

Roles of $ERR\alpha$ and TGF- β signaling in stemness enhancement induced by 1 μ M bisphenol A exposure via human neural stem cells

PANPAN DONG^{1*}, GANGHUI YE^{1*}, XINZHUO TU^{1*}, YING LUO², WEITONG CUI¹,
YUXIN MA³, LEI WEI¹, XUEWEN TIAN³ and QINGLU WANG^{1,3}

¹Key Laboratory of Biomedical Engineering and Technology of Shandong High School, Qilu Medical University, Zibo, Shandong 255213; ²Department of Clinical Laboratory, Zibo Central Hospital, Zibo, Shandong 255300; ³College of Sports and Health, Shandong Sport University, Jinan, Shandong 330013, P.R. China

Received June 10, 2020; Accepted October 6, 2021

DOI: 10.3892/etm.2021.11087

Abstract. Bisphenol A (BPA) is a common industrial chemical widely used to produce various plastics and is known to impair neural stem cells (NSCs). However, the effects of low-dose BPA exposure on the stemness maintenance and differentiation fate of NSCs remain unclear in the infant brain. The present study demonstrated that 1 μ M BPA promoted human NSC proliferation and stemness, without significantly increasing apoptosis. The Chip-seq experiments demonstrated that both the cell cycle and the TGF- β signaling pathway were accelerated after treatment with 1 μ M BPA. Subsequently, estrogen-related receptor α ($ERR\alpha$) gene knockout cell lines were constructed using CRISPR/Cas9. Further western blotting and chromatin immunoprecipitation-PCR experiments demonstrated that BPA maintained cell stemness by binding to an $ERR\alpha$ receptor and activating the TGF- β 1 signaling pathway, including the downstream factors Aurora kinases B and Id2. In conclusion, the stemness of NSCs could be maintained by BPA at 1 μ M through the activation of the $ERR\alpha$ and TGF- β 1 signaling pathways and could restrain the differentiation of NSCs into neurons. The present research further clarified the mechanism of BPA toxicity on NSCs from the novel perspective of $ERR\alpha$ and TGF- β 1 signaling pathways regulated by BPA and provided insights into potential novel methods of prevention and therapy for neurogenic diseases.

Introduction

Neurodevelopmental disabilities (NDDs), such as learning disabilities, developmental delay, cerebral palsy and cognitive dysfunction, are one of the major diseases that affect the quality of an individual's life from birth and harm the physical and mental health of children and adolescents; they also cause a heavy and far-reaching burden on families and society (1). NDDs are not only related to genetic factors (2,3) but also to medications taken during pre-pregnancy and rubella infection in early pregnancy infections (4,5). Environmental toxicants and industrial chemicals, such as lead (6), cadmium (7), arsenic (8), ethanol (9), methyl mercury (10), pesticides, organic solvents (11) and endocrine disrupting chemicals (EDCs) (12,13), which are abundant in the environment, affect the developing brain, alter neurogenesis and cause learning and memory deficits.

Neural stem cells (NSCs) exist in the nervous system which can self-renew and differentiate into neurons, astrocytes and oligodendrocytes (14). A moderate number of NSCs are needed for the normal development of the nervous system (15). The abnormal proliferation or development of NSCs can cause severe neurological developmental defects (15,16). Various factors, such as bisphenol A (BPA) interfere with the induction or occurrence of nerves by affecting the normal proliferation or differentiation of NSCs, thereby producing the neurodevelopmental toxicity expressed in severe NDDs (17).

NSCs change their response to external signals and their developmental differentiation potential with different developmental stages during neurogenesis (14). Some NSCs begin to express neuron-specific genes such as glial fibrillary acidic protein (GFAP) and microtubule-associated protein 2 (MAP2) to obtain neuronal fate under the action of some regional differentiation signals. However, Nestin and SRY-box 2 (Sox2) are expressed in the population of undifferentiated NSCs that still maintain pluripotency (18).

BPA is a synthetic phenolic compound widely used to produce polycarbonate and epoxy resins for food containers, such as cans and water dispensers (19). BPA can leach from some of these polymers into water or food products (19).

Correspondence to: Professor Qinglu Wang or Professor Xuewen Tian, College of Sports and Health, Shandong Sport University, 10600 Century Avenue, Jinan, Shandong 330013, P.R. China
E-mail: wql_zcq@126.com
E-mail: xuewentian1978@163.com

*Contributed equally

Key words: human neural stem cell, bisphenol A, stemness enhancement, TGF- β 1 signaling, estrogen-related receptor signaling

Humans are exposed to this compound through their diet and skin contact (20); BPA has been detected in >90% of the human bodies surveyed in population-representative samples (21,22). BPA exposure causes a wide range of neurodevelopmental disorders, including cognitive impairment, autism (23), neurodegeneration (24-26) and schizophrenia (27).

According to the development process of the vertebrate nervous system, NSCs are the starting point for differentiation into other nerve cells and are activated in neurodegenerative diseases; they also play an important role in the repair/replacement of nerve cells in lesion areas (28,29). Both *in vivo* and *in vitro* experiments confirm that BPA affects the proliferation and differentiation of NSCs (30), which triggers the development of neurodegenerative diseases (31).

Previous studies have looked into whether the high or low doses of BPA can influence NSCs, including the possible role it may exert in NSCs or whether it can regulate NSC proliferation and differentiation via the Wnt/ β -Catenin, TGF- β or the estrogen receptor signaling pathways (12,30,32). Several previous studies on ovarian cancer cells confirm that BPA can affect the TGF- β and the Wnt/ β -Catenin signaling pathway (33,34). Downstream Smads of TGF- β can activate c-myc through zinc finger E-box-binding homeobox 1 (ZEB)1, ZEB2, Snail zinc finger protein (SNAIL)1, Snail2, Twist-related protein 1 and PIM1, while c-myc can regulate the expression of aurora kinases B (AURKB) to promote cell proliferation (35). The TGF- β signaling pathway can also activate downstream factors to prevent cell differentiation by forming myc-max heterodimers to inhibit *Id2* expression (36). In order to further understand the mechanism of BPA-induced NDDs in infants, the current study revealed that low-dose BPA (1 μ M) enhanced the stemness of human NSCs via the estrogen-related receptor α (ERR α) (37) and the TGF- β signaling pathways.

Materials and methods

Cell culture and treatment. Human NSCs (ReNcell® CX Immortalized cells; cat. no. SCC007; MilliporeSigma) were provided by Dr Dan Lou (Shanghai Municipal Center for Disease Control & Prevention, Shanghai, China). Before cell resuscitation, Laminin (cat. no. L-2020; MilliporeSigma) was thawed, diluted with DMEM (Gibco; Thermo Fisher Scientific, Inc.) and plated on 35-mm petri dishes at 4°C for 2 h. Then, 1×10^4 cells were seeded in the laminin-coated 35-mm culture dishes filled with complete medium, which was prepared by adding basic fibroblast growth factor (bFGF; 20 ng/ml) and EGF (20 ng/ml) to maintenance medium (cat. no. SCM005; MilliporeSigma) for maintain cell stemness and cultured in a 37°C, 5% CO₂ saturated humidity incubator. The fresh cell culture medium was replaced every 1-2 days to form a stemness maintenance model. According to the manufacturer's instructions of ReNcell CX kit (cat. no. SCC009; MilliporeSigma), cell differentiation was identified by double immunofluorescence staining. The antibodies of β -tubulin III (1:500, ab215037; Abcam) and GFAP (1:50, ab279290; Abcam) were used to form a neural differentiation model after incubation overnight at 37°C.

A total of 1×10^4 NSCs with 3 ml culture medium were seeded into six-well plates in triplicate. The cells were randomly divided into four subgroups as follows: Control group (C), the

medium contained EGF and bFGF without BPA; NSC stemness maintenance and BPA stress group (SMB), the medium contained EGF and bFGF, and BPA was added to the medium to a final concentration of 1 μ M; differentiation control group (DC), the medium had no EGF and bFGF, and no BPA was added; and differentiation and BPA stress group (DBS), the medium had no EGF and bFGF, and BPA was added to the medium to a final concentration of 1 μ M.

CRISPR-knockout of ERR α in hNSCs. Based on the CRISPR-DO website (Version 0.1, cistrome.org/crispr/), a single guide (sg) RNA target site (5'-GACAGAGACCGAGCCTCCTG-3') was identified in the coding region of the *ERR α* gene. Subsequently, the knockout all-in-one vector pCMV-Cas9-GFP-ERR α and negative vector pCMV-Cas9-GFP (cat. no. CAS9GFPP) were constructed by Sigma-Aldrich (Merck KGaA). NSCs with *ERR α* -knockout were obtained by transfecting the pCMV-Cas9-GFP-ERR α all-in-one vector using a liposome (Lipofectamine 3000, Thermo Fisher Scientific, Inc.) at room temperature. The monoclonal cells were sorted 48 h post-transfection by flow cytometry with blue (488 nm) lasers (BD FACSAria; BD Biosciences) based on green fluorescent protein in hNSC and expanded to extract the genome to screen cell lines with *ERR α* gene frameshift mutation. The specific primers (forward, 5'-GGTTGAAGGTTGCCTGGGGGCTAC-3'; reverse, 5'-GACTCTTTCAGAGCCACTGTAGAAG-3') were designed according to the sgRNA targeting site for PCR to analyze the mutation of the sgRNA targeting site. The PCR product was sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing analysis.

Effects of BPA on NSC morphology. Wild-type hNSCs were inoculated into a six-well cell culture plate at 10×10^5 cells/ml and cultured to 80% confluence. Cells were treated with BPA (0, 0.1, 1 and 10 μ M) according to previous reports (12,30,32,38). Each concentration of the experimental group was set up in triplicate. After 24 h of exposure at 37°C, the cells were observed under a light microscope for morphology (scale bar, 20 μ m).

Effects of BPA on NSC proliferation. Cell proliferation was measured via the Cell Counting Kit-8 (CCK-8) assay. The NSC stemness maintenance and BPA stress group (SMB) and control group (C) were selected. A total of 10 μ l of CCK-8 (CA1210; Beijing Solarbio Science & Technology Co., Ltd.) was added to each well, and the cells were then incubated for 2 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.). Proliferation rates were evaluated at 0, 24, 48, 72 and 96 h after treatment with BPA.

Effects of BPA on NSC apoptosis by flow cytometry. Cells that reached 80% confluency were treated with BPA at 0, 0.1, 1, 2.5, 5 and 10 μ M for 24 h at 37°C, and then digested with Accutase™ (cat. no. SCR005; MilliporeSigma) for 3 min at 37°C to make cell suspension. The cell suspension was collected in a centrifuge tube and centrifuged at 300 x g for 5 min at room temperature. The supernatant was discarded and phosphate-buffered saline (PBS) was added to pipette the cells evenly. Subsequently, the cell suspension was centrifuged

at 300 x g for 5 min at room temperature, the supernatant was discarded again and the operation was repeated once. NSCs were diluted with deionized water at a ratio of 1:3, the diluent was pre-cooled at 4°C, rinsed twice with PBS and was centrifuged at 300 x g for 5 min at room temperature. Finally, NSCs were resuspended using 250 µl of binding buffer (E-CK-A151; Elabscience) in a liquid solution to adjust the concentration to 1x10⁶ cells/ml. A total of 100 µl cell suspension was collected in a 5-ml flow tube, 5 µl Annexin V-FITC and 10 µl PI staining solution were then used to stain the cells for 15 min at room temperature in the dark. Finally, 200 µl of diluted binding buffer was added to terminate the reaction and immediately detected by flow cytometry (BD FACSAria; BD Biosciences) and analyzed by FlowJo 10.8.0 (BD Biosciences).

Western blotting. The cell suspension was centrifuged at 300 x g for 5 min at room temperature, and the supernatant was discarded, total protein was extracted from cells using lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology) with protease inhibitors PMSF(ST506; Beyotime) and kept on ice for 30 min. Protein concentrations were determined by Bradford assay and then 20 µg each protein sample were boiled for 5 min, separated by 10% SDS-PAGE, transferred onto a PVDF membrane, blocked with 5% skim milk in TBST buffer (20% Tween) for 3 h at room temperature and then incubated with primary antibodies at 4°C overnight. Primary antibodies were Nestin (1:1,000, ab6320; Abcam), Sox2 (1:1,000, ab171380; Abcam), GFAP (1:1,000, ab279290; Abcam), Map2 (1:1,000, ab281588; Abcam), ERRA (1:1,000, bs-6998R; Bioss), TGF-β1 (1:1,000, ab215715; Abcam), α-tubulin (1:10,000, ab7291; Abcam), AURKB (1:10,000, ab45145; Abcam) and Id2 (1:500, ab90055; Abcam). The membranes were then washed three times with TBST and incubated with a secondary antibody (1:30,000, Goat Anti-Mouse IgG H&L/HRP, bs-40296G-HRP; 1:30,000, Goat Anti-Rabbit IgG H&L/HRP antibody, bs-40295G-HRP; Bioss) at room temperature for 1 h. Protein bands were visualized using ECL (MilliporeSigma).

Immunostaining. Cells were fixed with 4% paraformaldehyde for 15-30 min at room temperature. After washing with PBS for three times, the cells were covered with 4% BSA solution containing 0.1% Triton X-100 at room temperature for 1 h and then incubated overnight with 4% BSA-diluted primary antibodies β-tubulin III (1:500, ab215037; Abcam) and GFAP (ab279290, 1:50; Abcam). The samples were removed from the 4°C condition on the second day, reheated at room temperature for 30 min and then incubated with goat anti-rabbit IgG Cy5 (1:500, bs-0295G-Cy5; Bioss) or goat anti-mouse IgG FITC (1:500, bs-0296G-FITC; Bioss) at room temperature for 1 h. The solution was then discarded, and the samples were washed with PBS for three times. Nuclei were stained at room temperature with Hoechst 33342 (C0030; Beijing Solarbio Science & Technology Co., Ltd.). The NSCs differentiation were identified by detecting the expression of β-tubulin III and GFAP under a fluorescence microscope (scale bar, 20 µm; Zeiss AG).

RNA-seq and data analysis. Total RNA was isolated from hNSCs with TRIzol (Takara Bio, Inc.) and assessed using a Qubit™ 3.0 Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc) for total RNA quantity and integrity. RNA libraries were

constructed using a QuantSeq 3' mRNA-Seq library Prep kit FWD for Illumina (no. 016; Lexogen) following the manufacturer's protocol. mRNA was purified using Oligo(dT) magnetic beads and fragmented at 95°C for 8 min with fragmentation buffer. Using mRNA as a template, the first cDNA strand was synthesized with random oligonucleotides and reverse transcriptase under the following conditions: 5 min at 65°C, 2 min at 4°C, 1 h at 42°C and 10 min at 70°C. The second cDNA strand was synthesized by the addition of the buffer, dNTPs, RNase H and DNA polymerase I under the following conditions: 2.5 h at 16°C and 10 min at 70°C. Following purification, end repair and ligation of the sequencing adapter were performed according to the QuantSeq 3' mRNA-Seq Library prep kit manufacturer's protocol.

The PCR products were analyzed on 2% agarose gel and stained with ethidium bromide. The 150 nucleotide-long fragments were isolated and purified by using QuantSeq 3' mRNA-Seq Library prep kit and then analyzed by Beyotime Institute of Biotechnology on a Illumina HiSeq 2000 (Illumina, Inc.). FASTQ files were uploaded to the BaseSpace Suite (Illumina, Inc.) and aligned using the RNA-Seq Alignment application (version 1.0.0), in which STAR was selected to align the sequences and the maximum number of mismatches was set to 14 following the recommendation by Lexogen GmbH. Output files were analyzed using Cufflinks Assembly and DE application (version 2.1.0) in the BaseSpace Suite to determine the differentially expressed genes (DEGs), which were used to generate an expression heatmap. GO and KEGG analysis were performed by Beyotime Institute of Biotechnology using ClueGO (version 2.3.3) and CluePedia (version 1.3.3), which are Cytoscape software applications (version 3.5.1) (39).

Chromatin immunoprecipitation (CHIP)-PCR. A total of 10⁸ cells were used to isolate genomic DNA. The cells were resuspended in DMEM culture medium and fixed with 1.5% formaldehyde for 15 min at 4°C. The cell pellets were harvested and lysed by cell collection buffer [100 mM Tris-HCl (pH 9.4); 10 mM DTT with complete protease inhibitor cocktail; Roche Diagnostics] on ice for 20 min. The deposits were collected and washed twice with PBS. Cell pellets were pretreated with a nucleus/chromatin preparation buffer [buffer I: 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5), 0.25% Triton X-100; buffer II: 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5), 200 mM NaCl] and then lysed with a nuclear lysis buffer [10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1% SDS, complete protease inhibitor cocktail] on ice for 15 min. Sonication on ice was performed to generate a DNA fragment of 400-800 bp with a no. 2 microtip at a power output of 25% (on for 6 sec and off for 30 sec for 10 times). Chromatin quantified to 100 µg of DNA was utilized for further immunoprecipitation and mixed with immunoprecipitation (IP) buffer [2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1), 0.1% Triton X-100, complete protease inhibitor cocktail] in a total volume of 1 ml. The chromatin was diluted at a rate of 1:10 in IP buffer as the input. Protein A/G magnetic beads (cat. no. B23202; Bimake) were washed twice with TE buffer [10 mM Tris-HCl (pH 8.1), 1 mM EDTA]. Then, 1 ml chromatin (containing 100 µg DNA) was mixed for IP with 60 µl of prepared beads (30 µl beads A and 30 µl DNA beads G) in siliconized tubes and rotated at 4°C for 3 h. The supernatants were transferred to fresh

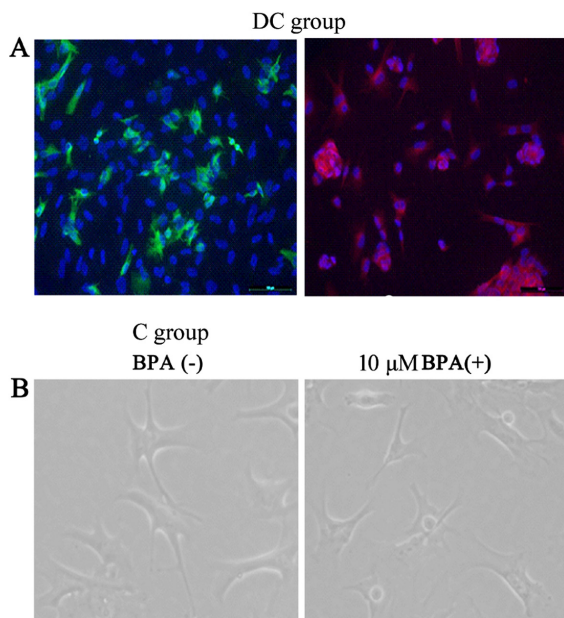


Figure 1. Morphology and differentiation of NSCs. (A) GFAP and β -tubulin III expression profiles were upregulated in NSCs after EGF and bFGF were removed from medium (blue staining indicates DAPI; green indicates GFAP; red indicates β -tubulin III). (B) Treatment with 10 μ M BPA did not affect the morphology of NSCs. NSCs, neural stem cells; GFAP, glial fibrillary acidic protein; bFGF, basic fibroblast growth factor; BPA, bisphenol A.

microcentrifuge tubes and 2 μ g of ERR α antibody (1:200, GTX108166; GeneTex) or IgG (1:500, GTX35035; GeneTex) was added to each sample and rotated overnight at 4°C. The following day, 40 μ l of protein A/G magnetic beads were added into each antibody-chromatin sample, and rotation was performed for 2 h at 4°C. The beads were harvested with a magnetic holder and washed in the shaker sequentially for 5 min at 4°C in 1 ml of wash buffer I [2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, 150 mM NaCl] x 2, wash buffer II [2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, 500 mM NaCl], and TE buffer 2X. The beads were resuspended in 250 μ l of extraction buffer (1% SDS and 0.1 M NaHCO₃) and shook (40 rpm) at room temperature for 30 min. The supernatants were collected, and this step was repeated. The confluent supernatants were chromatin eluted following the addition of 10 μ g of RNase A (cat. no. R5125; Sigma-Aldrich; Merck KGaA) for RNA digestion at 37°C for 2 h. All samples, including IP and input samples, were mixed with 120 μ g of proteinase K to reverse formaldehyde crosslinks at 65°C overnight. DNA was purified with a PCR purification kit (D0033; Beyotime) and the chromatin was dissolved in 120 μ l of TE buffer. Overall, 2 μ l of DNA solution per well was loaded for the PCR assay using TransTaq DNA Polymerase High Fidelity (AP131-11; Transgen Biotech). The primers were as follows: *TGF- β 1* promoter (target region), forward 5'-AAATTGGGGACAGTAAATGTATGGG-3', and reverse, 5'-TAGGAGAAGAGGGTCTGTCAACAT-3'; *TGF- β 1 ORF* forward (control region), 5'-CAACAATTCCTGGCG ATACC-3', and reverse 5'-GAACCCGTTGATGTCCACTT-3'. The thermocycling conditions were as follows: 95°C for 10 sec, followed by 30 cycles of 94°C for 10 sec, 50°C for 20 sec and 72°C for 30 sec, and 72°C for 1 min. PCR products were detected by electrophoresis on a 12% agarose gel and ethidium bromide staining.

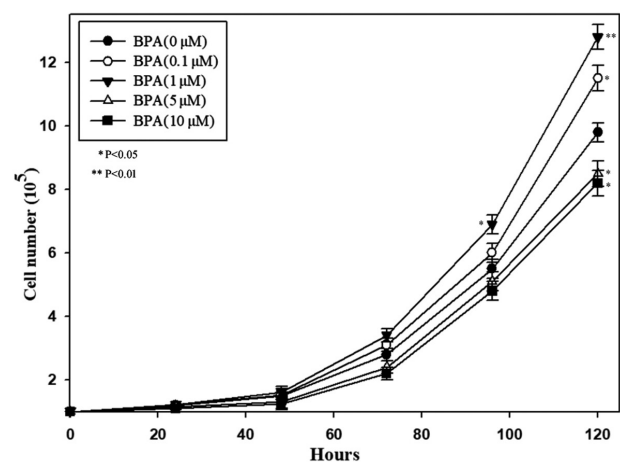


Figure 2. Effects of BPA on NSC proliferation. After BPA was added for 24 h, the cell number of all samples was counted using Cell Counting Kit-8 assays. * P <0.05, ** P <0.01. BPA, bisphenol A; NSCs, neural stem cells.

Statistical analyses. All experiments were performed a minimum of three times independently. Data are expressed as the mean of three repeats \pm standard deviation. Statistical comparisons of results from multiple groups (data from NSC proliferation, GO analysis, KEGG pathway analysis) were analyzed using one-way analysis of variance. P <0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed with the GraphPad Prism 6.0 program (GraphPad Software, Inc.).

Results

Low BPA concentration has no marked effect on cell morphology. Under the ReNcell CX kit instructions, EGF and bFGF were removed from the medium, which played roles in the maintenance of NSCs. A differentiation model of human NSCs was constructed, and the expression profiles of GFAP and β -tubulin III were revealed in cells, thereby indicating the formation of neurons (Fig. 1). The expression of GFAP and β -tubulin III indicated that the NSCs have the ability promote differentiate, and on this basis, the effects of different doses of BPA on its morphology were investigated. The cells were added with low doses (0.1, 1 and 10.0 μ M) of BPA. As a consequence of these experiments, these gradient doses of BPA exhibited no remarkable effect on cell morphology.

Low concentrations of BPA affects cell proliferation and apoptosis. According to the effects of BPA on NSC morphology, the effects of BPA on cell proliferation after cultivation at 0.1, 1, 5, and 10 μ M BPA concentrations were analyzed using a CCK-8 assay. It revealed that 0.1 and 1 μ M BPA markedly promoted cell proliferation (Fig. 2) and that 1 μ M BPA demonstrated a stronger promotion effect. However, it was also observed that a concentration >5 μ M could markedly inhibit cell proliferation.

Flow cytometry was used to analyze the effects of 0.1, 1, 2.5, 5 and 10 μ M BPA on NSC apoptosis (Fig. 3). Results revealed that the amount of apoptosis induced in NSCs by <1 μ M BPA (Fig. 3B and C) were similar compared with that of the C group (Fig. 3A); whereas 2.5, 5 and 10 μ M BPA caused a marked increase in NSC apoptosis compared with the C (Fig. 3D-F).

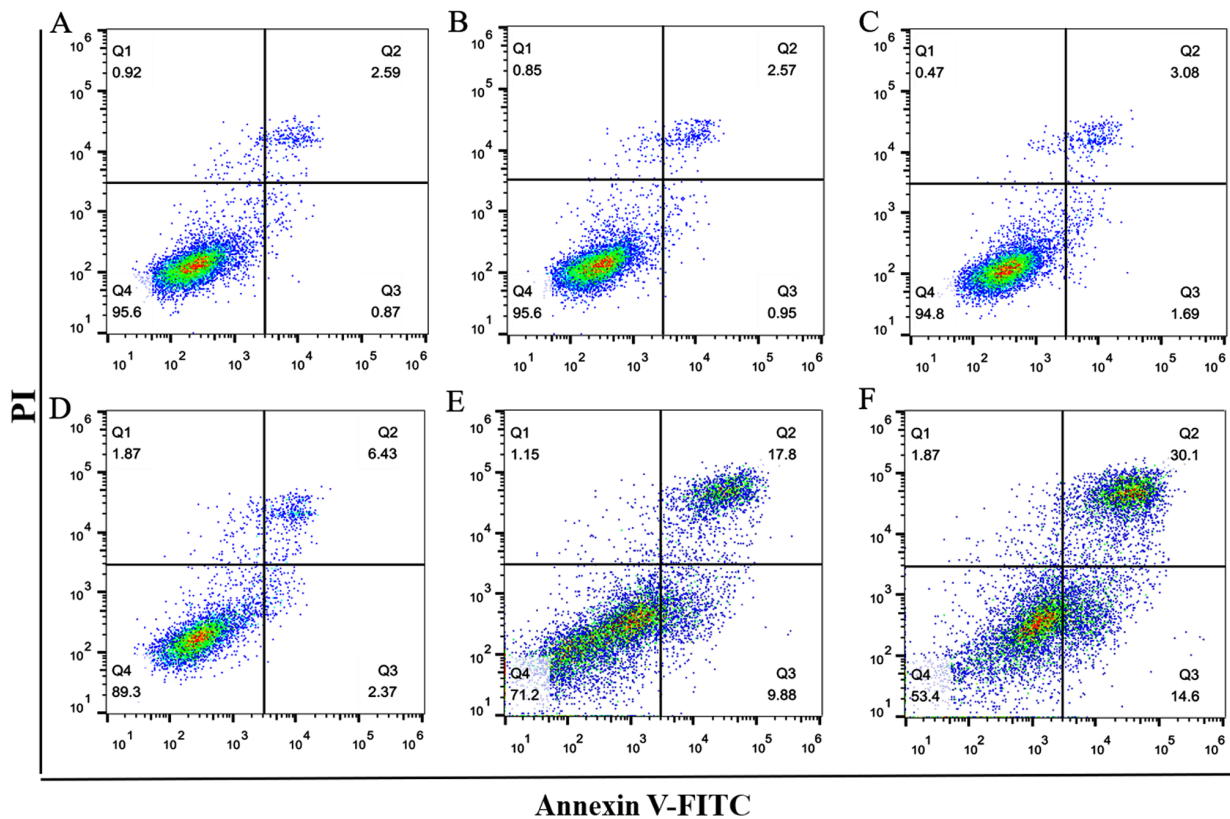


Figure 3. Effects of BPA on human NSC apoptosis. Cells were treated with BPA at (A) 0, (B) 0.1, (C) 1, (D) 2.5, (E) 5 and (F) 10 μM for 24 h, and apoptosis was analyzed using flow cytometry. Data demonstrated that $\geq 5 \mu\text{M}$ BPA induced NSC apoptosis, while BPA with a lower concentration had no effect on NSC apoptosis. BPA, bisphenol A; NSCs, neural stem cells.

Thus, 1 μM BPA was selected for subsequent molecular mechanism research after considering the highest BPA concentration in serum in the present population based on BPA characteristics (40).

BPA (1 μM) affects the key molecules of cell differentiation and proliferation. The NSC maintenance factors EGF and bFGF were removed from medium. The increase in β -tubulin III expression in DC group indicated the beginning of differentiation compared with C group. When 1 μM BPA was added to the cell culture medium, differentiation was inhibited, as seen by the decrease in β -tubulin III expression in DBS group compared with DC group (Fig. 4).

Western blotting was used to detect the expression of partial genes in NSCs after exposure to 1 μM BPA. These genes included maintenance marker genes, such as nestin and Sox2, and differentiation marker genes, such as GFAP and MAP2. Western blotting demonstrated that 1 μM BPA notably reduced GFAP and MAP2 expression levels in NSCs compared with the control; whereas 1 μM BPA considerably increased the expression levels of nestin and Sox2 (Fig. 5). The results indicated that the dose of 1 μM BPA affected the fate transfer mechanism of human NSC maintenance and neural differentiation.

BPA promotes cell cycle. To classify and characterize the DEG functions and pathways, a Gene Ontology classification was performed in addition to a functional annotation of molecular functions (MFs), cellular components (CCs) and biological processes (BPs). In contrast to the control group, 2,124 gene expression (Processed data GSE185138) levels were remarkably

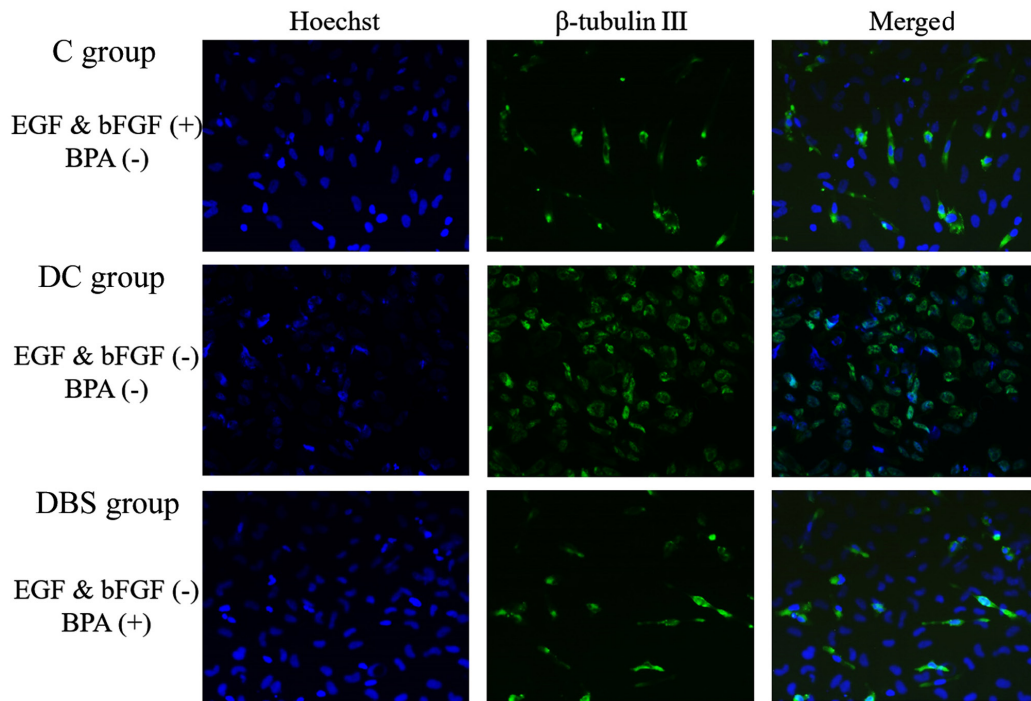
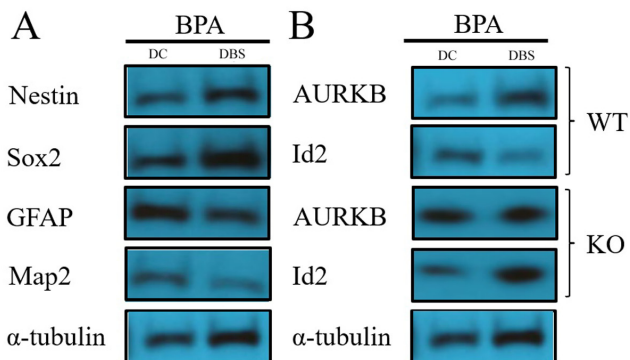
altered in the 1 μM BPA treated cells. Of these gene expression levels, 259 were attributed to MFs, 1,397 were attributed to BPs and the remaining 468 transcripts belonged to CCs. As presented in Fig. 6A, the GO function analysis results indicated that treatment with 1 μM BPA promoted 'cell cycle' (BPs).

BPA regulates the TGF- β pathway. The DEGs were used as input data, and KEGG pathway analysis was performed to visualize the effects of 1 μM BPA on specific cell signaling pathway-related gene expression changes (Fig. 6C) and to screen for biological pathways that may be potentially involved. The results, including the significantly enriched KEGG signaling pathway ($P \leq 0.05$), are presented in Fig. 6B. A total of seven signaling pathways were found with remarkable activation of low-concentration (1 μM) BPA: 'Homologous recombination', 'TGF- β signaling pathway', 'cell cycle', 'Fanconi anemia pathway', 'p53 signaling pathway', 'oocyte meiosis' and 'pathways in cancer'. Among these pathways, the TGF- β and p53 signaling pathways are closely related to cell differentiation fate (12,30,32). TGF- β gene can be activated by the estrogen signaling pathway (41). DEG analysis demonstrated that BPA had a significant influence on TGF- β and estrogen signaling pathway, and the associated genes are summarized in Table I.

ERR α and TGF- β 1 signaling pathways are regulated by 1 μM BPA. BPA can bind to the ERR receptor to activate gene expression (37). The present study designed the sgRNA sequence (5'-GACAGACCGAGCCTCCTG-3') and utilized CRISPR/Cas9 technology. In the current study, the

Table I. Associated genes of the TGF- β and estrogen signaling pathways.

Signaling pathway	Number of differentially expression gene	Number of significantly differentially expressed genes (P<0.05)	Gene name list	Classic Fisher	FDR
Estrogen	98	16	PRKACA, PLCB4, GABBR1, GPER1, SHC1, ADCY3, ATF4,GNAS, ATF2, CALM2, HRAS,ERRA, ADCY6, PLCB2, RAF1, CREB3L4	0.827	1
TGF- β	84	13	SMAD4, SMAD5, E2F5, SMAD1, TFDP1, RPS6KB2, AURKB, TGFB3, SKP1, TGFBR1, SMAD2, CREBBP, ID2	0.956	1

Figure 4. β -tubulin III expression is repressed after treatment with 1 μ M BPA. Immunostaining revealed that β -tubulin III expression in NSCs was downregulated when exposed to 1 μ M BPA. BPA, bisphenol A; NSCs, neural stem cells.Figure 5. Western blotting of key protein factors in NSCs. (A) Effects of BPA on the expression levels of human NSC maintenance and differentiation marker genes. (B) Expression levels of downstream factors in the TGF- β 1 signaling pathway. BPA, bisphenol A; NSCs, neural stem cells; Sox2, SRY-box 2, GFAP, glial fibrillary acidic protein; Map2, microtubule-associated protein 2; AURKB, aurora kinases B.

ERR α -knockout cells were obtained whose genomic coding region was inserted into the 'A' base (Fig. 7A). The wild-type cell line (WT) served as a control to analyze the *TGF- β 1* gene expression in the *ERR α* gene-deficient cell line (knockout type cell line, KO) under 1 μ M BPA stress. In WT cell lines (Fig. 7A), *TGF- β 1* expression level increased in the presence of BPA. In the *ERR α* -deficient cell line, even in the presence of BPA, the expression of *TGF- β 1* was not revealed to be obviously different. These results indicated that BPA might promote *TGF- β 1* gene expression by binding to the *ERR α* receptor and the promoter of *TGF- β 1*. CHIP-PCR results also revealed that *ERR α* could bind to the promoter of *TGF- β 1* under 1 μ M BPA exposure (Fig. 7B).

Changes in the TGF- β 1 signaling pathway downstream factors AURKB and Id2. AURKB and Id2 are downstream molecules of the TGF- β 1 signaling pathway, which promote cell proliferation and inhibits cell differentiation (35,36). Western blot

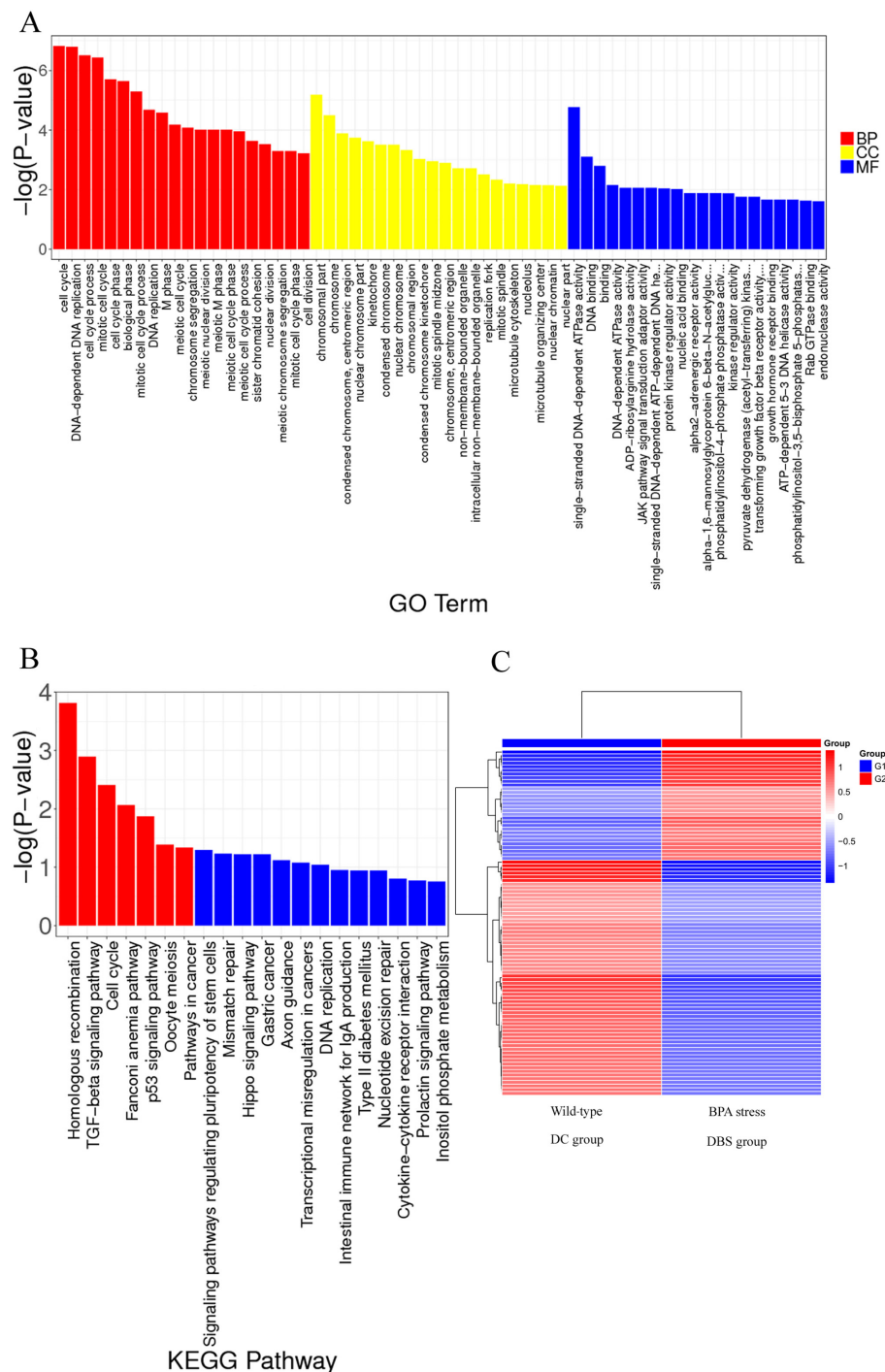


Figure 6. GO and KEGG analyses of DEGs. (A) GO analysis of the DEGs. The abscissa represents the negative logarithm (base 10) of the P-value which comes from enrichment analysis. The results indicated that 1 μ M BPA enhances 'cell cycle' and 'DNA-dependent DNA replication'. (B) KEGG pathway analysis. The abscissa represents the name of the significantly enriched KEGG pathway, and the ordinate represents -Log₁₀ (P-value). The red bars indicate significant pathways ($P \leq 0.05$), and the blue bars indicate non-significant pathways ($P > 0.05$). The results indicated that 1 μ M BPA activates the TGF- β and p53 signaling pathways. (C) Heatmap of DEGs. BPA (1 μ M) upregulated 31 genes and downregulated 64 genes. DEGs, differentially expressed genes; BP, biological processes; CC, cellular components; MF, molecular function; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

analysis to detect the protein expression levels of AURKB and Id2 in the NSCs exposed to 1 μ M BPA (Fig. 5). The results revealed of Fig. 5 revealed that 1 μ M BPA could markedly decrease *Id2* expression and increase *AURKB* expression in WT NSCs; moreover, the same result was obtained from the DEGs of RNA-seq analysis (Table I). In conclusion, the results in the current study indicated that 1 μ M BPA may activate the TGF- β 1 signaling pathway through *ERR α* to regulate the fate

transition of the dry maintenance/neurological differentiation of human NSCs (Fig. 8).

Discussion

BPA is found in breast milk and transferred from pregnant women to their fetuses, affecting a developing embryo and showing long-lasting deleterious effects during postnatal

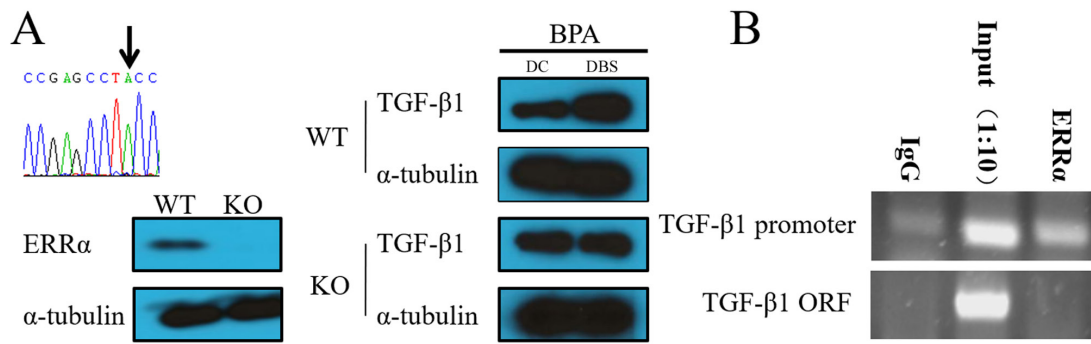


Figure 7. Analysis of the ERR α receptor-bound TGF- β 1 promoter. (A) TGF- β 1 expression was not affected by BPA. The ERR α -knockout cell line whose coding region of the *ERR α* gene was inserted into a deoxyadenylic acid was obtained using CRISPR/Cas9. ERR α -knockout cells were identified by western blotting and sequencing. (B) ERR α receptor can bind to the TGF- β 1 promoter. Chromatin immunoprecipitation-PCR results indicated that the negative control IgG group revealed no obvious bands while bands appeared in the positive control Input group. ERR α group results demonstrated that the ERR α receptor can bind to the TGF- β 1 promoter. ERR α , estrogen-related receptor α ; BPA, bisphenol A; ORF, open reading frame; WT, wild-type; KO, knockout.

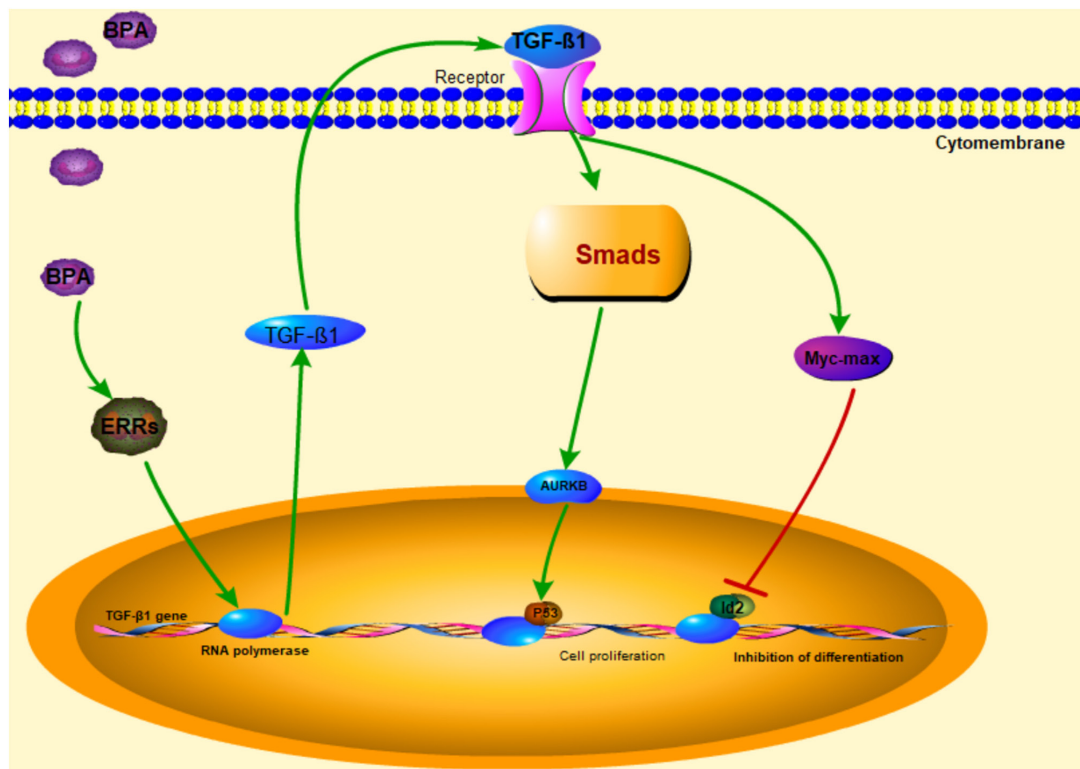


Figure 8. Schematic of BPA regulation mechanism that enhances stemness. Upon BPA exposure, the TGF- β 1 signaling pathway is activated via the ERR α -BPA complex that is bound to the TGF- β 1 gene promoter, resulting in the enhancement of human NSC stemness and the inhibition of cell differentiation. BPA, bisphenol A; NSCs, neural stem cells.

periods (42,43). Children born from pregnant women exposed to BPA have a much higher likelihood of attention deficit hyperactivity disorder (11.2%) and social impairment compared with children born to women without exposure to BPA (44).

BPA's deleterious effects on the nervous system are well understood (31); however, the effects of BPA on neurogenesis and the underlying cellular and molecular mechanisms are not completely clear. The median concentration of BPA in the urine of pregnant Chinese women is $4.8 \times 10^{-3} \mu\text{M}$ (45), and the concentration of free-BPA in serum is 4×10^{-6} – $9.1 \times 10^{-2} \mu\text{M}$ (40). Based on BPA hydrophobicity and the effects of low BPA concentration on morphology, proliferation and apoptosis, the present study analyzed the effects of $1 \mu\text{M}$ BPA on

human NSCs. The results revealed that $1 \mu\text{M}$ BPA enhanced human NSC proliferation but did not affect NSCs apoptosis. This implied that $1 \mu\text{M}$ BPA regulated NSC stemness via a molecular mechanism. However, the present study did not further explore the effect of BPA on cell proliferation changes via cell density, neurosphere diameter and cell cycle, as this study aimed to analyze the mechanism of $1 \mu\text{M}$ BPA on NSCs stemness maintenance.

At $1 \mu\text{M}$, BPA represses dopaminergic neuron differentiation from human embryonic stem cells by downregulating the expression of insulin-like growth factor 1 (38). In addition, 0.01 and 0.1 μM BPA promotes epithelial to mesenchymal transition via the canonical Wnt pathway in ovarian cancer cells;

the effect of 0.1 μ M BPA on the TGF- β pathway has also been confirmed by Chip-seq (33). These data suggest that the BPA signaling pathway may differ in different cell types (38). The aforementioned results imply that BPA can regulate different signaling pathways with different concentration. In the present experiments, BPA at 1 μ M concentration was used to activate the TGF- β 1 signaling pathway.

Estrogen-related receptors are present in embryonic stem cells and NSCs (46), and their subtypes, including ERR α , ERR β and ERR γ , can bind to BPA to achieve BPA regulation (37). In this process, ERR α can regulate cell proliferation and also directly bind to the *TGF- β 1* gene promoter to induce its expression (41). It is hypothesized that BPA may bind to the ERR receptor and activate *TGF- β* gene expression, thereby activating the *TGF- β* signaling pathway.

BPA is involved in disrupting epigenetic programming, thus altering brain development (47,48). BPA causes developmental toxicity by inhibiting the proliferation of neural progenitor cells and rat embryonic midbrain cells by suppressing the ERK, JNK, CREB and p53 signaling pathways (32,49). BPA induces reactive oxygen species (ROS) and oxidative stress in rodent liver, sperm and brains (50,51). Therefore, decreased NSC proliferation by BPA can be due to the ROS generation, as enhanced ROS levels significantly reduce neurogenesis (52).

The different signal pathway regulation regulated the differentiation and proliferation of NSC. The differentiated signal pathway activity is relatively low or inhibited when proliferation is enhanced (53). In the present study, 1 μ M BPA could promote human NSC proliferation and stemness. RNA-seq results revealed that 1 μ M BPA enhanced the cell cycle and activated the TGF- β signaling pathway. Further experiments confirmed that BPA maintains cell stemness by binding to the EER α receptor and activating the TGF- β 1 signaling pathway and downstream factors *AURKB* and *Id2*. Future *in vivo* studies investigating BPA's toxic inhibition may provide further insights into the mechanisms behind its activity.

Acknowledgements

The authors would like to thank Dr Dan Lou at Shanghai Municipal Center for Disease Control & Prevention (Shanghai, China) for providing the hNSC.

Funding

The present work was supported by the Natural Scientific Foundation of Shandong Province, China (grant no. ZR2018MH038); and the Zibo City Integration Development Project (grant no. 2019ZBXC320).

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the GEO repository (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE185138>).

Authors' contributions

QW and XT conceived and supervised the study. PD and GY designed the study. XT, YL, WC, YM and LW performed the

experiments and analyzed the data. PD and QW confirmed the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

References

1. Bellinger DC: Interpreting epidemiologic studies of developmental neurotoxicity: Conceptual and analytic issues. *Neurotoxicol Teratol* 31: 267-274, 2009.
2. Buxbaum JD and Hof PR: The emerging neuroscience of autism spectrum disorders. *Brain Res* 1380: 1-2, 2011.
3. Maussion G, Diallo AB, Gigeck CO, Chen ES, Crapper L, Thérout JF, Chen GG, Vasuta C and Ernst C: Investigation of genes important in neurodevelopmental disorders in adult human brain. *Hum Genet* 134: 1037-1053, 2015.
4. Arndt TL, Stodgell CJ and Rodier PM: The teratology of autism. *Int J Dev Neurosci* 23: 189-199, 2005.
5. Daniels JL: Autism and the environment. *Environ Health Perspect* 114: A396, 2006.
6. Jaako-Movits K, Zharkovsky T, Romantchik O, Jurgenson M, Merisalu E, Heidmets LT and Zharkovsky A: Developmental lead exposure impairs contextual fear conditioning and reduces adult hippocampal neurogenesis in the rat brain. *Int J Dev Neurosci* 23: 627-635, 2005.
7. Chow ES, Hui MN, Lin CC and Cheng SH: Cadmium inhibits neurogenesis in zebrafish embryonic brain development. *Aquat Toxicol* 87: 157-169, 2008.
8. Tyler CR and Allan AM: Adult hippocampal neurogenesis and mRNA expression are altered by perinatal arsenic exposure in mice and restored by brief exposure to enrichment. *PLoS One* 8: e73720, 2013.
9. Ieraci A and Herrera DG: Single alcohol exposure in early life damages hippocampal stem/progenitor cells and reduces adult neurogenesis. *Neurobiol Dis* 26: 597-605, 2007.
10. Radonjic M, Cappaert NL, de Vries EF, de Esch CE, Kuper FC, van Waarde A, Dierckx RA, Wadman WJ, Wolterbeek AP, Stierum RH, *et al*: Delay and impairment in brain development and function in rat offspring after maternal exposure to methylmercury. *Toxicol Sci* 133: 112-124, 2013.
11. Landrigan PJ, Lambertini L and Birnbaum LS: A research strategy to discover the environmental causes of autism and neurodevelopmental disabilities. *Environ Health Perspect* 120: a258-a260, 2012.
12. Kim K, Son TG, Park HR, Kim SJ, Kim HS, Kim HS, Kim TS, Jung KK, Han SY and Lee J: Potencies of bisphenol A on the neuronal differentiation and hippocampal neurogenesis. *J Toxicol Environ Health A* 72: 1343-1351, 2009.
13. Itoh K, Yaoi T and Fushiki S: Bisphenol A, an endocrine-disrupting chemical, and brain development. *Neuropathology* 32: 447-457, 2012.
14. Hirabayashi Y and Gotoh Y: Epigenetic control of neural precursor cell fate during development. *Nat Rev Neurosci* 11: 377-388, 2010.
15. Sanosaka T, Namihira M and Nakashima K: Epigenetic mechanisms in sequential differentiation of neural stem cells. *Epigenetics* 4: 89-92, 2009.
16. Temple S: The development of neural stem cells. *Nature* 414: 112-117, 2001.
17. Negri-Cesi P: Bisphenol a interaction with brain development and functions. *Dose Response*: Jun 17, 2015 (Epub ahead of print). doi: 10.1177/1559325815590394.

18. Klajn A, Drakulic D, Tosic M, Pavkovic Z, Schwirtlich M and Stevanovic M: SOX2 overexpression affects neural differentiation of human pluripotent NT2/D1 cells. *Biochemistry (Mosc)* 79: 1172-1182, 2014.
19. Kubwabo C, Kosarac I, Stewart B, Gauthier BR, Lalonde K and Lalonde PJ: Migration of bisphenol A from plastic baby bottles, baby bottle liners and reusable polycarbonate drinking bottles. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 26: 928-937, 2009.
20. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR Jr, Lee DH, Shioda T, Soto AM, vom Saal FS, Welshons WV, *et al*: Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. *Endocr Rev* 33: 378-455, 2012.
21. Vandenberg LN, Hauser R, Marcus M, Olea N and Welshons WV: Human exposure to bisphenol A (BPA). *Reprod Toxicol* 24: 139-177, 2007.
22. Chapin RE, Adams J, Boekelheide K, Gray LE Jr, Hayward SW, Lees PS, McIntyre BS, Portier KM, Schnorr TM, Selevan SG, *et al*: NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. *Birth Defects Res B Dev Reprod Toxicol* 83: 157-395, 2008.
23. de Cock M, Maas YG and van de Bor M: Does perinatal exposure to endocrine disruptors induce autism spectrum and attention deficit hyperactivity disorders? Review. *Acta Paediatr* 101: 811-818, 2012. Review.
24. Ooe H, Taira T, Iguchi-Ariga SM and Ariga H: Induction of reactive oxygen species by bisphenol A and abrogation of bisphenol A-induced cell injury by DJ-1. *Toxicol Sci* 88: 114-126, 2005.
25. Masuo Y and Ishido M: Neurotoxicity of endocrine disruptors: Possible involvement in brain development and neurodegeneration. *J Toxicol Environ Health B Crit Rev* 14: 346-369, 2011.
26. Huang B, Jiang C, Luo J, Cui Y, Qin L and Liu J: Maternal exposure to bisphenol A may increase the risks of Parkinson's disease through down-regulation of fetal IGF-1 expression. *Med Hypotheses* 82: 245-249, 2014.
27. Brown JS Jr: Effects of bisphenol-A and other endocrine disruptors compared with abnormalities of schizophrenia: An endocrine-disruption theory of schizophrenia. *Schizophr Bull* 35: 256-278, 2009.
28. Schoenfeld TJ and Cameron HA: Adult neurogenesis and mental illness. *Neuropsychopharmacology* 40: 113-128, 2015.
29. Reekmans K, Praet J, Daans J, Reumers V, Pauwels P, Van der Linden A, Berneman ZN and Ponsaerts P: Current challenges for the advancement of neural stem cell biology and transplantation research. *Stem Cell Rev Rep* 8: 262-278, 2012.
30. Tiwari SK, Agarwal S, Seth B, Yadav A, Ray RS, Mishra VN and Chaturvedi RK: Inhibitory effects of bisphenol-A on neural stem cells proliferation and differentiation in the rat brain are dependent on Wnt/ β -catenin pathway. *Mol Neurobiol* 52: 1735-1757, 2015.
31. Tiwari SK, Agarwal S, Tripathi A and Chaturvedi RK: Bisphenol-A mediated inhibition of hippocampal neurogenesis attenuated by curcumin via canonical Wnt pathway. *Mol Neurobiol* 53: 3010-3029, 2016.
32. Kim K, Son TG, Kim SJ, Kim HS, Kim TS, Han SY and Lee J: Suppressive effects of bisphenol A on the proliferation of neural progenitor cells. *J Toxicol Environ Health A* 70: 1288-1295, 2007.
33. Hui L, Li H, Lu G, Chen Z, Sun W, Shi Y, Fu Z, Huang B, Zhu X, Lu W, *et al*: Low dose of bisphenol A modulates ovarian cancer gene expression profile and promotes epithelial to mesenchymal transition via canonical Wnt pathway. *Toxicol Sci* 164: 527-538, 2018.
34. Kim YS, Hwang KA, Hyun SH, Nam KH, Lee CK and Choi KC: Bisphenol A and nonylphenol have the potential to stimulate the migration of ovarian cancer cells by inducing epithelial-mesenchymal transition via an estrogen receptor dependent pathway. *Chem Res Toxicol* 28: 662-671, 2015.
35. He J, Qi Z, Zhang X, Yang Y, Liu F, Zhao G and Wang Z: Aurora kinase B inhibitor barasertib (AZD1152) inhibits glucose metabolism in gastric cancer cells. *Anticancer Drugs* 30: 19-26, 2019.
36. Siegel PM, Shu W and Massagué J: Mad upregulation and Id2 repression accompany transforming growth factor (TGF)- β -mediated epithelial cell growth suppression. *J Biol Chem* 278: 35444-35450, 2003.
37. Tohmé M, Prud'homme SM, Boulahtouf A, Samarut E, Brunet F, Bernard L, Bourguet W, Gibert Y, Balaguer P and Laudet V: Estrogen-related receptor γ is an in vivo receptor of bisphenol A. *FASEB J* 28: 3124-3133, 2014.
38. Huang B, Ning S, Zhang Q, Chen A, Jiang C, Cui Y, Hu J, Li H, Fan G, Qin L, *et al*: Bisphenol A represses dopaminergic neuron differentiation from human embryonic stem cells through down-regulating the expression of insulin-like growth factor 1. *Mol Neurobiol* 54: 3798-3812, 2017.
39. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T: Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res* 13: 2498-2504, 2003.
40. Chen X, Bao HH, Wu WK, Yan SQ, Sheng J, Xu YY, Gu CL, Huang K, Cao H, *et al*: Exposure to bisphenol A during maternal pregnancy and the emotional and behavioral impact on their preschool children. *Chin J Epidemiol* 39: 188-193, 2018 (In Chinese).
41. Huang X, Wang X, Shang J, Zhaang Z, Cui B, Lin Y, Yang Y, Song Y, Yu S and Xia J: Estrogen related receptor alpha triggers the migration and invasion of endometrial cancer cells via up regulation of TGF β 1. *Cell Adhes Migr* 12: 538-547, 2018.
42. Nishikawa M, Iwano H, Yanagisawa R, Koike N, Inoue H and Yokota H: Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. *Environ Health Perspect* 118: 1196-1203, 2010.
43. Schönfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M and Chahoud I: Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ Health Perspect* 110: A703-A707, 2002.
44. Tewar S, Auinger P, Braun JM, Lanphear B, Kimberly Yoltan K, Epstein JN, Ehrlich S and Froehlich TE: Association of bisphenol A exposure and attention-deficit/hyperactivity disorder in a national sample of U.S. children. *Environ Res* 150: 112-118, 2016.
45. Zhang Z, Alomirah H, Cho HS, Li YF, Liao C, Minh TB, Mohd MA, Nakata H, Ren N and Kannan K: Urinary bisphenol A concentrations and their implications for human exposure in several Asian countries. *Environ Sci Technol* 45: 7044-7050, 2011.
46. van der Laan S, Golfetto E, Vanacker JM and Maiorano D: Cell cycle-dependent expression of Dub3, Nanog and the p160 family of nuclear receptor coactivators (NCoAs) in mouse embryonic stem cells. *PLoS One* 9: e93663, 2014.
47. Kundakovic M and Champagne FA: Epigenetic perspective on the developmental effects of bisphenol A. *Brain Behav Immun* 25: 1084-1093, 2011.
48. Dolinoy DC, Huang D and Jirtle RL: Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci USA* 104: 13056-13061, 2007.
49. Liu R, Xing L, Kong D, Jiang J, Shang L and Hao W: Bisphenol A inhibits proliferation and induces apoptosis in micromass cultures of rat embryonic midbrain cells through the JNK, CREB and p53 signaling pathways. *Food Chem Toxicol* 52: 76-82, 2013.
50. Bindhumol V, Chitra KC and Mathur PP: Bisphenol A induces reactive oxygen species generation in the liver of male rats. *Toxicology* 188: 117-124, 2003.
51. Kabuto H, Hasuie S, Minagawa N and Shishibori T: Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. *Environ Res* 93: 31-35, 2003.
52. Huang TT, Zou Y and Corniola R: Oxidative stress and adult neurogenesis - effects of radiation and superoxide dismutase deficiency. *Semin Cell Dev Biol* 23: 738-744, 2012.
53. Zhang L, Ye M, Zhu L, Cha J, Li C, Yao YG and Mao B: Loss of ZC4H2 and RNF220 inhibits neural stem cell proliferation and promotes neuronal differentiation. *Cells* 9: E1600, 2020.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.