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Advanced developmental toxicity test method based on embryoid body's area

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ABSTRACT

Embryonic stem cell test (EST) evaluates the embryotoxic potential of substances and measures the half inhibition in viability of mouse embryonic stem cells (ESCs), fibroblasts (3T3 cells) and in cardiac differentiation of ESC. In this study, we suggest the developmental toxicity test method (termed EBT) applying area of embryoid bodies (EBs) instead of cardiac differentiation of EST. In the assessment of 21 substances, EB area was logarithmically decreased in dose-dependent manner. Decline in EB area resulted in decrease of beating ratio during differentiation of ESCs. In classification by the EBT-based prediction model reflecting decline in cell viability and EB area, toxicity for 21 chemicals showed 90.5% accuracy. In the results of next generation sequencing, reduction in EB area resulted from cell cycle arrest mediated by HDAC2 and CDKN2A. Conclusively, EBT is advanced and is a useful tool to assess and classify various embryotoxicants in a short time with less effort.

1. Introduction

Since development of organisms produces rapid and complex changes within a relatively short period, some drugs must be administered to a mother during pregnancy under apparently appropriate conditions of time and dosage. Embryotoxicants can interfere with embryonic development through embryolethality, growth retardation or delayed organ growth, and result in persistent lesion and teratogenic effects. In the field of drug development, therefore, to evaluate the adverse effects of industrial chemicals on reproduction and development, multi-generation studies and/or screening tests have been performed in accordance with OECD Test Guidelines No. 414 (Prenatal developmental toxicity), No. 415 (One generation reproduction toxicology), No. 416 (Two generation reproduction toxicology), No. 421 (Reproduction/Developmental toxicity screening test), and No. 422 (Combined repeated dose toxicity study along with a reproduction/developmental toxicity screening test). However, these *in vivo* protocols are accompanied by sacrifices of large numbers of laboratory animals, and they are high cost and time consuming [1].

A wide spectrum of cell and tissue cultures have been presented as alternatives to the *in vivo* approach, and pharmaceutical and biotechnology industries are using more *in vitro* tests for high-throughput screening (HTS) of promising new compounds. To reduce the high attrition rate associated with *in vivo* testing in the later stages of drug development for animals or humans, there is an increasing demand for *in vitro* developmental toxicity tests for HTS systems in order to select "lead chemicals" in drug development [2]. Pluripotent embryonic stem cells (ESCs) can differentiate into any of the three germ layers through the formation of embryoid bodies (EBs), which are three-dimensional multicellular aggregates [3]. The ECVAM has suggested an *in vitro* embryonic stem cell test (EST) evaluating the cytotoxicity in mouse ESCs and fibroblasts (3T3 cells) and the inhibition in cardiac differentiation of ESC for predict-

Abbreviation: ESC, embryonic stem cell; EST, embryonic stem cell test; EB, embryoid body; EBT, embryoid body test; HTS, high-throughput screening; PM, prediction model.

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ing the embryotoxicity of chemicals [2]. However, to evaluate the inhibition of cardiac differentiation using beating requires a lot of time and labor and has a large error according to the experimenter.

In this study, a developmental toxicity test method (Embryoid Bodies Test, EBT) applying reductions in EB area instead of that endpoint using beating has been developed, and developmental toxicity of 21 chemicals was assessed. EBs with embryo-like structures act as the onset point for *in vitro* differentiation, recapitulate many features of differentiation during early mammalian embryogenesis, and therefore are useful as *in vitro* model systems for the study of mammalian embryogenesis. Since the quality of EBs affects the induction efficiency of EB derivatives in subsequent differentiation culture [4], a decrease in size or quality of EBs due to exposure to chemicals can result in growth retardation and abnormal differentiation during embryo development. The mechanism related to decrease in EB area due to toxicants was investigated by analyzing differentially expressed genes (DEG) *via* NGS. To establish the reliability and relevance of our EBT, three EBT endpoints were verified by performing experiments independently and repeatedly, and discrimination of EBT to embryotoxicants was determined by a biostatistical prediction model (PM).

2. Materials and methods

2.1. Chemicals

The tested chemicals were dissolved according to the solvent items listed in Table 1. All chemicals were purchased from Sigma-Aldrich and were prepared in molar concentrations (M).

2.2. Cell line and culture

Mouse ESCs (ES-E14TG2a) and 3T3 cells (Clone A31) were purchased from the American Type Culture Collection (Manassas, VA, USA). ESCs were cultured in culture medium (DMEM/F-12 (Gibco, Logan, UT, USA) supplemented with non-essential amino acids (NEAA, 1X; Gibco), 10% heat-inactivated and certified fetal bovine serum (FBS; Gibco), 2-mercaptoethanol (10^{-4} M), penicillin (100 U/mL), and streptomycin (100 μg/mL)) with mouse leukemia inhibitory factor (mLIF, 10 ng/mL; Millipore, Darmstadt, Germany) and grown on mitomycin C-treated mEFs in a 60 mm plate (Falcon, Glendale, AZ, USA) at 37 °C in a 5% CO_2 humidified tissue culture incubator (Sanyo, San Diego, CA, USA). For all experiments, ESCs used were between passage numbers 20 and 30. 3T3 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.3. Cytotoxicity

Cells were suspended in culture medium, and 50 μL of cell suspension containing 1.0×10^4 cells/mL were plated onto a well of 96-well plates. To each well was added 150 μL of culture medium with/without 1.333 times the final concentration of the tested chemical. Treated cells were grown in the same culture medium for 10 days. At the end of the treatment period, cells were incubated with a CCK solution (Dojindo Laboratories, Tokyo, Japan) in the dark for 1 h. To minimize the false positive effects associated with color interference, the medium containing a chemical was removed from the 96-well plate. After washing two times, medium with substrate was added. The absorbance of each well sample was read at 450 nm by using an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). The percentage of cell viability was determined from the absorbance of each well relative to the absorbance of the vehicle group which was set at 100%. The IC_{50} values were calculated according to the obtained concentration-response curve, and analyzed by using GraphPad Prism 6.01 (GraphPad Prism Software, San Diego, CA, USA).

2.4. Embryotoxicity using EB area

For the formation of EBs, a cell suspension containing 3.6×10^4 cells/mL in differentiation medium containing 15% FBS without LIF was prepared, and 90 drops with 20 μL of cell suspension per drop were plated on the lid of a 90 mm Petri dish (SPL, Pocheon, South Korea) and cultured as hanging drops with/without chemicals for three days after turning over the lids. D-PBS (7 mL) was added to the bottom dish to prevent drying of the drops. The EBs formed on the lids of two plates were transferred into a non-coated Petri dish with 6 mL differentiation medium with/without chemicals. The EBs were formed and evaluated at more than six concentrations of 21 chemicals separately.

Seven or eight pictures per concentration of chemicals were taken by phase contrast microscope (IX71; Olympus, Tokyo, Japan). An acceptable picture included five or more EBs, and the cross-sectional area of more than forty EBs per chemical concentration level was assessed. EBs were photographed so that the EBs had high contrast with the background by using a phase contrast microscope with 100 × magnification and phase 1. The photographic image files were opened in NIH Image J software (NIH, Bethesda, MD, USA). The images were converted to the 8-bit type so that the EB is black and fully visible by adjusting software 'Image → Adjust → Threshold' values, in that order. The adjusted images were saved as PNG files using the NIH Image J software macro in which the input and output file folder are selected and the format of output file is specified as PNG. The number of black pixels was obtained from the NIH Image J software, and the EB area was calculated by dividing the number of black pixels by the number of EBs. The obtained mean EB area was expressed as a relative percentage to the mean value of the vehicle group which was set at 100%. The half-maximal inhibition concentration value for the EBs (ID_{50} EB) was calculated from the dose-dependent reductions of EB area.

The following statements in macro:

```
run("8-bit");
setAutoThreshold("Minimum");
//run("Threshold...");
setOption('BlackBackground', false);
run("Convert to Mask");
saveAs("PNG","F:<\\output_folder_upper\\output
folder_lower\\Image.png>");
```

2.5. Analysis of molecular markers

2.5.1. Next generation sequencing (NGS)

Extraction of total RNA from harvested EBs was performed by using Trizol reagent (Ambion) according to the manufacturer's instructions, and mRNA was isolated by the polyA capture protocol with a bias to the 3′ transcript end. After mRNA fragmentation, cDNA libraries were constructed and sequencing of transcripts was performed on an Illumina HiSeq 2500 sequencer that can produce reads 50–150 bp in size and can sequence cDNAs of 200–300 bp in size by paired-end sequencing. Raw reads were purified by removing artifacts including the library adaptor sequence, contaminant DNA, and PCR duplicates. Refined reads were mapped on a reference genome by using the TopHat program, thereby producing aligned reads. DAVID, the web-based database (http://david.abcc.ncifcrf.gov/) for annotation, visualization, and integrated discovery, was used to interpret the DEGs. Genes were classified according to gene function information derived by the DAVID gene ontology tool (http://david.abcc.ncifcrf.gov/home.jsp). In the Venn diagram,