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Embryonic Stem Cells in Toxicological Studies

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1. Introduction

1.1 Toxicity assays

For most of their lives, humans are exposed to a variety of chemicals from different sources. The characterization of chemicals' toxicity implies the necessity to have available reliable testing methods to assess their capability to produce adverse effects on living organisms. Thus, it is necessary to acquire a battery of relevant internationally agreed methods to be employed by governments, industry and independent laboratories to assess the safety of chemicals. Presently, a set of validated tests is widely used to analyze the toxicity of chemicals and pharmaceutical drugs and to ensure their safety, and is recognized for this purpose (OECD, 2008; Stummann et al., 2009).

OECD Guidelines require *in vivo* studies to comply with the human health risk assessment process before registering and authorizing the use of chemicals. Toxicity guidelines characterize the adverse effects against the target organism that are attributable to exposure to the tested chemical with two main purposes in mind: the hazard identification and risk assessment deriving from exposure to the assessed chemical (Estevan et al., 2011).

Currently, the OECD has at least one *in vivo* validated guideline for testing the following endpoints: skin sensitization, skin and eye irritation and corrosion, mutagenicity, acute toxicity, target organ systemic toxicity (repeated daily dose for 28 days, 90 days, and for more than 12 months), carcinogenicity and toxicity to reproduction (screening, teratogenicity and two generations). All these OECD Guidelines have to be usually applied for a safe and consistent risk assessment, even when the chemical's hazard is not related to the tested target system. In this way, some of the most relevant tests are reproduction/developmental studies because the endpoints assessed in these protocols and the non-observed-adverse-effect levels (NOAELs) obtained are typically taken into account not only for reproductive toxicity testing, but also for the risk assessment of general systemic effects. For example, the NOAELs of systemic maternal toxicity in the teratogenicity study are frequently considered for risk characterization in short-term exposure scenarios.

Guidelines for testing toxicity to reproduction are also highly relevant because the effects of chemicals or drugs on germ cells or early embryos may lead to infertility or impaired development of pre-implantation embryos, and might result in embryotoxic or teratogenic

effects in the progeny (Spielmann, 2005; Krtolica et al., 2009). This is the reason why toxicity to reproduction is required in all government regulations for the authorization and regulation of most chemicals. For example, extensive screening and multi-generation studies (including teratogenicity, fertility and development) are required for registering agrochemicals.

1.2 Necessity of cellular assays in toxicology

It is obvious that the application of an *in vivo* test is very expensive, time-consuming and involves lots of ethical concerns relating to the use of animals for experimentation purposes. Nevertheless, we now go on to illustrate these concerns with some figures. Höfer and coworkers (2004) estimated the number of animals needed to apply each *in vivo* test. It can be easily concluded that the number of animals needed to test a single chemical with all the above-mentioned available tests is around 4,710. Fleischer (2007) also estimated that the cost of this set of tests would be around € 1,800,000.

Apart from this, the relevance of many *in vivo* toxicological assays is questionable as regards human health because there are times when the animal model is not representative because of inter-species differences in pharmacokinetics and toxicokinetics (Wobus and Löser, 2011). Such inter-species differences have been detected, for example, in the cases of 13-cis retinoic acid (Hendrickx, 1998) and thalidomide (Nau, 1993; Tzimas et al., 1994). Specifically, the dramatic consequences of the teratogenic effects of thalidomide on humans urgently lead to the need for new, optimized human-specific test systems.

Therefore due to the aforementioned economical, logistical and ethical concerns, it is obvious that the availability of fast, safe, reliable and high throughput alternatives to animal experimentation methods would be extremely welcomed by industry, researchers and regulatory bodies. Cellular methods fit all these requirements and, due to the availability of cellular cultures for all tissues, these methods are good candidates to be employed for testing toxicity in all the possible target organ systems (liver, skin, heart, reproduction, etc.).

1.3 Roles of stem cells in toxicological studies

Stem cells may play several relevant roles in cellular assays for testing toxicity, and these roles can be played in either embryotoxicity or in organogenesis for testing toxicity in adult tissues (Figure 1). Indeed, embryonic stem cells (ESC) or reprogrammed-induced pluripotent stem cells (iPSC) can be employed through forced directed differentiation protocols as a source of adult human cells cultures (hepatocytes, neurons, and others) that would be further used for the *in vitro* testing of respective target organ toxicity (hepatotoxicity, neurotoxicity, etc.). This application is useful because it avoids the sacrifice of animals for experimentation purposes, allows the use of human cells which usually have a limited availability and avoids the need for the above-mentioned interspecies extrapolation. This application of stem cells related with organogenesis will be commented and explained in more detail in another section of this chapter. Nonetheless, it is relevant to remark that the recent iPSC applications in toxicology and drug research provide new alternatives to the standard routine tests performed by industry and offer new chemical safety assessment strategies (Laustriat et al., 2010; Trosko and Chang, 2010).

The other role of stem cells in toxicological studies relates to embryotoxicity. Indeed, the testing methods relating to these applications take advantage of ESCs to differentiate in adult tissues and help the studies into how these differentiation processes are altered by exposure to

the tested chemical (Figure 1). The most important and relevant factor for embryotoxicity testing is that ESCs mimic early *in vivo* embryonic development processes and show tissue-specific expression profiles (Wobus and Löser, 2011). In combination with three-dimensional cultures supported by extracellular matrix proteins, stem cell-based systems can mimic the microenvironment of the *in vivo* niche, which is especially relevant in humans for testing embryotoxicity (Trosko and Chang, 2010) and for organogenesis (Miki et al., 2011; Lock and Tzanakakis., 2009) and further assay of toxicity on the generated cell cultures.

Developmental toxicity assays with stem cells occupy a prominent position within the available battery of cellular assays since some studies have demonstrated that stem cells can be used to understand the processes underlying organogenesis, as shown in the case of the heart (Miller et al., 2008). Therefore, the study of the interferences in stem cell differentiation caused by chemicals can be used to detect potential developmental toxicants. An additional advantage of these models is that genetic molecular approaches enable not only for an exhaustive analysis of which genes are affected by each substance, but also for the subsequent research into the mechanisms of action underlying the teratogenic or embryotoxic effects caused by the assessed chemical (Pamies et al., 2011).

Details of ESCs-based embryotoxicity testing methods will be provided in the following sections of this chapter, but can be divided into four categories (Table 1): the validated Embryonic Stem cell Test (EST); variations of the EST using molecular endpoints as indicators of altered differentiation; the ACDC method, and human cell-based embryotoxicity methods.

Methods	References
EST	ESAC, 2002; Glenschow et al., 2000, 2002 and 2004
Variations of validated EST with molecular end-points	Bremer et al., 2001; Buesen et al., 2009; Osman et al., 2010; Romero-Lucena., 2010; Seiler et al., 2004; Stummann et al., 2007; van Dartel et al., 2009; 2010a, 2010b, 2011a, 2011b
ACDC	Barrier et al., 2010; Jeffay et al., 2010
Human cell-based embryotoxicity	Addler et al., 2008a, 2008b

Table 1. Embryotoxicity testing methods employing embryonic stem cells

The cellular methods for testing embryotoxicity deserve special mention because, despite the validated *in vivo* OECD guidelines for testing toxicity to reproduction covering fertility, teratogenicity and development, there are no specific guidelines available for exclusively testing embryotoxicity (Pamies et al., 2011). Indeed, this is a major gap because a guideline for this purpose would allow the detection of developmental toxins in early development stages without awaiting teratogenicity.

There are other well-established alternative methods for testing embryotoxicity with endpoints relating to differentiation, such as the frog embryo teratogenesis assay on xenopus (Bantle et al., 1990), the chicken embryo toxicity screening test on chicken embryos (Jelinek et al., 1985), the micromass assay using mouse embryonic mesenchymal cells (Flint, 1993; Spielmann, 2005), and the mammalian whole embryo culture assay using mouse (Sadler et al., 1982) or rat (Schmidt, 1985; Cockroft and Steele, 1987) embryos. Nevertheless, all these methods are not based on stem cells and are not of interest in this book.

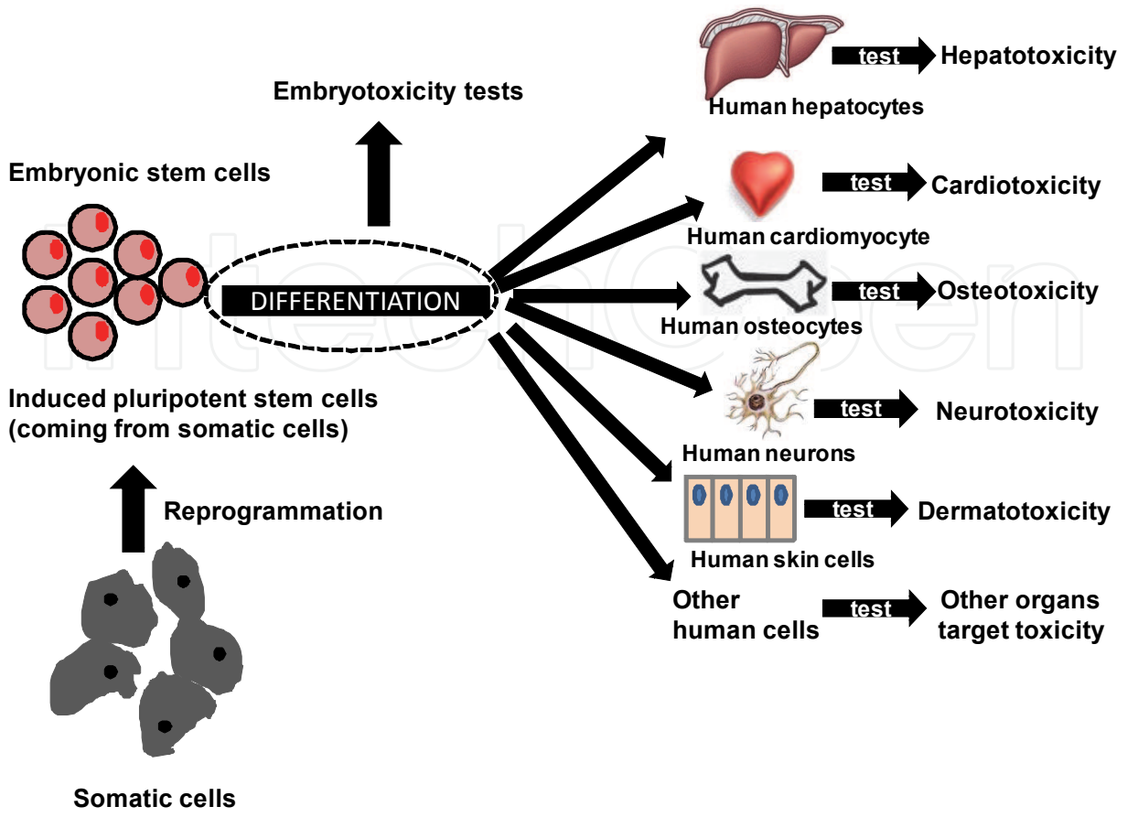


Fig. 1. Role of stem cells in toxicity testing.

1.4 Earlier toxicity assays with stem cells

Some of the earliest attempts for testing cytotoxicity, embryotoxicity or teratogenicity screening using mammalian cells date back to between two and three decades (Kimmel et al., 1982; Schwetz et al., 1991). The most important drawback of all these assays is that they rely on somatic cells, which do not entirely reflect the reaction of embryonic cells to toxic compounds (Wobus and Löser, 2011).

Embryotoxicity tests include the analysis of the toxic effects of chemicals on embryonic cells in differentiation, whereas cytotoxicity assays measure the chemical-induced alterations of metabolic pathways or the structural integrity of cells, which may or may not be directly related to cell differentiation (Wobus and Löser, 2011). Both cytotoxicity and embryotoxicity assays have been applied with pluripotent stem cells. The determination of cytotoxic effects of chemicals based on the colorimetric MTT test for cellular growth and survival (Mosmann, 1983) was performed with murine ESCs (Laschinski et al., 1991), showing greater sensitivity to selected embryotoxic agents than mouse adult cells (fibroblasts).

In order to overcome the limitations of cytotoxicity assays to determine embryotoxic effects, the ESC-derived embryoid body (EB) model was introduced (Wobus et al., 1988; Wobus et al., 1991). The ESCs differentiated in EBs for 5-7 days develop *in vitro* into the progenitor cells of all three primary lineages of ectoderm, mesoderm and endoderm (Wobus and Boheler, 2005). The fundamentals of the EB model were that early developmental processes simulated by EB differentiation would be affected by exposure to toxic chemicals and, consequently, specific differentiation processes would be inhibited or accelerated.

One of the first experiences to illustrate how embryonic cells could be used for testing embryotoxicity was the case of retinoic acid. There is evidence that retinoic acid *in vivo* acts as morphogen by forming a concentration-dependent gradient along embryonic axes, thus affecting the antero-posterior patterning of the body axis and limbs (Kessel and Gruss, 1991). High concentrations of retinoic acid applied to mouse ESCs during early EB differentiation (days 0-2) revealed increased neural-specific transcript levels and significantly induced neuronal differentiation (Fraichard et al., 1995; Strubing et al., 1995), whereas retinoic acid application at lower concentrations applied between days 2 and 5 induced mesodermal, specifically skeletal muscle and partially cardiac differentiation (Wobus et al., 1994). This is in line with the developmental processes in the EB where the maximum expression levels of neural plate morphogenesis genes like *brachyury* and the expression of genes associated with dorsal mesoderm specification were observed at around day 3 of EB formation (Wobus et al., 1994; Dani et al., 1997). Cardiac and vascular smooth muscle cells both originated (at least partially) from the lateral plate mesoderm, and were induced by retinoic acid when applied on days 5-7 and 7-11, respectively (Drab et al., 1997). Obviously, the specific temporal response of the different cell types within the EB to retinoic acid correlated with the retinoic acid receptor gene expression (Rohwedel et al., 1999). These early studies provided the basis to subsequently establish the so-called EST method.

2. The embryonic stem cell method test (EST)

In the past, several groups have used murine ESC to establish an *in vitro* embryotoxicity assay. Laschinsky et al. (1991) compared cytotoxicity in both ESC and mouse fibroblasts to assess the embryotoxic potential of teratogenic agents. The data showed that ESC were more sensitive to toxic agents than adult cells. Newall and Beedles (1994) measured both the cytotoxicity and the colony-forming potential of ESC after 7 days of culture in the presence of teratogenic agents. In these assays, only a few embryotoxic agents could be correctly classified. This was probably due to the fact that only two endpoints were selected for the biostatistical assessment in the ESC assays, which seems insufficient. To overcome the limitations of the previously mentioned ESC tests, a third endpoint value, alterations in cell differentiation, was introduced and linked to the two former ones (cytotoxicity in embryonic and adult cells). These three endpoints allow the test compounds to be classified into three *in vivo* embryotoxicity categories.

2.1 Basis of the method

The fundamentals for the test are that the *in vitro* tests of basal cytotoxicity are sufficiently predictive for the rodent *in vivo* LD_{50} assay (Ekwall, 1999; Spielmann et al., 1999) and that ESCs show alterations in their *in vitro* differentiation pattern when exposed to embryotoxic chemicals during EB differentiation (Wobus et al., 1994). The EST method is based on the potential of D3 mouse ESC to differentiate into beating cardiomyocytes and in determining how this differentiation is altered by exposure to chemicals, together with the cytotoxic insults in these cells and in a mouse adult cells model.

Two permanent mouse cell lines are used: D3 embryonic stem cells to represent embryonic tissue and 3T3 fibroblasts to represent adult tissue. The test was developed when D3 mouse ESC were discovered to be able to form EBs in the absence of the cytokine leukemia inhibiting factor which, after 10 days of culture, spontaneously differentiated into cardiomyocytes.

The basis of the EST protocol is summarized in Figure 2. The test comprised the morphological analysis of beating clusters in differentiating EBs (seeded in single wells of multiwell plates) and the determination of those concentrations of the test substance at which cardiac differentiation was inhibited by 50% after 10 days of exposure (ID_{50}). The cytotoxic effects on D3 and 3T3 cells were estimated by determining those concentrations of the test substance at which proliferation was inhibited by 50% after 10 days of exposure, yielding IC_{50} D3 and IC_{50} 3T3, respectively. On the basis of these 3 endpoints, a biostatistical prediction model formed by three different functions helped assign the tested chemical to one embryotoxicity category (strong, weak or non embryotoxic) (Genschow et al., 2000; Genschow et al., 2002; Spielmann et al., 2001) (Figure 1). This method is currently validated by the European Centre for Validation of Alternative Methods (ECVAM) as a screening assay for potentially embryotoxic chemicals (ESAC, 2002).

2.2 EST performance

The testing of the 20 test chemicals employed in the EST validation study provided 78% accuracy (correct classifications related to the *in vivo* data) (Genschow et al., 2004). The highest precisions were detected for weak and strong embryotoxic chemicals, which were correctly detected in 83% and 81% of cases, respectively (Genschow et al., 2000). The poorest precision was recorded for the detection of non embryotoxic compounds (70%). Finally, the predictability for strongly embryotoxic chemicals was 100%, while that for non- and weakly embryotoxic compounds was similar (70 and 72%, respectively), which is considered sufficiently high.

2.3 Limitations of EST

EST has several limitations. Since D3 mouse ESCs are differentiated as EBs in ''hanging drops'', this test is laborious and time-consuming. EST does not consider molecular endpoints to detect cardiac differentiation (formation of beating cardiomyocytes) (Schmidt et al., 2001; Piersma, 2004), and it is an assessment based exclusively on histological considerations which introduces a high degree of variability. The inclusion of differentiation markers of the ectoderm, mesoderm and endoderm lineages and the analysis of the tissue-specific gene expression have been recommended as additional endpoints for the purpose of improving EST's predictability on the basis of better differentiation assessment.

As with most *in vitro* methods, the EST is limited by lack of metabolic competence. This lack has been recognized as another potential factor to affect the EST's experimental outcome and the correct classification of tested substances (Verwei et al., 2006). Experiments which attempted to add the S9 mix, the mix usually applied as a source of exogenous metabolic activation enzymes, failed because it is toxic for D3 mouse ESC. Therefore, the consortium of the ReProTect project (Integrated Project of the European Union funded within the 6th Framework Programme, including 35 partners focused on alternatives to animal tests for reproductive toxicity with the scope of pre-validating and validating the brightest ones) recommended other test systems to analyze the potential toxicity of those compounds that require metabolic activation (Marx-Stoelting et al., 2009). Other relevant limitation of the EST is that this is also incapable of detecting teratogenic chemicals with mechanisms acting beyond the initial embryo differentiation steps.

This method needs an intermediate level of technical difficulty because it requires two endpoints and the cultures of two different cell lines. In addition, it does not yield

information about the morphological alterations caused by the teratogen as other methods do (e.g., whole embryo culture) (Pamies et al., 2011).

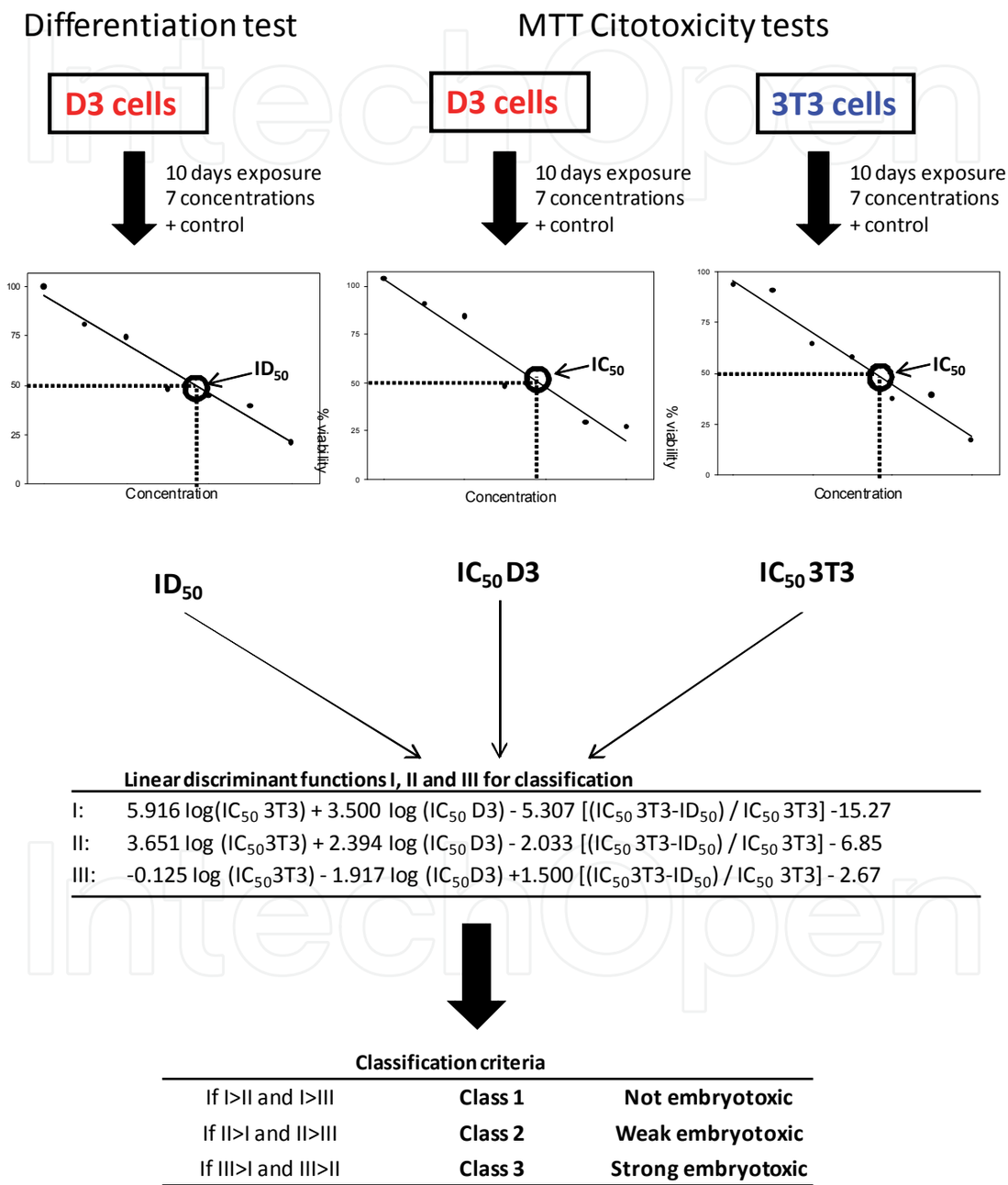


Fig. 2. Embryonic Stem cell test (EST) method.

3. Proposed variations of the EST based on molecular endpoints

As stated in the previous section, the EST has several limitations, one of which is the most relevant: lack of molecular endpoints for a reliable assessment of the degree of differentiation achieved by EBs. Indeed, ECVAM has recommended the inclusion of molecular endpoints in the EST with a view to improving its performance (Spielman et al., 2006). A great deal of work has been done to follow this recommendation in an attempt to introduce gene expression as biomarkers of differentiation. A second line of work has also been introduced, that of reducing the technical complexity of the EST using a single cell line.

3.1 Use of biomarkers of differentiations as endpoints

Previous studies about lineage-dependent effects of retinoic acid on ESC differentiation (Wobus et al., 1994) and the application of reporter gene constructs controlled by cardiac-specific promoters (Kolossov et al., 1998) have demonstrated that an ESC differentiation model is suitable to be analyzed at a cellular level. Genes coding for tissue-specific proteins are expressed in the course of ESC differentiation in a pattern which closely resembles the time pattern observed during mouse embryogenesis (Rohwedel et al., 2001). For example, cardiac-specific transcription factor Nkx2.5, expressed in cardiac precursor cells during embryogenesis *in vivo,* is the first cardiac-specific gene expressed during EB development in parallel to the α1 subunit of the L-type Ca^{2+} channel (Fässler et al., 1996), followed by the expression of α- and β-myosin heavy chain isoforms.

The recent progress made in improving the EST has underlined previous demands to implement new embryotoxicity testing strategies using ESCs by analyzing tissue-specific genes via the reporter gene expression, automated high-throughput screening for changes in gene and protein expression patterns using microchip arrays for transcriptome and proteome analyses, and the application of human ESCs (Rohwedel et al., 2001).

Reporter gene assays for developmental toxicity, specifically for cardiac toxicity (Bremer et al., 2001), have been included in the EST. Cardiac markers α-myosin heavy chain and α-actin (both quantified by fluorescence-activated cell sorting (FACS)) have been successfully employed instead of the microscopic observation of beating EBs to determine cardiac differentiation after exposing D3 EBs to model embryotoxic test substances (Seiler et al., 2004; Seiler et al., 2006). The FACS-based EST has been successfully adopted for assessing developmental toxicity (Buesen et al., 2009). While the molecular FACS-EST shows the same sensitivity as the validated EST for the classification of chemicals, test duration is reduced and almost identical ID_{50} values were obtained for ten representative compounds of the three classes, indicating that FACS analyses values can serve as a new EST toxicological endpoint.

Other potential biomarker genes of embryotoxicity that may be used in early development stages are *Pnpla6*, *a-fetoprotein, nestin and Vgfa*. All these genes displayed statistically significant reductions in their respective expressions after 5 days of exposure to a non cytotoxic dose of 50 ng/ml of embryotoxic 5-fluorouracil (Table 2) (Romero, 2010). These marker genes also offer an advantage over the conventional EST method as they are recordable after 5 days of exposure instead of the 10 days of differentiation needed for obtaining a beating cardiomyocytes. Alpha-fetoprotein has also been independently reported as a very sensitive biomarker of exposure to 5-fluorouracil (Pamies et al., 2010).

Gene	%
Pnpla6	80±4
AFP	26±7
Nes	54±11
Vgfa	46±17

Table 2. Expression of several marker genes after 5 days exposure of D3 mouse embryonic stem cells in differentiation to 50 ng/ml embryotoxic 5-fluorouracil. The gene expression was indicated as a percentage as regards a differentiated control under the same conditions, but in the absence of 5-fluorouracil. In all cases, differences in expression as regards the control were statistically significant for at least $p<0.05$. (Data taken from Romero, 2010).

Inclusion of additional differentiation endpoints, specifically of the neural lineage, has been requested by researchers and authorities (Spielmann et al., 2006). The purpose of using such systems is to correctly classify the substances that were not identified as embryotoxic in the conventional EST. Inclusion of parameters for neuronal differentiation into the EST would allow, for example, classification in accordance with the *in vivo* data of methylmercury as a highly embryotoxic, whereas the conventional EST failed to correctly classify this compound (Stummann et al., 2007). In order to increase the number of molecular markers as endpoints of embryotoxicity testing, real-time Taqman RT-PCR analyses have been adopted for the EST in pilot studies (zur Nieden et al., 2004).

Peters and co-workers determined the relative embryotoxic potential by using a modified EST screening system (Peters et al., 2008). In this study, 12 compounds were investigated in a modified EST performed in 96-well plates. Test substances were applied on day 3 of culture for 10 days, and the assay did not involve the preparation of EBs. This newly revised high throughput EST allowed the analysis of a larger number of substances, less manual work, yet it yielded data comparable to those obtained with the conventional EST.

Present efforts to improve the EST now address the application of additional lineage-specific markers to define additional toxicological endpoints. Other than cardiac markers, markers of neuronal, bone and cartilage development are included. However, this will prolong the test duration from 7-10 to 30, or even 32 days (Marx-Stoelting et al., 2009). In the future, novel molecular endpoints and reporter-based systems will have to be included in the EST (Rohwedel et al., 2001; Marx-Stoelting et al., 2009; Spielmann, 2009). Such improvements will also be required to establish EST-like tests with human ESCs.

3.2 Use of transcriptomics and proteomics as endpoints

The identification of embryotoxic compounds on the basis of analyzing the alterations they cause in the expression of genes and proteins relating to the differentiation of embryonic stem cells has been proved possible (van Dartel et al., 2009; 2010a; 2010b; 2011a; 2011b; and Oshman et al., 2010). By following this methodology in a procedure that includes a 4-day assay (three days for embryonic body formation and one additional day to expose cells to the assessed chemical), the set of genes called "van_Dartel_heartdiff_24h" was able to correctly predict the embryotoxicity of 83% of the assessed chemicals (ten correct predictions of a total of 12 assessed chemicals, where two non embryotoxic chemicals were wrongly

classified as embryotoxic) (van Dartel et al., 2011a). In the same study, the group of genes called "EST biomarker genes" correctly predicted the embryotoxicity of eight of the 12 assessed chemicals (67%). Here the mistakes corresponded to one non embryotoxic and three embryotoxic chemicals, respectively (van Dartel et al., 2011a).
Protein markers can also be used to detect exposure to embryotoxic chemicals. Osman and coworkers found that the embryonic bodies formed from day 3 of differentiation of D3 cells, which were further exposed to embryotoxic monobutyl phthalate for 24 hours, expressed 33 proteins in a differential way, including cardiomyocytes biomarkers (whose expression was repressed if compared with controls) and chromatin modulator enzymes (Osman et al., 2010).

3.3 The ACDC method

The Adherent Cell Differentiation and Cytotoxicity (ACDC) assay is a test to establish a model system to assess the chemical effect using a single cell culture (instead of two as the EST does) in order to improve feasibilities for throughput assays (Barrier et al., 2010). The ACDC assay uses quantitative markers for both the differentiation degree and cell proliferation. In this assay, pluripotent J1 mouse ESCs are plated in a 96-multiwell plate and further cultured in differentiation medium for 9 days. Afterward, each well is assessed for the cell number and differentiation to cardiomyocytes (using quantitative in-cell Western analysis for myosin heavy chain protein normalized with the cell number). This method has already proved suitable for testing the effects of haloacetic acids and their major metabolites (Jeffay et al., 2010), but has still not been validated.

4. Human stem cells for screening cytotoxicity and embryotoxicity

Development of human ESC-based *in vitro* systems for testing the embryotoxicity of chemicals implies significant progress. The use of human ESCs in *in vitro* methods would enhance their predictability and avoid problems associated with the interpretation of the results obtained with animal-based assays in a human context (Wobus and Löser, 2011). For example, species-specific differences between mouse and human pre-implantation development, as in DNA methylation, DNA repair and the expression of those genes involved in drug metabolism, may interfere with the correct interpretation of animal studies regarding their significance to humans (Krtolica et al., 2009). The use of human ESC-based test systems could avoid the incorrect classification of chemicals due to inter-species variations and would, consequently, improve consumers' safety.
Adler and co-workers (2008a) provided proofs that human ESCs are a relevant *in vitro* model for developmental toxicity testing. They studied the cytotoxic effects of well-known embryotoxicants on human ESC, human ESC-derived progenitors and human foreskin fibroblasts. As observed in the respective murine cells, all-trans retinoic acid and 13-cis retinoic acid had a stronger cytotoxic effect on pluripotent stem cells than on fibroblasts, while the mesenchymal progenitors deriving from human ESC displayed the strongest sensitivity to both compounds. While all-trans retinoic acid and 13-cis retinoic acid substances revealed comparable cytotoxic effects on human ESC in this study, only all-trans retinoic acid was seen to be cytotoxic in mouse ESC in previous analyses. This suggests the necessity of developing human ESC-based assays to assess human-specific developmental toxicity.

In a second study, Adler and co-workers arranged the test system according to the EST, but used human instead of mouse ESC (Adler et al., 2008b). The cytotoxic effects of the two well-known developmental toxicants, 5-fluorouracil and all-trans retinoic acid, on human ESCs and human fibroblasts were similar to those previously observed in mice systems. However, testing for the potential cytotoxic effects on pluripotent stem cells does not suffice to detect the developmental toxicity of chemicals that affect developmental processes (Rohwedel et al., 2001). These authors proposed markers of undifferentiated cells such as *Oct4, hTert and Dusp6*, as well as markers of neural plate morphogenesis and early cardiogenesis, such as *Brachyury* and *GATA-4* for monitoring cardiac differentiation, thus demonstrating that these markers may have the potential to serve as endpoints for developmental toxicity studies using a humanized EST (Adler et al., 2008b).

Despite the progress made, numerous problems have to be solved before human ESC-based assays can be implemented into routine procedures for the developmental toxicity testing of drugs and chemicals. These problems include the establishment of reliable and reproducible differentiation procedures that can be performed in a high-throughput format. The predictability, the sensitivity and specificity of the respective test systems for a wider panel of chemicals are still to be shown. In addition, very few substances have been tested in the different approaches. Furthermore, human ESC-based systems may have the capacity to predict human-specific embryotoxic effects which cannot be measured with mouse cell systems due to species-specific differences. However, the superiority of human ESC-based systems over existing animal *in vitro* tests for developmental toxicity has still to be shown and, in this context, the use of different human ESC lines would prove advantageous.

5. Induced pluripotent stem cells in toxicological studies

iPSC play a prominent role in their use as a tool in toxicity tests in the embryotoxicity field or as a source of cellular tissues for testing toxicity in adult tissues (Figure 1). These two applications are particularly interesting when used with human cells because they will eliminate uncertainties deriving from the necessary inter-species interpolation when animal cells are used.

5.1 Applications in embryotoxicity studies

The successful reprogramming of adult somatic cells into a ESC-like state through genetic manipulation (Yu et al., 2007; Takahashi et al., 2007) offers a new opportunity for toxicology assay development as reprogrammed somatic cells possess very similar characteristics to those of human ESC. Nevertheless, some questions arise as to the use of iPSC for toxicological assays. One is whether the different epigenetics of reprogrammed iPSC (Surani et al., 2008; Han and Sidhu, 2008) might influence responses to a toxic insult. The other challenge is that the derivation of iPSC involves permanent genetic modifications to somatic cells due to the use of the viral transduction of recombinant DNA (Yu et al., 2007; Takahashi et al., 2007). Integration of recombinant DNA into the genome of iPSC lines might lead these cells or their differentiated progenies to behave differently from normal human cells, particularly when exposed to toxic challenge.

The application of human iPSC in embryotoxicity testing might be a promising tool, as well as ESC (Heng et al., 2009). However on the basis of the recently reported inconveniences

caused by reprogramming, future work has to show whether human iPSC will be applicable and offer advantages over human ESC in the developmental toxicology field (Gore et al., 2011).

In the future, it is possible that iPSC lines for toxicology screening may also be derived from reprogramming primary explanted hepatocytes and cardiomyocytes because such somatic lineages have wide-ranging applications in both toxicology and pharmacology screenings (Heng et al., 2009).

5.2 Applications in organogenesis and target organ toxicity testing

The ability to generate iPSC from somatic cells by the forced expression of reprogramming factors Oct3/4 and Sox2, along with either Klf4 or Nanog and Lin28, raises the possibility of generating patient-specific cell types of all lineages. Differentiated cell types produced from patient's iPS cells have many potential therapeutic applications, including their use in tissue replacement and gene therapy (Si-Tayeb et al., 2010). They also provide a platform for drug toxicity screenings. Generation of hepatocytes from iPSC is a particularly appealing goal because this parenchymal cell of the liver is associated with several congenital diseases (Burlina, 2004) and is the main site of xenobiotic control.

Mouse iPSC were generated from fibroblasts (Si-Tayeb et al., 2010), and embryos were then produced from this iPSC tetraploid complementation using transgenic mice which ubiquitously express enhanced green fluorescent protein (EGFP). When embryos were generated from mouse iPSC, from which EGFP is absent, all the embryos, including their livers, lacked the EGFP expression except in extra embryonic tissues. A thorough examination of iPSC-derived embryos and their livers revealed that they appeared to be identical to controls. These livers were examined, and the expression of those proteins that are characteristic of specific cell types was identified to reveal that, like the control fetal livers, the iPSC-derived livers contained hepatocytes (HNF4a positive), endothelial cells (GATA4 positive), sinusoidal cells (LYVE1 positive) and Kuppfer cells/macrophage (F4/80 positive).

In the cardiogenesis field, the latest studies have revealed that mouse iPSC can differentiate into cardiomyocytes by EBs formation or through the use of collagen IV-coated dishes and feeder cells (Narazaki et al., 2008; Mauritz et al., 2008). However, it is unknown whether iPSC can differentiate into cardiomyocytes without EBs formation and without using collagen IV-coated dishes or feeder cells. In addition, myocardial cell differentiation efficiency has still not been determined as being uniform or diverse in different iPS cell lines (Kaichi et al., 2010).

The transcription factors, such as GATA4 and Nkx2.5, expressed in the lateral mesoderm play important roles in the following heart developmental processes (Molkentin et al., 1997; Kasahara et al., 1998). The activities of cardiac transcription factors are regulated, in part, by histone acetyltransferases and histone deacetylases (HDACs). In the treatment of ES cells with trichostatin A (TSA), a specific HDAC inhibitor has been reported which not only induces the acetylation of both GATA4 and histones, but facilitates their differentiation into cardiomyocytes (Kawamura et al., 2005).

In the very near future, it is expected that other cellular types other than hepatocytes and cardiocytes can be successfully obtained, as shown in Figure 1. These cell cultures would also be suitable for use in toxicity studies as platforms for testing *in vitro* toxicity on adult cells.

6. Conclusions and final remarks

Stem cells are an excellent platform for testing embryotoxicity *in vitro* on the basis of these cells' capacity to mimic the embryo formation process. Nevertheless, much effort and hard work is still needed to find good suitable endpoints for monitoring different differentiation stages. There is also the urgent need to go ahead with the characterization of those models employing human cells, either ESC or those from reprogrammed iPSC.

Stem cells also represent a good opportunity to generate adult tissues to test toxicity in such tissues, which again is especially relevant in humans. Nevertheless, the development of good differentiation protocols to allow the generation of a variety of cell types apart from hepatocytes and cardiocytes is still needed.

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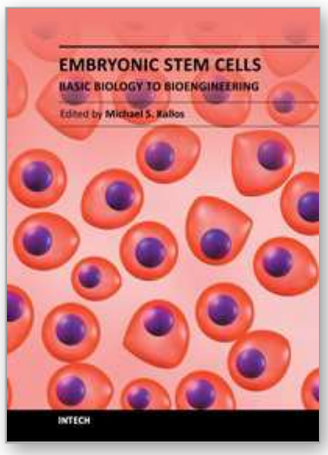
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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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