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A genomics-based framework for identifying biomarkers of human neurodevelopmental toxicity

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ABSTRACT

Human embryonic stem cell (hESC) neural differentiation models have tremendous potential for evaluating environmental compounds in terms of their ability to induce neurodevelopmental toxicity. Genomic based-approaches are being applied to identify changes underlying normal human development (*in vitro* and *in vivo*) and the effects of environmental exposures. Here, we investigated whether mechanisms that are shared between hESC neural differentiation model systems and human embryos are candidate biomarkers of developmental toxicities for neurogenesis. We conducted a meta-analysis of transcriptomic datasets with the goal of identifying differentially expressed genes that were common to the hESC-model and human embryos. The overlapping NeuroDevelopmental Biomarker (NDB) gene set contained 304 genes which were enriched for their roles in neurogenesis. These genes were investigated for their utility as candidate biomarkers in the context of toxicogenomic studies focused on the effects of retinoic acid, valproic acid, or carbamazepine in hESC models of neurodifferentiation. The results revealed genes, including 13 common targets of the 3 compounds, that were candidate biomarkers of neurotoxicity in hESC-based studies of environmental toxicants.

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1. Introduction

Environmental factors may underlie a variety of developmental anomalies, the majority of which arise during the early stages of pregnancy. Driven by the desire to reduce costs and animal usage in developmental toxicology studies, the transition from *in vivo* to *in vitro* testing is gaining momentum [1,2]. Cell culture models are an attractive alternative that enable high throughput evaluation of environmental factors that may contribute to developmental toxicity. Of note, less than 1% of the 80,000 Environmental Protection Agency (EPA)-registered compounds have been properly tested for their potential to cause neuro (developmental) toxicity [3]. Therefore, established *in vitro* models of neurodevelopment are needed to study the multitude of prevalent compounds with unknown toxicities.

Human embryonic stem cells (hESCs), which can be differentiated into neurons, have been proposed as screening tools for neurodevelopmental toxicity testing [4,5]. There are well-recognized differences in embryonic development [6], ESC properties [7] and chemical-sensitivity [8] between rodents and humans. Therefore, hESC models offer advantages over rodent ESCs for human hazard assessment, enabling extrapolation of data to our species. Several multi-step protocols have been established to induce hESC neural differentiation. For example, in a commonly used approach, pluripotent hESCs are suspended as aggregates (embryoid bodies (EBs)) in serum-containing medium, which initiates spontaneous differentiation of the three germ layers. These changes model the initial steps of differentiation that occur during gastrulation [9].

Added during the EB stage, defined media and growth factor cocktails are used to specify ectodermal, mesodermal or endodermal fates. As to neuronal development, growth factors/cytokines

Abbreviations: hESC, human embryonic stem cell; NDB, Neurodevelopmental Biomarker; DE, differentially expressed gene; RA, retinoic acid; VPA, valproic acid; CBZ, carbamazepine.

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and small molecules that work in specific pathways (*e.g.*, BMP2 and/or SMAD [10] inhibitors, LIF [11] and retinoic acid [RA] [12]) are added to direct an ectodermal fate. EBs are placed on select substrates [13], which promote neural rosette (NR) formation. Patterned NRs contain neural precursor cells (NPCs) that express markers that are upregulated in the neural tube during human embryogenesis [14,15]. Then multipotent NPCs are differentiated into cells of the neural lineage, and additional factors added to influence patterning and the emergence of neuronal subtypes [16]. The addition of environmental compounds at one or more stages of the hESC neural differentiation protocol enables testing of their effects on the major developmental transitions [17].

Genomics-based approaches are used to investigate the underlying molecular changes during human embryogenesis at cellular and organismic levels. For example, investigators have used hESC models and global transcriptomic approaches to profile neuronal progenitor states [18]. These studies suggest multiple genes and related-pathways are modulated during this process. Along these lines, using the UCSF4 hESC line, our group profiled pluripotent and NP cells with the goal of identifying key genes/pathways that are expressed in this model of human development [19]. At an organismal level, transcriptomic technologies have been employed at specific time periods during human embryonic development, ranging from the first cell divisions to the later stages of organogenesis [20–22]. The data provide valuable insights into the dynamic molecular transformations occurring at select stages. These investigations also enable cross-species analyses of shared mechanisms [23].

For assessing developmental toxicity of environmental chemicals, genomic responses, *i.e.*, toxicogenomic approaches, are more sensitive and compound-specific than classical morphological endpoints [24]. Furthermore, toxicogenomic effects can be evaluated across models. Specifically, responses *in vitro* and *in vivo* can be directly compared, providing context for using cell, organ and embryo culture models for risk assessment [25,26]. Therefore, genomics-based approaches may add tremendous value to standardized *in vitro* methodologies in screening for neurodevelopmental toxicity.

Recent toxicogenomic studies used a standardized (mouse) ESC differentiation model to study the developmental toxicity of various compounds [27]. The results identified genes that are differentially expressed as a function of differentiation (neural or cardiac) in culture and highlighted the effects of the test compounds on these patterns [28–31]. These studies support the notion that the altered expression of molecules that are coordinated with normal differentiation *in vitro* can successfully predict the potential developmental toxicity of environmental compounds. While these reports point to molecular correlates for the assessment of developmental toxicity, uncertainty remains over their relevance and specificity as applied to human development.

Over the past decade, researchers have produced volumes of genomic data under diverse conditions using human and non-human, *in vivo* and *in vitro* models. Submitted to repositories, such as NCBI (Gene Expression Omnibus (GEO)) [32] or EBI (ArrayExpress) [33], these publicly available datasets are a valuable resource for *post hoc* analyses. Taking advantage of this resource, recent comparison studies provide potential strategies for determining: (1) commonalities between *in vitro* and *in vivo* models [23]; and (2) the cross-model consistency of molecular responses to environmental exposures [34,35]. In this context, we devised an analysis framework that we used to integrate human transcriptomic datasets for identifying biomarkers of neurodevelopmental toxicity (Fig. 1). We conducted a meta-analysis of *in vitro* differentiation data, specifically for the hESC neural differentiation model. These transcriptomic datasets included results of experiments completed in our laboratory [19] and previously published studies. We compared the results to transcriptomic datasets that were acquired during the initial stages of human embryo development, including the period of neurogenesis. Thus, we identified a core set of differentially expressed (DE) genes which change during the early stages of human neurodevelopment, common to both *in vitro* and human embryos. In preliminary verification experiments, we demonstrated the utility of this gene set for studying the effects of environmental chemicals in hESC models of this process.

2. Material and methods

2.1. Transcriptomic data collection

Datasets were acquired via public NCBI (GEO) and EBI (ArrayExpress) repositories. The criteria for selection of transcriptomic datasets relevant to hESC neural differentiation were as follows. Search terms were defined as "human", "embryonic stem cell", "differentiation", and "neural" or "neuron". A minimum of 2 biological replicates per group and 6 microarray assays (Affymetrix or Illumina array platforms) was required. Fifty-two GEO and 8 ArrayExpress studies met these criteria. Secondary filters eliminated studies that used genetically modified lines, non-neural-related differentiation routes, and non-human cell lines. In total, we identified four unique hESC neural differentiation datasets [36–39]. We added a transcriptomic dataset that was generated in our laboratory that compared the UCSF4 hESC line in a pluripotent state and their NPC derivatives [19]. Using a similar approach, we searched the two repositories to obtain human embryo transcriptomic datasets. We selected studies that analyzed at least 6 stages and a minimum of 3 biological replicates per group. In total, 3 datasets from different stages of human embryonic development met these criteria [20–22].

2.2. Data processing and gene expression comparisons

Each transcriptomic dataset was downloaded and individually processed using the Affymetrix Expression Console [40] and Transcriptome Analysis Console (TAC) [41] software packages or BRB Arraytools (for Illumina arrays) [42]. Raw values were normalized via the Robust Multi-array Average (RMA) algorithm [43]. One-way-ANOVA (OWA) was independently applied within each of the five *in vitro* and three human embryo datasets to determine significance of differentially expressed (DE) genes across time. Average fold change (FC) values between hESCs and their derivatives (*in vitro*) or the earliest embryonic stage vs. later periods (human embryos) were determined. Datasets were annotated using the Affymetrix TAC or Illumina databases (10/1/14). For comparisons across studies, we used the Official Gene Symbol (OGS). In the case of multiple probes per gene, the one with the lowest *p*-value, *i.e.*, most significant changes over time, was used for comparison purposes. Datasets were merged using the R statistical package [44] via the OGS identifier. Significantly DE genes were defined as $p \leq 0.01$ OWA and FC ≥ 1.5 (absolute). To describe regulation over time across groups, Pearson correlation (PC) coefficients were calculated for each gene within each dataset across time using time in culture or human embryo stage as a categorical variable. The comparability of DE genes for the five *in vitro* studies was determined by making iterative comparisons of each dataset with the other four. Hierarchical clustering of FC values was computed using average linkage and Euclidean distance (TIGR MEV [45]). Finally, the DE genes that emerged from the five *in vitro* studies were used to identify similar expression patterns over time in the three human embryo datasets. Similar patterning of common DE genes between the two models was determined by comparing PC coefficients [*in vitro* (median of the five datasets)

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