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PERSPECTIVE

International Society for Cellular Therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for advanced phase clinical trials

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Abstract

Mesenchymal stromal cells (MSCs) as a pharmaceutical for ailments characterized by pathogenic autoimmune, alloimmune and inflammatory processes now cover the spectrum of early- to late-phase clinical trials in both industry and academic sponsored studies. There is a broad consensus that despite different tissue sourcing and varied culture expansion protocols, human MSC-like cell products likely share fundamental mechanisms of action mediating their anti-inflammatory and tissue repair functionalities. Identification of functional markers of potency and reduction to practice of standardized, easily deployable methods of measurements of such would benefit the field. This would satisfy both mechanistic research as well as development of release potency assays to meet Regulatory Authority requirements for conduct of advanced clinical studies and their eventual registration. In response to this unmet need, the International Society for Cellular Therapy (ISCT) addressed the issue at an international workshop in May 2015 as part of the 21st ISCT annual meeting in Las Vegas. The scope of the workshop was focused on discussing potency assays germane to immunomodulation by MSC-like products in clinical indications targeting immune disorders. We here provide consensus perspective arising from this forum. We propose that focused analysis of selected MSC markers robustly deployed by in vitro licensing and metricized with a matrix of assays should be responsive to requirements from Regulatory Authorities. Workshop participants identified three preferred analytic methods that could inform a matrix assay approach: quantitative RNA analysis of selected gene products; flow cytometry analysis of functionally relevant surface markers and protein-based assay of secretome. We also advocate that potency assays acceptable to the Regulatory Authorities be rendered publicly accessible in an "open-access" manner, such as through publication or database collection.

Key Words: *Mesenchymal Stromal cells, potency assays, release assays, matrix assays, immune functional testing, clinical trials, ISCT*

Culture-expanded mesenchymal stromal cells (MSCs) meeting minimal core identity for MSCs as defined by International Society for Cellular Therapy (ISCT) in 2006 [1] derived from marrow, adipose tissue, umbilical cord tissue and other sources from either autologous or allogeneic donor sources are being studied in clinical trials across numerous regulatory jurisdictions worldwide. The ailments targeted with this cell pharmaceutical platform fall roughly within two pathophysiological categories: immune/inflammatory and tissue repair/restoration [2]. It is now widely accepted that the pharmaceutical effect of MSC-like cells is predominantly mediated by paracrine and contact factors arising from intrinsic MSC physiological processes that are maintained after culture expansion. It is further accepted that following in vivo delivery, MSCs are further responsive to environmental cues encountered in situ leading to additional cellular functionalities [3]. Culture expanded MSC-like cells are unambiguously classified as a more-than-minimal-manipulated cellular and gene therapy (CGT) product regulated in the United States under section 351 of the Public Health Service Act (PHS Act) (42 U.S.C. 262). As a type of CGT product, MSC-like cells require an Investigational New Drug Application (IND) from the Food & Drug Administration (FDA) for conduct of clinical trials in the USA. The FDA further requires development of tests to measure potency as part of release criteria of advanced clinical trials designed to support marketing approval and registration. Similar requirements are made by the European Medicines Agency (EMA) for Advanced Therapy Medicinal Products (ATMPs), which include cell therapies, as defined by the European Regulation (European Commission [EC]) No. 1394 / 2007,[1] further strengthened on December 30, 2008, and directly enclosed in the legislation of each EU member nation with no need of other implementation. The EU Regulation is in compliance with the 2004/23/EC directive on donation, supply and testing of human cells and tissues and with directive 2002/98/EC on human blood and blood components.[2] The tripartite components of release criteria for MSC-like cellular products in early phase clinical trials—identity, viability and sterility—raise little practical controversy and the consensus on markers for identity of MSC-like cells, considering their intrinsic heterogeneity and phenotype plasticity, is also reasonably well defined [1,4]. However, the issue of potency testing remains largely open-ended and is informed by the putative mechanism of action (MOA) of MSC-like cells in a given indication. Care must be made in distinguishing curiosity-driven research as part of ancillary studies on cell products and release potency assays required to satisfy the Regulatory Authorities. Although pre-clinical MOA studies will necessarily inform the methods and reduction to practice of deployable potency assays, the latter have specific requirements for the following as part of assay validation: accuracy, precision, specificity, linearity and range, system suitability, and robustness.

International regulatory authority guidance on potency tests for cellular therapy products

The FDA has published guidance with recommendations for developing tests to measure potency for CGT products.[3] These recommendations are intended to clarify the potency information that could support

[1] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:EN:PDF.

[2] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:033:0030:0040:EN:PDF.

[3] http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM243392.pdf.

Table I. Challenges to potency assay development for MSC-like products.

Challenges	Examples
Inherent variability for starting materials	Autologous and allogeneic MSC donor variability Tissue source for MSCs (adipose, marrow, puerperal products)
Limited lot size and limited material for testing	Single-dose therapy using autologous cells suspended in a small volume
Limited stability	Viability of cell products Functionality of cell products at time of administration relative to banking (thawing)
Lack of appropriate reference standards	Autologous cell material
Complex MOA	Multiple potential effector functions of cells Multiple steps required for function
In vivo fate of product	Migration from site of administration Half-life of cellular product post administration Cellular differentiation or activation in to the desired cell type

Adapted from http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM243392.pdf.

an IND or a Biologics License Application. Because potency measurements are designed specifically for a particular product, their guidance does not make recommendations regarding specific types of potency assays nor does it propose acceptance criteria for product release. FDA guidance defines potency (strength is also synonymously used) as the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Regulatory Authorities are not prescriptive in what constitutes a definitive potency assay, and FDA regulations allow for considerable flexibility in determining the appropriate measurements of potency for each product. Potency is determined on the basis of individual product attributes; therefore, the adequacy of potency tests is evaluated on a case-by-case basis. The Regulatory Authorities also recognizes the inherent challenges in defining potency assays (Table I). Similar guidance has been published by EMA, which defines potency as the quantitative measure of biological activity based on the attribute of the product, which is linked to the relevant biological properties. Consequently, the assay demonstrating the biological activity should be based on the intended biological effect, which should ideally be related to the clinical response and aimed at investigating major cellular functions by using surrogate markers and appropriate technology.[4] The newly released guidelines of quality control of stem cell products by the Chinese National Health and Family Planning Commission[5] mainly emphasized guidance for facility requirements, biological contaminants, viability and product consistency. No specific criterion was given on the issue of potency assay, and the guidance limits its recommendation to assessment of potency according to disease indications.

There is no single test that can adequately measure product attributes that predict clinical efficacy. Taking into consideration this limitation, the potency assay should represent the product's mechanism of action (i.e., relevant therapeutic activity or intended biological effect). However, many CGT products, including MSC-like cells, have complex (e.g., rely on multiple biological activities) and/or not fully characterized mechanisms of action, making it difficult to determine which product attributes are most relevant to measuring potency. Indeed, it will be extraordinarily challenging to perform reductionist mechanistic experiments in human subjects that will conclusively define substantive MOA of MSC-like cells in vivo and meet modern standards of ethical conduct in clinical trials. Nonetheless, all attempts should be made to develop potency measurements that reflect the product's relevant biological properties and that can also serve as a measure of comparability between production lots [5]. Therefore, defining hypothesis-driven MOA based on correlative in vitro experiments and buttressed, where feasible, with comparative biology approach in animal systems will inform the choice of potency assays to be developed. The Regulatory Authorities anticipate that Manufacturers demonstrate clinical effectiveness by correlative "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 or proposed labeling thereof (section 505(d) of the FDC Act). 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 related to its specific ability to affect a given result and that also meets the criteria required by Regulatory Authorities (Table II).

Analytical methods to measure potency

Bioassays can provide a measure of potency by evaluating a product's active ingredients within a living biological system. Bioassays can include in vivo animal studies, in vitro organ, tissue or cell culture systems, or any combination of these. Development of a quantitative bioassay for MSC-like products may be

[4]http://www.ema.europa.eu/docs/en_GB/document_library/Presentation/2015/05/WC500187352.pdf.

[5]http://www.nhfpc.gov.cn/qjjys/s3581/201508/15d0dcf66b734f338c31f67477136cef.shtml.

Table II. Release testing of licensed biological product.

Release testing	Applicable FDA biologics and cGMP regulations
Indicate potency (biological activity/activities) specific to the product	21 CFR 600.3(s) and 610.10; and 21 CFR 210.3(b)(16)(ii)
Provide quantitative data	21 CFR 211.194; see also 21 CFR 600.3(kk); 21 CFR 211.165(d); 211.165(e)
Meet pre-defined acceptance and/or rejection criteria	21 CFR 211.165(d); see also 21 CFR 600.3(kk); and 21 CFR 210.3(b)(20)
Include appropriate reference materials, standards, and/or controls	21 CFR 210.3(b)(16)(ii) and 211.160
Establish and document the accuracy, sensitivity, specificity and reproducibility of the test methods used through validation	21 CFR 211.165(e) and 211.194(a)(2))
Measure identity and strength (activity) of all active ingredients	21 CFR 211.165(a); see also 21 CFR 210.3(b)(7))
Provide data to establish dating periods	21 CFR 600.3(l) and 610.53(a)
Meet labeling requirements	21 CFR 610.61(g)(3) and 610.61(r)

Adapted from http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/UCM243392.pdf.

complicated by properties of the product and/or technical limitations of certain assays (see Table I). In cases in which development of a suitable bioassay is not feasible, it may be necessary to identify a surrogate measurement of biological activity. For example, the use of non-biological analytical assays performed outside of a living system that is practical and demonstrates adequate performance characteristics for lot release. Examples of such analytical assays provided by the FDA include methods that measure immunochemical (e.g., quantitative flow cytometry, enzyme-linked immunosorbent assay), molecular (e.g., reverse transcription polymerase chain reaction, quantitative polymerase chain reaction, microarray) or biochemical (e.g., protein binding, enzymatic reactions) properties of the product outside of a living system. 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 measurement(s) can be substantiated by correlation to a relevant product-specific biological activity(s).

Assay matrix

The FDA states that a single biological or analytical assay may not provide an adequate measure of potency. The following are some potential reasons: (i) product has complex and/or not fully characterized mechanism of action, (ii) product has multiple active ingredients and/or multiple biological activities, (iii) limited product stability, or (iv) biological assay is not quantitative, not sufficiently robust or lacks precision. If one assay is not sufficient to measure the product attributes that indicates potency, then an alternative approach could be used, such as developing multiple complementary assays that measure different product attributes associated with quality, consistency and stability. When used together and when results are correlated with a relevant biological activity, these complementary assays should provide an adequate measure of potency. Such a collection of assays (referred to as an assay matrix) might consist of a combination of biological assays, biological and analytical assays or analytical assays alone. The assay matrix may include assays that give a quantitative readout (e.g., units of activity) and/or qualitative readout (e.g., pass–fail). If qualitative assays are used as part of an assay matrix to determine potency for lot release, stability or comparability studies, they should be accompanied by one or more quantitative assays. A concrete example of an assay matrix for MSC-like cells recognized by the FDA is detailed in a published report by Athersys Inc. (Cleveland, OH) outlining their effort in establishing an angiogenic potency assay for their MultiStem product based on measure of CXCL5, interleukin (IL)-8 and vascular endothelial growth factor (VEGF) production coupled to an in vitro cell-based angiogenic assay [6]. The correlative relationship between the surrogate measurement and biological activity may be established using various approaches, including comparison to preclinical/proof of concept data, in vivo data (animal or clinical) or in vitro cellular or biochemical data. The suitability of data used to support the correlative relationship between the surrogate assay and the biological activity of a MSC-like product will be evaluated on a case-by-case basis by the Regulatory Authorities and depends on or is influenced by the following: (i) type and relevance of the correlations being made, (ii) the amount of product information accumulated, (iii) how well the biological activity of the product is understood, and (iv) how well the surrogate measurements reflects biological activity.

Defining release potency assays for MSC-like cells developed for immunomodulation

The open-ended guidance from the Regulatory Authorities and the published precedent of a matrix potency release assay approach used by Athersys Inc. for Multistem in support of their advanced clinical studies informs a path forward on how to characterize a MSC-like product coupling an in vitro bioassay

interrogating the cellular secretome by enzyme-linked immunosorbent assay (ELISA) and a functional cell-based assay. Another industry sponsored example is that of Prochymal (Osiris Therapeutics, Inc.), which is an industrial-scale expanded MSC-like product derived from marrow collected from random donors that was studied as part of prospective randomized clinical trials. As a surrogate measure of potency, soluble TNFR1 was defined as a release criterion [7]. There are likely other potency release assays strategies that have been considered by the Regulatory Authorities for MSC-like ATMPs for use in immunomodulation, but these are unpublished and are not available for public consultation because Regulatory Authorities are not at liberty to publicly disclose otherwise confidential IND disclosure made by Manufacturers (academic or industrial). Nonetheless, these precedents inform us that a minimal set of assay components to be taken into consideration will likely require direct assay(s) of cell functionalities of MSC-like products and possibly a companion cell physiology assay on a responder lymphomyeloid cell population.

Functional in vitro assays with responder immune cells

Allogeneic random donor human peripheral blood mononuclear cell (PBMCs) serve as a useful tool to decipher MOA of human MSCs. However, there are serious limitations to their use as robust and reproducible potency release assay for human clinical trials [8]. Activated $CD3^+$ T cells provide the opportunity to measure inhibition of proliferation and cytokine production in vitro, yet it is unknown whether this assay accurately reflects the MOA of MSCs in vivo. Indeed, there are published pre-clinical data demonstrating that MSCs and other stem cell types can influence the cell physiology of monocytes, B-cells, natural killer cells and granulocytes, none of which is reflected in a classic mixed lymphocyte reaction (MLR)-like assay [9]. The use of unfractionated PBMCs collected from consenting normal human volunteers contains a mixture of lymphomyeloid cells that varies among human subjects and further complicates the reproducibility and interpretation of such assays. Finally, if the goal of the immunopotency assay is to highlight the MSC MOA that will be effective in a specific clinical setting, the use of purified immune effector cells that are involved in the disease pathogenesis rather than unfractionated responder PBMCs could be more informative. Considering the inherent shortcomings of using PBMCs in cell responder release assays, a reductionist perspective may provide guidance to robust and predictive MSC potency assays. One may rationally argue that if the mechanism by which MSCs suppress T-cell proliferation in vitro is defined, direct analysis of expression and induction of such as outlined earlier by a matrix assay approach may avoid the intrinsic variance in using random donor PBMC responder cells as part of descriptive release assay. The key will be defining functionalities that are theoretically causative of suppressor function in vivo so as to best inform on comparability of MSC-like platforms and also provide guidance on selection of clinical trial subjects which are poised to best respond based on hypothesis-driven mechanistic predictors of response.

Assay matrix for MSC-like cells to assay immunomodulation functionalities

The FDA provides guidance on assay systems that can serve as methods to ascertain potency/strength of cell therapy such as MSC-like cells. As an aggregate, these assays measure specific functionalities that can be correlated with in vitro cell-based systems. From a Manufacturer's perspective (academic or industrial), the assay matrix needs to comply with criteria required by the FDA (Table II) and possess certain elements of robustness, reproducibility and economy. Taking these in to consideration, workshop participants identified three preferred analytic methods that could inform a matrix assay approach: (i) quantitative RNA analysis of selected gene products, (ii) flow cytometry analysis of functionally relevant surface markers and (iii) protein-based assay of secretome. The analysis of MSC-like cells at time of lot release typically is performed on cells that are in log phase of growth within the boundaries of manufacturing parameters under study. In essence, this is a snapshot of steady-state functionalities. The presence of static cell markers (e.g., $CD73^+$, $CD90^+$, $CD105^+$, $CD45^-$) are useful to validate identity of MSC-like products, but they have not been demonstrated to act directly or predict for immune modulating function [9]. Within this context, the use of a "cell ruler" against which released lots are compared gains nominal importance [10,11].

Immune plasticity

The MSC committee of the ISCT released a position statement paper in 2013 that proposed immunological characterization of Multipotent Mesenchymal Stromal Cells [9]. Relative to their homeostatic resting state, MSCs undergo polarization toward inhibitory functionality on exposure to various pro-inflammatory cytokines such as interferon (IFN)γ, tumor necrosis factor (TNF)-α, IL-1α or IL-1β. In vitro MSC inflammatory "licensing" better recapitulates what likely happens in vivo once MSCs are transfused into patients with dysregulated immune responses or with systemic inflammation [12]. Measurable immunological features of MSCs, both at

Table III. FACS phenotype of resting and IFNγ-primed MSCs.

FACS phenotype	Resting	IFNγ activated
CD40	–	++
CD80	–	–
CD86	–	–
HLA-ABC	++	++++
HLA-DR	–	+++
CD274 (PD-L1)	+	++++
CD54 (ICAM-1)	+	++++

Adapted from Krampera et al [9].
FACS, fluorescence-activated cell sorting.

phenotypic and functional levels, depend on their activation status at the time of interaction with effector cells, although variability may be observed among different donors [13]. Thus, if these functional assays aim to assess the immune regulatory functions of MSCs for clinical purposes, comparing the results with both resting and licensed MSCs would be most informative, regardless of the tissue origin. Different protocols of MSC licensing are available from the literature [14], but normally the addition of IFNγ for 12–48 h is adequate to obtain MSC activation that allows for their analysis as part of an assay matrix. IFNγ-activated MSC-like cells readily increase expression of surface markers relevant to functional immune modulation and are amenable to routine clinical flow cytometric analysis (Table III). Similarly, quantitative RNA analysis of genes identified in the literature as likely related to MOA (Table IV) before and after IFNγ activation MSCs leads to increased transcription of an array of immune relevant genes among which a significant subset are increased more than a thousand-fold (e.g., IDO, CXCL9, CXCL10, CXCL11 and CIITA) [15]. Protein-based assays such as ELISA can further measure secreted factors produced directly by MSC-like cells, especially those amplified by in vitro IFNγ licensing, which can be reasonably postulated to play a role in immune modulation. The approach here highlighted does not exclude the use of alternate methods of in vitro MSC licensing such as the use of TLR agonists or distinct cytokines (TNF-α, IL-1, etc.), inasmuch as these deploy immune modulation functionalities linked to effector function. Further, open-mindedness toward emerging concepts in MSC cell physiology and function including, but not restricted to, the influence of exosomes and micro RNA, for example, may play a part of release potency assays as their mechanistic role becomes better defined.

Table IV. Genes significant to MSC immune-biology amenable to Fluidigm nanoscale qPCR array and/or ELISA.

IDO [13], CXCL10 [17], CXCL9 [18], CXCL11 [19], CIITA [20], ICAM-1 [21], CCL5 [22], TRAIL [23], TLR3 [24], CCL7 [25], VCAM-1 [21], HLADR [26], HGF [27], IL-6 [28], HLA-G 5 [29], CCL2 [30], PI9 [31], CCR7 [32], VEGF [33], PDL1 [26], CX3CR1 [34], COX-2 [35], AHR [36], TSG-6 [37], KGF [38], TLR4 [24], CXCL12 [39], CD46 [40], PDL2 [26], TGF-β [41], CXCR6 [18], CCR10 [42], TIMP-2 [43], CD55 [44], BCL-2 [45], ANGPT2 [46], A20 [47], HSP70A [48], IL-8 [24], ULBP-3 [49], HSP70B [48], CXCR1 [50], GAL-1 [51], CXCR4 [52], HO-1 [53], TIMP-1 [54], IL-1RA [55]

Adapted from Chinnadurai et al [15]. qPCR, quantitative polymerase chain reaction.

Controls

As with all well-designed experiments, developing a potency assay must include appropriate assay controls and a comparison to an appropriate product-specific reference material, when available. Running a product-specific reference material and/or control samples in parallel with the product helps ensure that the assay is performing as expected. In addition, controls help 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. The Manufacturer is expected to develop "in-house" reference material(s) as part of product development when feasible. These may include well-characterized clinical lots or other well-characterized materials prepared by the Manufacturer or another resource (e.g., a well-characterized cell line with a profile similar to MSC-like cells) that has been appropriately qualified. Taking this perspective in consideration, the objective of developing a universal MSC "cell ruler" has been addressed [16] and in itself represents a challenge almost as great as developing the potency assays for which they are to serve as a reference [10]. A complementary approach that may address the regulatory requirement for a reference material could include using "resting" MSCs as an internal "cell-ruler" control and their contemporaneous "activated" MSCs counterparts as the test [12]. In essence, each MSC lot serves as its own "control" when assaying immune plasticity.

Perspective statement: A focused analysis of selected markers robustly deployed by in vitro cytokine licensing (e.g., IFNγ) of MSCs and metricized with a complementary matrix of assays (fluorescence-activated cell sorting, quantitative RNA and proteomic [ELISA]) using "resting" MSCs as controls (from the same lot) should be responsive to requirements from Regulator Authorities regarding release potency assay (Table II). Candidate MOA surrogates outlined in Tables III and IV could serve as potential "universal" markers of strength for MSCs developed for their suppressive functionalities. It is also advisable to discuss potency development with the Regulatory Authori-

ties early on and before initiation of phase 3 trials when potency becomes a Regulatory Authority requirement.

Conclusion

The key deliverable for the translational community invested in developing MSC-like cells to their full clinical potential will be defining functionalities which are predictive of their tissue repair and immune and inflammation modulatory functions in vivo to best inform on comparability of MSC-like platforms and also provide guidance on selection of clinical trial subjects that are poised to best respond based on hypothesis-driven mechanistic predictors of response. Although this guidance is focused on release potency assays, these remain tightly coupled to companion identity assays that themselves likely require refinement from the original 2006 ISCT position paper [1] as new knowledge on MSC-like cells from various tissue sources is gained by the field at large. Furthermore, the concept of economy is of material importance. Although the cost of release testing of large industrial lots can be easily amortized on a per-unit cost, the same is not applicable to one patient/one product setting of autologous cell manufacturing or low passage allogeneic MSC doses (usually <10 doses) typically manufactured by academic health centers in support of clinical trials. Indeed, complex matrix assays using cutting-edge—and expensive—technologies can add substantial costs to manufacturing and release of personalized MSC units. Thus, an open mind-set by Regulatory Authorities in allowing for use of assays systems that are economical, especially in the setting of autologous cell therapies (e.g., one product per patient) or for allogeneic products with small number of cell does per lot (as is often done in academic health center sponsored allogeneic MSC clinical trials) would be useful. It is also desirable that unfettered open access of Regulatory Authority–approved release potency assay systems for MSC-like cells by Manufacturers engaged in clinical trials be encouraged and embraced by the cell therapy community. Public access of these elements can only be achieved by voluntary public disclosure by Manufacturers, and this shared regulatory data will further advance the field by allowing stakeholders to adopt rationally developed and validated common standards and assays.

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