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A statistical approach towards the derivation of predictive gene sets for potency ranking of chemicals in the mouse embryonic stem cell test

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HIGHLIGHTS

- We assessed concentration-dependent gene expression changes to four phthalates.
- We derived a 97-gene set for toxicity potency ranking of phthalate monoesters.
- The gene set describes and predicts developmental toxicity of phthalates.
- Gene expression results are comparable to those using the classical ID50.

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ABSTRACT

The embryonic stem cell test (EST) is applied as a model system for detection of embryotoxicants. The application of transcriptomics allows a more detailed effect assessment compared to the morphological endpoint. Genes involved in cell differentiation, modulated by chemical exposures, may be useful as biomarkers of developmental toxicity. We describe a statistical approach to obtain a predictive gene set for toxicity potency ranking of compounds within one class. This resulted in a gene set based on differential gene expression across concentration-response series of phthalatic monoesters. We determined the concentration at which gene expression was changed at least 1.5-fold. Genes responding with the same potency ranking *in vitro* and *in vivo* embryotoxicity were selected. A leave-one-out cross-validation showed that the relative potency of each phthalate was always predicted correctly. The classical morphological 50% effect level (ID50) in EST was similar to the predicted concentration using gene set expression responses. A general down-regulation of development-related genes and up-regulation of cell-cycle related genes was observed, reminiscent of the differentiation inhibition in EST. This study illustrates the feasibility of applying dedicated gene set selections as biomarkers for developmental toxicity potency ranking on the basis of *in vitro* testing in the EST.

1. Introduction

In the process of development of novel compounds, it is important to compare toxic potencies, in order to prioritize for development those compounds which are effective in their prospective application (e.g., as a pharmaceutical or as a plasticizer) in the presence of the lowest toxic potency. Classically, toxic potency is assessed in animal studies, which are costly, time consuming, and ethically less favorable. These aspects apply especially to animal-consuming developmental toxicity studies. It has been shown that toxic potency of a compound may also be inferred from concentration-responses of its gene expression changes in alternative test systems (Baken et al., 2007; Kopec et al., 2010; Rowlands et al., 2013; Schulpen et al., 2012; van Dartel et al., 2011a; van Kesteren et al., 2011; van Kol et al., 2012). Based on these findings we hypothesized that a suitable gene set can be used in order to predict the potency of un-ranked compounds within the same class. Here we applied a statistical approach to obtain a set of genes which can function to rank compounds within a chemical class, using monophthalates as an example, within the embryonic stem cell test (EST) as a model system. The EST is a widely studied alternative test, proposed for the assessment of developmental toxicity of chemicals and pharmaceuticals (Heuer, 1993; Scholz et al., 1999; Seiler and Spielmann, 2014). This test is based

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on the potential of mouse embryonic stem cells (mESC) to differentiate into contracting myocardial cells within a period of 10 days. The inhibitory effect of compounds on the formation of contracting cardiac myocyte foci is the morphological endpoint of the assay. Concentration response analysis within the EST allows for calculation of the concentration at which there is 50% inhibition of differentiation (ID50). The inclusion of gene expression as a readout parameter in EST has resulted in a reduced test period of four days (van Dartel et al., 2010; van Dartel et al., 2009a). Previous studies have shown that in the first twenty-four hours of exposure major gene expression changes occur, specifically in proliferation- and differentiation-related genes, and that gene expression is highly sensitive to compound exposure (van Dartel et al., 2009a). In a previous analysis, we showed that 668 genes responded in a concentration-response like fashion to each of three embryotoxic phthalates, (Schulpen et al., 2012). Overall gene expression patterns of this set were mostly consistent with the relative potency ranking of the phthalates. To explore this latter finding, we used the corresponding data in a new analysis, to derive a suitable predictive gene set that could be used as a manageable biomarker to predict relative potency within the chemical class of monophthalates. Toxicity data of phthalates obtained in existing animal studies (Fabjan et al., 2006; Janer et al., 2008) were used to determine *a priori* relative *in vivo* potency of phthalates for this investigation. Genes were selected based on the concentrations at which each phthalate gave a 1.5 fold expression change in an individual gene. A set of 97 genes was obtained for which the ranking of these concentrations was consistent with in vitro embryotoxicity ranking. Potency ranking could be predicted by the relative order in which the majority of the genes in the set were changed by at least 50% to the control level. The robustness of this approach was demonstrated with a leave-one-out cross-validation experiment, in which each of the four monophthalates was left out one by one, a gene set was derived based on the three remaining phthalates, and subsequently, data for the left out phthalate were used to predict its relative potency on the basis of the derived gene set. We characterized the gene set as to biological processes involved. Furthermore, we derived effective concentrations on the basis of gene expression and compared them with classical ID50 concentrations.

2. Methods

2.1. Embryonic stem cell culture

Mouse embryonic stem cells were continuously cultured on gelatin coated dishes in Dulbecco's modified Eagle's medium (DMEM,Gibco, Gaithersburg, MD, Cat#11960-044), supplemented with 20% foetal bovine serum (FBS, Hyclone, Logan, UT, Cat#SH30070.03), 1% non-essential amino acids, (Gibco, Gaithersburg, MD, Cat#11140-035), 2 mM L-glutamine (Gibco, Gaithersburg, MD, Cat#25030-024), 1% 5000 IU/ml penicillin/5000 g/ml strepto-mycin (Gibco, Gaithersburg, MD, Cat#773139), and 0.1 mM β-mercaptoethanol (Sigma–Aldrich, Zwijndrecht, Cat#31350-010), in the presence of leukaemia inhibitory factor (LIF, Millipore, Temecula, California), 1000 units/ml, to maintain pluripotency. ESC were kept for a maximum of 25 passages and were passaged to new coated dishes every other day.

2.2. Differentiation

To initiate cell differentiation, pluripotent stem cells were cultured in hanging drops of culture medium on the inside of a lid of a Petri-dish, containing 5 ml phosphate buffered saline (PBS), Ca^{2+} and Mg^{2+} free (Gibco, Gaithersburg, MD, Cat#14190-094) as shown in Fig. 1. Within three days, the cells proliferated and clustered to form cell aggregates, so-called embryoid bodies (EB). The EB were transferred to bacterial dishes containing 5 ml of culture medium in which they were cultured for 2 days. The EB were then plated individually in wells of a 24-well plate and cultured for 5 additional days in which the EB further differentiated and formed foci of contracting cardiomyocytes (van Dartel et al., 2009b).

2.3. Exposure

Exposure was started three days after initiation of differentiation, at the point at which the EB were transferred to dishes (van Dartel et al., 2009b). The cells were exposed to one of the four phthalate monoesters: mono-(2-ethylhexyl) phthalate (MEHP, CAS# 4376-20-9, Wakochemicals, Neuss, Germany), monobutyl phthalate (MBuP, CAS#131-70-4, TCI, Zwijndrecht, The Netherlands), monobenzyl phthalate (MBzP, CAS# 2528-16-7, TCI, Zwijndrecht, The Netherlands) or monomethyl phthalate (MMP, CAS#4376-18-5, Sigma–Aldrich, Zwijndrecht, The Netherlands) in 0.3% DMSO for 7 days, in equimolar concentrations (0.00441, 0.0143, 0.0441, 0.143,0.441, 1.43 and 4.41 mM). At day 10 cardiomyocyte differentiation was microscopically examined by monitoring the wells of an individual 24-wells plate containing contracting cell foci. Via a concentration-response curve the ID50 concentration was calculated using Proast (Slob et al., 2002).

2.4. Transcriptomics

The following concentrations of phthalate monoesters were tested using microarray analysis of whole cells: for the effect on gene expression of MEHP the effect of concentrations 0.00441, 0.0143, 0.0441, 0.143 and 0.441 mM was tested. For MBzP and MBuP concentrations 0.00441, 0.0143, 0.0441, 0.143, 0.441, 1.43 and 4.41 mM were tested. For MMP concentrations 0.143, 0.441, 1.43 and 4.41 mM were tested. Each concentration of each compound was tested in 8 replicates. One dish contained one concentration of a single monoesther phthalate. Exposure was initiated at day 3. After 24 h exposure the cells were collected in RNA-protect and stored at −20 °C, up to RNA extraction. RNA was extracted with the QIACube by using the RNA Mini-extraction kit (Qiagen). After extraction the RNA concentration was measured with a Nanodrop spectrophotometer (Thermo Scientific). The quality of the RNA was assessed by using a Bioanalyzer (Agilent). RNA samples with sufficient quality and purity, based on RIN values were selected for microarray analysis. Samples were randomized, blinded and further processed for array analysis at ServiceXS (Leiden, The Netherlands) using Affymetrix HT MG-430 PM array plates.

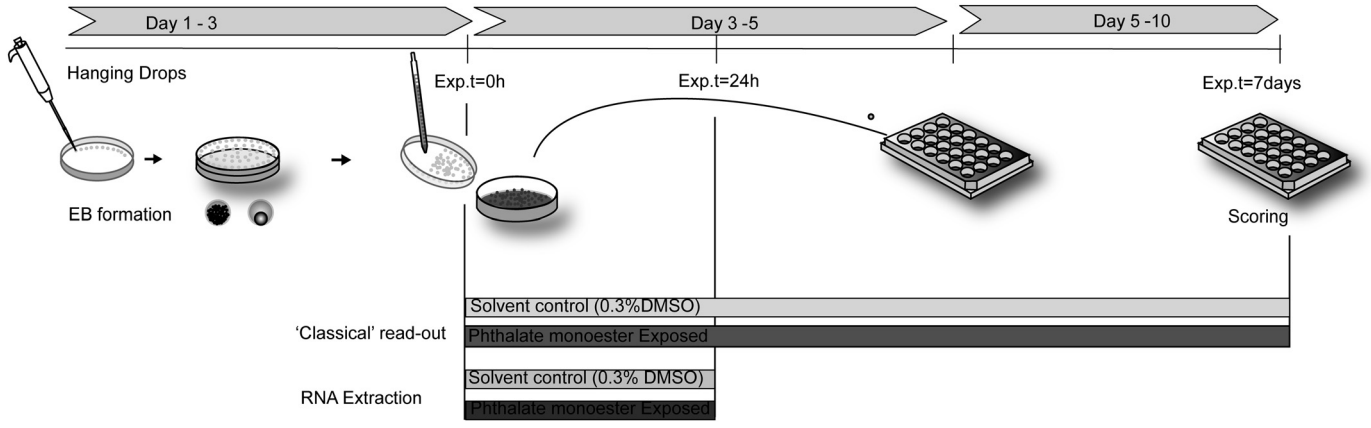

Fig. 1. Study design.
Mouse embryonic stem cells (mESC) were cultured in hanging drops on the inside of a lid of a Petri-dish for three days. At day 3, embryoid bodies (EB) had formed and were transferred to a bacterial Petri dish and cultured in culture medium containing the compound. For microarray analysis the EB were stored in RNA-protect at −20 °C after 24 h of exposure (day 4) and subsequently RNA was extracted. For morphological read-out and calculation of differentiation inhibition EB were individually transferred to a well of a 24-well plate at day 5 and cultured for five more days. At day 10 the cells were microscopically examined and wells containing contracting cell foci were scored.