Stem Cells and Predictive *In Vitro* Toxicity Testing

A HemoGenix® White Paper

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INTRODUCTION

The possibility that stem cell research could provide the most significant breakthrough in treating numerous diseases is the hope of investigators, physicians and patients alike. Just the words "stem cells" give patients and their families increased hope that a treatment for a disease may be on the way. A quick search on www.clinicaltrials.gov shows that there are more than 3,770 clinical trials listed that have either been performed or are underway using stem cells directly or differentiated cells derived from stem cells. Many of these stem cell cellular therapeutic applications involve the treatment of patients with conditioning drugs that themselves affect the patient's stem cells and may affect the stem cells used to treat the patient. Since the majority of these drugs target one or more steps in the proliferation process and the latter is one of the most basic properties of stem cells, it follows that stem cells are extremely sensitive to cytotoxic drugs.

For decades, it has been taken for granted that toxicity to normal cells is one of the many side effects of anti-cancer drugs. However, a compound does not have to be an anti-cancer drug to cause harm or affect stem cells. Virtually any compound might have an affect on stem cells in either a positive or negative manner. The important aspect is whether the compound has a differential effect on the targeted cells and normal cells. This therapeutic ratio or index has to be taken into account when considering potential toxicity, risk, safety and efficacy. It has been estimated that the attrition rate for all drug candidates is approximately 40% [1]. This proportion is much lower for marketed drugs, but the consequence that a drug is removed from the market due to unforeseen problems is much greater. This is one of the reasons why harnessing predictive toxicity testing is so important. As a result, drug companies have not only been searching for toxicity detection methods, but also new cell-based targets used with those methods. It is therefore not surprising that stem cells represent a natural fit as the new cell-based targets.

The development and potential use of human embryonic stem (ES) and human induced pluripotent stem (iPS) cells has fueled the hunt for new cell-based targets. According to the Drug Safety Executive Council (DSEC) and Cambridge Healthtech Associates (CHA), a number of organizations are either planning, currently evaluating, or, to a far lesser degree, actually incorporating the use of stem cells in routine testing for immunotoxicity, idiosyncratic or general liver toxicity, neural toxicity or cardiovascular effects [2].

Yet, with all the media hype regarding the use of ES and iPS cells, the path from basic research findings to proven and functional therapy is many years, sometimes decades, apart. It should be emphasized that even though ES and iPS cells may instill excitement and potential advancement in many areas, they are still the "new kids on the block" and to embrace the potential of these new stem cell systems, a greater understanding of the biology, physiology and regulation is required. Of the primary stem cell systems employed today for a myriad of applications, lympho-hematopoiesis is the best known and the most investigated. Other primary stem cell systems are the gut, skin, reproductive organs and the specialized cells in the eye. All of these primary stem cell systems exhibit a similar organization and hierarchy. Other primary stem cell systems that have received increased scrutiny include, but are not limited to, hepatic stem cells (oval cells), neural stem cells and lung stem cells. From a practical viewpoint, however, primary stem cells have one major disadvantage over ES, iPS and other stem cell lines; primary stem cells are usually present in minute numbers and are often difficult to obtain and use. This is a distinct handicap that limits the use of primary stem cells for toxicity testing. Yet, if ES, iPS and other stem cell lines are to be used reliably in any form of toxicity testing, they have to be directly compared to primary stem cell systems in order to demonstrate and validate their use as equivalent alternatives. In other words, do the new stem cell alternatives truly

represent the characteristics, properties and responses seen with primary stem cells?

WHAT ARE STEM CELLS?

For a cell to be a stem cell, it must demonstrate the following characteristics and properties:

1. Stem cells must demonstrate the capacity for self-renewal. This is considered the definition of a stem cell. Unlike all other cells, a stem cell, when it divides, can either (a) produce two identical cells, (b) produce one identical stem cell and another non-identical stem cell that would be slightly more mature than its parent and (c), two more mature stem cells. By producing an identical stem cell by division, the capacity for self-renewal is implied. Thus, stem cell self-renewal can be either symmetric or asymmetric depending on the types of stem cells produced. Actually measuring stem cell self-renewal is usually difficult to accomplish and would require a more lengthy article to describe and discuss than the present White Paper. Although stem cell self-renewal is the dominant hypothesis for maintaining the heterogeneous stem cell "pool", it is not the only theory. An animal, person or a stem cell system may be endowed with a finite number of stem cells that exhibit an aging structure, thereby obviating the need for self-renewal [3]. The probabilities of stem cell division also demonstrate the second characteristic of stem cells.
2. Stem cells usually exist in a hierarchy. The hierarchy is a continuum of stem cells in which a more primitive stem cell imperceptibly becomes slightly more mature than its predecessor. The most primitive stem cells are usually quiescent and are not in cell cycle. This does not imply that potentially toxic compounds do not affect quiescent stem cells. Small molecules can enter quiescent stem cells. When induced into cell cycle, the presence of the drug or compound may inhibit proliferation. Quiescent stem cells only enter cell cycle when they are required to do so by responding to feedback mechanisms from downstream elements of the cell lineage. The stem cell hierarchy acts as a buffer so that the stem cell compartment does not become depleted. If this were to happen, the system fed by the stem cells would cease to exist and die. In some cases the distance and time a stem cell must transverse the hierarchy may be very short. In other cases, it may last a lifetime.
3. Stem cells exhibit the greatest proliferation ability and potential of all cells in the body. This ability is also called stem cell "quality", a property that is becoming increasingly important in stem cell therapy. Another stem cell therapeutic property is provided by stem cell proliferation potential. The potential to proliferate increases with the "stemness" or primitiveness of the stem cell. In other words, the more primitive a stem cell, the greater its proliferation potential. Stem cell proliferation potential is a measure of stem cell potency.
4. Stem cells are undifferentiated cells. Stem cells only proliferate. They may demonstrate differentiation ability and potential, but a cell that has initiated the process of differentiation cannot be considered a stem cell because it will no longer possess the properties indicative of stem cells. As a stem cell continues its path from being a primitive stem cell to a mature stem cell, it eventually comes to a stage in which the molecular and cellular apparatus switches to initiate the differentiation process. Stem cell determination is the point at which this switch occurs. The proliferation process overlaps and may still exceed the differentiation process in the early stages, but eventually proliferation will cease altogether. The differentiation and maturation processes take over.
5. Stem cells have the capability of producing one or more lineages of mature, functional cells. The conditions under which a stem cell passes the point of determination and enters a particular lineage is dependent upon many factors, including, but not limited to, the genetic makeup of the stem cell, the environment in which the stem cell finds itself and the requirements to

produce specific cell types.

To be used as a model and predictor of potential toxicity for a particular biological system, the cells should exhibit the above characteristics and properties to be considered a stem cell system.

STEM CELL TOXICITY AS A PREDICTOR FOR SYSTEM TOXICITY

Toxicity is one of the leading reasons for drug failure. The paradigm for drug development is to produce targeted drugs that are safe with low or non-existent toxicity. The reason for extensively testing a drug prior to starting human clinical trials is to try and predict unforeseen deleterious effects before clinical trials begin. It is better to fail a drug candidate during the development pipeline than to fail it later when time, effort and costs increase almost exponentially. For this reason, the biopharmaceutical industry validates and invests in the best technology in order to try and predict potential failure. Yet the power of predicting potential failure rests squarely on the knowledge of one or more biological systems to which the drug has been targeted. If little is known about the biological system in question, then the procedures, tests and assays that should be developed for the specific application will not produce the desired results and the interpretation and conclusions will be erroneous or faulty.

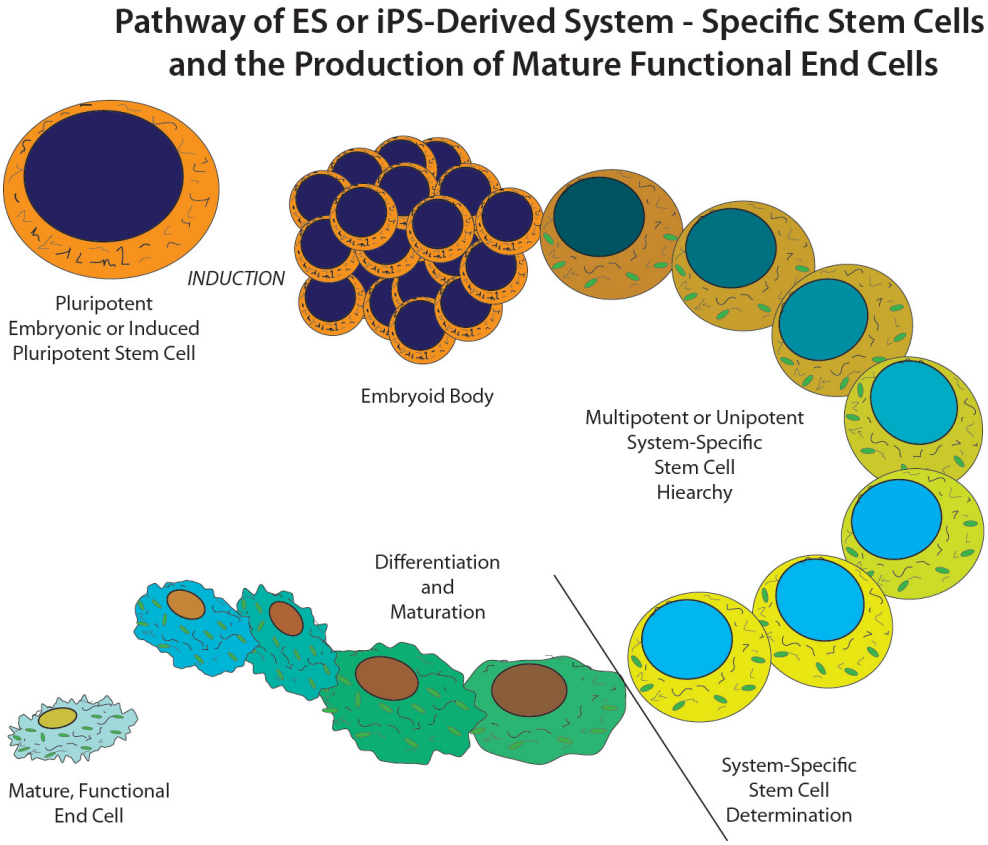
Drugs are usually targeted to a specific step or steps in a biological pathway. But stem cells may be the innocent bystanders that are also affected, often with severe consequences. The stem cells are responsible for producing the mature functional cells. Regardless of whether the stem cells are primarily active during development, partially active during tissue or organ regeneration or continuously active to maintain steady state conditions in the adult, damage to the stem cells will be amplified throughout the system they supply. The ability to detect and interpret changes in stem cell response is pivotal to employing stem cells as targets in predictive *in vitro* toxicity testing.

Stem cells have been associated or shown to be an integral part of many biological systems in the body. This is a clear indication that the use of traditional toxicity testing may need to be dramatically reconsidered [4,5]. Traditional ADME-Tox screening and lead optimization testing often do not predict responses during pre-clinical animal testing. And the results from pre-clinical animal testing are difficult to extrapolate to the human patient [4,5]. For these and other reasons, it is obvious that only the use of primary human cells or equivalent alternatives would be capable of predicting effects during human clinical trials. Given the limitation of availability of primary stem cells, it is clear that ES and iPS cells could provide a distinct advantage. But could ES- and/or iPS-derived cells provide equivalent alternatives to primary stem cells in order to predict toxicity?

Both ES and iPS cells are essentially cell lines and can change with time. They are maintained in a parental state and either differentiate spontaneously or under specific culture conditions into different cell lineage functional end cells. Prior to forming the end cells of a particular lineage, ES and/or iPS cells would probably pass through a secondary system-specific stem cell compartment. Whereas the ES or iPS cells are considered pluripotent because they have the capacity of producing all cell types in the body, system-specific stem cells would be considered multipotent or even unipotent because they produce cells for a specific cell system. This implies that when ES or iPS cells are induced to produce particular cell types, they would pass through a system-specific stem cell stage that should exhibit similar characteristics and properties to the respective primary stem cells (Figure. 1). Can these ES- and/or iPS – derived system-specific stem cells be expanded and maintained for use in culture for predictive *in vitro* stem cell toxicity testing? Do ES- and/or iPS-

derived lineage-specific end cells, such as cardiomyocytes, muscles cells and hematopoietic cells from the mesoderm, hepatocytes, lung and pancreatic cells from the endoderm and neural and epithelial cells from the ectoderm, exhibit similar characteristics, functions and responses as primary end cells? Do they demonstrate embryonic/fetal or adult phenotype? These are questions that have important significance in toxicity testing.

Figure 1



At the present time, the biology, physiology and regulation of ES and iPS cells and cell systems that can be derived from them is still in its infancy. One other aspect that has been known for many years, but has not played a serious role in drug development is the fact that many functions of primary end cells and the production of those cells from stem cells exhibit distinct circadian rhythms. Cells removed from the body will maintain their circadian rhythm. There appears to be a close circadian coordination between different tissue, for example the blood-forming system and the cells in the gut. Circadian rhythm plays a particularly important role in the toxicity and efficacy of drug therapy. Although different for many drugs, there are periods during the day when toxicity can be minimized and efficacy maximized. This is called chronomodulated drug delivery [6]. Determining the correct time of day to administer a drug, regardless of whether it is a conventional or cellular therapeutic drug, can be determined using *in vitro* assays. However, such assays are dependent upon the availability of donor samples to assess the circadian rhythms of the cell populations and other cellular functions involved. The use of ES- and/or iPS-derived stem cells and their lineage-specific cells might be extremely useful in this field. The question is whether ES- and/or iPS-derived tissue or organ specific stem cells and their immediate decedents demonstrate similar circadian rhythms

to those of primary cells. If the answer to this question can be confirmed, it would demonstrate that ES- and/or iPS-derived stem cell systems could indeed be equivalent to normal tissues and provide a further impetus for considering these sources as more than just potential lifesavers.

HEMOGENIX® TECHNOLOGY FOR *IN VITRO* STEM CELL PREDICTIVE TOXICITY TESTING

The purpose of this White Paper was to provide the reader with some of the scientific and practical aspects involved in using stem cells for predictive *in vitro* toxicity testing. There is certainly a significant potential for using ES and/or iPS cell systems, but there are many aspects and questions that need to be addressed and answered before they can be used to predict toxicity outcomes. Yet the field is exciting from many viewpoints. The mere fact that it might be possible to establish a complete stem cell system in the culture plate and to use specific parts of that system to investigate not only potential toxicity issues, but basic biological questions, cannot not be underestimated. In the meantime, it should be emphasized that some primary human stem cell systems can already provide *in vitro* predictive value even at the earliest stages of drug development. Implementing these systems and their associated methodology would not only save time and costs, but also reduce the need for costly and time-consuming pre-clinical animal studies.

The best example is the blood-forming or lympho-hematopoietic system. This continuously proliferating stem cell system is responsible for producing about 2 million red blood cells and 200,000 white blood cells every second of a person's life. With more than 70 years of knowledge accrued, the cellular, molecular and genetic aspects of the biology, physiology and regulation that make up the organization and hierarchy of this stem cell system have allowed *in vitro* hemotoxicity testing to be one of the most predictive areas in toxicology. HemoGenix® has been the leader in *in vitro* stem cell hemotoxicity screening and testing since it launched its HALO® Platform in 2002. Based originally on the "classic" colony-forming cell assay first published in 1966 [7,8], the HALO® Platform has developed into a highly advanced and validated *in vitro* stem cell toxicity screening system. It uses proprietary bioluminomics™ technology that incorporates the standardized measurement of intracellular ATP (iATP) and a bioluminescence luciferin/luciferase reporting system to measure cytotoxicity in up to seven different lympho-hematopoietic stem cell populations from human bone marrow, peripheral blood or umbilical cord blood as well as from seven other different species. Target cell sources are relatively easy to obtain and usually provide sufficient lympho-hematopoietic stem cells for high throughput toxicity screening. Results can, in part, be extrapolated to other continuously proliferating stem cell systems, especially the cells of the gut. HALO®-Tox HT has also been used to predict the best time of day to administer some anti-cancer drugs, the result of which correlate with chronomodulated clinical studies. A paradigm for predicting hemotoxicity using primary stem cells was developed using HALO® in 2005 [9]. In addition, HALO®-Tox HT has been validated and shown to provide greater than 80% concordance between *in vitro* and *in vivo* results [9-12]. A HALO® assay has also been developed for hematopoietic stem cell therapy to measure stem cell potency (HALO®-96 PQR), thereby distinguishing different stem cell populations by their proliferation potential [13].

Another primary stem cell system that is actively investigated and used in cellular therapy and regenerative medicine is the mesenchymal stem cell (MSC) system. Although MSCs have not yet found their place in toxicity testing, the cells of this system are responsible for producing stroma that provides the microenvironment to allow other systems to function properly, as well as chondrogenesis (development of cartilage), osteogenesis (development of bone) and adipogenesis

(development of fat cells). Toxicity to these and other cell systems derived from MSCs can result in considerable damage and the inability of tissues to function properly. Mesenchymal stem cells can be obtained from bone marrow, cord blood as well as other sources, including iPS cells [14]. The organization of the primary MSC system is, in many ways, similar to that of the lympho-hematopoietic system, although not as well documented. HemoGenix® developed the LUMENESC™-Tox HT Platform (similar to the HALO®-Tox HT platform) for *in vitro* MSC toxicity testing. Together with its strategic partner, Vitro Biopharma, MSC toxicity testing using the LUMENESC™ Platform incorporates high performance MSCGro™ culture or differentiation media as well as bioluminomics™ technology to provide the most advanced MSC toxicity testing system available.

In vitro toxicity testing for other primary explanted cell systems is covered by the LumiSTEM™-Tox HT Platform that can be used with cells from the lung, breast, kidney, prostate, bladder and brain to name but a few. In addition, LumiSTEM™ is an ideal *in vitro* assay system for ES- and iPS cells. Specific *in vitro* assays have been developed for iPS cells (LumiSTEM™-iPS) and primary and iPS-derived hepatocytes (LumiCYTE™-HT). In association with ArunA Biomedical, LumiSTEM™-NeuroSC incorporate ES-derived neural stem/progenitor cells, the media to grow the cells and ability to measure viability, cell functionality and proliferation or cytotoxicity using HemoGenix® bioluminomics™ technology.

Since all three *in vitro* toxicity detection platforms use the same standardized bioluminomics™ technology, the response between primary and ES- and/or iPS-derived stem cell systems could be directly compared over time. This provides the ability to ensure that an ES and/or iPS-derived system produces similar or equivalent results to a primary cell system.

CONCLUSIONS

Stem cells do not just include embryonic and induce pluripotent cells that may provide the cure for many diseases heard in news reports. Many biological systems in the body are stem cell systems. Understanding stem cell biology, physiology and regulation may be the key to understanding how this new era in science and toxicology can be put to use to help people in need. Drug development is just one area in which the extensive capacity and capability of stem cells can be put to good use. The technology to detect, measure and predict toxic side effects of new drug candidates is certainly available using several primary human *in vitro* stem cell systems. In time, the promise of ES and iPS cell technology could provide equivalent and perhaps better sources by which drug toxicity is 100% predictable.

REFERENCES

1. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates? Nat Rev Drug Discov. (2004) 3:711-715.
2. Drug Safety Executive Council. http://www.drugsafetycouncil.org.
3. Van Zant G, de Haan G, Rich IN. Alternatives to stem cell renewal from a developmental viewpoint. Exp Hematol. (1997) 25:187-192.
4. Toxicity Testing in the 21st Century: A vision and a Strategy. The National Academies of Science.
5. Kang K-S, Trosko JE. Stem cells in toxicology: Fundamental Biology and Practical Considerations. Toxicol Sci. (2011) (Suppl 1) S269-S289.

6. Ali J, Saigal N, Qureshi MJ, Baboota S, Ahuja A. Chronopharmaceutics: a promising drug delivery finding of the last two decades. Recent Pat Drug Deliv Formul. (2010) 4:129-144.
7. Bradley TR, Metcalf D. The growth of mouse bone marrow cells *in vitro*. Aust J Exp Biol Med. (1966) 44:287-299.
8. Pluznik DR, Sachs L. The induction of clones of normal mast cells by a substance from conditioned media. Exp Cell Res (1966) 43:553-563.
9. Rich IN, Hall KM. Validation and development of a predictive paradigm for hemotoxicology using a multifunctional bioluminescence colony-forming proliferation assay. Tox Sci (2005) 87:427-441.
10. Rich IN. *In vitro* hematotoxicity testing in drug development: A review of past, present and future applications. Curr Opinion Drug Discov Develop. (2003) 6:100-109.
11. Rich IN. High-throughput *in vitro* hemotoxicity testing and *in vitro* cross-platform comparative toxicity. Expert Opin Drug Metab Toxicol (2007) 3:295-307.
12. Olaharski AJ, Uppal H, Cooper M, Platz S, Zabka TS, Kolaja K. *In vitro* to *in vivo* concordance of a high throughput assay of bone marrow toxicity across a diverse set of drug candidates. Tox Letters (2009) 188:98-103.
13. Hall KM, Harper H, Rich IN. Hematopoietic stem cell potency for cellular therapeutic transplantation. In: Hematopoietic Stem Cells, Ed., RP Camacho. InTech Open Access Publisher. (In Publication).
14. Giuliani M, Oudrhiri N, Norman ZM, Vernochet A, Chouaib S, Azzarone B, Durrbach A, Bennaceur-Griscelli A. Human mesenchymal stem cells derived from induced pluripotent stem cells down-regulate NK-cell cytolytic machinery. Blood. (2011) 118:3254-3262.

About HemoGenix®, Inc

HemoGenix® is a privately-held Contract Research Service and Assay Development Laboratory based in Colorado Springs, Colorado. Specializing in predictive *in vitro* stem cell toxicity testing, HemoGenix® provides its services to small, medium and the largest biopharmaceutical companies in the world. The proprietary assays developed for its contract services are further developed into application-specific assay kits that are manufactured and produced in Colorado Springs and sold worldwide. HemoGenix® has been responsible for changing the paradigm and bringing *in vitro* stem cell hemotoxicity testing into the 21st century. It is also seeking to change the paradigm in cellular therapy by providing advanced, standardized and regulatory compliant, instrument-based stem cell quality control and potency assays for hematopoietic stem cell transplantation and regenerative medicine.

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