

## Critical elements in the development of cell therapy potency assays for ischemic conditions

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### Abstract

A successful potency assay for a cell therapy product (CTP) used in the treatment of ischemic conditions should quantitatively measure relevant biological properties that predict therapeutic activity. This is especially challenging because of numerous degrees of complexity stemming from factors that include a multifactorial complex mechanism of action, cell source, inherent cell characteristics, culture method, administration mode and the *in vivo* conditions to which the cells are exposed. The expected biological function of a CTP encompasses complex interactions that range from a biochemical, metabolic or immunological activity to structural replacement of damaged tissue or organ. Therefore, the requirements for full characterization of the active substance with respect to biological function could be taxing. Moreover, the specific mechanism of action is often difficult to pinpoint to a specific molecular entity; rather, it is more dependent on the functionality of the cellular components acting in a multifactorial fashion. In the case of ischemic conditions, the cell therapy mechanism of action can vary from angiogenesis, vasculogenesis and arteriogenesis that may activate different pathways and clinical outcomes. The CTP cellular attributes with relation to the suggested mechanism of action can be used for the development of quantitative and reproducible analytical potency assays. CTPs selected and released on the basis of such potency assays should have the highest probability of providing meaningful clinical benefit for patients. This White Paper will discuss and give examples for key elements in the development of a potency assay for treatment of ischemic disorders treated by the use of CTPs.

**Key Words:** *angiogenesis, cell therapy, potency assay, vasculogenesis*

### Introduction

The goal of a potency assay is to predict the therapeutic activity of a tested product in a quantitative and reproducible manner by measuring relevant biological function on the basis of the attributes that are linked to relevant biological properties. However, there is no single test that can adequately measure those product attributes that predict clinical efficacy. Manufacturers demonstrate clinical effectiveness by “substantial evidence,” that is, evidence that the product will have the effect it purports or is represented to have under the conditions of use prescribed, recommended or suggested in the labeling [1]. The therapeutic mechanisms of action (MoA) vary significantly between cell therapy products (CTPs). This variability is a result of factors such as the cell source, inherent cell characteristics, culture

method, administration mode and the *in vivo* conditions to which the cells are exposed. Because the cell is a living organism that responds to its environment in a complex multimodal manner, each set of conditions that the cell encounters can change its features and response, in some cases resulting in long-term epigenetic changes. Because the CTP MoA relies on multiple biological activities, it is especially challenging to determine which exact product attributes are most relevant to measuring potency. Nonetheless, it is imperative to develop potency measurements that reflect the product’s relevant biological therapeutic properties and provide a reliable measure of production batch-to-batch consistency; this has an inherent challenge for developing a potency assay to a CTP (also referred to as advance therapy product) before clinical trials.

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Only after clinical trials can one truly define a specific assay as a potency assay. This White Paper is aimed for cell therapy developers, giving them the tools, guidance and examples to develop potential assays for ischemic conditions that can mature to a potency assay once correlated to clinical data. This White Paper is part of the Alliance for the Regenerative Medicine and the International Society for Cell Therapy's efforts to provide developers with useful information to guide them through the potency assay development process. The paper focuses on CTPs that regenerate blood flow to ischemic tissues. Several MoAs underlying this reperfusion therapeutic effect will be described, and guidance for development of MoA-relevant potency assays—including specific examples of assays currently in use or under development—will be provided. For the purpose of this paper, the term “angiogenesis” will encompass all processes involved in assembling new blood vessels. The term “vasculogenesis” will be used to describe processes involved in induction of collaterals and natural bypasses and/or repair of existing vessels. One should take into consideration, during all development stages, that an assay can be truly defined as a potency assay only once correlated to clinical data at late-stage development. Therefore, it is highly recommended to develop several assays on the basis of several suspected MoAs during development.

#### *Requirements of potency assays*

Ideally, a potency assay should verify the presence of the relevant biological activity of a cell therapy on the basis of quantitative measure(s) of the factors and/or activities that are related to its mechanisms of action in each clinical indication. These measures would directly correlate with relevant clinical outcomes and would be feasible, repeatable, quantifiable and robust. To develop such an assay, it is necessary to determine the factors and/or activities that are representative of the presumed MoA and those that are necessary and sufficient for providing benefit in the clinical setting.

Both the Food and Drug Administration (FDA) and the European Medicines Agency recommend adherence to the International Conference on Harmonization guideline 6QB definition of potency as the quantitative measure of biological activity that is based on the attribute of the product, which is linked to its relevant biological properties. The assay demonstrating the biological activity should be based on the intended biological effect related to the clinical response. Likewise, according to the FDA's Center for Biologics Evaluation and Research guideline from 2011, potency (also referred to as strength) is defined

as the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended to affect a given result. Potency tests represent the product's MoA (ie, relevant therapeutic activity that is based on the intended biological effect) [1]. Along with a number of other tests ensuring the quality and reproducibility of the product, potency assays are used to demonstrate that only product batches and lots that meet predefined specifications or acceptance criteria are administered to patients.

Regulations require that tests for potency will consist of *in vitro* and/or *in vivo* settings that have been specifically designed for each CTP to show its biological activity in a quantifiable manner. The traditional approach for assessing the potency of biological products is to develop a quantitative biological assay (bioassay) that measures the activity of the product. In cases in which development of a suitable bioassay is not feasible, it may be necessary to identify a surrogate measurement of biological activity related to its specific ability to effect a given result [1]. Major cellular functions such as viability, self-renewal (also referred to as multiplication), delayed death (included apoptosis), secreted molecules and differentiation are pivotal to the quality, function and sustainability of the product and may need to be at release with the use of validated assays. It should be ensured, however, that the method of characterization is relevant to the intended biological effect *in vivo*. Thus, as a complementary validation (also referred to as an assay matrix) of the *in vitro* test, *in vivo* assays for potency may also be useful, especially when experimental animal models are available.

Regulations allow for considerable flexibility in determining the appropriate measurement(s) of potency for each product. Potency is determined on the basis of individual product attributes; therefore, the adequacy of potency tests is evaluated by the regulator on a case-by-case basis. In addition, potency assay results may be used to establish a correlation between product efficacy and the potency measurement. Furthermore, it is acknowledged that the amount of information needed to make that assurance will vary with the phase of the investigation, the proposed duration of the investigation, the dosage form and the amount of information otherwise available.

Potency assays used for release testing of licensed biological drug products must comply with applicable biologics and current Good Manufacturing Practice regulations, including:

- Measure identity and strength (activity) of the product, for example, indication of biological

activities specific to the product with results that correlate to an effect in a relevant animal model and human trials

- Provide quantitative data
- Include appropriate reference materials, standards and controls
- Establish and document the accuracy, sensitivity, specificity and reproducibility of the test methods used
- Meet pre-defined acceptance and/or rejection criteria
- Provide data to establish dating periods
- Meet labeling requirements

Although the FDA does not require, nor is it feasible, to test all of the biological functions of a CTP, potency testing should include its most relevant biological activities.

*Considerations in the development of potency assay on the basis of a CTP's reperfusion biology (angiogenesis, vasculogenesis and arteriogenesis)*

Reperfusion therapy is a medical treatment that restores blood flow to a previously ischemic tissue or organ. The most commonly referred to reperfusion therapy is angioplasty or bypass surgery, which restores blood flow in large and medium-sized blood vessels. Stem cell-based therapeutic reperfusion, however, derives from restoration and improvement of small to medium-sized blood vessel function. Induction of reperfusion to tissues and organs through induction of new vessels and/or repair of existing vessels has been identified as a key component in providing clinical benefit through cell therapy in a wide variety of vascular diseases, including:

1. Cardiovascular disease, such as acute myocardial infarction and chronic heart failure, whether of ischemic or non-ischemic etiology, as well as refractory angina and chronic myocardial ischemia
2. Acute and chronic cerebrovascular disease such as stroke and vascular dementia
3. Peripheral vascular disease such as wound healing, peripheral arterial disease and limb ischemia

The MoA for reperfusion of a tissue by restoration of blood flow can be based on generation of vascularization (also referred to as natural bypasses) and on improving blood flow within existing arteries and arterioles. The biological processes underlying these effects involve the therapeutic activity of the CTP and soluble molecules such as vascular

endothelial growth factor (VEGF) produced and secreted by it that affect the ischemic site.

Identifying the suitable CTP *in vivo* MoA is central to choosing and developing a relevant potency assay. This is particularly true because there are several distinct biological processes that can result in reperfusion [2,3]. The following biological processes are central to restoration of blood flow:

1. Angiogenesis is defined as new blood vessels sprouting from pre-existing vessels. This process consists of (i) degradation of basement membrane; (ii) proliferation and migration of endothelial cells (EC) into the connective tissue; (iii) formation of a solid EC core; and (iv) formation of a lumen (capillary) concurrent with formation of new basement membrane and recruitment of pericytes [2]. Angiogenesis is promoted primarily in response to hypoxia, and the primary cells participating are local EC and pericytes. In most cases, the end result of angiogenesis is an increase in capillary density.
2. Vasculogenesis is defined as the formation of blood vessels, usually by sprouting from existing vessels, primarily by recruitment of circulating endothelial progenitor cells (EPCs) originating from the bone marrow. This process consists of (i) secretion of cytokines; (ii) mobilization of EPCs from bone marrow and recruitment of EPCs to local ischemic areas; and (iii) integration of EPCs into the area and formation of new capillaries that sprout from existing vessels in which EPCs differentiate into mature ECs [3–6].
3. Arteriogenesis is defined as lumen enlargement of arteriolar anastomoses (connection of two arteries) to collateral vessels. Arteriogenesis may occur in a non-hypoxic environment in response to increased flow shear stress as a result of vascular occlusion. This process consists of (i) increase of adhesion proteins on ECs; (ii) activation, adhesion and migration of monocytes to the endothelium; (iii) perivascular infiltration of monocytes/macrophages; (iv) secretion of growth factors and cytokines by the monocytes/macrophages; and (v) vessel remodeling by proliferation of smooth muscle cells and possibly EC/EPC recruitment. The end result, as detected by means of intravital microscopy assay, is a significant increase in vessel diameter, in some cases sufficient to take over the role of large arteries [7–10].

Although the degree to which these processes are distinct and separate remains under investigation, it is clear that there are multiple biological processes

that occur *in vivo* to address ischemic conditions and increase perfusion. The MoA of different cell therapies may act by differentially promoting one or more of the above processes. Therefore, understanding which of these processes is central to the MoA of a given therapy is crucial in choosing a relevant potency assay.

One example would be a potency assay that is based on relevant cytokine secretion. The capacity of a CTP to produce cytokines is determined by testing its conditioned medium (CM) which is the culture media in which the cells grow that contains the secreted cytokines. As previously reported, VEGF is central to angiogenesis (EC proliferation) [9], whereas granulocyte macrophage colony-stimulating factor (GM-CSF) is central to vasculogenesis (as a promoter of EPC mobilization from BM) [4,10]. Thus, one optional relevant cytokine to measure would be VEGF when the CTP's MoA is angiogenesis and GM-CSF when the CTP's MoA is vasculogenesis. Yet, secretion of a specific cytokine as VEGF or GM-CSF measurement is not sufficient to prove potency/biological activity and is not regarded as a biological assay. In addition to secretion measurements, a relevant *in vitro* potency assay showing the effect should be performed. Nevertheless, a correlation between cytokine secretion profile and assay results is essential for potency and MoA assumption.

When vasculogenesis is the CTP's MoA, a potential functional test assessing the capacity of CM or cell co-culture to induce BM cell migration will typically be used, whereas the endothelial cell proliferation test will be applied in the case of angiogenesis.

Furthermore, in many cases, more than one MoA is involved for the same CTP; therefore, multiple potency assays should be developed early on in clinical phases to characterize the main MoA for each CTP and increase the chance to correlate them to the clinical findings.

*Considerations in development of potency assay on the basis of CTP cell biology (secretomes, differentiation and engraftment)*

Given the fact that each CTP is unique, the MoA of cell therapies can be roughly divided into two main categories: (i) secretome-based MoA and (ii) differentiation, engraftment and tissue integration-based MoA. Typically, mesenchymal stromal cells (MSCs) may depend more on paracrine, secretome-based mechanisms, whereas BM-derived hematopoietic cell MoAs may be dependent, in addition to the secretome paracrine effect, on engraftment and differentiation into effector cells.

In secretome-based cell therapy, the cells function as responsive protein factories that act thorough

a paracrine effect. The MoA of these cells is to deliver a milieu of proteins, growth factors, chemokines and cytokines that induce self-healing of the injured tissue. In many cases, the delivered cells remain in the area for days to weeks and can respond to the *in vivo* environment by altering the composition of their secretome accordingly. An example would be the use of MSCs to promote *in vitro* and *in vivo* angiogenesis [11–13].

CTPs with differentiation, engraftment and tissue integration MoAs, in addition to their secretomes, have the capacity of substituting injured tissue and thus become a permanent replacement of dead or dying host cells [14,15]. Autologous or allogeneic cells with an appropriate differentiation capacity are extracted, cultured and differentiated *in vitro* before administration or differentiate *in vivo* after their administration. These cells such as hematopoietic stem cells are intended to integrate in the target tissue and replace or augment injured tissue function for the long term (years).

One must consider that although these MoAs differ, they may also be intertwined; cells injected with the goal of differentiation, engraftment and integration may also exert an effect through secretion of proteins. Conversely, in the secretome-based paracrine MoA, small numbers of delivered cells may remain as functional cells for the long term, although this is less probable and can be tested by means of *in vivo* biodistribution experiments. In both MoAs, the *in vivo* test would consist of the ultimate capacity of the CTP to induce tissue reperfusion. However, the *in vitro* potency assay design used for these two MoAs will differ significantly.

In the secretome-based MoA, cells would be tested for their capacity to produce and secrete certain levels of relevant proteins and for the capacity of the cell-conditioned media to induce various *in vitro* aspects of angiogenesis such as endothelial cell proliferation.

In the differentiation, engraftment and integration MoAs, additional tests would be needed to test for the cells' appropriate differentiation, multiplication (EPC colony-forming units), engraftment, integration and survival capacity.

*Considerations in the development of multimodal potency assay for reperfusion*

Assays confirming different aspects of reperfusion activity of CTPs can be based on existing or novel *in vitro* tests such as cell paracrine mechanisms, cell multiplication and proliferation, cell migration, cell differentiation or tube formation (TF) or on *in vivo* angiogenesis tests such as matrigel plug assay or corneal angiogenesis assay [16].

Although these assays could be used as a potency assay directly, they can also be used to identify the activity of pro-angiogenic secreted factors by which CTPs drive vascularization. Identification of the pro-angiogenic factors required for CTP biological activity has several advantages. First, the importance of these factors for CTP potency can be directly tested by knocking out or blocking activity of the factors through the use of inhibitors or antibodies in *in vitro* or *in vivo* assays. These types of experiments will establish whether each factor alone or a combination of factors is necessary and/or sufficient for the activity. Second, minimum required levels of these factors can be established by adding back recombinant factors, at least in the *in vitro* setting, allowing for a quantitative measure of potency. Finally, the relationship between cell dosage and potency can be tested by measuring the effects of increasing the levels of one or more pro-angiogenic factors alone or in combination in *in vitro* or *in vivo* assays [17,18].

In their work on development of a surrogate angiogenic potency assay for a CTP of BM-MSC like cells (MAPCs), Lehman *et al.* [17] showed that the cytokines, chemokine (C-X-C motif), ligand 5 (CXCL5), interleukin 8 (IL-8) and VEGF, were required for human umbilical cord vein endothelial cells (HUVECs) derived from *in vitro* and *in vivo* angiogenic activity.

First, they compared the HUVEC maximal TF achieved in the presence of endothelial growth media to the TF resulting from the CTP CM. The experiments subsequently demonstrated that depletion of any of these factors from the media prevented the HUVEC TF (Figure 1A). Addition of increasing concentrations of each cytokine created a dose-response curve enabling the determination of the minimum level of VEGF, CXCL5 and IL-8 required for angiogenesis (Figure 1B). The data from this study provide confidence that the enzyme-linked immunosorbent assay (ELISA)-based measurement of these factors in clinical supernatants correlates with the performance in the vascular endothelial tube formation assay and hence represents a surrogate measure of the CTP's angiogenic potential [17].

One disadvantage of this approach is that the CTP MoA is multimodal, and each biological activity of the cells (such as angiogenesis) is probably dependent on a combination of multiple factors. Therefore, care must be taken to determine which factors and activities can be assayed as surrogate markers for the induction of the angiogenic biological process. By establishing a few activities and factors as measures of potency, the relevant tests that contribute to the correlation with the clinical outcomes could be retained as potency assays. However, because of this complexity of the CTP MoA, in many

cases the specific factors that are responsible of the biological activity will not be fully identified, in which case more general *in vitro* or *in vivo* assays may be necessary.

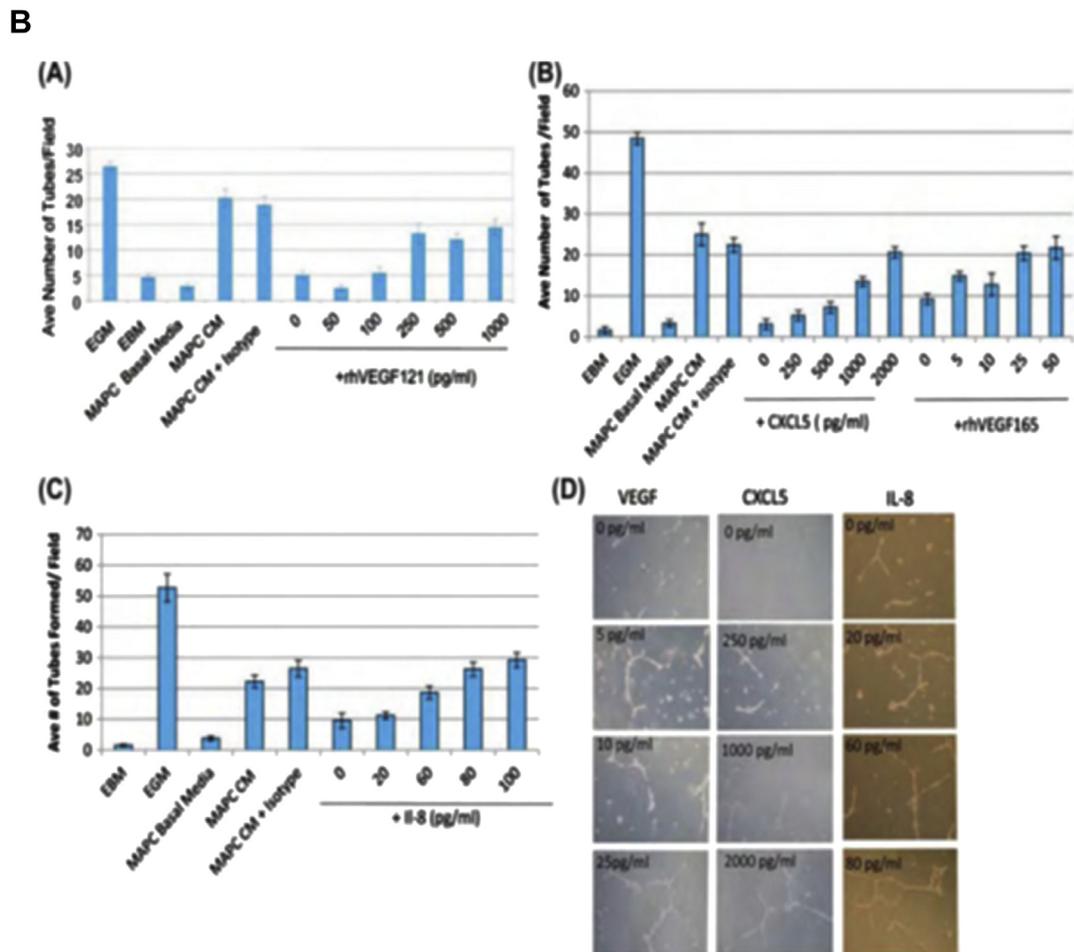
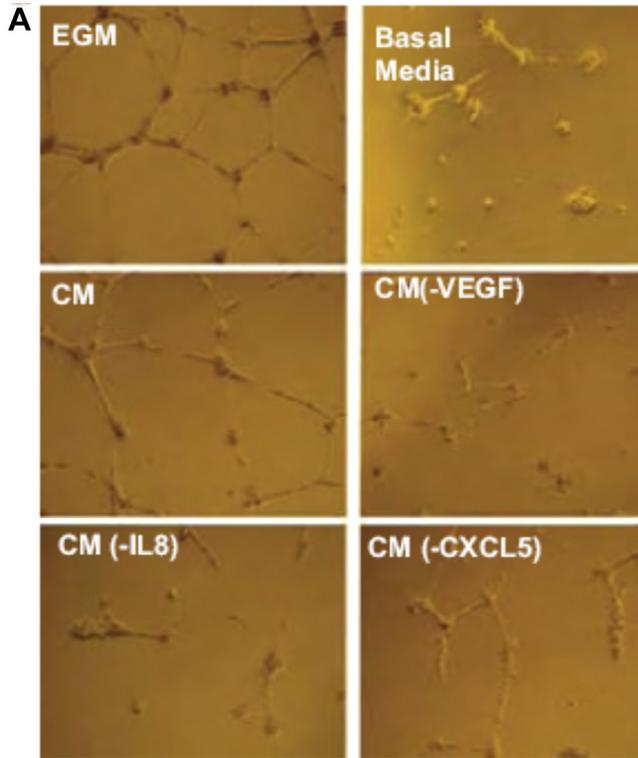
#### *Controls-based validation of potency assay*

Potency assays are important to verify the biological activities of CTPs to guarantee the reflection of the capacity to produce an effect (efficacy) of the CTP product. In addition, potency assays should measure the consistency of the manufacturing process and demonstrate efficiency-relevant differences between batches, therefore becoming a critical release criterion. Potency assays are also important to determine the product stability. In stability programs, it is essential to track after differences in potency results throughout the shelf life of the product, thereby predicting its clinical efficiency. Deviate results such as out of spec or out of trend might imply a decrease in product stability. Additionally, potency assay should be used for comparability experiments to determine the effect of production changes such as critical raw material changes, process changes, scale up or manufacturing location changes. It is essential that the assays chosen to measure biological activity be quantitative, reproducible and economical. Once an assay is chosen on the basis of its relation to the mechanism of action of the CTP, it must be validated for use as the product matures in its clinical development.

Potency assay should include proper controls, reference materials and sufficient replicates to ensure that the assay is performing as expected. Assay-specific controls will depend on the product being analyzed as well as the assay used.

Appropriate assay controls as reference sample (a representative batch of product) helps to ensure that the assay is performing as expected and can be used as system suitability indicators for equipment and reagents. Additionally, accumulated data of reference sample (continues trend) can increase confidence that results are credible and reproducible. General principles for reducing variability include the use of qualified reagents, qualified and calibrated equipment and adequately trained and qualified operators. Assay variability can also be substantially reduced by adherence to detailed standard operating procedures and having appropriate controls and statistical ranges in place (acceptance criteria), which will allow compliance with established specifications and standards.

Validation should demonstrate that the assay is reproducible with different operators, on different days and with different lots of reagents. Reproducibility can be especially challenging for *in vitro* or



*in vivo* functional assays in which biological raw materials such as extra cellular matrix are used and complicated measurements such as 3-dimensional microscopic evaluation are applied. For example, the TF assay, a well-established measure of EC alignment, elongation and ability to form 3-dimensional networks or tubes, requires the use of ECs such as HUVECs and matrigel. Both of these reagents are biological products with significant lot-to-lot variability. Thus, given the variability of the systems, this assay, along with other *in vivo* assays, such as the corneal angiogenesis assay, would be challenging to perform in a reproducible manner and would require significant controls and robust reference materials resembling reference standard (RS), if possible, for validation. One approach would be to normalize the results to well-established reference samples or negative controls. Each new batch and lot of reagents could then be recalibrated to the standards and controls to ensure its reproducibility.

As with all well-designed experiments, development of a potency assay should include appropriate assay controls and a comparison to appropriate product-specific reference materials (resembling the RS) when available. Running a product-specific reference material and/or control samples in parallel with the product helps to ensure that the assay is performing as expected. In addition, controls help to establish that the equipment and reagents are working within established limits. A well-designed set of control samples can substantially increase confidence that results are meaningful and reliable. Because in-house reference materials will be used at various stages of CTP development and characterization, these reference materials also should be subjected to stability studies in parallel with the product stability studies and should be assigned appropriate retest or expiration dates. Moreover, each new batch of reference material should be characterized and compared with the original to qualify and eventually validate new reference materials. Validation also requires testing of a representative population of CTP, resembling the RS as much as possible, to ensure that the entire batch meets specifications. For CTPs produced in single batches, such as autologous therapies, which are specific to each patient, the potency assay must be easily performed on a small amount of product and should be able to be

performed in a short time frame. In these instances, any type of *in vivo* testing may not be possible. Therefore, testing for the presence of surrogate biomarker(s) of activity, such as a set of cellular marker expression signatures or secretome secretion profile of pro-angiogenic factors (such as VEGF) determined by quantitative polymerase chain reaction or ELISA, may be an option.

An example of reproducibility (with different operators, on different days and with different CTP lots) is given by an ELISA test that measures the secretion of a panel of three cytokines shown to be crucial for angiogenesis activity. With the use of this surrogate marker-based test, Lehman *et al.* [17] assessed the variability and range of results of VEGF, CXCL5 and IL-8 expression in 15 separate manufacturing MAPC lots, each tested by two different operators and on two different days. The resulting coefficient variant (%CV) among all variants was less than 20%, indicating a well-controlled, reproducible assay performance that could thus reliably detect the cytokine levels in the tested samples (Figure 2) [17].

In addition, it is important to establish that these surrogate measures are not merely a reflection of the characterization and purity of the cells but rather are measurements of factors demonstrated to be essential for the CTP's angiogenic activities. This is especially critical because physicochemical characteristics of CTPs may not correlate well with potency. In the sample presented here, the cytokines' relevancy and their meaningful concentrations were pre-determined in the TF angiogenesis test *in vitro* and *in vivo* as part of a set of experiments justifying their use as surrogate markers [17].

It is also important to establish that acceptance criteria for the assay will eventually be based on the biological properties of the cells and performance in animal models and clinical outcome. The acceptance criteria should be based on the minimum level of activity or factors needed to induce the required biological activity *in vivo*, if possible. In accordance with the regulatory requirements, it is important to continue to collect data and to adjust assay design to continue fit-for-purpose optimization. This includes incorporating a sufficient number of replicates to allow for statistical analysis, with the use of sample randomization to reduce biases. Variability of

Figure 1. Surrogate angiogenic potency assay for a CTP of BM-MSCLike MAPCs, based on cytokine derived HUVEC angiogenic activity. (A) VEGF, CXCL5 and IL-8 are required for MAPC-induced angiogenesis. Immunodepletion of VEGF IL-8 and CXCL5 reduces angiogenesis induced by MAPC CM. (B) Determination of the minimum level of VEGF, CXCL5 and IL-8 is required for angiogenesis. VEGF, CXCL5 and IL-8 were individually immunodepleted from the CM. These factors were subsequently added back in a dose-response curve to identify the minimum level needed to restore the angiogenic response (two isoforms of VEGF were tested, VEGF 121 and VEGF 165) [17].

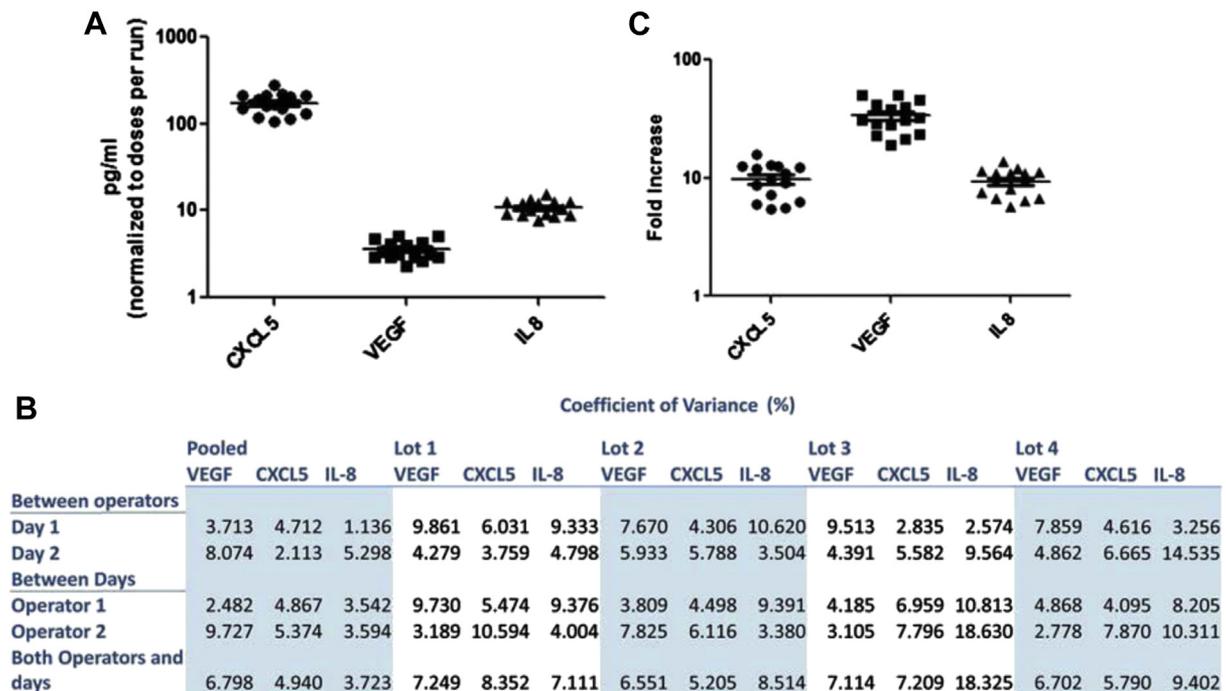


Figure 2. Clinical manufacturing lots show consistent cytokine expression. (A) Analysis of VEGF, CXCL5 and IL-8 expression in 15 separate manufacturing lots demonstrated consistent production of these factors across lots. The expression level was normalized to the cytokine production by number of doses produced per run to eliminate variability in cell number and media volume. (B) Quantification of the ELISA assay demonstrated that the variability of results from two different operators performing the ELISA on two separate days was low for VEGF, CXCL5 and IL-8. A positive control made from pooled MAPC CM was validated for use in the potency assay. (C) The spent media from clinical production runs contained, on average, 32-fold more VEGF and approximately 9 times more CXCL5 and IL-8 than the minimum required for angiogenesis [17].

bioassays, even when reduced, is unavoidable and therefore should be balanced, measured and modeled.

Once the assay has been validated through repeated testing of known standards, it can then be tested on different lots and product samples previously validated in animal models. Samples that are within the criteria should be functional in preclinical models, whereas those outside of the criteria should not be sufficient to induce adequate angiogenesis. These limits should then be used as product lot release criteria.

#### Potency assay as a predictor of dose effect

In the cell therapy field, the correlation between dose and efficacy has been less than clear, with some trials demonstrating little dose dependency at the dose ranges tested, whereas others have shown clear dose-response curves.

For example, in the study of autologous CD34+ stem cells for intractable angina, Losordo *et al.* [19] selected the doses of CD34+ cells on the basis of results of preclinical studies showing that CD34+ cells exhibit increased potency and safety for therapeutic neovascularization after myocardial infarction [19,20].

Ideally, one function of an angiogenesis potency assay would be to correlate biological activity of CTP with the effective dose range in animal studies and clinical trials. Demonstrating that an ineffective dose range in an *in vitro* assay correlated to a reduced level of vessel formation and less benefit in animal models would allow for a more precise calculation of dosage for future trials as well as provide a screening method for optimizing the CTP development process. As more clinical data are collected, the relationship between *in vitro* potency assays and clinical outcomes could be further validated. It would also allow for the examination as to whether the cell therapy-induced functional angiogenesis can be gradual or whether a cutoff for activity exists such that a minimum cell dosage is required to induce any angiogenesis and whether increasing the dose above this minimum does not increase angiogenesis linearly.

For example, even though in their intractable angina study, which, on the basis of a dose-response study in an animal model, did not show significant dose-response, Losordo *et al.* [21] did show correlation to preclinical dose-response in their clinical study, “Study of autologous CD34+ cell therapy for critical limb ischemia” [21,22].

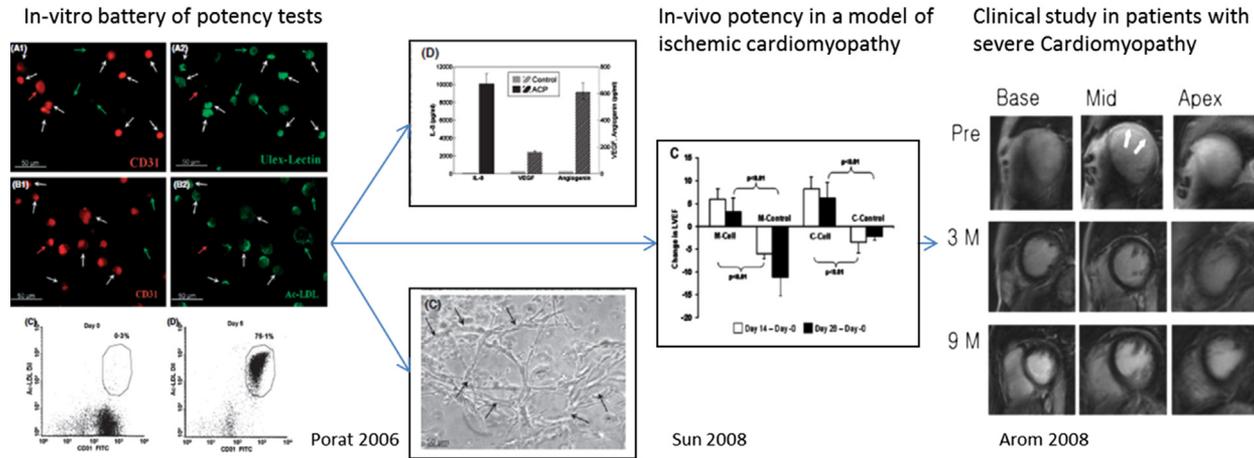


Figure 3. Potency assays for EPC-like CTP, based on cell attribute characterization and surrogate markers; potency of a complementary battery of *in vitro* tests to predict efficacy results of the CTP in an animal model of cardiac disease and in cardiomyopathy patients. FACS-based, rapid tests, based on a concomitant expression of surface marker identification with enzymatic activity, was developed for the characterization of EPC-like populations (70% concurrent expression of CD31 and uptake of Ac-LDL). The cells were subjected to additional *in vitro* functional tests including angiogenesis assessment with the use of a TF assay and FACS-based array cytokine secretion assay (TF grades 3–4; secretion of IL-8, VEGF and angiogenin). The *in vitro*-characterized CTP was then tested and for efficacy *in vivo* in the rat model of ischemic cardiomyopathy as a validation of the predictability of the *in vitro* assays. Cells selected and released according to the potency assays release criteria were found to be functional in cardiomyopathy patients [23–26].

Correlating biological activity with effective dose can be affected by several variables. Mode of administration and biodistribution can vastly affect the effective dose required for induction of angiogenesis. The duration of activity and cell retention also must be considered. Cells may be cleared quickly, especially in the allogeneic cell setting. The residence time required for the cells to induce angiogenesis could also affect the correlation between potency assay results and clinical dosing. Therefore, correlations of the effective dose *in vivo* to cell levels required *in vitro* should take these factors into account. For example, for assays demonstrating that paracrine effect by trophic factor expression

correlates with angiogenesis potential, the bio-distribution of the cells and retention times should be taken into account to determine the likely target exposure to the trophic factors in question. On the basis of quantitative measures of the levels produced by the cells *in vitro*, the minimum dosage of cells, and hence trophic factor required for activity, can be calculated and compared with the effective cell dosage *in vivo*. One caveat of this approach is that CTP can respond to their environment and subsequently change their expression and secretome secretion profile accordingly. *In vivo* methodology, such as laser capture, to examine the expression and secretion profile of the cells *in vivo* can help to further clarify the relationship between factor secretion and effective dose and hence further demonstrate the relationship between the potency assay and biological activity. Thus, it is possible to use non-biological analytical assays that are practical and demonstrate adequate performance characteristics for lot release.

#### Development of analytical potency assay on the basis of cell attribute characterization

Analytical assays can provide extensive product characterization data by evaluating immunochemical, biochemical and/or molecular attributes of the product. These attributes may be used to demonstrate potency if the surrogate measurements can be substantiated by correlation to relevant product-specific biological activities. In addition, if one assay is not sufficient to measure the product

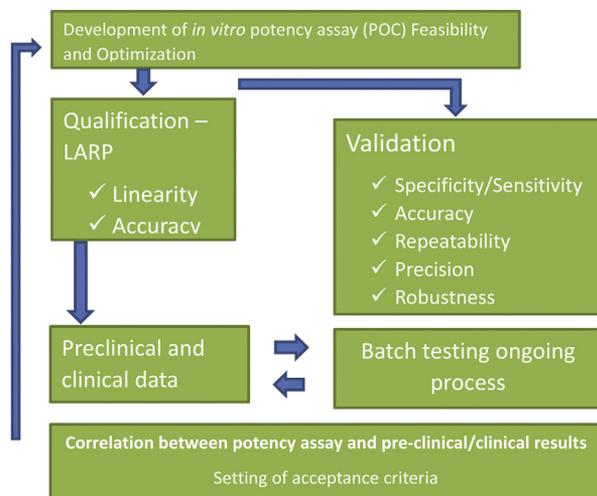


Figure 4. Schematic of *in vitro* potency assay development.

potency, then an alternative approach could be used, with application of an assay matrix that measures different product attributes associated with quality, consistency and stability.

The expected biological function of a CTP encompasses complex interactions that may range from a biochemical, metabolic or immunological activity to structural replacement of damaged tissue or organ. Therefore, the requirements for a full characterization of the active substance in terms of biological function could be taxing. Moreover, the specific mechanism of action is often difficult to pinpoint to specific molecular entity, but it is more dependent on the functionality of the cellular components acting in a “tissue-like” fashion as a whole.

Because there are many possible assays and many different types of CTPs, this document has focused on the principles and definitions that we believe should guide each sponsor. When considering the development of analytical potency assay that is based on cell attributes, the following issues should be taken into account:

1. The CTP intended effect: MoA and surrogate markers
2. The cell source: autologous cells versus allogeneic cells; treatment of a single patient versus multiple patients
3. Mode of administration and single versus multiple dosing
4. Cell performance: patient versus healthy donor
5. Manipulation level: minimally versus extensively manipulated cells and culture duration
6. Lineage differentiation
7. Viability and proliferative capacity of the cells
8. Cell organization, cells-cell interaction and cell-substratum interactions
9. Stability and time frame limits for distribution of fresh or preserved (frozen) CTPs

The characterization should be designed to allow setting up the routine controls that will be applied for release of the drug substance and final product as well as those to be performed at several steps of in-process control (IPC) to guarantee batch consistency.

#### *The CTP intended effect: MoA and surrogate markers*

Potency assays testing a surrogate marker should first correlate to (at least one) *in vivo* potency model. For example, in the case of a potency assay for secretome secretion profile (in most cases the effect of secreted factors is caused by a combination of factors rather than by a sole molecule), one can consider *in situ* molecular expression tests such as Western blot, gel-based, immunofluorescence and mass cytometry or

an assay assessing soluble secreted molecules, with application of single or multi-analyte assays such as ELISA, protein arrays/multiplex, enzyme immunoassay, chemiluminescent, immunofluorescence and enzyme-linked immunosorbent spot. In both the *in situ* and secretion-based assays, a correlation to the *in vivo* model should show that cells that produce/secrete different levels of the tested molecules will elicit a corresponding range of *in vivo* outcomes. A relevant model such as the hind limb ischemia or left anterior descending coronary artery ligation can be used with application of the relevant controls and testing various concentrations of the molecules (ie, *in vivo* potency). After proving the relevance of the molecules, they can be used as surrogate markers in a development of *in vitro* potency assays. When secreted molecules are tested as surrogate markers, it is recommended, if possible, to grow the cells in a serum-free medium and without cytokines for 24 to 48 h (starvation conditions) to test only the molecules secreted by the cells and not those that were added during culture. Alternatively, when *in situ* molecular expression is tested, there is a need to show during assay development that molecular expression can lead, under appropriate conditions, to the secretion of functional molecules.

As an example, potency tests that are based on a combination of marker identification with enzymatic activity have been developed for the characterization of EPC-like populations. Typical to EPCs, approximately 70% of these cells co-expressed binding of Ulex-Lectin and uptake of acetylated low-density lipoprotein (Ac-LDL). The assay developed demonstrated that cells known to express high levels of the surface molecule CD31 (defined as CD31Bright) also show uptake of Ac-LDL and are positive in the *in vitro* test of TF. Thus, concurrent expression of CD31Bright and uptake of Ac-LDL were consequently used to define the CTP with application of a fluorescence-activated cell sorting (FACS)-based assay [23]. In addition to the confirmation of cell identity by an FACS-based assay, a cytokine secretion assay to define the secretome was developed to test the attributes of the cells in the CTP. To this end, the cells were washed and cultured for 24 h under starvation conditions in medium, without serum or supportive cytokines. The resultant CM contained only cytokines secreted from the cells during the starvation period. An array test showing that the cells secreted IL-8, VEGF and angiogenin was used to assess the product potency and later to narrow the range of tested cytokines to IL-8, which was subsequently used as a release test for the CTP [23]. Validation of the surrogate marker-based tests was achieved when the CTP was tested and shown to be effective in an *in vivo* rat model of ischemic cardiomyopathy [24] and in

human patients with ischemic and non-ischemic cardiomyopathy [25,26] (Figure 3).

In this example, the surrogate marker test that uses concurrent expression of CD31Bright and uptake of Ac-LDL is a FACS-based assay that supports a rapid and reliable test, which allows the use of small cell samples. These features enable its use as an IPC assay as well as a release test for both autologous and allogeneic CTP.

*Cell source: autologous versus allogeneous and distribution to a single patient versus multiple patients*

According to risk assessment development paradigms, the risk of false-positive potency results for autologous and single-patient allogeneic products means that one patient will not receive a potent cell product, whereas for allogeneic products distributed and supplied to multiple patients (even hundreds or thousands), the risk is multiplied by the number of patients and doses administered with the product. Therefore, even though basically the cell source is not relevant to the potency assays that should be demonstrated on the basis of the assumed MoA, the requirements for allogeneic CTP are likely to be higher. Furthermore, the production of allogeneic CTP usually requires long-term culture, and the batches are typically much larger than autologous and single-patient allogeneic products. Thus, the availability of more product and more time in an allogeneic setting allows for more comprehensive testing (for example, testing several concentrations and time points).

*Mode of administration and single versus multiple dosing*

*In vivo* potency assays should reflect the clinical condition in humans and closely measure the effect of administration mode and dosing. Thus, in addition to selecting acceptable *in vivo* models such as hind limb ischemia and left anterior descending coronary artery ligation, the assay should be designed and performed with application of the intended administration mode such as single administration or a series of intramuscular injections or intravenous infusion as well as escalating and repetitive dosing over time. In addition, the delivery device used for the *in vivo* animal model experiments should be as similar as possible to the delivery device used in the clinic.

*Cell performance: patient versus healthy donor*

In autologous CTP, the raw material is a crucial issue that should be addressed. Raw material cells are usually taken from an unhealthy donor, whereas most of the development process is performed with

the use of samples from healthy donors. These two cell sources may not give rise to similar cells. As an example, circulating EPCs can be, in some instances, impaired in cardiovascular patients as compared with healthy individuals. It is therefore crucial to show that a potent product can be produced from patients' cells as well. The assay matrix of tests including viability and FACS-based potency tests described above (concurrent expression of CD31Bright and uptake of Ac-LDL) was used to assess differences between the ability of raw material obtained from healthy donors and cardiovascular patients to produce a final potent CTP [27]. Furthermore, correlation between samples from different sources (such as patients and healthy donors or samples from different ethnic populations) enabled definition of samples that will be used as a RS for the assays.

*Manipulation level: minimally versus extensively manipulated cells and culture duration*

Minimally manipulated cells are defined as natural, semi-purified or marker-based selected cells, from an autologous or allogeneic source. Such CTP may not need extensive product characterization and potency tests before administration to patients. However, all other CTP, regardless of their origin, are considered manipulated and should be tested for potency as part of their characterization. On the basis of a risk assessment development paradigm, the more the cells are manipulated and the longer they are cultured *in vitro*, the more IPC tests of potency should be conducted. The determination of the extent of IPC and whether the potency test should be implemented not only on the final product but also on in-process intermediate points, such as a frozen intermediate cell stock lots, depends largely on the type of cell, degree of manipulation and duration of *in vitro* culture.

*Lineage differentiation*

On the basis of putative MoA, undifferentiated and differentiated, lineage-specific CTP cannot be tested with the use of the same potency tests. For example, hematopoietic culture-derived EPCs are expected to express surface markers such as VEGFR2 and show the uptake of Ac-LDL, whereas CD34 hematopoietic stem cells are not expected to do so. However, it is assumed that although these cells are *in vivo*, they will mature and differentiate to support tissue reperfusion in response to *in vivo* signals. Thus, *in vivo* studies will be required to test the reperfusion potency of the cells as well as *in vitro* studies to demonstrate the product's capability to differentiate into lineage specific cells on exposure to typical cytokines.

*Viability and proliferative capacity of the cells*

Viability and proliferative capacity of the cells are usually discussed as part of the safety attributes of a CTP, but potency assays also depend on these features. Viability is an important consideration in potency assay development because cell cycle status correlates to the CTP's capability to perform their putative MoA. For example, apoptotic cells will not produce nor secrete cell markers and cytokines. Numerous methods and assays are available to test viability, proliferation and cell cycle, including application of fluorescent dyes (such as 7-aminoactinomycin D, propidium iodide, carboxyfluorescein diacetate succinimidyl ester, SYTOX and others) and colorimetric enzymatic reactions (such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and alamar blue).

*Cell organization, cells-cell interaction and cell-substratum interactions*

It is well recognized that cell-cell and cell-substratum interactions, even though not fully understood and analyzed, influence the biological activity of a given cell population and thus, the biological effect and potency of cellular products. Therefore, *in vitro* and *in vivo* potency assays may be designed and performed while taking into consideration cell-cell interaction as well as cell-substratum interactions.

*Stability and time frame limits for distribution of fresh or preserved (frozen) CTPs*

The cell product to be tested for potency should be the final product; thus, depending on if the product is fresh or frozen, the potency of either fresh or thawed cells must be tested on the appropriate samples. According to regulatory guidelines, cell products should be released by the manufacturer only after completion of tests for conformity with standards applicable to such product, which include tests for potency, sterility, purity, and identity. These requirements apply to all biological products, including autologous and single-patient allogeneic products, in which a lot may be defined as a single dose. Thus, for cell products that are administrated fresh, the duration of the proposed potency assays is crucial. For allogeneic and autologous products that can be frozen and stored for long periods before use, there is time to perform relatively complex assays. This is usually not the case for autologous and single-patient allogeneic products. Thus, for freshly delivered products, a relatively quick and simple assay must be developed. However, there is still a need to demonstrate during assay development the correlation of the quick and simple assay to more

representative and longer *in vitro* assays as well as to the *in vivo* tests. In the case of cryopreserved products, an integral aspect of determining product stability is examining all potency and release tests at the relevant time points after cryopreservation.

Specifically, activity of each batch of cells at the end of the production process is the basis for testing the cellular product activity. However, it is not enough for a cell population to show activity at the end of the production process because it will be delivered to the patient after a certain time gap. Thus, it must be proven that activity and stability of the cell product is maintained throughout the entire handling, shipping and *in vivo* delivery process by use of exactly the same set of tests as the fresh product. For example, if a cell product is frozen and thawed, flown to another destination or re-suspended to a new volume, each of these points should be tested and compared with the original activity of the drug product. Furthermore, if the cells are administrated with the use of a certain device, then stability tests should also assess the product potency after passing in the device. Once the potency and viability of the cells is tested several times at all of these time points and after handling stages, the cells can be routinely tested at an earlier time point such as after freezing at the production facility. In one example, such an assessment with application of a set of tests including viability, ELISA and FACS-based was used to test an autologous cellular product with a distribution chain that includes packaging in a chilled container that will keep the ready-to-use cell suspension packed in syringes for several hours to allow international shipment. The stability of the EPC-like cells was 35 h of preservation under these conditions as was demonstrated by recovery of 80% to 100% of all tested parameters including viability, number of cells, FACS-based potency and identity tests and ELISA-based potency tests [27].

Specifically, the stability of the cells after preservation was further challenged by passing the cells through a catheter to mimic administration conditions and ensuring that cells do not adhere to the catheter tube walls [27].

*Development of in vivo potency assay*

The use of an *in vivo* model as a routine potency assay is time-consuming, expensive and difficult to validate. Therefore, *in vivo* models are mostly used to validate *in vitro* predictability as part of the assay development process. *In vivo* testing can help in selecting the most promising *in vitro* potency assay as well as allow fine-tuning of the potency assay parameters (eg, identifying the correct pass/fail result for potency). Applying *in vivo* potency assays enables

the assessment of efficacy as well as safety of a CTP. *In vivo* relevant models such as the hind-limb ischemia in normal (eg, Balb/C, C57/Black), nude (eg, CD-1 nude, SCID) and diabetic (eg, db/db) mouse and rat (eg, Sprague-Dawley rat) are widely used to assess CTP potency and MoA.

One of the major challenges regarding *in vivo* potency assessment is the variability in the spontaneous reperfusion in the animals (as a result of revascularization or angiogenesis) (Figure 2). As a result of spontaneous reperfusion, it is difficult to assess whether the CTP-induced reperfusion is a reliable, reproducible and quantitative. To overcome these biological and technical challenges, an *in vivo* potency assay should be designed in a rigorous manner. Good controls should be applied to define the spontaneous versus CTP-induced reperfusion, including sham and vehicle control treatments. Furthermore, one should consider immune-related or immune-inflammatory-related non-CTP-specific xenogeneic responses that might mask CTP-induced effects.

In view of kinetics of response, vast reference literature work and availability of immune-compromised and immune-component strains, working with mice as opposed to other animal models, should be preferred when possible.

It is critical to use animals that have completed the rapid growing phase (at least 9 weeks of age) and within a limited range of body weight.

It might be necessary to demonstrate a significant angiogenic effect in two independent mice strains (immune-compromised: nude or SCID mice; and immune-component: Balbc or CB57 Black). In view of the potential effect of vehicle electrolytes or other components on cell survival or inflammation (both affecting local angiogenesis processes), vehicle control should be used as a reference control. In addition, wherever possible, a relevant control of unprocessed cells should be tested to demonstrate the CTP-specific effect.

A surgical procedure that creates stable hind-limb ischemia should be standardized as well as laser Doppler measurements, tissue sampling and assessment. These measurements should be standardized among all development groups wherever possible. It should be noted that the use of different surgical approaches is associated with different outcomes (decrease in blood flow, rate of spontaneous recovery rate of amputation, etc).

It is critical to use a surgical procedure that significantly decreases blood flow to a degree that even after spontaneous reperfusion, when blood flow is stabilized, the degree of reduction in blood flow remains within the range of 50% to 80% of the contralateral non-operated limb. One should also consider that most of the biological variability in

blood flow restoration occurs during the early phase of spontaneous recovery. Hence, for potency assays and for assessment of clinical relevancy, one may consider cell administration starting from day 15, when the spontaneous non-TCP-specific reperfusion has been stabilized.

Lack of information as to CTP MoA and the complex dose-response relationship require cell dose-range finding to be included as a supportive assay. Another consideration relates to the mode of cell administration (for example, local or systemic administration) and the per-site and total cells injected volume. It is also important to mimic the intramuscular administration in patients as much as possible. Most models include one or two sites of administration and an injectable volume within the range of 25 to 50  $\mu$ L per site.

Another consideration is standardization of time point measurements and duration of measurement with respect to the variability in the kinetic of response. Whereas in some cases, improvement in blood flow after CTP administration can be observed starting from day 15, in other cases, the effect can be observed only at day 21 or later. Furthermore, reversibility of perfusion after a single administration should also be considered.

Repeated measurements starting from day 15 after cell administration until day 28 or later (days 15, 21 and 28) and definition of maximum angiogenic response can be used for potency assessment in a comparative manner.

Several tools can be used for potency assessment such as angiography, histology, immunohistochemistry, laser Doppler flowmetry and near infra-red spectroscopy. The use of laser Doppler flowmetry, with or without near infra-red spectroscopy, is useful for non-invasive quantitative *in vivo* measurements of reperfusion activity. Because these measurements by themselves do not explain mechanisms underlying the reperfusion effect, it is advised to support findings with histological and immune-histochemical methods such as co-localization of fluorescein isothiocyanate-dextran with angiogenic/stem cell markers (CD31/CD34).

## Conclusions

The goal of a potency assay of CTP addressed to treating ischemic conditions is to predict its therapeutic activity. This is especially challenging because of numerous degrees of complexity stemming from factors such as the complex multifactorial MoA, cell source, inherent cell characteristics, culture method, administration mode and the *in vivo* conditions to which the cells are exposed. Because the cell is a living organism that responds to its environment in a

complex multimodal manner, each set of conditions that the cell encounters can change its features and response. The CTP cellular attributes can serve as surrogate markers for the putative MoA. The surrogate markers (including cell markers, protein production and secreted molecules) can be used for the development of quantitative and reproducible analytical potency assays.

A central aspect of validating any potency assay is verifying that it is a good indicator of the therapeutic activity of the therapy in humans. Before initiating human clinical trials, an integral component of examining the relevance of potency assays is testing the *in vitro* assay results relative to the therapeutic activity in an animal model of the clinical indication. Only once larger (usually phase III) clinical trials are initiated and clear end points and biomarkers for efficacy are identified can the potency assay be validated as being effective in predicting clinical outcome in humans. However, the utility of a potency assay in predicting a biological response is valid independent of a clinical setting. As the therapy enters commercialization, additional data should be evaluated; much of which may have slight variations in potency assay results can be assessed for any differences in clinical efficacy, thus truly examining the robustness of the potency assay and its pre-set acceptance criteria as it relates to the beneficial effect to human patients. However, it is important to note that to date, there are no cell therapies that are at the advanced clinical use stages that would allow the back-evaluation of a potency assay in light of significant clinical data. Looking forward, it will be interesting and crucial to see whether the current biological potency assays (*in vitro* and *in vivo*) that are considered to be the gold standard to test cell therapy potency are indeed good indicators of therapeutic activity in humans.

Key elements in the development of a potency assay (Figure 4) include basic understanding of the CTP MoA; reliable performance provided by application of appropriate controls, study design and strict standard operating procedures; establishment of accepted criteria for batch release that are based on correlation to *in vivo* activity; and relevancy to real conditions that the CTP will encounter in clinical settings if possible. Potency should be used not only as a release criterion for clinical use but during production changes, stability during preservation and trend analysis. CTP selected and released on the basis of such potency assays should provide consistent clinical benefit for patients [28–30].

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