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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

distance and Ward linkage. Commonalities among genes significantly regulated during differentiation in both assays and after VPA exposure were calculated and displayed in Venn-diagrams, using Venny [25].

2.7. Functional analysis

Significantly enriched GO terms of specifically selected gene sets ($P < 0.01$) during either mESTn- or hESTn differentiation were analyzed using DAVID [26]. Human tissue enrichment of the 14939 genes was analyzed using Tox-profiler [27]. For each sample the enrichment was calculated with a significance of T-value ≥ 3.5 and $E \leq 0.05$. The T-test is a value obtained by a T-test between the expression changes for a defined set of genes *versus* all other genes [27]. The E-value is the associated two-tailed P-value with Bonferroni correction for the number of gene sets tested. If a GO-term or human tissue specific gene set was significantly regulated by at least one experimental group, it was selected for further analysis and other experimental groups were included in the comparison. The effects on GO-term- and human tissue enrichment after VPA exposure of the 14939 genes, were analyzed using Tox-profiler.

3. Results

3.1. Comparison of time-related gene expression changes in mESTn and hESTn

During mESTn differentiation 3774 genes were significantly regulated across time. For these mouse genes and their human homologs measured in the hESTn, FC at each time point for each gene was calculated as compared to the species average FC of each particular gene over time, and illustrated in a heat map using hierarchical clustering (Fig. 1A). Six clusters could be identified based on their gene expression pattern and are graphically represented (Fig. 1A). The enrichment for GO biological processes of genes present in each cluster was studied using DAVID. Clusters 1–4 showed a down regulation of genes over time in mESTn, which was less clear in hESTn. Clusters 2 and 3 contained significantly enriched GO-terms. Cluster 2 involved developmental processes like gastrulation, pattern specification and embryonic morphogenesis. Gene expression in this cluster peaked at day three in mESTn. Cluster 3 contained significantly enriched GO-terms involved in DNA and RNA processing. In mESTn, both clusters 5 and 6 showed an up regulated gene expression with time in which (neuro) developmental processes, such as neuron differentiation, pattern specification, skeletal system development and embryonic morphogenesis, were highly enriched. A similar gene expression change with time was observed in hESTn in cluster 6.

In hEST, 1975 genes were significantly differentially regulated over time. The FC per gene was calculated relative to the average expression per gene and compared to expression changes of homologous genes in mESTn (Fig. 1B). Visualization in a heat map and hierarchical cluster analyses resulted in eight clusters identified based on gene expression patterns (Fig. 1B). Out of the down regulated gene clusters in hESTn (1–4), clusters 3 and 4 contained significantly enriched GO-terms associated with cell cycle and proliferation, like cell cycle, mitosis and nuclear division. The mESTn displayed a similar trend in cluster 3, whereas in cluster 4 hardly any expression changes occurred in mESTn. Clusters 5–8 included genes which were up-regulated over time. Significantly enriched GO-terms in cluster 5 were involved in development and differentiation related processes, including neuron differentiation, pattern specification, embryogenesis and cartilage- and skeletal development. A similar expression pattern was observed for mESTn in cluster 5. The GO-terms observed in cluster 6 were linked to (macro) molecular factors, such as extracellular matrix organization, macromolecular complex- and plasma lipoprotein particle remodeling. Cluster 7 contained GO-terms including blood coagulation, hemostasis and response to wounding. In mESTn, cluster 7 barely showed regulation of gene expression.

Between mESTn and hESTn, 950 homologous genes were significantly regulated in both assays (Fig. 1C). These genes were not confined to specific locations in Fig. 1A and 1B. After heat map visualization and hierarchical clustering analysis we identified 7 clusters for further analysis (Fig. 1D). Clusters 1–3 and 5 showed an opposite pattern of gene expression regulation in both assays. However, no GO-terms were found significantly enriched in either of these clusters. Cluster 4 showed genes that were up-regulated across time in both assays. GO-terms associated with (neural) development, like neuron differentiation and development, axonogenesis, and skeletal system development were highly enriched. In contrast, genes in cluster 6 showed an overall down regulation with time, some after an initial up-regulation during the first days into the assay. Early developmental processes associated GO-terms, like pattern specification, embryonic development, gastrulation, endodermal development, where significantly enriched in this cluster. Individual genes regulated in the most responsive gene clusters with time in Fig. 1C, up-regulated genes in cluster 4A and down-regulated genes in cluster 6, are shown in Fig. 1E and F.

Tissue-correlated gene expression was analyzed using Tox-profiler. Per experimental group the significantly regulated tissue-related gene expression (Fig. 2) was determined, based on whole genome gene expression changes. Using hierarchical clustering, 4 clusters (A–D) were identified based on regulation patterns over time. Cluster A exclusively contains tissues of ectodermal origin, including specific brain tissues, of which characteristic gene sets were significantly up-regulated in both assays and all tissues. Cluster B contains several mesodermal originated tissues (B1) showing steep up-regulation of characteristic gene sets in hESTn, and three endodermal tissues (B2) of which characteristic gene sets were al significantly regulated in the hESTn with time, with a lower relative magnitude of regulation in mESTn. Cluster C contains 8 tissue gene sets of which 6 related to ectodermal tissues, which were significantly enriched in the hESTn throughout the differentiation period. Cluster D is a mixture of meso-, endo- and ectodermal originated tissues. The responses for cluster C and D tissues were less prominent in mESTn than in hESTn.

3.2. Comparison of VPA-mediated gene expression changes in mESTn and hESTn

After VPA exposure, in the mESTn 3281 genes were significantly differentially expressed (Fig. 3). Matching these mouse genes with available human homologues and selecting those genes present on both mouse and human gene expression arrays resulted in 2941 genes available for comparison. Exposure to VPA in the hESTn resulted in 3354 differentially expressed genes. There were 988 homologous genes regulated in both assays by VPA exposure. Between assays, 143 genes were commonly regulated both over time and after VPA exposure (Fig. 3, field *G*). GO-term analysis of this selection of genes revealed processes involved in cell proliferation and regeneration. In fields *A*, *B*, *C*, *E* and *F* of the Venn diagram in Fig. 3, developmental and neural GO-terms were significantly enriched. Genes exclusively regulated in hESTn over time (*I*), were involved in GO-terms describing blood (coagulation), wound healing and hemostasis. VPA-regulated genes in both mESTn and hESTn which were not regulated over time in both assays (*N*) contained GO-terms associated with the pharmaceutical mode of action of VPA, including histone acetylation and modification, and chromatin modification. The latter was also significantly enriched in the gene group not regulated over time and exclusively regulated

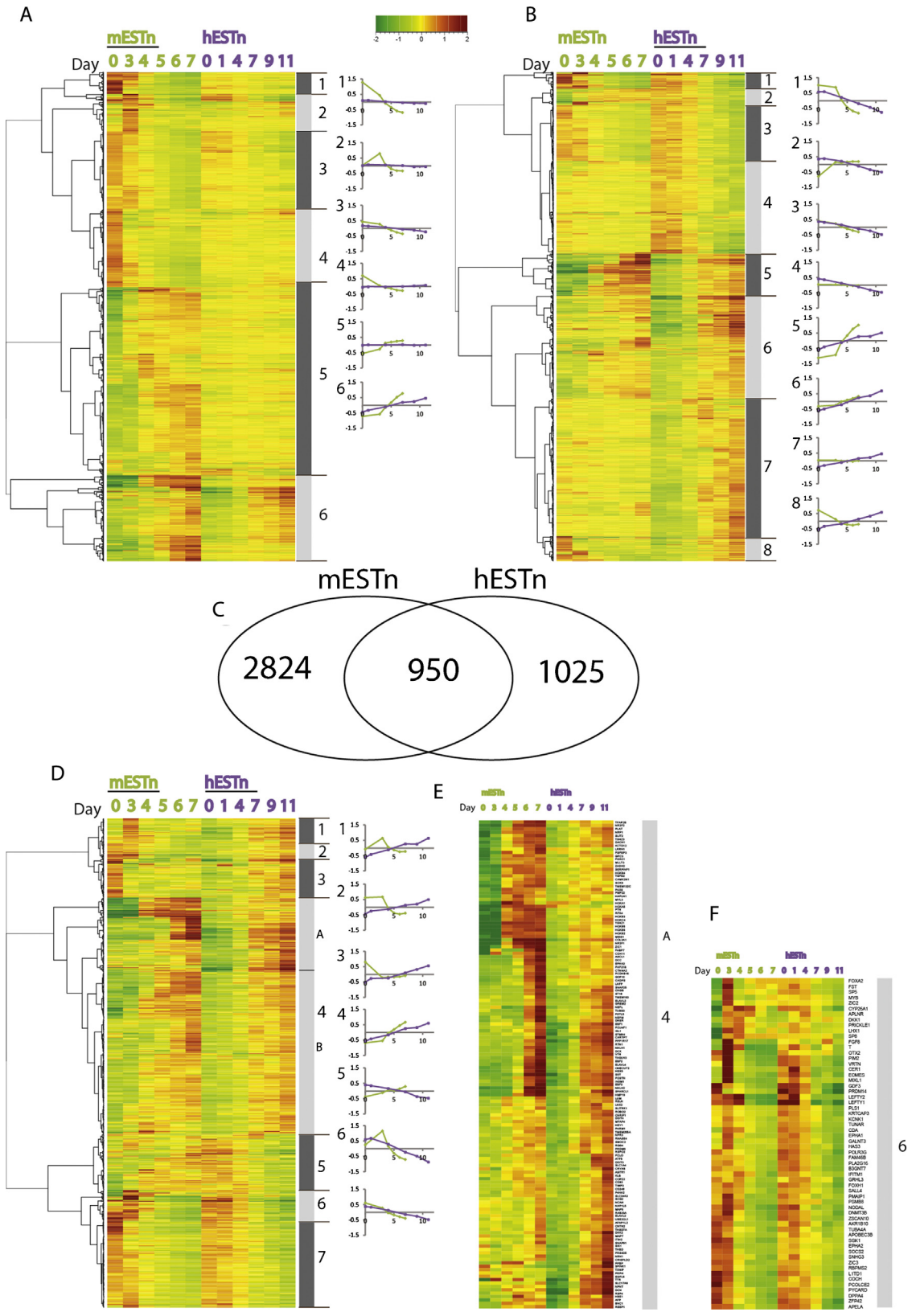

Fig. 1. Heat map comparing gene expression changes with time in mESTn and hESTn. (A) heat map of 3774 mouse genes significantly regulated in mESTn compared with the expression changes of their human homologs in hESTn; (B) heat map of 1975 human genes significantly regulated in hESTn compared with the expression changes of their mouse homologs in mESTn; (C) Venn diagram showing 950 genes of which homologs were regulated over time in both mESTn and hESTn; (D) Heat map of 950 homologous genes commonly regulated in both mESTn and hESTn; (E) and (F) enlarged representation with gene name details of clusters 4A and 6 in D.

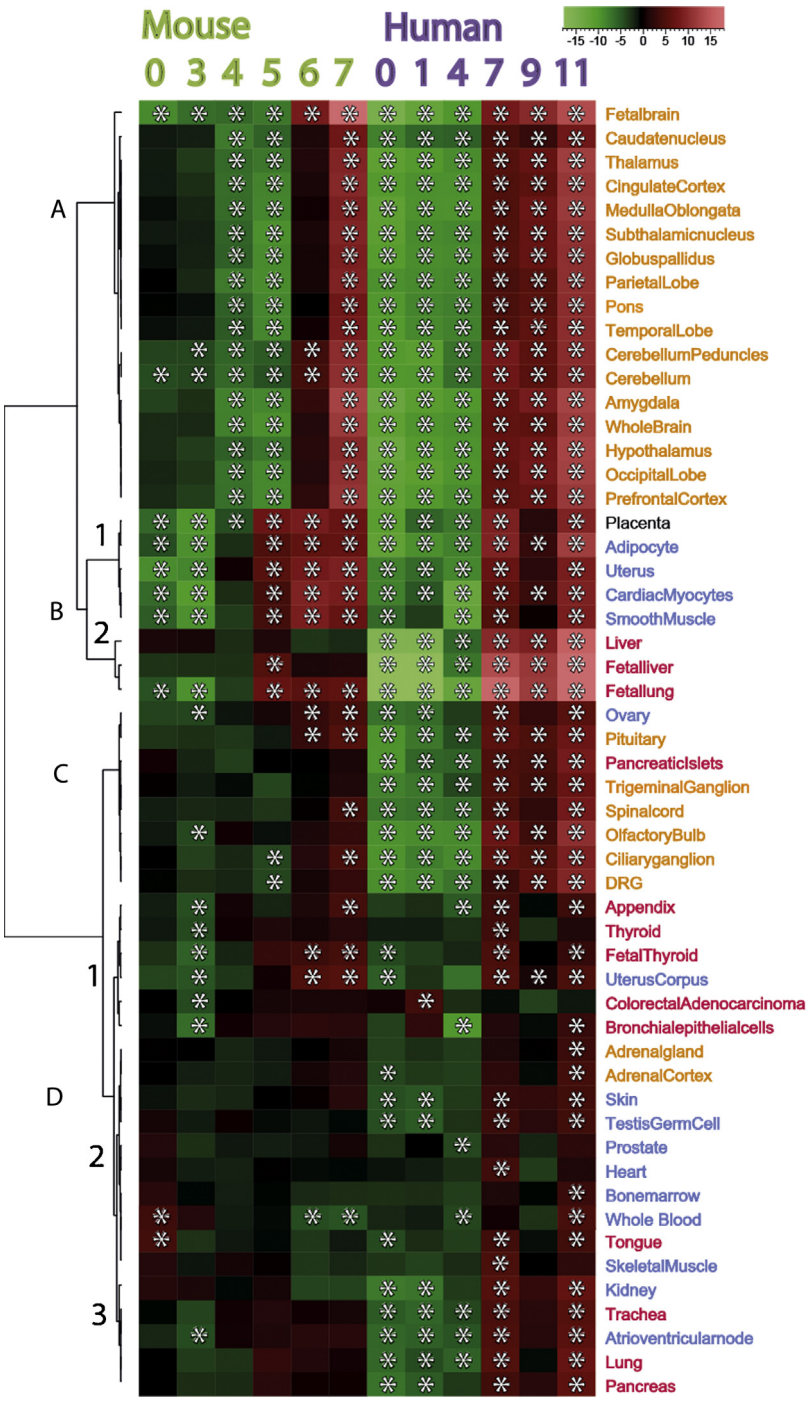

Fig. 2. Hierarchical clustering of tissues significantly enriched according to Tox-profiler analysis (*T-value ≥ 3.5 and $E \leq 0.05$) during differentiation in the mESTn (green header) and in the hESTn (purple header). Tissue names on the right are color coded for their primary germ layer origin: ectodermal (yellow), mesodermal (blue) and endodermal (pink) tissues. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in hESTn after VPA exposure (O). The converse gene group not regulated by time but exclusively in mESTn after VPA exposure (M), was characterized by the GO-terms protein localization and cell cycle/proliferation processes. An overview of regulated GO-terms is given in Supplementary Table 2.

Heat map and cluster analysis of concentration-dependent VPA effects resulted in two main groups of regulated genes, characterized by either down- or up-regulation with increasing concentrations, respectively, as observed in the hESTn (Fig. 4, clusters 1–3 down *versus* clusters 4–6 up). However, these trends in some regions conflicted with findings in mESTn. Cluster 2, containing genes associated with chromatin modification and remodeling and (positive) regulation of gene expression, showed a down regulation in the hESTn, whereas mESTn showed partly down- and partly up-regulation of homologous genes. Cluster 3 showed concentration dependent down regulation in both assays. Go-terms significantly enriched in this cluster were associated with sterol biosynthetic process and cell death [28,29]. Cluster 4 included genes related to neuron developmental processes, including neuron development and differentiation, together with catabolic and metabolic processes, characterized by up-regulation, which showed a stronger effect on FC observed in hESTn than in

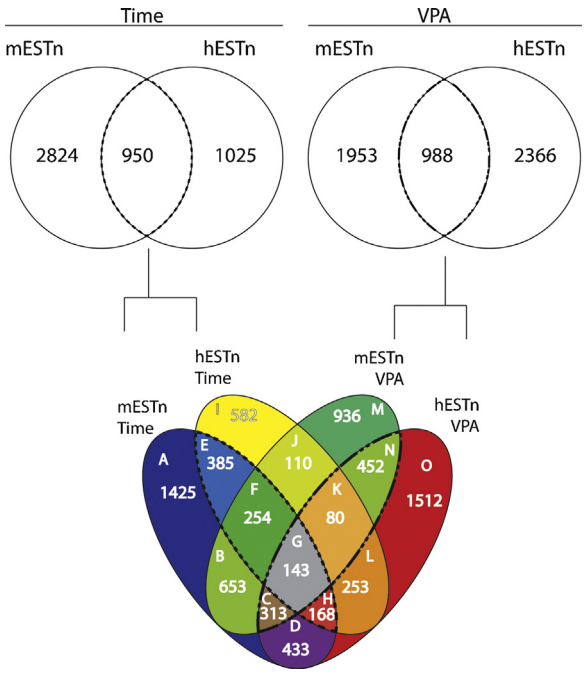

Fig. 3. Venn diagram: numbers of commonly and uniquely regulated genes (and their homologs) over time during differentiation in the mESTn and hESTn (top left); numbers of commonly and uniquely regulated genes (and their homologs) after VPA exposure in the mESTn and hESTn (top right); combined distribution of time and VPA regulated genes (and their homologs) in mESTn and hESTn. (bottom).

mESTn. Cluster 5 showed an up-regulation in both mESTn and hESTn. The GO terms associated with this cluster where involved in metabolic process, oxidation and chemical homeostasis. Cluster 6, with GO terms related to neurogenesis, such as axonogenesis, neuron projection development and cell morphogenesis involved in differentiation, was strongly up regulated in the hESTn, and to a lesser extent in the mESTn.

The effect on gene expression after VPA exposure was studied based on enrichment of GO-processes and of human tissues. In the heat map, illustrating GO-term T-scores, four clusters (1–4) could be distinguished (Fig. 5, left). Cluster 1 includes neural processes, which showed concentration dependent up-regulation in both assays. The second cluster (2) contains cellular processes involved in cell division and isoprenoid-/cholesterol biosynthetic process. The latter two are involved in the regulation of transcription events affecting lipid synthesis, meiosis and developmental patterning [30]. VPA exposure caused a significant down regulation of these processes in the mESTn, which was not observed in homologous genes in the hESTn. The third cluster (3), included GO-processes involved in chromatin modification, showing down-regulation. The fourth cluster (4) contained GO-terms involved in nuclear processes and replication. The GO-terms in cluster 4 showed a concentration dependent down-regulation.

The effects observed on the enrichment on the level of tissues, was similar between both assays (Fig. 5, right). Three clusters could be distinguished in the heat map (1–3), representing *T*-scores of the human tissue gene sets. The strongest effect in both assays was observed on neural systems (cluster 1). Here gene groups representing specific brain areas were strongly up-regulated in both assays with increasing concentrations of VPA. Cluster 2 also included neural tissues like thymus, dorsal root ganglion (DRG) and spinal cord, in which thymus-related genes were only regulated in the hESTn. Other tissues, like sex-organ associated tissues, heart, blood and kidney were up-regulated in hESTn. Segment 3 was mainly characterized by the strong down-regulation after 0.1 mM

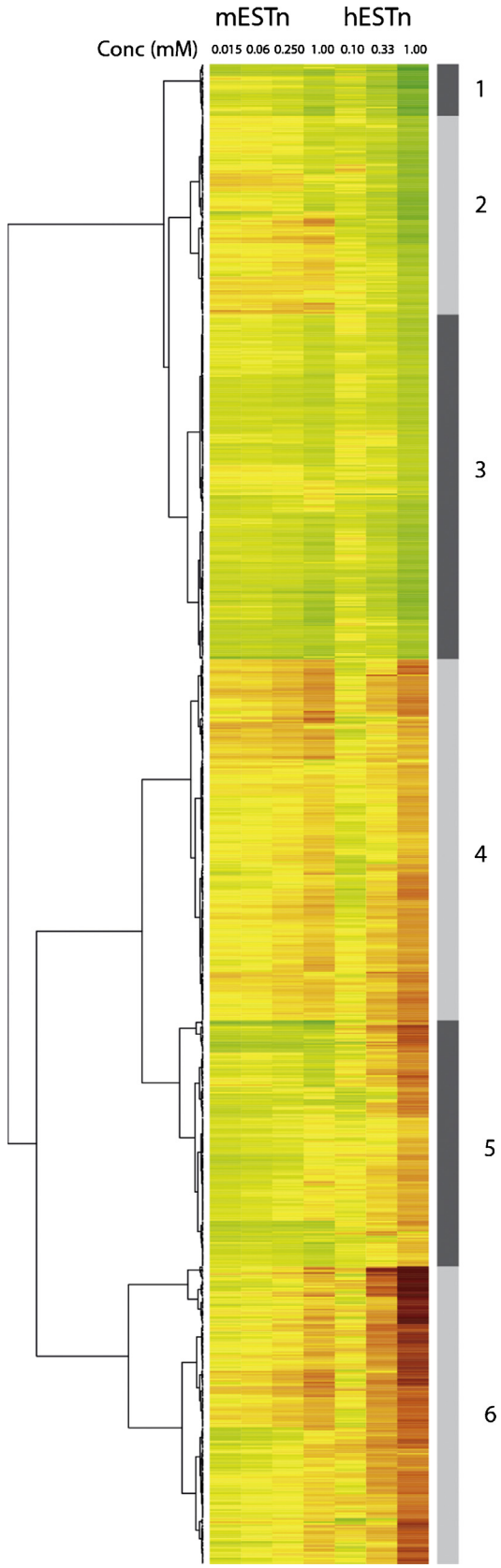

Fig. 4. Heat map representation after hierarchical clustering of homologous genes regulated by increasing concentrations of VPA exposure in both the mESTn (left) and hESTn (right).

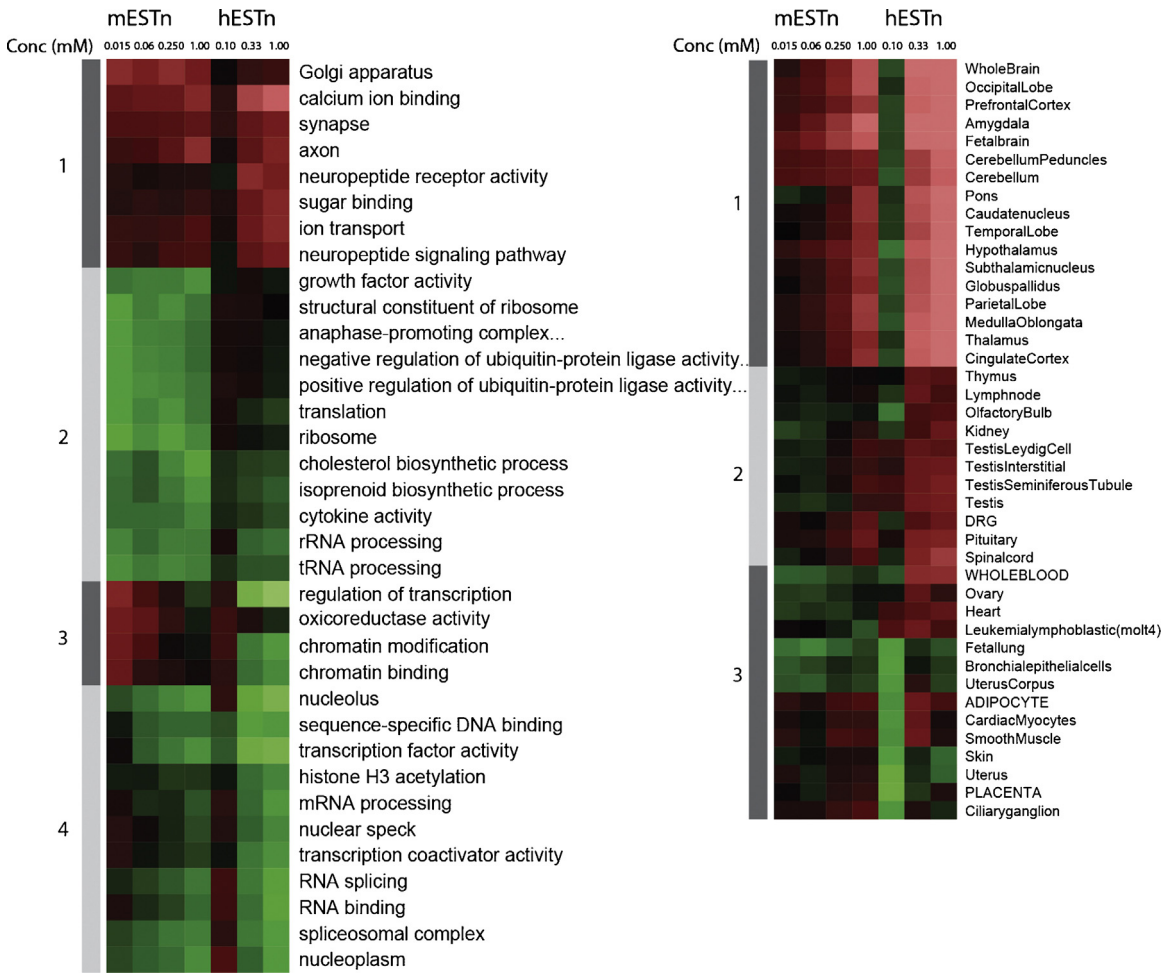

Fig. 5. Enriched GO-terms (left) and tissues (right) after exposure to increasing concentrations of VPA in the mESTn and hESTn based on 14939 genes using Tox-profiler.

VPA in the hESTn. Regulated tissues included adipocyte, cardiomyocytes, smooth muscle and fetal lung.

An overview of regulated GO-terms and tissues is given in supplementary Table 3.

4. Discussion

4.1. Embryonic stem cell tests

Human embryonic stem cell based assays for developmental toxicity testing of chemicals have gained interest in the past decade. This interest stems from the hypothesis that human hazard is probably best assessed in assays with human biological systems, which is one of the key messages from the NAS landmark report on toxicity testing in the 21st century [31]. In the past, primarily mESC were used for the stem cell test [32] which provided ground work for development of the EST [33]. The EST was designated more recently as one of the most promising validated *in vitro* tests for developmental toxicity prediction [10]. However, it has been shown that different species may respond differently as to developmental toxicity [34]. Perhaps the most striking example is the difference in response to thalidomide which caused severe malformations during human development, whereas it caused fetal death in rodents [34,35]. The use of human embryonic stem cells would avoid interspecies extrapolation in alternative assays.

Human embryonic stem cells can be manipulated *in vitro* to differentiate into several cell types *in vitro* like, neuronal, skin, adrenal and keratinocyte [36–38], blood, endothelial, kidney, bone, muscle, heart [39–43], pancreas and liver cells [44–46], cardiomyocytes [42,47,48], and fetal ventricular myocytes [49], as summarized by Pellizzer [50]. Human embryonic stem cells have been suggested to be "toxicology's new best friends" [51]. In 2008 the use of hESC for developmental toxicity testing was published [10,52]. Since then several protocols have been published on the use of hESC for developmental toxicity testing [34,53–55]. *In vitro* hESC neural differentiation was used to study neurodevelopmental toxicity of ethanol and retinoic acid [56,57]. Several other studies using hESC for neurodevelopmental toxicity testing have been published [13,58–60].

4.2. Comparing cell differentiation in mESTn and hESTn

We have developed experience with both mouse and human ESTn, specifically with regard to gene expression regulation during neural differentiation as well as to the effects of compounds thereupon [7,18]. These data allowed us to compare mouse and human EST, in order to identify commonalities and differences, possibly indicating conserved genes responsive in both assays that could be used as prioritized predictive markers for developmental neuro toxicity (DNT). We are aware that differences observed between both assays are caused by an intimate interplay between cell characteristics and culture conditions, which are partly interdependent. Whereas the mESC employed can be cultured relatively easily on gelatin coated surfaces in standard media containing LIF [61,62], hESC employed in these studies required feeder cells and sub culturing in cell clusters for retaining stem cell characteristics [18].

This causes necessary differences in their differentiation induction protocols as well, with an initial aggregation step needed for mouse stem cells but not for human stem cell clusters before differentiation is initiated. In order to standardize between assays as well as possible, we have adapted the assay protocols such that both share an 11 day differentiation protocol, with a 24 h period of exposure to test compounds, initiated at the start of cell differentiation, followed by analysis of gene expression. We hypothesize that commonalities in the expression regulation of homologous genes in both assays, that persist in the presence of differences in species, cell lines and assay conditions, may indicate genes and functionally related gene groups that might provide robust biomarkers for neurodevelopmental toxicity.

Common gene expression regulation in mouse and human ESTn were related to early development, involving genes which were down regulated over time (Fig. 1D and F, cluster 6). Among them was PRICKLE, involved in neural outgrowth and associated with neural tube defects [63,64]. EPHA1 is a receptor tyrosine kinase implicated in guidance of the migration of axons and cells in the nervous system [65,66]. EOMES is required for specification and proliferation of intermediate progenitor cells and their offspring in the cerebral cortex [66]. CER1 plays a role in anterior neural induction and somite formation during embryogenesis [67]. FGF8 is required for normal brain, eye, ear and limb development during embryogenesis [68,69]. LHX1 plays a role in neurogenesis [70]. Additional genes, like Brachyury (T) and MIXL1, are both involved in regulation of mesoderm formation and differentiation [62,71–73]. MIXL1 is also involved in endodermal cell differentiation [74]. LEFTY1 and -2,both play a role in left-right asymmetry of organ systems during development [75].

Common gene expression changes were observed in neural differentiation related genes up-regulated over time (Fig. 1 D and E, cluster 4). DNER is an activator of the NOTCH1 pathway, which plays a role in neural progenitors and neuronal differentiation. Furthermore, DNER acts as and mediator of neural-glia interactions during astrocytogenesis and developing cerebellum [76]. DCX is a micro-tubule-binding protein regulates cytoskeletal dynamics and neuronal morphogenesis [77]. RELN plays a role in layering neurons in the cerebral cortex and cerebellum [78,79]. Pou4F1 is involved in the regulation of specific gene expression within a subset of neuronal lineages and development of the sensory nervous system [80]. Furthermore, Tubb3, the major constituent of microtubules [81] and MAPt, which promotes microtubule assembly and stability [82], were regulated in both assays with differentiation. Thus, significant functional commonalities were observed between homologous genes related to neural differentiation in both assays.

Examination of tissue-specificity of gene regulation during differentiation demonstrated numerous neural- and brain tissue related gene sets commonly significantly regulated between both assays (Fig. 2, cluster A1). In addition, especially in hESTn an additional number of gene sets related to ectodermal tissues were significantly enriched, whereas gene sets related to tissues from other germ layers were significantly regulated as well. This wider array of differentiation in hESTn as compared to mESTn may in part be due to methodological differences between assays. In the mESTn, but not in hESTn, differentiation was induced by retinoic acid (RA) addition, a major physiologic neural differentiation inducer [83,84]. However, the presence of cell types outside the primary differentiation route studied in a given EST, whilst not informative in terms of the specific end point measured, may enhance the sensitivity of the system through cell–cell interactions among different cell types that may modulate differentiation. For instance, in the original mESTc, monitoring murine cardiac muscle cell differentiation, at day 7, 17% MHC, 6% actinin positive cardiac myocytes were found [61]. At day 10, 76.7% Myh6 positive cells have been reported by others [62]. Cardiomyocytes are mesoderm-derived, a germ layer that is formed by induction between ectoderm and endoderm. Therefore, stimuli representing both these germ layers are likely represented in the assay to allow mesoderm differentiation. Likewise, in hESTn, effects on differentiation of non-neuronal cell types may affect neural differentiation patterns and thereby influence sensitivity of the assay.

4.3. Comparing VPA responses in mESTn and hESTn

The data for this comparative study originates from different experiments with different designs. However, in both cases the concentration response was based on 96 individual samples spread over the concentration response, covering the therapeutic range. The 988 genes commonly regulated after VPA exposure in the hESTn and mESTn were enriched in processes associated with its pharmacological action, such as histone acetylation, chromatin modification, lipid biosynthetic process and cholesterol biosynthetic process. These gene sets were regulated by VPA but not by time in the assays, and were indeed enriched in section N of the Venn-diagram (Fig. 3). Neurodevelopmental processes were mainly enriched among genes appearing in section C and 3F (Fig. 3), indicating statistically significant regulation of neural related GO terms by VPA being observed in mESTn but not hESTn. The wider array of differentiation occurring in the hESTn, discussed above, may have caused less statistically significant enrichment of the neural differentiation associated GO processes in the hESTn. Differences and commonalities between assays were observed in the directionality of gene expression regulation after VPA exposure. Genes up-regulated in the hESTn showed either a common or opposite expression change with time in the mESTn (Fig. 4, cluster 4 and 6, respectively). A different expression directionality was observed *e.g.* in genes significantly regulated in chromatin and histone modification, which play a role in pharmacological mode of action of VPA (Fig. 4, cluster 2) [85,86].

The 143 genes regulated both by time and VPA exposure in both assays, although selected based on a single tested compound only, may represent promising differentiation-related biomarkers for species-independent compound-mediated DNT. Significantly enriched GO-terms represented by genes among the 143 genes were regulation of cell proliferation and regeneration. In addition, the 143 genes regulated in both assays and after VPA exposure contained important genes involved in neural associated GO-terms like (regulation of) neuron differentiation and nervous system, neural plate development and neurofilament cytoskeleton organization, including TUBB3, HOXA1, ADM, LHX1, EPHA2, GAP43, STMN2, MAP2, NEFL, CHRNA3, METRN, DNMT3B, T, NODAL, INA, ZIC3 and CTGF. Furthermore, important genes involved in general development were enriched in processes like embryonic- and cell morphogenesis, ectodermal development, (anterior and posterior) axis- and pattern formation, such as FGF8, MYC, TXNIP, ANXA1, CTGF, COL1A2, MLLT3 and RHOU. These 25 genes are potential candidate marker genes for DNT. GAP43, INA and LHX, have been published before as candidate markers for DNT *in vitro* [34,38,39] which supports their universal responsiveness in such assays.

5. Conclusion

Both systems nicely illustrated successful neural differentiation of ESC *in vitro*, with both commonalities and unique gene expression changes occurring in response to time and VPA exposure. The mESTn assay clearly shows a more specific neurodevelopmental differentiation pattern, whereas the hESTn also showed differentiation of cell types originating from other germ layers as well. Both these assays have their advantages, for instance as to specificity and species of origin. With further optimization of the human assay,

e.g. removal of the need for a feeder layer for undifferentiated hES cells and opening the possibility for subculture from controlled cell suspensions, the assay may become technically easier. This analysis identifies a number of genes and their homologues in the other species that respond similarly in both assays. This provides support for the application of these genes and their representative GO terms as possible biomarkers in neurodevelopmental toxicity testing in ESTn.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2015.06.043

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