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Research Article

# Low-dose BPA and its substitute BPS promote ovarian cancer cell stemness via a non-canonical PINK1/p53 mitophagic signaling

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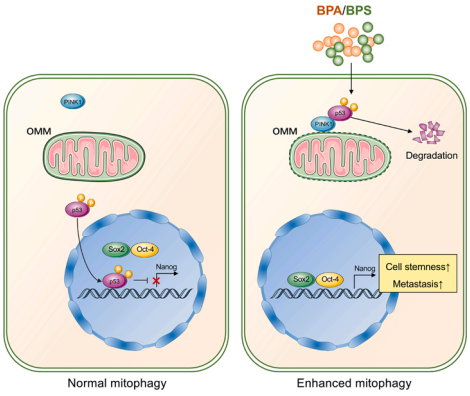
## HIGHLIGHTS

- Environmental relevant BPA/BPS exposure enhances ovarian cancer cell stemness.
- Low-dose BPA/BPS upregulates PINK1 expression.
- p53 rather than Parkin pathway is activated under low-dose BPA/BPS exposure.
- BPA/BPS promotes ovarian cancer in vivo metastasis in a mitophagy-dependent manner.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The environmental toxicity of bisphenol A (BPA) and its analog like bisphenol S (BPS) have drawn wide attention, but their roles in cancer progression remain controversial. Here, we investigated the effect of BPA/BPS on the development of ovarian cancer. Human internal BPA/BPS exposure levels were analyzed from NHANES 2013–2016 data. We treated human ovarian cancer cells with 0−1000 nM BPA/BPS and found that 100 nM BPA/BPS treatment significantly increased Cancer Stem Cell (CSC) markers expression including OCT4, NANOG and SOX2. Cancer cell stemness evaluation induced by BPA/BPS was notably attenuated by the knockdown of PINK1 or Mdivi-1 treatment. The activation of PINK1 initiated mitophagy by inhibiting p-p53 nuclear translocation in a non-canonical manner. In vivo studies validated that BPA/BPS-exposed mice have higher tumor metastasis incidence compared with the control group, while mitophagy inhibition blocked such a promotion effect. In addition, CSC markers such as SOX2 had been found to be overexpressed in the tumor tissues of BPA/BPS

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exposure group. Taken together, the findings herein first provide the evidence that environmentally relevant BPA/BPS exposure could enhance ovarian cancer cell stemness through a non-canonical PINK1/p53 mitophagic pathway, raising concerns about the potential population hazards of BPA and other bisphenol analogs.

## 1. Introduction

Bisphenol A (BPA) is extensively utilized in food, beverage containers, cigarette filters and many other usages production[66]. As a kind of xenoestrogen, BPA has the potential to promote the progression of many estrogen-related tumors including ovarian and breast tumors [57]. Previous studies have verified that BPA can affect estrogen and/or androgen concentrations by inhibiting gonadal hormone synthesis and metabolism [74]. Increasing concerns about BPA for its possible adverse effects on human health have promoted the emergence of "BPA-free" products. Some of the structural analogs like bisphenol S (BPS) and bisphenol F (BPF), however, are also bisphenols (BPs) and their toxicity profile is largely unknown [30].

The occurrence of BPA and bisphenol analogs in foods, consumer products and human fluids has been documented [38,53], of which BPS was highly detected among bisphenol substitutes [4]. Determined from measurable concentrations of BPA of normal people in body tissues and fluids, the concentration ranging from 0.5 to 10 ng/ml has been regarded as a theoretical internal concentration [65]. Studies suggested that the detectable BPS concentration in human plasma is basically equal to or slightly less than the BPA [22]. To be more specific, the mean plasma detected concentration of BPA and BPS were 0.67 μg/L and 0.78 μg/L respectively [22]. While in breast milk, ND∼0.548 μg/L and ND∼0.683 μg/L [43] BPA/BPS were detected. Moreover, in some tissues like the placenta, BPA could also be measured (0.53 μg/kg) which means bisphenols exposure during pregnancy may affect their offspring[24]. For occupationally exposed people, epidemiological data indicated that occupational exposure would cause higher urine levels of BPA than general individuals about 70 times [17]. It is noteworthy that numerous toxicological and epidemiological studies have shown that BPs including BPA and BPS exhibit a variety of toxic effects, such as reproductive toxicity [2,7], neurotoxicity [47,55] and metabolic toxicity [63]. Whether an internal dose of BPA or BPS could cause the development of tumors remained to be controversial.

Among the estrogen-related tumors of women reproductive system, ovarian cancer is the third prevalent but deadliest of the gynecological malignancies [12], fewer than half of the patients survive beyond 5 years [64], of which posing a serious threat to women health worldwide. It is reported that the occurrence and development of ovarian cancer are closely related to EDCs exposure, especially exposure to environmental estrogen such as BPA[18]. However, the molecular mechanism by which environmental dose BPA or its analog exposure promotes the development of ovarian tumors has not been clarified yet.

The mechanism of Cancer Stem Cell (CSC) is adopted to explain tumor development in recent years, which has indicated that the growth and metastasis of tumors depend on a small number of tumor stem cells with "self-renewal" ability in tumor cells [1]. Accumulating evidence has also supported that the poor prognosis of tumors was essentially ascribed to the acquired stemness properties of cancer cells [23,33,60]. Our previous study [20] has found that low-dose BPA could promote epithelial-mesenchymal transition (EMT) of cancer cells via canonical Wnt pathway , which tends to be associated with the acquisition of stemness phenotype [25]. Nevertheless, the underlying mechanism between BPs and estrogen-related tumor stem cells (such as the ovarian cancer stem cells) has not been intensively elucidated.

Mitochondrial autophagy (mitophagy) is a key mitochondrial quality control pathway that eliminates impaired, aged or dysfunctional mitochondria through autophagy [72], which is essential in maintaining cellular homeostasis and stemness [11,40,46]. Mitophagy plays a critical role during tumor progression [5]. In the late stage of tumorigenesis, cancer cells could tolerate adverse conditions (low nutrient levels and hypoxia) better under enhanced mitophagy, which is involved with canonical PINK1/Parkin signaling or non-canonical pathways independent of Parkin [41,68,9]. Furthermore, mitophagy is highly indispensable in maintaining stem cell characteristics as well as alterations in the metabolic phenotype during cell fate transition in normal and cancer cells [39,42]. For instance , the mitochondrial fission factor FIS1 promotes stemness of human lung cancer stem cells via mitophagy [31], while mitochondrial fission factor (MFF) enhances stemness and tumor-initiating capability via promoting mitochondrial fission in non-liver cancer-initiating cells [62]. However, whether BPs could regulate the stemness of ovarian cancer via canonical or non-canonical mitophagic signaling has remained to be unclear.

In this present study, we aimed to determine whether environmental BPs could enhance ovarian cancer cell stemness in vitro/in vivo and the underlying mechanism. Western blotting, qRT-PCR and immunofluorescence staining were conducted to assess ovarian cell stemness phenotype induced by BPA/BPS. Our established ATdb tool [6], bioinformatics analyses, nucleocytoplasmic separation and Co-IP assays were used to screen the underlying mitophagic pathway which accounts for the mechanism of how BPA/BPS regulates the stemness of ovarian cancer cells. Additionally, histopathological analyses and immunofluorescent staining were implemented among the in vivo models after BPA/BPS exposure. Overall, this study firstly provides new evidence that how environmental concentrations of BPA and BPS regulate cancer cell stemness and tumor metastasis via a novel mitophagic pathway.

## 2. Materials and methods

### 2.1. Study population

Our analyses were based on cross-sectional data from the National Health and Nutrition Examination Survey (NHANES) 2013–2016. In our research, we extracted data from 5337 participants aged 20 years and older enrolled in two NHANES cycles (NHANES 2013–2014 and 2015–2016). 541 participants were not involved in further research due to the lack of urinary concentration of BPA and BPS. A total of 4796 individuals were brought into our main analyses. The method for measuring phenols uses online solid phase extraction coupled with HPLC/MS detection technology. Internal standards labeled isotopically were used as standard quality control, the detection limits in 100 μl of urine are 0.1 – 0.2 micrograms per liter (μg/L), sufficient for measuring urinary levels of phenols. The limits of detection (LODs) were 0.20 and 0.10 μg/L for BPA and BPS.

### 2.2. Chemical treatments and cell culture

BPA (>99.0%), BPS (>98.0%) and Mdivi-1 (>98.0%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The stock solution of BPA ( 20 mM ) , BPS ( 20 mM ) and Mdivi-1 (25 mg/ml) were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Human ovarian cancer cell lines SKOV3 were purchased from the Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and epithelial ovarian cancer cell lines A2780 were purchased from iCell Bioscience Inc (Shanghai, China). SKOV3 and A2780 cells were cultured in McCoy's 5 A (Gibco, USA) and DMEM (Gibco, USA) media, respectively.

### 2.3. Animal study

5-week-old female BALB/c nude mice were purchased from

Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai China). All procedures were performed according to the Ethics Committee of Animals Using in Zhejiang University (ZJU20220208). The ovarian tumor xenograft metastasis model in BALB/c nude mice establishment was outlined in previous studies [26]. Animals were randomly divided into 6 groups (each group containing 6 members): (1) control group (2) BPA group (3) Mdivi-1 group (4) BPA+ Mdivi-1 group (5) BPS group (6) BPS+ Mdivi-1 group. BPA/BPS was dissolved in sesame oil and given by gavage administration at a dose of 50 μg/kg body weight/day [21,50,75], control mice were exposed to the same dose of sesame oil without BPA/BPS. The experimental mice received 25 mg/kg/day Mdivi-1 intraperitoneally [77], while the control mice received saline treatment. The mice were sacrificed after 4 weeks before taking representative images of the metastasis tumors. Then the number and weight of metastatic lesions were measured. The metastases nodes were fixed with formalin for subsequent analysis.

### 2.4. EdU cell proliferation assay

EdU-594 Cell Proliferation Kit (BeyoClick™) was used to detect cell proliferation ability and carried on as previously explained [35]. We used a cell density of $5 \times 10^4$ per well as a starting point in a 12-well plate. After being cultured for 24 h, ovarian cancer cells were exposed to different concentrations of BPA/BPS for 24 h. Fluorescence imaging was collected by fluorescence microscope (Nikon, Japan).

### 2.5. SDS-PAGE and western blot analysis

SKOV3 or A2780 cells were harvested and lysed in cold RIPA (Beyotime) buffer for 30 min. After centrifuging and collecting the supernatants, cell lysates were then subjected to SDS-PAGE analysis. Nitrocellulose filter membranes (0.22 or 0.45 μm) were used for protein transferring and 5% non-fat milk in Tris-buffered saline was used for membrane blocking. Then, the membranes were incubated with the primary and secondary antibodies and visualized by ChemiScope 3300 Mini (Clinx, China) with the help of ECL substrate (Cyanagen, Italy). Experiments were performed in triplicate. A list of antibodies was itemized in Table S1.

### 2.6. Immunofluorescence staining

After 24 h BPA/BPS treatment, tumor cells were fixed by 4% paraformaldehyde in PBS for 15 min, followed by permeabilized with 1% Triton X-100 (Sangon Biotech, China # A110694) for 10 min and then blocked with 5% bovine serum albumin (BSA) for 1 h. The primary and secondary antibodies used there are listed in Table S1. After incubated with the antibody, the coverslips were stained with DAPI (1 μg/ml, Beyotime, China #C1002) for 5 min. Images were captured and merged using bx63 Olympus microscope. Experiments were performed three replicated times.

### 2.7. RNA extraction and Real-Time PCR

SKOV3 or A2780 cells were treated as mentioned above. Total RNA was extracted using Trizol reagent (Sangon Biotech, China #B511311) and was reversed for cDNA synthesis by Takara Prime Script™ RT reagent Kit. Applied Biosystems 7500 fast System was carried for qPCR analysis, with an SYBR Green PCR master Mix Kit (Takara, Otsu, Shiga, Japan). The necessary primers were synthesized by Sangon Biotech (Sangon Biotech, Shanghai, China). Primer sequences of β-actin, Sox2, Oct-4, Nanog and PINK1 are shown in Supplementary Materials (Table S2). The $2^{-\triangle\triangle Ct}$ method was applied to analyze the expression levels of each gene, which was normalized with β-actin.

### 2.8. siRNA transfection

PowerFect reagent (SignaGen, USA #SL100569) was utilized to conduct small interfering RNA (siRNA) transfection. A diluted mixture of si-PINK1 and PowerFect reagent (SignaGen, USA) was added to the culture medium, and the cells were cultured for another 4–6 h. Following medium refreshment, the cells were cultured for 24 h accompanied with BPA/BPS. Negative control siRNA, siRNAs targeting PINK1 were designed and synthesized by GenePharma (China). The siRNA sequences were listed in Table S3.

### 2.9. Nucleocytoplasmic separation

The cells were collected and lysed with cell lysis buffer (Beyotime). Nucleocytoplasmic separation of cultured ovarian cancer cells was separated by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's protocol.

### 2.10. Co-Immunoprecipitation (Co-IP)

SKOV3 cells were harvested in NP40 lysis buffer containing PMSF, and cell debris is removed by centrifugation (12,000xg for 10 min at 4 ℃). The cell lysate was first pre-cleaned with IgG mixed with Protein A/G PLUS-Agarose (Santa Cruz, #G1321) at 4 ℃ for 30–60 min. Then anti-PINK1 or IgG antibodies were added into cell lysate and incubated at 4 ℃ overnight. On day 2, 20 μl resuspended volume of Protein A/G PLUS-Agarose was supplemented to the cell lysate. Further, the protein was separated by SDS-PAGE. Western blot was conducted to assess the degree of p53 and p-p53. Detailed information was shown in supporting information.

### 2.11. Survival analysis

Overall survival of TCGA and GSE13876 ovarian cancer patients was determined by Kaplan-Meier analysis. Hazard ratio (HR) and corresponding 95% confidence interval (CI) were calculated with an optimal cutoff value.

### 2.12. Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded (FFPE) tumor sections were prepared for immunohistochemistry (IHC) analysis. Images were captured and merged using Olympus FV1000 microscope or bx63 Olympus microscope. Experiments were performed three replicated times. The detailed methods are stated in the supporting information.

### 2.13. Transmission electron microscopy (TEM)

Tumor cells were imaged at the Research Center of Diagnostic Electron Microscopy at Zhejiang University using Thermo Scientific Talos L120C. Cells were fixed with 2.5% glutaraldehyde and stained with uranyl acetate.

### 2.14. Statistical analyses

GraphPad Prism 9 and R 4.1.0 were applied for statistical analyses in our research. Pearson correlation coefficients were calculated to measure the linear relationship. Scatter plots were drawn with R package ggpubr. Three independent experiments results were presented as mean ± SD. Two-tail student's t-test and one-way analysis of variance (ANOVA) were adopted for two or multiple groups comparisons, correspondingly. A p-value $< 0.05$ was considered statistically significant.

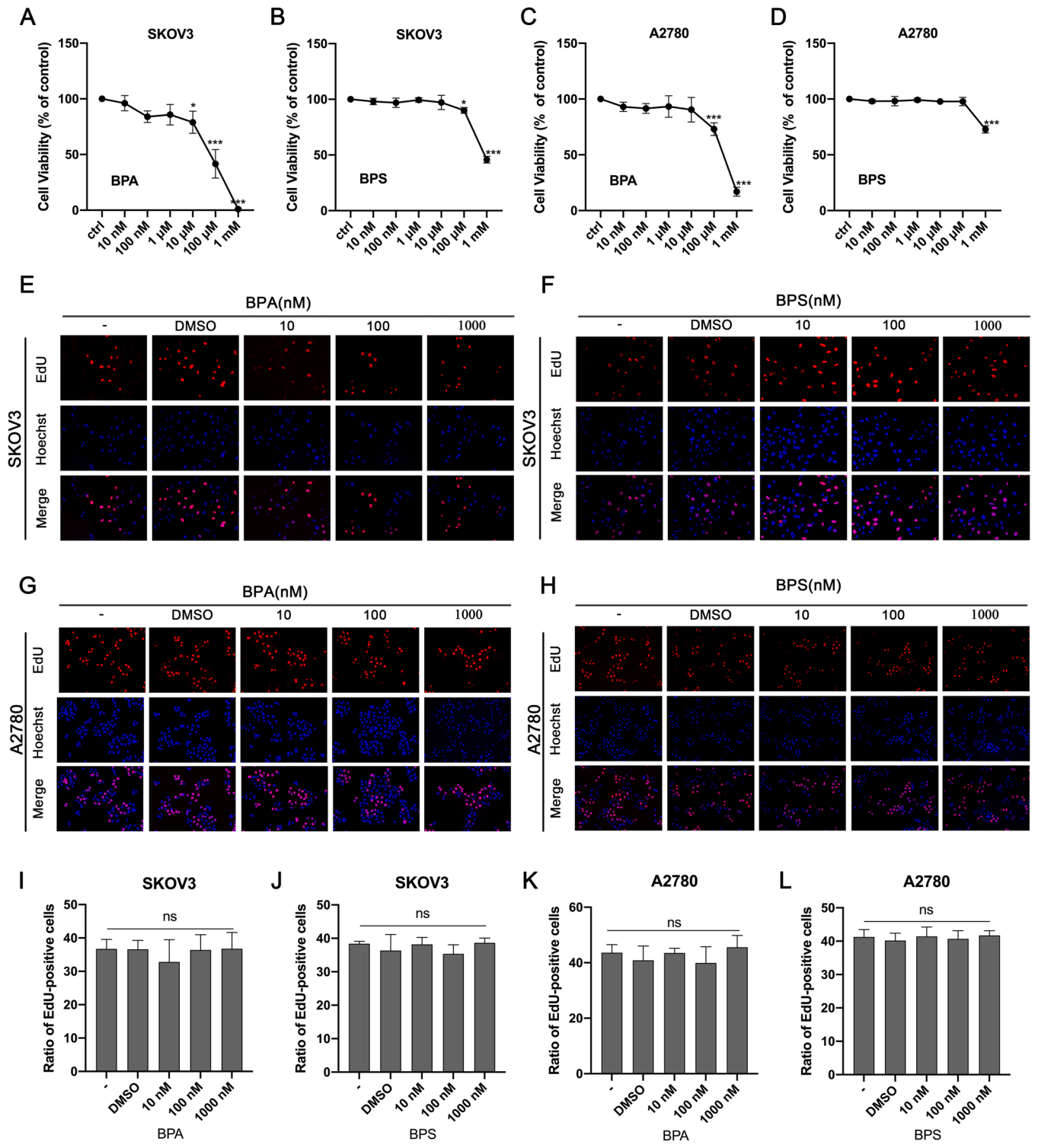


**Fig. 1.** Effects of BPA/BPS exposure on ovarian cancer cell proliferation. (A-D) SKOV3 and A2780 cell viability was determined after treatment with DMSO control or 10 nM/100 nM/1 μM/10 μM/100 μM/1 mM BPA/BPS for 24 h by CCK-8 kit. (E-H) EdU proliferation assay in BPA/BPS treated SKOV3 and A2780 cells. (I-L) The results for EdU-positive cells were graphed and statistically analyzed. ns, no statistical significance, * $p < 0.05$, *** $p < 0.001$, by one-way ANOVA with Dunnett's test compared with the control group ($n = 3$). Error bar indicates the mean ± SD.

## 3. Results

### 3.1. Effects of BPA/BPS exposure on ovarian cancer cell proliferation

To investigate the cell toxicity of BPA/BPS on ovarian cancer cells, SKOV3 and A2780 were exposed to concentration gradients of BPA/BPS. As shown in Fig. 1A, no significant decrease in viability was observed in SKOV3 cells following BPA exposure lower than 10 μM, while in A2780 cells the threshold was 100 μM (Fig. 1C). BPS was characterized by lower cell toxicity (Fig. 1B, D). Then we chose 10–1000 nM of BPA and BPS as concentration gradients accompanied by blank control and solvent control. In EdU cell proliferation assays, 24 h BPA/BPS treatment at

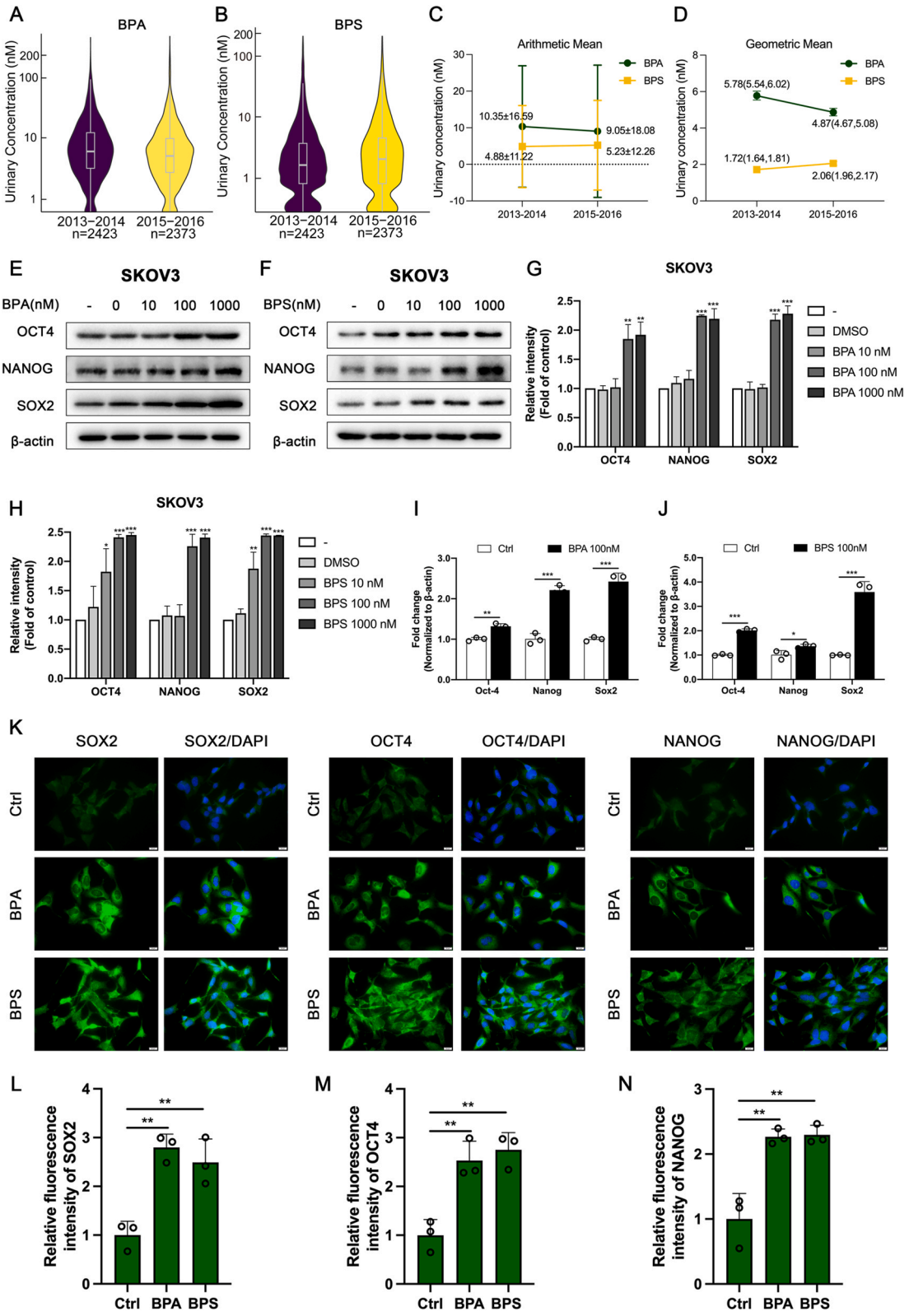


**Fig. 2.** BPA/BPS upregulated ovarian cancer cell stemness. (A, B) The level of urine BPA and BPS concentration was analyzed in NHANES database (2013–2016, n = 4796). (C, D) The arithmetic mean and geometric mean change of BPA/BPS. (E, F) Representative western blot showing the expression of cancer stem cell (CSC) related proteins OCT4, NANOG and SOX2 after BPA/BPS treatment at concentrations of 0,10,100,1000 nM with blank control for 24 h in SKOV3 cell. β-actin served as an internal control. (G, H) Quantification of western blot detection of OCT4, NANOG and SOX2 in SKOV3 cells after treatment with BPA/BPS 24 h.The relative intensity was analyzed with Image J software and calculated by the ratio relative to the β-actin intensity. (I, J) mRNA expression levels of Oct-4, Nanog, Sox2 were measured by qPCR. The gene β-actin was used as an internal control. (K) SKOV3 cells were exposed to DMSO or 100 nM BPA/BPS for 24 h. OCT4, NANOG, SOX2 expression were detected by Immunofluorescence. (L-N) Quantification of immunofluorescence detection of SOX2, OCT4 and NANOG in SKOV3 cells after treatment with BPA/BPS 24 h.The immunofluorescence intensity was analyzed with Image J software and calculated by the ratio relative to the Ctrl group. Scale bar: 20 μm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by Student's t-test compared with control group (n = 3). Error bar indicates the mean ± SD.

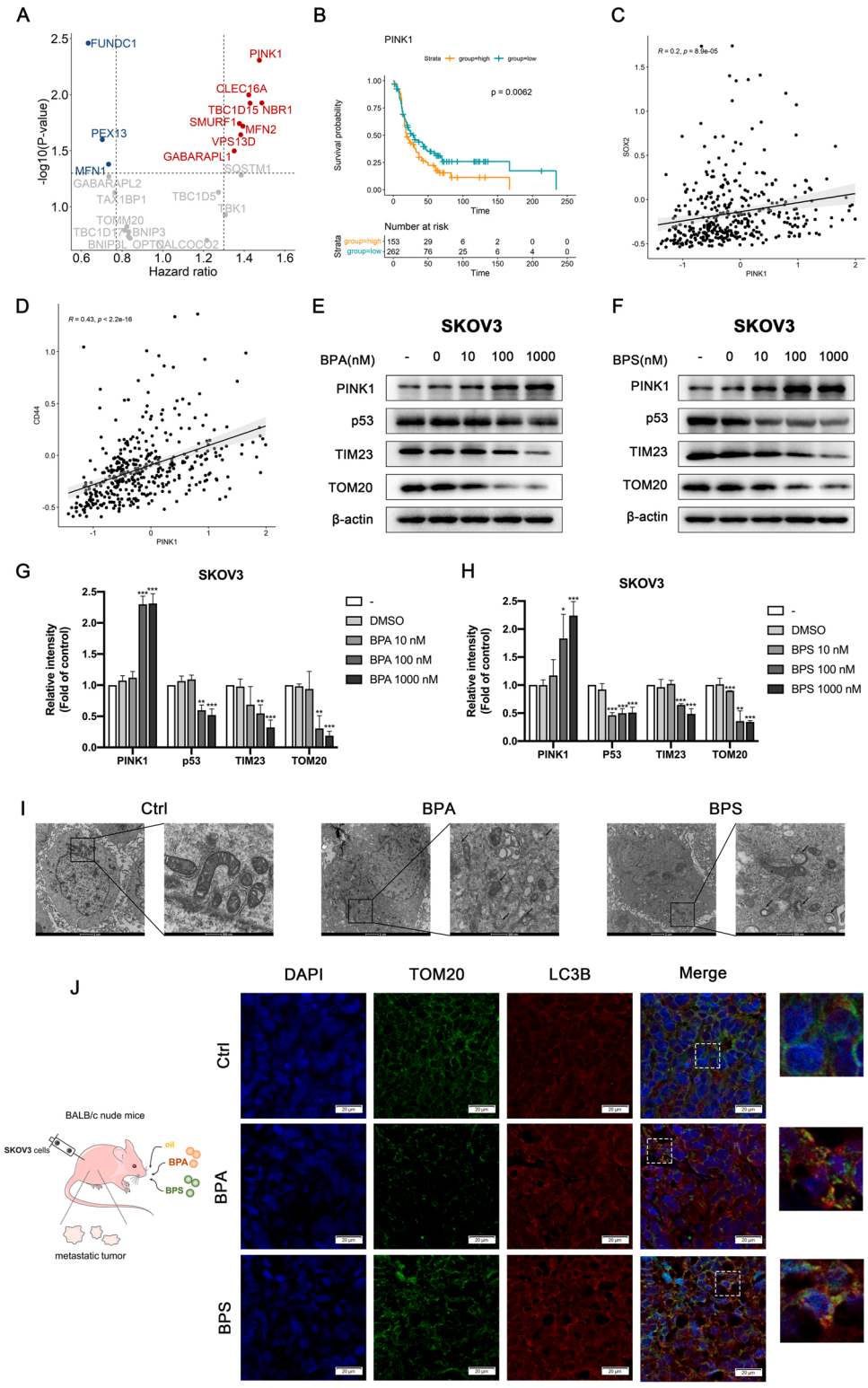


**Fig. 3.** Low-dose BPA/BPS enhanced mitophagy by upregulating PINK1. (A) The correlation between mitophagy-related protein with ovarian cancer patients' prognosis. (B) Kaplan-Meier analyses for overall survival of GSE13876 ovarian cancer patients. The optimal cutoff value was automatically chosen. n = 415, p = 0.0062. (C) The correction analysis between PINK1 and SOX2 expression in ovarian cancer patients was determined via Spearman rank correlation analysis. n = 415. (D) The correction analysis between PINK1 and CD44 expression in ovarian cancer patients was determined via Spearman rank correlation analysis. n = 415. (E, F) Representative western blot showing the expression of PINK1, p53, TIM23, TOM20 after BPA/BPS treatment at concentrations of 0,10,100,1000 nM for 24 h in SKOV3 cell. β-actin served as the internal control. (G, H) Quantification of western blot detection of PINK1, p53, TIM23 and TOM20 in SKOV3 cells after treatment with BPA/BPS 24 h.The relative intensity was analyzed with Image J software and calculated by the ratio relative to the β-actin intensity. (I) SKOV3 cells were treated with 100 nM BPA/BPS for 24 h accompanied with control and analyzed by transmission electron microscopy (TEM). Insets (black boxes) show mitochondria and the autophagosomes engulfing mitochondria. Scale bars, 2 μm; insets: Scale bar, 500 nm. (J) Representative immunofluorescent images of xenograft tumors from control, BPA and BPS groups. Boxes: co-localizations of TOM20 with LC3B. DAPI was used to tag the nucleus. Scale bar: 20 μm.

different concentrations, however, showed no significant change in cell proliferation ability (Fig. 1E-L). The results above indicated that ovarian cancer cell proliferation ability was not obviously altered under low-dose Bisphenols administration.

### 3.2. BPA/BPS upregulated ovarian cancer cell stemness

To determine the actual BPA/BPS exposure level in humans, we analyzed data from 4796 participants aged 20 years and older enrolled in two NHANES cycles (NHANES 2013–2014 and 2015–2016) for our main analyses. The results showed that in recent years, the internal exposure concentration of BPA in the general population exhibited a certain downward trend while its substitute BPS showed a contrary trend, the mean BPA/BPS internal concentration was around 10 nM and the upper limit of these bisphenols was about 200 nM (Fig. 2A-D; Table S4-6). In our early study, Hui et al. found that low-dose BPA could

promote Epithelial to Mesenchymal Transition (EMT) via canonical Wnt pathway [20]. Cells that have undergone EMT also acquire resistance to several drugs and chemotherapeutic agents [8], which is similar to cancer stem-like cells (CSCs) [36]. Based on these concepts, we determined to explore the association between BPA as well as its analog BPS and cancer cell stemness. As expected, BPA upregulated the expression of stemness biomarkers OCT4, NANOG and SOX2 in a dose-dependent manner in both cell lines (Fig. 2E, Fig. S1A). The intensity qualification was shown in Fig. 2G and Fig. S1C. It was perturbing that BPS treatment exhibited a similar manner with BPA and SOX2 showed an obvious enhancement even under 10 nM BPS treatment (Fig. 2F, H; Fig. S1 B, D). Since 100 nM BPA/BPS exposure could obviously promote stemness, which is also comparable with human environmental exposure level [27,28,65], 100 nM was taken as the working concentration subsequently. The transcription level changes of the stemness biomarkers were detected by qPCR. Under 100 nM BPA/BPS treatment, the mRNA level of Oct-4, Nanog, and Sox2 were significantly promoted (Fig. 2I, J; Fig. S1E, F). Similar alterations were also observed by immunofluorescence of which 100 nM BPA/BPS treatments dramatically stimulated the expression of CSC markers in SKOV3 and A2780 (Fig. 2K-N; Fig. S1G-J). In addition, nuclear accumulation of OCT4, NANOG and SOX2 was found after BPA/BPS exposure, while SOX2 presented the most evident nuclear translocation effect. Altogether, these results suggested that BPA/BPS exposure in a concentration comparable to the environmental level could promote cancer cell stemness.

### 3.3. Low-dose BPA/BPS enhanced mitophagy by upregulating PINK1

Mitophagy is a distinct autophagic process that can selectively clear redundant or damaged mitochondria via autophagic lysosomes [29]. It was not controversial that mitophagy plays a momentous role in the maintenance and remodeling of stem cell populations through metabolic reconfiguration for better adaption to the tumor microenvironment [48, 49]. Enriched cancer stem cell further results in a worse prognosis. We used ATdb [6] database to screen the mitophagy-related proteins associated with worse prognosis of ovarian cancer patients, and found that the high expression of PINK1 was highly correlated with the poor prognosis of ovarian cancer patients (Fig. 3A). In GSE13876 database, ovarian cancer patients with higher PINK1 level showed significantly worse prognosis (Fig. 3B), which was also verified in TCGA database (Fig. S2C). Moreover, correlation analysis revealed that the expression of PINK1 was positively correlated with SOX2 and CD44 (Fig. 3C, D), both of which are important stemness makers in ovarian tumors. Patients with higher SOX2, OCT4 and NANOG expression levels also exhibited worse prognoses (Fig. S2F-H). As expected, our results showed that PINK1 expression was extremely elevated under 100 nM BPA/BPS treatment (Fig. 3E-H; Fig. S2A, B; D, E). Since PINK1 is an initiating kinase that activates cellular mitophagy, we further determined the expression of mitochondrial membrane markers in ovarian cancer cells. Results showed that BPA/BPS treatment decreased TOM20 and TIM23 protein levels in ovarian cancer cells, which are translocases associated with outer and inner mitochondrion membranes, respectively [14] (Fig. 3E-H ; Fig. S2A, B; D, E). Electron microscopy also confirmed that 100 nM BPA/BPS treatment for 24 h drove mitophagy compared with the control group (Fig. 3I). Finally, we evaluated whether environmental BPs exposure triggered mitophagy in vivo. The results on immunofluorescent staining showed that the co-localization of TOM20 and LC3B was significantly enhanced in BPA/BPS-stimulated tumor tissues (Fig. 3J). These results provided evidence that low-dose BPA/BPS enhanced mitophagy by upregulating PINK1 in ovarian cancer cells.

### 3.4. BPA/BPS increased mitophagy in ovarian cancer in a Parkin-independent manner

We have verified that low-dose BPA/BPS enhanced mitophagy by upregulating PINK1, then we decided to investigate possible molecular contributions to the process. Since PINK1/Parkin-mediated mitophagy is a canonical mitophagy mechanism, we wonder whether Parkin could involve in this pathway. Unexpectedly, based on the sample data retrieved from Human Protein Atlas (HPA) and TCGA [54], in estrogen-related tumors including ovarian tumors, Parkin was expressed at a low level or even not detected (Fig. 4A). Moreover, the immunohistochemical results showed that the liver tumor tissue exhibited a high positive rate of Parkin, while the ovarian cancer tissue showed not (Fig. 4B). In tumor cell lines, ovarian cancer cells substantially did not express Parkin either, compared to SH-SY5Y cells as a positive control (Fig. 4C). In order to have a better understanding of the downstream proteins that may interact with PINK1 besides of Parkin, we screened the non-canonical mitophagy-related proteins that directly interact with PINK1 in the NCBI database, and drew gene mutation fingerprint for the 16 interacting proteins. The result showed that p53 was top-ranked (Fig. 5A). Co-IP also verified the crosstalk between PINK1 and p53, p-p53 (Fig. 5B). Treating SKOV3 cells with BPA/BPS, which enhanced mitophagy, decreased p53 protein level (Fig. 3E-H) and significantly inhibit the nuclear translocation of p-p53, which was reversed by the knockdown of PINK1 expression (Fig. 5C-H). Whereas no conspicuous changes were observed in cytoplasmic under BPA/BPS treatment. The conclusion was also verified in A2780 cells (Fig. S2A, B; D, E; Fig. S3E-J).

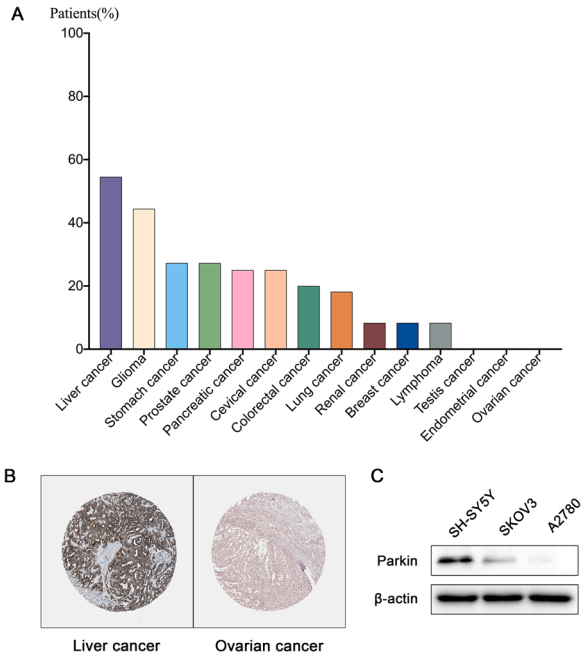


**Fig. 4.** Parkin relative expression level in different tumor tissues and cell lines. (A) Relative PRKN expression level in different tumor tissues. (B) Representative IHC image from ovarian cancer patient and liver cancer patient. (C) Representative western blot of SH-SY5Y, SKOV3, A2780 cell showing the expression of Parkin. β-actin served as the internal control.
The above data are compiled from The Human Protein Atlas, the original data can be obtained from https://www.proteinatlas.org/ENSG00000185345-PRKN/pathology.

### 3.5. Role of PINK1 in BPA/BPS-induced ovarian cancer cell stemness

Next, siRNAs were adopted to knock down PINK1 expression and further studied its role in BPA/BPS-induced ovarian cancer stemness. According to knockdown efficiency, siRNAs si-2 were adopted in subsequent experiments (Fig. S3A-D). Knockdown of PINK1 significantly reversed the increased expression of OCT4, NANOG, SOX2 enhanced by BPA/BPS. This phenotype was verified in both SKOV3 and A2780 cell lines (Fig. 6A-H; Fig. S4A-H). Immunofluorescence staining revealed a

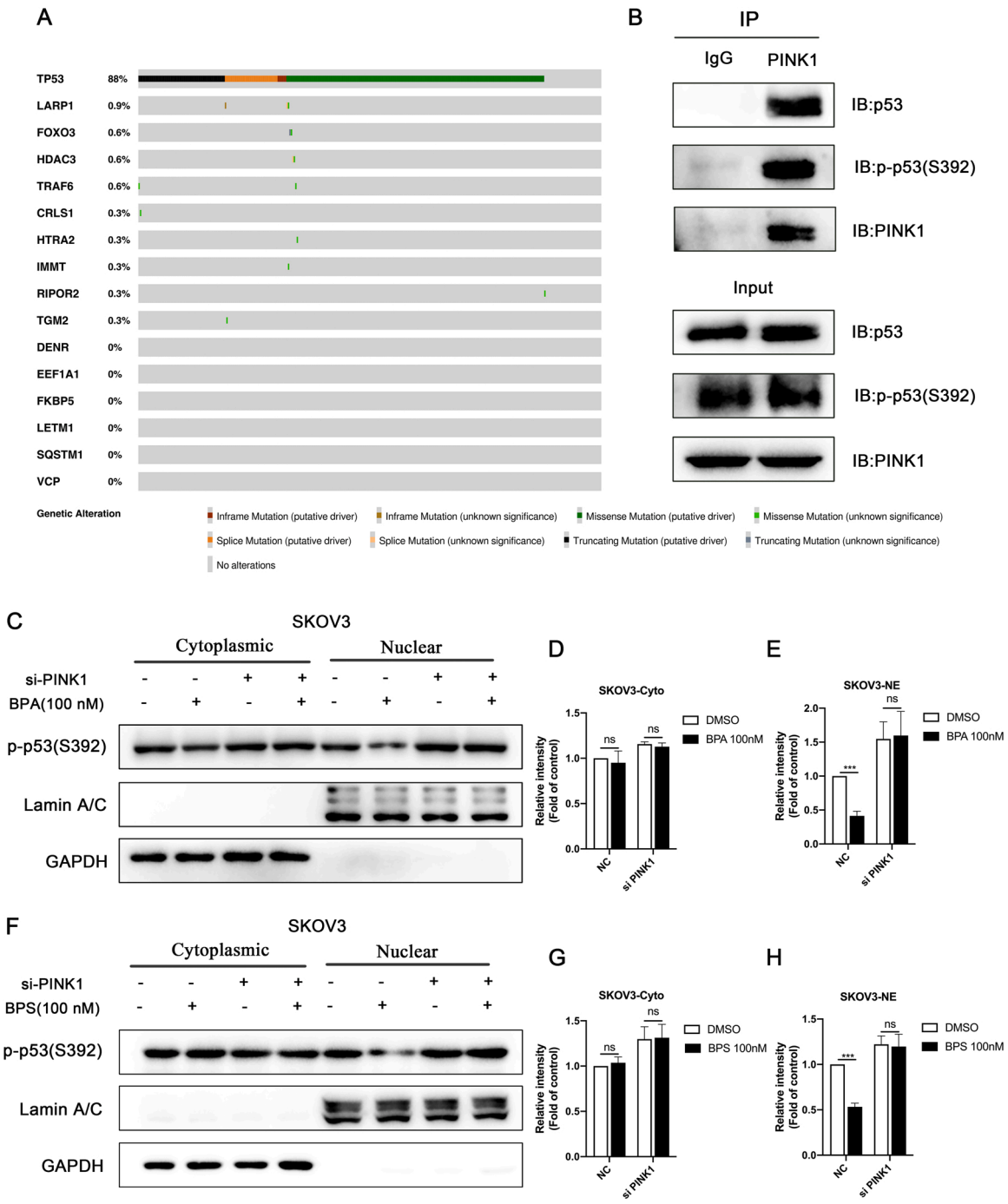


**Fig. 5.** BPA/BPS increased mitophagy in ovarian cancer in a Parkin-independent manner. (A) Profile of fingerprinting of non-canonical mitophagy-related proteins directly interacting with PINK1. (B) Co-immunoprecipitation of PINK1 with p53 and p-p53. (C-H) SKOV3 cells transfected with NC or si-PINK1 were treated with DMSO or 100 nM BPA/BPS for 24 h, and then the protein expression level of p-p53 in cytoplasmic extracts and nuclear extracts were detected by western blotting and then quantified. GAPDH and Lamin A/C were adopted as the cytoplasmic and nuclear loading control, respectively. ns, no statistical significance, *** $p < 0.001$, by Student's t-test compared with the control group ($n = 3$). Error bar indicates the mean ± SD.

similar conclusion (Fig. 6I-L; Fig. S4I-L). These findings demonstrated the pivotal role of PINK1 in the stemness of ovarian cancer under low-dose BPA/BPS exposure.

### 3.6. Mdivi-1 alleviated environmental BPA/BPS-induced cancer cell stemness via inhibiting mitophagy

Having established the concept that low-dose BPA/BPS could enhance ovarian cancer cell stemness via PINK1/p53 involved non-canonical mitophagy pathway, a specific mitophagy inhibitor Mdivi-1 was used to determine the particular effect of mitophagy on environmental BPA/BPS-caused cell stemness. The results showed that in the presence of BPA/BPS, p-p53 protein level decreased while that of CSC biomarkers (SOX2, OCT4, NANOG) increased. However, treating ovarian cancer cells with Mdivi-1 (10 μM) alleviates such effect (Fig. 7A-D; Fig. S5A-D). All in all, our results verified that environmental BPA/BPS exposure caused cell stemness via triggering p53 involved non-canonical mitophagy.

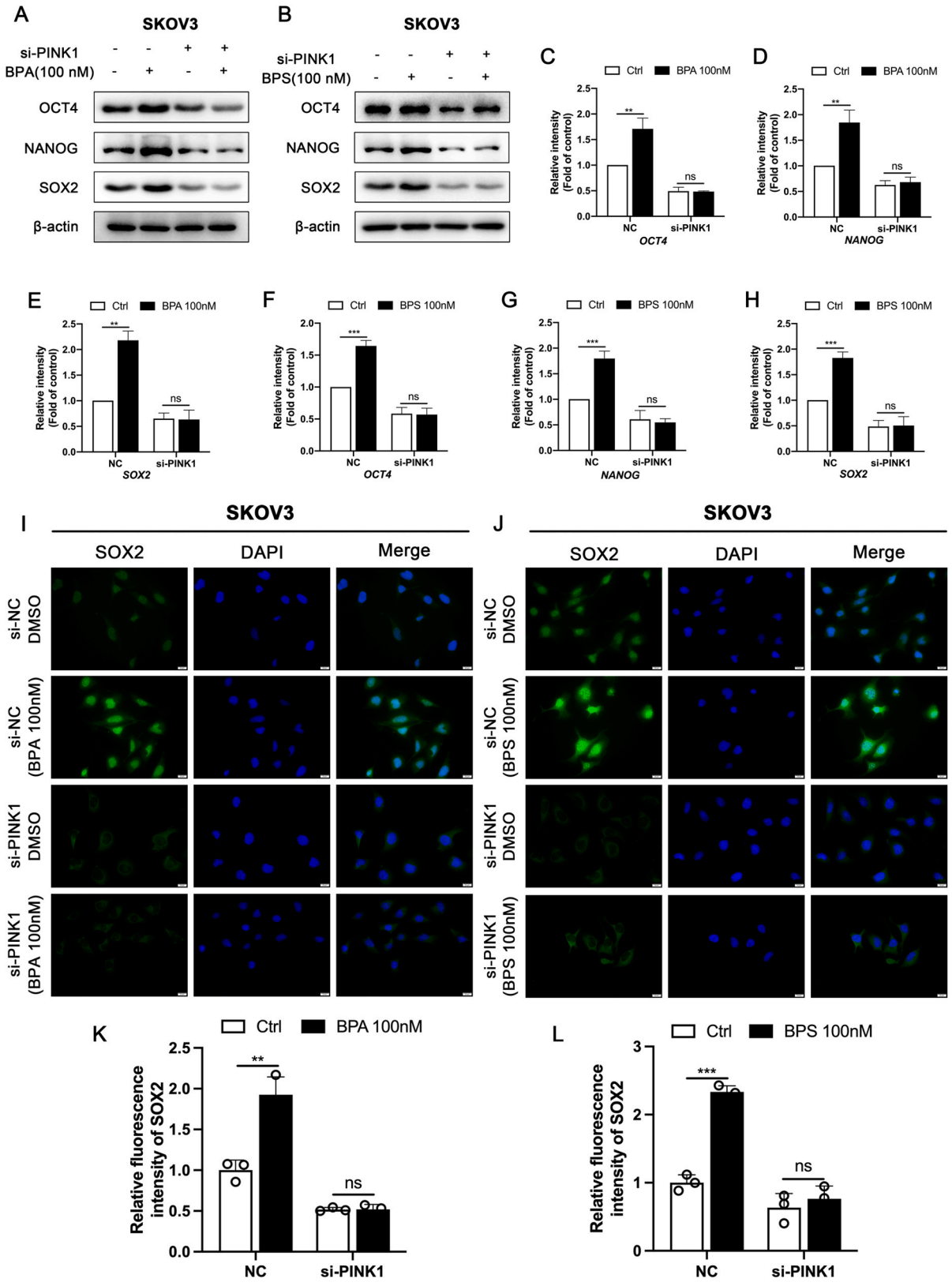


**Fig. 6.** Role of PINK1 in BPA/BPS-induced ovarian cancer cell stemness. (A, B) Representative western blot of SKOV3 cell showing the expression of OCT4, NANOG and SOX2 after 100 nM BPA/BPS treatment combined with PINK1 knockdown. β-actin served as the internal control. (C-H) Quantification of western blot detection of OCT4, NANOG and SOX2 in SKOV3 cells after treatment with 100 nM BPA/BPS combined with PINK1 knockdown. The relative intensity was analyzed with Image J and calculated by the ratio relative to the β-actin intensity. (I, J) Representative immunofluorescent images of SKOV3 cells under BPA/BPS (100 nM) treatment combined with PINK1 knockdown. (K, L) Quantification of immunofluorescence detection of SOX2 in SKOV3 cells under BPA/BPS (100 nM) treatment combined with PINK1 knockdown. The immunofluorescence intensity was analyzed with Image J software and calculated by the ratio relative to Ctrl group. Scale bar: 20 μm. ns, no statistical significance, ** $p < 0.01$, *** $p < 0.001$, by Student's t-test compared with control group ($n = 3$). Error bar indicates the mean ± SD.

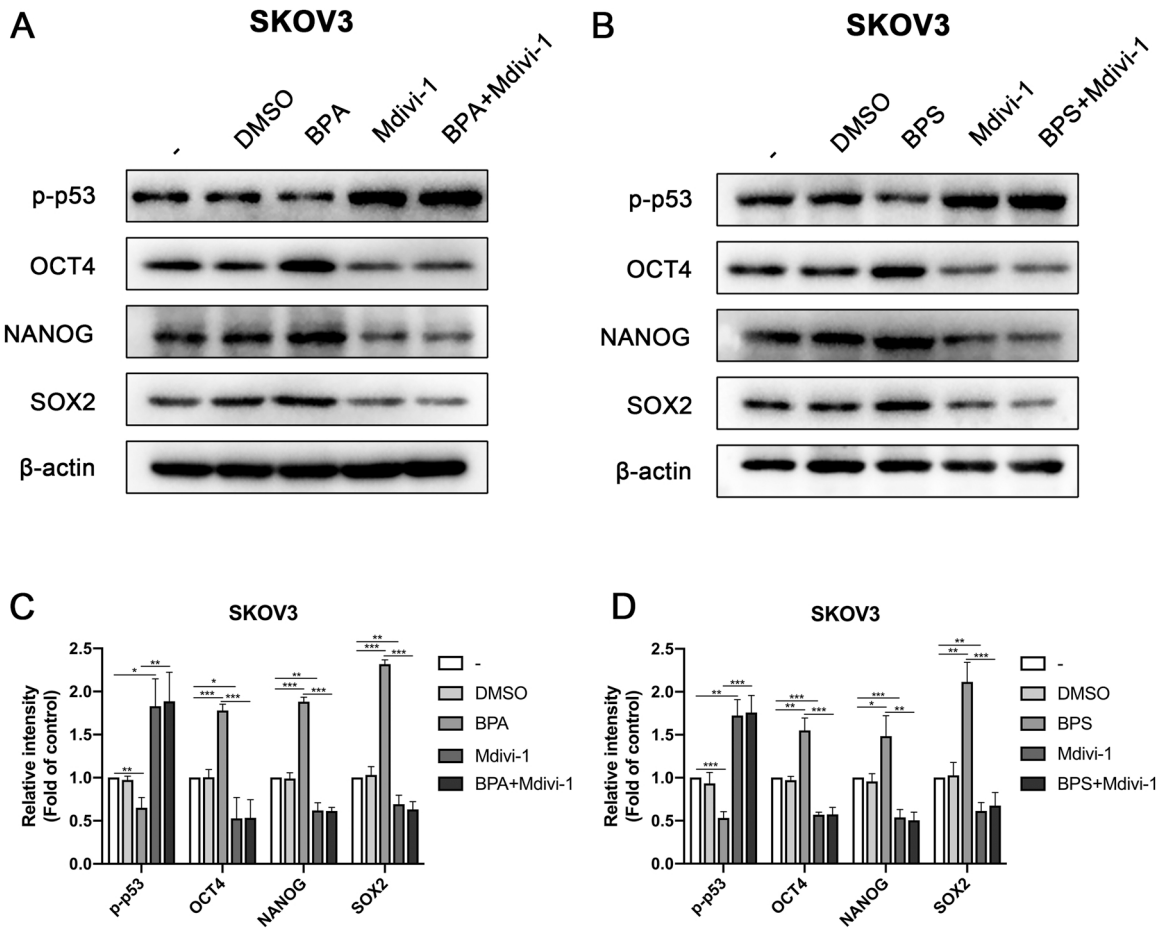


**Fig. 7.** Mdivi-1 alleviated environmental BPA/BPS-induced cancer cell stemness via inhibiting mitophagy. (A, B) Representative western blot of SKOV3 cell showing the expression of p-p53, OCT4, NANOG and SOX2 after treatment with BPA/BPS 24 h with or without pretreatment of Mdivi-1(10 μM) for 2 h. β-actin served as the internal control. (C, D) Quantification of western blot detection of p-p53, OCT4, NANOG and SOX2 in SKOV3 cells after treatment with BPA/BPS 24 h with or without pretreatment of Mdivi-1(10 μM) for 2 h. The relative intensity was analyzed with Image J software and calculated by the ratio relative to the β-actin intensity. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by Student's t-test compared with control group ($n = 3$). Error bar indicates the mean ± SD.

### *3.7. Low-dose BPA/BPS promoted ovarian cancer in a non-canonical mitophagy-dependent manner in vivo*

As mentioned above, BPA/BPS may promote tumor metastasis via a non-canonical mitophagy pathway in vitro. To test our hypothesis that exposure to BPA/BPS could also contribute to the development of ovarian cancer in vivo, an ovarian cancer xenograft metastasis model was established. The mice were euthanized 5 weeks after tumor cell injection (Fig. 8A). As shown in representative photographs of mice (Fig. 8B), the metastatic tumors could be observed in many organs such as intestine, liver and peritoneum which was also similar to the metastasis pattern of human ovarian cancer. The mice fed with BPA/BPS led an augmented number and weight of metastatic lesions compared with those in the control group, which indicated that BPA/BPS could promote ovarian cancer metastasis in vivo. Conversely, the administration of 25 mg/kg Mdivi-1 daily significantly attenuated BPA/BPS-induced increase of metastatic nodes (Fig. 8C, D). The evaluation of biomarkers for cell stemness showed that BPA/BPS treatment increased the expression level of SOX2 in tumor tissues, whereas Mdivi-1 could inhibit such effect (Fig. 8E; Fig. S6C). OCT4 and NANOG expression in the tumor also exhibited similar trends (Fig. S6A, B; D, E). These findings further confirmed that environmental BPA/BPS exposure could promote ovarian cancer development in a mitophagy-dependent manner in both vitro and vivo.

## 4. Discussion

The ubiquitous use of BPA and its substitute BPS leads to numerous adverse effects in humans such as reproductive system disorders [2,7], neural toxicity [47], endocrine disruption and metabolism alteration [45], but the role of BPA/BPS in tumor progression has remained to be explained. Based on studies from NHANES [19] and several Asian countries [75], the average exposure concentration of BPA and BPS in the general population is about 1–10 nM, and the upper limit is 25–140 nM [13,22]. In this present study, we found that low-dose BPA/BPS treatment upregulated the expression of OCT4, NANOG and SOX2 in a dose-dependent manner in both protein and mRNA levels, which are all key CSC regulators. It was noticed that 10 nM BPS could already increase SOX2 protein level while BPA showed such effect in 100 nM, which intimated that BPS may have stronger cancer stem cell inducing ability. In fact, although BPS was less estrogenic than BPA[4], BPS was characterized by more serious EDCs ability in many diseases [45]. BPS exhibited stronger catalytic efficacy on 17α-hydroxyprogesterone (17α-OH progesterone) [52].

Cancer stem cell concept was first reported 40 years ago, which was considered to be closely related to tumor growth and renewal of healthy tissues [1]. CSCs were first identified in acute myeloid leukemia [3] and then in various tumors including pancreatic [16], colon [44,51], ovarian [73], lung [10] and brain cancers [59]. Ovarian cancer patients with higher CSCs markers expression (SOX2, POU5F1, NANOG) also showed worse prognosis (Fig. S2 F-H). The relationship between EMT progress

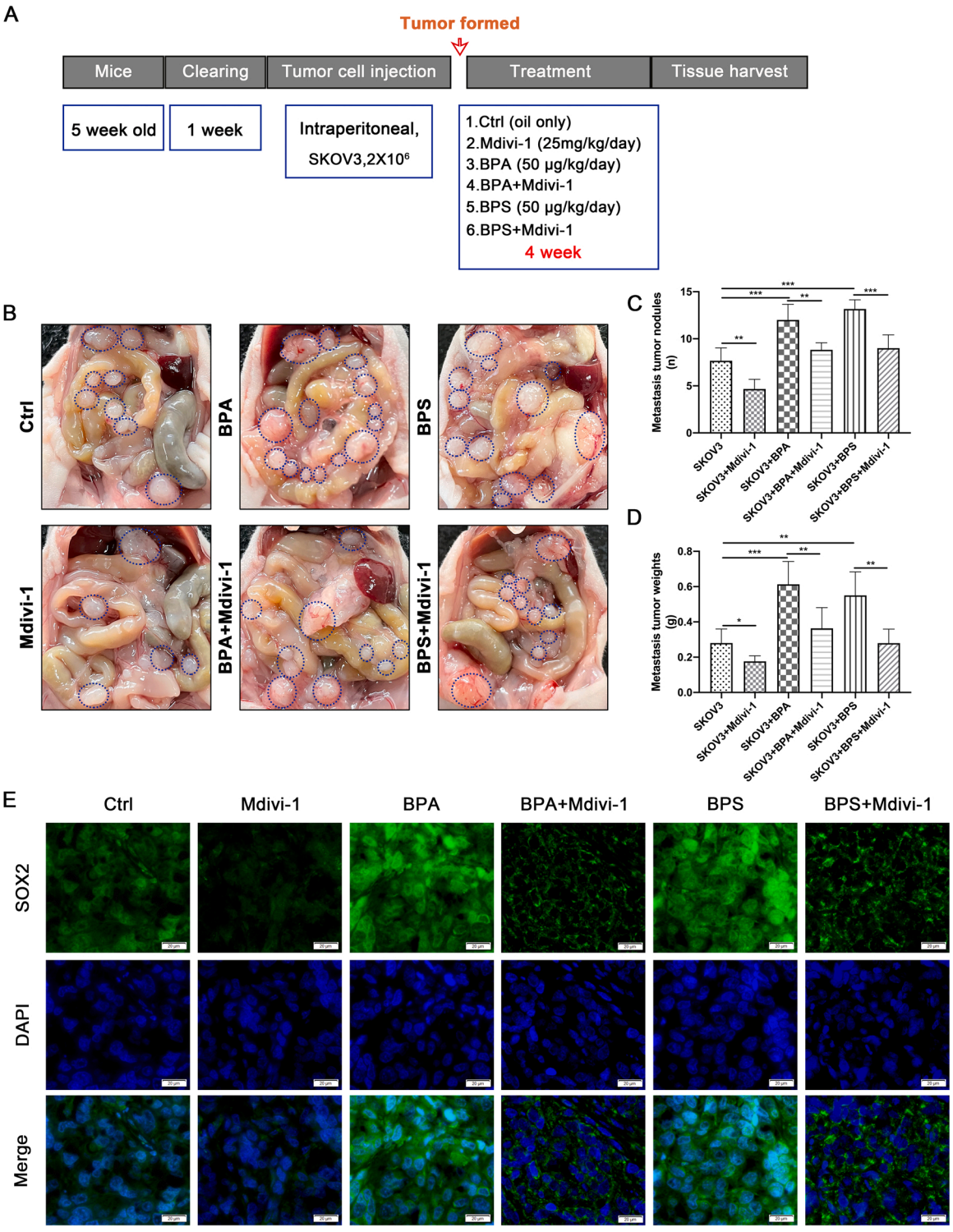


**Fig. 8.** Low-dose BPA/BPS promoted ovarian cancer in a non-canonical mitophagy-dependent manner in vivo. (A) Experimental scheme for BPA/BPS exposure period. (B) Representative images of the metastatic lesions in each group, marked with circles. (C) The average number of the metastatic lesions per mouse in each group. (D) The average weight of the metastatic lesions per mouse in each group. (E) IHC of SOX2 expressed in the metastatic tumor sections. Scale bar: 20 μm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by Student's t-test compared with control group ($n = 6$). Error bar indicates the mean ± SD.

and the cancer cell stemness has been primarily evaluated in the situation of cancer pathogenesis. Following the study in 2008 which implicated this procedure in the formation of breast CSCs [37], EMT has since been linked to CSCs in a variety of solid malignancies [58]. Apart from this, TGFβ/SMADs, Wnt/β-catenin and NF-κB pathways which have been proven essential for EMT program are also known to contribute critically to the "stemness" of cancer cells [34,56,69,71]. Therefore, the role of BPA in inducing stemness of ovarian cancer cells in the present study is consistent with our previous research, which indicated that a low dose of BPA could promote EMT of ovarian cancer cells significantly [20]. Given the perturbing evidence that BPS had a similar ability in cancer cell stemness inducing, we further examined the underlying mechanism.

Recently, some novel mechanisms that contributed to CSC

development have emerged. For instance, CSCs are usually characterized by a dysregulation of mitophagy [42]. Inhibition of DRP1 (a key mitophagy regulator) inhibits mouse embryonic fibroblast reprogramming after being provided with the Yamanaka factors (SOX2, OCT4, KLFF4) [67]. Another study showed that a novel inhibitor of mitophagy liensinine could sensitize breast tumors to chemotherapy in a DNM1L-dependent way [76]. Since we uncovered that BPA/BPS could enhance CSC property, then we continued to explore the underlying mechanism. Interestingly, our established ATdb tool was used to find that the progression of ovarian cancer was significantly associated with the critical key mitophagy activating kinase PINK1, which could be upregulated in ovarian tumor cells after BPA/BPS exposure. Therefore, those phenotypes mean BPA/BPS may increase tumor stemness via PINK1-involved mitophagy.

Previous studies proposed that PINK1/Parkin cascade mediated mitophagy is regarded as one of the most well-characterized pathways [15,72,9]. Once mitochondria are damaged under specific stimulation, the initiating molecular PINK1 stabilizes at the outer mitochondrial membrane (OMM) and recruits its direct substrate E3 ubiquitin ligase Parkin, which is an amplified signaling molecule for canonical mitophagy. PINK1/Parkin cascade activation can remove damaged mitochondria to maintain cell homeostasis [41], while the non-canonical mitophagy pathway only requires the initiation of PINK1 to recruit other substrate proteins to complete [68]. Although our results confirmed BPA/BPS-induced PINK1 activation, Parkin was not involved in our further investigation due to the expression of parkin was undetectable in both SKOV3 and A2780 cells according to our experiments (Fig. 4C). Furthermore, the immunohistochemistry analyses of HPA database also confirmed parkin expression level is almost unobserved in estrogen-related cancers, especially in ovarian cancer. (Fig. 4A, B). Therefore, we screened the non-canonical mitophagy-related proteins that have been reported directly interacting with PINK1 in the NCBI database of the past ten years, and drew a gene mutation fingerprint map (cBioPortal) for 16 candidate genes, TP53 was top-ranked. Recent studies also suggested that PINK1 may act in its mitophagy regulation effects via p53. In murine colon tumor cells, PINK1 overexpression promoted mitophagy by decreasing glycolysis and increasing mitochondrial respiration potentially via p53 activation [70]. In addition, Liu. et al. showed that PINK1 binds to p53 on mitochondria and phosphorylates p53 at serine-392 [32]. But no research yet was focused on the relationship between EDCs like BPA/BPS and cancer stem cells through non-canonical mitophagy. Based on this, we furtherly carried out Co-IP experiments, which strongly confirmed the endogenous interaction between PINK1 and p-p53, p53 protein in ovarian cancer cells. We creatively noticed the critical role of non-canonical PINK1/p53 mitophagic signaling in cancer stemness induced by low-dose BPs.

Exposure to BPA and BPS enhanced tumor cell stemness in both vitro and vivo. It should be noted that in ovarian cancer cell lines, our present study proposed enhanced mitophagy induced by PINK1 is essential for ovarian tumor stemness which can be attenuated by whether PINK1 knockdown or Mdivi-1 treatment. Mdivi-1 is a mitophagy inhibitor that prevents mitochondrial fission via targeting DRP1[61], and the accumulation of DRP1 on mitochondria leads to a dramatic mitochondrial division which is similar to PINK1 OMM stabilization. In our ovarian cancer xenograft metastasis model, intraperitoneal injection of Mdivi-1 also declined BPA/BPS-induced SOX2 overexpression.

In summary, our present study firstly investigated the regulation effect of BPA and its substitute BPS on the stemness of ovarian cancer cells through PINK1/p53 induced non-canonical mitophagic pathway. Our data showed that BPA/BPS-promoted ovarian cancer cell stemness was tightly associated with enhanced mitophagy which was driven by PINK1 activation. In addition, the orchestrated activation of PINK1 by BPA/BPS may be an important process for inhibiting p-p53 nuclear translocation. Interestingly, the blockade of PINK1 appeared to retard the CSC phenotype. Correspondingly, mitophagy inhibitor Mdivi-1 could also attenuate BPA/BPS-induced cancer cell stemness in vitro and tumor metastasis in vivo. These findings may have important implications for understanding the biology of cancer cell stemness and the role of mitophagy in ovarian cancer progression induced by BPA/BPS, which could provide novel evidence for the evaluation and preventive strategy of Bisphenols.

## 5. Conclusions

In summary, the present research demonstrated for the first time the effect of BPA/BPS exposure on ovarian cancer cell stemness. BPA/BPS treatments evaluated CSC marker expression including OCT4, NANOG and SOX2, such effect was notably attenuated by the knockdown of PINK1 or Mdivi-1 treatment. The findings from in vivo experiments supported that environmentally relevant BPA/BPS exposure promoted ovarian tumor metastasis. This study provides evidence that environmental concentrations of BPA/BPS regulate cancer cell stemness and tumor metastasis via a novel PINK1/p53 involved non-canonical mitophagic pathway.

## CRediT authorship contribution statement

**Xiaoyu Yuan:** Project administration, Formal analysis, Methodology, Writing – original draft. **Kelie Chen**: Data curation, Software, Methodology, Writing – original draft. **Fang Zheng:** Methodology, Formal analysis, Validation. **Sinan Xu**: Methodology, Formal analysis, Writing – review & editing. **Yating Li**: Formal analysis, Validation. **Yuwei Wang:** Data curation, Software, Methodology. **Heng Ni**: Validation, Methodology. **Fang Wang**: Software, Formal analysis, Investigation. **Zhenyan Cui**: Project administration. **Yuheng Qin**: Project administration. **Dajing Xia**: Conceptualization, Supervision, Project administration, Funding acquisition. **Yihua Wu**: Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

We declare we have no actual or potential competing financial interests.

## Data Availability

Data will be made available on request.

## Acknowledgments

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*Environmental Implication*

There are many sources for Bisphenol A (BPA) and its substitute Bisphenol S (BPS) exposure such as food, beverage containers and many other usages production. The environmental level of BPA/BPS exposure is still a threat to public health, yet understanding the potential tumor progression impact of bisphenols (BPs) remains challenging. Here, we found a novel pathway accounting for the promotion effect induced by BPA/BPS in ovarian cancer. Our findings contributed to the understanding of the effects and mechanisms of BPA/BPS exposure and further raised concerns about the potential population hazards of

bisphenol analogs.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.131288.

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