Assessment of Bisphenol A (BPA) neurotoxicity *in vitro* with mouse embryonic stem cells

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ABSTRACT

The adverse effects of environmental pollution on our well-being have been intensively studied with many *in vitro* and *in vivo* systems. In our group, we focus on stem cell toxicology due to the multitude of embryonic stem cell (ESC) properties which can be exerted in toxicity assays. In fact, ESCs can differentiate in culture to mimic embryonic development *in vivo*, or specifically to virtually any kind of somatic cells. Here, we used the toxicant Bisphenol A (BPA), a chemical known as a hazard to infants and children, and showed that our stem cell toxicology system was able to efficiently recapitulate most of the toxic effects of BPA previously detected by *in vitro* system or animal tests. More precisely, we demonstrated that BPA affected the proper specification of germ layers during our *in vitro* mimicking of the embryonic development, as well as the establishment of neural ectoderm and neural progenitor cells.

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Introduction

During our everyday life we are constantly exposed to many artificial substances created in numerous industrial processes. Many of these materials tend to accumulate in the environment. As a consequence, human exposure to these chemicals, and the potential adverse health effects caused by them, may occur even in the absence of direct use of these industrial products. This has raised a growing concern about the effects of environmental pollution on human health and prompted the urgency and necessity of validated and comprehensive toxicity tests to address the potential hazardousness of these pollutants. Stem cell toxicology is a very powerful alternative to animal tests or traditional *in vitro* assays because it allows to test *in vitro* the acute and developmental toxicities of a pollutant of interest, quickly, thoroughly, and cost-effectively (Faiola et al., 2015; Jennings, 2014). In fact, embryonic stem cells (ESCs) offer the advantage that they can be derived easily and also cultured indefinitely in dishes. Therefore, they can be employed without problems for cytotoxicity assays like any other kind of cells. In addition, ESCs can be utilized in developmental toxicity assays. For instance, they can differentiate *in vitro* as three dimensional aggregates so-called embryoid bodies (EBs) which mimic the early stages of embryonic development *in vivo*. Moreover, ESCs can virtually differentiate specifically into any type of cells of an adult organism. Those cells can then be used

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for cell function toxicity assays as any other derived primary cell type (Wobus and Loser, 2011; Liu et al., 2013; Mori and Hara, 2013).

One of the most studied and dreaded environmental pollutants to date is a chemical called Bisphenol A (BPA). It has been employed since the 1950s to make polycarbonate plastics in a variety of products. BPA has been also used to coat the inner surface of beverage and food containers, regardless of the fact that it has been recognized as an endocrine-disrupting chemical (EDC) since 1936 (Dodds and Lawson, 1936). Indeed, countless studies in the last two decades have documented BPA-related effects on fertility, genital and behavioral abnormalities, heart disease, diabetes, and obesity (Rochester, 2013). However, in those reports, many different *in vitro* and *in vivo* systems have been employed, but almost none utilized stem cells. In addition, when BPA was tested with mouse ESCs, little or no toxic effects were detected (Panzica-Kelly et al., 2013; Kong et al., 2013).

In this study, we employed the known developmental toxicant BPA to test its effects on mouse ESCs with our stem cell toxicology system. Contrary to previous reports, we were able to detect BPA toxicity *in vitro*, particularly towards the neural ectoderm specification.

1. Materials and methods

1.1. Cell culture

All cell culture reagents and plasticware were purchased from Gibco (Life Technologies, NY, USA) and Corning (USA), respectively, unless otherwise indicated. J1 mouse ES cells were acquired from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured at 37°C in humidified air with 5% CO_2 in high glucose KnockOut DMEM medium supplemented with 15% fetal bovine serum, 2% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), 1% nucleosides, 1% glutamine, 1% non-essential amino acids, 10^{-4} mol/L β-mercaptoethanol and 10^3 U/mL leukemia inhibitory factor (LIF) (Merck Millipore, Darmstadt, Germany). All plates were coated with 0.1% gelatin (Sigma-Aldrich, MO, USA) before use.

1.2. Cytotoxicity assay

For cytotoxicity assays, mESCs were pre-incubated with 1–10 μmol/L BPA (or DMSO solvent control) for 24 hr, then seeded in 96-well gelatin-coated plates, and incubated with BPA/DMSO for seven days. Cell viability was determined by the AlamarBlue (AB, Sigma-Aldrich, MO, USA) fluorescence assay. Briefly, cells were incubate with the AlamarBlue reagent for two hours at 37°C, and fluorescence was measured in a multiwall fluorometric reader (ThermoFisher Scientific, MA, USA) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

1.3. Alkaline phosphatase (AP) staining

To visually detect undifferentiated mESCs, an AP staining kit (Sigma-Aldrich, MO, USA) was used according to the manufacturer's instructions.

1.4. Embryoid body (EB) differentiation assay

For EB differentiation assays, 4×10^6 mESCs were resuspended by trypsinization and seeded in 10-cm petri dishes in ES medium without LIF, to allow aggregation and formation of EBs. Media were replaced every other day and samples collected at days 0, 2, 4, 6, 9, 12, and 20, for RNA extraction and qRT-PCR measures. One day before the start and along the differentiation process, cells were incubated with 10 μmol/L BPA or DMSO control.

1.5. mESC differentiation into neural progenitor cells (NPCs)

mESCs were pre-treated with 10 μmol/L BPA or vehicle control for 24 hr, and then allowed to form EBs as described above. At day 4 of EB formation, a final concentration of 5 μmol/L retinoic acid (RA, Sigma-Aldrich, MO, USA) was added to the medium to stimulate neural ectoderm specification. At day 8, EBs were dissociated into single cells by trypsinization and passage through a 40 μm nylon cell strainer (BD, USA), and seeded into plates coated with laminin/poly-L-lysine (Roche, USA, and Sigma-Aldrich, USA, respectively) in N2 medium (DMEM-F12, N2 supplement, 1% GlutaMAX, and 2% Pen/Strep antibiotics). Media were replaced after 2 hr. Samples were collected at days 0, 4, 8, and 10 for RNA extraction and qRT-PCR analyses.

1.6. Adherent cell neuroectoderm differentiation

For the differentiation of mESCs into neural ectoderm in monolayer conditions, cells were pretreated with 10 μmol/L BPA or DMSO control as above. Then, RA was added to the medium to stimulate neuroectoderm differentiation. Samples were collected every other day from day 0 to day 10 for RNA extraction and gene expression analyses by qRT-PCR.

1.7. RNA extraction and qRT-PCR analyses

Total RNA was extracted with Trizol (Life Technologies, NY, USA), following the instructions of the manufacturer. RNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining. RNAs were converted to cDNAs with the PrimeScript RT Master Mix Kit (Takara, Japan) according to the manufacturer's recommended procedures. qPCR was performed with the SYBR Premix Ex Taq kit (Takara, Japan). Cycling conditions were executed forty times, except initial denaturation and final cycle, as following: initial denaturation, 95°C 30 sec; denaturation, 95°C 5 sec; annealing, 60°C 30 sec; final cycle, 95°C 5 sec, 60°C 1 min, 95°C 30 sec. The comparative C_t methods was used to calculate the relative gene expression normalized to the *gapdh* gene. Measures were obtained in triplicates. Results are shown as mean ± standard deviation (SD). Primers employed are listed in Table 1.

1.8. Statistical analysis

Statistical significance was determined by multiple *t*-test using the Holm-Sidak method, with alpha = 5.000%. Each row was analyzed individually, without assuming a consistent SD.

Table 1 – List of primers used in qPCR assays.			
Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
gapdh (F)	CGTCCCGTAGACAAAATGGT	*brachyury* (F)	GTCTAGCCTCGGAGTGCCT
gapdh (R)	TTGATGGCAACAATCTCCAC	*brachyury* (R)	CCATTGCTCACAGACCAGAG
pax6 (F)	CGGGACTTCAGTACCAGGG	*nestin* (F)	AGATCGCTCAGATCCTGGAA
pax6 (R)	CTTCATCCGAGTCTTCTCCG	*nestin* (R)	GAGTTCTCAGCCTCCAGCAG
fgf5 (F)	GCTGTGTCTCAGGGGATTGT	*sox1* (F)	AGTGGAAGGTCATGTCCGAG
fgf5 (R)	ACAGTCATCCGTAAATTTGGC	*sox1* (R)	TGTAATCCGGGTGTTCCTTC
gata6 (F)	TACACAAGCGACCACCTCAG	*sox3* (F)	ACTGGAAACTGCTGACCGAT
gata6 (R)	TGTAGAGGCCGTCTTGACCT	*sox3* (R)	CCGGTACTTGTAGTCCGGGT
sox17 (F)	TGGAACCTCCAGTAAGCCAG	*krt14* (F)	GGCCCACTGAGATCAAAGAC
sox17 (R)	TCAGATGTCTGGAGGTGCTG	*krt14* (R)	GATCTGCAGGAGGACATTGG
hand1 (F)	TGAACTCAAAAAGACGGATGG	*cdx2* (F)	GAAACCTGTGCGAGTGGATG
hand1 (R)	CTTTAATCCTCTTCTCGCCG	*cdx2* (R)	TCTGTGTACACCACCCGGTA
F: forward primer; R: reverse primer.			

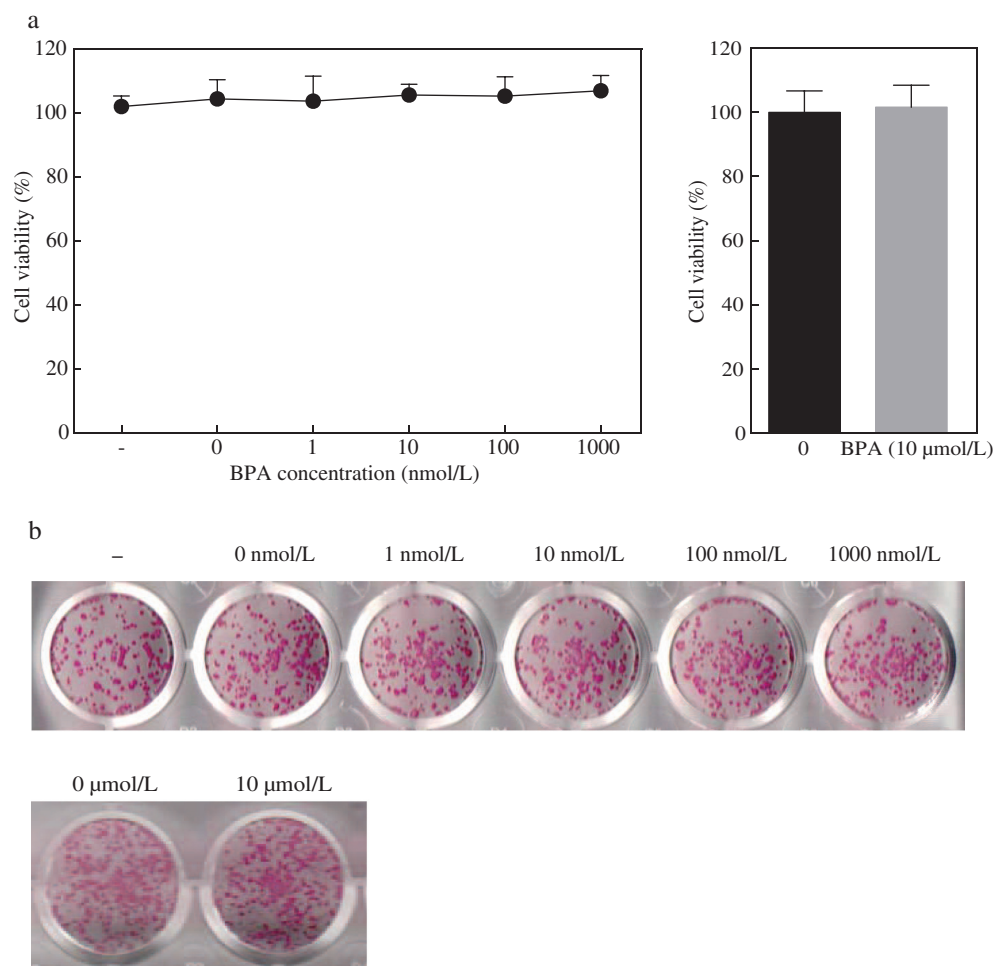## 2. Results and discussion

2.1. BPA cytotoxicity

In order to employ appropriate BPA concentrations during our developmental toxicity assays, we first determined the cytotoxicity properties of BPA with mESCs. Since our differentiation procedures would last at least one week, we measured BPA-dependent acute toxicity upon seven-day incubation with the chemical. Fig. 1a (left panel) shows that mESC viability was not significantly affected by BPA concentrations ranging from 1 nmol/L to 1 μmol/L, as compared to DMSO solvent control. To assess whether ESCs were not only alive but also in the undifferentiated state, we stained the cells with alkaline phosphatase (AP) at the end of the cytotoxicity assay. Only live and self-renewing cell would stain dark red, while differentiating cells would stay unstained or only partially stained. As depicted in Fig. 1b (top panel), similar amounts of ESC colonies were stained red for AP in all wells, suggesting similar levels of undifferentiated ESCs upon incubation with BPA or DMSO. We also incubated our cells with 10 μmol/L BPA, or solvent vehicle, for a week and measured cell viability and AP staining. Fig. 1a (right panel) and B (bottom panel) shows no effects on cell viability and self-renewal at this BPA concentration. These results agree with the two previously published studies (Panzica-Kelly et al., 2013; Kong et al., 2013). We also passaged our ESCs in the presence of 10 μmol/L BPA for several times, and observed no noteworthy effects on cell viability and self-renewal (data not shown). Thus, we were confident we were using concentrations of BPA far from lethal.

2.2. BPA *affected mESC embryoid body (EB) differentiation*

To test if BPA behaved as a toxicant in our stem cell system, we mimicked embryonic development *in vitro* by differentiating mESCs *via* EB formation. In fact, when ESCs aggregate in suspension to form EBs in differentiating conditions, they start specifying the three primary germ layers, endoderm, mesoderm, and ectoderm, similarly to what would happen *in vivo*. ESCs were pre-incubated with 10 μmol/L BPA, or vehicle, for one day, and then allowed to aggregate in suspension in ES medium deprived of LIF (day 0). EB samples were then collected approximately every other day for RNA extraction and qRT-PCR analyses. We then checked for the expression of markers for endoderm (*gata6*, *sox17*), mesoderm (*brachyury* (T), *hand1*), ectoderm (*fgf5*, *krt14*), and trophectoderm (*cdx2*). As shown in Fig. 2a, there was a slight reduction in the expression of the two endoderm markers tested when cells where incubated with BPA, as compared to DMSO control. The trophectoderm marker *cdx2* seemed to be affected by BPA exposure as well, with an initial BPA-dependent increase (day 4 and day 9) followed by a pronounced reduction at days 12 and 20 of EB formation (Fig. 2d). This interesting behavior is under further investigation and will be described in a future study. Conversely, mesoderm specification appeared not to be altered by the presence of BPA (Fig. 2c). This could explain why in a previous study (Kong et al., 2013) they did not detect any noteworthy BPA toxicity, since they only looked at mESC differentiation into beating cardiomyocytes, which had derived from mesoderm. When we analyzed BPA effects on ectoderm specification, we did not notice any noteworthy down-regulation in the expression of the early ectoderm marker *fgf*5 (Fig. 2b). Interestingly, *fgf*5 exhibited a quick up-regulation at early stages (up to day 4), and gradually decreased afterward, and BPA treatment slightly increased its transcription. On the contrary, the expression of the non-neural and late ectoderm marker *krt14* (one of the keratins in skin epithelia) appeared to be negatively affected by the presence of BPA, at least at day 20 of the differentiation procedure. These observations suggest that BPA may stimulate the initial specification of ectoderm, but negatively affects the later generation of tissues, such as the skin, which are derived from ectoderm.

Numerous studies have demonstrated the BPA neurotoxic effects with *in vitro* and *in vivo* systems (Beronius et al., 2013). Thus, we examined the neural ectoderm specification during our EB differentiation assays upon incubation with BPA. Our molecular read-out was the expression of four neuroectoderm markers: *sox1*, *pax6*, *sox3*, and *nestin*. As depicted in Fig. 3, all four genes showed reduced expression in the BPA-treated samples, relative to DMSO control ones. This implied a BPA-dependent neurotoxicity during embryonic development, and that our stem cell toxicology system was able to detect it.

Fig. 1 – BPA did not alter mESC viability at the concentrations tested. (a) Viability plot for cells incubated with 0–1000 nmol/L BPA (left panel), or 10 μmol/L BPA (right panel) for seven days. (b) Alkaline Phosphatase staining at day 7 of representative wells for cells incubated with the indicated concentrations of BPA. – indicates no treatment; 0 nmol/L refers to DMSO only.
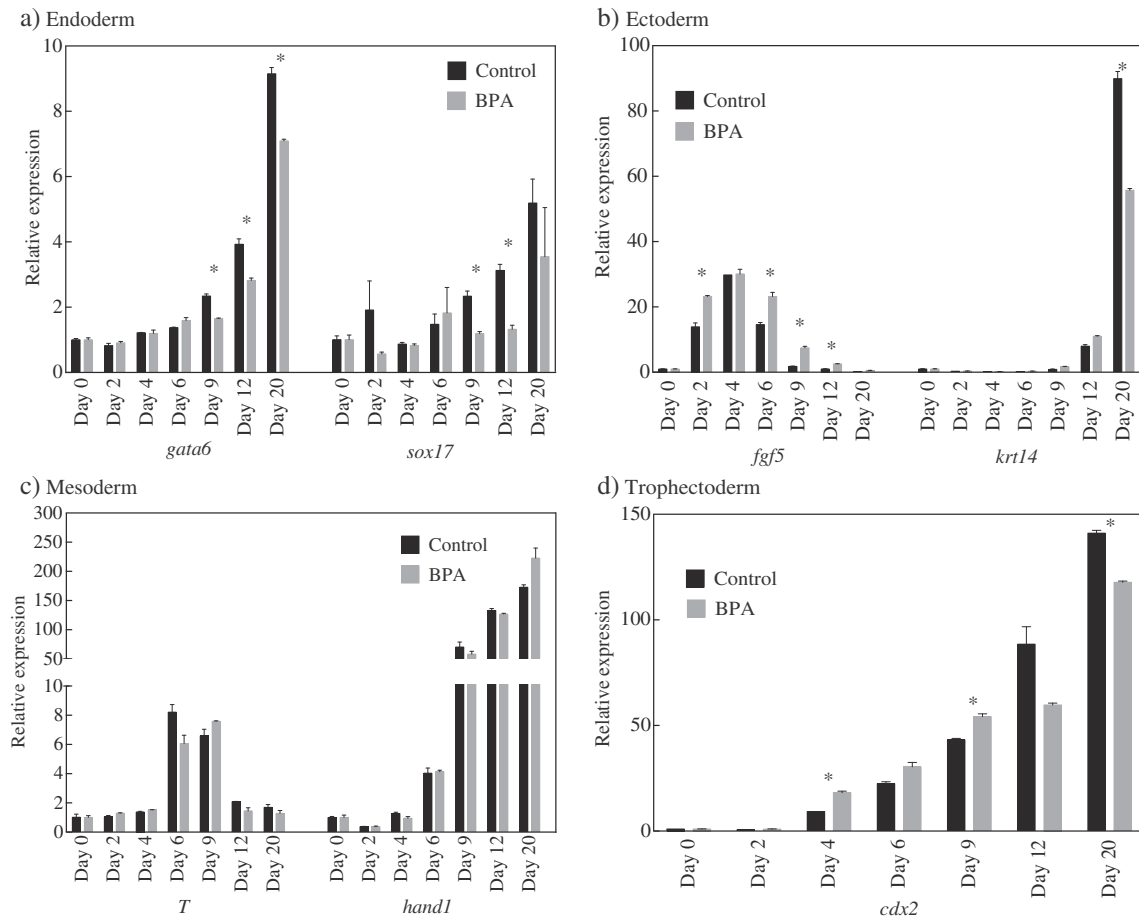
2.3. BPA significantly impaired EB-based neural differentiation of mESCs

Intrigued by the substantial alteration of all the neural ectoderm markers checked during mESC EB differentiation in the presence of BPA, we decided to utilize a differentiation procedure to specifically differentiate ESCs into neuroectoderm first, and then into neural progenitor cells (NPCs). This would allow a more precise assessment of BPA neurotoxic abilities. Thus, we incubated the cells with 10 μmol/L BPA, or DMSO solvent, and add retinoic acid (RA) starting from day 4 during the EB differentiation process to induce neural ectoderm. At day 8, we dissociated the EBs and transferred the cells to plates coated with laminin/poly-L-lysine to culture NPCs. We then collected samples at different time points and checked for the expression of specific neural markers. As depicted in Fig. 4a, all the markers checked were significantly affected by BPA incubation, as compared to DMSO control. It is also noteworthy that a higher expression of the markers was observed in this differentiation process as compared with the global EB differentiation of Fig. 3. These results suggested an effect of RA in neuroectoderm formation. We also looked at the early ectoderm marker *fgf*5 and the late
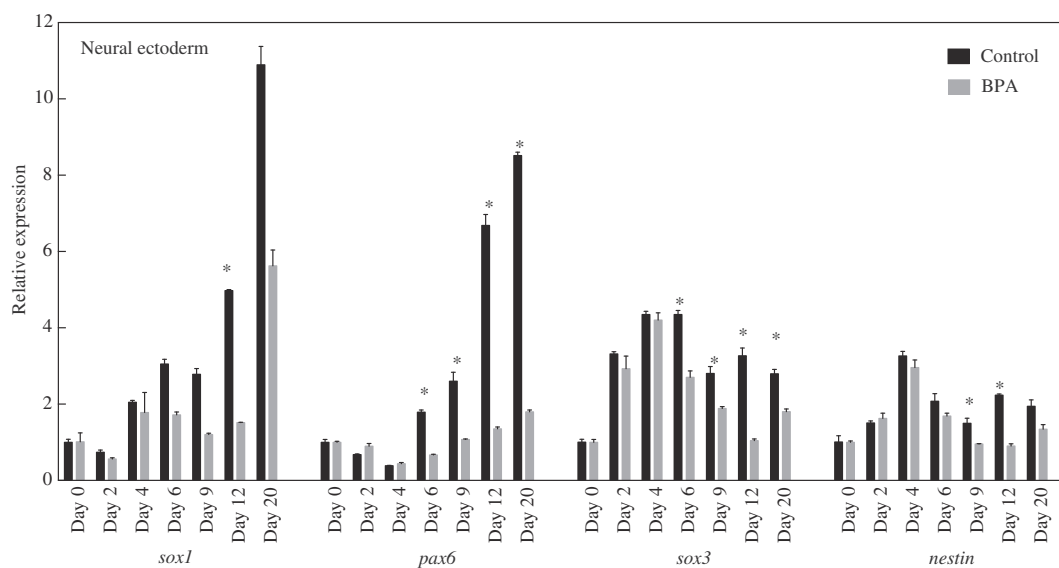
non-neural ectoderm gene *krt14*. *Fgf*5 was, as expected, activated only at early stages (up to day 4), and became quickly repressed afterward. Exposure to BPA reduced its expression. Conversely, *krt14* was never expressed (Fig. 4b). These data clearly confirm the deleterious effects of BPA on the neural lineage specification we observed during the global differentiation of mESCs.

2.4. Adherent neuroectoderm differentiation of mESCs was also compromised by BPA

To differentiate mESCs into neural ectoderm, we employed an alternative and simplified protocol based on monolayer conditions. Also in this case, we removed LIF and added RA to stimulate the differentiation. Although the efficiency of this procedure is generally not as good as the one from the EB-based process, the majority of the cells differentiate towards neuroectoderm. Upon 10 μmol/L BPA incubation, we determined the levels of expression of three markers, *sox1*, *pax6*, and *nestin*. Fig. 5 shows that *sox1* and *pax6* expression were impaired in the presence of BPA, as compared to DMSO control, while transcription of *nestin* was not affected by the chemical. These results confirmed the effects of BPA on the
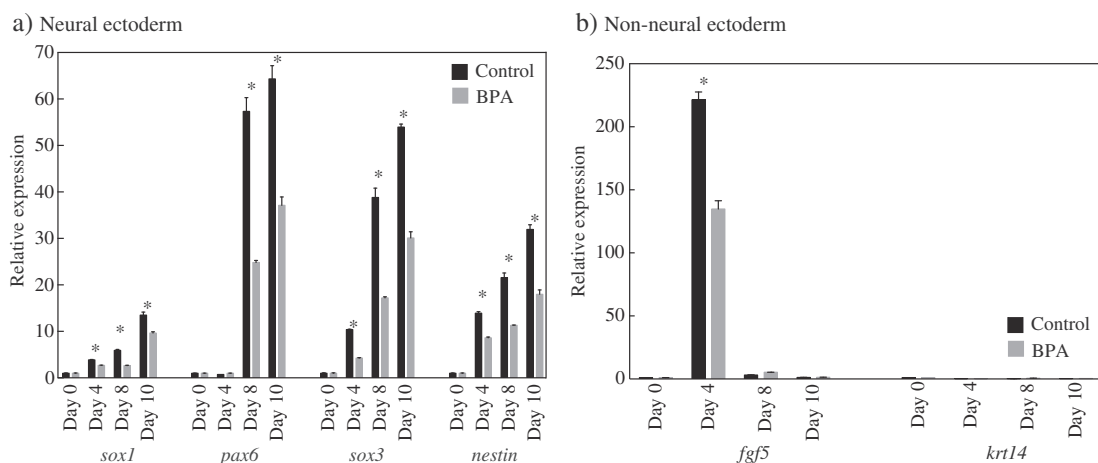
Fig. 2 – Expression profiling of lineage specific markers during embryoid body-based differentiation of mESCs upon incubation with BPA or DMSO vehicle. Specific marker expression for (a) endoderm (*gata6*, *sox17*), (b) ectoderm (*fgf5*, *krt14*), (c) mesoderm (*T*, *hand1*), and (d) trophectoderm (*cdx2*), respectively. * indicates a statistical significant difference in gene expression between BPA and control samples.

Fig. 3 – BPA treatment affected neural ectoderm specification during EB differentiation. Expression of four typical neuroectoderm markers was analyzed by qRT-PCR. * indicates a statistical significant difference in gene expression between BPA and control samples. The statistical analysis for *sox1* day 6, 9, and 20 samples was not performed because the number of replicates was equal to 2.
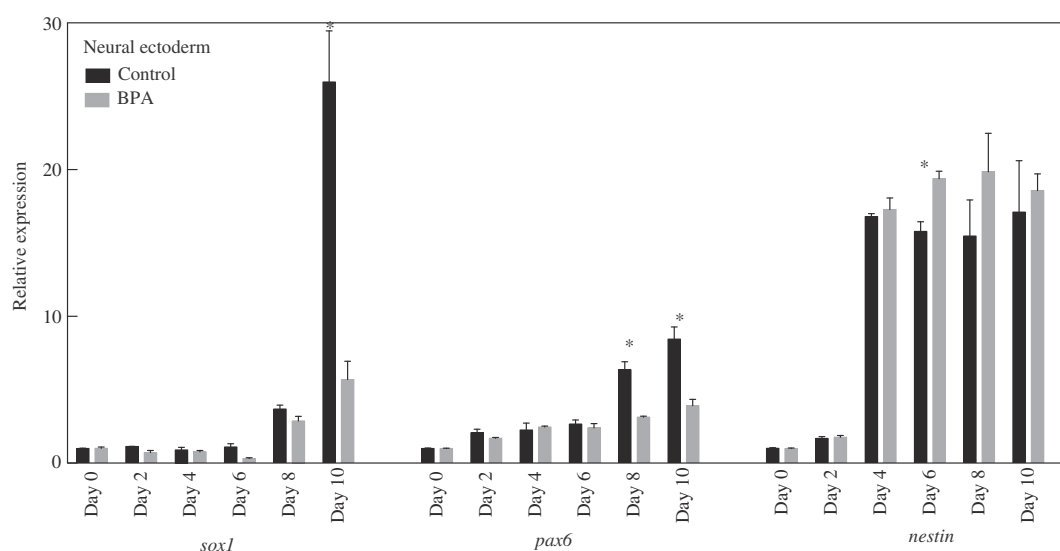
Fig. 4 – Differentiation of mESCs towards neural progenitors cells (NPCs) was compromised by BPA. (a) Expression of four typical neural markers: *sox1*, *pax6*, *sox3*, and *nestin* upon BPA or DMSO treatment; (b) expression of the early ectoderm marker *fgf5*, and the non-neural ectoderm marker *krt14*, upon treatment with BPA or DMSO. * indicates a statistical significant difference in gene expression between BPA and control samples.

expression of the two transcription factors *sox1* and *pax6*, similar to what we observed above. The discrepancy about BPA effects on *nestin* expression between the two differentiation procedures can be explained by the fact that the adherent differentiation, although quicker and less technically challenging, is not as efficient and specific as the one based on EBs. As a result, more elusive gene expression differences may not be easily detected.

3. Conclusions

In this study, we demonstrated that stem cell toxicology could be a powerful system to detect the developmental toxicity of environmental pollutants. We employed BPA as a test chemical

owing to its toxicity established with many other *in vitro* and *in vivo* systems. With our EB-based differentiation process that mimics *in vitro* embryonic development, we showed that BPA slightly altered the expression of endoderm and trophectoderm markers, and significantly impaired the neural ectoderm specification. The neurotoxic effects of BPA were also evident in our two additional differentiation procedures specifically directed towards neural ectoderm and/or neural progenitor cells, *via* EB formation or in monolayer conditions. In sum, we proved that our stem cell toxicology system could serve as a noteworthy improvement over traditional stem cell toxicity assays, which were previously shown to detect either no BPA toxicity (Panzica-Kelly et al., 2013), or just slight embryotoxic effects (Kong et al., 2013). Compared to the first study, our combination of several differentiation procedures (EB-based or in monolayer

Fig. 5 – BPA impaired the neural ectoderm differentiation of mESCs in monolayer conditions. The time course expression profiling of the three markers *sox1*, *pax6*, and *nestin* is shown. * indicates a statistical significant difference in gene expression between BPA and control samples.

conditions) with gene expression analyses over a time course, proved to be more sensitive and reflective of the physiological toxicity effects of BPA, not detected by single-point analyses (Panzica-Kelly et al., 2013). In addition, our approaches were able to examine the effects of BPA on the development of all three germ layers, instead of just one, mesoderm (Kong et al., 2013). Most importantly, our methodology was much more sensitive and efficient in detecting BPA as a toxicant by using concentrations well below lethality. It also argues against the choice of a single time point during the differentiation process to assess toxicity. The improved sensitivity and efficiency established by our system will be of tremendous use for testing the effects of other hazardous chemicals on human health.

Acknowledgments

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