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A comparative transcriptomic study on the effects of valproic acid on two different hESCs lines in a neural teratogenicity test system

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HIGHLIGHTS

- A test for neural teratogenicity detection was designed.
- Two hES lines were exposed to valproic acid (VA) and gene expression compared.
- Genes commonly differentially expressed were indicative of teratogenic effect of VA.
- Differences were linked to the intrinsic variance between hES lines.

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ABSTRACT

A number of in vitro toxicity assays based on human embryonic stem cells (hESCs) are under development in order to provide alternative methods for the screening of chemicals and drugs and to reduce the number of animals needed for developmental toxicity assessment. The major challenge is to demonstrate the reliability of these in vitro methods by correlating the in vitro produced results to the available in vivo data. In this context transcriptomic approaches associated to toxicogenomic database analysis give the possibility to screen, annotate and cluster high numbers of genes and to identify the molecular changes that univocally mark the toxicity induced processes or are indicative of the early initiating events that lead to cellular toxicity. In this retrospective study we compare microarray transcriptomic data derived from two different hESCs lines (HUES1 and H9) exposed to valproic acid (VA) while applying the same differentiation protocol. We present the results of this comparative analysis in light of the known teratogenic effects of VA. The results show molecular changes in the processes of neural development, neural crest migration, apoptosis and regulation of transcription, indicating a good correspondence with the available in vivo data. We also describe common toxicological signatures and provide an interpretation of the observed qualitative differences referring to known biological features of the two hESCs lines.

1. Introduction

The need for in vitro alternatives to animal testing and for safer and human-specific in vitro testing methods has promoted the use of human embryonic stem cells (hESCs) for the development of toxicological tests. In particular, since hESCs easily and efficiently undergo neuronal differentiation and many protocols exist for the

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derivation of regionalised neuronal derivatives, the topic of developmental neurotoxicity is one of the most interesting and promising area of research for the design of in vitro methods. Moreover, neurodevelopmental defects are within the most common birth defects affecting on average 0.5–2 newborns per 1000 births (Greene et al., 2009) and in vivo the process of neural tube formation and closure is a critical step for embryonic development. We and others (Lee et al., 2007; Colleoni et al., 2010) have demonstrated that hESCs are able to differentiate into early neuroectodermal cells that represent an in vivo primitive neural stem cell state and recapitulate the early neurulation events that bring to the formation of the neural tube and to the emergence of the neural crest cells (Lazzari et al., 2006; Pankratz et al., 2007; Elkabetz et al., 2008). Therefore, the formation of neural rosettes in vitro represent are a powerful tool for developmental neurotoxicity screening since they represent the earlier stage of the neural development from which both CNS and PNS originate (Colleoni et al., 2010). We have also demonstrated that neural rosettes derived from HUES1 show a specific regionalised identity that makes these cells a suitable instrument for the focused detection of neural crest/neural tube toxicity (Colleoni et al., 2011). However, it is well known that marked differences exist between different hESCs lines in terms of differentiation ability (Osafune et al., 2008), linked to the different culture conditions but also to their intrinsic differentiation propensity. The possibility to find molecular changes that univocally mark the toxicity induced processes or are indicative of the early initiating events that lead to cellular toxicity is a challenge for the development of in vitro test methods. In the context of the FP7 project entitled: Embryonic Stem cell-based Novel Alternative Testing Strategies ESNATS (www.esnats.eu), that was focused on prenatal neurodevelopmental toxicity, a number of methods for neural teratogenicity testing have been designed using several different hESCs lines. Within this project, a first effort to compare different test methods for neurodevelopmental toxicity has been done in a recently published paper (Krug et al., 2013), in which transcriptomic data from different ESNATS test systems, mostly H9 hESC line-based, were compared. Despite a little overlap between differentially expressed individual genes across the different tests there was a large common set of enriched transcription factor binding sites (TFBS) and also TFBS that where specific for each test system and chemical. Therefore, the authors suggest that the commonly affected TFBS could provide a general indication of toxicity while TFBS specific for each test system and chemical could be used as signature for related chemicals belonging to the same class. In another comparative study (Theunissen et al., 2013), in which two mouse ES cells based tests (ESTc and ESTn, murine embryonic stem cells tests for cardiac and neural toxicity) were challenged with seven different compounds, it was observed that in each system there were compound-specific and/or system-specific transcriptional changes. The effects of valproic acid (VA) exposure was particularly investigated because in both systems this compounds induced the highest gene expression changes with cardiac genes dominating in ESTc and neural genes in ESTn. Results of this study indicate that combining toxicogenomics data from two assays provides complementary mechanistic informations on specific test compounds.

The aim of this paper is to present an additional comparative study focused on the transcriptional changes observed following VA exposure on human ES cells during induction of neural differentiation. In particular this study is a retrospective analysis of microarray data derived from two different hES cells lines (HUES1 and H9) subjected to the same neurodevelopmental toxicity test protocol with the aim of refining the mechanistic data on the effects of VA on differentiating human ES cells. The test protocol per se was already demonstrated to be predictive for prenatal neural teratogenicity in the context of the ESNATS project, for a limited set of compounds (Colleoni et al., 2011, 2012; Vojnits et al., 2012; Krug et al., 2013). We analysed the genes differentially expressed and their relative gene ontology terms with the aim to find a common thread specific for VA, taking advantage of the well-documented transcriptomic changes induced by this teratogenic compound (Massa et al., 2005; Jergil et al., 2009). The availability of a flexible test system adaptable to many different cells lines would be a powerful tool for the detection of prenatal neurodevelopmental toxicity. Moreover the identification of VA specific dysregulated genes and pathways could constitute the basis for predictive toxicology methods and for the extrapolation from in vitro to in vivo data for chemicals belonging to the same class.

2. Materials and methods

2.1. HUES embryonic stem cell culture and neural differentiation

Human ESCs (HUES-1 cell line) (Cowan et al., 2004) were cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) inactivated by mitomycin C (Sigma–Aldrich, Milan, Italy, www.sigma-aldrich.com) treatment. Cells were cultured in KO-DMEM (Gibco Invitrogen, Milan, Italy, www.invitrogen.com) supplemented with 10% serum replacement (Gibco), 4.3 mg/ml bovine serum albumin (BSA) (Sigma–Aldrich), 2 mM glutamine (L-alanyl-L-glutamine, Sigma–Aldrich), 1% non-essential amino acids (Gibco), 0.055 mM beta-mercaptoethanol (Gibco), 50 units/ml penicillin and 50 μg/ml streptomycin, and 10 ng/ml bFGF (Peprotech). The medium was changed daily and cells were passaged with 0.05% trypsin/EDTA on average every 3–4 days. For the derivation of neural rosettes, growing hESCs were incubated with collagenase (1 mg/ml collagenase type IV, Gibco) at 37 °C for 1 h and then the cell monolayer was gently pipetted to detach hESCs colonies from the feeder. The colonies were recovered and cultured in suspension in hESCs medium without growth factors in 25 cm^2 flasks (Corning, NY, USA, www.corning.com) to induce the formation of embryoid bodies (EBs). After 4 days the EBs were singly plated in matrigel coated (1:100 dilution, Matrigel, BD, Milan, Italy, www.bd.com) 96 well plates (Nunc, Roskilde, Denmark, www.nuncbrand.com) and cultured in DMEM-F12 supplemented with 0.6% glucose, 3 mM sodium bicarbonate, 2 mM glutamine, 5 mM hepes, 25 μg/ml insulin, 60 μM putrescine, 20 nM progesterone, 100 μg/ml transferrin, 30 nM sodium selenite, 2 μg/ml heparin and 20 ng/ml bFGF (Colleoni et al., 2010) EBs were cultured in these conditions for 8 days during which time neural rosettes formation was observed.

2.2. H9 embryonic stem cells culture and neural differentiation

NIH-registered H9 hESCs (WiCell, USA) were cultured undifferentiated in 60 mm cell culture dishes (TPP, Switzerland) at 37 °C and 5% CO_2 on a layer of mitomycin C inactivated primary mouse embryonic fibroblast (pMEF, CF-1 strain Millipore USA) in standard maintainance medium for undifferentiated hESCs (DMEM-F12 supplemented with 20% KO serum replacement, 1% non essential amino acids, 2 mM glutamine, 0.1 mM beta-mercaptoethanol and 4 ng/ml human recombinant bFGF (all from Invitrogen). Cells were expanded weekly by microdissection and further propagated on a feeder layer. A modification of a previously published protocol was applied for differentiation toward early neuroepithelial precursors (Stummann et al., 2009). Briefly, intact 6 day-old H9 colonies were detached by exposure to 1 mg/ml collagenase (Gibco, Invitrogen) and left in suspension culture dishes for 3 days in hESCs medium without bFGF to allow the formation of the EBs. Subsequently, EBs were transferred onto single wells (one EB per well) of 96-well plates coated with 10 mg/ml laminin in water (Sigma) containing neural induction medium (DMEM/F12 supplemented with 1% non essential amino acids, 1%