Available online at www.sciencedirect.com

ScienceDirect

www.journals.elsevier.com/journal-of-environmental-sciences

JOURNAL OF ENVIRONMENTAL SCIENCES
www.jesc.ac.cn

Studying developmental neurotoxic effects of bisphenol A (BPA) using embryonic stem cells

*Jinhua Li, Katherine Z. Fu, Sai Vemula, X. Chris Le, Xing-Fang Li**

Division of Analytical and Environmental Toxicology, Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB T6G 2G3, Canada

ARTICLE INFO

Available online 21 August 2015

Keywords:
Bisphenol A (BPA)
Embryonic stem cells (ESCs)
Embryoid bodies (EBs)
Environmental toxicology
Developmental toxicity
Neural ectoderm
Neural progenitor cells (NPCs)
Neurotoxicity

There is little to no toxicity information regarding thousands of chemicals to which people are exposed daily. In fact, of the 84,000 chemicals listed in the United States Toxic Substances Control Act Inventory, there is limited information available on their effects on neural development (Betts, 2010; US EPA, 2015). The number of chemicals tested remains low due to the high cost of conducting multi-generational animal studies and the lack of alternative testing methods.

In addition to high costs, inter-species variation has led to efforts to limit the use of animal models (mammalian and non-mammalian), and instead, turn to using alternative strategies to test toxicity. The goals of "Toxicity Testing in the 21st Century: A Vision and a Strategy", the 2007 report of the U.S. National Research Council, are to use high-throughput assays to identify endpoints and pathways linked to toxicity and apply predictive models to prioritize chemicals of interest for further testing (Kavlock et al., 2012; NRC, 2007; Tice et al., 2013; Krewski et al., 2014). Ultimately, new testing paradigms based on computational models and *in vitro* assays have the potential to eliminate the need for traditional animal tests (NRC, 2007). The main challenge of traditional *in vitro* systems is to simulate the complex nature of biological systems (Knudsen et al., 2015; Wu et al., 2014).

Studying the differentiation of stem cells is a promising model to measure the impact of environmental chemical exposure on embryonic development (Mori and Hara, 2013). Mouse embryonic stem cells (mESCs) were first isolated and cultured *in vitro* by Evans and Kaufman (1981). In 1998, Thomson et al. (1998) first isolated and characterized human embryonic stem cells (hESCs). Since then, embryonic stem cells (ESCs) have been used in a variety of fields to study tissue regeneration and to better understand normal development and to screen drugs and toxicants (Hoffman and Carpenter, 2005; Faiola et al., 2015). ESCs are derived from preimplantation stage embryos, a process that involves culturing embryos to the morula or blastocyst stage (Hoffman and Carpenter, 2005). ESCs are pluripotent, meaning cells can differentiate into the three germ layers: ectoderm, mesoderm, and endoderm (Thomson et al., 1998). During development, the endoderm eventually forms the gut epithelium; the mesoderm forms cartilage, bone, smooth muscle, and striated muscle; and the ectoderm forms neural epithelium, embryonic ganglia, and stratified squamous epithelium (Thomson et al., 1998), as shown in Fig. 1.

In a recently published study in the *Journal of Environmental Sciences*, Yin et al. from the State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, used mESCs to study the developmental neurotoxicity of bisphenol A (BPA). BPA, one of the most widely known environmental pollutants (Fromme et al., 2002; Jiang et al., 2012; Chen et al., 2014; Zhang et al., 2014), is a monomer found in polycarbonate plastics and in the lining of many food and beverage containers. Heat and contact with either basic or acidic compounds can accelerate leaching of BPA from food storage containers and cans (vom Saal and Hughes, 2005). In a literature review on BPA

* *Corresponding author*. E-mail: xingfang.li@ualberta.ca (Xing-Fang Li).

http://dx.doi.org/10.1016/j.jes.2015.08.002

exposure, vom Saal and Hughes (2005) stated the need for new risk assessment methods to test the toxicity of BPA. The review emphasized the extensive literature of sub-lethal toxic effects, widespread human exposure due to leaching from food and beverage containers, high levels of BPA reported in human blood and tissue, and epidemiological studies linking BPA exposure to adverse effects in women as contributing to this need for further research.

One of the advantages of using ESC toxicity tests is the ability to observe the differentiation of mESCs *via* the formation of embryoid bodies (EBs). EBs are three-dimensional cell aggregates, which simulate the developing embryo *in vitro*, and are able to differentiate into all three germ lineages: endoderm, ectoderm, and mesoderm, and further differentiate into all the cell types (Desbaillets et al., 2000). Yin et al. (2015) first measured the gene expression of markers for each germ lineage to explore whether BPA affected differentiation of mESCs. Their results showed that BPA affected gene expression markers of the endoderm, ectoderm and trophectoderm, but not of the mesoderm. The lack of effect on gene expression of the mesoderm was consistent with the observation of a previous study that focused on the mesoderm (Panzica-Kelly et al., 2013). However, the study of Yin et al. (2015) found that BPA affected the gene expression of the other germ lineages. To test whether BPA affected neurodevelopment, Yin et al. further examined the neural ectoderm specifically during EB differentiation. The expression of four neuroectoderm markers, *sox1*, *pax6*, *sox3*, and *nestin*, was significantly decreased in BPA-treated samples compared to the controls. These results showed direct evidence of BPA neurotoxicity.

To improve the precise assessment of BPA developmental neurotoxicity, Yin et al. (2015) utilized two protocols to drive the differentiation of mESCs specifically into neural ectoderm first, and then into neural progenitor cells (NPCs). The first protocol was *via* the formation of EBs, and the second was *via* cells grown under monolayer conditions (Fig. 2). The results showed again that BPA significantly reduced the expression of the four neural ectoderm markers based on the EB formation method. BPA also impaired the expression of *sox1* and *pax6* based on cells grown in monolayer conditions. These data further supported the conclusion of the developmental neurotoxicity of BPA.

In summary, Yin et al. (2015) demonstrated that mESCs are a good model to detect developmental toxicity of environmental substances. One advantage of their method is its higher sensitivity, enabling the detection of BPA neurotoxicity at lower concentrations (*e.g.*, 10 μmol/L). The concentrations of BPA used by Yin et al. are significantly lower than the median lethal concentration (IC_{50}, 29 μg/mL or 127 μmol/L) reported by Kong et al. (2013). The other advantage is that they observed the effects of BPA on gene expression over a time course, rather than at a single time point that is usually used in other toxicological studies (Mori and Hara, 2013). In fact, gene expression during ESC differentiation is a dynamic process, and not all the genes are expressed at the same time.

The probable daily intake (PDI) of BPA, as estimated by the Health Products and Food Branch (HPFB), Health Canada in 1995, is 0.18 μg/kg body weight (bw)/day for adults and 2.63 μg/kg bw/day for infants (Health Canada, 2008). The provisional tolerable daily intake (pTDI) established by Health Canada in 1996 is 25 μg/kg bw/day. The U.S. Environmental Protection Agency (US EPA) and the European Food Safety Authority (EFSA) have also established pTDI values for BPA. The US EPA expresses this as an "oral reference dose (RfD)" set as 50 μg/kg bw/day (US EPA, 1993) and the EFSA has recently revised the value of the "tolerable daily intake" from 50 μg/kg bw/day to a "*temporary* tolerable daily intake" of 4 μg/kg bw/day (EFSA, 2015). The estimated daily intake by Canadians is much lower than these guideline or reference values. However, BPA has since been characterized as an endocrine disrupting agent and has shown

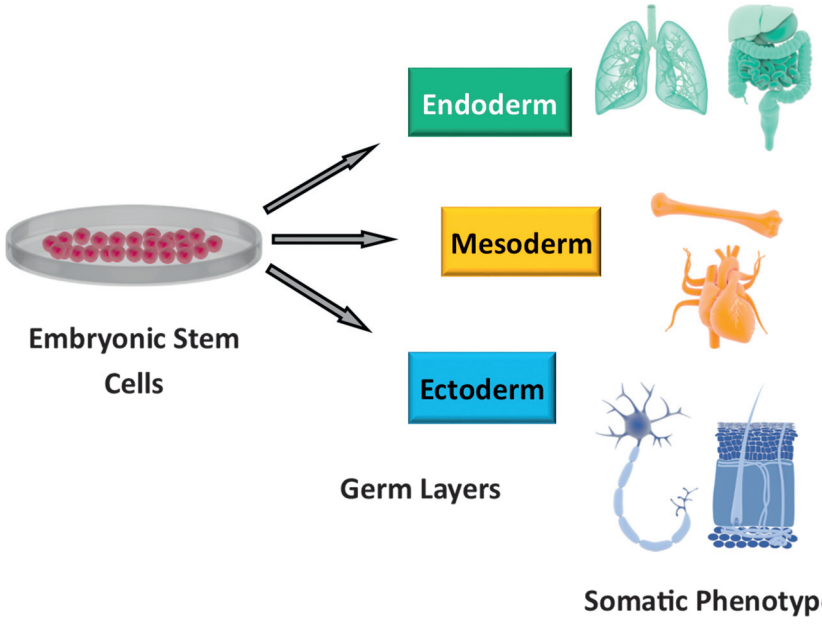

Fig. 1 – Differentiation of embryonic stem cells into three primary germ layers: endoderm, mesoderm, and ectoderm.

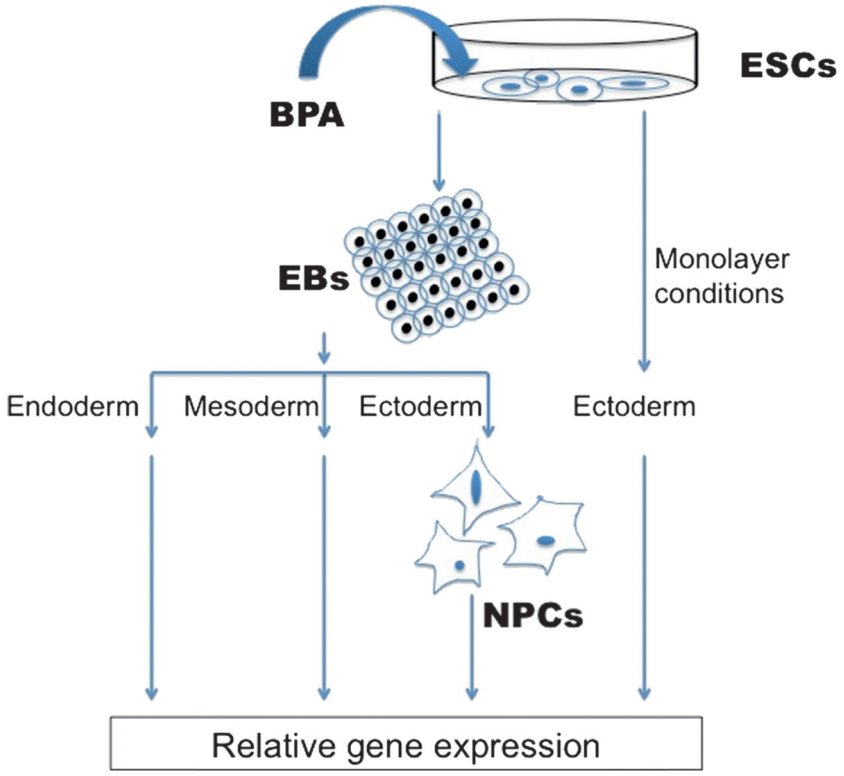

Fig. 2 – Experimental design for studying the effects of bisphenol A (BPA) on the differentiation of embryonic stem cells (ESCs). The first protocol to drive the differentiation of mESCs was *via* the formation of embryoid bodies (EBs), and then into neural progenitor cells (NPCs). The second protocol involved growing cells under monolayer conditions. mESCs: mouse embryonic stem cells.

reproductive and neurobehavioral effects at concentrations lower than the pTDI (the lowest concentration, causing estrogen interruptions, being 0.02 μg/kg bw/day). The NTP CERHR Expert Panel Report on BPA (2007) expressed "some concern" for these studies in rodent models and recommended further research in characterizing human relevance (Shelby, 2008). In 2007, the HPFB determined the average concentration of BPA in canned liquid infant formulas to be 5.2 μg/L, or 0.0227 μmol/L, which is three orders of magnitude lower than the test concentrations used in the study of Yin et al. (2015). Crain et al. (2007) reviewed studies measuring environmental BPA concentration and found that BPA levels varied based on proximity to sources, but that concentrations in stream and river samples fell below 21 μg/L. The highest BPA concentration in stream and river water, therefore, is two orders of magnitude lower than the test concentrations.

The potential of this *in vitro* model in toxicity studies can be further developed by using human embryonic stem cells (hESCs) instead of mESCs, while still looking at appropriate neuronal endpoints. This is especially relevant in expanding the embryonic stem cell test and its uses in neurotoxicity studies, as calibration across species is required in *in vitro* models as well as in *in vivo* models. Moreover, using *in vitro* toxicity testing has proven to be pivotal in predicting hazards, and the consequences of failing toxicity examinations is reflected in well-known examples such as thalidomide and rofecoxib (Khan et al., 2006). The successful establishment of hESCs provides the means for studying diverse neuronal endpoints, while limiting the number of laboratory animals needed for hazard and risk assessments (Stummann et al., 2009).

Yin et al. have centered their analysis on one main method for mESC maturation and differentiation. To assess progressive maturation and neuronal differentiation of the mESCs to the germ layers, EBs, and NPCs (*via* both differentiation methods), they used quantitative reverse transcription polymerase chain reaction (qRT-PCR) as the quantitative determinant. The study could benefit from including a comparison of protein expression, in addition to the comparison of gene expression at the RNA level. Protein quantification could further strengthen the evidence of the effects of BPA on gene expression. The complement of gene and protein expression has often been employed in neurotoxicity studies (Lou et al., 2014).

Another extension of this BPA study could include a functional neurodevelopmental assay or a functional toxicity assay that is focused on fully differentiated neural cells (*e.g.*, neurons, astrocytes, oligodendrocytes). Such investigations have been reported with various environmental compounds (Baek et al., 2012; Ivanov et al., 2013). Currently, neurodevelopmental toxicity is assessed in several ways, with little agreement on which developmental stage or which cell lineage can best assess a hazard threshold. Using multiple neuronal endpoints, such as combining the examination of germ layer formation and functional differentiation, could help develop a specific and verified model for neurotoxicity testing. Future work could also benefit from evaluating developmental toxic effects in neuroglial differentiation. The current work

opens up opportunities for these developments and for diverse exciting applications to environmental toxicology.

Acknowledgments

We thank Alberta Health, Alberta Innovates, the Canada Research Chairs Program, the Canadian Institutes of Health Research, and the Natural Sciences and Engineering Research Council of Canada for their support.

REFERENCES

Baek, D.H., Kim, T.G., Lim, H.K., Kang, J.W., Seong, S.K., Choi, S.E., et al., 2012. Embryotoxicity assessment of developmental neurotoxicants using a neuronal endpoint in the embryonic stem cell test. J. Appl. Toxicol. 32, 617–626. http://dx.doi.org/10.1002/jat.1747.

Betts, K.S., 2010. Growing knowledge using stem cells to study developmental neurotoxicity. Environ. Health Perspect. 118, A433–A437. http://dx.doi.org/10.1289/ehp.118-a432.

Chen, R., Yin, P.H., Zhao, L., Yu, Q.M., Hong, A.H., Duan, S.S., 2014. Spatial-temporal distribution and potential ecological risk assessment of nonylphenol and octylphenol in riverine outlets of Pearl River Delta. Chin. J. Environ. Sci. 26 (11), 2340–2347. http://dx.doi.org/10.1016/j.jes.2014.09.019.

Crain, D.A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G.A., et al., 2007. An ecological assessment of bisphenol-A: evidence from comparative biology. Reprod. Toxicol. 24, 225–239. http://dx.doi.org/10.1016/j.reprotox.2007.05.008.

Desbaillets, I., Ziegler, U., Groscurth, P., Gassmann, M., 2000. Embryoid bodies: an *in vitro* model of mouse embryogenesis. Exp. Physiol. 85, 645–651. http://dx.doi.org/10.1111/j.1469-445X.2000.02104.x.

EFSA (European Food Safety Authority), 2015. Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. European Food Safety Authority. EFSA Journal. 13 (1), 3978. http://dx.doi.org/10.2903/j.efsa.2015.3978.

Evans, M.J., Kaufman, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156. http://dx.doi.org/10.1038/292154a0.

Faiola, F., Yin, N.Y., Yao, Y.L., Jiang, G.B., 2015. The rise of stem cell toxicology. Environ. Sci. Technol. 49, 5847–5848. http://dx.doi.org/10.1021/acs.est.5b01549.

Fromme, H., Kuchler, T., Otto, T., Pilz, K., Muller, J., Wenzel, A., 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water Res. 36, 1429–1438. http://dx.doi.org/10.1016/S0043-1354(01)00367-0.

Health Canada, 2008. Health Risk Assessment of Bisphenol A from Food Packaging Applications. Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch, Ottawa, Canada (http://www.hc-sc.gc.ca/fn-an/securit/packag-emball/bpa/bpa_hra-ers-eng.php. (Accessed July 31st, 2015]).

Hoffman, L.M., Carpenter, M.K., 2005. Characterization and culture of human embryonic stem cells. Nat. Biotechnol. 23, 699–708. http://dx.doi.org/10.1038/nbt1102.

Ivanov, V.N., Wen, G., Hei, T.K., 2013. Sodium arsenite exposure inhibits AKT and Stat3 activation, suppresses self-renewal and induces apoptotic death of embryonic stem cells. Apoptosis 18, 188–200. http://dx.doi.org/10.1007/s10495-012-0779-1.

Jiang, W.W., Yan, Y., Ma, M., Wang, D.H., Luo, Q., Wang, Z.J., et al., 2012. Assessment of source water contamination by estrogenic disrupting compounds in China. J. Environ. Sci. 24 (2), 320–328. http://dx.doi.org/10.1016/S1001-0742(11)60746-8.

Kavlock, R., Chandler, K., Houck, K., Hunter, S., Judson, R., Kleinstreuer, N., et al., 2012. Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. Chem. Res. Toxicol. 25, 1287–1302. http://dx.doi.org/10.1021/tx3000939.

Khan, S.A., Rao, P.G.M., Rodrigues, G., Surulivelrajan, M., Heda, A., 2006. From thalidomide to rofecoxib: can we afford to wait and watch? Int. J. Risk Saf. Med. 18, 219–230.

Knudsen, T.B., Keller, D.A., Sander, M., Carney, E.W., Doerrer, N.G., Eaton, D.L., et al., 2015. Future Tox II: *in vitro* data and *in silico* models for predictive toxicology. Toxicol. Sci. 143, 256–267. http://dx.doi.org/10.1093/toxsci/kfu234.

Kong, D., Xing, L.N., Liu, R., Jiang, J.J., Wang, W.Y., Shang, L.Q., et al., 2013. Individual and combined developmental toxicity assessment of bisphenol A and genistein using the embryonic stem cell test *in vitro*. Food Chem. Toxicol. 60, 497–505. http://dx.doi.org/10.1016/j.fct.2013.08.006.

Krewski, D., Westphal, M., Andersen, M.E., Paoli, G.M., Chiu, W.A., Al-Zoughool, M., et al., 2014. A framework for the next generation of risk science. Environ. Health Perspect. 122 (8), 796–805. http://dx.doi.org/10.1289/ehp.1307260.

Lou, Q.Q., Cao, S., Xu, W., Zhang, Y.F., Qin, Z.F., Wei, W.J., 2014. Molecular characterization and mRNA expression of ribosomal protein L8 in *Rana nigromaculata* during development and under exposure to hormones. J. Environ. Sci. 26 (11), 2331–2339. http://dx.doi.org/10.1016/j.jes.2014.09.018.

Mori, H., Hara, M., 2013. Cultured stem cells as tools for toxicological assays. J. Biosci. Bioeng. 116, 647–652. http://dx.doi.org/10.1016/j.jbiosc.2013.05.028.

NRC (National Research Council), 2007. Toxicity Testing in the 21st Century: a Vision and a Strategy. National Academy Press, Washington, DC.

Panzica-Kelly, J.M., Brannen, K.C., Ma, Y., Zhang, C.X., Flint, O.P., Lehman-McKeeman, L.D., et al., 2013. Establishment of a molecular embryonic stem cell developmental toxicity assay. Toxicol. Sci. 131, 447–457. http://dx.doi.org/10.1093/toxsci/kfs293.

Shelby, M.D., 2008. NTP-CERHR monograph on the potential human reproductive and developmental effects of bisphenol A. National Toxicology Program. U.S. Department of Health and Human Services. NIH (Publication No. 08–5994).

Stummann, T.C., Hareng, L., Bremer, S., 2009. Hazard assessment of methylmercury toxicity to neuronal induction in embryogenesis using human embryonic stem cells. Toxicology 257, 117–126. http://dx.doi.org/10.1016/j.tox.2008.12.018.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., et al., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147. http://dx.doi.org/10.1126/science.282.5391.1145.

Tice, R.R., Austin, C.P., Kavlock, R.J., Bucher, J.R., 2013. Improving the human hazard characterization of chemicals: a Tox21 update. Environ. Health Perspect. 121, 756–765. http://dx.doi.org/10.1289/ehp.1205784.

US EPA, 1993. Bisphenol A. Integrated Risk Information System (IRIS).(http://www.epa.gov/ncea/iris/subst/0356.htm (Accessed August 27, 2015)).

US EPA, 2015. Chemical Assessment and Management Program. United States Environmental Protection Agency, Washington DC (http://www.epa.gov/champ/pubs/programs.html (Accessed July 7, 2015)).

vom Saal, F.S., Hughes, C., 2005. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ. Health Perspect. 113, 926–933. http://dx.doi.org/10.1289/ehp.7713.

Wu, Y., Li, K., Zuo, H.X., Yuan, Y., Sun, Y., Yang, X., 2014. Primary neuronal-astrocytic co-culture platform for neurotoxicity

assessment of di-(2-ethylhexyl) phthalate. J. Environ. Sci. 26 (5), 1145–1153. http://dx.doi.org/10.1016/S1001-0742(13)60504-5.

Yin, N.Y., Yao, X.L., Qin, Z.F., Wang, Y.L., Faiola, F., 2015. Assessment of bisphenol A (BPA) neurotoxicity *in vitro* with mouse embryonic stem cells. J. Environ. Sci. 36, 181–187.

Zhang, A., Li, Y.M., Chen, L., 2014. Distribution and seasonal variation of estrogenic endocrine disrupting compounds, N-nitrosodimethylamine, and N-nitrosodimethylamine formation potential in the Huangpu River, China. J. Environ. Sci. 26 (5), 1023–1033. http://dx.doi.org/10.1016/S1001-0742(13)60530-6.