HemoGenix®

Changing the Paradigm

The Science and Biology of Hematopoietic Stem Cell Potency, Quality and Release Criteria for Transplantation

This White Paper accompanies and enhances the webinar presented by HemoGenix® on:
How To Determine…
Potency, Quality and Release of Mobilized Peripheral Blood and Cord Blood for Stem Cell Processing Laboratories:
The Science and Assays

A HemoGenix® White Paper
October, 2010

Ivan N. Rich, PhD
HemoGenix®, Inc
1485 Garden of the Gods Road, #152
Colorado Springs, CO 80907
Tel: (719) 264-6250
Fax: (&19) 264-6253
E-mail: info@hemogenix.com
Website: www.hemogenix.com

INTRODUCTION

Stem cell transplantation is based on the biology and physiology of the hematopoietic system that dates back prior to 1950. It is not the purpose of this article to review or discuss the history of hematopoietic stem cell transplantation. Instead, this article will demonstrate how the concepts of basic biology and our wealth of scientific knowledge concerning the hierarchical organization and regulatory mechanisms underlying the functioning of the hematopoietic system, have and can be used to develop assays that provide cellular therapists with reliable and meaningful results that can help reduce risks and improve efficacy to the patient. This article emphasizes the science underlying stem cell potency and quality, which together define release criteria for a product destined for transplantation into a patient. Although focused on mobilized peripheral blood (mPB), umbilical cord blood (UCB) and bone marrow (BM) stem cell products, the same concepts and science can be applied to virtually all cellular therapeutic products, including mesenchymal stem cells (MSC) and numerous other new and emerging therapies in which cells are transplanted into patients.

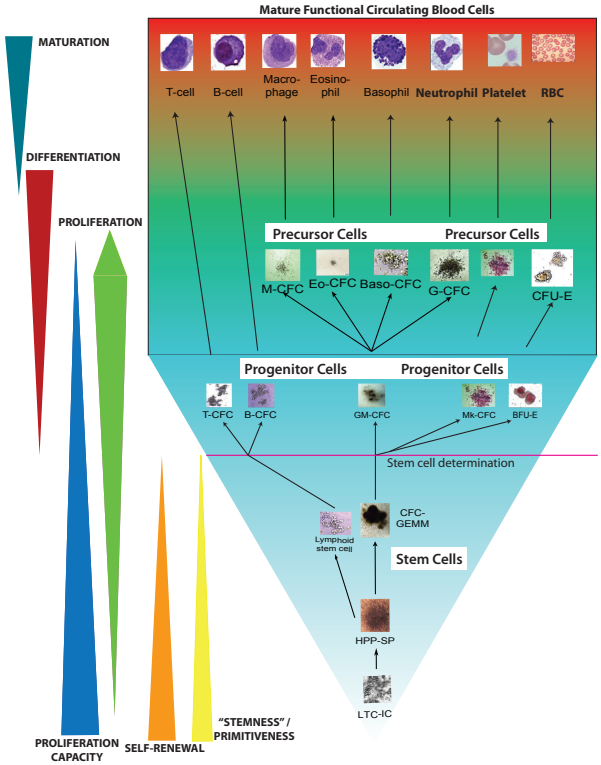
STEM CELLS: An absolute requirement for engraftment and reconstitution

Stem cells are an absolute requirement for the engraftment and reconstitution processes after transplantation of a stem cell therapeutic product. No other cells are relevant. If stem cells are not present in the product, if they have a low potency and quality or if they are dead, no engraftment or reconstitution will take place. The reasons for this are:

- Stem cells have the capacity for self-renewal.
- Stem cells are undifferentiated.
- Stem cells have the greatest proliferation potential of all other cells in the body.
- Stem cells have the capability of producing one or more lineages of mature, functional cells.

All of these functions and properties are shown in Fig. 1. It therefore follows that if the assays do not measure stem cells and the properties that are relevant to the engraftment and reconstitution process, the results obtained could provide a false assessment of the product.

Figure 1. THE ORGANIZATION AND HIERARCHICAL TREE OF THE BLOOD-FORMING SYSTEM



PROLIFERATION AND DIFFERENTIATION: The basic cellular processes underlying stem cell engraftment and reconstitution.

Proliferation is the expansion of cells by the continuous division of single cells into two identical daughter cells. In contrast, differentiation is the process whereby an undifferentiated cell acquires the features of a specialized cell. This means that proliferation must occur prior to differentiation and that the latter is a default process of the former. Once stem cells have been infused into the patient, they will home to the bone marrow, seed and begin the process of proliferation. This is the first requirement of engraftment; the stem cells must first proliferate in order to attain the initial pool of stem cells that will be responsible for reconstitution. The initial stem cell proliferation process will occur by those stem cells that are relatively mature (Fig. 1). They will be responsible for short-term engraftment. These mature stem cells will, in turn, account for short-term hematopoietic reconstitution that will give rise to the first wave of differentiated and mature cells seen in the circulation and measured as "time to engraftment". However, the transplanted stem cell product must also contain more primitive, usually quiescent stem cells that will also seed in the bone marrow, but will take a longer time to be effective. These more primitive stem cells are responsible for long-term engraftment and reconstitution (Fig. 1). In fact, it is these primitive stem cells that will be responsible not only for long-term hematopoietic, but also immune reconstitution. The reason for this is because lymphopoiesis and hematopoiesis diverge at a very early stage in the stem cell compartment and it can take a considerable amount of time for these early stem cells to become determined into one or other lineage-specific pathway (Fig. 1). These are some of the basic scientific principles of lympho-hematopoietic regulation by stem cells. They also clearly illustrate that for the transplantation procedure to be successful, the following minimal requirements must be met:

1. Proliferation of the stem cells must occur prior to any differentiation (Fig. 1).
2. Both mature and primitive stem cells must be present in the product.
3. The stem cells must exhibit biological functionality and therefore must be shown to be able to proliferate.
4. The different stem cells present in the product must be shown to exhibit different potentials for proliferation in order to account for both short- and long-term engraftment and reconstitution.

These minimal conditions are based on a huge body of scientific evidence. But, how can these requirements be met and furthermore, how can they be measured?

It is important to realize that although the proliferation and differentiation processes overlap (Fig.1), they are, from an assay standpoint, two completely different measurements. This is because the endpoints and therefore the readouts for proliferation and differentiation are different. As a result, a proliferation assay cannot be used to measure differentiation and visa versa. Engraftment is a proliferation-dependent process that occurs prior to reconstitution. The latter requires both proliferation and differentiation. It follows that both engraftment and reconstitution are dependent upon the presence and biological properties of stem cells. Only a proliferation assay will detect the presence of different stem cells and their respective biological activities that reliably and quantitatively relate to stem cell potency and quality.

BIOLUMINOMICS™: Designing a Stem Cell Proliferation Assay Appropriate to Measure Potency, Quality and Release

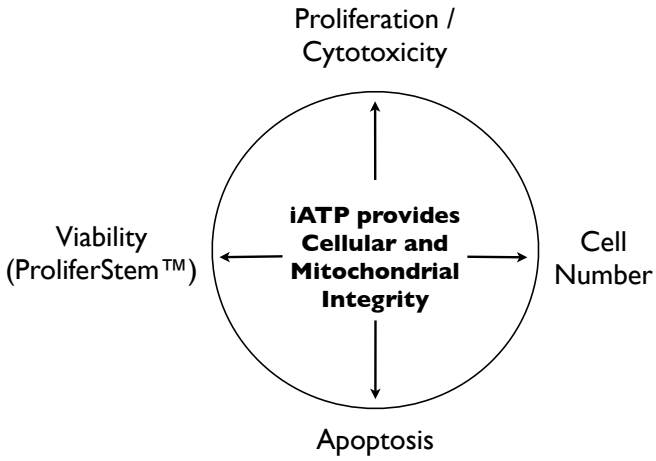
Bioluminomics™ is the quantitative, standardized and validated measurement of cell viability and functionality by detecting the intracellular ATP (iATP) concentration when it reacts with luciferin and luciferase to produce bioluminescence. The latter is produced as photons of light, which are detected in a plate luminometer.

The concept underlying bioluminomics™ is simple. All cells produce iATP as an energy source. If a cell is producing iATP, it will demonstrate cellular and mitochondrial integrity and will therefore be viable (Fig. 2). However, when cells are stimulated to proliferate in the presence of growth factors, cytokines or other agents, the iATP concentration will increase manifold above the basal level of iATP production. This increase in iATP concentration is not only dependent on the concentrations of the stimulants, but also on the cell dose, both of which correlate directly with iATP. This concept has been applied to several applications [1-5]. As demonstrated in the sections below, it is the simple correlation of iATP concentration with cell dose that plays a fundamental role in the assay that has been developed to determine stem cell potency, quality and release [6].

Figure 2

The Concept of Bioluminomics™

iATP - A Biochemical Marker with Multiple Readouts



This assay is called HALO®-96 PQR (Potency, Quality, Release) [6]. It was launched in December 2007, prior to the EMEA Guidance on potency in May 2008 [9] and before the FDA draft guidance on potency was published in October 2008 [10]. HALO®-96 PQR not only incorporates a bioluminomics readout, but uses methylcellulose-free Suspension Expansion Culture (SEC) technology. This unique and powerful combination allows for easier and faster setup and handling, more rapid turnaround time, increased accuracy and significantly lower variation with coefficients of variation (CV) of 15% or less. In contrast to other *in vitro* culture assays (e.g. the colony-forming cell (CFC) assay) and flow cytometric assays (e.g. CD34, ALDH), all HALO® bioluminomics™ assays are quantitative, standardized and can therefore be validated according to regulatory requirements [7]. The importance of this cannot be underestimated. Without these characteristics, potency, quality and release of cellular therapeutic products cannot be reliably performed.

PROLIFERATION STATUS AND PROLIFERATION POTENTIAL: Understanding the Information Provided by the Cells

The results shown in Fig. 3A and 3B compares the results of 7 different cell populations derived from bone marrow or cord blood, (each stimulated with a specific growth factor/cytokine cocktail that distinguishes one cell population from another) and setup with 5,000 cells/well and cultured for 5 or 7 days. This protocol detects the proliferation status of cell populations [4]. It asks the question, how much proliferation can each cell population perform? If, on the other hand, the CFC assay were used, differentiation status or ability would be detected. From the results in Fig. 3, it clear that the primitive (HPP-SP) and mature (CFC-GEMM) stem cells exhibit the greatest amount proliferation. The hematopoietic progenitor cell populations (BFU-E, GM-CFC, Mk-CFC) all exhibit similar proliferation ability, while the two lymphopoietic cell populations (T-CFC and B-CFC) exhibit the lowest proliferation status. These results also demonstrate that for the stem cells and BFU-E, the proliferation status increases approximately 3 fold by culturing the cells an extra 2 days from day 5 to day 7. This demonstrates an extremely important difference between the CFC assay and HALO®. If the CFC assay were used, the number of colonies counted on day 7 would be similar to the number of colonies counted on day 10, 12 or 14 [1], but the size of the colonies would change. An increasing size of the

colonies with time represents an increase in proliferation. Unfortunately, this information is lost in the CFC assay because it cannot be measured. (Even measuring the diameter or size of a colony would not provide this information). In contrast, since proliferation correlates with the iATP concentration and the latter, in turn, correlates with cell number (as seen in Fig. 4), HALO® can be easily used to quantify cell expansion or amplification.

The capacity of cells to expand is a function of the cell dose. Whereas proliferation status or ability is measured using a single cell dose, additional information is provided when a cell dose response is performed. This is shown in Fig. 4. Although only bone marrow is used in this example, the same cell populations are being examined as seen in Fig 3A. From the results it is evident that the 7 populations can be divided into 3 separate clusters. The lymphopoietic cluster is represented by the T- and B-CFC cell dose response curves with the lowest slope. The two stem cell populations (HPP-SP and CFC-GEMM) represent the cluster with the steepest cell dose response slopes, while the three hematopoietic cell populations show intermediate slopes. The pattern of results seen here for bone marrow also occurs for mobilized peripheral blood and umbilical cord blood.

From this simple study using cell dose response curves, it is now possible to formulate the 4 rules to understanding the information provided by these cell dose response curves.

Rule 1: The slope of the linear regression of the cell dose response is equal to the proliferation potential of the cell population measured.

Rule 2: The steeper the slope of the cell dose response, the greater the proliferation potential.

Rule 3: The steeper the slope of the cell dose response, the more primitive the cells.

Rule 4: The steeper the slope of the cell dose response, the greater the potency.

Figure 3A
Proliferation Status of Bone Marrow on Day 5 or Day 7 of Culture

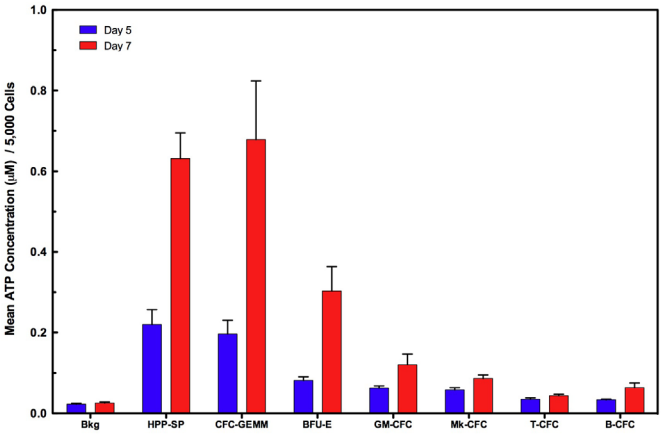


Figure 3B
Proliferation Status of Cord Blood on Day 5 or Day 7 of Culture

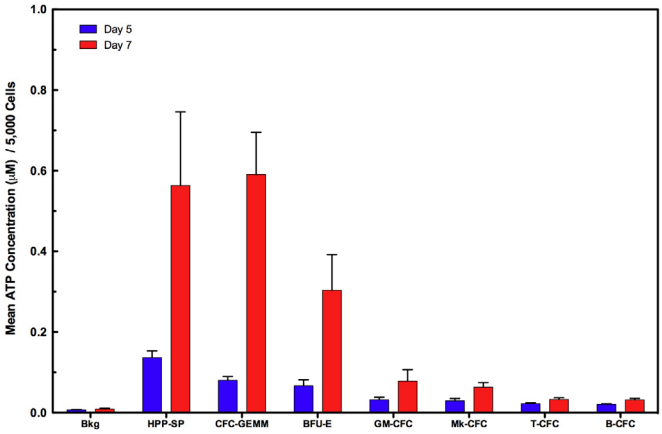
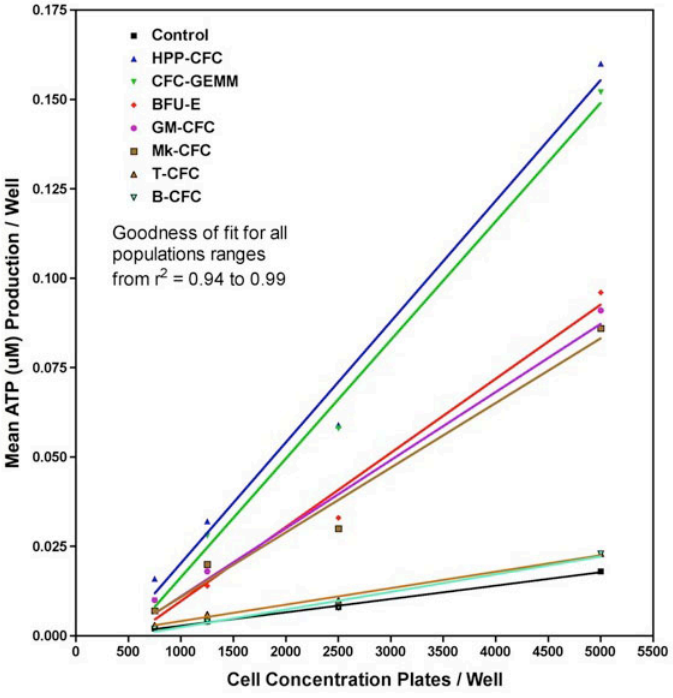


Figure 4
Cell Dose Response for Human Bone Marrow Populations

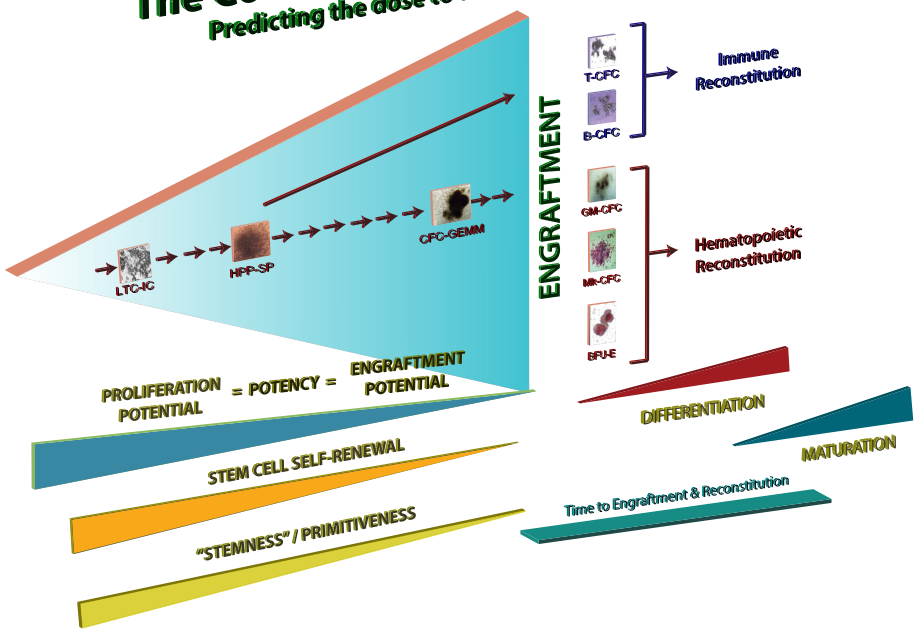


THE CONCEPT OF STEM CELL POTENCY

Figure 5 shows the concept of stem cell potency in graphical terms. Reconstitution after transplantation involves both hematopoietic and immune reconstitution. To achieve this, stem cells must be transplanted and they must engraft. Whether engraftment occurs depends on many factors. Two of the factors that can be physically controlled prior to transplantation are stem cell potency and quality, which together allow the release criteria to be defined (see below). From the previous section, performing a simple cell dose response will provide all the information required to measure the three parameters, potency, quality and release criteria. As seen in Fig. 5, stem cell potency has nothing to do with time to engraftment. Stem cell potency depends on the proliferation process. Time to engraftment depends on the differentiation process and is the result of engraftment, not the cause of it.

Before demonstrating the 3 steps in measuring potency, quality and release, it is worth briefly summarizing the importance and meaning of potency and the requirements of a potency assay.

Figure 5
The Concept of Stem Cell Potency
Predicting the dose to achieve engraftment



POTENCY – Importance, Meaning and Requirements

As it applies to drugs (and the trend is for cellular therapeutic products to be considered as drugs [8]), potency is important because:

- It ensures consistency during production and manufacture.
- It demonstrates product stability.
- It predicts product performance and assurance.
- It allows for evaluation and/or correlation with a clinical response.
- It avoids product failure or toxicity due to improper potency.

Each of these points is linked to the regulatory requirements that all potency assays must demonstrate [10]. These requirements are:

- To indicate biological activity specific to the product (the definition of potency in its simplest form).
- To provide results for release of the product.
- To provide quantitative data.

- To meet pre-defined acceptance/rejection criteria.
- To include reference materials, standards and controls.
- To demonstrate validation.
- To measure the identity and activity of all "active ingredients".

A living cell and, in particular, a living stem cell, is more complex than a traditional compound drug, vaccine, growth factor etc. as far as potency is concerned. Proliferating systems, such as the lympho-hematopoietic system, are continuously in flux and present technology does not allow identifying and measuring every single stem cell "active ingredient". Nevertheless, the same principles and concepts that have been developed for measuring potency of a compound drug, can be applied to stem cells in that:

1. An appropriate and validated assay should be used.
2. A reference standard of the same material should be present and compared to the sample.
3. A dose response relationship of the "active ingredient(s)" should be measured.

Before demonstrating how HALO®-96 PQR, is used to measure stem cell potency and quality and define release criteria, it is necessary to first describe how the potency ratio is calculated and what the potency ratio means.

POTENCY RATIO: How it is calculated and what information does it provide?

As mentioned in the previous section, cells are more complex than a drug or other compound. As a result, the normal method of calculating the potency ratio using parallel dose response curves [11] is difficult, if not impossible, to apply to cells. A cell dose response is still performed, but for stem cells, the ratio of the slopes of the cell dose responses will provide the potency ratio as follows:

Stem Cell Potency Ratio = Slope of the Sample Linear Regression Cell Dose Response /
Slope of the Reference Standard Linear Regression Cell Dose Response

The potency ratio of the reference standard is always 1. This means that a potency ratio less than 1 indicates that more cells or a greater cell dose, is required to achieve the same response as the reference standard. A potency ratio greater than 1 indicates that fewer cells or a lower dose, is required to achieve the same response as the reference standard.

3 STEPS TO MEASURING STEM CELL POTENCY, QUALITY AND RELEASE USING MOBILIZED PERIPHERAL BLOOD AND UMBILICAL CORD BLOOD SAMPLES.

All of the following information was obtained from purchased samples of cryopreserved mobilized peripheral blood (mPB) and umbilical cord blood (UCB). A total of 90,000 cells are required to perform a minimum 3-point cell dose response of 2,500, 5,000 and 7,500 cells/well using 6 replicates/dose. The 3 cell doses suggested take into consideration that for mPB and especially UCB, the amount of sample available for testing in a frozen vial or segment, may be extremely limited. It is important, however, to ensure that a minimum 3-point cell dose response is performed, although the actual cell concentrations can vary. This would be the case if, for example, a purified ($CD34^+$) product was used. A reference standard of mPB or UCB is also required, but establishing a series of reference standards is a topic for another White Paper and will not be discussed here. Using HALO®-96 PQR, the 3-step procedure is as follows:

Step 1: Perform a 3-point cell dose response for two stem cell populations from the samples and reference standard and calculate the potency ratio based on the slope of the linear regression of the cell dose responses.

Step 2: Correlate ATP concentration at a specific dose with the slope of the cell dose response linear regression for each stem cell population from each sample.

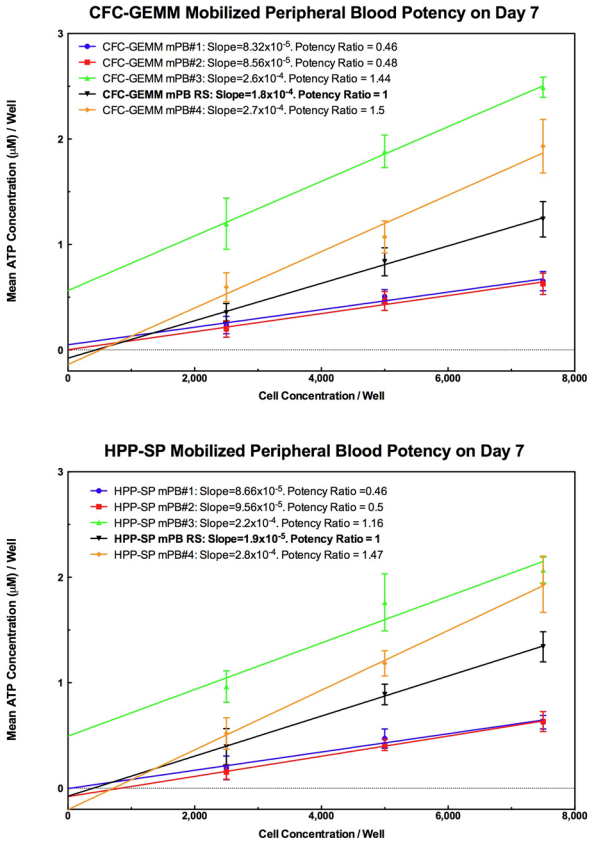
Step 3: Combine the results from stem cell proliferation status and stem cell cumulative potency

to determine the release criteria of the samples.
For a more detailed protocol of HALO®-96 PQR, please consult "Cellular Therapy: Principles, Methods and Regulations" [6].

A. Mobilized Peripheral Blood

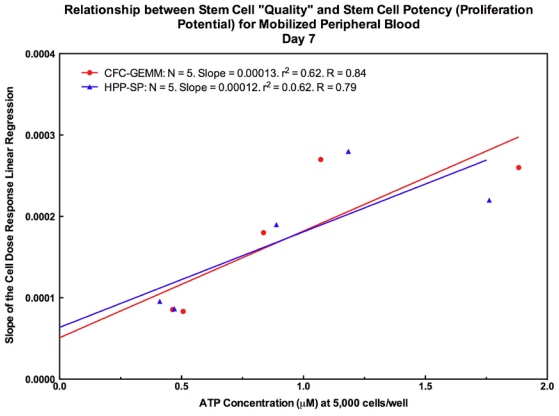
Figures 6A, 6B and 6C show the 3 steps and results for measuring potency, quality and defining release criteria for mobilized peripheral blood using HALO®-96 PQR. Step 1 is the only hands-on part of the procedure; the other steps use the results obtained from this first step. Step 1 is to perform the 3-point cell dose response for both the CFC-GEMM and HPP-SP stem cell populations for several samples. This will also allow historical data to be accumulated. In Fig. 6A this has been performed for 4 mPB frozen samples plus a mPB reference standard. The results are shown after 7 days of culture. A 7-day culture period can be more easily adapted to a routine processing laboratory than a 5-day culture period. The graphs and potency ratios show that two mPB samples exhibit potencies below and two above the reference standard. Examination of the graphs will indicate that at any cell dose along the X-axis, as the ATP concentration increases there is, in general, a concomitant increase in the slope of the linear regression cell dose response.

Figure 6A



Step 2 of the procedure is to plot the ATP concentration at a specific cell dose, e.g. 5,000 cells/well, against the slope of the cell dose response. The results are shown in Fig. 6B and indicate a direct correlation between these two parameters. Since stem cell quality is measured at a single cell dose and stem cell potency is measure by the slope of the cell dose response, this correlation demonstrates that there is a direct relationship between stem cell quality and potency. However, a stem cell quality assay cannot be used to determine potency, because only a single point is measured. In contrast, a stem cell potency assay can be used to determine both stem cell quality and potency in a single assay.

Figure 6B



The relationship between quality and potency is then used in the final step to define release criteria (Fig. 6C). Previously, it had been thought that the results from a stem cell quality assay could be used to arbitrary define the acceptance/rejection cutoff. This was set at 0.04µM ATP, because below this concentration, cells were either dead or could not sustain proliferation [14]. However, as seen in Fig. 6C, all four mPB samples exhibited ATP concentrations above this cutoff value and all would have been considered acceptable for release. Because of the relationship between quality and potency, it is also necessary to take cumulative potency of both stem cell populations into account. When

this is combined with the stem cell quality data, it is clear that mPB samples 1 and 2 exhibit potencies for both stem cell populations below the reference standard. Since both CFC-GEMM and HPP-SP are responsible for short- and long-term engraftment and reconstitution respectively, it is obvious that neither sample 1 nor 2 would be able to fulfill this requirement and would be deemed unacceptable. Samples 3 and 4, however, exhibit both high quality and potency for both stem cell populations and providing other parameters proved favorable, could be accepted for use.

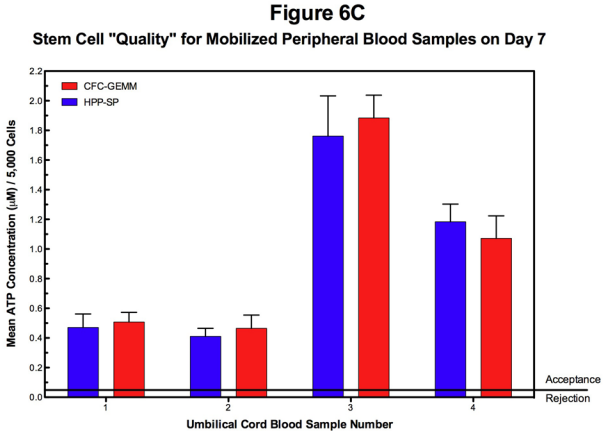
B. Umbilical Cord Blood

In this case, 5 cryopreserved cord blood samples were used plus a cord blood reference standard. Exactly the same 3-step procedure was used as described above. The results are shown in Figs. 7A, 7B and 7C. From the ATP concentrations and the potency ratios as defined by the slopes of the linear regression cell dose responses (Fig 7A), the same relationship between stem cell quality and potency exists as for mPB (Fig. 7B). This allows the release criteria to be assessed (Fig. 7C). For the UCB samples tested, it is apparent that sample 4 would be unacceptable. However, samples 1 and 2 pose a slight dilemma. Stem cell quality would be acceptable. But the potency of the CFC-GEMM is approx. similar to that of the reference standard in both samples. It would be preferable if the potency of CFC-GEMM would be greater than the reference standard as it is in samples 3 and 5, because this population would be responsible for short-term hematopoietic engraftment and reconstitution. Nevertheless, the potency of the HPP-SP population for samples 1 and 2 is greater than the reference standard and since HPP-SP can feed into and produce CFC-GEMM, there is the possibility that these two samples, together with other favorable parameters, could be acceptable.

CONCLUSIONS AND SUMMARY

This White Paper has described the science and biology underlying hematopoietic stem cell potency and quality and how release criteria might be assessed. The correlation of these parameters with engraftment has not been described although, at the time of writing, HALO®-96 PQR demonstrated an accuracy and prediction of

Figure 6C



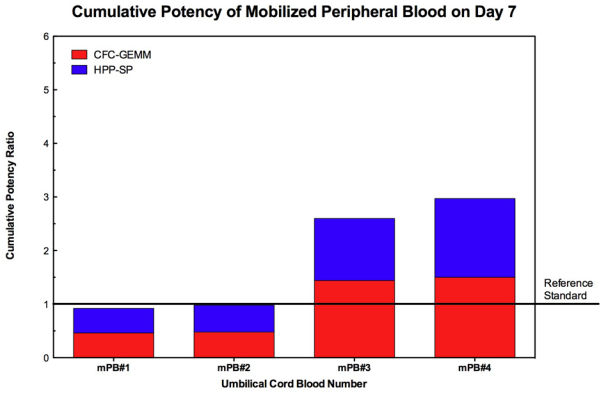
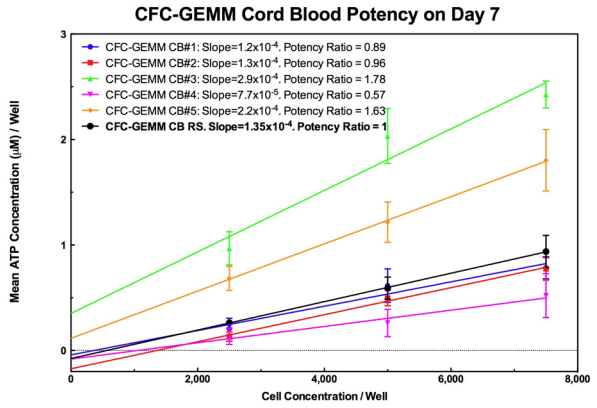


Figure 7A



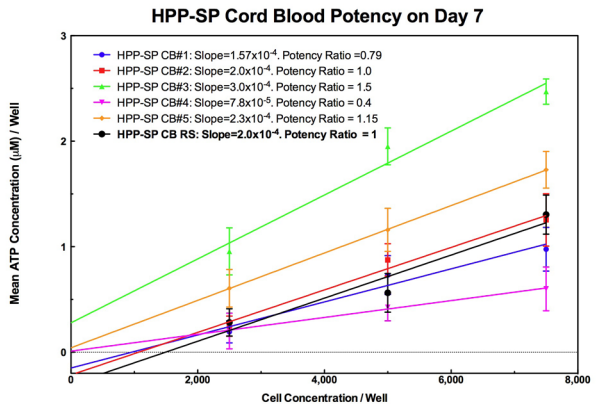


Figure 7B
Relationship between Stem Cell "Quality" and Stem Cell Potency (Proliferation Potential) Day 7

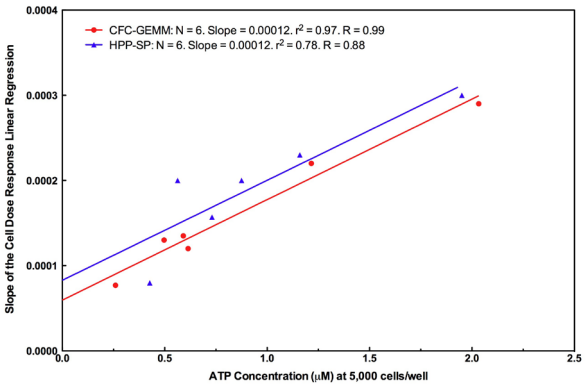
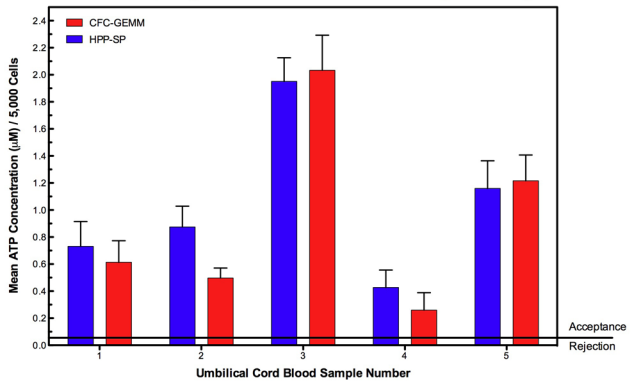
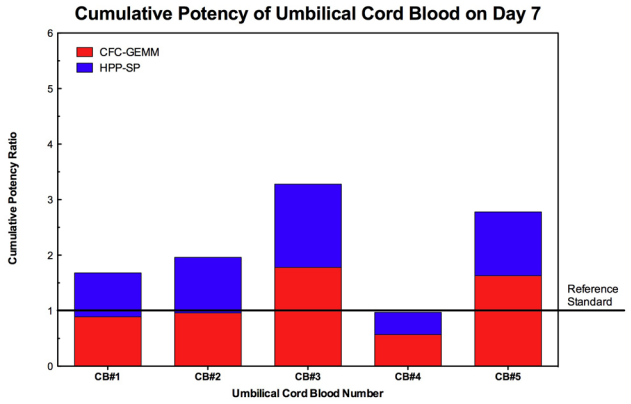


Figure 7C
Stem Cell "Quality" for Umbilical Cord Blood Samples on Day 7





greater than 85% with engraftment for a much larger cord blood sample set. Obviously more studies are needed to reinforce and extend the assay's predictive power. HALO®-96 PQR is now available for mobilized peripheral blood, umbilical cord blood and bone marrow. For mesenchymal stem cells (MSC), LUMENESC™-96 PQR is also available. All are for research purposes only, but together, they provide the first reference standard-based potency, quality and release assays for cellular therapeutic products. The advanced technology incorporated into these assays does not make then more difficult or costly to use. In fact, it takes two days or less to learn how to use HALO® and often costs significantly less than presently used assays (e.g. CFC assay), since few extra supplies are needed and both time and labor costs can be dramatically reduced due to instrument-based technology.

HALO®-96 PQR is part of a family of assays specifically designed for the hematopoietic stem cell transplantation and banking field. HALO®-96 SPC-QC is a stem cell quality control assay specific for the CFC-GEMM and HPP-SP populations. In contrast to PQR, the SPC-QC assay is for routine stem cell quality assessment such as determining the quality of the stem cells prior to cryopreservation, or assessing stability and consistency for other procedures. HALO®-96 PMT was designed to monitor the proliferation of the hematopoietic stem and progenitor cells or, in addition the primitive stem cell and lymphopoietic cells. The results shown in Fig. 3 used the HALO®-96 PMT 7-Population platform. Together, these assays can provide a wealth of information that has never been previously available.

Hematopoietic stem cell transplantation has relied on the same tests and assays for over 30 years. New technology incorporating better science, quantitative and validated procedures and stricter regulations might, at first, be difficult to embrace, but is inevitable. HemoGenix® has always advocated a greater understanding of the scientific principles that are the foundation of application-specific assays to provide superior laboratory tools that can ultimately reduce risk and improve efficacy to the patient.

LITERATURE CITED

1. Rich IN. In vitro hematotoxicity testing in drug development: A review of past, present and future applications. Current Opin Drug Disc Develop 2003; 6:100-109.
2. Rich IN, Hall KM. Validation and development of a predictive paradigm for hemotoxicology using a multifunctional bioluminescence colony-forming proliferation assay. Toxicol Sci 2005; 87:427-441.
3. Rich IN. High-throughput in vitro hemotoxicity testing and in vitro croos-platform comparative toxicity. Exp Opin Drug Metab Toxicol 2007; 3:295-307.
4. Reems J-A, Hall KM, Gebru LH, Taber G, Rich IN. Development of a novel assay to evaluate the functional potential of umbilical cord blood progenitors. Transfusion 2008; 48:620-628.
5. Olaharski AJ, Uppal H, Cooper M, Platz S, Zabka TS, Kolaja KL. In vitro to in vivo concordance of a high throughput assay of bone marrow toxicity across a diverse set of drug candidates. Toxicol Let 2009; 188:98-103.
6. Hall KM & Rich IN. Bioluminescence assays for assessing potency of cellular therapeutic products. In: Cellular Therapy: Principles, Methods and Regulations. Eds: Areman EM and Loper K. AABB, Bethesda, MD (2009); pp 581-591.
7. FDA Guidance for Industry. Bioanalytical method validation. 2001. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf.
8. FDA Guidance for Industry. Minimally manipulated, unrelated allogeneic placental/umbilical cord blood intended for hematopoietic reconstitution for specified indication. 2009. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM187144.pdf.
9. European Medicines Agency (EMEA) Guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer. 2008. http://www.tga.gov.au/docs/pdf/euguide/bwp/27147506en.pdf.
10. FDA Draft Guidance for Industry. Potency tests for cellular and gene therapy products. 2008. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm078687.pdf.
11. Gottschalk PG, Dunn JR. Measuring parallelism, linearity, and relative potency in bioassay and immunoassay data. J Biopharm Stat 2005; 15:437-463.
12. Rich IN, Hall KM, Harper H. A stem cell potency and release criteria assay specifically designed for umbilical cord blood transplantation that is compliant with regulatory guidelines. Cytotherapy 2009; 11:13.

About HemoGenix®, Inc

HemoGenix® is a privately-held Contract Research Service and Assay Development Laboratory that produces and sells its services and assay kits in the U.S.A. and countries worlwide. HemoGenix® specializes in developing predictive in vitro assay platforms for primary human and animal target cells. These assays have been specifically developed for contract research services and as assay kits for in-house use. HemoGenix® has been responsible for changing the paradigm and bringing stem cell hemotoxicity testing into the 21st century, by developing HALO® to allow biopharmaceutical companies to screen, test and predict the effects of compounds on multiple cell populations from up to 8 different species simultaneously. HemoGenix® is also changing the paradigm in cellular therapy by providing advanced and standardized, instrument-based stem cell quality control and potency assays. HemoGenix® prides itself on bringing the best possible in vitro assay tools to its clients and customers.

Trademarks and Patents

HemoGenix® and HALO® are registered trademarks of HemoGenix®.
HALO® is protected by U.S. patents 7,354,729; 7,354,730; 7,666,615; 7,700,354; 7,709,258 and other patents pending both nationally and internationally.