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Potency, Proliferation and Engraftment Potential of Stem Cell Therapeutics: The Relationship between Potency and Clinical Outcome for Hematopoietic Stem Cell Products

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Abstract

This review describes why potency is important and how potency can be measured to release stem cell cellular therapeutic products for transplantation into patients. Particular attention is paid to umbilical cord blood as a stem cell product and the misconception that potency must correlate with clinical outcome. The case is made that present day tests and assays used in the cord blood industry to provide only basic cell characterization. Although these tests and assays may correlate with time to engraftment as a clinical outcome, they do not measure the potency of the stem cells that are responsible for, and correlate with, the engraftment response. It is suggested that present cell therapeutic product characterization be supplemented with more advanced assays that incorporate accepted concepts and principles of potency testing and can actually measure stem cell potency in accordance with regulatory requirements.

Keywords: Potency; Quality; Stem cells; Proliferation potential; Engraftment potential; Time to engraftment; Transplantation; Umbilical cord blood; Bone marrow; Peripheral blood

Introduction

In the biopharmaceutical industry and the growing cellular therapeutic and regenerative medicine field, potency is a measure of the strength of the effect a drug or other product will produce. The United States Food and Drug Administration (FDA) considers "strength" to be equivalent to potency, that is, "the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data ….." [1]. That clinical data or perhaps a clinical outcome can be used to measure potency is misleading since potency is a prospective or predictive measurement. It cannot be used retrospectively because the information obtained from a potency assay has to be used to release the lot or batch of a drug. Thus, there appears to be a misconception that for some cellular therapeutics and hematopoietic stem cell therapy is a prime example; the potency must correlate with clinical outcome. For most cell-based therapeutics, this may rarely occur. The results of a potency assay may correlate with a response, but need not correlate with clinical outcome. Indeed, this communication will argue that the reason for this misconception is because many, if not all, of the assays presently used in the field of hematopoietic stem cell therapy do not and cannot measure potency.

Reasons for Measuring Potency

The importance of measuring product potency cannot be underestimated. Indeed, it is probably one of the most important properties that can and should be measured, since without it, administration of a drug is trial and error.

Potency is used to measure consistency in the manufacturing process and demonstrate stability of the product. It is also used to avoid product failure or toxicity due to improper potency. Engraftment failure of a stem cell therapeutic is a specific example of this. Potency predicts whether the product can be released for use and can provide information on the dose required to ensure the product will perform as intended. Since all of these aspects are predictive, it follows that potency of a drug is measured prior to use in the patient. Furthermore, since a cellular therapeutic or regenerative medicine product might result in a systemic effect on the patient, knowledge of at least one or more biological properties of the product would be incorporated into a potency assay. A potency assay should, therefore, be one of the first exercises in developing a cell-based therapeutic.

Both the European Medicines Agency (EMA) [2] and the FDA [1] stress the need to quantitatively measure biological activity of a product as a basis for a potency assay. Other required assay characteristics include, but are not limited to, the establishment of pre-defined acceptance/rejection criteria, incorporation of reference materials, standards and controls that are needed to validate the assay and identifying and measuring the activity of the "active ingredients" or constituents that are responsible for the intended effect or response. Although the focus of this communication will be on hematopoietic stem cell therapeutics, and cord blood stem cell therapy in particular, many of the concepts and principles discussed can be applied to other stem cell products and proliferating cell populations in general.

Traditional Drug Potency

The potency of a traditional drug is determined using the "paradigm of parallelism". This is a requirement by both the U.S. Pharmacopeia (USP) and the European Pharmacopeia (EP) [3]. The principle is shown in (Figure 1). Traditional drugs, when tested on their target tissue or cells, will exhibit either a negative or positive sigmoidal dose response indicating an inhibitory or stimulatory effect, respectively. If

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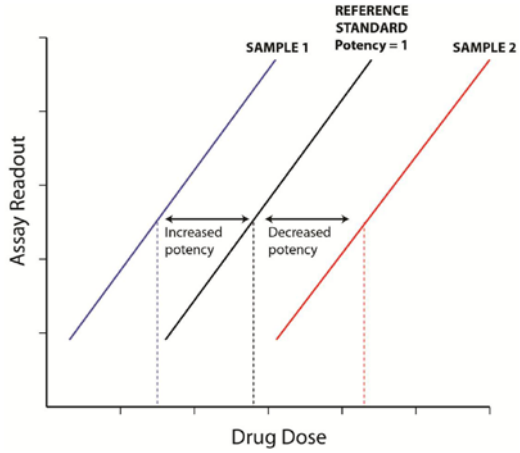

Figure 1: Measurement of potency using the "paradigm of parallelism" for a traditional drug.

For drugs, growth factors vaccines etc, the linear portions of the sample dose response curves must be statistically parallel with that of the reference standard of the same material. The ratio of the linear displacement by the sample from the reference standard provides the potency ratio.

the manufacture of a compound is consistent, variations in the potency between lots or batches will be minimal. However, since a potency assay is meant to determine manufacturing consistency, comparison with a known or international reference standard of the same compound being tested will determine whether this is actually the case.

The measurement of potency is the potency ratio (PR). To determine the PR, the dose response for the sample lot is compared to that of its reference standard (RS). The potency of the RS is always one [1]. As seen in Figure 1, if the linear portion of the dose response of the sample is statistically parallel [3,4] to that of the RS, the potency ratio can be calculated by dividing the horizontal displacement of the sample by the potency of the RS. If the resulting ratio is greater than one, then the potency of the sample will be greater than that of the RS and the linear portion of the dose response curve will be displaced to the left of the RS. In addition, since the activity or specific activity of the RS will be known, a sample with higher potency will exhibit a greater activity and therefore less of the compound will be required to produce the same response as the RS. The opposite is also true, in which case the sample will demonstrate a dose response displaced to the right of the RS. If the linear portion of the sample dose response is not statistically parallel with that of the RS, an impurity or contaminant can be implied. Thus, the potency assay designed and developed for the compound must be specific and must measure not only the active ingredients, but indicate the presence of other constituents that might affect the potency.

Correlation with a clinical response and/or outcome is usually a dose-dependent phenomenon. For example, the response of adding erythropoietin (EPO) to bone marrow cells in vitro or administering EPO to a patient is to increase erythropoiesis there by increasing the production of red blood cells. In a clinical setting, for example in patients suffering from anemia of chronic renal disease, administration of EPO results in a dose-dependent clinical outcome, namely the alleviation of the anemia [5,6]. In other cases, such as the anemia associated with some cancers, the administration of EPO may not have any effect and therefore a clinical outcome may not be observed [7]. In this case, potency will not correlate with clinical outcome.

Applying the "Paradigm of Parallelism" to Cellular Therapeutic Products?

Living, viable cells exhibit cellular and metabolic functions that are continuously in flux. As a result, it is improbable that any cell preparation or population can be obtained in a "pure" state. For a continuously proliferating, definitive stem cell system such as lympho-hematopoiesis, individual cells in a cohort developing through the system will display slightly different properties that indicate different degrees of primitiveness or maturity. This, in turn, depends on the state of proliferation, differentiation or both. Lympho-hematopoiesis is viewed as a continuum of cells in which one state changes imperceptibly into the next. As a result, a cell suspension, even if it is considered "pure", will consist of cells in different developmental states.

Within the lympho-hematopoietic stem cell compartment this continuum [8-10] means that the potency of stem cells is continuously changing. If it is accepted that only the stem cells are responsible for engraftment and reconstitution, then not only must the stem cells be the only "active" constituents, but also the only cells for which measuring potency is required.

If the corresponding analogy to measuring potency of traditional drugs is applied to lympho-hematopoietic stem cells, mesenchymal stem/stromal cells or any other proliferating cell population, it will be seen that the "paradigm of parallelism" [3,4] will not be applicable for most, if not all, stem cell products. There will certainly be some cases in which the cell dose response for a specific stem cell population will demonstrate parallelism with an RS of the same material. In such cases the displacement to the left or right will not indicate an increased or decreased potency, but rather a greater or lower concentration of stem cells with the same potency as the RS.

Since the "paradigm of parallelism" cannot be universally applied to all stem cell products, it follows that one or more specific biological properties of stem cells must be employed to measure potency. One property that is common to all stem cells is proliferation. Stem cell primitiveness or "stemness", self-renewal and engraftment are all dependent upon the potential and ability of stem cells to proliferate.

To identify and quantitatively measure stem cell proliferation, an appropriate assay is required for which validation characteristics can be measured. This can only occur if the assay readout can incorporate the proper standards and controls. Taking this into account, there are three primary considerations for developing a potency assay.

Development of an Assay to Measure Stem Cell Potency

Assay readout

The readout of any assay is probably the most important aspect in developing a test for a specific application. There are several methods to measure cell proliferation, the easiest being to count the number of cells before and after a treatment. This can be done manually or with an instrument. Suffice is to say that there are numerous reasons why cell counting cannot be used as a stem cell potency assay and these will be discussed later. Measuring cell proliferation can also be performed using flow cytometry or using absorbance/colorimetric assays such as the MTT assay [11] or fluorescence metabolic assays. Many of these assays are also metabolic viability assays. Some of these instruments-based readouts have been used specifically to measure the proliferation of hematopoietic cell populations, but the ability to incorporate standards and controls, a necessary prerequisite for potency assays, is rather difficult.

In contrast, the ability to incorporate all of the regulatory requirements for a potency assay was achieved by detecting changes in the cell's energy source, intracellular adenosine triphosphate (iATP), as a biochemical marker that correlates directly with proliferation status [12]. To measure changes in iATP, the latter is released from the cells in culture and reacts as a limiting substrate for a luciferin/luciferase reaction to produce bioluminescence. The light produced is measured in a plate luminometer. Incorporation of an external ATP standard and controls allows the instrument to be calibrated and the assay standardized prior to measuring any sample. Assay standardization also allows results to be compared over time, a property that is especially important in establishing reference standards within and between laboratories. This readout technology has been incorporated into a stem cell quality assay [13] and two potency assays, one developed for lympho-hematopoietic cells (HALO-96 PQR) and one for mesenchymal stem/stromal cells (LUMENESC-96 PQR). Both assays use the same concepts and principles described here to measure potency and quality and defining acceptance limits for releasing the product for use [14,15].

Besides the physical and validation parameters [15], the acceptance/rejection limits must be pre-defined for a potency assay. For both potency assays using the ATP signal detection system, the ATP concentration above which cells can sustain proliferation was found to be approximately 0.04 μM. As shown later, this level of iATP, together with the potency ratio, will determine the release criteria for the product.

Measuring the "active" constituents

Identifying and measuring the "active" constituents responsible for the intended effect of the product is a fundamental requirement of a potency assay. In primary organs and tissues, stem cells represent a minute proportion of the total cellularity and in many cases are difficult, if not impossible, to morphologically identify from other cells. This is because stem cells are undifferentiated. It has been known for many years that to identify specific lympho-hematopoietic stem cell populations, different cocktails of growth factors are necessary to induce the cells to proliferate. This functional property can be used to grow colonies of cells derived from different stem, progenitor and precursor cell populations under clonal conditions in a semi-solid, viscous medium such as agar, plasma clot or methylcellulose [16-20]. As discussed later, this colony-forming cell (CFC) assay requires that the target cells proliferate to produce a colony that is identified by the mature cells in the colony. Although proliferation is necessary to form a colony, the assay does not measure proliferation. Instead, it detects differentiation ability. By using growth factor cocktails that stimulate stem cells at different stages of primitiveness and measuring the changes in iATP concentration at a time when only the numbers of stem cells are increasing, not only are specific "active" stem cell populations identified, but both their proliferation ability and potential can be measured simultaneously. The time at which the iATP concentration is measured after stimulating the stem cells is critical because the stem cells should not be allowed to pass through the "gate of determination". At this point, the cells cease to be stem cells and enter a differentiation lineage.

Although the growth factor cocktail will determine the cell population that is stimulated, it is the measurement of proliferation potential that actually identifies the stage of primitiveness and therefore the stem cell population. Proliferation potential or the capacity for proliferation is greatest for the most primitive stem cells and decreases as the stem cells mature. Similarly, the proliferation potential of primitive hematopoietic progenitor cells is greater than that for cells that have developed to the precursor stage and only have limited proliferation potential. The slope of the linear regression of the cell dose response determines proliferation potential [15]. It would therefore be expected that the steeper the slope, the greater the proliferation potential and the more primitive the cell. As a result, when stem cells are stimulated with different cocktails of growth factors, their primitiveness and thus their proliferation potential can be quantitatively measured by the slope of the cell dose response. It is the slope of the cell dose response that also defines the potency, because the more primitive the stem cell, the greater its potency [15].

When the iATP concentration of a stem cell, or any other proliferating cell population for that matter, is determined at a single cell dose, the parameter being measured is proliferation ability. For stem cells, proliferation ability is equivalent to stem cell "quality". It follows that from the cell dose response used to measure stem cell proliferation potential and potency, it is also possible to measure proliferation ability or "quality" at the same time [14,15].

Reference standard (RS)

The final component of developing a stem cell potency assay is the RS that allows calculation of the potency ratio. Establishing the RS for a cellular therapeutic is probably the most difficult aspect of any potency assay. A reference standard is essentially a batch of material that is divided into small aliquots and used to compare with the activity of a sample from a new batch of the same material. For practical reasons, several RS batches are usually established, the potency of each batch being measured against its predecessor [21]. A reference standard of a traditional drug may be made available and distributed by an international agency, such as the World Health Organization (WHO) or by another organization such as the United States or European Pharmacopeia.

In contrast to traditional drugs, cellular therapeutic products are only available in very small quantities. In addition, reference standards of cells would have to be cryopreserved, the process of which can vary drastically from one laboratory to another. The lack of process, procedure and assay standardization has contributed to the overall difficulty in being able to compare results between laboratories.

For potency testing, there are two ways to overcome these difficulties. The first is to use assays that have or can be validated and that have been developed in such a way as to allow direct comparison of results within and between laboratories. The HALO and LUMENESC assays are examples. The second is to establish in-house reference standards that are used to measure potency and determine if the results allow release of the product for patient use. It follows that assay and the establishment of a reference standard for potency testing goes hand in hand, since the result is decision-making information that could have important consequences.

Measuring the Potency Ratio

The measure of potency is the potency ratio. This ratio can only be obtained by comparison to an RS of the same material as the sample. By dividing the slope of the linear regression of the stem cell dose response for the sample by that for the same population from the RS, the potency ratio for that specific stem cell population can be calculated. The resulting potency ratio will either be greater or less than the RS. Since potency can be used to predict the dose required for the intended effect or response, it should theoretically be possible to predict the stem cell dose that will predict engraftment potential, i.e. the intended effect. Unfortunately, this is not as easy as that for a traditional drug. To predict

the stem cell dose, it is necessary to determine the "stem cell activity" of the reference standard that will elicit engraftment. From the previous discussion and the clinical involvement requirement, it is obvious that this is impractical. Nevertheless, it is possible to quantitatively predict whether a stem cell product will engraft. That information alone is significantly greater than any information obtained using present tests and assays.

One of the requirements for a potency assay is that all "active ingredients" or components should be measured. As discussed above, measuring the potency of all stem cell populations is not only impractical, but also a physical impossibility with present technology. However, measuring the potency of a single stem cell population is also unwise. This is because the correlation between stem cell primitiveness, proliferation potential and potency also extends to engraftment and therefore reconstitution potential. Mature stem cells would be expected to exhibit a lower potency than primitive stem cells and therefore a lower engraftment potential. From a practical viewpoint this means that mature stem cells would be expected to demonstrate short-term engraftment [22-25] and reconstitution while more primitive stem cell would demonstrate long-term engraftment and reconstitution [22,25,26]. As a result, measuring the potency of just a single stem cell population, especially a mature stem cell population, could result in a false interpretation and conclusion. For this reason, the potency of a minimum of two stem cell populations, one mature and one primitive, can provide most (but not all) of the information required to predict whether a cellular product will engraft and therefore could be released.

The Relationship between Stem Cell Potency and "Quality"

Since stem cell proliferation potential or potency and proliferation ability or "quality" can be determined using the same assay, it follows that a relationship between these two parameters should exist. From a study of 28 frozen cord blood samples for which both stem cell potency and "quality" were measured, it was found that when the potency, as determined from the slope of the stem cell dose response linear regression, was plotted against the ATP concentration at a specific cell dose, a direct and positive correlation occurred. For the mature hematopoietic stem cell population represented by the CFC-GEMM population (colony-forming cell–granulocyte, erythroid, macrophage, megakaryocyte), the regression coefficient (R) was 0.853, while for the primitive lympho-hematopoietic stem cell population, represented by the High Proliferative Potential–Stem and Progenitor Cell (HPP-SP), the R value was 0.87. These are highly significant values and have an equally significant consequence. The correlation indicates that neither parameter alone is sufficient to release the product for use; both parameters must be taken into account for both stem cell populations.

Using Stem Cell Potency and Stem Cell "Quality" to Define the Release Criteria for Cellular Therapeutic Products

Table 1 shows the individual stem cell "quality" or iATP concentrations at 5,000 cells plated (proliferation ability) and the potency ratios (calculated from the individual slopes of the linear regression dose response curves) for both CFC-GEMM and HPP-SP stem cell populations from 28 cryopreserved umbilical cord blood samples. The release criteria for a product would be based on these two parameters, namely that the sample must exhibit a stem cell "quality" greater than the pre-defined acceptance limit of 0.04 μM ATP for both stem cell populations and that the cumulative potency ratio for both

Sample UCB Unit Number	Stem Cell "Quality" (iATP/5,000 cells)				Cumulative Potency Ratio
	CFC-GEMM	HPP-SP	CFC-GEMM	HPP-SP	
1	0.172	0.156	2.91	0.70	3.61
2	0.128	0.118	3.12	0.69	3.80
3	0.064	0.041	1.40	0.31	1.72
4	0.128	0.096	2.07	0.95	3.02
5	0.122	0.122	2.18	0.81	2.99
6	0.047	0.082	0.92	0.56	1.48
7	0.087	0.057	2.23	0.52	2.76
8	0.103	0.070	2.48	0.46	2.94
9	0.058	0.043	1.50	0.30	1.81
10	0.167	0.183	-	-	-
11	0.071	0.030	1.75	0.29	2.05
12	0.175	0.131	2.81	0.62	3.43
13	0.076	0.065	1.08	0.23	1.31
14	0.057	0.028	0.81	0.11	0.92
15	0.043	0.038	0.66	0.15	0.81
16	0.110	0.077	1.25	0.32	1.57
17	0.057	0.060	1.80	0.60	2.39
18	0.223	-	3.05	-	-
19	0.107	0.226	1.99	1.39	3.38
20	0.134	0.173	2.50	1.00	3.49
21	0.082	0.160	1.53	1.03	2.56
22	0.138	0.159	2.29	1.03	3.32
23	0.057	0.082	1.19	0.53	1.72
24	0.085	0.109	1.62	0.70	2.31
25	0.074	-	1.45	-	-
26	0.091	0.160	2.10	0.85	2.95
27	0.065	0.086	1.35	0.54	1.90
28	0.061	0.075	1.49	0.68	2.16

Table 1: Stem cell "quality" and stem cell potency both contribute to the release of the product prior to use.

stem cell populations is greater than 1 (RS). Under these conditions, all 28 samples would pass for the CFC-GEMM population. For primitive HPP-SP stem cell population, samples 11, 14 and 15 would not pass and sample 3 would be questionable. Samples 18 and 25 had insufficient cells to perform the assay. Analyzing the individual potency ratios for each stem cell population, samples 6, 14 and 15 demonstrate a lower value than the RS, while only 4 HPP-SP samples indicate a greater potency than the RS. There are two primary reasons for this. The first is that there are always fewer primitive stem cells than mature stem cells. The second is that primitive stem cells are usually quiescent and not proliferating. As a result, the cumulative potency for both stem cell populations can be taken in account. In this case, only samples 14 and 15 exhibited a cumulative potency ratio lower than the RS. For sample 10, insufficient cells were available to perform a 3-point cell dose response for both stem cell populations and for samples 18 and 25, insufficient cells were available for the primitive stem cell population. Samples 10, 18 and 25 had insufficient data to make a decision, so that other test parameters would have to be taken into account. Therefore, from the 25 remaining samples, two (samples 14 and 15) would not be released since they did not meet the required release criteria and would not be expected to engraft.

This small case study [15] was a retrospective study. All 28 cord blood units were transplanted into patients, and all engrafted. Since potency assay could only be completed on 25 samples and two of those would not have been released for transplantation purposes, the accuracy of the assay was approximately 92%.

It has been shown that between 15 and 20% of all cord blood stem cell transplants do not engraft [27-29]. From the results described here, it would appear that this high percentage rate of graft failure could be reduced by at least 10% thereby significantly improving efficacy and reducing unnecessary risk to the patient.

The Misconception that Potency must Correlate with Clinical Outcome

From the previous assay description, emphasis has focused on the predictive ability to measure stem cell engraftment potential. This is the predictive capacity of a product, which, when administered or in this case, transplanted, can elicit the engraftment process. The engraftment process is the response of the transplantation procedure. It is important to distinguish this from clinical outcome detected by time to engraftment. This is the result of the response to produce a specific number of cells, usually functionally mature cells, demonstrating that initial reconstitution has occurred. It is obvious that time to engraftment as a clinical outcome, is a retrospective assessment. The FDA Guidance on Potency Tests for Cellular and Gene Therapy Products [1] states that, "Efficacy data from well controlled clinical investigations can provide evidence that a product has biological activity, and thus is potent. However, use of clinical study data may not be a practical method to quantitatively test for potency to release a lot". This is because "clinical data may not be available prior to release of individual lots". Herein lays the misconception that potency must correlate with clinical outcome. Potency must correlate with the response, but need not correlate with clinical outcome. Since reconstitution is a result of the response, the clinical outcome as measured by time to engraftment, is not a valid measure of potency.

This conclusion is quite different to that presently being proposed in the field of hematopoietic stem cell transplantation and especially in the cord blood community. One of the reasons for this discrepancy lies with the entrenchment of tests and assays used to characterize the product and that has been shown to correlate, to different degrees, with the need to measure time to engraftment as the clinical outcome. Stem cell potency and clinical outcome are two separate biological processes. As described above, stem cell potency is measured by the proliferation potential of stem cell populations. Clinical outcome is detected by the differentiation capability of the lineage-specific progenitor cells, derived from the engrafted stem cells, to produce functionally mature cells. Since proliferation and differentiation cannot be measured using the same assay readout, stem cell potency and clinical outcome require different endpoints performed at different times.

Ideally, a potency assay should be in place prior to beginning the manufacturing process. This, in turn, requires that the biological function(s) of the cells that will result in the intended effect can be measured using a standardized and validated assay so that steps in the manufacturing process can be compared and controlled. When hematopoietic stem cell therapy became a routine procedure in the 1970s with the pioneering work of Thomas et al. [30], the biological activity of the stem cells as a question of potency, was hardly considered. Indeed, the regulatory considerations and assays for measuring potency had not been developed. At the beginning of the 1970s, the Colony-Forming Unit (CFU) assay had just been applied to human progenitor cells [31], and in vitro stem cell assays were several years away from being introduced [32-35]. Although separation of mononuclear cell suspensions by density gradient centrifugation was introduced in 1968 [36], other procedures such as centrifugal elutriation, the basis of present day apheresis, and cell separation using magnetic beads, have taken precedence. The introduction of flow cytometry in the 1970s, the production of monoclonal antibodies and the detection of the CD34 membrane antigen [37] as a "stem cell marker" as well as other cell markers has revolutionized clinical hematology. These tests and assays may be trusted by the community, but it doesn't mean that they are potency assays.

The Science of Cord Blood Potency

Umbilical cord blood was designated a drug in 2009 by the FDA [38]. Like bone marrow and mobilized peripheral blood, cord blood results in a systemic effect. However, cord blood, as a source of stem cells, is virtually never used fresh, but must be cryopreserved and stored, often for many years prior to use. Instead of applying its own established requirements and regulations for measuring potency, the FDA recommended that Total Nucleated Cell count (TNC), viability and the number of viable CD34 positive cells were acceptable purity and potency requirements. Cell counting or cell content determination and viability are not only two of the most basic procedures performed in any laboratory using cell-based assays, but the number of cells and their viability are also required for potency assays. In hematopoietic stem cell processing it is common to rely on the Total Nucleated Cell (TNC) count instead of the mononuclear cell (MNC) count. The difference is that the TNC count includes dead and contaminating cells, which not only play no role in the engraftment process, but also dilutes the stem cells present in the product. The consequence of using TNC is that larger volumes and greater numbers of cells must be used than would otherwise be necessary with a more concentrated MNC fraction.

Most viability assays employ dye exclusion using trypan blue, propidium iodide (PI), 7-aminoactinomycin D (7-AAD) and acridine orange to name but a few. They all rely on cell membrane permeability and therefore detect membrane integrity. No information is provided on cellular and metabolic integrity. The consequence is that dye exclusion viability can produce false positive results that can lead to a conclusion that the cells may be viable when they are actually metabolically dead. By extension, using 7-AAD to measure viable $CD34^+$ cells can also produce false positive results that can have serious implications in a clinical situation. In addition, the CD34 "stem cell marker", not only identifies a subset of stem cells [39-42], but also other cells, especially progenitor cells, that are not stem cells [43-46]. Another "stem cell marker", aldehyde dehydrogenase (ALDH) has also been considered as potency assay readout for hematopoietic cell products, but this too is not specific since the enzyme is found in many other cell types [47-51]. Thus, none of these tests and assays quantitatively measures the biological activity of the active stem cell constituents.

Although not recommended as a potency assay, it has been shown that the CFU assay, in the manner in which the results are enumerated, demonstrates one of the highest correlations with time to engraftment. The CFU assay has been a requirement for cord blood stem cell processing laboratories to obtain Net Cord-FACT accreditation. Results from the CFU assay must also be entered into the primary cord blood database (CordLink), administered by the National Marrow Donor Program (NMDP), to search for potential cord blood units for transplantation. Reporting or documenting CFU assay results represents a curious situation. Results can be documented either as a "growth–no growth result" or the total number of colonies is counted, but only reported as the number of granulocyte-macrophage (GM) colonies. The focus is on the number of GM colonies produced since this correlates with time to neutrophil engraftment [52,53,28]. The growth factor cocktail used mostly for these assays include Erythropoietin

(EPO), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Interleukin-3 (IL-3) and Stem Cell Factor (SCF). In some cases, Granulocyte Colony Stimulating Factor (G-CSF) may be included to potentiate the production of neutrophils. Since this combination of factors does not include Thrombopoietin (TPO) that is responsible for stimulating the production of megakaryocytes, the stem cells actually detected are more mature than the CFC-GEMM described above and similar to those stem cells that would be responsible for the shortest engraftment and reconstitution. As a result, the number of GM colonies will not, and does not, correlate with the active stem cell components that are responsible for and correlate with the process of stem cell engraftment [15].

For years, these four parameters, TNC, viability, CD34 and CFU, have been the only results acceptable for characterizing a cord blood stem cell product. It has been almost heresy to suggest anything different. The reason or reasons for obtaining certain clinical outcomes, notably lengthy engraftment periods and engraftment failure, have been previously discussed in detail [54,55], and have been considered almost exclusively in terms of these four limited parameters. As far as lengthy engraftment periods are concerned, this has been addressed in an interesting manner by transplanting two cord blood units [56-58]. However, in a recent publication by de Lima et al. [59], more rapid time to engraftment was achieved by transplanting in vitro expanded cells of one of the two cord blood units on mesenchymal stromal cells. After expansion, the TNC had increased more than 12 fold. Although the number of $CD34^+$ cells had expanded more than 30 fold, this expansion appeared to be exclusively due to increased monocytes and granulocytes as also indicated by the 17.5 fold expansion of CFU-C or GM progenitor cells. This would imply that the shorter time to engraftment might be due solely to the increased number of $CD34^+$ GM progenitor cells. Unfortunately, a stem cell potency assay on the expanded cells was not performed. If it had been, there is a good chance that due to the expansion pressure applied to the stem cells present in the cord blood unit during the procedure (stimulation with SCF, Flt3-ligand, TPO and G-CSF for 14 days), loss of primitive and therefore more potent stem cells, may have occurred that would leave the primitive stem cell compartment virtually empty and result in relapse. Of the 31 high-risk patients treated, 10 remained alive after 12 months, but 4 patients have relapsed, the remainder dying from other causes.

The reason for the high engraftment failure has been attributed to low cord blood unit potency [28]. This, in turn, has been assumed to be a problem with the tests and assays used to characterize the cells. Based on this conclusion, Page et al. [29] proposed a statistical scoring system using TNC, MNC, viability, CFU and $CD34^+$ content of pre- and post-cryopreserved samples to optimize use of specific cord blood units for transplantation. That the knowledge of stem cell biology might provide some information for diminished potency was never considered. That the tests and assays were not standardized, let alone validated, so that results from different units could be directly compared with each other, was also never considered. The idea that measuring the cells responsible for engraftment might provide a reasonable clue to the problem was never discussed. Finally, that the assays might not be measuring potency at all was never taken into account.

These are just two examples of many that clearly illustrate that present tests and assays do not provide the necessary release criteria that would help reduce engraftment failure. The need for changing present procedures is known. In a publication by Spellman et al. [60], the NMDP discussed the limitations of the assays currently used and proposed guidelines for cord blood potency assays and the expectations of new assays. These expectations included "standardized methodologies, reproducibility with limited variability between testing sites, automated testing outputs, high throughput for UCB banks, rapid turnaround time and single sample tests for transplant centers". All of these expectations have been met by the assay described above and in detail elsewhere [14,15,61]. Yet, the NMDP has not yet exercised its expectations within the framework of its own guidelines. Private and federally funded cord blood banks and transplantation centers worldwide now represent an individual growing industry within the exponentially growing stem cell industry. The focus of the cord blood industry is to provide high quality and high potency stem cell products for those who require the potential life-giving benefits of this type of therapy. If none of the tests and assays actually measures the cells that are supposed to provide this life-giving benefit, let alone the potency and quality of the cells to release them for use, then the time has come for the industry to rethink how cord blood characterization is performed and what is needed to reduce the high engraftment failure. This might be realized in 2013 when NetCord-FACT finally removes the shackles of requiring cord blood banks to perform the CFU assay to allow alternative and replacement assays to be performed that are non-subjective, validated and more informative. Similarly, NMDP should demonstrate its flexibility to allow results from assays, other than the CFU, to be entered into its database. Continually reinventing the wheel will not help the situation. If these changes take effect, then the industry might start asking the necessary questions that are needed to move to a more advanced level of cell processing, testing and characterization that will benefit all involved, especially the patients.

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