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Concentration-dependent gene expression responses to flusilazole in embryonic stem cell differentiation cultures

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ABSTRACT

The murine embryonic stem cell test (EST) is designed to evaluate developmental toxicity based on compound-induced inhibition of embryonic stem cell (ESC) differentiation into cardiomyocytes. The addition of transcriptomic evaluation within the EST may result in enhanced predictability and improved characterization of the applicability domain, therefore improving usage of the EST for regulatory testing strategies. Transcriptomic analyses assessing factors critical for risk assessment (i.e. dose) are needed to determine the value of transcriptomic evaluation in the EST. Here, using the developmentally toxic compound, flusilazole, we investigated the effect of compound concentration on gene expression regulation and toxicity prediction in ESC differentiation cultures. Cultures were exposed for 24 h to multiple concentrations of flusilazole (0.54–54 μM) and RNA was isolated. In addition, we sampled control cultures 0, 24, and 48 h to evaluate the transcriptomic status of the cultures across differentiation. Transcriptomic profiling identified a higher sensitivity of development-related processes as compared to cell division-related processes in flusilazole-exposed differentiation cultures. Furthermore, the sterol synthesis-related mode of action of flusilazole toxicity was detected. Principal component analysis using gene sets related to normal ESC differentiation was used to describe the dynamics of ESC differentiation, defined as the 'differentiation track'. The concentration-dependent effects on development were reflected in the significance of deviation of flusilazole-exposed cultures from this transcriptomic-based differentiation track. Thus, the detection of developmental toxicity in EST using transcriptomics was shown to be compound concentration-dependent. This study provides further insight into the possible application of transcriptomics in the EST as an improved alternative model system for developmental toxicity testing.

Introduction

The current regulatory guidelines for toxicological hazard identification require large numbers of experimental animals. As a consequence of the implementation of the new EU regulation for Registration, Evaluation and Authorization of Chemicals (REACH) in 2007, the potential toxicity of thousands of chemicals needs to be assessed over the coming years. To reduce the number of experimental animals needed within REACH, alternative methods for toxicity testing are required. Since more than 60% of experimental animals in REACH will be used for reproductive and developmental toxicity assessments (van der Jagt et al., 2004), the demand for *in vitro* alternative test methods is particularly high for these areas of testing. Several alternative methods for the identification of developmental toxicity have been developed, varying from whole embryo cultures of rat or zebrafish, and organ cultures, to continuous cell-line based assays, such as the embryonic stem cell test (EST) (Flint and Orton, 1984; Genschow et al., 2004; Piersma et al., 2004; Rubinstein, 2006). None of these assays have achieved regulatory acceptance, due in part to their uncertain predictive capacity as well as their ill-defined applicability domain, i.e. the biological endpoints that are incorporated and the chemical categories for which developmental toxicity can correctly be predicted.

The EST is a cell-line based standardized *in vitro* assay that has been established to classify compounds with respect to their developmentally toxic potential (Genschow et al., 2004). The EST

Abbreviations: EB, embryoid body; ESC, embryonic stem cells; EST, embryonic stem cell test; FLU, flusilazole, FR, fold ratio; GO, Gene Ontology; GOID, gene ontology identifiers; PCA, principal component analysis.

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measures the inhibition of murine ESC differentiation into contracting cardiomyocytes upon exposure to compounds. The EST is considered a promising alternative assay for developmental toxicity prediction (Marx-Stoelting et al., 2009). However, it still has several weaknesses that need improvement to allow successful regulatory acceptation. These aspects include the relatively long culture duration, subjective and laborious endpoint scoring and the limited characterization of its applicability domain and predictability (Marx-Stoelting et al., 2009).

Since the emergence of transcriptomics in the 1990s (Schena et al., 1995), many studies have shown that this technique provides a powerful tool to study toxicological mechanisms and toxicity profiles at the level of gene expression. The use of transcriptomics in predictive toxicology has been extensively studied for the prediction of carcinogenicity, using liver tissue of *in vivo* exposed experimental animals (Kramer et al., 2004; Nie et al., 2006; Fielden et al., 2007; Ellinger-Ziegelbauer et al., 2008; Uehara et al., 2008). These data indicate that a gene expression-based predictive model can be an effective tool for identifying carcinogens. In addition, promising results have been published in other fields of toxicology, including developmental toxicology (Yang et al., 2007).

The implementation of transcriptomics in the EST may validate this test as a successful *in vitro* model for predicting developmental toxicity. We recently showed that compound-induced gene regulation could be observed shortly after the initiation of compound exposure, resulting in an abbreviated test protocol (van Dartel et al., 2009a; van Dartel et al., 2010b). Furthermore, gene expression changes pertain not to cardiac muscle cells only but to all cell types present in the culture. Therefore, gene expression endpoints may improve the predictability and applicability domain of the model. We have identified ESC differentiation-related genes and showed that developmental toxicants could be identified by the differential expression of these genes (van Dartel et al., 2010a).

So far, most transcriptomics studies have investigated the toxicity of a single test compound concentration. Only few studies have addressed concentration-dependent gene expression changes, often by testing only a low and a high dose (Ezendam et al., 2004; Goetz and Dix, 2009), but also more comprehensive concentration–response studies have been described (Naciff et al., 2005, 2010; Robinson et al., 2010). Mostly, the selection of these concentrations has been based on their observed phenotypic/morphological effects in the model system. Overall, compound concentration appeared to relate to both the magnitude of gene induction and the number of differentially expressed genes. Several studies have shown that dose–response assessment of gene expression is of great importance for correct prediction of toxic properties (Naciff et al., 2005; Baken et al., 2007; Yang et al., 2007; Auerbach et al., 2010). In a recent study, we showed the interdependence of morphological effect size and the extent of gene expression changes, suggesting that effect prediction through gene expression is modulated by compound concentration (van Dartel et al., 2010a).

The present study was designed to investigate the effect of compound concentration on gene expression in ESC differentiation cultures. Six concentrations of flusilazole were tested, which makes this study one of the most extensive studies in concentration-related gene expression responses to date. Flusilazole was selected due to its known developmental toxic properties, causing growth retardation and skeletal abnormalities in rodent models (Farag and Ibrahim, 2007) and its relevance for human health risk in view of its global use as a fungicide in agricultural practice (Zarn et al., 2003). In the present study, gene expression profiling confirmed concentration-dependent transcriptomic changes in differentiating ESC exposed to flusilazole. Gene expression-linked developmental-related processes appeared to be most sensitive to flusilazole exposure. Genes related to cell division as well as processes related to sterol metabolism, the pharmacological mode of action of flusilazole, were found to be affected. Furthermore, we applied principal component analysis (PCA) using gene sets related to ESC differentiation to describe the dynamics of differentiating ESC in the EST protocol, previously defined as the differentiation track (van Dartel et al., 2010b). These analyses showed that the dose-dependent effects on development were also evident by the extent of deviation of flusilazole-exposed cultures from the differentiation track. This study provides further insight into the use of transcriptomics in ESC differentiation as a model system for developmental toxicology.

Materials and methods

Pluripotent embryonic stem cell culture and differentiation. Pluripotent murine D3 embryonic stem cells (ESC; ATCC, Rockville, MD) were cultured in monolayer in DMEM-based (Gibco BRL, Gaithersburg, MD) medium supplemented with leukemia inhibitory factor (LIF; Chemicon, Temecula, CA), as described previously (Anon., 1999; van Dartel et al., 2009b). Induction of cardiomyocyte differentiation was performed according to the INVITTOX protocol (Anon., 1999) with few adaptations (De Smedt et al., 2008; van Dartel et al., 2010b).

Resazurin cell viability assay. To facilitate interpretation of the gene expression data, we assessed the effect of flusilazole (CAS No. 85509-19-9, Sigma-Aldrich, Zwijndrecht, The Netherlands) exposure on cell viability. We used resazurin dye reduction as a measure for the number of viable cells, as described previously (van Dartel et al., 2009b). Flusilazole was dissolved in DMSO and ESC differentiation cultures were exposed to final DMSO concentrations of 0.2%, which did not affect cell viability. Control cultures were also exposed to 0.2% DMSO for comparability reasons. ESC differentiation cultures were exposed to flusilazole during the first 3 days of the EST protocol, since effects on cell viability are most pronounced during this early phase of the EST protocol (van Dartel et al., 2009b). Flusilazole was tested at concentrations ranging from 0.1 to 300 μM in three independent runs. The resazurin reduction of exposed cultures relative to solvent control cultures was calculated and plotted against the flusilazole concentrations using PROAST software (Slob, 2002). The cell survival at the concentrations tested in the transcriptomics experiment and the concentration at which cell viability is reduced by 50% (IC_{50}) were calculated on the basis of the curve fit.

Inhibition of ESC differentiation by flusilazole exposure. Compound concentrations tested in the present transcriptomics study were selected on the basis of our dose-range finding studies on ESC differentiation inhibition. We selected a flusilazole concentration range that was equally spaced on a logarithmic scale and that covered the complete concentration–response range. Six concentrations of flusilazole (0.54, 1.8, 5.4, 18, 27 and 54 μM) were selected and we exposed ESC differentiation cultures from the embryoid body (EB)-stage at day three onwards. Cultures were microscopically evaluated at day 10 of the culture to assess the inhibition of cardiomyocyte differentiation, while parallel cultures were used for gene expression analysis at earlier time points as explained below. In each experiment, ESC differentiation inhibition was evaluated by calculating the mean scores of 24 exposed cultures per compound concentration relative score to the mean scores of 24 control cultures (van Dartel et al., 2011). The ESC differentiation at the concentrations tested in the transcriptomics experiment and the concentration at which ESC differentiation is reduced by 50% (ID_{50}) were calculated on the basis of the curve fit.

RNA isolation and whole genome gene expression profiling. RNA from flusilazole-exposed cultures was sampled 24 h after the start of exposure (culture day 4, 8 replicates per group), and RNA from DMSO-exposed control differentiation cultures was sampled after 0, 24 and 48 h from the EB-stage at day 3 (culture day 3, 4, and 5, 8 replicates per group). RNA isolation and preparation of the corresponding RNA