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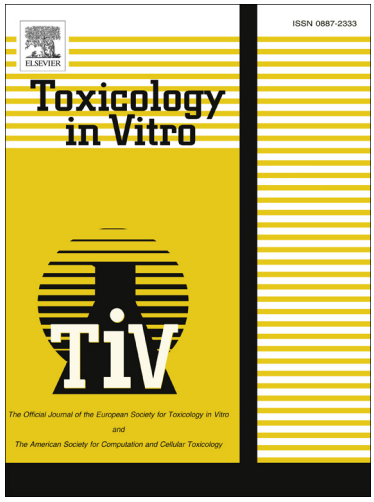

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Effect of bisphenol A on pluripotency of mouse embryonic stem cells and differentiation capacity in mouse embryoid bodies

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Abstract

Bisphenol A (BPA) poses potential risks to reproduction and development. However, the mechanism of BPA's effects on early embryonic development is still unknown. Embryonic stem cells (ESC) and embryoid bodies (EB) provide valuable *in vitro* models for testing the toxic effects of environmental chemicals in early embryogenesis. In this study, mouse embryonic stem cells (mESC) were acutely exposed to BPA for 24h, and general cytotoxicity and the effect of BPA on pluripotency were then evaluated. Meanwhile, mouse embryoid bodies (mEB) were exposed to BPA up to 6 days and their differentiation capacity was evaluated. In mESC and mEB, we found that BPA up-regulated pluripotency markers (*Oct4*, *Sox2* and *Nanog*) at mRNA and/or protein levels. Moreover, BPA increased the mRNA levels of endodermal markers (*Gata4, Sox17*) and mesodermal markers (*Sma, Desmin*), and reduced the mRNA levels of ectodermal markers (*Nestin, Fgf5*) in mEB. Furthermore, microRNA(*miR)-134,* an expression inhibitor of pluripotency markers including *Oct4*, *Sox2* and *Nano*g, was decreased both in BPA-treated mESC and mEB. These results firstly indicate that BPA may disturb pluripotency in mESC and differentiation of mEB, and may inhibit ectodermal lineage differentiation of mEB while *miR-134* may play a key role underlying this effect.

Keywords:

Bisphenol A; Mouse embryonic stem cells; Mouse embryoid bodies; Pluripotency; Differentiation; MicroRNA

Abbreviations:

BPA, bisphenol A; mESC, mouse embryonic stem cells; mEB, mouse embryoid bodies; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-*di*-phenytetrazoliumromide; AP, alkaline phosphatase.

1. Introduction

Bisphenol A (BPA), a manufactured chemical, is a component of polycarbonate plastics and epoxy resins and is found in medical devices, canned food and other food and drink packaging (Jurewicz and Hanke, 2011). BPA is one of the highest-volume chemicals produced globally with > 8 billion pounds produced each year (Vandenberg et al., 2010). Human exposure to BPA is widespread. The primary route of exposure to BPA for the human in daily life is through the diet (Jurewicz and Hanke, 2011). BPA can be detected in human blood, plasma, urine, saliva, amniotic fluid, placental tissue, follicular fluid, breast milk, and adipose tissue (Vandenberg et al., 2010). Many studies have shown that BPA poses potential risks to reproduction and development due to its estrogenic effects (Susiarjo et al., 2007; Itoh et al., 2012). Aoki and Takada showed that BPA might affect ovarian and testicular development as well as germ cell differentiation, and appeared to induce genes responsible for ovary development (Aoki and Takada, 2012). Moreover, experimental and animal studies have demonstrated that BPA negatively affected early embryonic development (Berger et al., 2007; Ehrlich et al., 2012).

However, the exact mechanism of BPA's effects on early embryonic development is still limited.

Mouse embryonic stem cells (mESC), derived from the inner cell mass of blastocysts, have unlimited capacity for self-renewal, pluripotency, and differentiation. Some markers of stem cell pluripotency, including *Oct4*, *Sox2*, *Nanog, and* alkaline phosphatase (AP), are important for maintaining self-renewal and pluripotency (Chambers and Smith, 2004; Wang et al., 2006). mESC were isolated from pre-implantation embryos and can be cultured in an undifferentiated status. Thus, it is an appropriate model for pre-implantation toxicity screening. Many studies have stated that embryonic stem cells (ESC) have been widely used in the studies of early embryonic development and the pluripotency state (Moorthy et al., 2005; Vallier and Pedersen, 2005; Sroczynska et al., 2009; Balmer et al., 2012).

Upon withdrawal of feeder cells and LIF in suspension cultures on non-adhesive dishes, mESC spontaneously differentiate and form mouse embryoid bodies (mEB), which consist of the three primary germ layers (ectoderm, mesoderm, and endoderm). The formation of mEB *in vitro* is similar to the early post-implantation stage of embryogenesis *in vivo* (Sajini et al., 2012). They are often used l to study the differentiation and gene expression during early embryonic development (O'Shea, 2004; Deshpande et al., 2012).

Previous literature has stated that ESC and EB are valuable models in toxicological studies and can be used to assess the adverse effects of environmental chemicals on early embryogenesis (Kim et al., 2006; Jung et al., 2010; Talbot and Lin,

2011; van Dartel et al., 2011). However, previous studies have not comprehensively examined the effects of BPA on mESC or mEB.

MicroRNAs (miRNAs) play a central role in a wide variety of key biological processes including the maintenance of stem cell pluripotency and differentiation. Recently, microRNA-134 (*miR-134*) was shown to target and down-regulate *Oct4*, *Sox2* and *Nanog* in mESC and is a powerful inducer of pluripotent stem cell differentiation (Gaughwin et al., 2011; Tay et al., 2008a; Tay et al., 2008b).

In the present study, in order to understand the mechanism of BPA effects on early embryonic development, we tested the effects of BPA on general cytotoxicity, pluripotency and differentiation using mESC and mEB as models, and further explored the potential role of *miR-134* in BPA-induced effects.

2. Materials and methods

2.1. Chemicals and reagents

BPA (CAS NO. 80-05-7, 99.5% purity) was purchased from Dr. Ehrenstorfer GmbH (Bürgermeister-Schlosser, Augsburg, Germany). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), diethylpyrocarbonate (DEPC), 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). BPA was first dissolved in DMSO and then adjusted to a concentration of 100mM with mESC medium as stock

solution which was further diluted to lower concentrations for testing. In formal experiments, the final DMSO concentration was 0.1%. All chemicals were of analytical grade.

2.2. Cell culture and BPA treatment

D3 mouse ES cells were a gift from Cell Institute of Shanghai, Chinese Academy of Sciences (Shanghai, China). Undifferentiated mESC were grown on mitomycin C-treated mouse embryonic fibroblast feeder cells (MEF) in knock-out Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 20% ES qualified fetal bovine serum (Gibco BRL), 0.1mM nonessential amino acids (Gibco BRL), 0.1mM β-mercaptoethanol (Sigma Chemical, St Louis, MO), 2mM L-glutamine (Gibco BRL), 1mM pyruvate sodium, 100 unit/ml penicillin/streptomycin (Gibco BRL) and1000 U/ml of leukemia inhibitory factor (LIF) (Millipore, Billerica, MA). Cells were maintained at 37°C and 5% CO_2 in humidified air, received fresh medium every day, and were passaged every 3 days. Cells were dislodged using trypsin/EDTA (Gibco BRL). Before the start of experiments, the ES cells cultures were depleted of feeder cells by incubating trypsinized cells in complete ES cells medium on cell culture dishes for 30 min, during which time feeder cells attached to the dish. Feeder cells were again depleted before counting and plating at the appropriate density. The ES cells were then plated on gelatin-coated dishes, and cultured overnight at 37°C in a 5% CO_2 incubator with a

relative humidity of 95%. To induce EB formation, feeder depleted ES cells were dissociated into a single-cell suspension, and cultured in ES cell medium without LIF. ES cells were plated in non-adhesive dishes at a density of about 5×10^5 per well in 6-well dishes.

For chemical treatment, ES cells were plated in 6-well, 24-well or 96-well culture plates and grown for 1 day. Then, ES cells were treated with BPA (0.04μM, 1μM, 25μM, and 100μM) dissolved in DMSO for 24h. As a negative control, ES cells were also exposed to 0.1% DMSO alone. mEB were cultured with BPA (0.04μM, 1μM, 25μM, and 100μM) or 0.1% DMSO (control) in non-adhesive dishes up to 6 days and used for gene and protein expression analysis on days 2, 4 and 6.

2.3. Cell viability assay and morphological study

Feeder depleted ES cells were seeded on gelatin coated plates at a density of about 1.5×10^4 per well in 96-well plates and 1×10^6 per well in 6-well plates and incubated overnight. ES cellular viability was evaluated using the MTT proliferation assay in a dose range of 0.04-100μM. MTT (5 mg/ml) was dissolved in PBS, sterilized by filtration through a 0.22 μm Millipore® filter and stored at 4 °C. After exposing the cells to BPA at different concentrations, the cells were washed twice with PBS. Then 25μl of MTT were added to each well, and the cells were incubated for another 4h at 37°C to allow MTT metabolism. The medium was replaced with 150μl DMSO, and plates were shaken for 10 min. The absorbance was determined at

490 nm. Results were presented as percentage of the control. The cells (ESC and EB) were examined and recorded morphologically using phase contrast microscope (Olympus, CK41, Japan) after exposure to BPA (0.04μM, 1μM, 25μM, and 100μM) or control medium (0.1% DMSO).

2.4. Cell cycle analysis and apoptosis assay

To determine if BPA could affect the cell cycle and induce apoptosis of mESC, flow cytometric analysis was used to determine the state of cell cycle and the DNA fragmentation. The feeders were depleted twice, and ES cells were seeded on gelatin coated 6-well plates at a density of about 1×10^6 cells per well. Cells were incubated overnight and subsequently exposed to BPA (0.04μM, 1μM, 25μM, and 100μM) and control medium containing 0.1% DMSO. After 24h, cells were washed with PBS and harvested with trypsin/EDTA. Cells were fixed in 75% ethanol for 2h or washed in cold PBS, and then stained with propidium iodide (PI) and annexin V for 30min protected from light. The fixed/stained cells were analyzed by FACSCalibur Flow Cytometry (BD Biosciences, NJ, USA) to quantify cell cycle or cell apoptosis.

2.5. Alkaline phosphatase staining and activity assay

Alkaline phosphatase (AP) staining was performed with the Alkaline Phosphatase Complete Kit (Sidansai Corporation of Biotechnology, Shanghai, China)

according to the manufacturer's protocol. The cells were washed twice with PBS and fixed for 2 min with fixing solution at room temperature. Then, the cells were washed with PBS and incubated with AP staining solution for 30 min, protected from light. After washing with PBS, the cells were photographed using a microscope (Olympus, CK41, Japan).

AP activity was determined from whole cell lysates by analyzing the rate of p-nitrophenyl phosphate disodium hexahydrate (pNPP) hydrolysis. AP activity was normalized to cellular protein levels determined using the Bradford Kit (Beyotime Institute of Biotechnology, Shanghai, China). The pNPP product was generated using a p-nitrophenol phosphate stock (Cell Biolabs, Inc., San Diego, CA, USA) and analyzed according to the manufacturer's instructions to produce a soluble yellow end product that was determined spectrophotometrically. The pNPP reaction was then terminated using the stop solution and immediately analyzed at 405 nm on a microplate reader and compared against a standard curve.

2.6. RNA isolation and quantitative real-time PCR assay

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Dried RNA pellets were dissolved in ddH_2O containing 0.1% DEPC, and quantified by measuring the absorbency at 260 nm. All real-time PCR reactions were carried out on ABI7900 Fast Real-Time System (Applied Bio systems, Foster City, CA, USA) according to the manufacturer's instructions for quantification of gene expression.

cDNA synthesis for coding genes was performed with 1 μg of total RNA according the manufacturer's instructions (Takara, Tokyo, Japan). mRNA levels of pluripotency markers were measured with TaqMan probes and PCR Master Mix, and mRNA levels of differentiation markers were measured using SYBR PCR Master Mix reagent kits (Takara). The housekeeping gene *GAPDH* was used as an internal control. cDNA synthesis for miRNAs was performed with 1 μg of total RNA according to the manufacturer's instructions (Applied Biosystems, CA,USA). TaqMan®MicroRNA Assays (Applied Biosystems, CA, USA) were used as the probe for *mmu-miR-134* and U6 which acted as an internal control. All primer sequences are given in Supplemental Table S1.

2.7. Western blot analysis

The total cellular proteins (60μg) were solubilized in the sample buffer (25 mMTris, pH 6.8, 1% SDS (w/v), 5% β-mercaptoethanol(v/v), 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue), and then fractionated by electrophoresis on a 12.3% polyacrylamide-SDS gel at 90V for 3 h. The proteins were then transferred to a polyvinyldiene difluoride membrane (PVDF, Bio-Rad, Hercules, CA). The membrane with transferred proteins was incubated in buffer containing specific rabbit polyclonal antibodies for Sox2/Nanog or goat polyclonal antibodies for Oct4 (Abcam, Kendall square, MA, USA, 1:1000 dilution), followed by incubation with goat anti-rabbit or donkey anti-goat secondary antibody conjugated with horseradish peroxidase at

1:1000. The specific signals were detected by the enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Life Science Limited). The amount of GAPDH (34 kDa) in each lane was used as a control to correct the expression of Oct4 protein (45kDa); Sox2 (43kDa) and Nanog (35kDa) protein.

2.8. Data analysis

Values are expressed as mean ± standard error of the mean (S.E.) for all experiments. Statistically significant differences between the treatments and the control were determined by one-way ANOVA, followed by Dunnett's multiple comparison test. All tests of statistical significance were two-sided, and the statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of BPA on cell viability and morphology in mESC

To examine the effect of BPA on cell viability and morphology, D3 mESC were exposed to various concentrations of BPA for 24h and 48h. Cell viability was assessed quantitatively using the MTT assay. As shown in Fig.1A, after 24h of exposure, BPA treatment did not significantly affect cell viability in the range of the concentrations used in this study. However, after 48h of exposure, a significant decrease in viability

was observed at doses of 5μM, 25μM and 100μM (Fig.1B). Since cytotoxic effects were not observed during 24h of treatment, in all of the following experiments, cells were exposed to BPA for 24h. As shown in Fig.1C, after 24h of BPA treatment, the morphology of mESC with and without BPA treatment was similar.

3.2. Effects of BPA on cell cycle and apoptosis in mESC

We examined the effect of BPA on the cell cycle and apoptosis during 24h of exposure using flow cytometery. We found no significant differences in cell cycle between treatment groups and the control group (Fig.S1 A and B), nor were there significant differences in apoptosis between treatment groups and the control group (Fig.S1 C and D).

3.3. Effects of BPA on alkaline phosphatase staining and activity in mESC

To determine if BPA influenced pluripotency, we stained mESC with alkaline phosphatase (AP) and quantified AP activity. Differentiation of mESC is characterized by loss of AP staining and appearance of cells with a flattened cellular morphology (Ivanova et al., 2006). In our experiments, mESC treated with BPA for 24h were similar to the control with deep staining and full colony morphology (Fig.S2A). Additionally, the *in vitro* assay showed no significant differences in AP activity between the treatment and control groups (Fig. S2B).

3.4. Effects of BPA on the pluripotency state in mESC

To determine if mESC maintain their pluripotency during BPA exposure, the expression levels of pluripotency-associated transcription factors were evaluated at the mRNA and protein levels. The mRNA levels of *Oct4* and *Sox2,* but not *Nanog,* were significantly increased after BPA treatment (Fig.2A-C). The levels of Oct4 and Sox2 proteins were likewise increased in a dose-dependent manner in response to BPA treatment. Yet, when compared with the control, Nanog only showed a significant increase at the protein level (Fig. 2D).

3.5. Effects of BPA on the expression of miR-134 in mESC

Previous studies revealed that miRNAs play a major role in silencing pluripotency markers (Sinkkonen et al., 2008)**.** To explore the mechanism by which BPA disturbed the pluripotency in mESC, miRNA expression was evaluated in mESC with and without BPA treatment. As shown in Fig.2E, the expression of *miR-134* was dose-dependently decreased by BPA exposure.

3.6. *Effects of BPA on differentiation capacity in mEB*

The effects of BPA on differentiation in mEB were examined using the

suspension method. The presence of BPA in the medium did not prevent mEB formation. mEB of control and BPA treated groups appeared morphologically similar (Fig.S3).

mEB were collected after 2, 4 and 6 days of culture in various concentrations of BPA (0.04μM, 1μM, 25μM, and 100μM) or DMSO as control. The expression levels of the pluripotency markers (*Oct4, Sox2 and Nanog)* were analyzed by qRT-PCR and Western blotting (Fig.3A-D). Treatment with BPA increased *Oct4 and Nanog* mRNA levels at 2 days and 6 days in a dose-dependent manner. However, a significant increase of *Oct4 and Nanog* mRNA levels at 4 days was only observed at the highest concentration group (100μM). *Sox2* mRNA levels were significantly increased by BPA treatment at 4 days and 6 days only at the highest concentration group (100μM). Oct4 protein levels were dose-dependently increased at 2, 4 and 6 days. Nanog and Sox2 protein levels were increased significantly after 4 days and 6 days, but not after 2 days of treatment. In addition, the mRNA levels of the three germ layers markers were analyzed by qRT-PCR (Fig.3E). The mRNA levels for endoderm markers (*Gata4 and Sox17*) and for mesoderm markers (*Sma and Desmin*) were significantly increased dose-dependently by BPA treatment. In contrast, the mRNA levels for ectodermal differentiation markers (*Nestin and Fgf5*) were decreased during treatment.

3.7. Effects of BPA on the expression of miR-134 in mEB

To explore the molecular mechanism for the change of differentiation capacity, similar to mESC, we also analyzed the expression of *miR-134* in mEB. As shown in Fig.3F, BPA at 25μM and 100μM significantly reduced the expression of *miR-134* after 2 days and 6 days. At 4 days, this reduction was only observed at the highest concentration group (100μM).

4. Discussion

This is the first study to comprehensively explore the effects of BPA on mESC and mEB, and the possible underlying mechanism at concentrations that did not induce a response in the MTT assay or increase apoptosis. This provides us a deeper understanding on the mechanism of BPA's effects on early embryonic development. Exposure to BPA increased pluripotency markers at the mRNA and/or protein levels in mESC and mEB, and decreased expression levels of ectodermal markers in mEB. *miR-134* may play an important role in modulating mESC pluripotency and mEB differentiation through its ability to regulate the expression of pluripotency markers (*Oct4, Sox2* and *Nanog)* (Tay et al., 2008a; Tay et al., 2008b).

Oct4 (the POU transcription factor encoded by Pou5f1), *Nanog* (the natural killer-2 class homeobox transcription factor,) and *Sox2* (the SRY family member), are master regulators of pluripotency. Recent studies suggest that *Oct4,* Sox2, and *Nanog* form feedforward loops that maintain pluripotency in mESC (Niwa, 2007; Chen et al., 2008). The precise level of Oct4 governed the fate of ES cells, and up- or

downregulation induced divergent developmental programmes (Niwa et al., 2000). Some endocrine disruptors, such as BPA, estradiol (E2), octylphenol (OP) and nonylphenol (NP), increased *Oct4* expression at the transcriptional level (Jung et al., 2010; Aoki and Takada, 2012). Similarly, in the current study, the mRNA and protein levels of pluripotency markers (*Oct4* and *Sox2)* in mESC were significantly increased following acute exposure to BPA. While *Nanog* mRNA levels did not increase, the protein levels did, which may due to the post-transcriptional inhibition by *miR-134*.This finding is consistent with the prior report (Tay et al., 2008b). BPA, an endocrine disruptor, could mimic the action of estrogen by binding to non-classical nuclear receptors (Takeda et al., 2009; Vandenberg et al., 2009). It is possible that the increased *Oct4* and *Nanog* levels induced by BPA may involve estrogenic activation of orphan nuclear receptors, such as the estrogen-related receptor beta (Esrrb), which is involved in embryonic pluripotency in mESC through coordination with *Nanog* and *Oct4* (Zhang et al., 2008). Thus, a further investigation is required to clearly elucidate the possible molecular mechanism.

EB closely mimic the post-implantation stage of mouse embryonic development and also serve as the starting point of differentiation of stem cells into various lineages (Tripathi et al., 2011). Stages equivalent to the beginning of gastrulation occur within the first 2 days of EB differentiation. EB (3~5 days differentiation) contain cell lineages found in embryos during gastrulation at 6.5 to 7.0 days post-conception. After 6 days in culture, EB are equivalent to early organogenesis-stage embryos (Leahy et al., 1999). During embryogenesis, the balance

between pluripotency and differentiation in time and space is very important. We observed the effect of BPA on mEB up to 6 days. The expression of pluripotency markers (*Oct4*, *Sox2* and *Nanog)* at the mRNA and/or protein levels were significantly increased in BPA-treated mEB. However, some studies have demonstrated the expression of pluripotency markers decreased following EB formation from ESC during differentiation (Desbaillets et al., 2000; Choi et al., 2005). BPA treatment might disturb differentiation of mEB by keeping high expression levels of pluripotency markers in mEB. These results are in line with a report in which the investigators found that EtOH could delay the decline of pluripotency markers during differentiation (Arzumnayan et al., 2009). Our findings showed that BPA increased the expression levels of endodermal markers (*Gata4, Sox17*) and mesodermal markers (*Sma, Desmin*), and reduced the mRNA levels of ectodermal markers (*Nestin, Fgf5*). These data suggest that BPA enhances differentiation toward endoderm and mesoderm, and inhibits differentiation to ectodermal lineages. Our data support the studies showing that prenatal exposure to BPA resulted in aberrant neuronal network formation in mouse fetuses apparently due to a decrease in the number of neural stem/progenitor cells after BPA exposure (Nakamura et al., 2007; Komada et al., 2012). Niwa et al. stated that increases of *Oct4* in expression caused differentiation into primitive endoderm and mesoderm which was also consistent with our data (Niwa et al., 2000).

miRNAs are thought to regulate more than a third of all protein coding genes, and they have been implicated in the control of virtually all biological processes,

including processes in stem cells (Mallanna and Rizzino, 2010; Lin et al., 2012; Liu et al., 2012; Yu et al., 2012). miRNAs are important parts of the core ES cell transcriptional regulatory network and play critical roles in regulating the self-renewal and pluripotency (Marson et al., 2008; Wellner et al., 2009). *miR-134* is transcribed from a neuronally expressed miR gene cluster at the mouse Dlk1-Gtl2 domain (Seitz et al., 2004; Khudayberdiev et al., 2009). During mouse embryogenesis *in vivo*, *miR-134* expression increases between E13.5 and E17.5 which was correlated with neuronal proliferation, migration and differentiation (Tay et al., 2008b). Recently, *miR-134* has been identified as a potential regulator of dendritic spine volume and synapse formation in mature rat hippocampal neurons (Schratt et al., 2006).*miR-134* can target and down-regulate *Oct4*, *Sox2* and *Nanog* in mESC (Tay et al., 2008a; Tay et al., 2008b). Our data demonstrated suppression of *miR-134* and increasing expression levels of Oct4, Sox2 and Nanog in BPA-treated mESC. Moreover, *miR-134* promoted ectodermal lineage differentiation of mEB (Tay et al., 2008b). Interestingly, our study found that reduced levels of *miR-134* was corresponding to the decreasing mRNA levels of *Fgf5* and *Nestin* markers associated with differentiation toward ectoderm in BPA-treated mEB. Our data supported the idea that BPA disturbed differentiation of mEB, and specially inhibited ectodermal lineage differentiation of mEB by reducing *miR-134* level.

BPA levels in biotic tissues vary widely from 0 to 1934.85 ng/ml. In our study, we chose 0.04μM and 1μM as the exposure doses which represented occupational and non-occupational exposure of human, respectively (Wang et al., 2012; Chen et al.,

2013). According to our results, these two exposure concentrations could cause some adverse effect. Moreover, when the exposure concentrations were increasing, the increase of Oct4 and Sox2 proteins levels and the decrease of *miR-134* expression level were found in a dose-dependent manner. Our data showed BPA could affect the pluripotency of mESC and differentiation capacity in mEB and explored the related mechanisms. Previous work indicted that BPA exposure is ubiquitous and likely occurs daily, and may exist in a relatively pseudosteady state over the course of months or years (Mahalingaiah et al., 2008). Given the long-term exposure to BPA for human body, the effect of BPA on human may be still underestimated in vitro. Therefore, the biological significance of our findings regarding the effects of BPA on pluripotency of mESC and differentiation capacity in mEB with different levels should be taken into consideration.

In conclusion, we report the first observation that down regulation of *miR-134* is associated with up-regulation of pluripotent marker genes including *Oct4, Sox2,* and *Nanog* upon BPA treatment in mESC and mEB. Additionally, BPA specifically inhibits expression of ectodermal lineage marker, and increases the expression of mesodermal and endodermal markers in mEB. These findings enhance our understanding of mechanism of BPA effects on embryonic development.

Conflict of interest statement

The authors declare no conflict of interest with the study or preparation of the manuscript.

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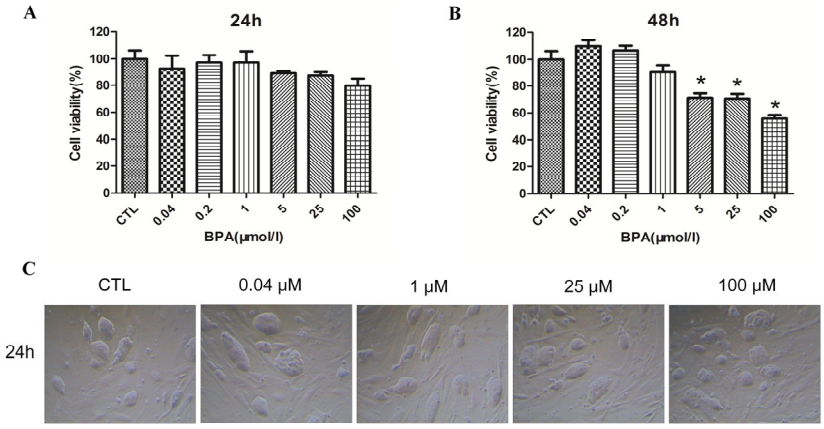
Figure Legends

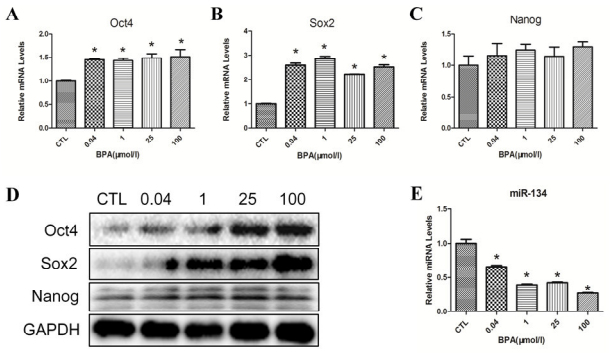
Fig.1. Effects of BPA on cell viability and morphology in mESC. (A and B) Cell viability was determined by MTT assay after exposure to various concentrations of BPA for 24h and 48h. (C) Expose D3 mESC to BPA, cell morphology was observed. Magnification, 100X. Cells were cultured with various concentrations of BPA (0.04μM, 1μM, 25μM, and 100μM) or DMSO as control for 24h. Values of the experiment were represented as the percentages of cell viability compared with that of the control and expressed as means ± S.E. from five separate experiments in which treatments were performed in quadruplicate. * indicates significant difference when the values were compared to that of the control at $p < 0.05$.

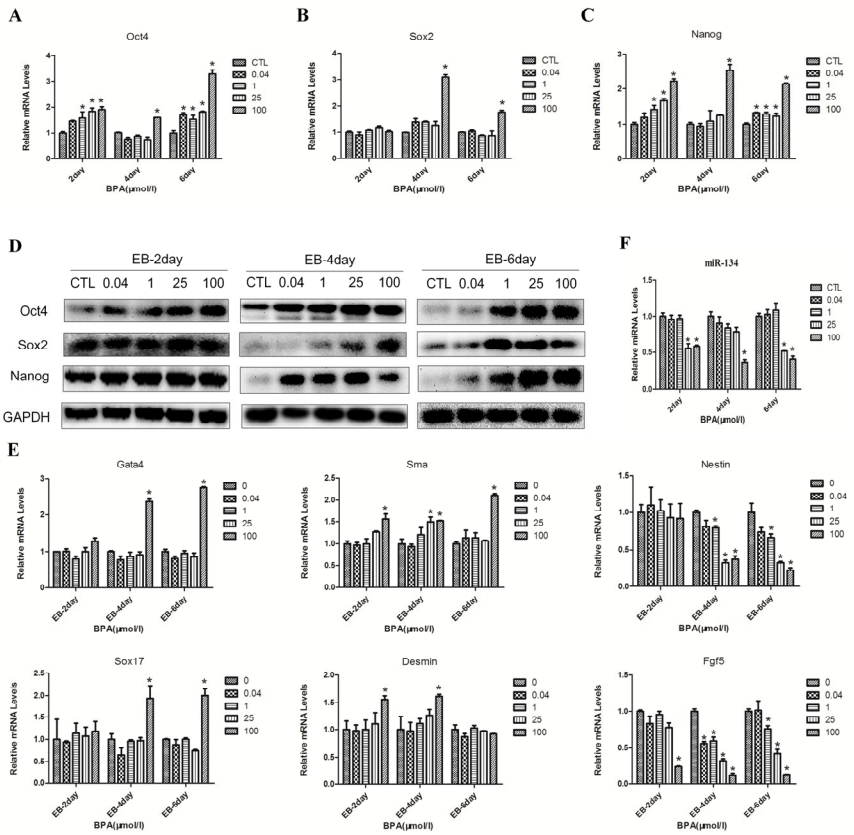
Fig.2. Effects of BPA on pluripotency and expression of *miR-134* in mESC.

Cells were cultured with various concentrations of BPA (0.04μM, 1μM, 25μM, and 100μM) or DMSO as control for 24h. (A-C) *Oct-4 /Sox-2/Nanog* mRNA levels were determined by quantitative real-time PCR using a housekeeping gene *GAPDH* as an internal control. (D)The protein levels of Oct-4/Sox-2/Nanog were determined by Western blot analysis using GAPDH as an internal control. (E) miRNA-TaqMan assay. miRNA was analyzed using total RNA from mESC with probe specific for *mmu-miR-134* and were normalized to U6 as an internal control. Each data point was normalized to the control (DMSO) and represented the means ± S.E. from three independent experiments. * indicates significant difference when the values were compared to that of the control ($p < 0.05$).

Fig.3. Effects of BPA on differentiation capacity and expression of *miR-134* in mEB. mEB were cultured with various concentrations of BPA (0.04μM, 1μM, 25μM, and 100μM) or DMSO as control up to 6day. (A-C) *Oct-4 /Sox-2/Nanog* mRNA levels were determined by quantitative real-time PCR using *GAPDH* (housekeeping gene) as an internal control. (D)The protein levels of Oct-4/Sox-2/Nanog were determined by Western blot analysis using GAPDH as an internal control. (E) Endodermal markers (*Gata4, Sox17*), mesodermal markers (*Sma, Desmin*) and ectodermal markers (*Nestin, Fgf5*) mRNA levels were determined by quantitative real-time PCR using *GAPDH* as an internal control. (F) miRNA-TaqMan assay. miRNA was analyzed using total RNA from mEB with probe specific for *mmu-miR-134* and were normalized to U6 as an internal control. Each data point was normalized to the control (DMSO) and represented the means ± S.E. from three independent experiments. * indicates significant difference when the values were compared to that of the control ($p < 0.05$).







- BPA disturbed the expression of pluripotency markers in mESC and mEB.
- BPA enhanced differentiation toward endoderm and mesoderm in mEB.
- BPA inhibited differentiation to ectodermal lineages in mEB.
- The expression of *miR-134* was decreased in both BPA-treated mESC and mEB.