoma. Further investigation of the EED impact on neuroblastoma and other childhood cancers is required to improve preventive methods of pediatric tumorigenesis.

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