Mouse and Human Embryonic Stem Cells: Can They Improve Human Health by Preventing Disease?

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Abstract: Given the vast number of chemicals that are released into the environment each year, it is imperative that we develop new predictive models to identify toxicants before unavoidable exposure harms the health of humans and other organisms. *In vitro* models are especially attractive in predictive toxicology as they can greatly reduce assay costs and animal usage while identifying those chemicals that may require further *in vivo* evaluation. With the derivation of both mouse and human embryonic stem cells, new opportunities have developed that could revolutionize the field of predictive toxicology. Stem cells themselves can be used to model pre-implantation development, or they can be used during or after differentiation model the earliest stages of development. Because embryos and fetuses are usually more sensitive to environmental toxicants than adults, stem cells provide an unique tool for studying the prenatal phase in our life cycle. The embryonic stem cell test (EST), which has been validated for use with mouse ES (mESC), is an accurate predictor of embryotoxic compounds, particularly those that are highly embryotoxic. Human embryonic stem cells (hESCs), although not yet incorporated into a validated test, are a particularly attractive platform for toxicological testing as they can give us direct information on humans and avoid concerns about species variation in response. This review discusses toxicological studies and strategies that have been used with embryonic stem cells during the past five years and possible directions that could lead to improvements in the development of predictive assays in the future.

Keywords: *In vitro* assays, embryonic stem cells, predictive toxicology, toxicological testing, drug testing, embryonic stem cell test, cigarette smoke, harm reduction cigarettes.

INTRODUCTION

The United States releases over 1,500 new chemicals into the environment each year, and this number is much higher worldwide [1]. Most of these chemicals have not undergone adequate toxicological testing prior to their release, and their potential to adversely affect human health is largely unknown. In addition, drug companies screen numerous chemicals each year for toxic or undesired side effects prior to developing them into potential products. Our ability to monitor and identify toxic chemicals and drugs before they cause harm to humans and other species has been important for many years, and numerous strategies have been developed to deal with this problem. However, methods to evaluate the toxicity of both environmental chemicals and potential drugs need to be modernized and improved to deal with the growing need to protect human health yet make sophisticated progress toward development of new industrial products and medicines. In developing new assays, it is important to consider that the unborn are generally the most sensitive to chemical exposure, and leading toxicologists recently advocated that future evaluation of chemicals be done on prenatal stages of development so that the most vulnerable in our population will be protected from exposure [2].

Most chemical testing is currently done using laboratory animal models, usually mammals such as rats or mice or non-mammalian species such as the zebrafish [3]. The cost of performing animal studies is very high, requires a large number of animals for reliable data, often requires a significant amount of time to complete, and is based on non-human species that may not respond the same way to a test chemical as humans. There is clearly a need to develop new methods for evaluating toxicity without complete reliance on animal testing.

The value of using *in vitro* models for measuring toxicity of environmental chemicals and drugs has been appreciated for many years and discussed in recent reviews [3-4]. In contrast to laborious *in vivo* models that require many animals and may not be accurate predictors for humans, *in vitro* models potentially enable high throughput screening of chemicals and drugs and can be done using human cells, which should be better predictors of human health effects than models based on non-human species. Chemicals found to be toxic *in vitro* in initial screens could be further evaluated in more elaborate *in vitro* assays, such as metabolizing assays, or *in vivo* in an animal model. Moreover, *in vitro* studies are generally less expensive and may be conducted more rapidly than experiments in animal models. Numerous *in vitro* models using both mammalian and non-mammalian sources have been developed over the past 30 years [3]. In general, these have been good predictors of developmental toxicity, although they vary considerably in the time and difficulty required to perform them. With the introduction of mouse embryonic stem cells in 1981 [5], it became apparent that these cells could be an excellent model for early embryonic development, and they have subsequently been used in

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toxicological testing [6]. Human embryonic stem cells, which were first derived in 1998 [7], extended the opportunity to develop *in vitro* assays based on human cells that model embryonic development. The purpose of this review is to summarize and discuss work that has been achieved in developing and using embryonic stem cells in toxicological testing during the past 5 years.

EMBRYONIC STEM CELLS (ESC) – WHAT ARE THEY?

All stem cells have the ability to self renew indefinitely and to produce daughter cells that can differentiate into another type of cell(s). Stem cells come from various sources, and they are usually named according to their source. For example, stem cells that are isolated from the inner cell mass of blastocysts are called embryonic stem cells (ESC), while cells isolated from fat are called adipose stem cells and are an example of an adult stem cell. Adult stem cells are more limited in their ability to differentiate and are usually restricted to the lineages that they normally give rise to *in vivo*. Moreover, adult stem cells generally do not divide as quickly *in vitro* as embryonic stem cells, are often hard to obtain, and can usually not be passaged indefinitely. These factors make adult stem cells less attractive than ESC for use in toxicological assays. As a consequence, most toxicological studies involving stem cells have been done with ESC or cells differentiated from ESC. ESC were first isolated in 1981 from the inner cell mass of mouse blastocysts [5]. It was not until 1998 that similar lines of embryonic stem cells were derived from human blastocysts [7]. Embryonic stem cells are especially attractive candidates for toxicological testing as they are pluripotent, meaning they can develop into any cell type in an embryo and they can be passaged many times *in vitro*.

DERIVATION OF EMBRYONIC STEM CELLS

ESC are generally derived from whole blastocysts obtained from the reproductive tracts of mice or from spare blastocysts offered for research purposes by patients undergoing *in vitro* fertilization. Trophoblast cells, which give rise to the placenta, are removed either by microdissection or immunodissection, and the inner cell mass is plated on a layer of mouse embryonic fibroblasts to generate a new cell line Fig. (**1**). Embryonic stem cell per se model the inner cell mass (mESC) or the epiblast (hESC), while cells that have under gone differentiation model later stages of development. In the pluripotent state, lines of hESC vary from each other, and some lines have a greater propensity to differentiate into certain lineages than others [8]. It is important to consider this variation in toxicological work with this model. Multiple lines could be used to obtain the best overall information, or alternatively, generation of a line that represents the "gold standard" for toxicological testing may be possible.

There are several ways in which stem cells can be used to evaluate chemical toxicity Fig. (**2**). In the first strategy, chemicals can be added directly to stem cells and their effects evaluated on endpoints such as maintenance of pluripotency, proliferation, apoptosis, survival, and growth. The endpoints can be evaluated using morphological or molecular criteria. For example, apoptotic blebbing and activation of caspase 3 are good morphological and molecular markers for apoptosis, respectively. This strategy models either preimplantation or epiblast development. Alternatively, embryonic stem cells can be cultured using conditions that favor differentiation. This is often done by first forming embryoid bodies and then allowing the embryoid bodies to further differentiate into a particular lineage or differentiate spontaneously. Chemicals can be added either before or after embryoid body formation or at both times. Endpoints can include embryoid body formation, proliferation, or differentiation depending when the chemical is added. This strategy models the post-implantation embryo when differentiation of cells has begun. Finally, specific cell types, such as cardiomyocytes or hepatocytes, can be differentiated from embryonic stem cells and chemicals added to the differentiated cells. The differentiated cells can be fully or partially differentiated to enable comparison of fetal and adult counterparts. Endpoints can include apoptosis, survival, and retention of the differentiated state. This strategy is attractive as it allows mass production of large numbers of a specific cell type for testing (such cells may be difficult to obtain from a human source). Moreover, these cells can be fully or partially differentiated.

Induced pluripotent stem cells (iPSC) are the newest entry into the pluripotent stem cell arena[9]. These cells are usually derived from differentiated adult cells that have two to four genes ectopically expressed in culture. The first genes that were used to reprogram to a pluripotent state included Oct4, Sox-2, Klf4 and c-myc. In subsequent studies, it has been possible to reduce this list to just Oct4 and Sox2 [10]. A small percentage of cells transfected with these genes reverts to a pluripotent state and becomes similar to embryonic stem cells. Although the possibility of using iPSC for toxicological studies is interesting, we need to have a better understanding of how close the iPSC model represents the pre and post implantation embryo and how faithfully cells differentiated from iPSC represent their *in vivo* counterparts before they can be used with confidence in predictive toxicology and drug discovery. Nevertheless, this avenue should certainly be explored and potentially represents an outstanding opportunity for future developments in toxicological testing and drug screening.

TOXICOLOGICAL STUDIES USING MOUSE EMBRYONIC STEM CELLS (mESC)

The Mouse Embryonic Stem Cell Test (EST)

The mouse EST is an important assay that was developed in Germany in the 1990s [11]. It is the only validated toxicological assay that uses stem cells and does not require animals (except for the initial derivation of the stem cell line). Its development was motivated by the movement in Europe to re-evaluate about 30,000 existing chemicals by the year 2015 [12]. To perform this re-evaluation and to carefully evaluate any newly released chemicals would require extensive financial and human resources and enormous numbers of laboratory animals. Movement toward highly predictive *in vitro* assays that can be used in conjunction with high throughput screening technology will be necessary to achieve this goal, as well as future evaluation of new chemi-

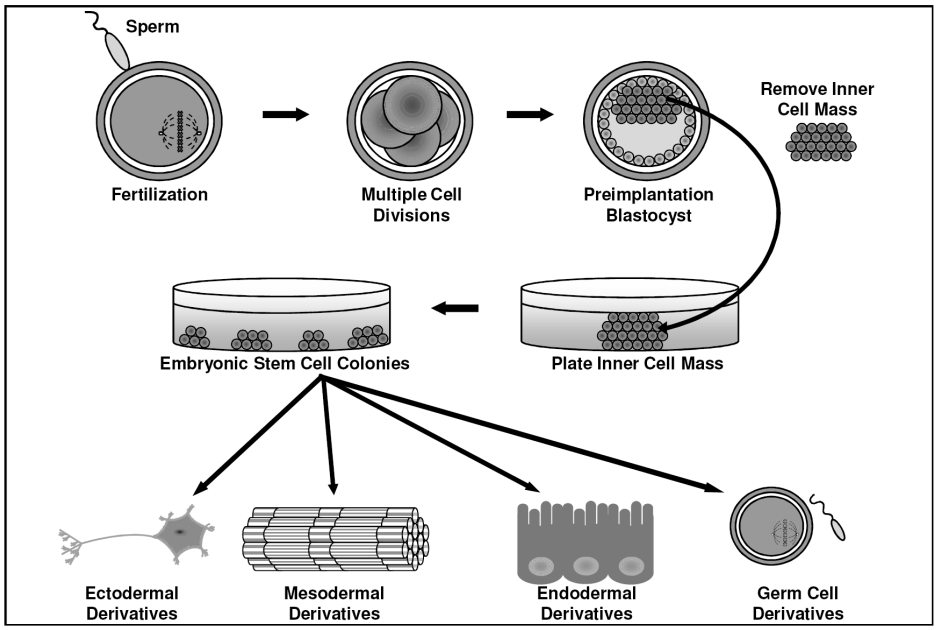

Fig. (1). Diagram showing the relationship between human embryonic stem cells and human preimplantation embryos. Human embryonic stem cells, which are derived from the inner cell mass of preimplantation embryos, are the best model currently available for studying the pre and post-implantation stage of human development.

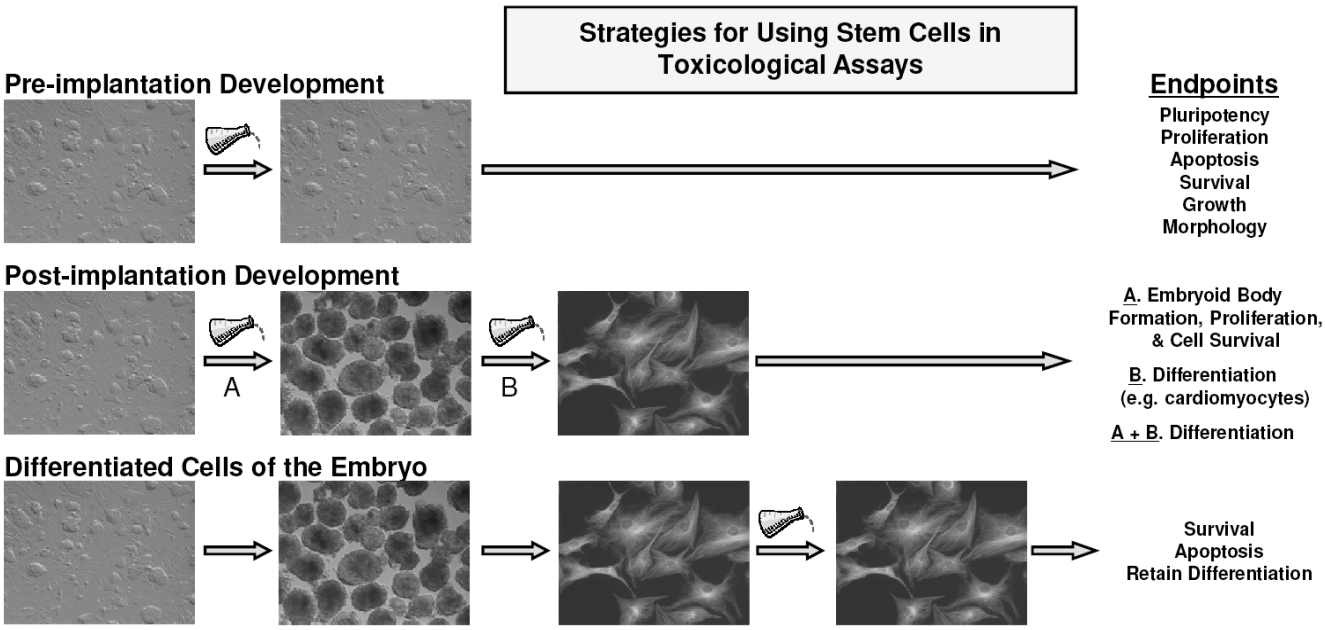

Fig. (2). Diagram showing various strategies for using embryonic stem cells in toxicological studies. To model pre-implantation development, mESC cells can be treated directly with test chemicals and various endpoints monitored. To model post-implantation development, ESC can be made into embryoid bodies and then differentiated either spontaneously or using a method to control lineage. Test chemicals can be added before embryoid body formation (**A**), after embryoid body formation (**B**), or at both times. The endpoints examined will depend on when the chemicals are added to the culture. Finally, ESC can be differentiated into a particular cell type, such as cardiomyocytes or hepatocytes, and the test chemical added to the differentiated cells. These cells can be fully or partially differentiated and represent embryonic or fetal cells. Controlled differentiation is advantageous, and cultures should be as pure as possible.

cals. The EST is an evolving assay that may eventually be highly useful in solving the problem of screening large numbers of chemicals for toxicity. It continues to be modified and improved each year.

The EST is based on three endpoints Fig. (**2**). For the first two, cytotoxicity of the test chemicals is measured using an MTT assay for both mESC (an embryonic cell type) and mouse 3T3 fibroblasts (a differentiated cell type). Cytotoxicity is evaluated using dose response experiments that give

IC_{50}s for both the 3T3 fibroblasts and mESC. The third endpoint is based on the capacity of D3 mESC to differentiate into contracting cardiomyocytes. mESC are grown in the presence of leukemia inhibiting factor (LIF) that prevents differentiation. Cells are next allowed to aggregate into embryoid bodies in hanging droplets in the absence of LIF for 3 days, then transferred to low attachment dishes for 2 days, followed by plating on tissue culture dishes to allow outgrowth and differentiation of cardiomyocytes. By 10 days, some cells in the embryoid bodies will spontaneously differentiate into cardiomyocytes that are easily visualized microscopically without any further processing by their ability to contract spontaneously (a property not shared by other cell types). The differentiation assay is done at various doses, and an ID_{50} can be computed for each chemical. A biostatistical prediction model was developed based on the three EST endpoints and can place test chemicals in one of three categories of embryotoxicity (non-embryotoxic, weakly embryotoxic, strongly embryotoxic). The EST has been subjected to validation in an international study by the European Center for the Validation of Alternative Methods (ECVAM) in which 20 chemicals of known embryotoxicity were tested. Two other widely used *in vitro* assays (the micromass test on limb bud cells of mouse embryos and the post-implantation whole rat embryo assay) were compared to the EST in the validation study, and all performed favorably. The EST was as successful in predicting embryotoxicity of chemicals as the two other assays.

The main strengths of the mESC as it was originally developed are: (1) it has been validated with known embryotoxic chemicals and is currently the only *in vitro* assay based on stem cells that has been validated, (2) it is an excellent predictor of strongly embryotoxic compounds, (3) it can be done relatively easily in any lab set up for cell culture, and (4) it avoids the use of animals. The main disadvantages of the mouse EST are: (1) it is done with mouse cells that may not always accurately predict harm to humans, (2) as originally developed, it requires a long time to complete, e.g., about 10 days are required to do the differentiation phase of the assay, (3) it does not always correctly predict the toxicity of chemicals at the low end of the embryotoxicity spectrum, (4) it does not directly take into account maternal effects of the chemicals, (5) it does not measure toxicity of chemicals that may be deactivated or activated *in vivo,* and in its current form, embryotoxicants that are produced by metabolism will likely be missed (as an example of this problem, the EST missed cyclophosphamide, which is a strong teratogen that forms an active metabolite on first-pass metabolism [4b]), and (6) it is based on the differentiation of only one mesodermal cell type, a possible limitation as other lineages may respond differently to a particular chemical. The above issues are being addressed as discussed below, and the EST continues to evolve into a stronger platform that will have more robust characteristics in the future.

Improvements and Additional Evaluations of the Mouse EST

Since its introduction, the mouse EST has undergone a variety of enhancements. For example, the mechanics of testing have been improved by modifying the culture methods used to produce embryoid bodies [13]. Since the standard operating procedure for making embryoid bodies for the mouse EST often did not yield the required 21 out of 24 contractile embryoid bodies needed to use the data, experiments often had to be discarded and repeated. To minimize this problem, Smedt *et al.* (2008) developed a non-enzymatic method based on a buffered medium containing EDTA to obtain mESC ~~cells~~ for making embryoid bodies and they standardized the hanging droplet culture [13]. These modifications resulted in more tests having an acceptable number of differentiated embryoid bodies and produced embryoid bodies that were more uniform in size and quality and had stronger contractions, making evaluation of the differentiation endpoint more reliable.

Evaluating contractility in embryoid bodies requires considerable human labor. To accelerate counting of contractile embryoid bodies and remove human bias from the EST, Peters *et al.* (2008) developed an automated method for evaluating the fraction of contractility from video data [14]. While there was no statistically significant difference between data obtained using the automated video method vs. manual assessment, there was considerable run to run variability in contraction, which may complicate the use of video parameters. While automating contractility would be helpful and could help move the EST to a medium throughput level, more evaluation will have to be done regarding the usefulness of the video approach in this application.

The original EST was developed using D3 mESC. Other lines have since been used with success. For example, a DBA/1lacj line derived at Pfizer gave results that mirrored those obtained in the original validation study by ECVAM [15]. As was seen in the original study, the EST, when used with DBA/1lacj cells, predicted high risk compounds better than low risk counterparts. The study using the DBA/1lacj line also tested a number of receptor mediated pharmaceuticals with known *in vivo* toxicity. The EST did not predict the risk of these chemicals as well has it did the chemicals used to validate the assay, none of which were receptor-mediated drugs. The DBA/1lacj study, in which numerous compounds including some that overlapped the original validation study, confirmed that the EST was a good, but not perfect, predictor of embryotoxicity and developmental toxicity. Clearly improvements in predictability can be made in the EST in the future to enhance its ability to predict receptor mediated drugs and chemicals that are weakly embryotoxic.

Two ~~of the~~ major drawbacks of the original EST are the long time (10 days) required to reach the differentiation endpoint (contracting cardiomyocytes) and the reliance on only contracting cardiomyocytes, an endpoint that could be misinterpreted, required considerable human time to evaluate, and did not take into account possible effects on ectodermal and endodermal derivatives. In an effort to improve the EST and reduce the time required to reach a developmental endpoint, molecular markers have been introduced into the EST [16]. A new version of the EST, described as the FACS-EST, relies on fluorescence activated cell sorting (FACS) to quantify two key cardiomyocyte proteins, sacromeric myosin heavy chain and α-actinin [17]. In a side by side comparison of 10 of the chemicals used in the original validation study, the EST and FACS-EST compared very well in their ability to

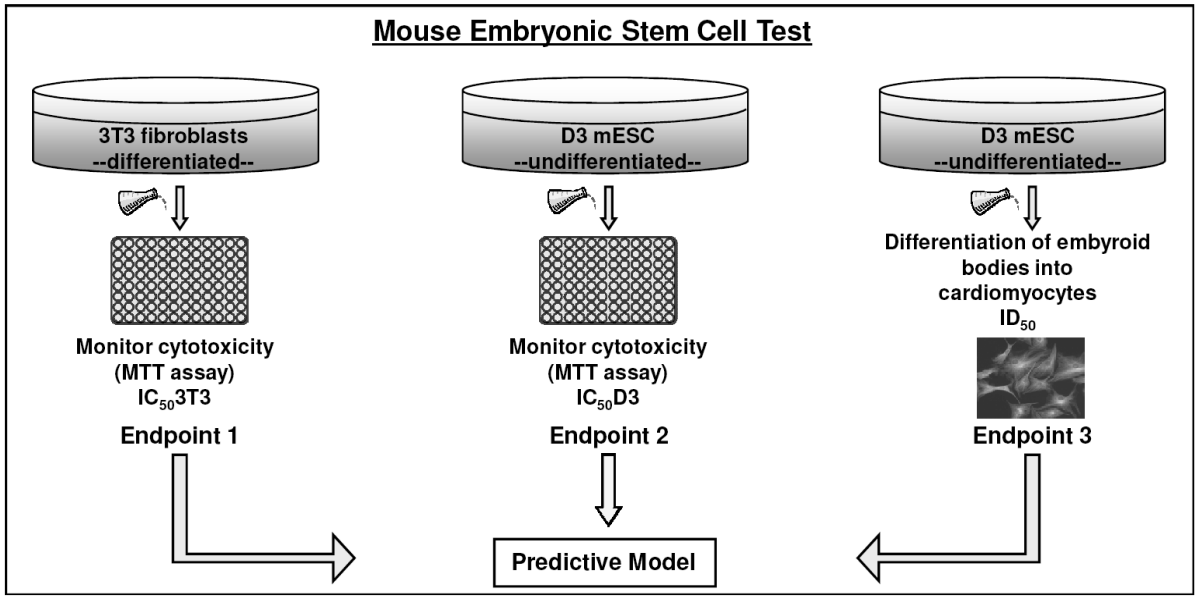

Fig. (3). Diagram showing the main features of the mouse EST. The test, as originally developed, relies on three endpoints. These are cytotoxicity of test chemicals using 3T3 fibroblasts which represent a differentiated cell type, cytotoxicity using D3 mESC which represent and undifferentiated cell, and differentiation of D3 mESC into contracting cardiomyocytes. Dose repose experiments are done, and IC_{50}s are computed for the cytotoxicity endpoints and ID_{50}s are computed for the differentiation assay. The IC_{50}s and ID_{50} can then be used in a predictive model to estimate the embryotoxicity of the test chemical. The predictive model classifies chemicals as non-embryotoxic, weakly embryotoxic, or strongly embryotoxic.

predict embryotoxicity. Addition of FACS to the EST reduced the time required to obtain differentiation data from 10 to 7 days and provided sound quantitative molecular data as the differentiation endpoint. The reduction in time required for the FACS-EST will make it more attractive to industries that need to screen chemicals and drugs for potential embryotoxicity, but it still may not be fast enough for medium and high throughput screening. One draw back to the FACS-EST is that in moving to molecular markers of differentiation at earlier times, the contraction endpoint is lost and with it valuable data on the physiology of the cardiomyocytes.

A recent study investigated the possibility of reducing the time required to access embryotoxicity with the EST even further by using transcriptomic techniques [18]. This study identified a set of 43 genes that are upregulated during the first 24 hours after plating embryoid bodies in differentiating conditions that favor production of cardiomyocytes. The effect on gene expression was then examined in embryoid bodies treated with monobutyl phthalate, a chemical that is known to produce birth defects, usually in structures of mesodermal origin. Monobutyl phthalate had the interesting effect of upregulating genes that are involved in pluripotency, proliferation, and non-mesodermal differentiation and downregulating the gene set expressed during cardiomyocyte differentiation. These data suggest that transcriptome analysis could be used to detect the earliest changes in cardiomyocyte development and that as early as 24 hours after plating embryoid bodies on low attachment plates, marker genes are expressed that could serve this purpose. While additional work would need to be done to validate this approach, shifting to transcriptome analysis could reduce the time to evaluate cardiomyocyte differentiation to 4 days rather than 7 days, which would represent a significant improvement in the application of this assay. However, the time and cost to analyze gene expression vs. contracting cardiomyocytes or FACS analysis of cardiomyocytes would need to be factored into this.

The evaluation of contracting cardiomyocytes is a suitable straightforward morphological assay for mesodermal differentiation. However, the EST could be improved by complementing cardiomyocyte contraction with molecular markers and by extending the EST to ectodermal and endodermal lineages, and to mesodermal derivatives other than cardiomyocytes [19]. In 2004, a modified version of the EST was introduced in which expression levels of markers for osteogenic, chondrogenic, neural, and cardiac differentiation were quantified after exposure to six toxicants that were non-teratogenic (penicillin), moderately teratogenic (diphenylhydantion, valproic acid, thalidomide), and strongly teratogenic (5-fluorouracil and retinoic acid) [20]. The molecular multiple endpoint EST (mme-EST) correctly classified each test chemical and additionally showed that some chemicals produced interesting differences in their effects on the three lineages. For example, retinoic acid inhibited osteogenic and chondrogenic differentiation, but not neural differentiation. In a related study that examined molecular markers for cardiac and bone differentiation in the presence of methotrexate, the expression of bone markers by mESC decreased, while cardiac marker expression remained at control levels [19]. These results are in agreement with known effects of methotrexate *in vivo*. Both the mme-EST and the methotrexate studies demonstrate the importance of using multiple endpoints that take into account different lineage markers as

lineage derivatives clearly differ in their response to the same chemical.

van Dartel *et al.* (2009) have shown that EST results can be affected by inhibition of both cell proliferation and differentiation, and they propose considering both of these parameters in the prediction model [21]. By exposing embryoid bodies from day 0 - day 10, the processes of proliferation and differentiation are not studied separately from each other. However, exposing cells from day 3 onward would give a better read-out for the effects of test compounds on differentiation. This suggestion has been recently made, and it is too early to know if it will be widely adopted.

It would also be highly desirable to increase the output of the EST. Progress toward this goal has been made recently by adapting the EST to 96-well low attachment dishes [22]. Twelve chemicals with known embryotoxicity were compared using the EST endpoints. This method predicted embryotoxicity in good agreement with the original EST and indicates that movement to a 96-well plate format is feasible and could enable large sets of data to be collected. The assay, however, is not fundamentally geared to high throughput as the time to reach the differentiation endpoint in this study was still 10 days. Combination of the 96-well format with an earlier molecular readout would help move the EST to a higher throughput mode.

ESC, which are pluripotent, provide an enormous benefit in that any cell type can potentially be differentiated from them. The EST has been expanded recently to include differentiation of endothelial cells. An efficient method was first developed to differentiate endothelial cells in embryoid bodies created in hanging droplets [23], and an assay based on the EST was then developed, except that the differentiation of endothelial cells, not cardiomyocytes, was monitored [24]. Differentiation of endothelial cells was accessed by examining expression of PECAM-1 and VE-cadherin with real time PCR. Six known embryotoxicants (all-trans-retinoic acid, 5-fluorouracil, diphenylhydantion, valproic acid, saccharin, and penicillin G) were tested in this assay which correctly classified the toxicity of each. With further work, this method could be developed to include a predictive model that would be useful for screening chemicals with unknown effects on embryos. It is probable that other differentiation endpoints will be added to the basic EST as it evolves. It would be helpful to expand this assay to also include differentiation of endodermal and ectodermal derivatives.

Finally, the EST could be combined with *in silico* assessment of risk to determine the *in vivo* effect levels for developmental toxicants. Some preliminary work has been done in this area, and the *in vivo* effect levels were correctly predicted for four of five tested toxicants [25]. If further refined, such an approach may help to further reduce the need for animals in toxicological assessment studies.

Use of the Mouse EST to Evaluate Toxicants that were not Tested During its Original Validation

The mouse EST has been employed by a number of laboratories to evaluate toxicity of compounds not originally tested during validation. Several examples will be given.

Since many test chemicals need to be dissolved in solvents for testing, Adler *et al.* (2006) have evaluated the effects of dimethylsulfoxide (DMSO) and ethanol on the ability of mESC to maintain pluripotency [26]. These two solvents are widely used to dissolve chemicals used in the EST. A reporter line of mESC that had been transfected with the mTert promoter coupled with the GFP gene was used. Analysis of solvent treated cells by flow cytometry and evaluation of Oct4 expression by semiquantitative RT-PCR revealed that DMSO could induce differentiation (loss of mTert and Oct4 expression). They recommended that these solvents be used in doses no higher than 0.1% for DMSO and 0.25% for ethanol to avoid induction effects.

To determine if the EST could accurately evaluate chemicals within a distinct chemical class, the toxicity of glycol ether alkoxy acid metabolites was compared using the EST and data obtained from *in vivo* studies [27]. At doses that were not cytotoxic, all tested compounds showed a dose dependent inhibition of cardiomyocyte differentiation. The hierarchy of potency from most to least potent was: methoxyacetic acid, ethoxyacetic acid, butoxyacetic acid, phenoxyacetic acid. The data obtained with the EST for this group of chemicals were in good agreement with *in vivo* data indicating that the EST can be used to predict toxicity of chemical groups of compounds. This study also found that variation between labs in performance of the EST was within acceptable limits.

Fluoxetine is an antidepressant that is often prescribed for women to treat mood disorders during pregnancy and lactation. Initial clinical trials and animal studies did not detect any adverse effects of fluoxetine on adults or prenatal development. However recent reports of increased incidence of several birth defects prompted a study of fluoxetine's effects using the EST, which showed fluoxetine adversely affected cell viability and differentiation of mESC into cardiomyocytes [28]. It was further found, by examining markers, that fluoxetine impaired mesodermal differentiation. These data in combination with recent reports of congenital defects in the offspring of fluoxetine users suggest caution in taking this drug during pregnancy and show that further study into its effect on developing young is needed. This is an interesting example of the mouse EST detecting toxicity of a chemical that was not found to be toxic in earlier work.

The only strongly embryotoxic chemical to be incorrectly classified as non-embryotoxic in the original EST validation study was methylmercury. This led others to hypothesize that the EST may not be able to correctly classify heavy metals. When cadmium and arsenic compounds that were known to be embryotoxic *in vivo* in mice were tested using the EST, the test failed to identify one cadmium and two arsenic compounds as embryotoxic [29]. When all tested heavy metals are considered together, the EST failed to correctly classify four out of seven. These data indicate the need for modifications or improvements to the EST so as not to miss important toxicants that have serious effects on embryos.

In an interesting application, the toxicity of eleven metals used in dental alloys were evaluated in the EST [30]. Mercury and chromium were classified by the EST as strongly embryotoxic, in agreement with other studies on these two metals [31]. Antimony, tin, and vanadium ions were weakly embryotoxic, while silver, cobalt, copper, nickel, palladium,

and zinc were not embryotoxic. However, as discussed above, the ability of the EST to identify toxic metals has been called into question [29], and the negative data in this study may need re-evaluation by an alternative method.

Studies that have Tested Toxicity Using mESC Without Using the EST

mESC have been used by a number of labs to evaluate toxicity of environmental chemicals without using the EST. For example, the effect of arsenic on the expression of selenoproteins in mESC was studied with the CGR8 cell line [32]. Selenoproteins play important roles in humans in antioxidation, redox regulation, and detoxification. Arsenic was found to up-regulate the expression of selenoproteins associated with antioxidation, while downregulating selenoprotein H and some of the selenoproteins located in the endoplasmic reticulum. Selenium was able to restore expression of the downregulated proteins in mESC.

Potassium dichromate [Cr(IV)], which is commonly used in laboratories and industry, is a widespread environmental toxicant. Its mechanism of action has been studied in depth using mESC to evaluate the signal transduction pathways activated by [Cr(IV)] [33]. [Cr(IV)] was shown to activate both p38 and JNK, but not ERK via MAP2K4 and MAP2K7. Phosphorylation of p38 produced cytotoxic effects in mESC, while activation of JNK inhibited cytotoxicity as well as differentiation of mESC into cardiomyocytes. This very thorough study approached the actions of [Cr(IV)] at multiple levels in the mESC model and provides a more complete understanding of the adverse effects of [Cr(IV)] on embryonic cells.

mESC stably transfected with the gene for green fluorescent protein under the regulation of the cardiac α-myosin heavy chain promoter were used to test various compounds in restorative dental materials [34]. This study also measured cytotoxicity of test compounds on mESC using the MTT assay. Chemicals were considered embryotoxic if they decreased expression of GFP without causing cytotoxicity. This assay was therefore similar to the EST except that transfected cells were used to monitor cardiac differentiation rather than contraction of cardiomyocytes and cytotoxicity was not compared to 3T3 cells. Prior to testing dental compounds, this assay (R.E. Tox assay) was validated using 20 reference chemicals and found to accurately predict embryotoxicity. When dental restoration compounds were then tested, three compounds were not cytotoxic and did not affect differentiation, while the remaining compounds produced various effects on both cytotoxicity and differentiation with methacrylic acid significantly stimulating differentiation at non-cytotoxic levels. This study demonstrates the potential to use GFP reporter cell lines in a modified version of the EST.

Paraquat, a highly toxic quick acting herbicide used in agriculture, was tested extensively using mES cells with a spectrum of biological assays [35]. When stem cells were examined after 24 hours of exposure, paraquat was found to stall cell proliferation, increase reactive oxygen species, and increase apoptosis and necrosis. These adverse effects could be prevented or reduced by inclusion of vitamin C in the culture medium containing paraquat. The data in this study are important in suggesting that paraquat may be dangerous to young embryos at concentrations that have not previously been considered harmful.

Valproic acid, a chemical known to cause defects in the heart and nervous system, was tested using mESC and cardiomyocyte differentiation, although the standard operating procedure for the EST was not used in this study. Valproic acid inhibited growth of embryoid bodies at levels that were not cytotoxic, decreased the number of contracting embryoid bodies, and decreased the area of contraction in plated embryoid bodies [36]. These effects coincided with increased levels of reactive oxygen species in valproic acid treated embryoid bodies. The effects of valproic acid on cardiomyocyte differentiation could be reversed by vitamin E treatment. In related studies, the anticonvulsants carbamazepine and valproic acid were studied using differentiation of contracting cardiomyocytes and the expression of marker genes for differentiation as endpoints [37]. In all assays including cytotoxicity, inhibition of expression of endodermal and mesodermal lineage markers, and induction neuronal differentiation, valproic acid was more potent than carbamazepine. These results were in good agreement with known *in vivo* data for these two anticonvulsants and demonstrate the usefulness of mESC to study and compare toxicity of therapeutic drugs.

Studies Using Differentiated Mouse Embryonic Stem Cells

In addition to the EST, numerous studies have been done in the past 5 years using mouse embryonic stem cells in other experimental designs [38]. One strategy, for example, is to differentiate specific types of cells from ESC and use the differentiated cells in toxicological testing. This would be a way, in principle, to obtain any cell type in large enough numbers to conduct toxicological experiments. Moreover, different lineages could be examined which is ultimately important since cardiomyocytes may not be responsive to all toxicants. Examples of some of this work will be presented. Most studies have allowed ES cells to differentiate into another type of cell, and then evaluated the toxicity of test chemicals.

While the EST relies on differentiation of cardiomyocytes, some labs have explored chemical toxicity using neurons differentiated from ESC. The idea of using neurons is attractive since the central nervous system is very sensitive to environmental chemicals throughout the prenatal period. In the original EST validation study, the EST failed to detect methylmercury as embryotoxic. This chemical produces malformations in brain development indicating the nervous system, not the heart, may be its major target. Stummann *et al.* (2007) examined the effect of methylmercury on the differentiation of mESC into neuronal-like cells and found downregulation of expression of Mtap2, a marker for neuronal differentiation [39]. However, several other neuronal markers were not affected by methylmercury indicating that multiple markers need to be used when gene expression is the endpoint. In a subsequent study using hESC, methylmercury inhibited differentiation of ESC into a neuronal precursor-like cell but was less effective at inhibiting maturation of the precursor cells into neuron-like cells [40]. With hESC,

the expression level of all markers except nestin decreased during methylmercury exposure, in contrast to the mESC experiment in which only Mtap2 decreased. This illustrates that different markers need to be used when evaluating toxicity with different species and further shows that results were highly dependent upon the stage of neuronal differentiation that was examined.

Endothelial cells differentiated from mES cells have also been tested with 5-fluorouracil, a chemical known to inhibit vasculogenesis [41]. Endothelial cells derived from mESC cells were somewhat more sensitive to 5–fluorouracil than adult mouse endothelial cells in a growth assay [41b], supporting the general idea that embryonic cells are more sensitive than adult cells to toxicants. This observation which is supported by diverse data is important as testing the most sensitive stage in the life cycle should arguably be the benchmark for toxically studies. Further work showed that the viability of endothelial cells derived from ES cells is significantly reduced by 24 hours of exposure to 5-fluorouracil (10 M), an effect that was reduced by simultaneous exposure to probucol (50 M) and that 5-fluorouracil also decreased proliferation and differentiation of endothelial cells and induced the G1/S phase arrest in the cell cycle [41b]. These authors suggest that endothelial cells differentiated from mESC cells could be a valuable model for screening for toxicity in new chemical compounds. Certainly interference with vasculogenesis or angiogenesis would be a significant problem prenatally and postnatally, as these are times when new vessel development is essential. Adaptation of this strategy to hESC would be beneficial.

Obtaining pure populations of differentiated cells for toxicological work is an area that needs attention. While controlled differentiation methods are continually improving and yields of specific cell types continue to increase, other strategies for obtaining pure cell types for assay development have also been investigated. For example, Chaudhary *et al.* (2006) have used laser microdissection and pressure catapulting (LMPC) to isolate contracting cardiomyocytes from differentiating embryoid bodies [42]. These isolates, which expressed cardiac markers and exhibited the functional characteristics of cardiomyocytes, can be transferred to 96-well plates for further study. They suggest that this method could be used to isolate homogeneous ESC-derived cell types for heterogeneous populations of differentiating cells.

Other novel strategies for measuring cytotoxicity using mESC cells have been developed. For example, Calabro *et al.* (2008) have measured transepithelial electrical resistance in monolayers of mES cells that were grown using differentiation conditions [43], and found that resistance decreased proportionally to increases in cytotoxicity. This model could be a developed into a valuable assay for measuring cytotoxicity with embryonic cells, although a number of cytotoxicity assays currently exist.

HUMAN EMBRYONIC STEM CELLS IN TOXICOLOGICAL TESTING

Examples of Studies that have Been Done

While usually thought of in the context of regenerative medicine, hESC also provide one of the best opportunities available for developing assays to assess toxicity of environmental chemicals, and thereby helping to prevent disease. hESC can be used to model the earliest stages of human development and can be differentiated into cells with characteristics of those found in embryos. Experiments can be designed to model undifferentiated hESC, differentiating hESC, or hESC that have differentiated into a progenitor or mature cell. Specific types of differentiated cells, such as hepatocytes and cardiomyocytes, would be especially valuable in testing toxicants, but in principle any type of cell could be produced and studied using hESC. So while hESC have great potential to treat and cure regenerative diseases, they also have equally great potential to prevent disease by identifying dangerous environmental chemicals and drugs before they cause harm.

In spite of the fact that hESC represent one of our best opportunities to develop methods for screening chemicals that may be toxic to humans, relatively little work has been done with them in this context. A human counterpart to the mouse EST has not yet been developed. This is in part because hESC are more difficult to work with than mouse cells and present certain unique challenges that must be overcome before well accepted assays are developed with them. For example, hESC grow slower than those from the mouse, they tend to clump, and they are more difficult to grow as single cells, a fact that could be important in assays involving cell quantification and homogenous distribution for cells in treatment groups. Also it has been difficult to efficiently and consistently differentiate hESC into contractile cardiomyocytes, but recent improvements in this technology [44] could facilitate development of a human EST patterned after the mouse model.

One recent paper developed the framework for a human Embryonic Stem Cell test [45]. Two embryotoxicants, retinoic acid and 5-fluorouracil, were used to develop a cytotoxicity assay comparing the sensitivity of hESC and human embryonic lung fibroblasts. In addition, quantitative RT-PCR was used to identify potential marker genes that could be used to monitor cardiac differentiation. The most useful markers identified in this study were brachyury and GATA-4 for cardiac differentiation and the late cardiac gene TNNT2, which was expressed between days 10 and 18. This study clearly shows that progress is being made toward the development of a human EST, but we are still far from completing this goal.

A second interesting study has evaluated the potential of hESC to provide information on toxic chemicals using two non-embryotoxic, two weakly embryotoxic, and two strongly embryotoxic chemicals [46]. Cytotoxic indices were determined using cells representing the embryo (hESC), fetus (human embryoid bodies) and adults (human foreskin fibroblasts). In addition, the effects of each chemical on differentiation were monitored using lineage specific markers. Both embryoid bodies and hESC were more sensitive to chemical treatment than the foreskin fibroblasts in the survival assay. In the gene expression assays, non-embryotoxic chemicals were without effect (penicillin) or only effective at high doses (saccharin), the weakly embryotoxic chemical, indomethacin, downregulated endodermal markers, while busulfan which is strongly embryotoxic downregulated most of

the markers. This study further correlated effective doses of each chemical with doses that were found in the serum of patients receiving these drugs and found that several were within the range present in human patients. These data showed that hESC and human embryoid bodies can be used to monitor toxicity of drugs and environmental chemicals; however, much further work is required to fully develop the potential of human stem cells for this purpose.

hESC were used recently to evaluate the ability of nonylphenol and octophenyl, two environmental contaminates that disrupt the reproductive and endocrine systems, to induce apoptosis [47]. Using a variety of methods, this study found that these test chemicals did induce apoptosis in hESC via a Fas-Fas ligand pathway in which caspase 8 and 3 activation increased following exposure to the test chemicals. hESC were also differentiated into neural progenitor cells which were found to be more sensitive to the two test chemicals than the hESC. This may be due to the presence in the hESC of ABC transporters which are able to protect these cells against stress.

Additional work supports the idea that differentiated or differentiating embryonic cells may sometimes be more sensitive to toxicants than hESC. In a survivability assay (MTT), hESC were more resistant to oxidative stress than fibroblasts that had differentiated from hESC [48]. Survivability was not enhanced for either cell type by heat-shock pretreatment or by preconditioning with low levels of oxidative stress. A similar finding has been reported for mESC which were more resistant to oxidative stress than embryonic fibroblasts [49]. In the mouse study, several antioxidant-related genes were down regulated as mESC differentiated into embryoid bodies. hESC and mESC thus appear to be more resistant to oxidative stress than newly differentiated cells, and their use in toxicological assays would need to consider this point.

In an interesting use of hESC to access arsenic toxicity, hESC were exposed to arsenic, then the ability of an arsenic antidote (monoisoamyl dimercaptosuccinic acid) to rescue the cells was evaluated using cytotoxicity and gene expression endpoints [50]. In addition, rats were treated with both arsenic and the antidote, and litter size and developmental defects were examined. The hESC assays were able to detect damage done by arsenic and reversal of damage by the antidote, and the *in vitro* observations with stem cells correlated well with the *in vivo* data on arsenic induced damage.

Differentiation of hESC and Toxicity Testing

Another strategy for using hESC is to first differentiate them into a specific cell type of interest then subject the differentiated cells to toxicological testing. Hepatocytes and cardiomyocytes could be useful for tools for this purpose, while other cells may also be highly interesting. For example, should it become possible to differentiate oocytes from hESC, they could be used to assess sensitivity of the germline to environment toxicants. Effects of toxicants on sperm and oocytes are important as they are fundamental to reproduction of our species and detection of damage may not occur for many years after exposure.

In a study on fibroblasts spontaneously differentiated from hESC, the cytotoxicity of mitomycin was measured using the MTT assay [51]. The differentiated population was heterogeneous, but this group argues that this will be satisfactory as the results they obtained were reproducible. This could be an important point as obtaining highly purified differentiated cells is not always easy and may not fit well into future high throughput operations. Nevertheless, improvements in directed differentiation are continually being made, and in the long term, the need to work with heterogeneous populations of differentiated cells should not be an issue.

Because the nervous system is very sensitive during all phases of prenatal life, finding ways to identify toxicants that affect its development is very important. Recently neurospheres derived from hESC have been used to evaluate neurotoxicity in a three dimensional model [52]. In this study, neurospheres were tested with teratogens classified as strong (hydroxyurea), weak (valproic acid and lithium), and non-teratogenic (acrylamide). The endpoints included cytotoxicity and neuronal protein marker expression. Valproic acid, which was the most effective chemical tested, reduced viability and decreased expression of neuronal markers, in agreement with studies using the mme-EST with mouse stem cells [20]. However not all applications of valproic acid have reached the same conclusion. In differentiation tests using mESC, valproic acid was found to promote neuronal differentiation, not inhibit it [37b, 53]. This discrepancy may be due to species differences or to the different methods that were used to induce neuronal differentiation, a factor that will need to be considered carefully when developing a general assay using stem cells for toxicological testing. In contrast to valproic acid, lithium did not effect viability or marker expression in the neurosphere study [52]. Hydroxurea and acrylamide reduced viability but did not alter marker expression. Thus, the neurospheres were useful in indentifying neurotoxicants, but the hierarchy of their potency obtained with the neurosphere test was not an exact match for their known *in vivo* toxicity. As often is the case, chemicals at the low end of the toxicity spectrum are the most difficult to classify. In this case acrylamide was weakly embryotoxic rather than non-embryotoxic. However, the fact that acrylamide did have an effect on viability may be important. *In vivo* assays that rely largely on anatomical changes and non-molecular endpoints may not detect subtle changes in cells that are revealed by *in vitro* assays. This begs the question - do we need to be more careful with chemicals that are classified as non-embryotoxic - we could be missing information that our traditional *in vivo* tests do not reveal.

Differentiated neurons are also of interest and can be obtained from hESC. It is difficult to grow and maintain neurons from the adult brain, but hESC provide a means to create an unlimited supply of differentiated neurons for toxicological and neurological studies. In a recent study, hESC were differentiated into dopaminergic derived neurons and treated with 1-methyl-4-phenylpyridium (MPP^+), which produces features of Parkinson's' disease in humans [54]. MPP^+ caused an increase in reactive oxygen species in treated neurons that was accompanied by an increase in apoptosis. Neurons could be rescued from the effects of MPP^+ by treatment with glial cell line derived neurotrophic factor. This study demonstrates the potential usefulness of hESC in differentia-

tion of cells that may otherwise be difficult to obtain for studies of toxicity.

USE OF ESC TO EVALUATE THE TOXICITY OF CIGARETTE SMOKE ON PRENATAL DEVELOPMENT

Cigarette smoke is a complex mixture of chemicals, many of which are known toxicants and carcinogens [55]. Nicotine, the major bioactive chemical in cigarette smoke, is addictive [56]. Numerous epidemiological studies have shown that *in utero* exposure to cigarette smoke decreases birth weight significantly and may produce other unwanted effects such as increased risk for placenta abruptio, stillbirth, ectopic pregnancy, preterm birth, and SIDS [57]. The epidemiological data are supported by animal studies, many of which have been done using *in vitro* technology [58]. Of additional concern are the recent findings that children exposed to smoke *in utero* have cognitive and learning problems after birth [57e, 59]. While it is not feasible to experimentally test the full range of effects of smoke on actual human embryos, it is possible to experimentally evaluate smoke's effect on human prenatal development by using ESC as an *in vitro* model for human embryos. Cigarette smoke can be studied both as mainstream smoke (MS), which is inhaled in each puff by active smokers, and as sidestream smoke (SS), which is the smoke burning off the tip end of a cigarette [60]. In addition, whole smoke or individual components in smoke can be studied using ESC-based assays. Thus ESC provide a model that can be used to gather information on how the earliest stages of human prenatal development are influenced by exposure to smoke. This is an important point as these stages are usually the most sensitive to environmental chemicals and are likely to be the most severely impacted by smoke.

The effects of MS and SS cigarette smoke from both conventional and harm reduction cigarettes have been studied using mESC [61]. Both MS and SS smoke inhibited attachment, survival, and proliferation of mESC dose dependently. Pretreatment of cells with smoke solutions, followed by washing and plating in control medium also reduced attachment of treated cells. Moreover, when mESC were first plated then treated with smoke, they detached from the substrate in a dose dependent manner. In side by side comparisons of traditional and harm reduction cigarettes (which are often claimed to be less toxic), smoke from the harm reduction brands was unexpectedly found to be more potent than smoke from the traditional brand when assayed with mESC. In addition, SS smoke was consistently more toxic than MS smoke from all brands. To verify that embryos respond similarly to smoke solutions, mouse pre-implantation embryos were recovered from oviducts and treated *in vitro* with MS and SS smoke solutions. In treated groups, blastomeres were often lysed, and caspases 3 & 7 were activated indicating the occurrence of apoptosis. These observations are important in calling attention to the sensitivity of the earliest stages of development to toxicants in both MS and SS and further highlight the need to better understand smoke from brands that are purported to reduce harm.

In a subsequent study, hESC were grown on Matrigel, detached using Accutase, then replated as single cells in control medium or medium containing a non-cytotoxic dose of SS smoke from a conventional brand (Marlboro Red), and followed using time lapse video in a BioStation IM. The percentage of attached cells over a 90 minute period was significantly higher in the control group than in the group treated with SS smoke solution Fig. (**4**). These data with hESC, while preliminary, are in agreement with the mouse study [61] and again show that smoke treatment impairs attachment to and spreading of embryonic cells on an extracellular matrix. Attachment of cells to extracellular matrices is important in all phases of embryogenesis, and factors that adversely affect attachment would be expected to impair normal development.

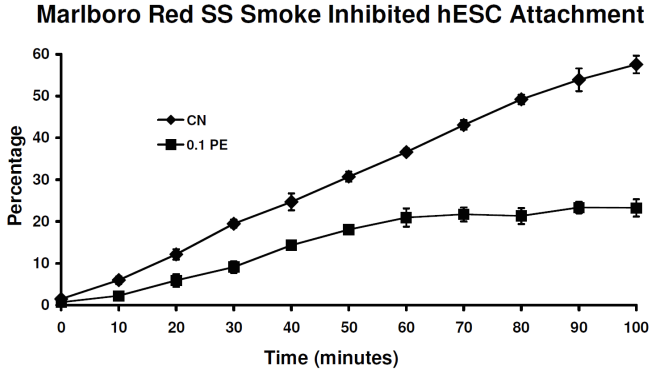

Fig. (4). SS smoke from Marlboro Red cigarettes significantly inhibited attachment and spreading of H9 hESC on Matrigel relative to control cells that were untreated. A non-cytotoxic dose (0.1PE) of smoke solution was used in this experiment. Multiple fields (N = 5) were monitored and assayed in both groups. Data were analyzed from time lapse videos collected with a BioStation IM.

In a related study using hESC, nicotine, the major bioactive component of cigarette smoke, dose dependently inhibited cell attachment to Matrigel, an effect that was reversed by tubocurarine, a nicotine antagonist [62]. The doses of nicotine that were effective (1.8 and 3.7 μM) were below the dose (6 μM) reported in the cervical mucus of the female reproductive tract [63]. The hESC responded morphologically to nicotine by undergoing extensive vesiculation which was reversible upon washout. Nicotine was also found to increase the percentage of apoptotic cells above control levels in both unattached and attached cells. Overall, nicotine affected a number of endpoints, and the reversibility of some of these effects by tubocurarine suggests that hESC have a receptor for nicotine. While these data show that nicotine is one agent in tobacco smoke that can adversely affect hESC, it is probable given the complexity of smoke, which contains well over 4,000 chemicals, that other chemicals also produce adverse effects on hESC.

The effect of nicotine on expression of pluripotency markers in embryonic stem cells is not yet clear. In mESC, nicotine doses that bracketed those found in human smokers were found using quantitative RT-PCR to increase expression of Oct-4 and Rex-1, two genes associated with pluripotency [64]. This effect was prevented by tubocurarine, a nicotinic acetylcholine receptor antagonist. In contrast, hESC

pluripotency markers appeared to decrease when cells were exposed to nicotine [62]. This discrepancy could be due to species differences (mESC vs. hESC) or culture conditions (mESC experiments were done on feeder layers, while those with hESC were done directly on Matrigel).

Preliminary data on embryoid bodies derived from D3 mESC further demonstrate adverse effects of cigarette smoke on differentiation. In a cytotoxicity assay using trypan blue, mESC were treated with 0.1 or 1.0 puff equivalents (PE) of MS or SS smoke from a harm reduction brand during formation into embryoid bodies. One PE equals the smoke in one puff that dissolves in 1 ml of medium. While neither dose of MS smoke affected formation and growth of embryoid bodies, the high dose of SS smoke (1.0 PE) inhibited aggregation of mESC into embryoid bodies, and most cells stained with trypan blue, indicating cell death had occurred Fig. (**5**). In a follow-up experiment using RT-PCR to evaluate gene expression, mouse embryoid bodies, which were formed and incubated in LIF containing medium, were exposed to 0.1 PE of MS or SS smoke solution from a harm reduction brand of cigarette (Advance Lights) for 14 days to determine if smoke treatment affected differentiation of the three germ layers and/or maintenance of pluripotency Fig. (**6**). Rex1, which is usually the first pluripotency marker to decrease as differentiation begins, was downregulated in cells treated with MS, but not SS smoke solution suggesting that MS smoke accelerated differentiation within the embryoid body. Other pluripotency markers remained high, probably because the medium contained LIF. Analysis of additional gene expression showed up-regulation of the endodermal (GATA-4 and α-fetoprotein) and mesodermal (T-gene) markers by both MS and SS smoke, while no effect was observed on the expression of the ectodermal markers (nestin and neuroD). The data showed that cigarette smoke exposure can alter the timing of gene expression in the early stages of development, a point that should be studied further.

Potential Pitfalls with Stem Cells

While stem cells provide one of the best opportunities to develop methods for toxicological testing, it is important to be aware of pitfalls connected with their use. The substrate used for ESC culture can affect the outcome of toxicological tests. For example, Matrigel outperformed gelatin in assays that measured maintenance of pluripotency, response of mESC to sodium arsenite, and percentage of cells that developed into cardiomyocytes [65]. Stem cells in culture may also undergo changes with repeated passaging on Matrigel. For example, mESC when repeatedly passaged in the presence of high LIF on Matrigel showed variable responses to caspase 3 activation by sodium arsenite [66]. Also passaging can lead to aneuploidy or more subtle chromosomal translocations or deletions, which may affect the outcome of toxicological tests. Stem cells are also subject to contamination by bacteria, fungi, and *Mycoplasma* which can cause set backs

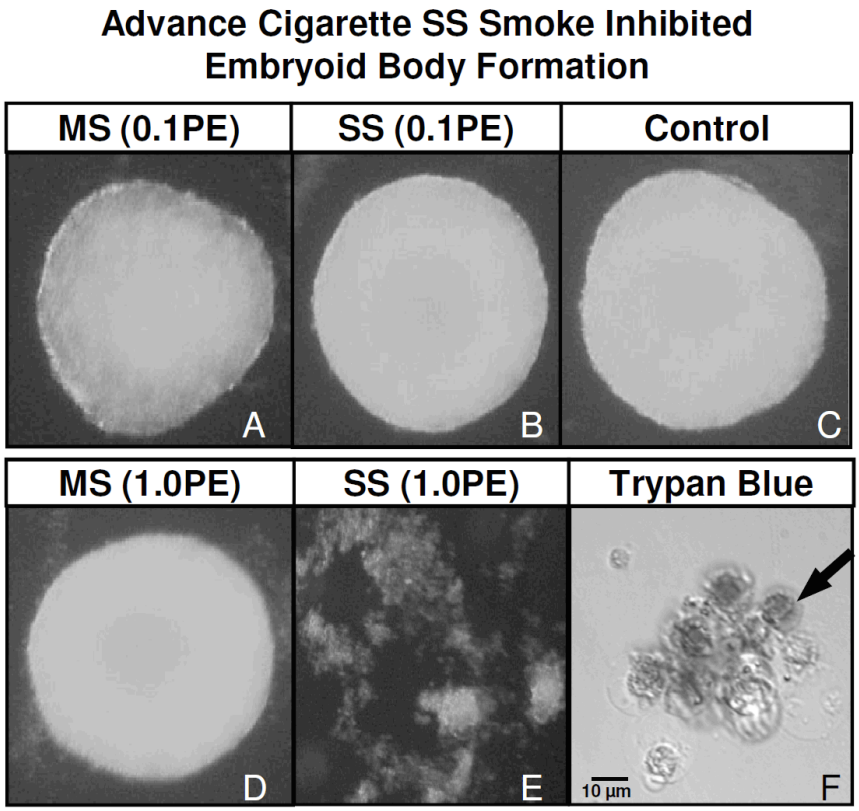

Fig. (5). Cytotoxic effects of MS and SS smoke solution on mouse embryoid bodies. Embryoid bodies were made using D3 mESC in 0.1 or 1.0 puff equivalents (PE) of MS or SS smoke from Advance harm reduction cigarettes. At 1.0 PE of SS smoke, embryoid bodies did not form and the cells appeared dead. Trypan blue was used to confirm cell death in cultures treated with 1PE of SS smoke. MS smoke at 1.0 PE did not kill the stem cells.

in performing assays, and are especially devastating when differentiating cells over long periods of time.

Some data clearly show that different labs can obtain similar results when using the EST; however, when standardized tests are not used, results can be variable was seen with valproic acid studies [20, 37, 52-53]. Standardization of differentiation protocols will be important to obtain meaningful data among different labs. It is also clear from studies that have already been done that the time of exposure can effect the interpretation of the results. Chemicals will generally act at a specific time in development, and if that time is not included in the assay, the toxicity of the tested chemical will be missed. We have also seen that not all markers are affected by a particular treatment. In assays that rely on differentiation markers, clearly more than one marker for a particular lineage needs to be used, and ideally derivatives of the three germ layers and germ cells would be studied.

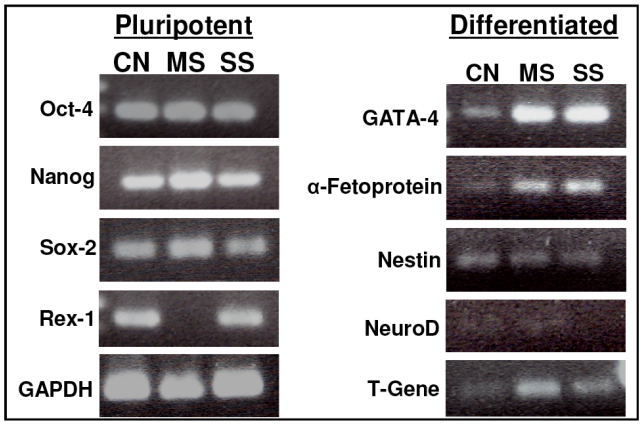

Fig. (6). RT-PCR gel showing the effect of MS and SS smoke from Advance harm reduction cigarettes on differentiation markers. Embryoid bodies made using mouse D3 cells were incubated in LIF containing medium with or without MS or SS smoke solution for 10 days. RT-PCR was performed on samples to determine if smoke treatment affected pluripotency or the rate of differentiation. Pluripotency markers were equivalent in all groups except for Rex-1 which was downregulated by MS smoke treatment. Differentiation markers for endoderm (GATA-4 and α-fetoprotein) and for mesoderm (T-gene) were upregulated by both smoke treatments relative to the untreated control, while markers for ectoderm (nestin and NeuroD) were unchanged. This experiment shows that smoke treatment alters the time of gene expression in mESC and could impact the developmental program of young embryos.

Future Improvements

The potential of hESC to be used in assays that predict toxicity of environmental chemicals and drugs is enormous. Investment in the development of new toxicological assays based on hESC could pay off enormously and help keep the planet healthy by preventing unwanted disease. hESC can meet an urgent need to develop new methods for toxicological testing that are faster, high throughput, cost effective, and based on human cells. In this closing section, some of the improvements and areas for future research will be considered.

Improved methods to culture and differentiate embryonic stem cells are developing rapidly and will enhance the options available for toxicological testing. More defined media have already been introduced for both mouse and human ESC [67], and it is likely that culture media will continue to improve. Interesting new hydrogels are becoming available that will provide three dimensional scaffolds for mimicking the *in vitro* environment more precisely than two dimensional matrices often used today [68]. Artificial hydrogels can also be prepared free of undefined growth factors that could influence the outcome of toxicological testing. The mouse EST is based on differentiating cardiomyocytes. It is clear from the literature that it is important to examine effects of chemicals on ectodermal and endodermal lineages as well. Adding new differentiation protocols to assay development will help improve their predictivity.

Pure cultures of differentiated cells, such as hepatocytes and cardiomyocytes, will be extremely valuable in the future in both drug and chemical testing. Hepatocyte-like cells differentiated from hESC show many characteristics of true hepatocytes, such as glutathione transferase activity [69], which is encouraging news. As the research effort in regenerative medicine evolves, improved protocols for differentiating stem cells into specific cell types will improve, and toxicologists can take advantage of the availability of this resource and integrate specific differentiated cells into platforms to screen for potential benefits or harm.

Validation of new assays is important. Although the mouse EST has been successfully validated, no assays based on hESC have yet been subjected to validation. While time consuming, validated assays will be important in the future of predictive toxicology. Validation can come only after a viable assay(s) has been developed and preliminarily tested. While validation will be a project for the future, planning for it can begin now.

Embryonic stem cells have an important advantage over adult cells in that they enable studies to be done on cells that represent stages in prenatal development. Because these stages are generally the most sensitive in our life cycle, it is often argued that the embryonic and fetal stages are the ones that should be used when assessing the risk of specific chemicals to humans [2]. As an additional bonus, stem cells provide a means to look at various stages of prenatal development (pre-implantation, epiblast stage, differentiation).

Consideration needs to be given to the metabolism of test chemicals which can inactivate or active toxicants in any assays that are used for human testing. The EST does not take into account maternal factors or metabolism of the test chemical and as originally developed will probably not be a good predictor of toxicants that are affected by metabolism. Combining the EST or hESC based assays with *in vitro* liver extracts, such as S9, may help improve their ability to identify the full range of chemicals that are embryotoxic.

Improvements in reducing the time required to collect data are important. Methods that enable medium or high throughput screens with ESC are needed and are in development [70]. Other strategies such as signalomics, which involves high throughput screens to reveal simultaneous alterations in signal transduction cascades in response to a test

chemical, could be valuable for future development of *in vitro* assays for drug and chemical testing [71]. With high throughput methods will come a need for sufficient numbers of both hESC and specific cell types differentiated from hESC. The ability to scale up production of hESC for use in high throughput assays will be an important adjunct to the development of the assays *per se*.

By moving to a human cell based platform, the potential to develop better, more accurate tools for predicting toxicological outcomes should greatly improve. As recently pointed out by Greaves *et al.* (2004), the dog, which is rarely used in toxicological studies, is a better predictor of human toxicities than the primate, rat or mouse, and even the dog misses about 37% of the chemicals toxic to humans [72]. Because animal studies are expensive, the transition to human stem cell based assays could be both cost effective and provide better predicative data than the currently used animal models.

The future of predictive toxicology has never looked better. With the potential to use hESC in the development of new assays and to be able to study specific cell types as well as cells that model young embryos, we can expect in the future to have vastly improved methods for screening chemicals before human exposure occurs and for screening potential drugs that may alleviate human disease.

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NOTE ADDED IN PROOF

Since submitting this manuscript, several groups have shown that hESC, while derived from the inner cell mass of blastocysts, in culture have properties similar to epiblast cells of implanted embryos [Nichols, J.; Smith, A. The origin and identity of embryonic stem cells. Development, 2011, 138 (1), 3-8]. In contrast, cultured mESC resemble inner cell mass cells of the preimplantation embryo. Therefore mESC and hESC model different stages of embryonic development.

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