# Prospects and Frontiers of Stem Cell Toxicology

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The development of stem cell biology has revolutionized regenerative medicine and its clinical applications. Another aspect through which stem cells would benefit human health is their use in toxicology. In fact, owing to their ability to differentiate into all the lineages of the human body, including germ cells, stem cells, and, in particular, pluripotent stem cells, can be utilized for the assessment, in vitro, of embryonic, developmental, reproductive, organ, and functional toxicities, relevant to human physiology, without employing live animal tests and with the possibility of high throughput applications. Thus, stem cell toxicology would tremendously assist in the toxicological evaluation of the increasing number of synthetic chemicals that we are exposed to, of which toxicity information is limited. In this review, we introduce stem cell toxicology, as an emerging branch of in vitro toxicology, which offers quick and efficient alternatives to traditional toxicology assessments. We first discuss the development of stem cell toxicology, and we then emphasize its advantages and highlight the achievements of human pluripotent stem cell-based toxicity research.

**Keywords:** stem cell toxicology, developmental toxicity, pluripotent stem cells, in vitro toxicology, embryonic stem cells

## Introduction

TOXICOLOGY, THE STUDY of the adverse effects of chemicals on living organisms, serves human society in many ways, to not only protect humans and the environment from the deleterious effects of toxicants but also facilitate the development of safer chemicals to be employed as clinical drugs, pesticides, food additives, and so on. Toxicity can be acute or chronic, comes from different routes of exposure, varies from one organ to another, as well as yields to different outcomes according to age, genetic background, gender, diet, physiological conditions, and the health status of the organism.

Many in vivo and in vitro testing methods are available in toxicology. Although animal tests have played a critical role in toxicology, a trend for an alternative in vitro toxicology, mostly consistent in cell-based assays, started in the 1950s with the publication of the 3R principle (Replacement, Reduction, and Refinement) [1]. In fact, in vitro toxicity tests take advantage of well-developed cell culture protocols and pre-validated cell-based toxicity tests with multiple biological endpoints. Besides, cell-based toxicity tests usually require shorter time and still allow for more replicates, than in vivo assays do [2].

Currently, the list of potentially hazardous chemicals we can get exposed to is continuously and rapidly updated, due to the exponential discovery and production of artificial materials. Therefore, it is extremely urgent to develop high-throughput experimental systems that will let us screen for the potential toxicity of all these substances.

Hence, in this review, we introduce stem cell toxicology, an emerging branch of in vitro toxicology, which offers effective and efficient alternatives to traditional toxicology assessments. We first discuss the development of stem cell toxicology, and then emphasize its advantages and highlight the achievements of human pluripotent stem cell (hPSC)-based toxicity research. We hope this review will be inspiring and innovative, and nurture more efforts into stem cell-based toxicology research.

## Development of Stem Cell Toxicology

Stem Cell Toxicology, originally inspired by the mouse embryonic stem cell test (EST), includes toxicology research on stem cells and stem cell-derived differentiated cells. Stemness allows for in vitro cell maintenance for many generations without loss of differentiation potential and without genetic manipulations. Moreover, contrary to somatic or cancer cells, stem cell-based toxicity models let us perform developmental toxicity assays, in particular with mouse pluripotent stem cell- and hPSC-based models. Therefore, those unique advantages of

stem cell-based toxicity models call for further development and utilization of this new crossover field.

## Mouse stem cell-based toxicology

After the successful derivation of mouse embryonic stem cells (mESCs) [3] and induction of mouse-induced PSCs (iPSCs) [4], the rapid development of stem cell technology also allowed for mESC-based toxicology research.

The first toxicity test based on mESCs was designed to test the cytotoxicity of potential teratogens [5]. It not only showed overall consistent results with in vivo data but also suggested that mESCs were more sensitive to tested chemicals, compared with mouse fibroblasts [5]. After this encouraging attempt, the mouse EST was proposed as the first complete and accepted in vitro toxicity test and has been continually improved and refined ever since [6]. The original EST consisted of, on 10-day toxicant exposure, MTT cytotoxicity assays on D3 mESCs and 3T3 mouse fibroblasts for the detection of IC50s (half maximal inhibitory concentrations), as well as a cardiogenic differentiation test with D3 mESCs, to determine the ID50 (the equivalent of IC50 for differentiating cells) [6].

Several efforts have been addressed to the optimization of the EST, particularly regarding its efficiency in experimental operation, accuracy, and objectiveness of endpoint determination [7], as well as toward high-throughput applications and extensive utilization (the main refinements of the mESC-based EST are summarized in Table 1). One early improvement was the introduction of molecular endpoints for gene and protein expression. For instance, the expression of the cardiac marker myosin heavy chain, quantified by flow cytometry, helped judge to which extent cardiogenesis was affected by tested chemicals [26–28] in a more objective and accurate way, compared with the original scoring of beating clusters of cardiomyocytes by microscopic observation [6]. In another example, groups of genes representative of the whole genome and that were involved in toxicity responses were selected for more predictive, reliable, and yet less laborious assays [10,20,29]. More recently, a shortened EST based on the detection of the cardiac and neural crest Hand1 gene expression, through a luciferase reporter assay, showed good reproducibility [22]. In addition, studies on the effects of flusilazole and monophthalates on mESC cardiogenesis convinced that low chemical concentrations, although not cytotoxic, compromised mESC cardiogenesis by down-regulating the expression of related genes in a dose-dependent manner [20,30]. These refinements allowed for the investigation of the underlying molecular events triggered by chemical exposure, especially for changes in molecular levels that could be relevant for development, instead of merely cell viability.

Another major strategy to improve the original EST has taken advantage of differentiation procedures besides cardiogenesis, such as neural differentiation, endothelial differentiation, and osteogenesis. Such strategies were motivated by the fact that in some cases the traditional EST underestimated the possibility that different developmental lineages might show different responses on exposure to the same chemicals [8,14,31]. The use of cardiogenic induction as the only differentiation procedure would not provide a comprehensive toxicity assessment. Therefore, to address this concern, the neural EST was introduced and successfully validated with six classical neurotoxicants with diverse mechanisms of toxicities [16]. Similar refinements were obtained by other groups [13,29,32]. There were also EST-inspired tests based on the differentiation of mESCs into endothelial cells [33] and osteoblasts [14,31], which showed to be effective and responsive to novel embryotoxic compounds [33], strongly validating the choice of using additional differentiation protocols in the EST.

Currently, mESC-based toxicity assays represent a powerful tool for the high-throughput toxicity screening of emerging organic chemicals [25], for chemical use and exposure guideline purposes. For instance, the US Environmental Protection Agency (EPA) and others developed a toxicity assay based on the monolayer differentiation of mESCs into cardiomyocytes, called mESC adherent cell differentiation and cytotoxicity (ACDC) assay [34], enabling the high-throughput screening of environmental pollutants, which also gives information on the underlying molecular mechanisms [35].

## Human stem cell-based toxicology

Both mESCs and human ESCs (hESCs) are necessary for stem cell toxicology research, but with different roles. In the perspective of stem cell biology, hESCs differ from mESCs in not only their morphology and expression of surface antigens but also gene markers and cytokines [3,36,37]. In addition, when derived from the inner cell mass of the blastocyst, mESCs are generally in the naive state (the highest pluripotency level) whereas hESCs are in the primed state (a lower pluripotency level) [38]. Thus, a rational corollary of these differences is that in toxicological terms, hESCs may be more appropriate than mESCs in terms of the representation of toxicity effects toward humans. In addition, hESC-based toxicity tests may be more reliable and indicative, since they avoid interspecies differences. Studies have found out hESCs were more sensitive to the adverse effects of a broad range of chemicals [39–42], and could be impaired by certain chemicals that were not necessarily toxic to mice, such as thalidomide [43], or chemicals, including ethanol and caffeine, which have raised health concerns [44,45].

The first hESC-based toxicity tests took advantage of the neuronal induction from hESCs. Exposure of hESC-derived neurons to specific dopaminergic neurotoxins resulted in apoptotic cell death, production of reactive oxygen species, and loss of neuronal functions [46]. Two years after this study, the European Center for the Validation of Alternative Methods (ECVAM) proposed the establishment of the human EST [47]. In this case, H1 hESCs and hMRC-5 embryonic lung fibroblasts were exposed to two well-known developmental toxicants, all-trans retinoic acid (RA) and 5-fluorouracil (5-FU), for either 4 or 10 days. MTT cytotoxicity tests and gene expression analyses for cardiogenic markers demonstrated that the toxic responses caused by RA and 5-FU were compatible with previous in vivo data. These two studies demonstrated already that hESCs could be employed in cytotoxicity, developmental toxicity, and functional toxicity assays.

To assess the vulnerability of the human nervous and cardiovascular systems, neonatally and postnatally, hESC- or human iPSC (hiPSC)-based toxicity research has been embraced with a variety of artificial chemicals, such as bisphenol A, perfluorooctane sulfonate, trichloroethylene, trimethyltin chloride, ibuprofen, and paraquat [48–50], as well as more complex materials including silver nanoparticles [51,52] and gold nanoparticles [53], for their effects on cardiogenesis and neural development.

TABLE 1. PRIMARY REFINEMENTS OF THE EMBRYONIC STEM CELL TEST

| Chemicals | Cell line | Exposure duration | Differences from Spielmann's original EST | Findings | Reference |
|---|---|---|---|---|---|
| 15 pharmaceuticals | DBA/11acJ murine derived ESCs, 3T3 A31 | Cytotoxicity assay: 7 days Differentiation assay: 10 days | Different ESC line | Other murine ESC lines can replace D3 in the EST; false positives and negatives can still be significant | 7 |
| Penicillin G, 5-FU, RA, diphenylhydantoin, valproic acid, thalidomide | Balb/c 3T3, D3 | Cell viability: 10 days Differentiation assays (contraction assay and collection of RNA samples): 10 days | Involvement of multiple marker genes of other lineages (neurogenesis, osteogenesis, and chondrogenesis markers) | First time to include multiple gene expressions in the EST; results are promising and open to further improvement and optimization | 8 |
| 6-Aminonicotinamide, 5-FU, methylmercury chloride, hydroxyl urea, valproic acid, boric acid, methoxyacetic acid, lithium chloride, penicillin G, saccharin, diphenhydramine, acrylamide | D3 | Cell viability assay: 6 days Differentiation assay: 10 days | Instead of being seeded in 24-well plates, mESCs are seeded and cultured in low attachment 96-well plate to facilitate high-throughput tests. Introduce relative embryotoxicity potency values that are derived from the ranking of the embryotoxic potential of test compounds relative to positive controls | No need for 3T3 to provide reference toxicity information. Toxicity ranking seems more reliable than the absolute classification of toxicity | 9 |
| Monobutyl phthalate, methoxyacetic acid, valproic acid, RA, 5-FU, penicillin G | D3 | Cell viability assay: 3 days Differentiation assay: 10 days | Use resazurin proliferation assay; involve whole-genome gene expression profiling | Several genes are very sensitive and significantly dysregulated after compound exposure, which are useful as additional endpoints in the EST | 10 |
| Triazoles, flusilazole, hexaconazole, cyproconazole, myclobutanil, triadimefon, triticonazole, 5-FU | D3 | Cell viability assay: 5 days Differentiation assay: 10 days | Compare the EST with in vivo tests, the rat postimplantation WEC, and zebrafish embryotoxicity test | The EST is able to represent developmental toxicity, and partially mimic the processes in utero | 11 |
| 9-cis-RA, 13-cis-RA, acitretin, ATRA, TTNPB, etretinate, retinol | D3 | Cell viability: 10 days Cell differentiation: 10 days | Compare the EST with in vivo tests, the rat limb bud micromass test, and the postimplantation rat WEC | The EST, although it could not completely imitate toxicological kinetics in vivo, was overall a promising in vitro alternative in toxicology | 12 |
| Methylmercury, valproic acid, AsV, AsIII, saccharin, isoniazid, ascorbic acid | D3, 3T3 | Cytotoxicity: 10 days Neural differentiation assay: 12 days | Neural differentiation instead of cardiac induction with exposure duration of 12 days. Use flow cytometry and detection of neural gene expression as endpoints | Neural differentiation could serve as an alternative process in the EST. The molecular endpoints introduced in the neural differentiation-based EST were *Tuj1* mRNA and protein levels | 13 |

TABLE 1. (CONTINUED)

| Chemicals | Cell line | Exposure duration | Differences from Spielmann's original EST | Findings | Reference |
|---|---|---|---|---|---|
| Mono-ethlhexyl phthalate, valproic acid, methotrexate, 6-aminonicotinamde, methoxyacetic acid, penicillin G | D3 | Cell viability: 5 days Osteoblast differentiation assay: 21 days. Cardiac differentiation assay: 10 days | Involvement of osteoblast differentiation and molecular endpoints to evaluate it. Comparison between osteoblast and cardiomyocyte differentiations on exposure to same chemicals | Osteoblast differentiation can be an alternative to cardiogenesis in the EST, and may give different results | 14 |
| Phenol, p-fluorophenol, p-heptyloxyphenol, p-mercaptophenol, p-methylketophenol | D3 | Cell differentiation assay: 10 days | Compare the EST with in vivo tests and the WEC assay | The EST gives toxicity rankings of tested phenols that are different from the rankings given by in vivo tests and the WEC assay; exposure doses in the EST have to consider the kinetics of in vivo absorption, metabolism, elimination, and excretion | 15 |
| Acealdehyde, carbamazepine, flusilazole, monoethylhexylaphthalate, penicillin G sodium salt, phenytoin | D3 | Cell viability test: 48 h Morphological scoring: 72 h Whole-genome expression profiling: 24 h | Neural differentiation Different exposure durations Resazurin cell viability assay Include genome profiling | The neural differentiation-modified EST is valid; transcriptomics provides mechanistic information | 16 |
| MeHgCl, monosodium L-glutamate, penicillin G, poly-L-ornithine, sodium arsenite, sodium valproate, chlorpyrifoe-ethyl, parathion-ethyl | D3 | Cell viability: 4 or 5 days Differentiation: 2 or 3 days | Differentiation to neural cells Cell proliferation tests are based on ELISA. Cell viability tests are based on CellTiter-Blue Cell Viability Assay. Involvement of βIII-Tubulin enzyme-linked immunosorbent assay | This method is suitable for high-throughput screening but does not necessarily represent relevant concentrations in vivo and is not applicable for acute and chronic toxicities | 17 |
| Bisphenol A, genistein, as well as combined with bisphenol A and 5-FU | D3, 3T3 | Cell viability test: 10 days Differentiation assay: 10 days | Cell Titer 96 Aqueous One Solution Cell Proliferation Assay for cell viability test; cells are exposed to two chemicals | Bisphenol A and genistein, to which we are exposed daily unintentionally, have combined embryotoxic effects that become synergistic at low concentrations | 18 |
| 38 teratogens 39 nonteratogens | D3 | Cell viability test: 72 h Cell differentiation assay: 96 h | Shorter exposure times; include gene expression analysis for 12 potential molecular endpoints | The Molecular Embryonic Stem Cell Developmental Toxicity Assay facilitates high-throughput screenings of potential teratogens with good predictivity and concordance with in vivo data | 19 |

(continued)

TABLE 1. (CONTINUED)

| Chemicals | Cell line | Exposure duration | Differences from Spielmann's original EST | Findings | Reference |
|---|---|---|---|---|---|
| Monobutyl phthalate, monobenzyl phthalate, mono-(2-ethylhexyl) phthalate, monomethyl phthalate | D3 | Cell viability: 5 days Differentiation assay: 10 days | Incorporate RNA microarray analyses as additional endpoints | A total of 668 commonly expressed genes are altered after exposure, proving the validity of transcriptomics in the EST | 20 |
| 5-FU, hydroxyurea, saccharin; silver nanomaterial, coated and uncoated zinc oxide, titanium and silica nanomaterials | D3, 3T3 | Cell viability: 10 days Cell differentiation: 10 days | Skip the step of EB formation in petri dishes and transfer EBs directly to 24-well plates. Add nanomaterial once to avoid continuous accumulation in cells | This simplified protocol shows to be more suitable to facilitate nanotoxicity research for medical or therapeutic nanomaterial uses | 21 |
| 6-aminonicotinamide, all-trans RA, 5-bromo-2′-deoxyuridine, dexamethasone, methoxyacetic acid, salicylic acid sodium salt, ascorbic acid, acrylamide, D-(+)-camphor, 5-FU | Linearized Hand1-promoter-Luc plasmid transfected C57BL/6 mice derived ESCs | Cell viability: 5 days Differentiation assay: 5 days | Monitor *Hand1* expression via Luciferase reporter assay, which at the same time indicates both proliferation and differentiation | The expression of *Hand1* by Luciferase reporter gene assay is reproducible and relatively accurate | 22 |
| Simvastatin | D3, 3T3 | Cytotoxicity: 10 days. Differentiation assay (both hanging drop method and monolayer differentiation): 10 days | Include both EB hanging drop method and monolayer differentiation. Molecular endpoints are maker genes for each germ layer | Genes of the mesodermal lineage are most sensitive to the two drugs; the hanging drop method and monolayer differentiation give rise to consistent results | 23 |
| Chinese herbal extracts from *Atractylodes macrocephala*, *Coptis chinensis*, *Radix isatidis*, *Flos genkwa* | OG2 mESCs, BALB/c 3T3 | Cell viability: 10 days Differentiation assay: 10 days | Cell viability assay: CCK8 assay Differentiation assay based on myosin heavy chain gene expression | *Atractylodes macrocephala* and *Radix isatidis* are non-embryotoxic, *Coptis chinensis* is weakly embryotoxic whereas *Flos genkwa* is strongly embryotoxic | 24 |
| 5-FU, RA, valproic acid, diphenhydramine, LiCl, saccharin, penicillin G | D3, 3T3 | Cell viability: 5 days Differentiation assay: 5 days | Based on only monolayer culture with 5-day exposure. Check 16 genes for the three germ layers as endpoints for differentiation | Monolayer culture is applicable in the EST with gene expression detection for three germ layers | 25 |

ATRA, all trans retinoic acid; EB, embryoid body; ESC, embryonic stem cell; EST, embryonic stem cell test; 5-FU, 5-fluorouracil; mESCs, mouse embryonic stem cells; RA, retinoic acid; TTNPB, (E)-4[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid; WEC, whole embryo culture.

In summary, stem cell toxicology offers great potential and is open to improvements and refinements in parallel with the development of stem cell biology. It would allow for toxicity tests to be designed for many different kinds of cells, with the ultimate goal of obtaining trustable toxic information relative to human health.

## Advantages of Stem Cell-Based Toxicity Tests

The unique properties of stem cells qualify them as one of the best materials for in vitro tests. Unlike primary cells, stem cells, such as ESCs and iPSCs, are able to proliferate extensively in vitro under defined conditions. Theoretically, ESCs and iPSCs can be passaged for indefinite generations without transformation, as established cell lines. Conversely, cancer or immortalized cell lines, although they can be also maintained in vitro indefinitely, are prone to queries as to whether or not they represent healthy or normal cells and are sensitive enough in toxicity assays. Stem cell-based toxicity tests avoid these issues and, consequently, stand out as promising alternatives to animal tests owing to their recapitulating of many key in vivo features.

Stem cells also hold the capacity to differentiate into other cell types. In other words, with proper differentiation conditions, it is possible to generate cells of all the different tissues of the body, including germ cells, in particular when starting from ESCs. These cells can then be used in toxicity studies without ex vivo derivation or ethical concerns. For example, hESCs are able to differentiate into cardiomyocytes, allowing for toxicity assays either during the process of cardiac differentiation or with the terminally differentiated cells [54], therefore permitting developmental and functional toxicity tests, respectively. Thus, stem cells offer the distinct possibility to study cellular, developmental, functional, and reproductive toxicities with various types of stem cells and stem cell-derived offspring, particularly when primary cell types are impossible to be derived directly.

Consistency between stem cell toxicity tests and live animal assays is necessary before concluding that by using stem cells in toxicity assays we can get rid of animal experiments altogether [55]. Summarized in Table 1 are the consistency assessments between EST-based stem cell toxicity tests and in vivo or other in vitro tests, such as the WEC (whole embryo culture) [11,12,15]. They revealed a general consistency between in vivo and in vitro results, advocating for the use of stem cell toxicity tests that would also allow for tests with multiple chemicals, doses, and durations at one time, with considerably less consumption of time, money, and labor.

Therefore, the advantages of stem cells mentioned earlier would help face the tremendous challenge of the numerous environmental pollutants, drugs, and industrial chemicals with vague toxicology information [11,39], by employing reliable and high-throughput experimental systems that will eventually contribute to the rise of stem cell toxicology [56].

## Major Achievements and Future Prospects of Human Stem Cell Toxicology

In this section, we focus only on the growing number of research studies recently reported for hESCs and hiPSCs related teratogen and drug screenings, with an emphasis on their potentiality for more comprehensive toxicity assays.

### hESC systems as powerful tools for developmental toxicity research

hESCs are pluripotent, as they have the capacity to differentiate into cells of all three germ layers, as well as germ cells. Thus, toxicity assays with hESCs can provide toxicity information at very early stages after fertilization, since differentiating ESCs mimic the dynamic process of embryonic development. Besides, hESC-derived cells can also serve as a useful alternative to study toxicity effects on a variety of somatic cells.

Cytotoxicity assays on hESCs involve the use of the chemical(s) of interest, to study not only cell death but also whether pluripotency of hESCs would be affected by the chemicals. For example, the embryotoxic compound 5-FU downregulated the expression of *Oct4* and *Nanog* in hESCs, as well as significantly dysregulated *HDAC9* (involved in neuronal, skeletal muscle and adipocyte differentiation), *DLK1* (involved in embryogenesis and differentiation of MSCs to chondrocytes), and *NFE2 L3* (involved in differentiation, inflammation, and carcinogenesis) [57], indicating that early exposure to 5-FU may result in multiple malformations. A similar study focused on how teratogens affected the hESC pluripotent state by observing changes in gene networks, and it proposed the explanation that adverse effects occurring during embryonic development might be originated from exposure during very early stages, even before ESCs in the blastocyst start differentiating [58]. Therefore, such a study helps understand how chemicals can influence the process of lineage fate decision in pluripotent cells.

Developmental toxicity tests with the hESC system benefit from the process of embryoid body (EB) formation, which recapitulates the key features of gastrulation [59]. Therefore, toxicity tests during EB formation yield information about which germ layer(s) will be affected and, therefore, which lineage differentiation will be impaired. For example, several natural and synthetic estrogens that readily cross the placenta, such as estradiol, estriol, and bisphenol A, negatively affected endodermal, mesodermal, and ectodermal differentiation during EB formation [49]. Moreover, a common component in toothpaste, fluoride, which had been shown to affect murine and human early embryogenesis, suppressed hESC proliferation and induced apoptosis, but also stimulated the generation of ectoderm and mesoderm at the expenses of endoderm, when administered at high doses [60]. Studies like these have drawn attention to the lack of toxicity information for emerging chemicals that have been widely used.

Toxicity assays for a variety of pharmaceuticals, materials, and environmental pollutants with hESC-derived somatic cells have been performed and validated, implying a great potentiality for these systems in functional toxicology. Cardiomyocytes are one of the earliest cell types being successfully derived from hESCs and have been already used in toxicity tests, for a few common drugs [54,61–63], and in a high-throughput screening for teratogens [8,44]. For instance, the chemotherapeutic agent doxorubicin caused acute cytotoxicity in hESC-derived cardiomyocytes, due to membrane damage [61,62]. Further, trichloroethylene, a ubiquitously detected industrial chemical, disrupted the transition from cardiac progenitor cells to cardiomyocytes [48]. Neural differentiation of hESCs, as another well-established in vitro process, has been introduced in stem cell toxicology [64,65]. Several studies

showed that a variety of chemicals or materials might impair the development of different types of neural cells [51,53,66,67], and they also deciphered the underlying mechanisms. For example, gold nanoparticles were toxic to neural hESC derivatives, due to the alteration of DNA methylation and hydroxymethylation levels during neural differentiation [53]. In addition, an immediate and significant increase in oxidative stress caused by silver nanoparticle on hESC-derived neural progenitor cells was due to dysregulation at both transcriptional and post-transcriptional levels [52].

There are also studies on more complex neural cell populations, such as neural rosette [68,69] and artificial neural constructs [50,70]. For example, highly consistent responses toward RA between in vitro neural rosette and in vivo systems confirmed the potentiality of neural rosette generation for developmental neurotoxicity tests [69]. One promising study focused on the generation of neural constructs, comprising different kinds of cells, in 3D structures mimicking the developing brain. That study obtained highly accurate toxicity results with known neural toxicants and controls [70], and together with another toxicity study based on 3D neural spheres [50], demonstrated the potential of 3D cell constructs or even organoids in toxicology research.

With the establishment of additional hESC-based differentiation protocols, other hESC-based models in toxicology have been validated, and several different kinds of toxicities from a variety of chemicals, materials, and environmental pollutants have been measured. For example, hESC-derived germ cell-like cells have been utilized in reproductive toxicity tests that facilitated and accelerated the screening of the remarkable number of potential endocrine disruptors to which we are continuously exposed [71]. A report on the perturbation of low-dose bisphenol A on the direct differentiation of hESCs into prostate organoids suggested that prenatal exposure could perturb the morphogenesis of the prostate [72].

Besides, systems biology methodologies, such as transcriptomics, proteomics, and metabolomics, have been also incorporated into stem cell toxicology research. For example, one group exposed hESCs to several teratogenic drugs and detected small molecules whose levels were significantly altered in response to those drugs, proving the convenience of metabolomics in toxicity assays [73]. A follow-up toxicity study based on metabolomics achieved an 83% predictive accuracy [74]. Another investigation using ornithine and cysteine, as indicators of developmental toxicity, identified novel potential teratogens with 77% accuracy [75], providing evidence for metabolomics effectiveness as an alternative method in toxicology. Optimally, integrating metabolomics, transcriptomics, and proteomics assays in stem cell toxicology would guarantee a more comprehensive analysis of the toxicity mechanisms.

In conclusion, from a toxicological point of view, hESCs have great potential. Novel and versatile toxicity screening platforms based on hESCs are advocated to advance the field of in vitro toxicology.

## hiPSCs as promising tools for personalized toxicology analyses

iPSCs have drawn attention and raised expectations in regenerative medicines, since their discovery. They are equivalent to ESCs in potency, although they are not derived from embryos. In fact, they are generated by reprogramming somatic cells, generally obtained from an adult individual, into the pluripotent state. Thus, this method could provide a large quantity of patient-specific pluripotent cells with no immune response issues for potential cell base transplantations and other clinical applications. Moreover, hiPSCs come with almost no ethical concerns compared with the derivation and use of hESCs. Besides regenerative medicine, iPSCs can potentially be employed for drug development and toxicology studies, as previously reviewed [76–79]. A study comparing the neurotoxicity effects of KG-501, an inhibitor of the CREB pathway, on hESC- and hiPSC-derived neurons and glia, verified the potentialities of hiPSCs in neural developmental toxicity assays [80]. Cardiomyocyte and hepatogenic differentiation from hiPSC has been also employed for drug screenings. Table 2 summarizes the findings in several drug screenings based on iPSC-derived cardiomyocytes and hepatocytes, proving the robustness of these models.

Reprogramming enables generation of iPSCs from patients as well as healthy people. In this respect, toxicology research can target different populations. For example, iPSC-derived cardiomyocytes from patients whose genetic backgrounds made them more prone to cardiac diseases resulted in more sensitivity to perturbations by cardio-toxic drugs, whereas cardiomyocytes from healthy people were more tolerant [85]. One investigation compared doxorubicin-induced toxicity in iPSC-derived cardiomyocytes from breast cancer patients who either suffered from doxorubicin-induced cardiotoxicity or did not. Results showed that cardiomyocytes derived from patients who experienced doxorubicin-induced cardiotoxicity were more sensitive to doxorubicin [89].

Further, iPSC-derived neural cells provide great research material, especially for patient-specific neurotoxicity information. Efforts on modeling neurotoxicity with iPSC-derived neural cell types are emphasized in one study about chemotherapy-induced toxicity [90], as well as in two others on embracing hiPSC neural differentiation in high-throughput screenings [91,92]. Those examples also clearly demonstrate that hiPSC-based assays are open to improvement and adaptation to personalized toxicology analysis for diagnosis and therapies.

Taking everything together, hiPSC applications in medical research and drug screenings support the idea of employing them as well as iPSC-derived cells in stem cell toxicology. In fact, those methods offer the possibility of a more personalized toxicology in that they allow to investigate whether patients or specific groups of healthy people with different genetic backgrounds respond differently to toxicants. However, there have not yet been a lot of persuasive reports on toxicity assays of environmental chemicals based on hiPSCs [93].

## Conclusive Remarks

Currently, toxicology still heavily relies on live animal tests, which are prone to errors due to interspecies inconsistencies [94] when we applied them to predict human health risks. Unlike drug screenings, which always include clinical trials with human volunteers, toxicology tests cannot rely on human subjects directly. In addition, we are continuously exposed to a variety of different chemicals,

TABLE 2. DRUG SCREENING AND TOXICITY ASSAYS BASED ON HUMAN-INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES OR HEPATOCYTES

| Differentiation lineage | Drug | Findings | Reference |
|---|---|---|---|
| Hepatocyte | Amiodarone Alatoxin B1 Troglitazone Ximelagatran | Amiodarone, Alatoxin B1 and Troglitazone cause dose-dependent toxic effects on hiPSC-derived hepatocytes after 14 days of exposure. There is specific induction of phospholipidosis and steatosis after exposure. hiPSC-derived hepatocytes show time-dependent toxicity effects from exposure to the drugs, suggesting that the model is applicable in long-term toxicity test and that certain drugs may have accumulative toxic effects on the liver | 62 |
| Hepatocyte | 24 drugs | 3D spheroid culture of hepatocytes not only produces high efficiencies but also allows cells to grow with better oxygen conditions, which gives rise to more functional hepatocytes with enhanced performance in drug screenings | 81 |
| Hepatocyte | 238 compounds from the Screen-Well™ Hepatotoxicity Library (Enzo Life Sciences) | High-content automated screening assays based on hiPSC-derived hepatocytes provide information on cell viability, nuclear shape and intensity, cytoskeleton integrity, mitochondrial potential, autophagy, and lipid accumulation by different staining methods | 82 |
| Hepatocyte | Staurosporine Acetaminophen | hiPSC-derived hepatocytes are highly similar to human primary hepatocytes in regulating and executing apoptosis after drug exposure, compared with two other human liver cell lines, suggesting that hiPSC-derived hepatocytes are a good alternative to primary hepatocytes | 83 |
| Cardiomyocyte | 24 drugs | hiPSC-derived cardiomyocytes show impedance changes after drug exposure. | 84 |
| Cardiomyocyte | Cisapride Nicorandil Alfuzosin Verapamil | Although iPSC-derived cardiomyocytes are not as mature as cardiomyocytes derived from the human body, based on their gene expression, iPSC-derived cardiomyocytes can be utilized in drug screenings, especially suitable for toxicity tests for high-risk populations | 85 |
| Cardiomyocyte | 131 drugs consisting of both cardiotoxic and cardio-safe ones | 384-well plate-based toxicity assays with good accuracy in classification of cardiotoxicity of drugs | 86 |
| Cardiomyocyte | Ponatinib | Ponatinib induces cell death, troponin secretion, and reactive oxygen species and lipid formation, inhibits ABL activation and survival pathways, and disrupts actin cytoskeleton structures and beating of hiPSC-derived cardiomyocytes, at medical significant doses | 87 |
| Cardiomyocyte | 23 drugs | Toxicity endpoints for hiPSC-derived cardiomyocytes cell viability and function provide robust evaluation of drug toxicity | 88 |
| Cardiomyocyte | Doxorubicin | iPSC-derived cardiomyocytes from patients experiencing doxorubicin-induced cardiotoxicity are more sensitive than the ones from patients who do not experience cardiotoxicity | 89 |

hiPSC, human-induced pluripotent stem cell.

including emerging environmental pollutants, which present potentially high risk for our health. Thus, reliable toxicity models based on the human physiology are needed to assess the adverse effects of all these chemicals on humans [95].

More than 20 years of exploration of ESC-based in vitro toxicity assays confirmed the importance of stem cells in toxicology research. Human PSCs enable toxicologists to study developmental toxicity, as well as functional toxicities on mature terminally differentiated cell types that are difficult to be derived directly from humans. The development of human stem cell toxicology benefited from all the efforts in the design and improvement of the mouse EST, which was the first stem cell-based developmental toxicity test to eliminate the use of pregnant animals. Nowadays, hPSC-derived

cardiomyocytes, neural cells, and hepatocytes are major lineages being employed. However, more stem cell-derived lineages, such as intestinal, gastric, and pulmonary cells, should be implemented in developmental and functional toxicity tests. In particular, differentiation of hESCs/iPSCs into primordial germ cells and gametes would broaden the scope of the in vitro reproductive toxicity investigations. In addition, advances in the synthesis of biomaterials, and conditions for 3D culture and differentiation of hESCs, have allowed the generation of more complex cell constructs, which provide a dynamic cell system resembling the in vivo situation more comprehensively than 2D systems. This would also make toxicology more predictive. The breakthrough of the generation of hiPSCs has also permitted drug screenings and toxicity

assays with cells derived from patients, building the foundations for a more personalized toxicology.

In conclusion, stem cell toxicology, although still developing, has already been proved to be a very comprehensive toxicity system, which allows for the assessment of embryonic, developmental, organ, reproductive, and functional toxicities. When fully evolved, it will definitely represent the gold standard for in vitro toxicology.

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## Author Disclosure Statement

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