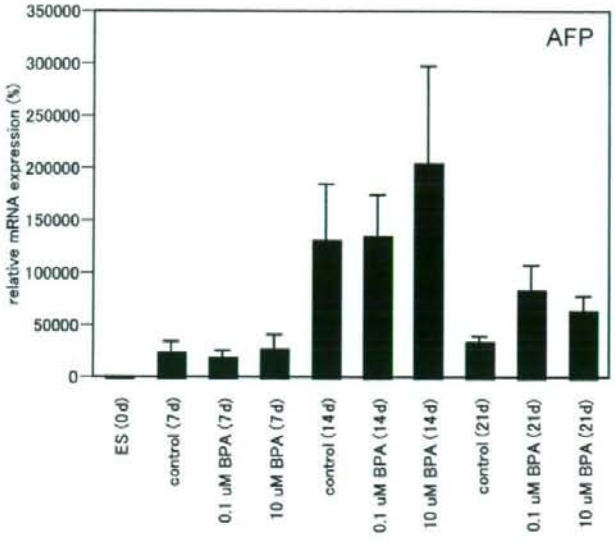

Fig. 4A. Effect of BPA on AFP mRNA expression in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

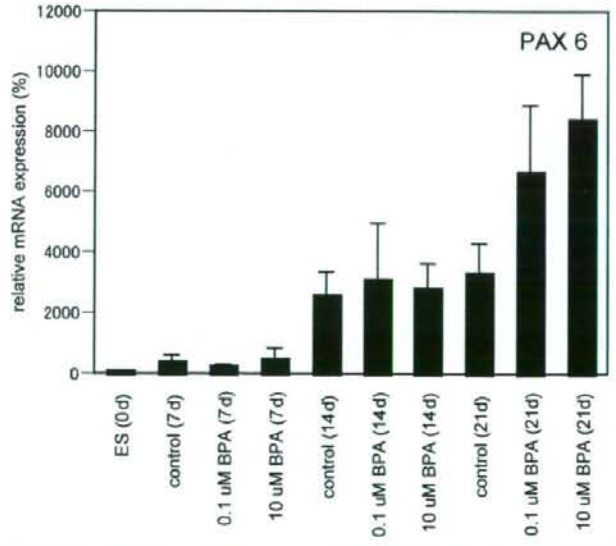

Fig. 4B. Effect of BPA on PAX-6 mRNA expression in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

not statistically significant (p=0.513). In 21-day EBs, the average mRNA expression in the presence of 0, 0.1 μM and 10 μM was 33,418% (reference category for p-value calculation), 80,100% (p=0.049) and 63,787% (p=0.049) respectively. The averages of three separate experiments of PAX-6 mRNA expression in the presence of 0, 0.1 and 10 μM BPA in 21-day EBs were 3,500%, 6,668% and 8,394%, respectively, compared with ES cells. The difference between doses of 0 and 10 μM was statistically significant (p=0.049). The difference between doses of 0 and 0.1 μM was not statistically significant (p=0.275). On Days 7 and 14, BPA did not show any evident effects on PAX-6 mRNA expression.

DISCUSSION

mRNA expression of Oct-3/4 was almost completely suppressed on Day 14, suggesting that ES cells reached differentiated status in around 14 days. E-cadherin, connexin 43, caveolin-1 and ASS were also consistently suppressed during EB differentiation (Table 2). The time patterns of their mRNA expression were similar to that of Oct-3/4. The cadherins are a class of transmembrane proteins that play important roles in cell adhesion. E-cadherin is first expressed in the 2-cell stage of mammalian development, and becomes phosphorylated in the 8-cell stage, where it causes compaction (Halbleib and Nelson, 2006). Connexin 43 is known to be related to gap junction-related protein, and gap junctions play significant regulatory roles in embryonic development (King and Lampe, 2005). Caveolin-1 has been shown to be the structural protein of plasmalemmal invaginations, termed caveolae, and functions as a tumor suppressor gene (Sotgia *et al.*, 2006). The tyrosine-phosphorylated form of caveolin-1 co-localizes with focal adhesions, suggesting that caveolin-1 plays a role in migration. Down-regulation of caveolin-1 leads to less efficient migration *in vitro*. ASS is an enzyme that catalyzes argininosuccinate synthesis from citrulline and aspartate, and is responsible for the third step of the urea cycle and one of the reactions of the citrulline-NO cycle (Husson *et al.*, 2003). ASS is highly conserved from bacteria to humans, and is present in large amounts in many tissues, including liver and kidney. It is difficult to discuss the significance of down-regulation of E-cadherin, connexin 43, caveolin-1, and ASS during EB differentiation, because they were selected by their ES/EB ratio in protein expression profiling. They may not be "general stemness marker genes" such as Oct-3/4, expression of which is always suppressed in any type of differentiation. However, they at least play a role, not only in maintaining the undifferentiated stem cell state, but also in readying stem cells for EB differentiation in response to deletion of signals from the MEFs. Reproducible results in some genes were obtained in separate experiments, indicating that this EB differentiation system could work as an embryotoxicity test.

Table 2. Average of mRNA expression of undifferentiated stem cell state-related genes.

	1d-ES	7d-EB	14d-EB	21d-EB
Oct-3/4	100%	3%	0.9%	0.4%
E-cadherin	100%	12%	20%	21%
Connexin 43	100%	32%	32%	27%
Caveolin-1	100%	17%	26%	21%
ASS	100%	10%	9%	7%

In response to BPA, expression of AFP andPAX-6 was increased at least temporarily. AFP is a glycoprotein that is produced principally in the fetal liver and gastrointestinal tract and is temporarily present during embryonic development. Estrogens were reported to modify AFP, exhibiting growth-suppressive properties (Vakharia and Mizejewski, 2000). BPA may interfere with the interaction between AFP and estrogen in EB differentiation. Recently, non-estrogenic effects of BPA on the central nervous system have been reported. In mice, prenatal and neonatal exposure to BPA induces a significant increase in the levels of dopamine D_1 receptor mRNA in the brain and increases central dopamine D_1 receptor-mediated activity (Suzuki *et al.*, 2003). In addition, expression of PAX-6 mRNA in embryos of *Xenopus laevis* was reported to be suppressed by treatment with 50 or 100 μM BPA from stage 10.5 to stage 35 (Imaoka *et al.*, 2007). PAX-6 is recognized as a master control gene for the development of eyes, sensory organs and certain neural and epidermal tissues that are usually derived from ectodermal tissues (Kondoh *et al.*, 2004). BPA may be related to one of the above functions in early development. The reason why the mRNA expression results for some genes were not reproducible may be that inappropriate primers were used for RT-PCR, that transfection with GFP may alter the characteristics of cells or that changes due to BPA may have occurred at the translational level rather than the transcriptional level.

In conclusion, our results indicated that this EB differentiation from cynomolgus monkey ES cells could work for detecting changes of gene expression in

response to BPA exposure, and could contribute to developing a primate ES embryotoxicity test in the near future.

ACKNOWLEDGMENT

The authors are grateful to Dr Colin R. Jefcoate (University of Wisconsin-Madison, USA), Dr Seishiro Hirano (National Institute for Environmental Studies), Dr Kunihiko Nakamura (National Institute for Minamata Disease) and Dr Koji Arizono (Prefectural University of Kumamoto) for their valuable comments, and to Ms Tomoyo Aratake and Ms Kiyoko Iwatsubo (National Institute for Minamata Disease) for their assistance in preparing the manuscript. This study was partly supported by a Grant (No. H16- REGENERATION-002) from the Ministry of Health, Labour, and Welfare of Japan.

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