Stem cell toxicology: a powerful tool to assess pollution effects on human health

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ABSTRACT

Environmental pollution is a global problem; the lack of comprehensive toxicological assessments may lead to increased health risks. To fully understand the health effects of pollution, it is paramount to implement fast, efficient and specific toxicity screening that relies on human models rather than on time-consuming, expensive and often inaccurate tests involving live animals. Human stem cell toxicology represents a valid alternative to traditional toxicity assays because it takes advantage of the ability of stem cells to differentiate into multiple cell types and tissues of the human body. Thus, this branch of toxicology provides a possibility to assess cellular, embryonic, developmental, reproductive and functional toxicity in vitro within a single system highly relevant to human physiology. In this review, we describe the development, performance and future perspectives of stem cell toxicology, with an emphasis on how it can meet the increasing challenges posed by environmental pollution in the modern world.

Keywords: stem cell toxicology, embryonic stem cells, mesenchymal stem cells, environmental pollution, environmental toxicology, health effects

INTRODUCTION

Environmental pollutants, including chemical and biological contaminants in air, water, food, soil, and radiation can have negative effects on human health. Economic globalization, which increases the interdependence of national economies across the borders through the exchange of resources and production, makes environmental pollution a common problem not restricted to a single country or city. Thus, because of globalization, the environmental problems of China are spread to other countries and vice versa [1,2]. However, China faces greater and more complex environmental challenges than other countries owing to its rapid economic development over the past few decades [1]. In addition, China is now experiencing an important period of socioeconomic transition characterized by an increasing demand for the assessment of health risks caused by industrial chemical waste, drugs, pesticides, food additives, cosmetics and atmospheric fine particles. To cope with this problem on a global scale, we urgently need to implement novel high-throughput and high-sensitivity systems that can provide rapid evaluation of the toxicity of environmental pollutants.

Currently, we still heavily rely on using live animals for toxicity testing, which is time-consuming, resource-intensive and raises ethical problems [3]. More importantly, the theory of ‘high fidelity fallacy’ postulated by Russell and Burch in 1959 has already warned us about inter-specific variations that make toxicity assays based on experimental animals not always translatable to human health [4]. In fact, there are numerous examples of drugs that passed animal testing but failed during clinical trials. Therefore, the 3Rs (Replacement, Reduction and Refinement) as the principles of alternative toxicology mostly based on in vitro experiments [4] may be more important today than ever before [3,5]. The accepted view is that, by implementing comprehensive in vitro tests based on human biology to identify relevant toxicity mechanisms at the cellular and molecular levels, we would eliminate the necessity for whole-animal testing and still provide adequate environmental- and health-friendly decision-making in the future [3].
The in vitro toxicological assays using human models are primarily based on cell cultures and have several limitations. For example, immortalized or cancer cell lines widely used for toxicity screening because of fast growth and expansion may no longer be representative of original cells because of accumulating mutations or altered cell functions. On the other hand, primary cells directly derived from human tissues are either impossible or difficult to obtain and the procedures are invasive; in addition, primary cells always need standardization prior to use and have limited ability to grow and proliferate in culture. Collectively, these issues can significantly limit the generation of the data, or affect their interpretation [6]. Most importantly, these in vitro assays are generally based on a single cell type, and cannot provide the information on toxicological responses at the tissue or whole-organism levels [3].

The emergence and development of stem cell biology have enlightened the enthusiasm of toxicologists. It is thought that contemporary stem cell technologies applied to the analysis of potential hazardous impacts of pollutants on human health can revolutionize the in vitro toxicology. Our group was the first to clearly define the term 'stem cell toxicology' to characterize a new promising trend in in vitro toxicology [7]. In this review, we describe the application and emphasize the vast prospects of stem cell toxicology, building upon and complementing other recent and excellent reviews, a few of which are listed here [8–11]. In addition, although embryonal carcinoma and cancer stem cells have been extensively studied, here we only focus on normal stem cells because they are more representative of microenvironments in vivo.

HISTORY OF STEM CELL BIOLOGY

The precise definition of stem cells remains a matter of debate. A commonly accepted one is offered by Douglas Melton [12]: ‘A working definition of a stem cell line is a clonal, self-renewing cell population that is multipotent and thus can generate several differentiated cell types.’ Taking into consideration the histological origin and differentiation potential of stem cells, we can roughly divide them into pluripotent stem cells (PSCs) and multipotent somatic stem cells (SSCs).

PSCs

Here, we describe the development of PSC biology focusing on the achievements that facilitated the emergence of stem cell toxicology (Fig. 1, top). The first evidence that PSCs have the capacity to differentiate into specialized cell types came from studying mouse embryonic carcinoma cells (malignant counterparts of embryonic stem cells) isolated from teratocarcinomas: tumors comprising cells from several differentiated tissues (reviewed in [13]). An important milestone in stem cell biology was set in 1981 by Martin Evans (Nobel Prize in Physiology or Medicine, 2007) et al., who were the first to establish an in vitro culture of non-malignant pluripotent cells from mouse embryos [14], which were later designated mouse embryonic stem cells (mESCs) by Gail Martin [15]. Later, the origin of mESCs was clearly proved to be the inner cell mass (ICM) of the pre-implantation blastocyst [16]. Interestingly, when ESCs were maintained in suspension, they formed small aggregates called embryoid bodies. During this step, cell types of all three germ layers (ectoderm, mesoderm and endoderm) differentiated and interacted with each other to produce different tissue-like structures. Pluripotent mESCs were also demonstrated to produce chimeric mice with germline transmission after blastocyst injection [17]. At that time, the maintenance of mESCs in vitro strictly required the presence of a feeder layer formed by fibroblasts; however, the introduction of leukemia inhibitory factor (LIF) in 1988 was a major contribution to the success of mESC culture because LIF addition to the medium prevented mESC differentiation and promoted their growth under feeder-free conditions, while LIF withdrawal resulted in EB formation [18]. After that, studies have focused on the identification of molecular mechanisms regulating mESC pluripotency, discovering Oct3/4 (POU5F1) [19,20] and other transcription factors such as Nanog, Sox2, Sall4, Esrrb, Tbx3, Klf4, Stella and Rex1 [21,22], which are essential for self-renewal and developmental potential of mESCs. Moreover, efforts have been directed to the establishment of protocols on ESC differentiation to different types of cells and tissues.

In 2006, Shinya Yamanaka and colleagues made a groundbreaking discovery in stem cell biology by reprogramming adult mouse fibroblasts into induced PSCs (iPSCs) through retroviral transduction of four transcription factors (Oct4, Sox2, c-Myc and Klf4) [23]. The generated iPSCs were virtually identical to ESCs but were of adult rather than embryonic origin. In that study, the authors combined the milestone achievements of John Gurdon on nuclear reprogramming (transplantation of somatic amphibian nuclei into oocytes) [24], and the important study demonstrated the possibility to convert one type of differentiated cells (fibroblasts) into another (muscle cells) through overexpression of the transcription factor MyoD [25]. In the follow-up investigation, Yamanaka’s and other groups
Figure 1. Important steps in the development of stem cell biology. The most significant achievements in stem cell biology (on top of the arrow) impacted on the development of stem cell toxicology. Note that the knowledge about human stem cells has been accumulating at a slower pace compared to that about mouse stem cells. Shown are also the stages in the development of somatic stem cell (SSC) biology using mesenchymal stem cells (MSCs) as an example (at the bottom of the arrow).

demonstrated that the gene expression, DNA methylation and chromatin status of murine iPSCs were similar to those of mESCs, and that iPSCs could produce competent germline chimeras by blastocyst injection [26,27]. In 2009, the laboratories of Qi Zhou and Shaorong Gao demonstrated that iPSCs could generate full-term mice via tetraploid blastocyst complementation, which indisputably established iPSCs as fully pluripotent cells [28,29] with a potential to substitute ESCs in all applications. For their breakthrough achievements in nuclear reprogramming, Shinya Yamanaka and John Gurdon were awarded the Nobel Prize in 2012.

The generation of human ESCs (hESCs) did not rapidly follow that of mESCs, and it took 17 years before James Thomson and his group [30] first obtained hESCs in 1998. In contrast to mESCs, hESCs do not respond to LIF, but require fibroblast growth factor 2 (FGF2) and transforming growth factor β1 (TGF-β1) or Activin A for their self-renewal [31,32], and are also more difficult to culture in vitro. Still, hESCs and mESCs are similar in terms of virtual immortality, marker gene expression and the ability to differentiate into all three primary germ layers. However, it is not possible to assess hESC pluripotency by blastocyst injection because of ethical and legal issues. In 2007, a year after the publication of their seminal article on mouse iPSCs, Yamanaka and his group reported that human somatic cells such as adult dermal fibroblasts could also be efficiently reprogrammed into human iPSCs (hiPSCs) by retroviral transduction of Oct4, Sox2, Klf4 and c-Myc [33]. Later the same year, James Thomson and colleagues showed that lentiviral transduction of a different set of transcription factors, including Oct4, Sox2, Nanog and Lin28, was sufficient to allow iPSC generation from human fetal and adult fibroblasts [34]. These findings were truly remarkable, as they opened a way to patient-specific regenerative medicine and, thus, can be qualified as one of the most significant events in the development of stem cell biology [35].

Also in 2007, a new cell line derived from the mouse epiblast (at a later developmental stage as compared to mESCs) and designated post-implantation epiblast-derived stem cells (EpiSCs) was demonstrated to share defining features with hESCs [36,37]. In fact, until that year, the differences between human and mouse ESCs/iPSCs were simply attributed to unknown species-specific genetic traits. EpiSCs are capable of differentiating into cells of all three germ layers in vitro or in teratoma assays, demonstrating pluripotency; however, they are inefficient in yielding chimeric animals once injected into pre-implantation embryos. For ethical reasons, no attempts have been made to obtain human equivalents of EpiSCs. In 2009, the two kinds of pluripotency were defined as naïve (mESCs) and primed (mEpiSCs and hESCs) [38]. Ground state naïve pluripotency is achieved in the pre-implantation epiblast and refers to a completed unrestricted cell population able to generate all the embryonic lineages. Upon implantation, the epiblast becomes ‘primed’ for lineage specification and commitment driven by signals from the extraembryonic tissues. Nevertheless, it is important to keep in mind that hESCs are not equivalent to mEpiSCs, which can be considered to be relatively less primed (reviewed in [39]). In 2010, hESCs and hiPSCs were proved to be maintained in a unique mESC-like pluripotent state by ectopic expression of Oct4, Klf4 and Klf2, and cultured in the presence of LIF and inhibitors of glycogen synthase kinase 3β (GSK3β) and mitogen-activated protein kinase (ERK1/2) (LIF/2i conditions) [40]. Since then, many studies have demonstrated that hESCs and hiPSCs could achieve naïve pluripotency under transgene-dependent or-independent conditions, although not as efficiently as mESCs or human ICM [39]. However, because of the limitations in performing chimeric analysis in humans, the accurate classification of the pluripotent state of isolated naïve hPSCs remains debatable.

Gametogenesis is also an import feature of the differentiation ability of PSCs. In 2003, two studies provided the first evidence that mESCs could potentially differentiate in vitro into germ cells, including oocytes and spermatocytes [41,42].
Primordial germ cells (PGCs) are another important type of pluripotent cells, which can generate germ cells, eggs and sperm. PGC-like cells can also be induced in vitro from mESCs or iPSCs through epiblast-like cells that are highly similar to pre-gastrulating epiblasts but distinct from EpiSCs [43]. Another important study demonstrated that naive hESCs and hiPSCs could be converted to PGC-like cells [44]. These findings are very useful for stem cell toxicology because PGC-like cells and germ cells could be employed in reproductive toxicity assays in vitro described below.

In conclusion, the basic characteristics of PSCs include: indefinite self-renewal under well-defined culture conditions, differentiation into cells of the three germ layers in vitro and in vivo, clonogenicity, a normal karyotype, and the ability to withstand freezing and thawing. Since a clear definition of pluripotency in vivo is still under debate, we determine it according to the species of origin and the level of pluripotency demonstrated in vitro. Thus, in animals, naive PSCs can be rigorously identified by observing whether blastocyst-injected cells can contribute to all somatic lineages and show germline chimerism. In addition, both naive and primed PSCs should be able to form teratomas in vivo, which contain differentiated cells from the three germ layers. However, in the case of hiPSCs, their in vivo pluripotency can only be assessed by teratoma assays because of ethical problems.

**Multipotent SSCs**

During the gastrulation stage of embryogenesis, pluripotent cells in the ICM are reorganized into the germ layers that eventually produce all the tissues of the body and, thus, become restricted in their potential to differentiate into all lineages. These new cells are less plastic and have the ability to generate a limited range of cells mostly within one or a few specific tissue types, so they are multipotent; some of them persist in adults and are called SSCs [45]. The existence of SSCs in adult tissues was first demonstrated in the hematopoietic system in 1960–1961 by a seminal work of McCulloch and Till, showing that cells from murine bone marrow could give rise to multilineage descendants while retaining the ability to self-renew (Fig. 1, bottom) [46,47]. Therefore, SSCs are also commonly referred to as adult stem cells; however, they can also be found in fetal tissues such as the umbilical cord and placenta, and are designated fetal stem cells. Moreover, SSCs are classified, according to their histological origin, into mesenchymal stem cells (MSCs), neural stem cells (NSCs), hematopoietic stem cells (HSCs), skin stem cells, etc. Here, although we recognize the importance of other SSCs, due to space limitation, we will only focus on MSCs (Fig. 1, bottom) as an example of SSCs.

Alexander Friedenstein is considered the first to put forward the concept of prototype MSCs. In 1968, he and his colleagues isolated, for the first time, adherent, fibroblast-like, colony-forming cells from mouse bone marrow with a high replication capacity in vitro, and demonstrated that those cells were able to differentiate into osteoblasts and reconstitute the hematopoietic microenvironment after subcutaneous transplantation [48]. When similar fibroblast colony-forming cells were detected in human bone marrow aspirates in 1980 [49,50] and showed a potential for differentiating into osteogenic, adipogenic, chondrogenic and myogenic mesenchymal lineages in vitro (reviewed in [51]), the term ‘mesenchymal stem cells’ coined by Caplan in 1991 [52] has been applied. By now, MSCs have been isolated from almost all fetal and post-natal tissues, including fat, dental pulp, periodontal ligament, tendon, umbilical cord, skin, placenta, amniotic fluid, muscle, liver and brain (reviewed in [53]). Multipotency has been demonstrated in some adult MSC lines generated from bone marrow [54,55] and adipose tissues [56]. However, because of their heterogeneity, not all of these plastic-adherent cells have comparable self-renewal and differentiation potential in vivo. Therefore, in 2005, the International Society for Cellular Therapy recommended the term Multipotent Mesenchymal Stromal Cells for the fibroblast-like plastic-adherent cells [57] and, in 2006, issued the minimal criteria to define human MSCs: adherence to plastic under standard culture conditions; expression of CD105, CD73 and CD90; no expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, CD10, and HLA-DR; and ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro [58].

Although there is still no agreement on the use of the terms ‘stem’ and ‘stromal’ [51,59], it did not affect the increasing application of MSCs in both basic research and clinical trials. In fact, MSCs can not only function as multipotent cell progenitors, but also modulate immune reactivity, support hematopoiesis and regulate the release of trophic factors in response to injury [51,53,59]. As of May 2016, a search of the website clinicaltrials.gov using ‘mesenchymal stem cells’ would yield a list of over 600 clinical trials covering a surprisingly vast array of pathological conditions.

Other commonly studied types of SSCs are also very important in the history of stem cell biology. For example, NSCs successfully isolated from both developing and adult brains can self-renew in vitro [60] and differentiate into the three major central nervous system lineages: neurons [61], astrocytes and...
oligodendrocytes [62]. Another example is HSCs, which are derived from bone marrow and undergo the process of hematopoiesis to generate all types of blood cells. However, they are not discussed here because of limited space.

**DEVELOPMENT OF STEM CELL TOXICOLOGY**

**PSCs in toxicology**

In 1991, 10 years after mESC cultures were established in vitro, Horst Spielmann et al. [63] (all the references from now on, in the development of stem cell toxicology section, are also included in Table 1), were the first to apply PSCs in toxicology by measuring cytotoxic effects of potential teratogens in vitro. The same group also developed an in vitro embryotoxicity test using differentiating mESCs [64,65]. Similarly, another assay using proliferating and spontaneously differentiating mESCs (induced by LIF removal) was reported in 1994 and 1996, for testing the teratogenic activity of 25 compounds; teratogens were identified if their IC$_{50}$ for differentiating cells was less than that for proliferating ESCs [66,67]. Although the assay was not very accurate and had poor prediction rate, at that time, it could potentially replace the traditional micromass test used to assess developmental toxicity. However, it was not developed any further.

In 1995–2004, the European Centre for the Validation of Alternative Methods (ECVAM) proposed, defined and validated the Embryonic Stem Cell Test (EST) [68–73] pioneered by Horst Spielmann as an in vitro assay for embryotoxicity [74,75]. In the EST, the effects of tested chemicals were assessed according to a prediction model based on three endpoints: inhibition of ES differentiation into the contracting myocardium analysed by EB formation (hanging drop technique) and cytotoxicity for ESCs and mouse 3T3 fibroblasts (control differentiated cells). The correlation between the EST and in vivo data (78%) was similar to that observed for other in vitro tests such as the micromass assay (70%) and the post-implantation rat whole-embryo culture assay (80%). However, the advantage of the EST was that it did not use embryos or primary embryonic tissues isolated from pregnant animals [74]; therefore, the establishment of the EST is considered a milestone in the history of stem cell toxicology. The EST has been evaluated for drug screening [76,77], developmental toxicity testing of panels of related compounds [78] and for embryotoxicity assessment of nanomaterials [79]. Nevertheless, it had several weaknesses such as limited differentiation assessment based solely on the detection of spontaneous myocardial differentiation (which in turn was subjectively evaluated by microscopic observation of beating areas), and the absence of metabolic analysis [80]. Moreover, in another EST evaluation study sponsored by the ECVAM and ReProTect, only 2 out of 13 previously untested compounds were classified correctly, highlighting the limitations of the EST prediction model [81].

To improve the EST performance, several modifications were proposed. Thus, instead of the visual microscopic evaluation of beating cardiomyocytes, more quantitative molecular endpoints were introduced, such as analysis of gene and protein expression of myocardial markers by reverse transcription quantitative PCR (RT–qPCR) and fluorescence-activated cell sorting (FACS) [82–87]. In addition, toxicity was assessed based on the differentiation to other cell types, including neuronal, skeletal muscle, pancreatic and osteogenic lineages [88–91]; for this, genetically engineered ESCs were introduced, which expressed green fluorescent protein (GFP)-labeled lineage-specific markers [92–94] or cardiac/neural-specific luciferase reporters [95]. Based on these improvements, a molecular multiple-endpoint EST was developed in 2004, which incorporated quantitative analysis by RT–qPCR and assessment of the differentiation to osteogenic, chondrogenic and neural cells in addition to the traditional cardiomyocyte differentiation [96]. Other improvements of the typical EST are mentioned in Table 1 [97–99]. An official ECVAM validation of the EST was published in 2011 in the journal Nature Protocol [100]. Moreover, ReProTect Inc. conducted a feasibility study with a modified EST method and showed correct prediction for 9 of the 10 blinded chemicals [101]. In another major global drug company, Roche, the EST has been routinely employed to detect potential teratogenic/embryotoxic liabilities during optimization of early pharmaceutical drug candidates [102].

The described ESTs utilized ESCs and differentiated fibroblasts (3T3 cell line). In order to simplify toxicity testing, procedures based only on ESCs were developed; they incorporated recent advancements in genomics and proteomics such as whole-genome and miRNA profiling by microarrays and mass spectrometry to enable high-throughput toxicological analysis. At the same time, more attention was directed towards understanding of the underlying molecular mechanisms on the one hand and the development of reproducible toxicity assays on the other [103–122].

Starting from the introduction of the EST, most of the developmental ESC-based toxicity tests analysed cell differentiation by EB formation. However, several later studies reported the assessment of ESC
### Table 1. Important studies for the development of stem cell toxicology.

| Study            | Reference # | Year    | Experimental design and/or conclusions                                                                 |
|------------------|-------------|---------|---------------------------------------------------------------------------------------------------------|
| Laschinski et al. | 63          | 1991    | They used mESCs for MTT cytotoxicity assays and demonstrated that ESCs were more sensitive than fibroblast cultures to known teratogens |
| Heuer et al.     | 64, 65      | 1993, 1994 | They utilized differentiating mESCs and demonstrated the inhibitory influence of the teratogen retinoic acid on blood and myocardial cell development in contrast to a stimulating effect on the nerve and skeletal muscle cell development |
| Newall et al.    | 66, 67      | 1994, 1996 | They used mESCs to design their stem cell tests. 25 potential teratogenic compounds were assessed by measuring cytotoxicity using the MTT assay, and differentiation by simply fixing and staining the cultures with Nile Blue and measuring their area using image analysis |
| Brown et al.     | 68          | 1995    | ECVAM suggested the further develop of *in vitro* methods based on mammalian ESCs                          |
| Spielmann et al. | 69          | 1997    | The embryonic stem cell test (EST) was officially defined                                               |
| Scholz et al.    | 70, 71      | 1999    | The EST was pre-validated according to the ECVAM pre-validation scheme                                  |
| Balls et al.     | 72          | 2002    | ECVAM released a statement on the scientific validity of the EST                                        |
| Genschow et al.  | 73, 74      | 2002, 2004 | ECVAM sponsored a formal validation of the EST with 20 test chemicals and demonstrated its validity for embryotoxicity assays |
| Bremer et al.    | 75          | 2002    | They discussed on-going projects to find novel endpoints for the validated EST                           |
| Whitlow et al.   | 76          | 2007    | They utilized the EST to predict the potential embryotoxicity of drugs in early development              |
| Paquette et al.  | 77          | 2008    | The ECVAM's EST protocol was used with several pharmaceutical compounds. They demonstrated its high false-positive rate but very low false-negative rate |
| de Jong et al.   | 78          | 2009    | The EST was used to compare the *in vitro* developmental toxicity of homologous compounds with the *in vivo*. They demonstrated a good correlation |
| Di Guglielmo et al. | 79      | 2010    | They utilized the EST to detect nanoparticle embryotoxicity                                               |
| Spielmann et al. | 80          | 2006    | ECVAM sponsored a validation of the EST as well as two other *in vitro* embryotoxicity assays, the mouse micromass and the rat whole-embryo culture tests. The EST was judged validated for routine use, but not ready to totally replace animal tests |
| Marx-Stoelting et al. | 81      | 2009    | EVCAM and ReProTect reviewed the EST and suggested further improvements, such as alternative endpoints |
| Bigot et al.     | 82          | 1999    | Introduction in the EST of molecular endpoints: gene expression by semi-quantitative RT–PCR               |
| zur Nieden et al. | 83         | 2001    | Introduction in the EST of molecular endpoints: gene expression by quantitative RT–qPCR                   |
| Seiler et al.    | 84, 85      | 2004, 2006 | They improved the EST by introducing molecular endpoints, such as FACS analyses of sarcomeric myosin heavy chain and alpha-actinin proteins |
| Riebeling et al. | 86          | 2011    | They used the EST to specifically assess the embryotoxicity of valproic acid (VPA). They also demonstrated the validity of a shortened EST based on flow cytometry of intracellular marker proteins |
| Buesen et al.    | 87          | 2009    | They introduced a new EST, FACS-EST, with molecular flow cytometry markers                               |
| Schmidt et al.   | 88          | 2001    | They utilized the EST with lithium chloride as a test chemical to show that cardiac differentiation alone was not sufficient to detect embryotoxic effects |
| Rolletschek et al. | 89       | 2004    | They showed how mESC differentiation in cardiac, neuronal and pancreatic cells could be exerted for toxicological assays |
| Pellizzer et al.  | 90, 91      | 2004    | They introduced selective target organ genes in the EST for a better prediction                           |
| Bremer et al.    | 92          | 2001    | They used engineered mESCs expressing a cardiac-specific green fluorescent protein (GFP) reporter to assess the effects of 15 chemicals on cardiac differentiation |
| Paparella et al. | 93          | 2002    | They employed an ESC line expressing an endoderm specific GFP reporter gene to perform toxicity assays |
| Kugler et al.    | 94          | 2015    | They used an ESC line with Bmp-mediated GFP, to identify teratogens                                      |
| Suzuki et al.    | 95          | 2012    | They defined the Hand1- and Cmya1-EST’s with genetically engineered ESCs expressing luciferase reporter genes, for embryotoxicity assays |
## Table 1. Continued.

| Study | Reference # | Year   | Experimental design and/or conclusions |
|-------|-------------|--------|----------------------------------------|
| zur Nieden et al. | 96          | 2012   | They developed the so-called molecular multiple-endpoint EST (mme-EST), which incorporated several improvements over the traditional EST, such as the quantitative analysis by RT–qPCR of the potential effects of selected chemicals on osteogenic, chondrogenic and neural differentiations, in addition to the traditional cardiomyocyte differentiation |
| Stummann et al. | 97, 98      | 2007, 2008 | They used an ESC neuronal differentiation-based EST. They reevaluated and correctly classified methyl mercury, cadmium, arsenite and arsenate compounds which were misclassified in the traditional EST (72) |
| Hettwer et al. | 99          | 2010   | They utilized a co-culture approach for EST embryotoxicity testing. Test compounds were pre-incubated with hepatocytes for metabolic activation prior to the EST test |
| Seiler et al. | 100         | 2011   | The protocol of FACS-EST published in ‘Nature Protocol’, marked acceptance by the scientific community |
| Schenk et al. | 101         | 2010   | The ReProTect Feasibility Study showed correct prediction of the EST for 9 of the 10 blinded chemicals. The partially correct prediction for one chemical was due to the fact that the effects in vivo depended on the route of administration |
| Peters et al. | 103         | 2008   | They established a high-throughput 96-well-based EST where they analysed the effects of 12 test chemicals on an EB-dependent ESC differentiation procedure |
| van Dartel et al. | 104–108     | 2009–2011 | They exerted transcriptomics techniques (microarrays) to monitor gene expression changes during early stages (24 h and 96 h) of mouse ESC differentiation |
| van Dartel et al. | 119         | 2014   | They applied whole-genome transcriptomics to characterize metabolic changes upon ESC early differentiation, to deal with the absence of metabolic evaluations in the traditional EST |
| zur Nieden et al. | 110         | 2010   | They combined microarray, IMAGE analysis and Ca\(^{2+}\) deposition assays as endpoints for the evaluation of the developmental osteotoxicity using a mESC model |
| Osman et al. | 111         | 2010   | They applied proteome profiling determinations of mESCs exposed to monobutyl phthalate to define markers for cell differentiation and embryotoxicity |
| Wang et al. and Neri et al. | 112, 113     | 2010–2011 | They characterized the molecular mechanisms and signalling pathways of the developmental toxic effects of dioxin on a mESC cardiomyocyte differentiation model by employing chromatin immunoprecipitation, microarray and ATP quantitation assays |
| Theunissen et al. and Pennings et al. | 114–117     | 2010, 2012 | They designed an in vitro murine neural embryonic stem cell test (ESTn) based on a 13-day neural differentiation protocol to assess neurodevelopmental toxicity through FACS and RT–qPCR analyses and microarrays |
| Aoki et al. | 118         | 2012   | They investigated the effect of BPA on the differentiation of mESCs focusing on the expression of germ cell marker genes as an indication of RT |
| Smirnova et al. | 119         | 2014   | They analysed changes in miRNome (analysed by miRNA expression microarray profiling) and transcriptome (whole-genome expression microarray profiling) during neural differentiation of mESCs exposed to the developmental neurotoxicant sodium valproate (VPA), and concluded that miRNA expression profiling was a suitable molecular endpoint for developmental neurotoxicity |
| de Jong et al. | 120         | 2014   | They published a novel embryonic stem-cell-based osteoblast differentiation assay (subsequently termed the ESTo), and suggested that incorporating the ESTo into a testing battery together with the traditional EST could improve the overall predictive value of the battery |
| Kroese et al. | 121         | 2015   | They combined the EST, the zebrafish embryotoxicity assay, the ReProGlo assay and the CALUX transcriptional activation assay in a battery to distinguish in vivo non- or weak developmental toxicants from potent developmental toxicants within groups of structural analogs |
| Li et al. | 122         | 2015   | They combined the EST with the BeWo transport model, mimicking the placental barrier, and provided a correct prediction for previously validated in vivo developmental toxicants |
Table 1. Continued.

| Study              | Reference # | Year  | Experimental design and/or conclusions                                                                 |
|--------------------|-------------|-------|--------------------------------------------------------------------------------------------------------|
| Romero et al.      | 123         | 2011  | They proposed a simplified EST in which the effects of chemicals on the differentiation of ESCs were assessed upon a five-day-long spontaneous differentiation of D3 ES cells in monolayer conditions instead of via EB formation |
| Barrier et al.     | 124         | 2011  | They developed an adherent cell differentiation and cytotoxicity (ACDC) assay using In-Cell Western analysis for mESC cardiomyocyte differentiation, which did not rely on EB differentiation |
| Chandler et al.    | 125         | 2011  | The efficiency of ACDC assay was confirmed through the evaluation of 309 environmental chemicals         |
| Zimmer et al.      | 126         | 2011  | They provided proof-of-concept for the suitability of differentiating mESC to detect chronic low-dose toxicity of MeHg to maturing neurons derived with a monolayer differentiation protocol |
| Baek et al.        | 127         | 2012  | They designed an adherent monoculture differentiation method to screen developmental neurotoxicants by detecting the neuronal marker Tuj-1 by flow cytometry, and demonstrated the predictability of this method |
| Kang et al.        | 128         | 2013  | They developed a hepatotoxicity assay using hepatic progenitor and hepatocyte-like cells derived from mESCs differentiated in monolayer conditions |
| Hayess et al.      | 129         | 2013  | They established a new in vitro assay using mESCs to predict adverse effects of chemicals and other compounds on neural development—the so-called DNT-EST. In this method, after treatment of differentiating stem cells for 48 h or 72 h, at two key developmental stages endpoint for neural differentiation, viability and proliferation were assessed. As a reference, undifferentiated stem cells were treated in parallel to the differentiating stem cells |
| Xu et al.          | 130         | 2013  | They evaluated mRNA and/or protein levels of pluripotency markers (Sox2, Nanog, Oct4) and level of microRNA (which can regulate pluripotency markers) in mESCs exposed with Perfluorooctane sulfonate (PFOS) |
| Chen et al.        | 131         | 2013  | They demonstrated the effects of BPA on mESC maintenance and differentiation                           |
| Cho et al.         | 132         | 2013  | They designed a high-throughput screening based on mESC differentiation in monolayer conditions        |
| Yin et al.         | 133         | 2015  | They employed mESC-based differentiation procedures via EBs and in monolayer to demonstrate the neural developmental toxicity of BPA |
| Cezar et al.       | 134         | 2007  | They demonstrated that the detection and identification of small molecules in hESCs and hESC-derived neural precursors (hNPs) by mass spectrometry-based metabolomics analyses, upon chemical exposure, could help define toxicity pathways |
| West et al. and    | 135–136     | 2010–11| They developed a more predictive developmental toxicity model utilizing metabolomics with the hESC system to discover biomarkers of developmental toxicity. They demonstrated that their model could correctly predict the teratogenicity of 88% of the eight drugs (127), and 83% of the 11 environmental toxicants tested |
| Kleinstreuer et al.|             |       |                                                                                                         |
| von Stechow et al. | 137         | 2013  | They performed metabolic profiling, by mass spectrometry, in hESCs treated with cisplatin for different time periods. Then, they integrated those metabolomics with transcriptomics analyses and connected cisplatin-regulated metabolites and metabolic enzymes to identify enriched metabolic pathways |
| Palmet et al.      | 138         | 2013  | They established a metabolomic biomarker assay for hESC-based developmental toxicity screenings, and identified potential developmental toxicants with 77% accuracy (57% sensitivity, 100% specificity) |
| Cao et al.         | 139         | 2008  | They utilized hESC-differentiated fibroblastic progenies for in vitro toxicology screenings and demonstrated that hESC-derived fibroblasts exhibited a more sensitive dose–response curve to mitomycin C compared to L929, a human lung fibroblast cell line |
| Adler et al.       | 140         | 2008  | They established an assay based on hESCs that was equivalent to the validated mouse EST                   |
Table 1. Continued.

| Study         | Reference # | Year | Experimental design and/or conclusions |
|---------------|-------------|------|----------------------------------------|
| Pal et al.    | 141         | 2011 | They evaluated the potential toxicity of several drugs, by analysing changes in cell cycle, germ layer-specific marker expression and hormone levels during hESC global differentiation through EBs |
| Jagtap et al. | 142         | 2011 | They proposed a transcriptomic approach in hESCs to monitor specific toxic effects of compounds during global EB differentiation |
| Meganathan et al. | 143      | 2012 | They demonstrated that a combination of transcriptomics and proteomics, in differentiating hESCs, enabled the detection of canonical and novel teratogenic intracellular mechanisms for thalidomide |
| Kim et al.    | 144         | 2013 | They investigated the effects of estrogen compounds on the proliferation and differentiation of short-term and long-term cultured hESC-derived EBs in vitro, and demonstrated that those compounds impaired endodermal and mesodermal differentiation |
| Colleoni et al. | 145      | 2011 | They designed a protocol based on the generation of neural rosettes from hESCs, with an emphasis on early neural development, for the detection of neural developmental toxicity |
| Krug et al.   | 146         | 2013 | They employed a transcriptomics approach in hESC-derived test systems for developmental neurotoxicity (DNT) and reproductive toxicity (RT). They were able to classify human DNT/RT toxicants on the basis of their transcriptome profiles |
| Stummann et al. | 147     | 2009 | They explored the hESC-based neuronal differentiation, and demonstrated that neuronal precursor formation was more sensitive to MeHg than later stages of neuronal differentiation |
| Zimmer et al. | 148        | 2012 | They generated hESC-derived neural crest (NC) cells, and assessed the impairment in their migration, caused by environmental toxicants, as well as the signal transduction pathways affected |
| Balmer et al. | 149        | 2012 | They used hESCs differentiating into neuroectodermal precursors as a model to investigate the modes of action of VPA, by analysing gene expression profiling and epigenetics changes |
| Hoelting et al. | 150    | 2013 | They developed a hESC-derived 3D in vitro neurosphere model that allowed testing the potential DNT of nanoparticles, which was named Nano-DNT |
| Pistollato et al. | 151    | 2014 | They compared the neuronal differentiation propensity of hESCs and hiPSCs and demonstrated that a CREB (cAMP-responsive element-binding protein) pathway inhibition could be involved in cellular and molecular neurotoxic effects, and qualified the use of hiPSC-derived neuronal model for studying chemical-induced neurotoxicity resulting from pathway perturbations |
| Waldmann et al. | 152   | 2014 | In an attempt to define universal rules for neurotoxicity tests, they tested the dose-dependent transcriptome deviations in an assay that recapitulated the development of hESCs into neuroectoderm, and suggested the use of the highest non-cytotoxic drug concentrations for gene array toxicogenomics studies. They argued that higher concentrations would yield wrong information on the mode of action of each chemical, and lower drug levels would result in low gene expression changes which might be difficult to detect |
| Schwartz et al. | 153  | 2015 | They cultured 3D neural constructs with hESC-derived neural progenitor cells, endothelial cells, MSCs and microglia/macrophage precursors on chemically defined polyethylene glycol hydrogels in serum-free medium to model cellular interactions within the developing brain. They also used linear support vector machines to construct a reliable predictive model from RNA-Seq data acquired from 240 neural constructs treated with 34 toxic and 26 nontoxic chemicals |
| Senut et al.  | 154        | 2016 | They assessed the effects, of gold nanoparticles (AuNPs) on the viability, pluripotency, neuronal differentiation ability and DNA methylation status of hESCs. They identified a type of AuNPs highly toxic to hESCs and demonstrated the potential of hESCs in predicting nanotoxicity and characterizing nanoparticle ability to alter DNA methylation and hydroxymethylation patterns in the cells |
| Study          | Reference # | Year | Experimental design and/or conclusions                                                                                                                                                                                                 |
|---------------|-------------|------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Ryan et al.   | 155         | 2016 | They measured the potential developmental neurotoxicity of 80 compounds, in a high-throughput, high-content assay using human neurons derived from iPSCs. Their studies further justified the use of human iPSCs in DNT/NT screenings. |
| Oh et al.     | 156         | 2016 | They showed that silver nanoparticles induced oxidative stress and dysfunctional neurogenesis at the molecular level in hESC-derived neural stem/progenitor cells (NPCs), through global expression profiles of genes and miRNAs. |
| Pallocca et al.| 157         | 2016 | They identified specific transcriptome signatures and biomarkers for potential developmental toxicants inhibiting human NC cell migration based on differentiating hESCs.                                                                 |
| Caspi et al.  | 158         | 2009 | They highlighted the possibility of a novel model for electrophysiological drug screening, in which human embryonic stem-cell-derived cardiomyocytes (hESC-CMs) were assessed with a combination of single cell electrophysiology and microelectrode array (MEA) mapping. |
| Braam et al.  | 159         | 2010 | They showed that electrophysiological properties and drug responses of hESC-CMs matched clinical observations on QT prolongation/shortening and arrhythmia at similar concentrations.                                                                 |
| Schaad et al. | 160         | 2011 | They generated a fibrin-based human engineered heart tissue (hEHT), which was a 3D force-generating cardiac tissue-like structure, using an unscreened population of differentiated human ESCs containing 30%-40% α-actinin-positive cardiac myocytes. They validated this system by detecting the effects of several proarrhythmic compounds. |
| Guo et al.    | 161, 162    | 2011, 2013 | They first reported in 2011 and then refined in 2013, a high-throughput functional assay employing a monolayer of beating human iPSC-derived cardiomyocytes (iPSC-CMs). This model could accurately detect drug-induced cardiac abnormalities via rapid cellular impedance technology cross-validated with microelectrode arrays. |
| Cerignoli et al. | 163     | 2012 | They exerted kinetic imaging cytometry (KIC) based on hESC- and iPSC-CMs for automated cell-by-cell analyses via intracellular fluorescence Ca^{2+} indicators, for toxicity tests.                                                                 |
| Sirenko et al.| 164         | 2013 | They further upgraded iPSC-CMs cardiotoxicity assays with a 384-well automated high-throughput analysis of KIC and other multi-parameters for beating cardiomyocytes, such as beat rate, peak shape (amplitude, width, rise, decay, etc.) and regularity. |
| Ting et al.   | 165         | 2014 | They developed an automated time-resolved video analysis and management system (TVAMS) for the evaluation of hESC differentiation to EBs-based CMs, but not terminally differentiated CMs. The TVAMS is a high-throughput non-invasive video-imaging platform that can be applied for the development of new CM differentiation protocols, as well as a tool to conduct CM toxicity assays. |
| Lagerqvist et al. | 166     | 2015 | They compared the differentiation towards cardiac lineages from both mouse (Nkx2.5^{GFP/w}) and human (NKX2.5^{GFP/w}) ESC reporter lines with live single cell high acquisition rate calcium imaging, and suggested that human Nkx2.5^{GFP/w} cells were less suitable for studies of compounds affecting cardiac pacemaker activity than mouse Nkx2.5^{GFP/w} cells, but very suitable for cardiac toxicity studies. |
| Medine et al. | 167         | 2013 | They reported that hESC- and hiPSC-derived hepatocytes (hESC-Hep and hiPSC-Hep) could achieve toxicity predictability in a manner comparable to the standard hepatotoxicity assays, representing a major advance in the field. |
| Holmgren et al.| 168         | 2014 | They described a long-term toxicity study using hiPSC-Hep hepatocytes, which were more sensitive than the human hepatocellular carcinoma cell line HepG2.                                                                                                                                                        |
| Sirenko et al.| 169         | 2014 | They reported that high-content automated screening assays using hiPSC-Hep cells were feasible, provided information about mechanisms of toxicity, and could facilitate the safety assessment of drugs and chemicals.                                                                                              |
Table 1. Continued.

| Study               | Reference # | Year | Experimental design and/or conclusions                                                                                                                                 |
|---------------------|-------------|------|---------------------------------------------------------------------------------------------------------------|
| Sjogren et al.      | 170         | 2014 | They compared toxic responses among hiPSC-Heps, primary cryopreserved human hepatocytes (cryo-hHeps) and the hepatic cell lines Heparg and Huh7, and demonstrated how hiPSC-Heps might be a good alternative to cryo-hHeps for compounds initiating apoptosis |
| Sengupta et al.     | 171         | 2014 | They showed that hESC-Heps in aggregate cultures displayed improved enzymatic inducibility and metabolic function, as compared to monolayer conditions. Nonetheless, the authors suggested that systems based on human ESC-derived hepatocytes would require further improvements to completely replace the ones utilizing primary human hepatocytes in drug development and toxicity screenings |
| Pradip et al.       | 172         | 2016 | They revealed that the hiPSC-Heps could serve as a platform for monitoring drug-induced steatosis and phospholipidosis by high-content analysis following mechanistic endpoints such as viability, nuclear changes, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) and plasma membrane permeability (PMP) |
| Yang et al.         | 173         | 2013 | They differentiated hESCs into mammary epithelial cells in 3D conditions to address the toxic effects of BPA and demonstrate how BPA low doses were toxic |
| Calderon-Gierszal et al. | 174  | 2015 | They provided the first direct evidence that low-dose BPA exposure perturbed hESC differentiation towards human prostate organoids, suggesting that the developing human prostate might be susceptible to disruption by in utero BPA exposures |
| Kameoka et al.      | 175         | 2014 | They employed a three-day monolayer-directed differentiation of hESCs and assessed the teratogenic risk of compounds by the reduction in nuclear translocation of the transcription factor SOX17 in mesendodermal cells. They also validated their method as a high-throughput screen with 71 drug-like compounds, 15 environmental toxicants and 300 kinase inhibitors: they named the human pluripotent stem cell test (hPST) |
| Pratt et al.        | 176         | 1982 | They prescreened environmental teratogens using cultured mesenchymal cells from the human embryonic palate |
| Scanu et al.        | 177         | 2011 | They were the first to evaluate the applicability of hMSCs as cell lines for in vitro cytotoxicity tests to correctly predict LDS0 values and the hazard category of each tested chemical, according to the globally harmonized system of classification (GHS). They demonstrated that hMSCs provided a more accurate modeling of in vivo conditions as compared to the validated 3T3 and NHK NRU test methods |
| Akhavan et al.      | 178         | 2012 | They demonstrated that reduced graphene oxide nanoplatelets (rGONPs) showed genotoxic effects on hMSCs through DNA fragmentations and chromosomal aberrations, even at concentrations as low as 0.1 mg/ml |
| Strong et al.       | 179         | 2015 | They exposed hMSCs with the endocrine-disrupting chemical (EDC) DDT (dichlorodiphenyltrichloroethane) and demonstrated profound alterations in self-renewal, proliferation, differentiation (both adipogenesis and osteogenesis) and gene expression, which could partially explain the homeostatic imbalance and increased cancer incidence among individuals exposed to long-term EDCs |
| Tamm et al.         | 180         | 2006 | They used the NSC line C17.2 and primary embryonic cortical NSCs (cNSCs) to investigate the effects of MeHg on the survival and differentiation of NSCs and showed how NSCs, in particular cNSCs, were highly sensitive to MeHg. The observed effects of MeHg on NSC differentiation could offer new perspectives for evaluating the biological significance of MeHg exposure at low levels |
| Buzanska et al.     | 181         | 2009 | They developed a human NSC line derived from umbilical cord blood (HUCB-NSC) for DNT tests, and investigated the effects of compounds on key neurodevelopmental processes like cell proliferation, apoptotic cell death, and neuronal and glial differentiation |
differentiation in cell monolayers, which could complement or replace that based on EBs. In fact, in 2011, using a simplified EST, the effects of chemicals were analysed by observing spontaneous differentiation of D3 ESCs cells in monolayers over 5 days and which provided faster, technically simplified analysis, while maintaining the same prediction rate [123]. Later that year, Barrier and colleagues developed the adherent cell differentiation and cytotoxicity (ACDC) assay using In-Cell Western analysis for mESC differentiation into cardiomyocyte lineage [124], which also did not rely on EB formation. The efficiency of the ACDC assay was confirmed by evaluating 309 environmental pollutants [125]. Further toxicity studies employing mESC monolayers [126–133] are described in Table 1.

An important limitation of toxicological analysis performed using mouse stem cells is that the results may not be directly applicable to humans because of inter-species variations. Therefore, toxicity testing based on hESCs is likely to generate more clinically relevant data. The first application of hESCs in toxicology was described in 2007 by Cezar et al. [134], who demonstrated that mass spectrometry-based small-molecule metabolite profiling of hESCs and hESC-derived neural precursors subjected to chemical stress could help elucidate molecular mechanisms of toxicity. Later, a more reliable developmental toxicity model based on the hESC system was developed [135,136]. By performing metabolomic profiling, they identified biomarkers of developmental toxicity and demonstrated that their model could correctly predict the teratogenicity of 7 out of 8 drugs (88%) and of 9 out of 11 environmental toxicants (83%). Further examples of metabolomics application in hESC-based toxicological assays [137,138] are described in Table 1. Another interesting study, published in 2008 [139], utilized hESC-differentiated into fibroblastic progenies for in vitro toxicology screening and showed that hESC-derived fibroblasts exhibited a more sensitive dose–response to mitomycin C compared to human lung fibroblast L929 cells. The same year, research from the ECVAM followed up on the successfully validated mouse EST and established an equivalent assay based on hESCs and human fibroblasts. By assessing cytotoxicity (IC_{50} values) and lineage marker expression (RT–qPCR) during EB differentiation, they demonstrated a predictability similar to that of the mouse EST [140]. The ability of hESCs to differentiate via EB formation was also shown by other research [141–144] (Table 1).

In several studies, hESCs were investigated with the aim to specifically assess toxic effects on neural differentiation. In 2009, a large-scale five-year European Union project titled Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNAT) was launched to establish novel prenatal developmental toxicity tests based on hESCs (http://www.esnats.eu). Within this project, in 2011, a protocol was designed based on the generation of neural rosettes from hESCs, with the emphasis on early neural development, for the detection of neural toxicity [145]. In 2013, several groups within the ESNAT project employed transcriptomics for the analysis of developmental neurotoxicity (DNT) and reproductive toxicity (RT) using hESCs; as a result, they were able to classify human DNT/RT toxicants on the basis of hESC transcriptome profiles [146]. Several other studies mentioned in Table 1 [147–152] also used hESCs to assess environmental neurotoxicity, and one of them employed epigenetic profiling to dissect the underlying molecular mechanisms [149]. In 2015, the group of James Thomson who first generated hESCs also published a toxicological study in which cellular interactions within the developing brain were modeled by culturing hESC-derived neural progenitor cells, endothelial cells, MSCs and microglia/macrophage precursors on chemically defined polyethylene glycol hydrogels in serum-free medium. As a result, they constructed, using linear support vector machines, a reliable predictive model based on RNA-Seq data acquired from 240 neural constructs treated with 34 toxic and 26 nontoxic chemicals [153]. In 2016, at the time of writing this review, four additional reports on using hPSCs for neurotoxicity testing were published [154–157] (Table 1).

Another popular direction in the development of human stem cell toxicology was the design of assays on cardiotoxicity [158–166] and hepatotoxicity [167–172] (Table 1). Thus, in 2013, it was reported that the approach based on hESC- and hiPSC-derived hepatocytes could predict toxicity in a way comparable to that of standard hepatotoxicity assays, representing a major advancement in the field [168]. Two other studies addressed toxic effects of bisphenol A (BPA), a chemical commonly used to harden plastics, on the reproductive systems using hESCs differentiated into mammary epithelial cells in 3D conditions [173] and human prostate organoids [174]. Another study published in 2014 used three-day monolayers of mesendoderm-differentiated hESCs to assess teratogenicity by the reduction in nuclear translocation of the transcription factor SOX17; the method named the hPSC test was validated by high-throughput screening of 71 drug-like compounds, 15 environmental toxicants and 300 kinase inhibitors [175].
SSCs in toxicology

The application of SSCs in toxicology can be first traced to 1982, when Robert M. Pratt and his colleagues prescreened environmental teratogens using cultured mesenchymal cells from the human embryonic palate [176]. After that, numerous studies were conducted on SSC-based toxicity assays; here, we will focus on those studies that used MSCs and NSCs. However, we recognize that there are plenty of toxicological studies with other SSCs, such as HSCs and so on. All these works are important in stem cell toxicology. Nevertheless, due to space constraints, we cannot address them thoroughly in this review.

In 2011, Cao and coworkers were the first to evaluate the applicability of hMSCs for \textit{in vitro} cytotoxicity testing to correctly assess LD$_{50}$ values and predict the hazard category of the tested chemicals according to the globally harmonized system of classification (GHS) [177]. Their findings indicated that hMSCs provided a more accurate modeling of \textit{in vivo} conditions compared to the validated 3T3 cell test and Normal Human Keratinocyte/Neutral Red Uptake methods. In 2012, Akhavan \textit{et al.} [178] demonstrated that low concentrations (0.1 mg/ml) of reduced graphene oxide nanoplatelets exerted genotoxic effects on hMSCs due to DNA fragmentation and chromosomal aberrations. Moreover, in 2015, Strong and colleagues exposed hMSCs to the endocrine-disrupting chemical dichlorodiphenyltrichloroethane (DDT) and revealed profound alterations in self-renewal, proliferation, differentiation (adipogenesis and osteogenesis) and gene expression, which could partially explain homeostatic imbalance and increased cancer incidence among the affected individuals [179].

In 2006, Tamm with coworkers demonstrated that neural stem cell line C17.2 and primary embryonic cortical stem cells were highly sensitive to methylmercury (MeHg) as evidenced by the effects on survival and differentiation, offering new perspectives for evaluating the biological consequences of MeHg exposure at low levels [180]. In 2009, Buzanska \textit{et al.} [181] established a human neural stem cell line from umbilical cord blood (HUCBNSC) and used it to test developmental neurotoxicity by analysing such parameters as cell proliferation, apoptosis, and neuronal and glial differentiation.

MAJOR FEATURES OF STEM CELL TOXICOLOGY

The studies described above mentioned many applications of stem cells in toxicology. However, in none of them was the term ‘stem cell toxicology’ used. Our group was the first to clearly define, in 2015, ‘stem cell toxicology’ as a new branch of toxicology [7]. In this section, we will then describe the major features of stem cell toxicology in more detail.

Pluripotent stem cell toxicology

Pluripotent ESCs and iPSCs are capable of differentiating \textit{in vitro} into virtually all the cell types of the adult organism, including germ cells (Fig. 2). This property defines the very core of stem cell toxicology and explains why only stem cells offer such a great potential in toxicity testing compared not only to other cell types used \textit{in vitro}, but also to experimental animals. Even a simple cytotoxicity assay can be more informative if performed with stem cells because of their pluripotency, which provides not only higher sensitivity compared to somatic cells, but also enables the assessment of harmful developmental effects. PSCs can mimic the early stages of embryogenesis \textit{in vitro} by forming EBs which, under differentiating conditions, give rise to the three primary germ layers and cell lineages and, thus, can be used to evaluate embryotoxicity or teratogenicity of environmental pollutants (Fig. 3). This is particular relevant for testing deleterious effects on human embryogenesis that cannot be reliably investigated using other experimental models.

PSC-formed EBs spontaneously differentiate into multiple cell lineages at the same time, which limits the sensitivity and specificity of toxicological assays, especially when subtle toxic effects on a specific tissue are masked by stronger responses of another tissue. To overcome these problems, protocols have been developed to promote preferential differentiation of EBs to a single germ layer and then to particular progenitor or somatic cells, which enables assessing tissues-specific toxic effects, such as neurotoxicity, cardiotoxicity, hepatotoxicity, etc. (Fig. 2). Many PSC differentiation protocols are based on monolayer conditions that facilitate the performance of developmental toxicity tests and provide faster data collection. Therefore, although differentiation assays conducted in cell monolayers do not reproduce \textit{in vivo} 3D conditions, they are very useful for quick preliminary screening of toxic compounds.

In addition to the analysis of developmental toxicity, stem cell toxicology provides functional assessment of PSC-differentiated cells in such cases when pollutants do not influence embryonic development and lineage commitment, but rather affect subsequent functional performance of differentiated tissues. In this respect, stem cell toxicology presents an advantage of analysing specific cell types
Figure 2. Features of stem cell toxicology. Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) formed at the blastocyst stage of mammalian embryonic development. ESCs can be cultured in vitro and induced to differentiate first into the primary germ layers (mesoderm, endoderm and ectoderm) and then into progenitor cells and virtually all cell lineages and tissues of the adult organism. ESCs are also potentially able to differentiate in vitro into primordial germ cells (PGCs) and then into gametes (eggs and sperm). Somatic (adult/fetal) stem cells (SSCs) are multipotent cells present in different tissues of the body, which can differentiate in vitro into every cell type of the corresponding tissue. Toxicity assays can be performed at any stage during embryonic and somatic stem cell differentiation to assess developmental and functional toxicity in different tissues: neuronal, cardiac, hepatic, etc.

FUTURE PERSPECTIVES OF STEM CELL TOXICOLOGY

Toxicology is a field that takes advantage of the development and technical innovations in many disciplines, including biology, chemistry, bioinformatics and engineering [185]. This is particularly true for stem cell toxicology because it has just recently emerged. In fact, for instance, stem cell toxicology relies on the constantly developing field of stem cell biology, especially regarding the further investigations of the molecular mechanisms underlying differentiation potential of stem cells to any cell type of the body, including neuronal cells, cardiomyocytes, adipocytes, osteoblasts and hepatocytes. Although without resorting to complicated and often invasive procedures of isolation from live tissues, which could be even practically impossible. Further applications of PSC toxicology include RT assessments because PSCs can generate in vitro PGCs as well as potentially terminally differentiated gametes and, thus, can be used to assess the impact of environmental contaminants on reproduction-related parameters (Fig. 2).

Somatic stem cell toxicology

Contrary to ESCs, SSCs cannot be used for teratogenic and embryotoxicity assays. However, SSCs can still self-renew and differentiate into somatic cells during infant and adolescent periods and, therefore, can be applied to the evaluation of environmental effects on the post-natal development into the adult organism. In adult tissues, SSCs are retained in a quiescent state until triggered to regenerate damaged cells/tissues through cycles of self-renewal and differentiation [182,183]. With physiological aging, tissue homeostasis is progressively disrupted and the ability of SSCs to repair injured terminally differentiated cells gradually declines [183]. Therefore, environmental pollutants can induce irreversible tissue damage that cannot be adequately repaired by SSC differentiation or directly target SSCs, causing their exhaustion and eventual premature aging and/or pathological conditions, including cancer [184]. Thus, primary tissue-derived or PSC-derived SSCs can be used for the in vitro assessment of harmful environmental effects on the development of infants and adolescents into adults (Fig. 4). SSC-based toxicology can also include the assays specifically designed to determine toxic effects of pollutants during tissue regeneration after injury or degenerative diseases, and assess the effects on stem cell exhaustion and aging (Fig. 4).
Figure 4. Special features of somatic stem cell toxicology. Multipotent somatic stem cells (SSCs) present in many adult tissues are generally quiescent in vivo but, under certain conditions, such as during tissue regeneration after injury, can start proliferating to maintain the number of stem cells and differentiating to replace damaged cells in the tissue. Pollutants accumulating in the body can negatively affect these processes, causing stem cell aging and exhaustion, which ultimately weaken tissue repair and may cause degenerative diseases. SSC-based toxicology enables testing the effects of toxicants on SSC self-renewal and differentiation in vitro.

A number of organ toxicity assays have been developed based on PSC differentiation protocols, further research should concentrate not only on the design of additional differentiation methods, but also on the improvement of established procedures to make them more reproducible, efficient, and less costly and labor-intensive. Those differentiation protocols should also include information about which stages we can freeze cells, to make stocks and subsequently thaw them. This would save time if toxicity assays need to be performed only at late stages of differentiation and there is no requirement to start from undifferentiated stem cells. An urgent need is the development of human stem cell toxicology, which is less advanced compared to the murine system, for several reasons. First, in vitro cultures of hESCs were established much later than those of mPSCs and, because of species-specific differences, the knowledge about the molecular mechanisms functioning in mESCs could not be directly applied to hESCs. Therefore, up to now, there are fewer standardized differentiation procedures available for hESCs than for mESCs. Second, it is more technically challenging to culture and differentiate hESCs, which is also a factor delaying the development of human stem cell toxicology.

A significant aspect of stem cell toxicology is the analysis of RT. ESCs can be first differentiated to PGCs and then to eggs and sperm, thus enabling the in vitro evaluation of toxic effects on the final commitment and function of germ cells. However, the development of differentiation procedures that would yield functional germ cells has been challenging and, up to now, a reliable protocol for the generation of mature eggs and sperm from ESCs in vitro has not been established, although mouse and human ESCs have been successfully differentiated into primordial germ-like cells in vitro. Consequently, stem cell-based RT assays can assess the effects on the development of PGCs, but not on that of functional gametes. Nevertheless, recent technological advancements indicate that the in vitro production of terminally differentiated eggs and sperm from stem cells is a perspective of the nearest future.

In addition, iPSCs could also be used in toxicology. This would avoid the ethical issues associated with human ESCs; however, iPSCs are not identical to ESCs in that they retain some epigenetic memory of the cell type of origin. Nevertheless, they may suffice for stem cell toxicology applications, provided more than one iPSC line, preferably originating from different somatic cell types and reprogrammed with different techniques, is used. Another advantage of iPSCs compared to ESCs is that a variety of iPSC lines representing different ethnic, clinical and environmental backgrounds are available, indicating a possibility for diversified toxicity assessment depending on the genetic and/or pathological conditions of the population. To accomplish this task, iPSC banks that would provide a panel of standardized iPSC lines corresponding to a specific toxicology assay or risk assessment need to be established.

For comprehensive representation of different in vivo microenvironments, 3D cell cultures are more suitable than monolayers, which is particularly relevant when differentiation of stem cells into tissues and/or organs is attempted. Current stem cell differentiation procedures are generally performed in monolayer settings, except when EBs are generated. Even in EB-based protocols, 3D conditions are only used in the early stages of differentiation when EBs are formed. The application of 3D cultures is especially beneficial in stem cell toxicology, as mentioned above, as it facilitates obtaining reliable data on developmental and functional effects of pollutants on early embryoogenesis. However, the application of 3D stem cell-derived culture systems, although very promising, is just at the initial stage [186–188]. Successful reconstruction of organoids/ organs ex vivo depends on careful selection of supporting matrices, either synthetic or derived from decellularized organs, and requires reproducibility in concerted cell assembly on scaffolds, which can only be achieved with automated bio-printing systems. Advances in 3D scaffold design and manufacturing, as well as bio-printing techniques, are paramount for the replacement of in vivo assays in stem cell toxicology. An ideal
situation would be simultaneous reconstruction of several human organs to mimic the whole organism during toxicity tests. This concept has been brought to life in the so-called ‘organ-on-a-chip’ technology that uses different miniature organs put together on the same chip and connected by an artificial vascular system providing nutrients and conducting metabolic signals (reviewed in [189]). This technology would allow more comprehensive toxicity evaluation by examining adverse effects on several organs at the same time. Nevertheless, microorgans described above cannot reliably represent real-sized organic systems; therefore, future efforts should be invested into organ-assembling technology based on the differentiation and maturation of whole-organ scaffolds.

An important technological revolution in toxicology was promoted by the completion of the Human Genome Project and recent advances in genome sequencing, transcriptomics, proteomics, metabolomics and global epigenetics, which allowed accumulation of a tremendous amount of relevant biological information carrying enormous potential for toxicity analyses, predictions and risk assessments. Genetic variations could explain why certain individuals and/or populations are more sensitive to a particular pollutant, while global transcriptomics, epigenetic signatures, protein expression analysis and metabolic profiling would help to identify toxicity mechanisms, screen potential toxicants and monitor human exposure to pollutants [34].

The technological revolution described above was accompanied by the development of cutting-edge informatics tools for comprehensive data analyses. Stem cell toxicology would greatly benefit from the accumulation and analyses of the ‘omics’ data, which should further improve our understanding of global molecular changes in stem cell self-renewal and differentiation elicited by drugs and/or environmental pollutants, especially in the human system. Recent technological developments include chemoproteomics [190,191] and chemical ChIP-SEQ [192] aimed at dissecting the interactions of host genes and proteins with small-molecular-weight compounds such as drugs, metabolites and environmental pollutants. The targeted host molecules would be then identified by mass spectrometry and chromatin immunoprecipitation followed by deep sequencing, providing the data on the molecular mechanisms affected by toxic substances and predicting, with a high degree of accuracy, potential toxicity of untested chemicals for further analyses. Moreover, once the interacting proteins and/or genes targeted by potential toxicants are identified, the information can be complemented using genome-editing techniques such as CRISPR/CAS9-mediated gene knock-out [193] to confirm the biological significance of the gene or signal transduction pathway for cell survival or sensitivity to a particular toxicant. The CRISPR/CAS9 system has been proved particularly beneficial for the evaluation of genome editing in hESCs that are not suitable for genetic analysis by other techniques [194].

CONCLUSIONS

Stem cell toxicology may become the gold standard in toxicity testing if fully validated physiologically relevant tests that are reproducible, relatively inexpensive, and not time- and labor-consuming are implemented on a global scale. In this case, stem cell toxicology would eliminate the need for whole-organism tests not allowed in humans, while providing the platform to evaluate a wide variety of untested chemicals to which we are continuously exposed. In addition, stem cells can differentiate into 3D organoid structures more closely recreating the in vivo microenvironments. Also, multiple SSCs and hiPSCs have been established to match clinical individual differences, with great potential applications in personalized toxicology. Consequently, stem cell toxicology would allow shifting from experimental animal systems that may not generate results fully applicable to human health because of species-specific differences, and may solve the problem of traditional in vitro toxicology that cannot reliably evaluate potential effects on the whole organism, bringing us a step closer to an ideal analytical system implemented exclusively in vitro.

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