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Teratogen screening using transcriptome profiling of differentiating human embryonic stem cells

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Abstract

Teratogens are substances that may cause defects in normal embryonic development while not necessarily being toxic in adults. Identification of possible teratogenic compounds has been historically beset by the species-specific nature of the teratogen response. To examine teratogenic effects on early human development we performed non-biased expression profiling of differentiating human embryonic and induced pluripotent stem cells treated with several drugs – ethanol, lithium, retinoic acid (RA), caffeine and thalidomide, which is known to be highly species specific. Our results point to the potency of specific teratogens and their affected tissues and pathways. Specifically, we could show that ethanol caused dramatic increase in endodermal differentiation, RA caused misregulation of neural development and thalidomide affected both these processes. We thus propose this method as a valuable addition to currently available animal screening approaches.

Keywords: human embryonic stem cells • teratogen • develpomental toxicity

Introduction

Toxicity testing is currently one of the major hurdles for many drug discovery processes. Currently, the main methods for identifying teratogens are either through epidemiological studies in human populations or by controlled exposure of animal models. Specifically, in order to test chemicals for developmental toxicity, OECD (Organization for Economic Co-operation and Development) guidelines require approximately 20 pregnant animals for the control group and each test group, in a minimum of three doses. Following gestation pregnant animals are killed and the litter examined thoroughly for abnormalities [1]. Thus, *in vivo* screening for teratogenic potential requires the use of a large number of laboratory animals and is very labour intensive and costly. Moreover, there are significant disparities between human beings and animal models in their response to chemical substances. Notably, thalidomide is a strong human teratogen that causes many severe congenital defects but was not identified prospectively as such in the mouse [2]. On the other hand, aspirin and caffeine are teratogenic in rodents but not in human beings [3, 4]. For many of the most prominent teratogens known, the *in vivo* 'no observed adverse effect level' (NOAEL) for mouse and rat, are more than two orders of magnitude higher than the lowest reported teratogenic dose in human. Thus, inferring teratogenicity from *in vivo* systems (other than primates) is problematic [2]. When a 'safe' margin from the animal NOAEL is often considered to be 1/100 or 1/1000 it is difficult in many cases to separate between actual teratogenic effects of compounds and non-specific effects, such as maternal toxicity. In this study we examined the potential of differentiating human embryonic stem cells (HESCs) and induced pluripotent stem cells (HiPSCs) as an *in vitro* model for teratogen screening in a human developmental setting, using physiologically relevant doses.

HESCs are cells derived from blastocyst stage embryos and have the unique traits of virtually limitless propagation in culture while retaining their inherent potential to differentiate into cells from the three embryonic germ layers and extraembryonic tissue. When detached from the dish, HESCs aggregate to form embryoid bodies (EBs). They thus begin a process of spontaneous differentiation that closely mimics early human embryogenesis [5, 6]. Previous studies using mouse [7–9] and human [10–12] ES cells have mainly focused on cytotoxicity assays to determine developmental toxicity. Our hypothesis was, that teratogenic effects at physiologically relevant doses would not necessarily result in cytotoxicity, but rather manifest as more subtle effects. Therefore, we decided to examine possible alterations in cellular differentiation causing deviation from the normal developmental program. In order to assess such effects, we performed non-biased transcriptome profiling on 7-day-old EBs.

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By this time EBs had undergone a phase of gastrulation-specific gene expression, and start to express early organogenesis genes [6, 13]. Changes in gene expression due to drug treatment were analysed for the presence of tissue specific genes and functional pathways. Thus we could identify in a single assay, both the teratogenic effect and related molecular events.

Here we set out to test for possible developmental effects of all-trans retinoic acid (RA), ethanol, lithium, thalidomide and caffeine. RA plays a major role in vertebrate development and is known to be a very potent human teratogen. Introduced as a treatment for severe acne, it was soon discovered to have caused substantial birth defects in over 25% of exposed foetuses [14]. Alcohol consumption during pregnancy has been shown to be the cause of a variety of neurodevelopmental disorders. Heavy drinking has also been shown to cause Foetal Alcohol Syndrome that may also include additional abnormalities such as growth retardation and microcephaly. Lithium is a broadly used psychiatric drug that was found to be teratogenic in the mouse [15], but its effect on the human embryo is yet unresolved [16]. Thalidomide is a very potent human teratogen that causes severe limb defects, heart malformations and spontaneous abortions. The mechanism of thalidomide teratogenicity is yet poorly understood. One possible explanation could be cellular damage induced by reactive oxygen species that are generated by thalidomide metabolites [17]. Importantly, the developmental toxicity of thalidomide does not manifest in mice and rats [2]. Thus, thalidomide was used by pregnant women for several years, during which time thousands of malformed babies were born. Caffeine on the other hand has been shown to be teratogenic in rodents [18], but does not pose a significant risk to human development.

Materials and methods

Cell culture

Human ES cells (HESCs, H9) [19] and HiPSCs (hTERT-BJ1-derived clone #28) [20], were cultured on Mitomycin-C treated mouse embryonic fibroblast feeder layer (obtained from 13.5 day embryos) in 85% knockout DMEM medium (Gibco, Invitrogen), supplemented with 15% knockout SR (a serum-free formulation) (Gibco, Invitrogen), 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 1% nonessential amino acids stock (Gibco, Invitrogen, Paisley, UK), Penicillin (50 units/ml), Streptomycin (50 μg/ml) and 4 ng/ml basic fibroblast growth factor (PeproTech-Cytolab, Rehovot, Israel). HiPSC#28 was previously derived and characterized as described by Pick *et al.* In brief, hTERT-BJ1 cells were infected with a combination of the human octamer-binding protein (OCT)4, sex determining region Y-box (SOX)2, v-myc myelocytomatosis viral oncogenes homolog (MYC) and Kruppel-like factor (KLF)4 genes packaged in pMXs retroviral vectors. Cells were passaged using trypsin-ethylenediaminetetraacetic acid (Biological Industries, Beit-Haemek, Israel). To obtain feeder-free cultures, cells were plated on laminin (1 $\mu g/cm^2$, Sigma-Aldrich) or gelatine (0.1%; Merck, Whitehouse Station, NJ, USA) coated plates and grown with medium conditioned by mouse embryonic fibroblasts. Differentiation *in vitro* into EBs was performed by trypsinizing confluent cultures using 0.25% trypsin-A in Dulbecco's phosphate buffered saline (DPBS) solution (Biological Industries) for 15 sec. to dissociate the colonies into large clumps. Clumps were then transferred 1:1 into non-stick Petri dishes and grown in regular HESC medium but without exogenous fibroblast growth factor 2.

Drug treatments

Embryoid body media were supplemented with the various treatment compounds on the first day and changed on alternate days. Ethanol 100% (Bio-Lab, Jerusalem, Israel) was used at 0.5%, 1.5%, 2% (v/v). Thalidomide (Sigma-Aldrich; T144) was dissolved in DMSO at 1000× stock solution and used at a final concentration of 10 μg/ml. all-trans-RA (Sigma-Aldrich; R2625) was dissolved in DMSO at 100,000× stock solution and used at a final concentration of 1 μM. Lithium chloride (J. T. Baker, NJ, USA, 2370) was dissolved in double distilled water (DDW) and used in a final concentration of 1 mM. Caffeine (Sigma-Aldrich; C0750) was dissolved in DDW and used in a final concentration of 50 mg/ml.

Microarray data analysis

Following 7 days in culture, EBs were harvested and RNA was extracted using RBC Bioscience (Taipei, Taiwan) total RNA extraction minikit (YRB50) according to the manufacturer's protocol. Hybridization to GeneChip Human Gene 1.0 ST expression arrays, washing and scanning were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Arrays were analysed using RMA probeset condensation algorithm (Expression Console, Affymetrix). Controls, RA, 0.5% ethanol and lithium treatments were performed in triplicate. Thalidomide was tested twice and caffeine once. 1.5% and 2% ethanol were each performed once and analysed together. Hierarchical clustering of the different treatments showed high reproducibility of the response to teratogens in either duplicates or triplicates (Fig. S1). All compounds were tested on H9 HESCs with the exception of RA that was tested also on HiPSCs. Adult tissue data [21] were obtained from Gene Expression Omnibus accession GSE9819. Tissue specific genes were identified by comparing the expression of each of the five most relevant tissues to the mean expression of all other tissues (testis, brain, heart, spleen, skeletal muscle, kidney, liver and breast). The top 200 genes by fold change for every tissue were regarded to be tissue specific genes.

Genes were considered differentially expressed when changed over 2-fold. To determine significance, up-regulated and down-regulated genes were classified according to the aforementioned tissue specific gene lists. Significance was calculated separately for each test using a chi-square independence test and corrected for multiple testing (Bonferroni). Chi-square tests were performed with the UCLA Statistics Online Computational Resource (SOCR) (http://www.socr.ucla.edu/). Functional classification and significance were performed with the DAVID online functional annotation tool (http://david.abcc.ncifcrf.gov/) using default settings, with a minimum *P*-value of 0.0001. General annotations with over 2000 members were removed from the analysis.

Immunofluorescent labelling

EBs were treated for 7 days with either 1.5% ethanol or 1 μM all-trans-RA (see Drug Treatment section). EBs were let to settle and perfused with 10% sucrose-phosphate-buffered solution (PBS) solution for 30 min. at 4°C,

followed by an additional step in 30% sucrose-PBS solution for 1 hr at 4°C, with gentle shaking. EBs were then frozen in Tissue-Tek OCT solution (Sakura Finetek, Alphen aan den Rijn, the Netherlands) and cut into 9 μm sections. Samples were fixed following sectioning with PBS buffered 4% formaldehyde solution for 3 min. Blocking was performed with PBS, 2% bovine serum albumin, 0.1% Triton X-100 solution for 1 hr at room temperature. Sections were incubated overnight at 4°C with the following primary antibodies; α-fetoprotein (AFP) at 1:50 dilution (SC-8399, Santa-Cruz Biotechnology, Santa Cruz, CA, USA); HOXA1 at 1:50 dilution (SC-17146, Santa-Cruz Biotechology); neural cell adhesion molecule (NCAM)-1 at 1:150 dilution (AF2408, R&D Systems); SOX17 at 1:400 dilution (AF1924, R&D Systems). The sections were then incubated for 1 hr with the appropriate secondary antibody (Cy-3, Jackson ImmunoResearch) at room temperature. Finally, sections were counterstained with Hoechst (B2883; Sigma-Aldrich) for 10 min. at room temperature.

Results

Treatment with human teratogens leads to major global gene expression changes in human embryoid bodies

In this study we treated developing EBs for 7 days with various teratogens at concentrations corresponding to documented human serum levels [8, 22]. Caffeine as a negative control was used at a concentration shown to induce malformations in mice [18]. Following treatment, gene expression was analysed using DNA microarrays. Expression results were analysed relative to their respective untreated controls and genes whose expression was significantly changed (over 2-fold) were counted. Lithium, caffeine and 0.5% ethanol produced little or no effect. Higher doses of ethanol (1.5% and 2%), RA and thalidomide had more dramatic effects with several hundred probesets changing in their levels of expression (Fig. 1A).

To determine specific developmental effects, we generated sets of tissue specific genes by cross comparison of microarrays from eight adult somatic tissues [21] (see 'Materials and methods'). We then classified the changed genes into the tissue specific gene sets from the five most relevant tissues. The fold enrichment of tissue specific genes was analysed separately for the up- and down-regulated genes. Significance was calculated using chi-square independence tests and corrected for multiple testing (Fig. 1B).

Treatment with RA had a dramatic effect on tissue specific gene expression. Brain specific genes were found to be enriched in genes both up-regulated and down-regulated by RA (2.6- and 13.2-fold, $P = 0.03$ and $P < 1 \times 10^{-15}$, respectively). Also down-regulated were heart specific genes (5.3-fold, $P = 1 \times 10^{-5}$). These results correspond to the major known malformations in infants induced by RA in the central nervous system and heart [14]. In EBs treated with high doses of ethanol there was significant enrichment in up-regulated liver (6.5-fold, $P < 1 \times 10^{-15}$) and heart (3.5-fold, $P = 1 \times 10^{-4}$) specific genes, congruent with the known teratogenic phenotype, there was also down-regulation of brain specific genes (6.4-fold, $P = 1 \times 10^{-5}$). Thalidomide caused up-regulation of brain and liver-specific genes (12.5- and 17-fold, $P < 1 \times 10^{-15}$ and $P < 1 \times 10^{-15}$, respectively) as well as down-regulation of liver specific genes (4.4-fold, $P = 0.015$).

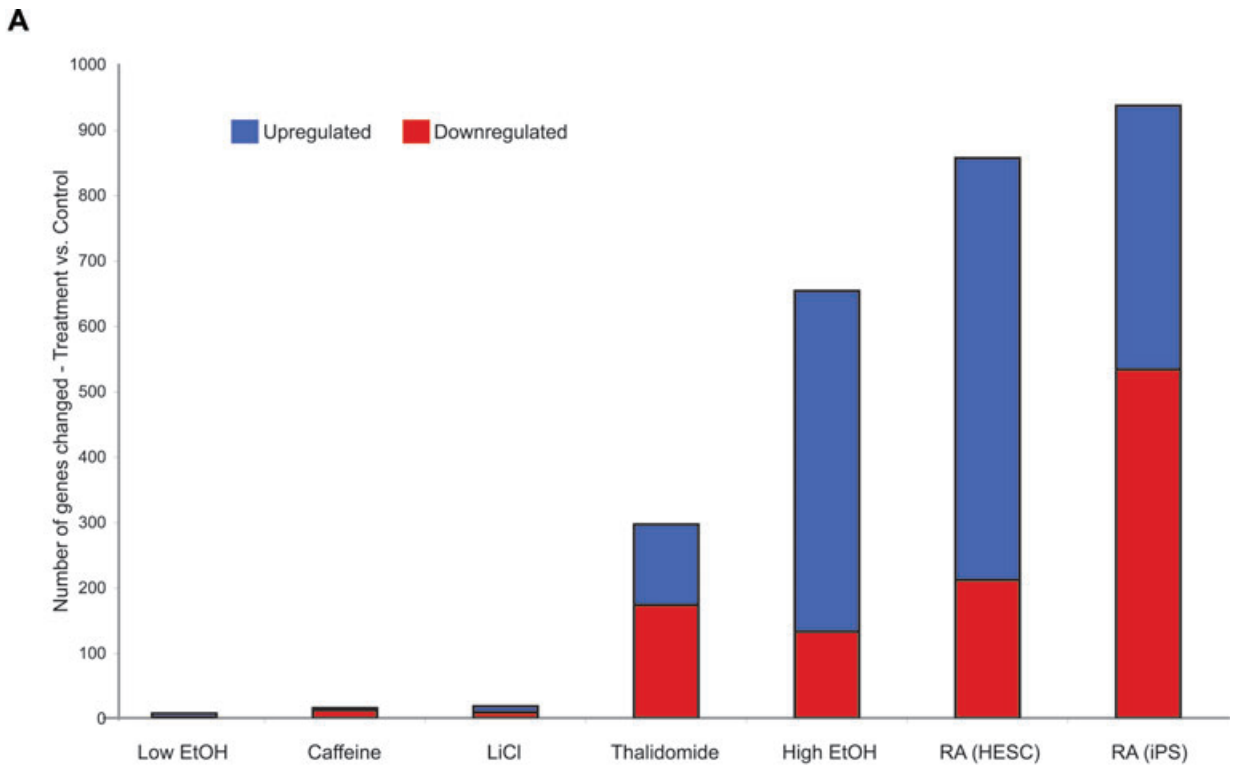
Functional analysis of retinoic acid, thalidomide and ethanol affected genes

To further characterize the molecular events induced by ethanol, RA and thalidomide, Gene Ontology based functional annotation was performed [23] (Fig. 2). Of the categories enriched in genes up-regulated by RA are the homeobox protein genes (14.1-fold above expected frequency). Hox genes of the Homeobox gene family are key regulators of many developmental processes and are known to be directly regulated by RA signalling [24]. Hox genes have been shown to be expressed in vertebrates along the dorsal axis in a spatially and temporally regulated manner, such that the 3′ located genes are expressed earlier and in the anterior regions, while the 5′ located genes are expressed later on and in the posterior parts of the embryo. In our analysis we show that the anterior HoxA and HoxB genes are specifically up-regulated by RA treatment both in HESC and HiPSC EBs (Fig. 3A). In the ethanol treated EBs the most significantly up-regulated functional group were liver protein genes (9.3-fold) (Fig. 2). These include several apolipoprotein and fibrinogen genes (Fig. 3B) as well as the early hepatic markers AFP and transthyretin. Later adult hepatic markers such as albumin and the alcohol dehydrogenase genes were not up-regulated. Thalidomide caused significant up-regulation of genes responsible for metabolism of xenobiotics (11.5-fold) and down-regulation of oxidative stress response genes (15.8-fold). This observation fits with previous reports connecting thalidomide activity with reactive oxygen species induced DNA damage. Additionally affected were several developmental related gene groups such as nervous system development genes (2.5-fold) and transforming growth factor β genes (29.6-fold) (Fig. 2).

Retinoic acid and ethanol alter differentiation of treated EBs as predicted by gene expression profiling

In order to validate the microarray results and characterize the observed effects at the cellular level, immunofluorescent labelling was performed on the treated EBs. Using this method we examined two major cellular effects previously highlighted by mRNA expression analysis. Specifically, we examined the protein levels of the foetal liver specific gene AFP and the earlier endodermal marker SOX17 to assess the effects of ethanol treatment. The effect of RA treatment on neural differentiation was determined using antibodies for the HOXA1 protein and the neuronal specific protein NCAM1.

Ethanol treatment clearly caused elevation of AFP staining, as was expected from the dramatic increase in mRNA levels shown by the microarray analysis. Interestingly, there was also dramatic



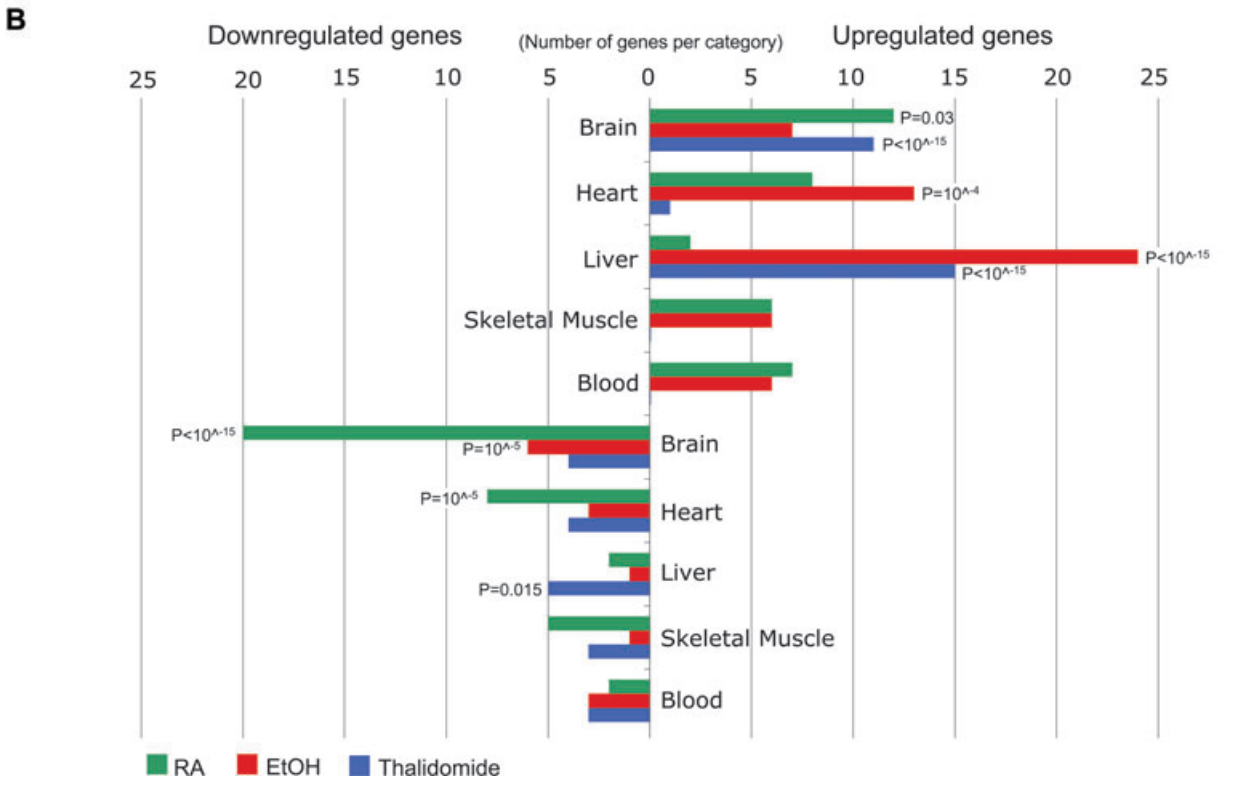


Fig. 1 Ethanol, RA and thalidomide treatments lead to major global gene expression changes in human EBs. (**A**) Histogram of the total number of up-regulated (blue) and down-regulated (red) genes induced by each of the treatments in the study. (**B**) Tissue classification of the affected genes. Shown separately are the up-regulated and down-regulated genes from those treatments showing significant expression changes, classified according to tissue specific genes (as described in 'Materials and methods'). Significance was calculated relative to the expected number of tissue specific genes using a chi-square independence test and corrected for multiple testing (Bonferroni). (For the complete list of genes see Table S1.)

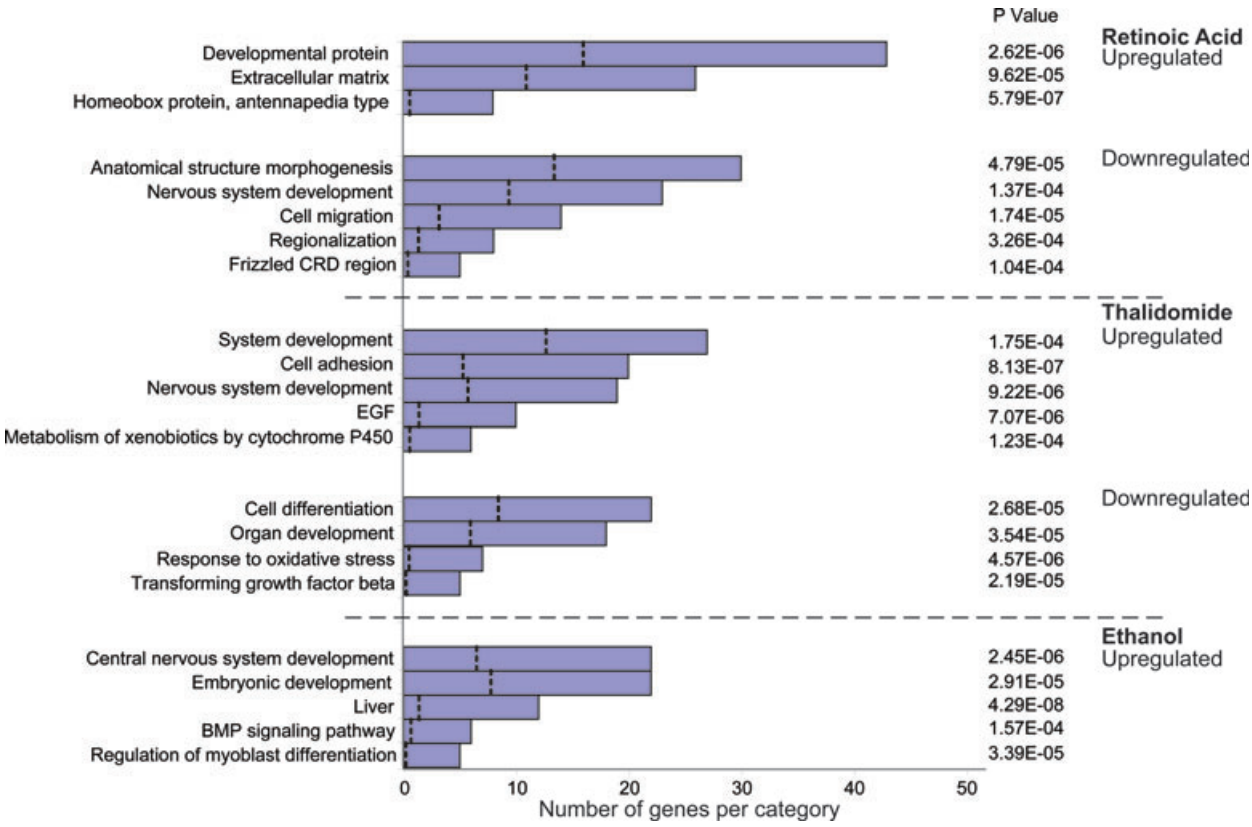


Fig. 2 Functional analysis of RA, thalidomide and ethanol affected genes. Bars represent the observed number of genes per category. The internal dashed line indicates the expected number of genes, representing the frequency on the array. Shown are the top ranking non-redundant annotations for each category by *P*-value, with a cutoff of 2-fold enrichment. Classification and significance were performed with the DAVID online functional annotation tool (http://david.abcc.ncifcrf.gov/). (For the complete list of enriched annotations and genes see Table S2.) There were no significantly enriched functional annotations for the genes down-regulated by ethanol.

increase in SOX17 staining (Fig. 4A). This would suggest that ethanol acts to increase differentiation of HESCs into the endodermal lineage, a subpopulation of which continues to differentiate into AFP-expressing early hepatic-like cells. The most dramatic effect of RA treatment was in the significant overexpression of the Hox genes (Fig. 3), and the misexpression of brain specific genes (Fig. 2), such that there was both significant up- and down-regulation of these genes. HOXA1 in particular was highly overexpressed at the mRNA level (13.6-fold, *P*-value = 0.0003). Indeed, at the protein level we can see similar increase in HOXA1 abundance, together with NCAM1 (Fig. 4B), indicating significant effects of RA on neuronal differentiation.

Discussion

In this study we compared the effects of five known and potential teratogens on HESCs. Previous studies have examined the toxic effects of drugs on mouse ES cells with decent success [7–9]. Notably, the embryonic stem cell test has gained much recognition [7]. This test focuses mainly on the observed higher sensitivity of ES cells to the cytotoxic effects of toxins, relative to fibroblasts. Recently, similar studies have been published using HESCs [10–12]. In the present study we chose to examine the global developmental effects of teratogenic compounds that pregnant

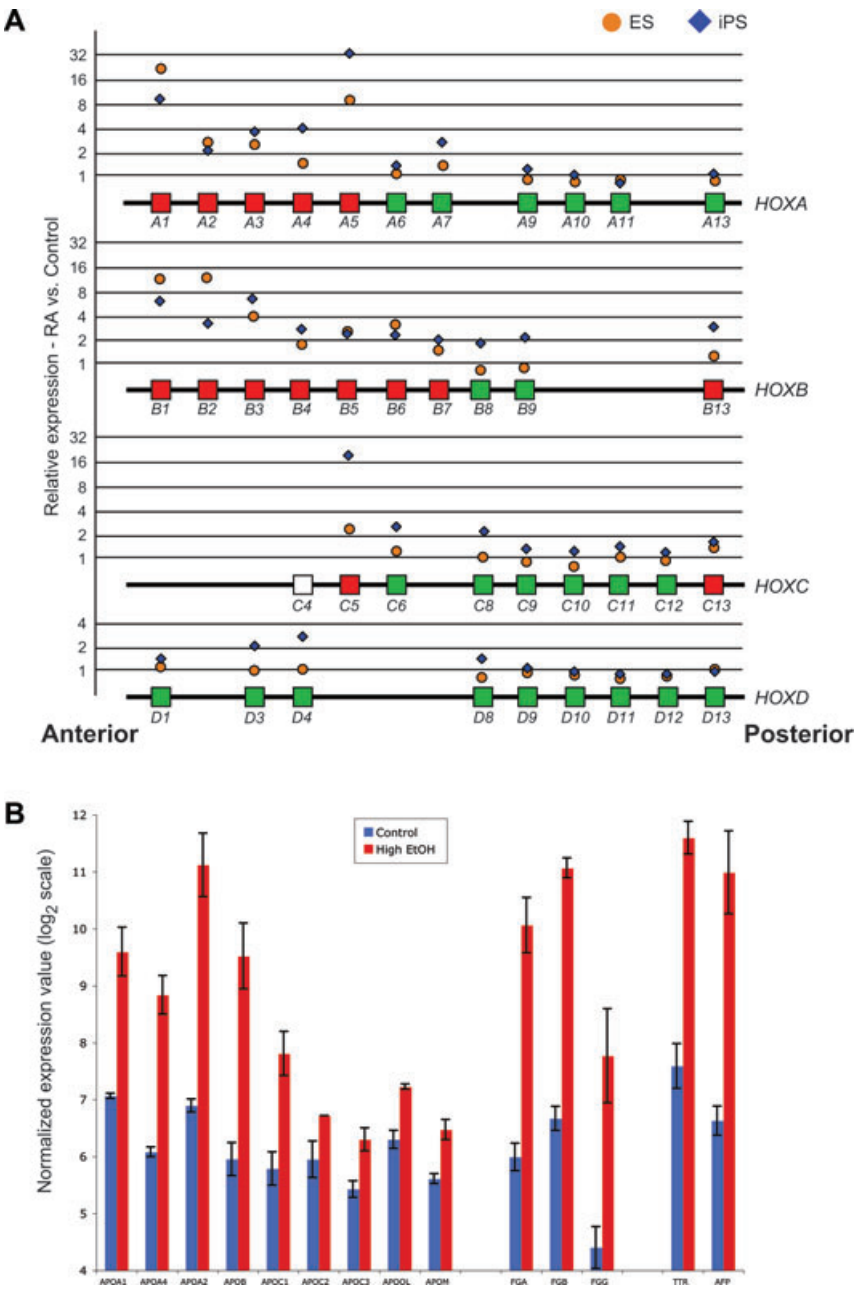


Fig. 3 (**A**) Anterior homeobox genes are up-regulated by RA. Shown is a schematic representation of the homeobox gene clusters and fold induction by RA relative to control, in both HESC and HiPSC EBs. Red boxes indicate significance of $P < 0.05$ in HESC as judged by two-tailed Student's t-test. (**B**) Early hepatic markers are specifically up-regulated following ethanol treatment. Shown are the expression levels of the AFP and transthyretin genes as well as genes from the apolipoprotein and fibrinogen gene families that have been found to be significantly ($P < 0.05$) up-regulated by high ethanol (1.5% and 2%) using two-tailed Student's t-test, error bars represent S.D.

women may be exposed to and, in some cases, the nature of the teratogenic risk they pose is currently unclear.

Using a non-biased expression profiling approach we were able to identify and characterize the teratogenic effects of RA, thalidomide and high levels of ethanol. Interestingly, the effect of ethanol on gene expression was highly dose sensitive, with a 3-fold increase in concentration causing an 'all or none' effect. Similar dose-dependant concentrations of ethanol were shown to induce Foetal Alcohol Syndrome phenotypes in a recent *Xenopus Laevis* model [25]. Lithium, a debated human teratogen under standard clinical concentrations [26], was found in this case to elicit only very minor gene expression changes. Similarly, caffeine had no significant effect despite being teratogenic in the mouse.

One of the unique strengths of the global gene expression profiling method for evaluation of teratogenicity is that it does not rely on the specific response of any individual gene. Thus, we defined a set of statistical tests of enrichment of tissue specific genes and molecular pathways that would indicate aberrations in the normal

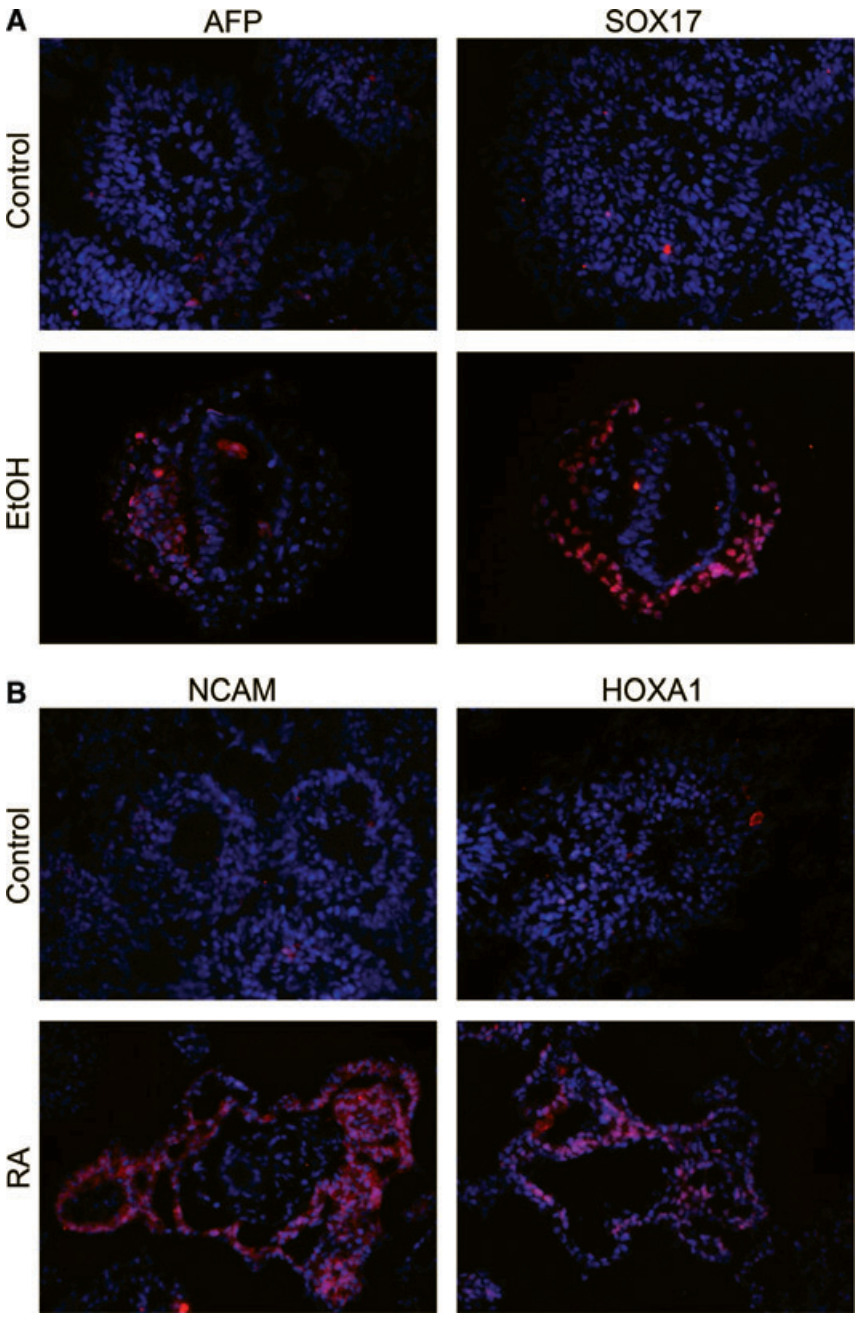


Fig. 4 RA and ethanol treatment affect the differentiation of EBs. (**A**) Immunofluorescent labelling of the endodermal lineage shows dramatic increase in AFP^+ and $SOX17^+$ cells as a result of ethanol treatment. (**B**) Immunofluorescent labelling of the Hox family member HOXA1 and neuronal specific protein NCAM1 demonstrate higher differentiation into the neuronal lineage as a result of RA treatment.

developmental program. Using these tests, we were able to classify and provide functional relevance to the gene expression changes observed. In the case of RA, we could see that the EB model mimics to great extent both the teratogenic phenotype, with gross changes in brain and heart specific genes, as well as the molecular phenotype, with the specific up-regulation of the anterior Hox genes. We further showed that the effects of RA can also be recapitulated in HiPSC-derived EBs (Figs 1A and 3A). We therefore suggest that the assay could be executed using either HESCs or HiPSCs.

Another measure of the similarity of the response of HESCs and HiPSCs treated with RA can be obtained from unsupervised hierarchical clustering of the gene expression profiles. Hierarchical clustering demonstrated that the expression profiles of these two different pluripotent stem cell lines treated with RA closely resemble each other, and cluster apart from the untreated controls (Fig. S1). Despite the close similarity in the effects of RA on different pluripotent stem cells, it is possible that for certain teratogens there will be variation in the response between different cell lines.

Thus, clinical interpretation of teratogenicity from any pluripotent stem cell based assay will most probably require the analysis of two or more independent cell lines.

The teratogen-induced gene expression changes observed, could be a direct effect on specific molecular pathways as in the example of RA-induced Hox gene expression and chemical response genes by thalidomide. Conversely, these changes may reflect altered levels of differentiation into the different cell types comprising the developing EB. For example, the overexpression of liver specific genes in the ethanol treated EBs could indicate an increase in differentiation into early hepatic cells or primitive endoderm.

The overexpression of Hox genes by RA, and endodermal markers by ethanol were further investigated at the protein level (Fig. 4), showing complete concordance with the mRNA analysis. Thus, we showed that ethanol had very dramatic effects on the progression of endodermal differentiation; suggesting that indeed, the gene expression changes observed were the result of alteration in the developmental processes taking place within the EBs. RA affected neural differentiation such that both HOXA1 and NCAM1 staining were dramatically increased, suggesting, as in the previous case, significant divergence from the normal developmental program of the EBs.

Both these examples highlight the accuracy of global gene expression analysis in identifying teratogenic potential and correctly assessing the effect of compounds on biological processes and cellular differentiation. Moreover, the large number of developmental genes affected, suggest that examining the EBs after a longer period of time could reveal even larger effects.

In future, additional studies will need to be performed in order to further establish this technique on a wider library of potential teratogenic compounds and even environmental factors. Additional results will also enable to further refine the assay, possibly allowing the use of focused microarray platforms. We believe these findings will provide a new approach of using pluripotent stem cells to prospectively screen for possible teratogenic effects, complementing existing animal *in vivo* and *in vitro* models.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Hierarchical clustering of gene expression data from treated and untreated EBs. Note the similarity in the effect of RA on the differentiation of HESCs and HiPSCs. Height represents distance by Pearson Dissimilarity measure.

Table S1 Tissue specific genes classification. Genes determined to be tissue specific are presented along with the gene expression data from the adult tissues as well as of the various treatments used in the study. Enrichment scores of the various treatments for tissue specific genes are also presented. Genes upregulated and downregulated 2-fold by the treatments are analyzed separately. Significantly enriched categories found by Chi-Square analysis following multiple test correction (Bonferroni) are highlighted in yellow.

Table S2 Functional annotation of genes changed by teratogen treatment of differentiating HESCs. Presented are the complete lists of enriched functional categories by DAVID analysis using default parameters. Genes upregulated and downregulated 2-fold by the treatments are analyzed separately. Shown are categories with fewer than 2000 members passing a minimum threshold of $P < 0.0001$.

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