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Genome-wide gene expression analysis of mouse embryonic stem cells exposed to *p*-dichlorobenzene

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Because of the limitations of whole animal testing approaches for toxicological assessment, new cell-based assay systems have been widely studied. In this study, we focused on two biological products for toxicological assessment: mouse embryonic stem cells (mESCs) and long noncoding RNAs (lncRNAs). mESCs possess the abilities of self-renewal and differentiation into multiple cell types. LlncRNAs are an important class of pervasive non-protein-coding transcripts involved in the molecular mechanisms associated with responses to chemicals. We exposed mESCs to *p*-dichlorobenzene (*p*-DCB) for 1 or 28 days (daily dose), extracted total RNA, and performed deep sequencing analyses. The genome-wide gene expression analysis indicated that mechanisms modulating proteins occurred following acute and chronic exposures, and mechanisms modulating genomic DNA occurred following chronic exposure. Moreover, our results indicate that three novel lncRNAs (Snora41, Gm19947, and Scarna3a) in mESCs respond to *p*-DCB exposure. We propose that these lncRNAs have the potential to be surrogate indicators of *p*-DCB responses in mESCs.

[**Key words:** Non-coding RNA; Mouse embryonic stem cells; Deep sequencing; *p*-Dichlorobenzene; Toxicological assessment]

The need for a more efficient method for toxicity testing in vitro has been widely acknowledged. Although it is not yet possible to replace in vivo testing completely, we can refine and reduce the number of animals used in such analyses (1). Numerous studies focus on acute exposure of chemicals and environmental samples to cells followed by DNA microarray-based transcriptome analysis (2–6). For example, Koike et al. (3) and Omura et al. (4) reported genome-wide expression changes in rat alveolar epithelial cells following exposure to an organic extract, the *n*-hexane-soluble fraction, *n*-hexane-insoluble fraction, or dichloromethane-soluble fraction of diesel exhaust particles (DEP) using DNA microarray. These studies revealed that DEP extracts up-regulate expression of genes related to drug metabolism, antioxidant enzymes, the cell cycle/apoptosis, and coagulation (3,4). Hara-Yamamura et al. (6) reported genome-wide expression changes in human hepatoma HepG2 cells following exposure to wastewater effluent from membrane bioreactors and the activated sludge process . This study revealed upregulation of genes related to lipid metabolism, responses to endogenous stimuli, and responses to inorganic substances. It is important to note that these studies mainly used immortalized cell lines exposed at a single time point or short durations. Moreover, the conventional DNA microarray-based transcriptome analysis used in these studies was limited to messenger RNAs (mRNAs). More recent studies have employed microarray technologies for analysis of long noncoding RNAs (lncRNAs) (7,8).

Cell types are critical to monitor cellular stress responses. Immortalized cell lines are genetically altered, typically aneuploid, and may exhibit irrelevant toxic responses to compounds. In contrast, cells isolated from animal tissues lose their in vivo phenotype, can exhibit high variability among isolations, and can often only be expanded by dedifferentiation (9). Many of these limitations can be overcome using mouse embryonic stem cells (mESCs). mESCs have three important attributes (10): (i) normality, regarded as native cells; (ii) pluripotency, the ability to differentiate into ectodermal, mesodermal, and endodermal lineages; (iii) self-renewal, the ability to undergo numerous cycles of cell division while remaining undifferentiated in culture. These characteristics make mESCs a promising choice for assessment of toxicity.

Recent studies have uncovered numerous noncoding RNAs (ncRNAs) that account for a large proportion of RNAs in eukaryotic transcriptomes (11). Eukaryotic ncRNAs can be roughly classified into three groups: small ncRNAs (20–30 nt) such as microRNAs (miRNAs), intermediate-sized RNAs (30–200 nt) such as small nuclear RNAs, and long RNAs (>200 nt) such as long intergenic ncRNAs. lncRNAs are an important class of pervasive non-protein-coding transcripts involved in various biological functions. However, their functions remain largely unknown (12–14). Although lncRNAs involved in cellular stress responses are beginning to be investigated, there are few examples of lncRNAs whose expression

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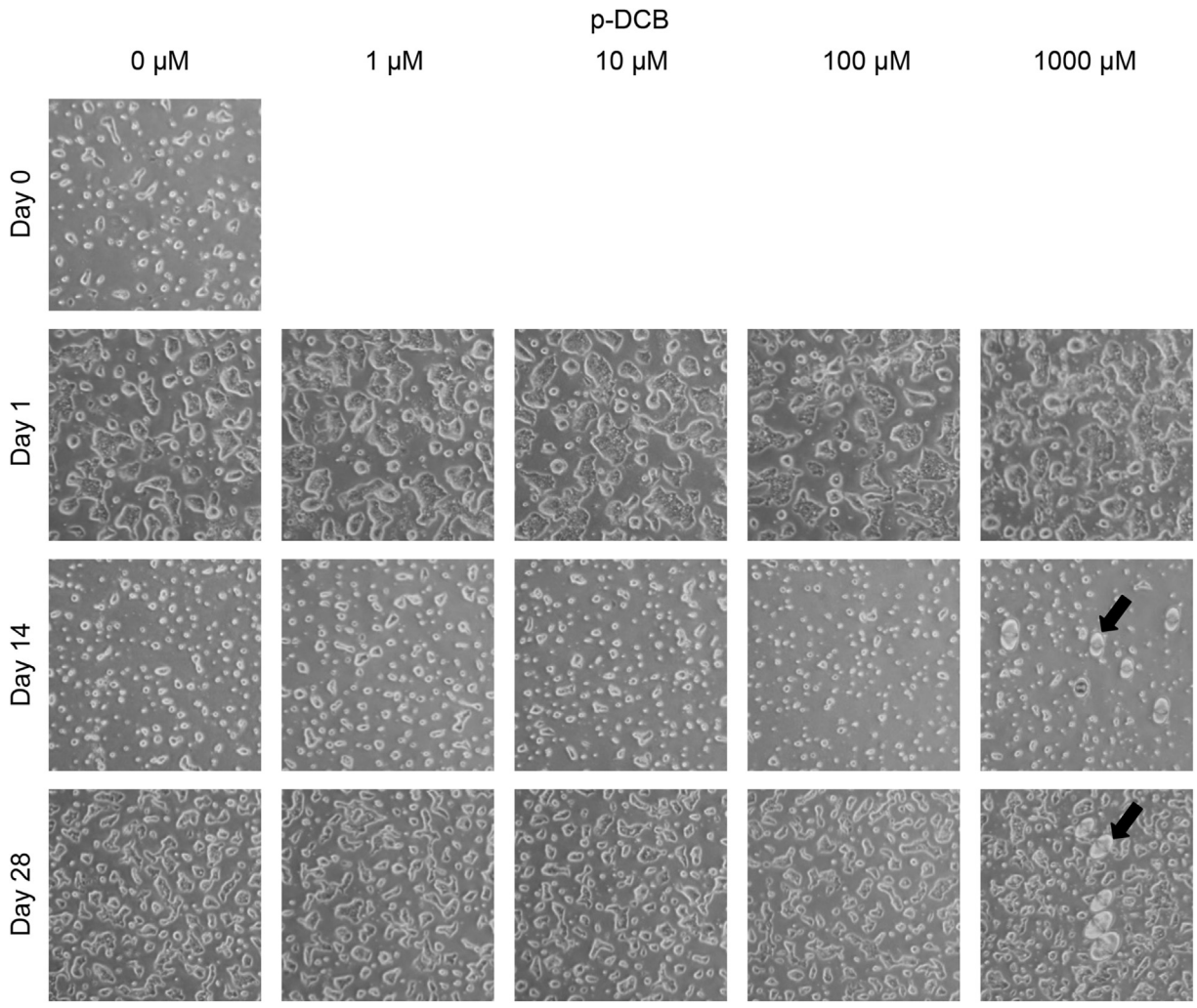

FIG. 1. Phase-contrast microscopic observation of mESCs. mESCs were treated with *p*-dichlorobenzene (*p*-DCB) at the indicated concentrations for 0, 1, 14, or 28 days. Arrows indicate changed cell shapes.

is altered by chemical stresses (15–18). These studies suggest that lncRNAs are important and tightly controlled in response to stress, and have profound effects on transcription, RNA processing, and translation.

In this study, we chose an exposure period of 28 days because many toxicity studies on mice have used this time point (19). We used mESCs because they are pluripotent and can differentiate into a variety of cell types such as cardiomyocytes, neurons, and hepatocytes. Using mESCs allows assessment of the exposure risk in a variety of tissues and cell types. In this study, we provide the basic framework for using mESCs and not differentiated cells derived from mESCs. We exposed mESCs to *p*-dichlorobenzene (*p*-DCB) for 1 or 28 days (daily dose), isolated the total RNA, and performed deep sequencing analyses (RNA-seq) to determine the effects of *p*-DCB exposure on genome-wide gene expression including lncRNAs. We chose *p*-DCB because it is listed in the Japan Pollutant Release and Transfer Register as a class I designated chemical substance. *p*-DCB is important because of high emission rates and hazardousness. Moreover, our data will be useful to assess the agreement of in vitro and in vivo studies, because there has been an in vivo study performed on mice (20) and several extensive risk assessment reports have been published by international organizations (19). DNA microarray analysis is limited to known RNAs with sequence data. However, RNA-seq can be used to detect known and unknown RNAs in genome-wide analysis. Thus, we used RNA-seq in this study.

MATERIALS AND METHODS

Chemicals *p*-DCB (Cas. No. 047-01315) was obtained from Wako Pure Chemical Industries (Osaka, Japan). *p*-DCB was dissolved in dimethyl sulfoxide and diluted in culture medium at a 0.1% vol/vol final concentration.

Cell culture The mESC line H-1 was isolated from C3H/He mice by Kitani et al. (21). mESCs were maintained in Dulbecco's modified Eagle's medium (4.5 g/l glucose) with L-glutamine, without sodium pyruvate, liquid (Nacalai Tesque, Kyoto, Japan) supplemented with 15% fetal bovine serum, 1000 U/ml StemSure Leukemia Inhibitory Factor (mouse, recombinant, solution; Wako), 0.1 mM StemSure 2-mercaptoethanol solution, and Penicillin-Streptomycin (Life Technologies). The mESCs were grown on mitomycin C-treated mouse embryonic fibroblast feeder cells (C57BL/6J) at 37°C in a humidified incubator with 5% CO_2. For chemical stress treatments, mESCs were cultured in ESGRO complete plus serum-free clonal grade medium (Merck Millipore) on gelatin-coated dishes without feeder cells.

Chemical stress treatments for 1 day Cells were seeded at 3.8×10^5 cells per well (6-well plate) in 2 ml medium or at 1.9×10^4 cells per well (96-well plate) in 100 μl medium. The cells were incubated overnight at 37°C with 5% CO_2. Cells were treated with *p*-DCB (final concentrations of 1, 10, 100, or 1000 μM) for 1 day. Total RNA was extracted from cells in the 6-well plate with RNAiso Plus (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The number of viable cells in the 96-well plate was counted using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions.

Chemical stress treatments for 28 days Cells were seeded at 3.8×10^5 cells per well (6-well plate) in 2 ml medium. The mESCs were treated with *p*-DCB (final concentrations of 1, 10, 100, or 1000 μM; Wako) for 28 days. Cells were passaged at 1:4–1:6 split ratios. At day 27, the cells were seeded at 3.8×10^5 cells per well (6-well plate) in 2 ml medium and at 1.9×10^4 cells per well (96-well plate) in