Chapter 10

Pluripotent Stem Cells in Toxicity Testing: An Omics Approach

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Abstract

Traditional approaches to toxicological testing are expensive and time consuming usually involving exposure of chemicals to large numbers of animals during the crucial period of organ development. In order to provide cost-efficient and high-throughput methods, various in vitro test systems have been proposed to access toxicity for environmental toxicants and many drugs. Although effective, these platforms are based on in vitro cell cultures and ex vivo models using embryo cultures and often do not accurately interpret results for human safety because of interspecies difference and/or the inability to reproduce human physiology. To address this problem, a humanized system, pluripotent stem cells were introduced to study toxicity of drugs.

Keywords: pluripotent stem cells, toxicity platform, toxicogenomics, differentiation

1. Introduction

Pluripotent stem cells are endowed with the capacity to self-replicate, to keep their pluripotent state, and ability to differentiate into specialized somatic cells [1–5]. The ability of pluripotent stem cells to differentiate into various types of somatic cells opened up a new era of in vitro toxicity testing. It has been recognized that pluripotent stem cells are not only promising for a potential cell therapy of degenerative diseases but also can be applied for toxicological in vitro assays during drug development. Omics technologies have been used to decipher the networks of signaling events and perturb molecular signaling to identify the new therapeutic targets [6]. In particular, the combination of pluripotent stem cells and "-omics" technologies is extremely promising as a model system for toxicology since at every time point of differentia-

tion, thousands of genes are differentially expressed, thereby significantly increasing the probability of identifying sensitive mRNA or protein markers of toxicity [7, 8].

2. Pluripotent stem cells and its somatic cells differentiation during toxicity testing

Embryonic stem cells (ESCs) are pluripotent cells; when yamanaka factors [9] that maintain them as pluripotent and provide suitable environment to differentiate are removed, these ESCs can differentiate into endodermal, mesodermal, and ectodermal lineages. During embryogenesis the developmental processes occurring in vivo can be partly recapitulated by the use of cultured ESCs in vitro. Progressive-directed differentiation leading to the formation of tissue-specific cells, for example, cardiac, neural, and hepatic, is controlled by differential gene expression, apoptosis, intercellular communication, and cell-matrix interactions [1, 2]. The temporal gene expression and morphological changes of the cultured ESCs occur hierarchically, first developing epiblast cells, then germ layers, and finally somatic cells. Progressive differentiation of human ESCs is coordinated by multiple genes and pathways involved in biological processes, such as cell proliferation and cell death. A balance between these mechanisms is essential for normal embryonic development in vivo. In vitro models combined with appropriate experimental protocols allow the identification of genes participating in developmental processes in vivo. Differentiation of ESCs can partially reproduce early human embryonic development [1, 2]. Therefore, stem cells are a suitable tool to assess toxicant profiles to understand and predict the damage caused by potential therapeutic agents (**Figure 1**).

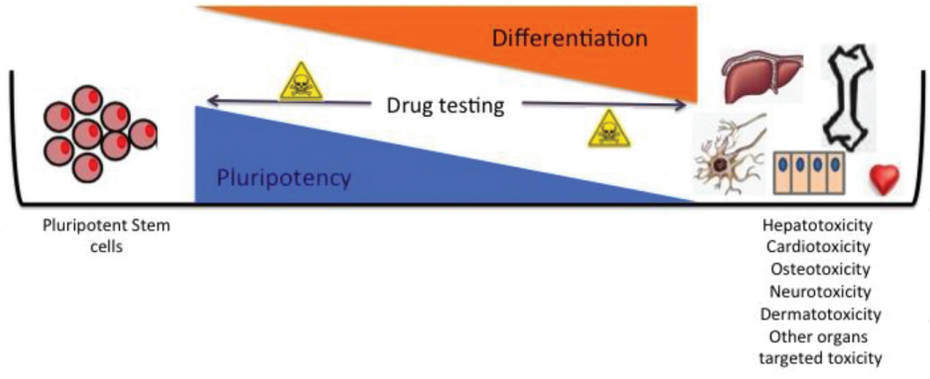

Figure 1. Graphical representation of in vitro toxicogenomics approach using pluripotent stem cells.

Traditional approaches to toxicological testing typically involve exposure of large number of animals to chemicals during the crucial period of organ development and further investigations of fetuses for visceral and skeletal developmental defects. These approaches are expensive and time consuming [3–4, 10]. A magnitude of in vitro test systems have been proposed in order to provide cost-efficient and high-throughput methods to assess the developmental toxicity of candidate drugs and environmental toxicants in the past 20 years. These platforms

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include primary in vitro cell cultures and ex vivo models using embryo cultures [11]. ESCs-based differentiation systems toward neuronal, cardiac, hepatic and, in general, multiple lineage differentiation have been utilized to monitor the toxic nature of known developmental toxicants on either a mechanistic or functional level [1, 2, 12–16].

3. Directed differentiation of pluripotent stem cell (embryoid-body-mediated differentiation)

In order to uncover multiple embryonic development perturbations in presence of potential toxicants, it is critical to determine optimal differentiation of ESC. Protocols for different cell lineages have been described that exhibit variable success. In most cases, the in vitro differentiation recapitulates the stepwise stages of embryological development for the cell type of interest. In vitro differentiation of stem cells to three germ layers mimics the sequential stages of embryonic development, a brief description of the key steps is provided, and factors in multilineage development are highlighted below in **Figure 2** [1, 2, 12].

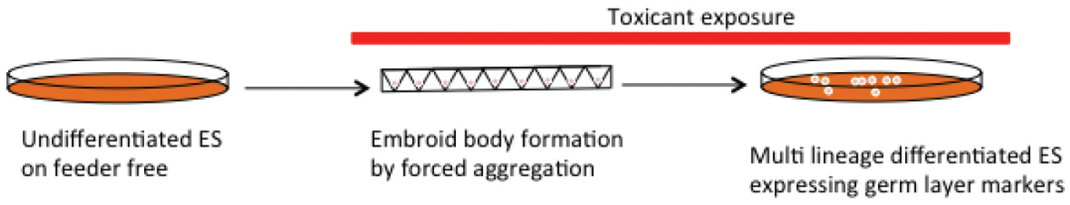

Figure 2. A schematic representation of multilineage differentiation.

hESC were cultured and passaged on irradiated mouse embryonic fibroblasts in knockout-DMEM-F12, KO serum replacement, nonessential amino acids, penicillin/streptomycin, b-mercaptoethanol supplemented with basic fibroblast growth factor. Prior to differentiation, the cells were maintained for 5 days on hESC-qualified matrix-coated tissue culture plates. Cultures were passaged and maintained in feeder-free conditions with conditioned medium supplemented with 8 ng bFGF.

For multilineage differentiation, embryoid bodies were formed by forced aggregation and hESC colony aggregates typically formed rounded discrete structures and acquired an embryoid-body-like appearance within 2–3 days [1, 2]. For differentiation, embryoid bodies were cultured in DMEM-F12, KO serum replacement, nonessential amino acids, penicillin/, streptomycin, and β-mercaptoethanol. These multilineage-differentiated embryoid bodies were analyzed for the presence of markers of the three germ layers. Multilineage differentiation of hESCs in embryoid bodies resulted in downregulation of pluripotency markers, such as POU5F1 and NANOG, and subsequent increase in the germ layer markers. The germ layer markers expressed on day 6 gradually developed and became stronger upon reaching day 12 and day 14. Markers for endoderm (FGA, AFP and DCN), ectoderm (SOX3 and MAP2), and mesoderm (HAND1 POSTN, PITX2) were expressed in these embryoid bodies.

4. Neuronal differentiation

Developmental neurotoxicity (DNT) and many forms of reproductive toxicity (RT) often manifest themselves in functional deficits that are not necessarily based on cell death but rather on minor changes relating to cell differentiation or communication. The fields of DNT/RT would greatly benefit from in vitro tests that allow the identification of toxicant-induced changes of the cellular proteostasis or of its underlying transcriptome network. Despite its high relevance, DNT is one of the least studied forms of toxicity [13, 17]. It is also particularly difficult to study because DNT is not necessarily caused by cell death. In fact, chemically induced changes in the proportions of neural cells, positioning, or connectivity may be sufficient to cause DNT [13, 18]. ESC-based systems recapitulate early neuronal development in vitro, including neural patterning, neurogenesis, and gliogenesis.

For neural differentiation, the cells were thawed and cultured in suspension in T75 flasks with N2B27 medium. From day 2 to day 7, the cells were incubated in N2B27 medium supplemented with 10 μM anti-TGF-beta and 2 μM dorsomorphin. From day 8 to day 32, medium replacement was performed with N2B27 medium only. On day 33, the generated spheres were dissociated as single cells and cultured in N2B27 medium in poly-ornithine and laminin-coated 6-well plates. On day 36, the cells were detached and frozen in N2B27 medium in different aliquots. To test neurotoxicity of chemical compounds, an aliquot was thawed in PLO and laminin-coated 6-well plates. The cells were cultured in a neuronal differentiation medium made of neurobasal medium, B-27 supplement as well as BDNF, recombinant human glial cell-derived neurotrophic factor, and 10 μM ROCK inhibitor. After 1 day of recovery, the cells were incubated with the neurotoxicant in neurobasal medium without ROCK inhibitor for 2 days and then the material was collected for analysis (**Figure 3**).

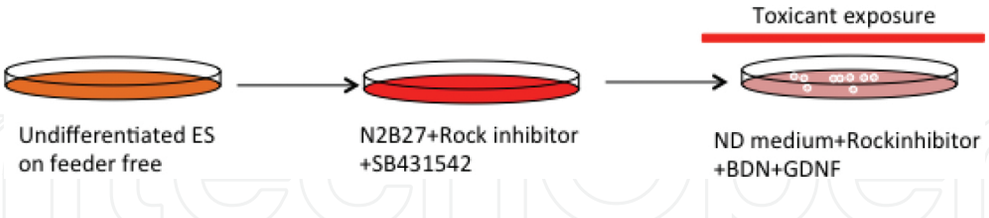

Figure 3. A schematic representation of the early neurogenesis differentiation.

The neural induction differentiation is represented in **Figure 4**. ESCs were differentiated by dual SMAD inhibition. ESCs were plated as single cells with mitomycin C-inactivated mouse embryonic fibroblasts, containing 10 μM ROCK inhibitor Y-27632 and 10 ng/ml bFGF. Differentiation was initiated 3 days after replating on the day of differentiation by changing the medium to knockout serum replacement medium supplemented with 35 ng/ml noggin, 600 nM dorsomorphin, and 10 μM SB-431642. From day 4 onward, KSR was replaced stepwise with N2 medium starting with 25% N2 medium on day 4. To assess the chemical effects on RNA expression, the cells were differentiated in the presence or absence of the chemicals.

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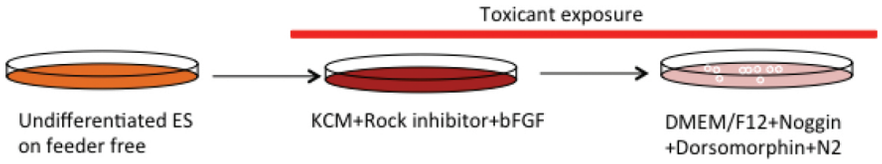

Figure 4. A schematic representation of the neural induction.

5. Cardiac and hepatic differentiation

Liver and cardiovascular disease modeling, mechanisms and therapies can be enhanced using ESC technology. Once pluripotency is established, ESCs can be differentiated into cardiomyocytes [15, 19] and hepatocyte-like cells [20, 21] by using various methods. Further, an embryoid-body-based technique that uses a defined proprietary medium, in addition to ascorbic acid, hypoxic conditions, and a rigorously defined growth factor regiment can be a way to differentiate cardiac cells [19]. Another method claims ESCs are cultured directly on stromal cells, such as murine OP9 or visceral endoderm-like cells [22, 23], and a third approach is a monolayer-based method that uses a medium consisting of RPMI 1640, the supplement B-27, and growth factors activin A and BMP4 with high-density hESC cultured on Matrigel [24, 25]. In all the above techniques, contracting cells are witnessed after 8–12 days.

The only cure for a critically failing organ is liver transplant, despite major breakthrough in liver diseases. The use of ESCs can be explored to developing new treatments leading to a better understanding of the disease process, offering new treatment or preventative strategies.

The protocol [20, 21] describes efficient differentiation of pluripotent stem cells to hepatocytes different tissue culture. Upon hepatic specification, cells express albumin, display cytochrome P450 activity and upregulation of "liver transcription factors," such as CAR, FXR, and PXR.

In summary, the in vitro testing systems demonstrate the ability of ESCs to be used as an alternative to traditional toxicity testing on animals. ESCs not only better reflect the human physiology but also help avoid interspecies differences making it an ideal tool to explore disease mechanism, cures, and to predict toxicity of the drugs.

6. Omics approaches to screen toxic drugs and their advantages to decipher molecular mechanism and signaling pathways perturbed during exposure of drug

A single in vitro assay system may not be able to mimic the complex interactions during human early development. For which, high-density technologies scrutinizing the genome, transcriptome and proteome such as microarray-based technologies and next-generation sequencing technologies come into play, having impressively enriched our knowledge on dynamics related to effects of toxicants during early development [26].

6.1. Detection of different transcriptional responses to toxicity modeling using ESCs

In vivo, the susceptibility of the developing embryo and fetus to chemical exposures during prenatal and early postnatal life may result in important effects on gene expression, thus ensuing functional defects and increased risks of disease later in life [27].

These chemicals are often environmental factors or therapeutic drugs. Since exposure to chemicals during development may cause irreversible effects, it is important to understand their specific mechanisms of toxicity [28].

Toxicogenomics and toxicoproteomics focus on modulation of gene expression in response to exposure and explore the basic mechanisms of toxicity and assessment of the proteome in organs and biofluids such as liver and blood, respectively. DNA microarrays can be used to measure the differential expression of thousands of genes at the same time. Cellular response and organ structure induced by the toxicant well correlates with changes in gene expression levels. A variety of array platforms such as cDNA arrays, high-density oligonucleotide and oligonucleotide bead arrays have been used [29, 30].

The discovery of key modified proteins mainly utilizes toxicoproteomics as early biomarkers for the prediction of an adverse effect. Although time consuming, using high-density LC-MS/MS platforms and its complementary methods can accelerate the toxicoproteomics analysis.

Multilineage differentiation expresses all the three germ layer transcripts. Expression of germ line markers during multilineage differentiation is influenced by the toxicant, resulting in potential prediction of developmental toxicity. The time course multilineage differentiation expression patterns reveal temporal changes in gene expression. Further, upregulation of the markers involving germ layer lineage, such as FGA, FGB, AFP, SLIT2, COL5A2, MYL4, COL6A3, MSX1, MYL7, BMP4, COL1A1, and COL5A1, is seen [1, 2]. Cytosine arabinoside, a strong teratogen, induces developmental toxicity in murine and rat embryonic development. A high concentration of cytosine arabinoside treatment produced cleft palate and lip abnormalities in vivo. Toxicogenomics study revealed cytosine arabinoside repressed gene expression of MSX1, COL3A1, COL1A1, COL1A2, AXUD1, MGP, CDH11, SPARC, POSTN, and BMP4 during bone morphogenesis and osteogenesis in multilineage differentiation of ESCs. Further, cytosine arabinoside could stimulate the expression of neuronal genes and pathways that consequently result in increased neurogenesis and in parallel suppress expression of mesodermal markers [1].

In another study, thalidomide, a teratogen and developmental toxicant, was found to perturb heart and limb development after analyzing microarray data. Thalidomide toxicity affected multilineage differentiation to reveal an upregulation of skeletal, neuronal, and respiratory development and 33 proteins along the reelin pathway. The ESCs differentiation and -omics assisted to uncover inhibition of RANBP1, which participated in the nucleocytoplasmic trafficking of proteins and inhibition of glutathione transferases (GSTA1, GSTA2) as a novel mechanism for thalidomide toxicity [2].

A relationship between cytotoxic response and developmental neurotoxicity-specific transcriptome is analyzed during neuronal differentiation [12]. Toxicity of methyl mercury and

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valproic acid was accessed using different neuronal differentiation protocols that employed transcriptomics to further investigate the technical feasibility of using transcriptomics as a major endpoint to characterize responses of ESCs-based test systems. Statistically overrepresented GO terms were identified to obtain an overview of the biological processes and displayed for each test system and condition. The genes downregulated by valproic acid in each of the neuronal differentiation system pointed to effects of the toxicant on RNA processing, and on chromatin modification/histone acetylation, consistent with the known activity of valproic acid as a histone deacetylase inhibitor. Transcriptome changes during differentiation were also able to identify upregulation of axonogenesis and ventral forebrain-associated genes, such as SLIT1, SEMA3A, DLX2/4, and GAD2. Further, valproic acid induced expression of miR-378, which was identified to target forebrain markers [12, 16].

Toxicity testing in vitro increasingly utilizes high-throughput screening (HTS) assays. Although HTS assays can test many chemicals, they have a limited use in the regulatory arena. This is because of the need to undergo rigorous, time-consuming formal validation. These HTS assays are additionally used to identify high-concern chemicals. The high-concern chemicals could then be tested sooner, and validation process would ensure the dependability and significance of assays (**Table 1**).

Toxicity	Differentiation	Chemicals	Readout	Markers	Ref.
Developmental toxicity	Multilineage	Cytarabine arabinoside	Microarray, RT-PCR	Induction of neurons and mesoderm inhibition.	[1]
		Thalidomide	Microarray, proteomics, RT-PCR	perturbed heart and limb development	[2]
	Undifferentiated human fibroblast	Arsenic + inhibitor, Busulfan, hydroxyurea	RT-PCR, cyQuant proliferation assay	POU5F1, NANOG, TDGF1, NES, NEFH, TUBB3, CCND1, CCRK, BAX, CASP3, DNMT3B, BCL2	[31, 32]
Neurotoxicity	Early neurogenesis	Methyl mercury, valproic acid	Microarray, RT-PCR		[12]
	Neural induction	Methyl mercury, valproic acid	Microarray, RT-PCR		[12]
	Dopaminergic neurons	pirenzepine, amiodarone, selamectin, clofoctol, perhexilline, griseofulvin, chloroactoxy quinoline, menadione, hexetidine	ATP measurement	OCT4, NESTIN, TH, beta-III tubulin	[33]
Cardiotoxicity	Cardiomyocytes, Na and Ca channel functions	TTX, diltiazem, nifedipine	MEA, Patch clamp	Expression of Na, Ca and HCN channel RNA	[34]

Toxicity	Differentiation	Chemicals	Readout	Markers	Ref.
	Cardiomyocytes, long QT model	cisapride, nifedipine, pinacidil, ranolazine	MEA, Patch clamp	Troponin I, alpha actinin and connexion 43 staining, expression of NKX2-5, MYL2, MYH6, MYH7, KCNH2	[35]
Hepatotoxicity	Hepatocytes	Not tested	accumulation of glycogen, metabolism of indocyanine green, accumulation of lipid	80% of cells expressed albumin, expression of hepatocyte-specific genes	[36]

Table 1. Differentiation protocols and test systems for toxico-omics relevant models.

7. Concerns: challenges and unresolved issues in stem cells toxicity testing

A species difference during animal and human study is a key important factor endpoints in toxicity study suffer (e.g., cardiotoxicity, hepatoxicity, developmental toxicity, neurotoxicity). To avoid such interspecies, variance toxicity tests that are based on human stem cells have been developed. A high-throughput toxicity screening demands unlimited supply of homogeneous population of cells, which is yet another challenge. Stem cells and its differentiation, being of nonmalignant origin and human origin, have the advantage to generate several types of cells of toxicological relevance. Although pluripotent stem cells have enormous potential for toxicity testing, many unresolved issues need to be addressed. The toxicity dose prediction for humans using in vitro system and challenges to yield consistent somatic cell types from pluripotent stem cells are in their preliminary stages. However, integration of the stem cells testing in combination with omics technology and in vivo animal testing could save millions of dollars and years of time in drug development and could demonstrate the mechanism of the toxicity.

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