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Comparison of gene expression regulation in mouse- and human embryonic stem cell assays during neural differentiation and in response to valproic acid exposure

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ABSTRACT

Embryonic stem cell tests (EST) are considered promising alternative assays for developmental toxicity testing. Classical mouse derived assays (mEST) are being replaced by human derived assays (hEST), in view of their relevance for human hazard assessment. We have compared mouse and human neural ESTn assays for neurodevelopmental toxicity as to regulation of gene expression during cell differentiation in both assays. Commonalities were observed in a range of neurodevelopmental genes and gene ontology (GO) terms. The mESTn showed a higher specificity in neurodevelopment than the hESTn, which may in part be caused by necessary differences in test protocols. Moreover, gene expression responses to the anticonvulsant and human teratogen valproic acid were compared. Both assays detected pharmacological and neurodevelopmental gene sets regulated by valproic acid. Common significant expression changes were observed in a subset of homologous neurodevelopmental genes. We suggest that these genes and related GO terms may provide good candidates for robust biomarkers of neurodevelopmental toxicity in hESTn.

1. Introduction

A variety of *in vitro* methods have been developed for the study of mechanisms involved in embryonic development. These methods can be employed to elucidate effects of compound exposure correlated to toxicity. Ultimately, these test methods could be used for developmental toxicity screening of compounds and may contribute to the reduction of experimental animal use. Embryonic stem cells (ESC) can differentiate *in vitro* into different cell types, enabling the study of mechanisms of differentiation and developmental toxicity [1–3]. Several assays have been developed in which mouse embryonic stem cells (mESC) differentiate in to various cell types like cardiomyocytes [4,5], neural cells [6,7] and osteoblasts [8]. Processes involved in development and regulated as a response to compound exposure may differ between species on the molecular level [9]. Therefore, test systems based on cells of human origin are preferred for human risk evaluation. During the last decade, the application of human embryonic stem cells (hESC) in toxicity testing has been extensively studied and differentiation assays have been developed [10].

We have previously developed ESC based neural differentiation assays, with cells from either mouse (mESTn) [7] or human (hESTn) origin [11]. Both methods were based on the same principles. First, differentiation was initiated by changing the culture conditions that maintain pluripotency to differentiation stimulating conditions. In both methods, ESC aggregates were used to facilitate the differentiation process, and the morphological endpoint was reached after 11 days, at which clear neurological structures are abundantly present. Gene expression changes have been demonstrated to provide a sensitive and informative readout in these assays to study the developmental toxicity of substances [12–14]. Compound exposure during differentiation causes concentration specific gene expression changes. Having extensive gene expression data available on both mouse and human EST cell differentiation, as well as of the effects of substances on that process, we

Abbreviations: DNT, developmental neuro toxicity; ESC, embryonic stem cells; EST, embryonic stem cell test; FC, fold change; GO, gene ontology; hESC, human embryonic stem cells; hESTn, human neural embryonic stem cell test; mEST, mouse embryonic stem cell test; VPA, valproic acid.

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have the unique opportunity to directly compare both methods. It can be hypothesized that homologous genes and gene pathways, regulated in both mouse and human EST, may be among the most robust and predictive candidate biomarkers of neurodevelopmental toxicity. Therefore, we compared mESTn and hESTn as to gene expression changes during neural differentiation. Furthermore, we compared the effects on gene expression in mESTn and hESTn of valproic acid (VPA), an anticonvulsant and neurodevelopmental toxicant *in vivo* [15–17].

2. Method

2.1. Stem cell culture and neural differentiation

mESC culture and neural differentiation was performed according to the protocol published by Theunissen et al. [7]. Briefly, mESC (ES-D3) were routinely cultured on gelatin coated culture dishes, in presence of leukemia inhibiting factor (LIF) and were sub-cultured every 2–3 days. The mESC culture medium (mCM) contained DMEM, supplemented with 20% fetal bovine serum, 1% nonessential amino acids, 1% penicillin/streptomycin, 2 mM L-glutamine and 0.1 mM β-mercapto ethanol. Neural differentiation was initiated by culturing the ESC in hanging drop culture, in which the cells were cultured in droplets of stem cell medium where they formed embryoid bodies (EB). After 3 days the cells were transferred to bacterial dishes and cultured in suspension of mCM, supplemented with 0.5 μM retinoic acid (RA). On day 5 EB were plated on laminin coated dishes and cultured in mCM containing 10% FBS and supplemented with 2.5 μg/ml fibronectin. On day 6, the mCM was replaced by ITS medium, containing DMEM/F12, supplemented with 0.2 μg/ml insulin, 50 μg/ml apo-transferrin, 30 nM sodium selenite, 1% penicillin/streptomycin, 2 mM L-glutamine and 2.5 μg/ml fibronectin. On day 7 the EB were replated on poly-L-ornithine and laminin coated dished and cultured in DMEM/F12 medium, supplemented with 0.2 μg/ml insulin, 1% penicillin/streptomycin, 30 nM sodium selenite, 50 μg/ml apo-transferrin, 20 nM progesteron, 100 μM putrescine and basic fibroblast growth factor (bFGF). The medium was replaced every other day for 7 days, until day 11.

hESC culture and neural differentiation were performed according to the protocol published by Schulpen et al. [18]. Briefly, hESC were cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts in hESC culture medium hCM, containing DMEM-F12 supplemented with 20% Knock Out Serum Replacement (KOSR), 1 mM L-glutamine, 0.5% 5000 IU/ml penicillin/5000 μg/ml streptomycin, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol and 0.2 μg/ml bFGF. The hESC cells were sub-cultured 1–3 times per week and hCM was refreshed every day. To initiate neural differentiation, the hESC clusters were enzymatically dissociated, transferred to bacterial dishes, and cultured in suspension in hCM. At day 4 the cell aggregates were transferred to poly-D-lysine and laminin coated dishes containing DMEM-F12 supplemented with 1% 5000 IU/ml penicillin/5000 μg/ml Streptomycin, 1.5 mM L-glutamine and 10% ITS premix. After 2 days the medium was refreshed. At day 7 the medium was replaced by Neurobasal medium, supplemented with N-2 premix, B27 premix and 1% 5000 IU/ml penicillin/5000 μg/ml streptomycin. After 2 days the medium was refreshed.

2.2. VPA exposure

VPA exposure data was obtained in earlier studies of the mESTn [19] and hESTn [20]. Exposure in both assay systems was started at the onset of differentiation initiation in cell aggregates. The VPA concentrations tested were based on human pharmacological relevant concentrations with >80% cell viability *in vitro*, as determined in earlier studies [18,19]. Optimal exposure concentrations were determined in earlier individual studies, performed independently, resulting in comparable concentrations of VPA exposure [18,19]. mESC had been exposed for 24 h, from day 3 in the protocol, to either 0.015 mM, 0.06 mM, 0.25 mM or 1.0 mM VPA. Each concentration contained 8 replicates ($n = 8$). hESC had been exposed for 24 h, from day 0 of the protocol, to either 0.1 mM ($n = 2$), 0.33 mM ($n = 6$) or 1.0 mM ($n = 6$) VPA. Existing data from these studies were used in the present comparative investigation. All replicates were individually analyzed. For calculations the average per experimental groups was calculated and compared to their corresponding control. Statistics were based on one way ANOVA including all experimental data, avoiding power issues of individual groups.

2.3. RNA extraction

Cells ready for RNA extraction were harvested and stored at −20 °C in RNA protect (Qiagen Benelux, Venlo, The Netherlands). Differentiating mESC were collected at days 0, 3, 4, 5, 6 and 7. Each control group contained 8 replicates, except for day 0, which contained 4 replicates. Differentiating hESC were collected at day 0 ($n = 6$), 1 ($n = 5$), 4 ($n = 4$), 7 ($n = 6$), 9 ($n = 2$) and 11 ($n = 4$). Mouse and human ESC exposed to VPA were collected at day 1 and 4, respectively. RNA was extracted using the manufacturer's protocol. The extracted RNA was eluted in RNase free water and stored at −80 °C, until analysis.

2.4. Microarray analysis

Mouse- and human RNA samples were randomized and processed for hybridization to whole Mouse Genome 430 2.0- or human HT HG-U133 + PM Affymetrix genechips, respectively and further processed as described in Theunissen et al. [7] and Schulpen et al. [18].

2.5. Data analysis and statistics

Quality control and normalization of Affymetrix CEL files was performed using either RMAexpress [21] for mouse Affymetrix genechips, or ArrayAnalysis website (http://www.arrayanalysis.org/) (Maastricht University, The Netherlands) [22] for human Affymetrix gene chips, using the Robust Multichip Average (RMA) algorithm [23] and MBNI custom CDF version 15 [24]. Subsequently, normalized data was Log2 transformed. For further analysis, mouse gene ID were transformed in human gene homologues, using R-software (version 2.15.0) and data downloaded from NCBI homoloGene (http://www.ncbi.nlm.nih.gov/homologene). Additionally, the genes which were present both on mouse- and human Affymetrix gene chips were selected. This resulted in a gene set with a total number of 14939 genes, which were used in this study for all further analyses (Supplementary Table 1).

2.6. Significant differentially expressed gene expression

Differentiation was studied by calculating the significantly regulated genes for each sample using a one-way ANOVA (OWA) analysis with a significance threshold of $P \leq 0.001$ and a maximum absolute fold change (FC) across time points ≥2, using R. For each significant gene the FC was calculated compared to the average fold change across all time points per species, using R. VPA significantly regulated genes were calculated using R with a significance threshold of $P \leq 0.001$ and FDR ≥ 5%. Heat-map visualization and hierarchical clustering was performed using Genemath XT (applied Maths, Sint-Martens-Latem, Belgium), using Euclidean