Stem Cells in Predictive Toxicology

CIRM Workshop Report
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1. EXECUTIVE SUMMARY

The California Institute for Regenerative Medicine (CIRM) is charged with furthering the cure or mitigation of human diseases using stem cells. Use of stem cells in drug discovery and toxicology testing could lead to safer, more customized pharmaceuticals. Similar use of stem cells in assessing the impact of environmental contaminants on human health could help identify chemicals that contribute to disease. In July of 2008, CIRM organized a workshop in Berkeley, California to assess the current and potential use of stem cells in drug discovery and in toxicity testing of environmental hazards. Scientists working in pharmaceutical and biotechnology industries, research universities and government research centers were invited to present their work and to discuss the potential impact of stem cells in predictive toxicology.

OUTCOMES OF THE WORKSHOP

Potential for Stem Cells and Drug Discovery

Pharmaceutical and biotechnology industries are in need of improved model systems for predicting potential drug toxicity, both to decrease the rate of drug-related adverse reactions as well as to reduce attrition rates of promising compounds. Human stem cells are potentially attractive reagents for predictive toxicology, particularly if they can be shown to be a reliable, large-scale source of differentiated human cells. First, stem cells and their cellular derivatives could form the basis of *in vitro* assays that can be miniaturized and adapted to high throughput screening platforms. Use of cell-based toxicity assays during the early phases of drug development could decrease the cost of attrition at a later stage in development, and would also provide the opportunity to optimize a chemical's safety profile through targeted medicinal chemistry. Second, the use of human cells could increase the correlation between safety studies and clinical trials, an important benefit since conventional animal models of toxicity are not always predictive of human responses. Finally, stem cells that are generated from adult tissues (iPS cells) could allow models to be created from individuals with a diverse range of drug susceptibilities, resistances or disease, which could reduce the rate of adverse effects within patient subpopulations. These "clinical trials in a dish" could help inform and optimize further trials in humans. Workshop participants agreed that the use of stem cell-based assays in the process of drug discovery has the potential to reduce late-stage attrition, to lower the cost of drug discovery, and to help understand the genetic contribution to drug susceptibility. These features would improve understanding of the molecular basis of toxicity and lead to the production of safer or more customizable drugs for human consumption.

Contribution of Stem Cells to Environmental Health

Federal and state agencies that are charged with protecting human health are eager to use predictive toxicology models to uncover the risks posed by pollutants, pesticides, personal care products, and other chemicals in the environment. Many of the same assays and approaches that are used in the pharmaceutical industry are being employed for assessing environmental toxicity, and several multi-agency consortia, such as the Environmental Protection Agency's ToxCast™ program, are providing a framework for developing and validating high throughput methodologies for testing chemicals. Environmental toxicity screens would derive the same benefits from stem cells as would toxicology studies in the drug discovery process, namely the improved relevance of a human model, a diverse repertoire of potential assays, and the ability to incorporate high throughput methodologies. As with drug studies, iPS cells may be useful for elucidating

mechanisms of chemical toxicity and understanding the genetic and environmental factors that contribute to individual responses, particularly when combined with human surveillance and biomonitoring studies that are being used to estimate exposure levels to environmental chemicals.

Status of Stem Cell-based Assays

Stem cell based models are in their infancy but offer tremendous promise for assessing toxicity. Differentiation procedures for the cell types most susceptible to drug toxicity – hepatocytes, cardiomyocytes, and neural, among others – are being developed. Although it is still not clear that these procedures yield fully mature, functional cells, some basic *in vitro* assays are already being tested and used for drug discovery and could potentially be adapted to the environmental health field. Supporting technologies, such as 3-dimensional tissue engineering and sophisticated bioreactors, are allowing researchers to more closely mimic the environment of these cells in the human body, and might ultimately result in viable *in vitro* organs. Given current work in the field, it appears that stem cells might soon represent a reliable and reproducible source of otherwise unavailable human cells for cell-based assays.

Challenges

Despite their promise, the extent to which *in vitro* assays using stem cells will be useful for predicting toxicity in humans remains unclear. There was general consensus among participating drug discovery and environmental health researchers that it is critical to validate the results from stem cell-based *in vitro* models of toxicity before these will become useful. Overall, participants agreed that correlating *in vitro* assays with responses in humans would be preferable to correlating them with existing animal models of toxicity. As will be discussed in the report, the factors identified as essential to validate stem cell-based assays are reliable cell lines ("Olympiad" cell lines), reproducible differentiation protocols, and a panel of compounds with known human toxicities to be used as a benchmark.

Once proof-of-concept experiments have been conducted and have demonstrated that stem cell-based assays are of value for predicting toxicity in humans, there will be further bottlenecks to overcome, such as maintaining reliable stem cell stocks, scaling the assays, and further correlating the results of *in vitro* toxicity assays with human endpoints by conducting human surveillance and biomonitoring studies. These issues were mentioned during the workshop, but were not discussed in detail.

Exciting new methods to derive stem cells from adult somatic tissues (induced pluripotent stem cells, or iPS cells) are providing tools for deriving cells with diverse genetic information. If iPS techniques prove robust, participants agreed that they could be of tremendous value for deriving cells from a diverse sample of the human population, including individuals with particular disorders or drug susceptibilities. To achieve these goals, a concerted effort will be needed to identify the appropriate human populations and produce reliable iPS cells from them. The ability to derive cells from individuals with known sensitivities would add tremendous value to *in vitro* toxicology assays, and would provide resources for elucidating the molecular basis of variable human responses to chemical and drug challenges.

In conclusion, this workshop confirmed that the use of human stem cell systems might dramatically increase our ability to predict toxic responses in humans. Stem cells could increase the predictive power of high-throughput cell-based assays by providing large quantities of reliable, high-quality human cells for assay development and mechanistic studies. These studies could allow for more effective medicinal chemistry that

would produce lead compounds that would have lower toxicity profiles, and would decrease the need for extensive toxicity tests in animals. Furthermore, new methods to derive stem cells from adult somatic cells (induced pluripotent stem cells, or iPS) could allow incorporation of genetic diversity into stem cell models, a feature that might help elucidate the molecular basis for variable human responses to drug or environmental exposure. Finally, increased use of human *in vitro* models of toxicity could, in the long term, decrease the use of animals in safety and risk assessment studies. Together, these approaches offer the potential to dramatically enhance our understanding of the molecular basis of toxicity, leading to improved assays and models for predicting biological response to drugs and environmental chemicals.

2. INTRODUCTION

The need to more accurately predict adverse health outcomes from exposure to drugs or environmental pollutants has brought Predictive Toxicology to the forefront of both public and scientific awareness. Underlying this new focus is the growing concern that current methods for screening and identifying potential toxic substances are both insufficient and imprecise. These concerns have led to a call for new methods to increase the predictability of studies to assess the human safety of both pharmaceuticals and chemical hazards.

The primary area of focus of predictive toxicology has been drug safety studies, an important part of the pharmaceutical development process. Traditionally, any drug that is intended for use in humans must first pass through rigorous trials in one or more living animal models. Unfortunately, there is often poor correlation of toxicity from one species to another, a phenomenon that leads to unforeseen and adverse health effects from drug exposure, costly drug recalls, and possibly the premature shelving of potentially beneficial medicines. These facts are illustrated by reports estimating that about 40% of drug candidates are abandoned throughout the development pipeline due to unforeseen adverse reactions in test subjects (1). Even though these potentially harmful candidates are screened out during testing, adverse drug reactions are still the 4th leading cause of death in the United States, indicating that current testing and regulatory methodologies are not adequately predicting toxicity. This is particularly true for subsets of individuals that have particular genetic susceptibilities (2). In addition to these human costs, the economic impact of drug failure is significant, nullifying the considerable time, labor, and financial investments that have gone into each candidate.

A second focus for increased interest in predictive toxicology lies in the growing awareness of chemical hazards in the environment and their potential effects on health and human development. Years of ongoing industry, agriculture and other human activities have resulted in the leaching of thousands of pollutants into the environment, many of which have not been scrutinized for potential toxicity. Unlike drugs intended for human consumption, these substances are not necessarily subjected to the same criteria for determining risk. Furthermore, exposure is usually unintentional, and thus the parameters that typically contribute to toxicity, such as dose, length of exposure, and presence of additional mitigating factors, cannot be assessed with reasonable confidence.

The discovery of stem cells and their ability to differentiate into diverse cell types has created opportunities to rethink the way chemicals are evaluated for risk to human health. Furthermore, it is now possible for stem cells to be produced by reprogramming adult fibroblasts, a technology that could greatly facilitate the derivation of multiple differentiated cell types from individuals with diverse genetic backgrounds and environmental histories. If certain challenges can be met, it might be possible to improve or replace many conventional models of toxicity screening with more relevant human systems, resulting in improved toxicology prediction and a better understanding of how genetics and environmental exposure contribute to an individual's sensitivity to drugs and susceptibility to disease.

The Workshop

The objectives of this workshop were to **a)** determine whether *in vitro* studies with human stem cells or their derivatives would be useful tools for assessing toxicity of candidate drugs and environmental pollutants in humans; and **b)** identify the key challenges for incorporating such methodologies into current practice. To best meet these goals, CIRM assembled a body of experts including toxicology researchers and other representatives from industry, academia and government to discuss the most reasonable and potentially

fruitful avenues for hastening the advancement and integration of stem cell methodologies into mainstream use.

The workshop took place over two days and was organized into a series of panels, each of which addressed questions regarding the use of stem cells and their derivatives in clinical toxicology studies and environmental health assessment. Panelists gave brief presentations and participated in moderated discussions.

Day 1: Early presentations, part of a panel chaired by Dr. Lee Rubin from the Harvard Stem Cell Institute, gave an overview of stem cells and some of the ways in which they are being used to predict toxicology and understand disease mechanisms. Panelists discussed the major limitations and liabilities of the current animal-based models that are used to screen drug candidates, and described how studies with stem cells might improve the accuracy, efficiency, and lower the cost at which drugs are determined to be safe for human use. Also discussed were the key target areas in which stem cell-based models have been and are likely to be useful. Panelists provided real examples of toxicity analysis using stem cells, identified the model systems where *in vitro* studies would be of greatest utility, and described technological requirements and bottlenecks that must be achieved or overcome in order for these studies to be practical.

Dr. Tracey Woodruff from UCSF chaired the second panel, which included a series of presentations which gave an overview of environmental health issues and how stem cells could be used to address risk assessment and ultimately predict toxicity from environmental pollutants. Representatives from federal and state agencies described deficiencies in current models and relayed their vision for a paradigm shift that would adapt methodologies and knowledge from preclinical drug development efforts to the specialized needs of environmental health assessment. Current federal and state initiatives were described that are developing and testing new models for predictive toxicology in the environmental health arena.

Dr. Marie Csete from CIRM chaired a third panel highlighting emerging technologies that could address the bottlenecks that currently prevent stem cell technology from being widely incorporated. Several devices were described that could provide improved culturing and growth conditions, thereby improving our ability to acquire large amounts of pure cell populations with relevant functionalities. Other tools, such as computational, *in silico*, and systems-based approaches were described that could be used in combination with stem cell data to make better predictive models. Additional sources of stem cells, including induced pluripotent cells from adult tissues, were described that might overcome limitations of primary embryonic stem cell lines and enable elucidation of genetic and environmental contribution to disease phenotypes and drug sensitivities.

The first day concluded with a presentation of the *in vitro* toxicity models that are currently being developed using stem cells, including models for cardiac, hepatic, neural and developmental toxicity. This concluded with a discussion on these models, chaired by Dr. Jane Lebkowsky from Geron.

Day 2. The second day began with an overview of potential procedures for scientific validation and eventual U.S. regulatory acceptance of stem cell based toxicology models. Dr. Melissa Carpenter led attendees in a discussion of the guidelines and goals that have been set forth by federal agencies and considered how they might shape the course of future research. Next, Nancy Koch from CIRM and Jeff Karan from Proteus Ventures led a series of discussions focused on defining the scientific scope of toxicology projects and determining which stem cell models, tools, and technologies would be most important for filling these needs. In the final discussions led by Dr. Patricia Olson from CIRM, attendees considered which areas of research should be targeted in the near term and what funding models might be most appropriate for a given project. Representatives from both industry and government offered insight into aspects of collaboration they would be willing to undertake and what would determine their level of interest in new technology. Panelists offered

advice on how CIRM could foster interaction, facilitate sharing of information, and avoid issues that could arise from intellectual property disputes.

3. STEM CELLS AND PREDICTIVE TOXICOLOGY IN DRUG DISCOVERY AND DEVELOPMENT

3.1 Deficiencies of Existing Models

Current methods for testing the safety of drugs that are in development for human use have several drawbacks, largely due to the poor correlation of toxicity patterns observed in animal models and eventual human response. This imprecision leads to high attrition of promising candidates, resulting in a significant waste of resources and contributing to the ongoing escalation of drug prices. Furthermore, animal models are themselves slow, expensive, labor-intensive and in the opinion of some, ethically questionable. It is widely viewed that an ability to perform toxicology tests in human systems, particularly using *in vitro* methodologies, would allow for greater accuracy and much wider variety of tests to be performed, resulting in increased safety and lower costs for drug development and discovery. It is estimated that lowering the attrition rate by only 10% would result in savings of $100 million per year (3), prevent wasted effort at earlier stages of the developmental pipeline, and minimize the need for animal studies. In addition to the economic impact, the ability to perform more comprehensive and relevant assays should translate into safer drugs and a better understanding of the relationship between toxicity, dosage, and individual human susceptibility.

In addition to whole animal studies, *in vitro* assays are often employed to predict the effects of drugs on specific cell types and whole organs. Unlike *in vivo* models, *in vitro* studies are amenable to miniaturization and use in high throughput screening platforms, including comprehensive, global approaches that offer insight into disease and toxicity mechanisms. Typical *in vitro* experiments make use of either transformed, immortalized cell lines or primary cells that are isolated directly from animal tissues. Although easily maintained and readily available, transformed cells exhibit abnormal behaviors, are usually aneuploid, and do not ideally recapitulate the phenotypes and mechanisms that would be observed in their normal cell counterparts. Primary cells, on the other hand, offer a more relevant model system for predicting toxicology but are limited in quantity and suffer from batch-to-batch variation, as cells must continuously be isolated from whole animals or tissues for further study.

Stem cells and their derivatives represent a promising opportunity for developing *in vitro*, human cell assays that would ultimately replace, enhance, or surpass the current models that are used for predictive toxicology. First and foremost, the ability to test drugs in a human system should increase the relevance and accuracy of predicting toxicological outcomes, resulting in less expensive and safer drugs emerging from the clinical pipeline. Second, the ability of stem cells to differentiate into a variety of cell types and develop into organ systems could allow them to replace transformed cell lines and primary cells for *in vitro* studies, eliminating irreproducibility and supply limitations and improving the relevance of predictive assays. Third, an ability to derive stem cells from individual human subjects would offer unprecedented opportunities to analyze the contribution of genetic background and other mitigating factors that affect susceptibility to toxicity and disease. A summary of currently used toxicology models, their drawbacks, and the potential advantages of human stem cells is presented in Table 1.

Table 1. Comparison of Various Model Systems for Predictive Toxicology

System	Supply	Relevance	Expense	Versatility
In vivo animal models	Limited by growth, reproductive rates	Only 30-80% correlation with human drug response, depending on system	More expensive: maintenance and care; husbandry requirements; ethical concerns	Limited to what can be observed and recapitulated in the particular non-human species
In vitro model, cultured primary cells	Cells must continually be isolated; cells isolated at different times can be variable	Poor correlation with human phenotype for some systems, better for others	More expensive than transformed cell lines, in some cases necessitates use of animals to provide cells	Amenable to high throughput screening, comprehensive approaches; genetic engineering of cells is limited to the lifespan of the cells
In vitro model, cultured transformed cell lines	Unlimited; generally easy to maintain	Cells are abnormal, aneuploid; may not exhibit normal cell behavior or response	Less expensive; robust immortal lines can be maintained indefinitely	Amenable to high throughput screening, comprehensive approaches; genetic engineering is possible
In vitro model, human stem cells*	Potentially unlimited, highly reproducible	Most relevant: genetically identical populations of human stem cells from single source could be used, or sources could be genetically customized	Unknown, but potentially low; more accurate and efficient screening could offset potential costs	Amenable to high throughput screening, comprehensive approaches; genetic engineering may be possible

* potential benefits if technology is sufficiently developed, as discussed in this report.

3.2 Current Uses of Stem Cells in Drug Research, Discovery and Development

Researchers from industry, academia and government agencies offered multiple examples of how stem cells and their derivatives, from human or other origins, have been successfully used to screen for drug toxicity or investigate disease mechanisms in a variety of systems. The approaches fell into two broad and complementary categories, both of which demonstrate the potential of this technology to improve our understanding of biological mechanisms and how they are impacted by drugs and disease. In the first type of investigation, specific cell models were directly screened by various methodologies, including computational approaches, for perturbation by drugs or correlation with disease phenotypes. In the second type, cells were used as models for investigating the mechanisms of particular diseases or toxicity pathways, the knowledge of which can be applied to improve the predictability of current toxicity studies as well as enable the design of more accurate and focused approaches for the future.

In the first category, a prototypical toxicity screen was described in which undifferentiated embryonic stem cells were treated with chemicals and examined for perturbation of subsequent development and

differentiation. Dr. Bob Chapin and Dr. Don Stedman described how this approach is currently being adapted at Pfizer, Inc. for assessing potential teratogenicity and other forms of developmental toxicity in their proprietary compound libraries (4). In a second example, Dr. Lee Rubin from the Harvard Stem Cell Institute described a screen in which tagged reporter molecules and fluorescence microscopy are being used to visually observe the effects of potential toxic substances on neurons that have been derived from stem cell progenitors (5). While both of these studies used murine cells, there is general scientific consensus that analogous experiments will succeed with human embryonic stem cells (hESC). As a third example, Dr. Gabriela Cezar described how metabolic profiling of human stem cells is successfully being used to identify new biomarkers, predict toxicity from known controls, and uncover potential mechanisms for uncharacterized forms of toxicity (6). It was clear from these lines of investigation that stem cells and their derivatives have proven value in predicting toxic outcomes from drug exposure and are leading to new avenues of investigation for drug and disease research.

In addition to direct screens, examples were given where cell-based *in vitro* assays were used to elucidate the mechanisms that underlie adverse cellular, or even physiologic responses to drugs or disease. In one example, a bioinformatics analysis led to correlation of a mouse single nucleotide polymorphism (SNP) pattern with resistance to acetaminophen or irinotecan toxicity. Metabolic profiling resulted in the narrowing of a list of 200 possible genetic loci to the 3 or 4 most relevant candidates. This combination of computational and *in vitro* experimentation enabled a study that would have taken up to 5 years to complete by traditional methods to be performed in a matter of days by a few individuals, further demonstrating the power of cell-based approaches for addressing *in vivo* toxicological outcomes. Although these particular studies used liver extracts, the ability of stem cells to differentiate into hepatocytes could greatly expand the breadth and scope of these assays, as will be discussed in the following section. In a second example of how stem cells can be used to advance our understanding of toxicity mechanisms, it was reported that stem cell-derived cardiomyocytes, or heart cells, can differentiate into the appropriate cellular subtypes, express the correct suite of protein markers and ion channels, and even recapitulate some of the functions of an intact organ, such as an ability to beat *in vitro* (Dr Bruce Conklin, Dr. Kendrick-Parker, Dr. Bjorquist, Dr. Snodgrass). These features are allowing scientists to design experiments to address the mechanisms underlying arrhythmias such as Long QT syndrome, one of the most serious and common toxicities that result from drug exposure. Additional stem cell derivatives, such as hepatocytes and neural cells, are expected to be similarly powerful additions to the repertoire of tools for predicting toxicity.

3.3 Key Areas of Interest

There is general consensus that stem cells offer potentially unprecedented advantages over existing models for predictive toxicology at various stages of drug discovery and development. The areas that would benefit the most from such tools are discussed below.

3.3.1 Predictive Hepatotoxicity and Cardiotoxicity

The liver plays a central role in processing and metabolizing drugs and other substances in the bloodstream and is therefore is particularly susceptible to the effects of toxic substances that are targeted to its vicinity. Not surprisingly, hepatoxicity is the most common form of adverse drug reaction, the leading cause of drug recall, and a major contributing factor to high attrition of new drug candidates (7). To further complicate matters, the byproducts of drug metabolism often possess their own toxic properties, leading to potential liver damage and/or other forms of pathology due to perturbation of cellular function.

A second major route by which drugs my induce toxicity is through perturbation of cardiac function. Long QT syndrome, a type of arrhythmia that can be induced in susceptible individuals, has famously led to major drug recalls over past few years and is a leading contributor to drug attrition from the developmental pipeline. Because the majority of adverse drug responses are due to cardio-and hepatotoxicity, the two most relevant cell types for use in cell-based toxicity profiling experiments are judged to be hepatocytes and cardiomyocytes.

The most common experimental systems for studying hepato- and cardiotoxicity are *in vitro* studies with primary liver or heart cells and *in vivo* animal tests, as described in Section 3.1. Meeting participants provided data showing that it is possible to derive both cardiomyocytes and hepatocytes from hESCs. However, further work is needed to achieve all of the cell types represented in adult liver and heart. Populations of genetically identical, mature hepatocytes or cardiomyocytes that could be reliably derived from an established progenitor human cell line would greatly increase the number and types of toxicity assays that could be performed while streamlining and simplifying their interpretation. Removing the batch-to batch variability of primary cells and replacing them with appropriate stem cell models, particularly cell types that could be genetically manipulated, should enable much more meaningful comparisons to be drawn from a wider variety of assays.

3.3.2. Predictive Neurotoxicity

Another important form of toxicity is that which affects the nervous system and its associated processes. Currently, there are no *in vitro* models for assessing neurotoxicity, and scientists rely on observational screens of animals to identify neurological and behavioral perturbation. Although some of these studies have been useful, animal models are especially ill suited for studying complex human traits that have no known counterparts in animal behavior, such as autism or various psychopathologies. In addition to providing more relevant models, neurobiologists are hopeful that cultured neurons will enable studies to be performed *in vitro* that are impractical or impossible to achieve *in vivo*. For example, certain classes of neurotoxins are known to cause axonal demyelinization in the spinal cord, but it is difficult to determine whether this is due to defects in glial cell growth, perturbation of oligodendrocyte function, or abnormal myelinization/remyelinization activity. A functional neural culture would allow such questions to be addressed *in vitro* using the standard techniques of molecular biology including targeted mechanistic studies, genetic analysis, and comprehensive cellular profiling methodologies.

Predictive *in vitro* models for neurotoxicity have not yet been realized, largely due to the complexity of recreating authentic neural units in culture. Unlike hepatocytes and cardiomyocytes, neurons require support cells (astrocytes and oligodendrocytes) and appropriate molecular cues in order to recapitulate normal function. Although it is currently possible to derive these cells from hESC, further research is needed to reproduce some of the results that have been achieved using primary cells. Dr. Aaron Chuang described how, despite the additional challenges, an *in vitro* model for neurotoxicity that is based on human cells would be extremely valuable for toxicology prediction, and efforts to recapitulate human neural activities in cell based assays are an active area of pursuit in the field.

3.3.3. Predictive Developmental Toxicity

Developing embryos can be exquisitely sensitive to the effects of drugs and chemicals, and the possibility of fetal damage due to parental exposure is a major concern for both drug developers and consumers. The effects of toxic substances on embryos can be profound and may lead to a variety of adverse outcomes including death, birth defects, biological abnormalities, and even psychological and behavioral problems throughout an individual's lifetime. Even drugs that have proven to be safe and effective in adults can have devastating consequences on a fetus when taken during pregnancy. For these reasons, there is a

pressing need to develop more accurate tools for predicting developmental toxicity in drug candidates, not only to produce safer medicines, but also to provide doctors and patients with comprehensive guidelines for using a medicine safely and effectively.

Undifferentiated embryonic stem cells could provide an ideal model system for studying the effects of known compounds on embryonic development and differentiation, and companies such as Pfizer are already implementing such assays, as described previously. In addition to assessing toxicity, these screens could help to identify the developmental pathways that are perturbed by toxic substances, elucidate the mechanisms that underlie toxicity, and define the windows of time in which embryos are susceptible.

3.3.4. Toxicity and Individual Susceptibility

Even the safest drugs may cause adverse reactions in certain individuals with a specific genetic background or environmental history. Unfortunately, there are currently no methods to predict individual susceptibility to a particular drug. Perhaps the most powerful models of predictive toxicology could be achieved using stem cells derived from adult tissues, or induced pluripotent stem (iPS) cells. iPS cells derived from individuals with known susceptibilities or resistances to various drugs or diseases could offer unprecedented opportunities to uncover the personal suite of genetic factors and potential epigenetic influences that contribute to variable drug response.

3.4 Technological Requirements for Implementation and Expansion

The potential for stem cells to revolutionize the field of predictive toxicology is based on established scientific precedent as well informed expert opinion. However, for each area of interest, there are significant technological gaps that must addressed in order for the full potential of this technology to be realized.

3.4.1. General Technological Needs

In order for stem cells to provide a superior, predictive model system, it is important for there to be robust cell lines that are plentiful, easy to maintain, differentiate appropriately, recapitulate known *in vivo* and *in vitro* responses, and produce reproducible results that can be independently validated. In addition, it is necessary to have a library of end point markers that can be used to verify the differentiated state of each cell type that will be used. While some of these criteria have been met with certain models, others have special limitations that need to be addressed through additional research, as described in 3.4.2. Finally, the sheer amount of data that would be produced by the comprehensive and high throughput screening methods will necessitate significant investment in the development of bioinformatics and statistical analysis tools. Multiple new technologies are under development that will address and potentially overcome these issues, and are discussed in Section 5.

3.4.2. Specialized Technological Needs

Each of the key areas described in Section 3.3 has different technical criteria and constraints that must be met before these cell types will be acceptable as a valid toxicological model. For example, to recapitulate primary cell data, stem cell derivatives must express a suite of proteins or transcription factors (end point markers) to prove they are functionally equivalent to the cells they will replace. The main technological requirements and bottlenecks for each desired model system is outlined below.

Hepatocytes: Ideally, hepatocytes that are generated from stem cells should be functionally equivalent to adult human hepatocytes, i.e. they should express liver genes of terminally differentiated hepatocytes, possess the correct suite of transporter proteins, reflect the genetic diversity of a population, including gender

differences, and be amenable to *in vitro* manipulation. Several groups have been working on methods to differentiate hESCs into hepatocytes, and meeting participants presented data that these hepatocytes express some of the enzymes necessary for liver function. However, Dr. Stephen Strom highlighted that most hepatocytes that have been produced from stem cells appear similar to fetal rather than adult liver cells, and participants agreed that there are insufficient numbers of cell lines to adequately represent a genetically diverse population. To produce mature hepatocytes, it will be necessary to improve culturing and differentiation protocols, likely by providing an extracellular matrix scaffold and possibly including additional cell types (co-culture) to better recreate the environment under which authentic hepatocytes develop. To recreate gender and genetic differences, multiple cell lines need to be established from a diversity of individuals.

Cardiomyocytes: Cardiomyocytes obtained from stem cells and other sources express endpoint markers that suggest they have some mature characteristics and can differentiate into the major subtypes (ventricular, atrial, nodal) that are required for heart function (8, 9, and data presented at this meeting). These cardiomyocytes beat in culture and exhibit electrophysiological activity, thereby reflecting an *in vivo* phenomenon that can be perturbed and tested *in vitro*. Stem cell-derived cardiomyocytes might thus enable powerful new approaches to be developed for elucidating both disease and toxicity mechanisms that perturb heart function. Such approaches could be the key to unraveling the contributing factors of Long QT Syndrome (LQTS), a potentially fatal heart arrhythmia. As mentioned previously, LQTS is one of the most prevalent forms of drug toxicity and a leading contributor to high attrition rates of drug candidates in the developmental pipeline. In addition to the drug-induced form, LQTS also results from heritable or spontaneous genetic mutations. While several of these have been mapped to loci that encode subunits of the HERG potassium channel, the majority of causes remain unknown. Interestingly, individual susceptibility to both the genetic and drug-induced forms of LQTS varies considerably, most likely due to additional genetic differences that are poorly understood. As cardiomyocytes derived from mouse stem cells have already been used to analyze the effects of genetic mutation on heart arrhythmia in mice (10), it is likely that analogous studies could be used to investigate LQTS mechanisms in human cardiomyocytes. An *in vitro*, beating human cardiomyocyte system from healthy and/or susceptible individuals could be used to screen drug candidates for toxicity, to identify genetic differences that contribute to susceptibility, and to directly investigate the electrophysiological and molecular features that define this syndrome.

To improve and expand the accuracy and versatility of cardiomyocyte studies, it will be necessary to generate large amounts of homogenous populations of cells and devise practical methods for scaling up growth. It would be especially desirable to have engineered cardiomyocytes with inducible genes or marked proteins to allow controlled differentiation and subsequent identification of developmental stages and phenotypes.

Neural Cells: Stem cell differentiation into authentic neural units has been uniquely challenging, as normal neural function is dependent on a delicate interplay of neurons and support cells (oligodendrocytes and astrocytes). Recently, it has been possible to produce 90% pure populations of each of the cell types, and progress is being made in the purification of subtypes. To fully realize the potential of a predictive neural assay system, however, it will be necessary to generate heterotypic cultures, and it might also be necessary to include brain slices in the culture medium. Thus, technological advances are needed in co-culturing techniques to recreate an appropriate context for neural function and to generate large quantities of pure populations of the various neural subtypes.

iPS: Every model system described thus far stands to benefit from an ability to create stem cells from individuals with diverse genetic backgrounds, environmental histories, disease phenotypes, and variable susceptibility to toxicity. iPS cells have recently been created from human somatic cells and were demonstrated to possess many features and behaviors of authentic embryonic stem cells, including the ability to differentiate into cells of all three germ layers, including beating cardiomyocytes (11, 12). While still in its infancy, iPS technology offers the potential to elucidate genetic and epigenetic contributions to disease and drug susceptibility. Furthermore, individuals experiencing unexpected, adverse reactions can be flagged for retroactive analysis, thereby allowing potential elucidation of toxicity pathways and leading to improved predictive models for future drug testing.

4. STEM CELLS AND PREDICTIVE TOXICOLOGY IN ENVIRONMENTAL HEALTH ASSESSMENT

4.1. Deficiencies of Existing Models

The ability to predict toxicological outcomes from chemical exposure is of utmost importance to federal and state agencies that are charged with assessing potential risk of environmental pollutants on public health. Traditionally, the Environmental Protection Agency (EPA), the National Institutes of Environmental Health Sciences, and the California –EPA and other state health agencies have relied on data from mechanistic studies, animal models, and epidemiological evidence in order to assign hazard classification to given chemicals. Unfortunately, these approaches suffer from many of the same drawbacks that plague toxicity assessment in the drug industry, such as poor correlation of phenotype from animal to human and differential sensitivity of individuals due to genetic factors and variable environmental histories. Risk assessment from environmental pollutants is further complicated by the fact that unlike the carefully controlled evaluations that are performed for clinical drugs, it is often impossible to gauge the dose, the duration, or mitigating effects from other substances that come from an accidental exposure.

The true scope of the challenge faced by environmental health agencies can best be summed up by the mantra "Too many chemicals, too little time". In the US alone, around 87,000 chemicals are made on a continuous basis, 3000 of which are categorized as "high production" (more than 1 million lbs/year). As of 1998, there were no toxicology data for 43% of these substances and only 7% have been comprehensively evaluated for toxicity (13, 14). Around 150 of these chemicals are found at detectable levels the US population. Increasing public attention to these issues has raised awareness of the potential correlation between exposure and risk of cancer, rising autism rates, developmental defects, and other adverse health outcomes. Environmental health experts concur that a "new paradigm" is needed, one that allows hazard assessment to move from an observational to a predictive science. At the state level, the California EPA has recommended the development of a program that would combine a comprehensive suite of tests, both general and targeted, with computational modeling and biosurveillance programs. The timeline for implementation is 20 years, and it is estimated that the basic research infrastructure required for such an endeavor would be $200-400 million/year (15). At the federal level, multiple consortia have already been assembled, and programs such as those desired by Cal-EPA have been initiated, as will be discussed below.

4.2. Developing Predictive Methods for Environmental Health Assessment

The needs and methods for predictive toxicology in environmental health assessment have many parallels to those of the pharmaceutical industry, and it has not escaped notice that current and future technologies that are useful for preclinical drug development could be used for environmental health assessment (EHA). Thus, several multi-disciplinary consortiums have been assembled to develop plans that will combine the experience and technology of the drug industry with the needs and goals of EHA. One outcome of these collaborations is the ToxCast™ program, a comprehensive effort by the EPA to develop a "cost-effective approach for prioritizing the toxicity testing of large numbers of chemicals in a short period of time" (16). In the first phase of this effort, over 300 well characterized pesticides and other chemicals will be studied by a variety of approaches including computational chemistry, high-throughput screening (HTS), and toxicogenomic profiling (17). Most of these chemicals have been previously evaluated using traditional toxicity models and thus serve as benchmarks for validation of the newer methodologies. For the second phase of this effort, a broader array of chemicals will be screened to evaluate the predictive potential of the

pathways that were identified during the first phase of study. Bob Kavlock, the Director of the EPA's Department of Computational Toxicology, reported at this workshop that ToxCast™ has already initiated proof-of-concept experiments with contracted laboratories using a library of ~ 800 characterized substances, such as pesticides and antimicrobials, and the first progress report is expected to be published in fall of 2008.

While the experimental approaches of ToxCast™ are similar to those used for preclinical drug development, the needs and priorities are somewhat different. For example, false negatives are of little concern to the drug industry, as the goal of HTS screening is to find a few potential leads for future optimization and study. On the other hand, as incorrectly identifying a hazardous compound as safe is unacceptable for the EHA due to its impact on public health. Furthermore, toxicology assays for EHA must ultimately be useful for predicting adverse reactions under scenarios where exposure to one or even multiple chemicals is accidental, and thus dosage and other parameters cannot be precisely known. In contrast, toxicology assays for preclinical drug development are designed to comprehensively evaluate the effects of substances with characterized structures and carefully controlled exposure parameters.

A second multidisciplinary effort to improve the predictability of toxicology assays is coordinated by the National Toxicology Program (NTP) and National Institute of Environmental Health Sciences (NIEHS). This "Tox 21" program, or High Throughput Initiative for Toxicology in the 21st Century, provides both a vision and a strategy to "support the evolution of toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused upon a broad array of target-specific, mechanism-based biological observations" (18). In the broadest sense, The Tox21 Roadmap describes a new paradigm where data from high throughput screens from disease and toxicity studies are combined with those obtained from traditional rodent models and medium throughput assays, such as genomic and metabolomic profiling, in order to develop a mechanistic understanding of toxic outcomes, and to identify biological pathway perturbations that could act as predictors of toxicity in uncharacterized compounds. To complement these approaches, efforts will be made towards development of analytical tools for data integration, expansion of endpoint markers for *in vivo* studies, and use of nonmammalian model systems (worms and zebrafish, see Section 7.6.) to perform targeted studies.

In February 2008, a memorandum of understanding was established between NIEHS/NTP, the NIH National Human Genome Research Institute/ Chemical Genomics Center (NHGRI/CGC) and US EPA that enables the sharing of resources for implementing Tox21, including the use of robotics for screening, chemical libraries for evaluation, and toxicology data from previous investigation. In the near term, plans are in place to test ~7600 compounds, map their toxicity pathways, develop capabilities for metabolic analyses and populate databases that will cross-reference one another. Structurally enhanced pathway enrichment analysis (SEPEA) will be used to achieve both qualitative and quantitative data, as dosage is highly relevant in evaluating the effects of environmental exposure. Longer term goals include elucidating the effects of multiple inputs on toxicity profiles and studying how the combination of genetic background and environmental history contribute synergistically to toxic substance sensitivity. The outcomes of these efforts will be made freely available to the public, government, scientific and medical communities.

4.3. Biomonitoring and Human Surveillance

As mentioned previously, predicting toxicity from environmental pollutants is complicated by the fact that the nature of exposure is usually unknown in terms of dosage and duration. Furthermore, individual susceptibility to toxic substances can vary greatly due to a combination of genetic differences, environmental history, and confounding factors such as exposure to chemical mixtures rather than to a single substance.

Finally, exposure to pollutants is often low-dose and chronic, variables that are difficult to recreate in a clinic and thus are inherently more difficult to evaluate.

In recent years biomonitoring, or the analytical measurement of biomarkers in the body or its fluids, has become an increasingly important way to gauge the dosage and duration of an environmental exposure. Studies by the EPA, Center for Disease Control, and other organizations, along with the improved knowledge of biomarkers and screening methodologies, have led to the growing availability of large amounts of biomonitoring data (19). It is widely believed that continued efforts for biosurveillance will form an integral component of the development of predictive assays for evaluating environmental toxins, as this is currently the best way to address the unknowable parameters that contribute to an individual's response.

4.4. Stem Cells and Environmental Health Assessment

The potential for stem cell models to add relevance and scope to predictive toxicology in preclinical development was discussed extensively in Section 3, and these same principles hold true when applied to the field of environmental health assessment, particularly when combined with data from biomonitoring. The areas of greatest priority differ slightly from those of the pharmaceutical industry, mainly due to the need for EHA to protect human health from a wider range of toxic compounds with limited knowledge of the exposure parameters. In addition, exposures to multiple chemicals simultaneously can lead to synergistic adverse effects that can be difficult to interpret.

4.4.1. Replacing or Enhancing Current *in vitro* Models

Christopher Portier, the Associate Director for the NTP/NIEHS, described several ways in which hESCs are envisioned to advance the goals of the Tox21 initiative. First, stem cells and their derivatives would form the basis of high throughput screening methods, both to enable new types of screens as well as to replace currently used primary cells and transformed cell lines. The disadvantages of transformed cell lines were listed in Table 1. Second, stem cells could be used to form tissues for new *in vitro* assays and for creating chimeric animals models with humanized organs. Finally, stem cells could be subjected to comprehensive profiling experiments to map the transcriptomic, proetomic, and epigenetic changes that occur during development.

In the near term, hESCs might provide superior models for ongoing EHA screens that are utilizing rodent and human cell lines to screen thousands of compounds for toxic effects. In the future, stem cells could potentially be used to generate *in vitro* organs for even more powerful assays.

4.4.2. iPS Cells

The ability to create iPS cells is a high priority for EHA. If iPS technology proves reliable, cell lines from different ethnicities, genders, ages, etc. could be incorporated into toxicology screens so that the contribution of genetic background and environmental exposure could be assessed. Such systems, particularly disease-specific lines, may also enable elucidation of disease mechanisms and evaluation of the relative contribution of genetic and environmental factors to disease etiology. The use of iPS in combination with biomonitoring data could enable a better understanding of the suite of environmental exposures that an individual has incurred, and could offer unprecedented opportunities to identify the factors that contribute to environmental toxicity. Thus, iPS has tremendous potential to improve risk management in the future.

4.4.3. Reproductive and Developmental Toxicity

Current environmental toxicity assays are reasonably robust for predicting the effects of exposure in adults. There are growing concerns from both the government and public, however, about the level of

environmental pollutants and their potential effects on human development. The rising frequency of cancer, autism, and changes in pubertal timing seem to underscore this apprehension. Existing assays are limited in their ability to predict early development effects arising from *in utero* and early life exposure. Use of undifferentiated stem cells for screening environmental toxicity could lead to mapping of developmental pathways and ultimately provide benefits beyond the field of toxicology, such as uncovering the cellular perturbations in early development that underlie poorly understood diseases.

4.4.4. Genotoxicity, Carcinogenesis and Immunotoxicity

Elucidating toxicity pathways that affect DNA repair or other genetic processes could offer new insights into cancer mechanisms and aid in the identification and classification of carcinogens. Dissection of immunotoxicity pathways could lead to a better understanding how environmental exposures to pesticides are affecting asthma rates, allergies, cancers, and other diseases that stem from abnormal immune function. For these latter studies, hematopoietic cells derived from hESC and iPS cells would be very useful.

4.4.5. Organ Toxicity

Advances in tissue engineering are allowing for toxicity testing in 3-dimensional structures that are beginning to mimic organ structures. As seen with preclinical drugs, many environmental toxins damage organs such as the liver, the heart and nervous tissue. In addition, many environmental toxins are metabolized in the liver. Generating organs using tissue engineering might therefore be useful in the future for designing assays to assess environmental hazards, and in fact Raymond Tice (NIEHS/NTP) indicated that the National Institute of Health plans to issue a Roadmap for three dimensional tissue engineering. Stem cells could play a key role in achieving functional model organs and tissues for EHA and other avenues of investigation, including disease research and tissue regeneration.

5. TECHNOLOGICAL ADVANCES IMPACTING STEM CELL USE AND IMPLEMENTATION

As described above, there are limitations in current stem cell methodologies that must be addressed in order for the full potential of this technology to be realized. Although these needs vary somewhat by cell system, the same fundamental issues are at the base of each hurdle. Fortunately, there are several new tools and technologies under development that should be generally applicable for both improving and optimizing the use of stem cells in predictive toxicology, regardless of cell type.

5.1 Technologies that Address Culturing and Growth Bottlenecks

Unlike terminally differentiated cell lines, human embryonic stem cells (hESCs) have proven to be somewhat difficult and expensive to propagate and grow in large numbers. There are several explanations for this phenomenon, the main one being the challenging task of recapitulating the appropriate environmental context, or orchestrated interaction of multiple cell types and growth cues, under which hESCs naturally develop. In addition, standard tissue culture conditions are two-dimensional and do not comprise scaffolds, extracellular matrices, or other mechanical factors that establish the three-dimensional architecture that is likely to be necessary for proper cell maintenance and differentiation into functionally relevant tissues. As a corollary, there is evidence that the natural byproducts of cell propagation, such as acids, can inhibit the growth and potency of stem cells when they accumulate in the growth medium. There is also evidence that final differentiation of cells is greatly facilitated in an *in vivo* environment. Workshop participants agreed that an optimal culturing environment for most types of stem cells would likely include the presence of more than one cell type (co-culture), a scaffolding system for three-dimensional growth architecture, and an ability to control the cellular microenvironment, including such parameters as pH, temperature, osmolarity, and oxygen concentration.

Several new technologies are being developed that address the deficiencies in standard culturing practices and show promise for overcoming the bottlenecks in cell growth, scale-up, and establishment of functional relevance. For instance, microelectromechanical systems (MEMS) technology can be used to build specialized slides or culture dishes that allow researchers to generate oxygen gradients across the surface of a field of cells (20). Similar devices could be used to control the level of DNA, RNA, proteins, chemicals, or a variety of other factors that could be used to modify or optimize the microenvironment for improved cell growth, differentiation and potency. Another new device that could prove beneficial for overcoming certain culturing bottlenecks is the benchtop microreactor. Microreactors are simply miniature versions of bioreactors, which are specialized chambers for scaled-up cell growth that allow constant surveillance and oversight of growth conditions such as pH, oxygen concentration, temperature, etc. A 24-well microreactor has been designed that can fit on a benchtop, can be used for small volumes, and is 50 times less expensive than a traditional model (Applikon, Inc.). This device could be used to test many conditions simultaneously, reducing the time and improving the efficiency at which culture conditions could be optimized.

In addition to improved control over growth conditions, new tools have been developed for enabling co-culture, scaffolding, and three dimensional cell organization to occur *in vitro*. One such technology allows cells to be mixed and applied to a semiporous membrane, where they can establish a three dimensional, organ-like growth architecture (Capsant, UK). These “Hi-Spots” can be expanded to 96 well format, enabling high throughput applications and assays to be devised. Other promising tools include collagen

nanofiber scaffolds, which can be used to promote cell alignment, and devices for applying shear stress to cell layers, thereby promoting tissue organization.

5.2 Computational, Systems-Based and *in silico* Strategies

As described earlier, applying high throughput screening methodologies to stem cells should provide a powerful and relevant model for predicting toxicology. However, as is the case with analogous studies, a significant portion of research would be devoted to developing and utilizing bioinformatics and biostatistics to analyze, integrate, and interpret the data. To meet these needs, computational approaches are being devised not only to analyze raw data but for other useful applications such as cross-referencing of multiple data sets, identifying meaningful patterns in complex data, enabling prioritization of efforts based on likeliness of relevance, and suggesting new avenues of research that are likely to succeed. For example, Dr. Michael Reed presented a "virtual" physiologic model system created from combined data from the public literature from animal studies and clinical data (Entelos, USA). Virtual experiments can be performed using this model system, and the results can be used to direct, focus, or prioritize efforts in a conventional experimental model. Dr. Richard Brennan described a second *in silico* method in which statistical algorithms and machine learning programs were used to analyze canonical biological pathway maps, public databases, and gene expression data in order to identify functional descriptors of toxicity. The resulting computational tool can integrate predictive toxicogenomic data with that from mechanistic analyses, and was successfully used to correlate nephrotoxicity with a particular functional perturbation (21). This technology has also shown promise for predicting other forms of toxicity including oxidative stress, skin irritation, phospholipidosis, and hepato- and ocular toxicity (GeneGo, Inc.).

5.3 Chimeric Model Systems

Dr. Gary Peltz described a slightly different way that stem cells could contribute to predictive toxicology Rather than using human cells for an *in vitro* assay, a "humanized" animal model was created for clinical studies (22). Immunocompromised mice were created that will accept human liver cells after their own have been destroyed. This approach suggests that stem cell technology might eventually allow replacement of other organs in model systems with human versions. It is thought that these hybrid models might offer more relevant toxicology data to be obtained from more conventional, *in vivo* methods.

6. VALIDATION AND ACCEPTANCE

6.1. Industry Acceptance and Integration

Given the enormous costs of drug attrition, industry representatives are interested in assays that could improve the predictability of toxicity assays, even if said assays were more expensive up front than high-throughput screening assays that are in use currently. Moreover, demonstrating toxicity that is not revealed by an animal screen or flagging drugs as potentially toxic before animal studies are initiated would offer additional benefits, including the ability to modify a candidate drug to reduce its toxicity early in the development process. To integrate or replace standard toxicology models in drug discovery and development, there are two important milestones that must be achieved. First, new assays must be scientifically validated to ensure that they are more relevant, accurate, or efficient than current methodologies. Second, strategic guidelines must be in place for eventual regulatory acceptance so that candidate medicines that result from new assays will be available for human use.

6.1.1. Method Validation

Scientists and industry representatives indicated that they would be eager to integrate *in vitro*, stem cell-based toxicology models into their screening procedures, provided certain criteria are met. Most importantly, new assays must be validated by several independent lines of investigation to prove that they are physiologically and scientifically relevant for the system under study. This means that in order for hESC-derived cells to replace primary cells, they must be examined for a sufficient number and variety of endpoint markers to suggest that they are functionally equivalent. In addition, stem cell derivatives should recapitulate known results from mechanistic studies using authentic tissue cells, or should yield data that corroborates results obtained from one or more independent lines of investigation, including mechanistic studies or tests in alternative model systems.

For *in vitro* assays, the ability to correlate an observed pathway perturbation with an *in vivo* effect would be the most desirable form of validation. Achieving this end, however, is complicated by the deficiencies of current models. For example, how much weight should be given to an assay that successfully predicts toxicity in animal studies (which can be easily be validated by conventional models) when the goal is to improve predictive toxicology for humans? In other words, should the gold standard for validation be prediction of toxicity in animals or humans? Clearly the latter is more relevant, but independent *in vivo* validation in humans may not be possible due to ethical considerations and experimental impracticality. Workshop participants suggested the need to identify and make available compounds with known human toxicities for testing and validation purposes. In fact, drug companies have already started to make these chemicals available for such efforts. Roche recently signed an agreement to share two sets of 25 well-characterized kinase inhibitors with Cellular Dynamics International, a company that is developing stem cell-based assays for cardiotoxicity. In addition, several pharmaceutical companies have come together under the Stem Cells for Safer Medicine consortium in the United Kingdom to share reagents (see Section 8). These collaborations promise to yield important validation tools for stem-cell based assays.

Nonetheless, it is probable that stem cell -based toxicology assays must necessarily be compared with the standard animal safety studies in order to determine whether or not they are superior at predicting adverse outcomes. An essential validation approach will be to correlate toxicity in stem cell-based models using sets of control compounds that have been characterized with an existing animal model. Another form of

validation could be pursuing further mechanistic understanding of pathways and how they are perturbed by drugs, then performing targeted tests in alternative models to generate corroborating evidence.

Due to the complexity and heterogeneity of drug responses, it is likely that no one validation strategy will be broadly applicable, but rather that each test will demand its own set of standards that will be determined by the nature of the investigation. While industry toxicologists will likely determine these criteria themselves, the National Institute of Environmental Health Science (NIEHS) and the National Toxicology Program (NTP) have jointly sponsored an initiative to derive validation guidelines for general toxicological testing I EHA. These measures also define criteria for regulatory acceptance and are discussed below.

6.2. Government Acceptance and Integration

Although imperfect, the current model systems for predicting toxicology and classifying environmental hazards have been the "gold standard" for years, and replacing or enhancing them with cell-based approaches will require extensive and convincing validation from both a scientific and regulatory perspective. To develop guidelines and establish criteria for such matters, several government agencies have come together to create the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) (23)**.** To address regulatory issues, NICEATM established the Interagency Coordinating Committee on the Validation of Alternative Methods, or ICCVAM, a collaboration of 15 federal research and regulatory agencies whose goals are summarized in Table 2. In addition to administering ICCVAM, NICEATM provides technical support, facilitates communication between methods developers and government agencies, resolves potential conflicts of interest, organizes workshops and study groups, and conducts validation studies to evaluate new methods that may reduce, replace, or refine animal use for toxicity testing. An additional goal of NICEATM is to harmonize their efforts with additional "VAM" committees that have been established internationally, thereby reducing potential trade barriers and facilitating the dissemination of potentially beneficial information. As is the case with other health initiatives, NICEATM activities are transparent to scrutiny and allow input from interested members of the public. Ultimately, ICCAM goals are designed to take into account not only scientific and economic considerations for replacing current methods but also the ethical considerations that are important to both the public and members of the research community, particularly in regards to the use of animals in testing. The combination of the ICCVAM mission and public interest will allow NICEATM to strategize and ultimately make recommendations that will facilitate the incorporation of new model systems into scientifically sound and governmentally-acceptable formats.

Table 2: Purpose of ICCVAM

- Increase the efficiency and effectiveness of U.S. Federal agency test method review
- Eliminate unnecessary duplication of effort and share experience among U.S. Federal regulatory agencies
- Optimize utilization of scientific expertise outside the U.S. Federal government
- Ensure that new and revised test methods are validated to meet the needs of U.S. Federal agencies
- Reduce, refine, and/or replace the use of animals in testing where feasible

7. FUNDING MODELS AND PROJECT STRUCTURE

To identify funding models and partnering options that could facilitate and expedite the development of stem cells for predictive toxicology, CIRM solicited the experience and advice of members of government, industry, and academia. CIRM also asked participants to provide an overview of the intellectual property landscape and other potential barriers to progress.

7.1 Collaborations, Consortia

In general, collaborations between academia and industry can be very productive and are considered to be excellent ways to expedite progress in a desired field. Academic researchers possess the basic expertise and the willingness to take risks in their endeavor to advance scientific knowledge and build careers, while industry provides materials, experience, and infrastructure for large-scale testing and assay evaluation. Before such collaborations can proceed, it is necessary to resolve issues surrounding current and future intellectual property (IP). Workshop participants suggested that CIRM could facilitate the development of academic/industry partnerships by funding research teams, and providing incentives for resolving potential issues with intellectual property and data sharing.

Another type of collaboration that could benefit from CIRM involvement is a partnership between a small biotechnology company and a large pharmaceutical corporation. Smaller companies often have interesting new technologies but lack the compounds or infrastructure required for testing. On the other hand, pharmaceutical companies are reluctant to buy into an untested technology if assays are not "ready to go". To bridge this gap, CIRM might establish "precommercial grants" to allow development and validation of assays that can then be handed over to drug companies for subsequent testing.

A third type of collaboration can be in the form of contract, or fee-for service arrangement. Several pharmaceutical corporations would like to capitalize on the potential of stem cells for *in vitro* toxicology screens, yet do not have in-house facilities for doing so. This type of contracted funding model is very well suited for projects that have clear beginning and end points, such performing a specific screen or assay with a finite set of criteria. CIRM could help support the development of facilities providing these services.

To illustrate the benefits of collaboration, two examples were offered where pharmaceutical companies are partnering with other entities to expedite the development and implementation of *in vitro*, stem cell based assays for improved toxicology prediction. As alluded to previously, Cellular Dynamics International (CDI) and Roche Palo Alto recently came together to develop and test new models for predicting toxicity using hESC-derived cardiomyocytes. Under the terms of this agreement, Roche will provide CDI with two sets of 25 well-characterized kinase inhibitors for testing and validating CDI's current toxicology platforms. CDI will generate data from a wide variety of assays, including genomic profiling and electrophysiology experiments, while Roche will assess cytotoxicity using biochemical and high content imaging analyses. Shared data from these approaches should be useful both for validating CDI's assays and for enabling Roche to develop improved models for predicting toxicity.

While Roche and CDI initiated their own interaction, the Stem Cells for Safer Medicine organization (SC4SM) in the United Kingdom was established in 2007 in order to facilitate such cooperation, with the goal of developing superior toxicology models from human stem cell sources. SC4SM is a nonprofit corporation that has attracted financial investment from both the public and private sector, including three major pharmaceutical companies, AstraZeneca, GlaxoSmithKline and Roche. In addition to providing funds, Dr. Phillip Wright revealed that these companies have shared proprietary data for over 200 compounds that failed clinical trials due to unforeseen toxicity. Both academic and small business enterprises are eligible for funding, and SC4SM provides scientific oversight in addition to establishing guidelines for validation,

ethical considerations, and management of intellectual property. The results of the first SC4SM-sponsored efforts are scheduled for publication in the summer of 2008.

7.2 IP Arbitration

A key concern that was repeatedly brought to the attention of CIRM was the fact that intellectual property issues and trade secrets are potentially huge barriers to successful collaboration. For example, several pharmaceutical companies have already created, tested and validated thousands of compounds from their own chemical libraries. Much of this data could be helpful for designing or testing predictive assays for EHA screens, providing controls for new screens, for populating pathway databases, for predicting the toxicity of unknown environmental chemicals, etc. However, because the drugs' structures are proprietary, it has not been possible to share information, despite the potential for future benefit to public health. As a neutral party, CIRM might be able to "get the ear" of the highest level industry representatives and facilitate a limited amount of information sharing, perhaps allowing for more efficient use of resources for developing, executing and validating predictive assays. In essence, industry representatives felt that efforts from CIRM to broker and negotiate IP arrangements in advance would do much to establish a safe harbor in which potential collaborators could focus on the science rather than the fear of losing assets.

8. TARGET AREAS OF PRIORITY FOR CIRM

The culmination of two days of presentations and discussions led to several interesting suggestions and a reasonable consensus on the key areas of study that would offer the most potential for high impact, shorter term benefits in improving stem cell technology, and enabling its use for predictive toxicology in the preclinical and environmental health assessment fields. First, validation of stem cell based toxicology models will be of the utmost importance for establishing proof of principle and for demonstrating their advantages over the currently used standards. For these methods to become mainstream, it will also be necessary to develop improved protocols for cell growth, maintenance, and differentiation, and to define *in vitro* phenotypes that correspond to relevant human endpoints. A second critical goal will be the derivation of iPS cell lines from a diversity of individuals, including those with known disorders or disease susceptibilities, to allow for the development of comprehensive, customizable approaches for correlating toxicity mechanisms with variable individual response. Additional priorities include the use nonmammalian models and nonhuman stem cells in order to bridge the transition of animal-based toxicology models to those based on humans, and integration of stem cell based assays into preclinical development as a tool for reducing early drug attrition. Finally, strategies for establishing multi-organizational partnerships, particularly with regards to current and future intellectual property concerns, will be important for removing any potential hurdles that could prevent the necessary progress that is required before stem cells could revolutionize the field of predictive toxicology.

8.1. Tools for Assay Development and Validation

8.1.1. "Olympiad[1]" Cell Lines and Gold Standard Compounds

Meeting participants indicated that a toolbox for assay development and validation is the most pressing need in order to bring stem cell based toxicology assays into mainstream use. The basic components of this toolbox were identified as "Olympiad" cell lines, "gold standard" compounds and robust differentiation procedures.

Cell lines to be used for validating *in vitro* assays should be robust, grow vigorously in culture, show reproducible behavior and differentiation, and preferably derive from human sources. Participants suggested that efforts should be directed towards screening existing stem cell lines for these favorable properties. If no suitable lines can be found, new cell lines could be made or alternative sources of stem cells could be tested (see below). The outcome of this research would be a set of robust human cell lines with fully characterized differentiation profiles that would be made freely available in large quantities ("Olympiad" lines). It was also suggested that that a cell banking facility could maintain and distribute these materials to interested parties. Finally, participants agreed that more research was needed on the molecular mechanisms that determine terminal differentiation of specific cell types, particularly hepatocytes (see below).

In addition to the Olympiad lines, participants expressed a need for a "Gold Standard" set of reference compounds with known *in vivo* toxicity profiles, both positive and negative, to be used as benchmarks for development and validation of new assays. These compounds should represent several forms of toxicity and should also be freely available to the scientific community.

[1] Dr. Bruce Conklin, UCSF

8.1.2. Improved Methods for Differentiation

While it is currently possible to derive specific cell types from embryonic stem cells *in vitro*, it is not clear that the resulting derivatives possess the same terminal differentiation endpoints that define their primary tissue counterparts. To develop improved methods for controlling differentiation, it will necessary to have a comprehensive library of markers that can be used to monitor the differentiation process and ensure that cells are sufficiently expressing indicators that are stage-appropriate. Further, key transcription factors and regulatory proteins are needed so that differentiation can be controlled and the various stages scrutinized. Metabolic profiling and mechanistic studies comprise two key areas that are contributing to the identification of markers, which can be tested in authentic tissues and compared with those derived from stem cells.

8.1.3. Culture Conditions and 3-D Environment

Many of the workshop participants felt that appropriate culturing conditions and three-dimensional environment might be important for optimal stem cell growth, differentiation, and possibly maturation, particularly in the case of hepatocytes and neural cells. The consensus is that research in this area is needed to resolve bottlenecks and improve the accuracy and relevance of stem cell based screens. Participants believed that multidisciplinary collaboration is required to overcome these hurdles, for example collaborations of engineers, chemists, biologists, etc.

8.1.4. Alternative Sources of Stem Cells for *in vitro* Screens

Thus far, the majority of research efforts have focused on stem cells that are derived from blastocysts, and hESC-derived hepatocytes and other derivatives may display characteristics of fetal rather than mature tissue. Efforts could be applied towards screening differentiated cells from alternative pluripotent cell sources to determine whether they possess features that might be more appropriate for current screens or for creating Olympiad cell lines. One potential source of stem cells comprises the progenitor cells that are found in organ tissues. For example, hepatic stem cells (HpSC) are found in fetal, neonatal or adult liver, can be cultured, and express markers that are indicative of, and appropriate to hepatic cells. Stem cells are also found in amniotic fluid, placenta and umbilical cord blood. Encouragingly, amniotic fluid cells (AFC) have already been shown to differentiate into bone, liver, heart, and other cell types and express markers that are appropriate for those lineages. Finally, stem cells could be created from differentiated tissues or somatic cells (iPS cells). Although these alternative cell sources have not been studied as extensively as have embryonic stem cells, it is possible that these lines or their derivatives will prove more robust, more reproducible, or more relevant for a given assay.

8.1.5. Correlating *in vitro* phenotypes with *in vivo* effects

To be useful for predicting toxicology, the output from *in vitro* assays must correlate with defined human endpoints. For example, specific electrophysiological perturbations in beating cardiomyocytes can be correlated with certain types of arrhythmia. However, for other forms of toxicity, it is not always clear that a straightforward correlation will be apparent. One promising way to identify toxicity phenotypes is to use stem cell models for screening known toxic substances, and identifying the pathway perturbations that result from this exposure. These data could be used to assign a "toxicity signature" that could then be used to potentially identify the mechanism of an unknown compound. Another method for identifying useful *in vitro* phenotypes is through mechanistic studies of specific biological pathways. The effects of toxins often mimic the symptoms of a known disease, and these similarities may provide hints as to which biological mechanisms are affected. This information can be used to steer the course of assay development by providing hypotheses that can be directly tested. As reported in Section 5, several new *in silico* technologies

and systems-based approaches are essentially using this strategy by integrating and synthesizing data from multiple lines of investigation and using this information to suggest specific experiments. Finally, it is possible that information from human surveillance might be used to identify *in vivo* endpoints that correspond to *in vitro* phenotypes. For example, persons with known susceptibility to a particular drug might have a distinguishable metabolic signature that could be revealed through biomonitoring. This information could then be used to design assays for detecting this signature as an indicator of toxicity or a predictor of adverse response.

8.2. A Diverse Bank of Induced Pluripotent Stem (iPS) Cells

iPS cells represent a most desired resource for multiple lines of investigation in all aspects of predictive toxicology. The ability to derive stem cells from adult tissues offers unprecedented opportunities to investigate genetic and environmental contribution to adverse reactions from drug or environmental exposure. Similarly, iPS cells could be used to perform retroactive studies when new adverse reactions are identified. In addition to their scientific value, iPS may resolve lingering ethical issues with use of embryonic sources for stem cells and potentially avoid certain hurdles surrounding intellectual property. The most useful iPS cell lines would be those derived from a range of backgrounds, known drug susceptibilities, and diseased states. Available donor tissues should be selected based on the type and extent of diversity that is required for a particular assay. At the least, iPS should represent differences in gender, age, ethnicity, and relevant disease or drug sensitivity, if known.

8.3 Studies with Animal Stem Cells and Nonmammalian Model Systems

Stem cell-based assays are envisioned to ultimately improve, enhance or replace current animal models for toxicology prediction. All new technologies, however, must be validated for accuracy by comparison with data obtained by conventional methodologies. To bridge the transition from animal to human, it could be useful to develop assays based on stem cells derived from traditional model organisms, such as rodent, canine, rabbit, etc. This way, data from *in vitro* analysis could be directly compared to those achieved from *in vivo* studies to maximize their potential for recapitulating a functionally relevant response Such translational studies have already been initiated in Europe, both to decrease the number of animals that are used for research as well as pave the way to more convincing validation strategies for predicting human outcomes.

Another type of study that could complement efforts to derive functionally relevant *in vitro* assays for toxicology prediction are mechanistic studies in *Caenorhabtitis elegans* and *Danio reria* (nematode and zebrafish). Both of these model organisms are extremely useful for investigating developmental mechanisms and could be used to identify potential pathways for targeted assay development in stem cells. The NIEHS/NTP initiatives described in Section 4 called for increased use of these models for investigating environmental toxicity, and many research groups in academia have extensive experience and expertise in these areas. A potentially promising avenue for expediting assay development and validation would be to provide these laboratories with Olympiad lines, allowing them to adapt promising new technology for their own needs while providing new and relevant uses for predictive stem cell models.

8.4. *In vitro* Preclinical Trials

One idea that generated considerable interest during the CIRM workshop is the concept of a "preclinical trial" in a dish. In essence, promising leads could be assessed for toxicity using a battery of *in vitro* assays before initiation of animal and/or human trials. Chemicals that show potentially detrimental effects could be weeded out at earlier stages of development, saving money, time, and lowering attrition rates. Chemicals that behave poorly at earlier stages could be reevaluated and possibly modified for a second round of testing. Finally, leads that are tested on cells derived from individuals with diverse genetic and environmental histories might reveal differences in individual drug susceptibility, allowing for more appropriate testing to be performed in the later stages of development. Such methods could result in the production of safer, more customized pharmaceuticals.

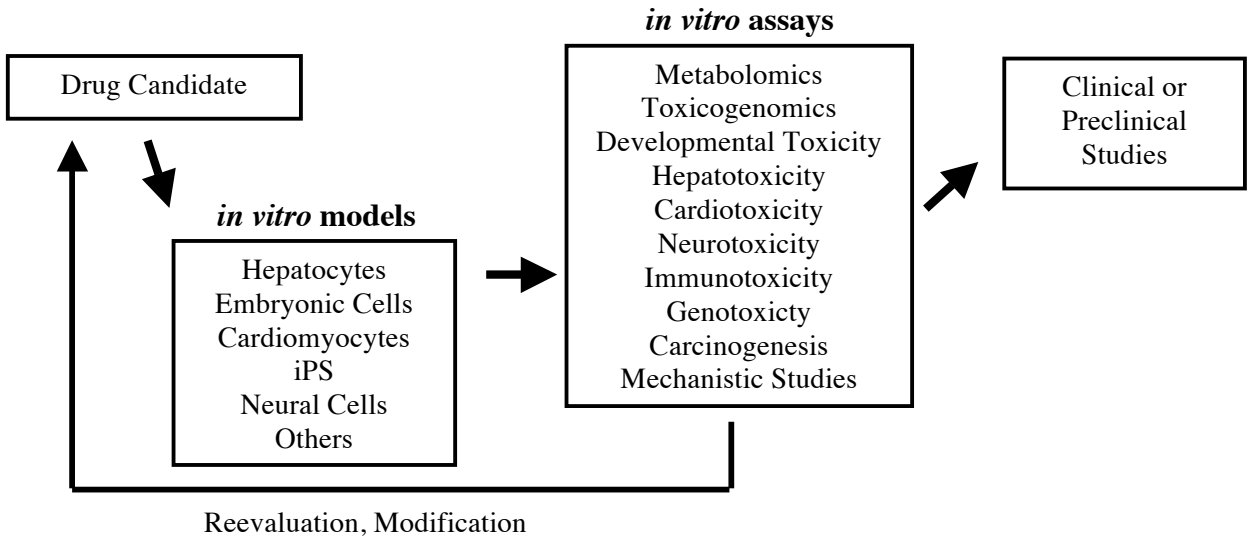

Figure 1. *In vitro* preclinical trials. Drug candidates could be tested in various model systems with a variety of assays. Toxicity positives could be withdrawn, reevaluated or modified for subsequent screens.

8.5. Multidisciplinary Team Coordination and Information Fostering

It is clear that collaborative, multidisciplinary efforts are key to resolving the current technological bottlenecks that prevent stem cell technology from being widely disseminated. CIRM could be useful in establishing collaborations, providing guidance and facilitating the necessary cooperation between teams. Including members from both Pharma and EHA on advisory boards could foster information sharing and ensure that predictive model improvements meet concerns from both areas.

9. WRAP UP

The goal of the Stem Cells in Predictive Toxicology Workshop was to gather expert knowledge and experience in order to evaluate the potential for stem cells and their derivatives to provide predictive toxicology tools for drug discovery and environmental health assessment. Topics included a discussion of the limitations and deficiencies of the current toxicology approaches and how human stem cell based *in vitro* assays might overcome them; reports of how stem cells are being used and how they could eventually be adapted for predictive toxicology in drug discovery and environmental health assessment; a discussion of the most useful stem cell models and the areas in which they are most likely to be of use; identification of the technological limitations that prevent stem cells from being integrated into standard practice, and an overview of new tools and technologies that may overcome them; a discussion of the technical and regulatory requirements for validating stem cell based toxicology models and integrating them into mainstream practice; and the types of projects and collaborations that are most useful for transforming cell based toxicology models into realty. At the conclusion of the workshop, participants offered a prioritized list of the areas in which CIRM could make the most substantial contributions to the development and implementation of stem cell based models for predictive toxicology. A summary of the key points is listed below.

Value:

• *In vitro* toxicology assays based on human stem cells and their derivatives would offer significant advantages over current animal models and assays based on primary and/or transformed cell lines, largely due to improved relevance and greater versatility.

• More relevant assays should translate into safer drugs, lower attrition rates, and a better understanding of disease and toxicity mechanisms.

• Stem cells derived from adult tissues (iPS cells) would allow assays to be designed where the contribution of an individual's genetic background or environmental history to toxicity response can be determined.

Uses:

• Stem cells and their derivatives have already proven valuable for screens and mechanistic studies including analysis of neurodegenerative disease, metabolomic profiling and screens for developmental toxicity.

• iPS cells offer the potential to unravel factors contributing to variable drug response, i.e. ethnicity, gender, age, developmental stage, environmental exposure history, genetic variation, etc.

• hESC could be used to map developmental pathways and produce predictive models for fetal and reproductive toxicity.

• Stem cells and their derivatives could provide virtually unlimited sources of tissue for a wide range of toxicity models.

• Stem cells could be used to replace organs in conventional animal models with more relevant, humanized versions.

• Comprehensive profiling of hESC (genomics, proteomics, transcriptomics, metabolomics, etc.) could be used to elucidate pathway perturbations that underlie toxicity and disease, enabling the development of predictive assays for adverse drug response.

• Stem cell based approaches, combined with data from human biosurveillance, offer the potential to predict adverse response from exposure to environmental pollutants, resulting in improved labeling and usage guidelines.

Needs:

• To develop predictive assays, scientists must have access to unlimited quantities of stem cells with robust and reproducible behaviors. Protocols must be available for scaling, differentiation and validation of the differentiated state.

• The two most important stem cell derivatives for toxicity screening in preclinical drug development are hepatocytes and cardiomyocytes, as liver and heart toxicity are the leading contributors to adverse human response and high drug attrition. Hepatocytes must be metabolically active, as it is often the byproducts of drug metabolism that result in toxic outcome.

• hESC assays for predicting toxicity must be validated at both the scientific and regulatory level. By necessity, this entails comparison with data from current animal models, generation of new data that can be corroborated by other methods, and possibly the development of several species-based stem cell systems (rabbit, guinea pig, canine, etc.) to bridge the transition from animal to human-based models.

• For new toxicity screens, it will be necessary to identify *in vitro* phenotypes that correspond to human, *in vivo* outcomes. Two ways in which this might be achieved are the mapping of pathway perturbations via comprehensive profiling experiments, and elucidating the molecular mechanisms of specific toxicities disease. These lines of investigation may be expedited by use of systems based and *in silico* tools described in Section 5.

• iPS cells from specific populations are needed to elucidate the factors that contribute to variable responses to drugs and environmental exposure. Potential sources include tissues from patients that suffered adverse drug responses, and individuals representing a diversity of gender, ethnicity, age and environmental histories.

Challenges:

• Current hESC derivatives recapitulate some, but not all of the features of authentic differentiated tissues. Additional research is needed to produce hESC-derived hepatocytes with mature liver function, and to produce neural cultures with the appropriate neuron/glial interaction.

• Efforts are needed to identify end point markers and procedures that can be used to verify the authenticity of tissues derived *in vitro* from stem cell progenitors.

• New procedures and technologies are needed to improve the scalability, survival and authenticity of hESC cultures. Providing an appropriate growth environment could allow the establishment of three dimensional tissue architecture, a more accurate representation of the natural landscape under which stem cells differentiate.

• Computational, systems based and bioinformatics procedures will be needed to integrate, analyze and interpret the massive amounts of information that will be generated from the use of high throughput, cell based assay for predictive toxicology.

• For hESC, the intellectual property landscape is currently restrictive. Use of iPS cells may avoid these concerns.

Solutions:

• Multidisciplinary collaborations are effective tools for overcoming technical limitations and may be key to resolving bottlenecks in stem cell culturing and scaling techniques.

• Bioreactors and MEMS technology show promise for optimizing culture conditions and promoting robust cell growth and maintenance.

• Alternative sources of stem cells have been discovered (HpSC, amniocytes, iPS) and may prove easier to culture and maintain than hESC lines.

• Metabolomic and other profiling methodologies show promise for identifying biomarkers that can be used to validate the differentiated state of stem cell derivatives; same experiments can be used to map pathway perturbation in response to drugs, identify *in vitro* phenotypes that might correspond to *in vivo* outcomes.

• Systems biology approaches and *in silico* methodologies could be useful for integrating and synthesizing data from multiple lines of investigation, and determining the most relevant course of subsequent research.

• Collaboration between drug companies and smaller research entities (academic, biotech, government) is a promising route to developing and validating new stem cell assays; companies could provide compounds and accompanying toxicity data for testing technologies under development.

• Increased use of human surveillance and biomonitoring studies could facilitate the design and analysis of predictive assays for environmental toxicity, where exposures levels are unknown and unintentional.

Outcomes and CIRM Priorities

1. Participants suggested that a toolkit for assay development and validation should be assembled and made freely available to the scientific community. Components include:

• "Olympiad" cells lines. Currently available hESC lines should be obtained and screened for the properties of robust growth and reproducible behavior. The differentiation profiles should be characterized, and standard protocols should be developed for maintenance and differentiation. Cell lines could potentially be distributed through the American Type Culture Collection (ATCC) or other bioresource center.

• Gold Standard Compounds. A library of compounds with well-characterized toxicity profiles, both positive and negative, should be made available for assay development and validation; the library should include all accompanying data including structural and mechanistic information, if known. Gold Standard Compounds could be obtained from NIEHS/NTP, which has around 45 freely available chemicals, and potentially from pharmaceutical companies, by donation or contract, on the Stem Cells 4 Safer Medicine.

2. A series of *in vitro* assays need to be designed and tested in order to validate stem cell-based toxicology models. Although the ultimate goal will be to correlate these with effects in humans, assays using animal stem cells should also be tested in order to bridge the transition from animal to human model. As traditional toxicity data has been obtained from various animal models, it important for validation that data from new assays be reconciled with an accumulated body of knowledge derived from decades of prior research.

3. iPS cells will potentially revolutionize the field of toxicology by enabling variability in drug susceptibility to be incorporated into predictive models. To fully realize this possibility, more research must be done to assess the reliability and reproducibility of these cell lines. In addition, iPS lines need to be derived from a diversity of individuals representing various genders, races, ages, and known susceptibilities to drugs and disease. The precise number and nature of these samples should be determined by toxicologists and/or other experts.

4. "Preclinical trials in a dish" could be incorporated into the drug development pipeline by utilizing *in vitro* toxicity assays based on iPS cells, hESC or their derivatives for preclinical testing. Toxic leads could be removed at an earlier stage of development or reevaluated for future study. Eliminating potential toxic substances at earlier stages of development will save labor, time, and reduce the potential for adverse human outcomes. Preclinical trials in a dish using iPS cells need to be designed and tested in order to enable more customizable drugs to be developed for persons with drug susceptibilities.

5. Sharing of knowledge and resources, including libraries of compounds with known structures and toxicity profiles, offer the most expedient and efficient route for designing, testing, and validating new assays. Due to intellectual property concerns, CIRM could move the field forward by establishing a safe harbor in which the concerns of collaborating parties can be met, ensuring that each collaborator has a goal that is well defined and tied in to both their own and their partner's success.

In summary, workshop participants were extremely enthusiastic about the potential for stem cells to provide superior model systems for predicting toxicity in drugs and environmental pollutants. While technological and cultural hurdles exist, experts were optimistic that these could be overcome. Even in a limited capacity, incorporation of human cell based assays into drug discovery efforts and environmental toxicity screens offers the potential for safer, more customized medicines, reduced costs of drug discovery, reduced or refined use of animal models, and more accurate risk assessment for environmental pollutants. Efforts from CIRM to establish resources for stem cell assay development and validation could expedite these ends.

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