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RAPID COMMUNICATION

Soliciting Strategies for Developing Cell-Based Reference Materials to Advance Mesenchymal Stromal Cell Research and Clinical Translation

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The mesenchymal stromal cell (MSC) field continues to rapidly progress with a number of clinical trials initiated and completed, with some reported successes in multiple clinical indications, and a growing number of companies established. The field, nevertheless, faces several challenges. Persistent issues include the definition of a MSC and comparability between MSC preparations. This is because of inherent cell heterogeneity, the absence of markers that are unique to MSCs, and the difficulty in precisely defining them by developmental origin. Differences in the properties of MSCs also depend on the site of tissue harvest, phenotypic and genotypic characteristics of the donor and the isolation, and storage and expansion methods used. These differences may be sufficient to ensure that attributes of the final MSC product could differ in potentially significant ways. Since there are currently no gold standards, we propose using a reference material to establish methods of comparability among MSC preparations. We suggest four possible ''ruler scenarios'' and a method for global distribution. We further suggest that critical to establishing a reference material is the need to define protocols for comparing cells. The main purpose of this article is to solicit input in establishing a consensus-based comparison. A comparative approach will be critical to all stages of translation to better clarify mechanisms of MSC actions, define an optimal cell manufacturing process, ensure best practice clinical investigations, extend the use of an MSC product for new indications, protect an MSC product from imitators, and develop uniform reimbursement policies. Importantly, a reference material may enable a consensus on a practical definition of MSCs.

Introduction

Friedenstein et al, in a series of seminal studies in the 1960s and 1970s [2], showed that the osteogenic potential of bone marrow (BM) cells was associated with a minor subpopulation of cells in the BM. These cells were distinguishable from most hematopoietic cells by their rapid adherence to tissue culture vessels and the fibroblast-like appearance of their progeny in culture, pointing to their origin from the stromal compartment of BM. While now known to be technically incorrect, the current colloquial term ''mesenchymal stem cell'' dates back to 1991 [3]. A work by Darwin Prockop and others [4] further defined the cells and their multilineage capability. The ability to grow and expand them efficiently and relatively easily and the wide variety of functions they have now been described to perform (Fig. 1) led to the birth of an entire subfield of cell therapy. The marrow stromal cell field expanded very rapidly, and the potential use of these cells in therapy is being tested worldwide for many indications. The potential utility of the cells lies in their multifunctional properties: They modulate the immune system, enhance engraftment of hematopoietic stem cells, promote tissue healing, and contribute to structures such as bone, cartilage, and fat. In addition, as culture-expanded cells, they may provide critical trophic support for normal tissue maintenance and protection and recovery from tissue injury. In this article,

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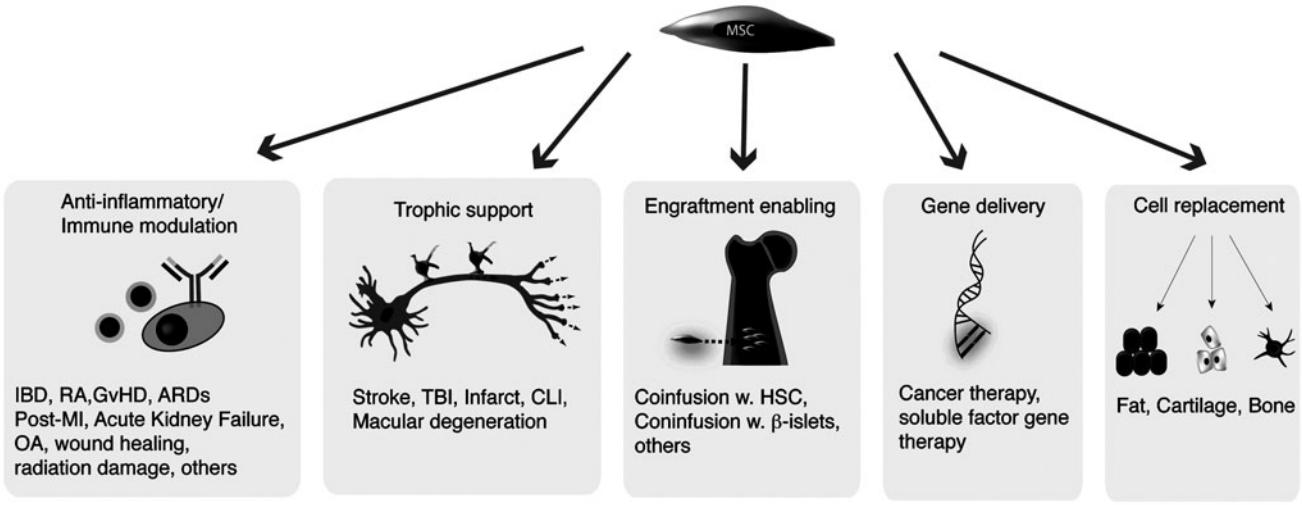

FIG. 1. Multiple modes of action attributed to MSCs include IBD, inflammatory bowel disease; RA, rheumatoid arthritis; GvHD, graft versus host disease; ARDs, acute respiratory distress syndrome; MI, myocardial infarction; OA, osteoarthritis; TBI, traumatic brain injury; CLI, critical limb ischemia; HSC, hematopoietic stem cells; MSC, mesenchymal stromal cell.

we have chosen to employ the term ''mesenchymal stromal cells'' (MSCs) rather than ''mesenchymal stem cell,'' which better describes the characteristics of the cells that may be independent of their stem cell properties. More than a thousand trials have been run, and more than fifty companies offering some variant of a mesenchymal cell are in existence. The first commercial cell therapy products that use MSCs are now available with regulatory permission in a number of countries, including Canada, New Zealand, and South Korea (Table 1), and more than a dozen companies have commercial products in late-stage clinical trials.

The rapidity with which the MSC field has advanced and the commercial potential of these cells has led commercial and academic investigators to seek unique attributes of MSCs and, consequently, has resulted in the isolation of MSC-like cells from a variety of sources, including fat, different parts of the placenta, the umbilical cord, skin, and a variety of other organs and tissues (Fig. 2). Furthermore, other groups have used culture or cell sorting techniques to isolate specific MSC populations from the same starting material, arguing that MSC isolates are heterogenous, and that subpopulations may have different functions and roles.

The problem with distinguishing cells as unique, particularly when dealing with a potentially heterogenous cell population whose composition may change with every passage, is fraught with difficulty. In addition to this is the observation that no single marker or even groups of markers clearly and uniquely defines this population. Indeed, most markers used to define MSCs are present on many mesodermal derivatives, including fibroblasts [5,6]. Further, there is little consensus on even a functional definition of a mesenchymal cell or even whether it is truly a stem cell [7,8] (Table 2). This is particularly complex, as the specific function and expected clinical actions of MSCs are different for many disease indications. Indeed, ''stemness'' may not be a necessary functional attribute of MSCs depending on the context of their potential use. Therefore, a definition of the anticipated disease-specific actions and the potency of any given MSC preparation needs to be considered.

Table 1. Commercially Available Mesenchymal Stromal Cell Products

Companies	*Commercial products*	*Description of product*	*Indication*	*Market*
AlloSource (United States)	Allostem	Allogeneic bone matrix with adipose-derived MSCs	Orthopedics applications	United States (21 CFR Part 1271)
Orthofix (United States)	Trinity Evolution	Allogeneic bone matrix with MSCs	Orthopedics applications	United States (21 CFR Part 1271)
Mesoblast (Australia)	Prochymal	BM-MSCs allogeneic	Pediatric GvHD	2012 in Canada & New Zealand
Medipost (S. Korea)	CartiStem	UCB-MSCs allogeneic	Degenerative arthritis	2012 in S. Korea
Pharmicell-FB (S. Korea)	Hearticellgram-AMI	BM-MSCs autologous	AMI	2011 in S. Korea
Anterogen (S. Korea)	Cupistem	Adipose-MSCs autologous	Anal fistula	2012 in S. Korea

SC, stem cells; POC, proof of concept; BM, bone marrow; UCB, umbilical cord blood; AMI, acute myocardial infarction; MSC, mesenchymal stromal cell.

	BM - MSC	UCB - MSCs	UCT- MSCs	AT - MSC	DP - MSCs	P- MSCs
Isolation	Gradient separation	Enzyme/mechanical dissociation/ centrifugation	Enzyme/mechanical dissociation/	Enzyme/ mechanical dissociation	Fresh tissue dissection/enzyme	Membrane separation (optional), tissue dissection, enzyme, centrifugation
Markers	CD105, CD73 and CD90, CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II	BM - MSCs markers + Oct4, Nanog, Sox-2 (low levels)	BM - MSCs markers + higher levels of CD146	BM - MSCs markers + higher levels of CD146; STRO-1 negative	BM - MSC markers + STRO-1, CD146	BM - MSC markers + SSEA-1, SSEA-4, Oct4, Nanog; Higher levels of CD49d, CD10, and CD56 than BM - MSCs
Limitations	Rare cell type	Low yield	Developing field	Heterogeneous populations	Low yield; fresh processing	Cumbersome to procure, properties need to be better understood

FIG. 2. Sources of MSC and methods of isolation and differences in culture conditions and usual markers used. BM-MSCs, bone marrow MSCs; UCB-MSCs, umbilical cord blood MSCs; UCT-MSCs, umbilical cord tissue MSCs; AT-MSCs, adipose tissue MSCs; DP-MSCs, dental pulp MSCs; PMSCs, placental MSCs.

The Importance of Consistency

Several arguments can be made for consistency in MSC application and use. From a translational and mechanistic standpoint, comparison to an (arbitrary) reference material is critical when assessing source, expansion and passage number, manufacturing, potency, and other attributes. A reference material will enable an intercenter comparison of results among research labs. With regard to product manufacturing, it is critical to ensure biological and functional equivalence between product lots; thus, a reference material will enable intra-center consistency. This is particularly important with autologous cellular therapy in which the starting sample is, by definition, different in each manufacturing run. Without some way to establish sufficient consistency in desired actions and potency between each product, it will be very hard to predict results with confidence, may potentially restrict use of the cells, and be difficult to build on positive results.

A second important reason to establish consistency in a product, and its manufacturing process, is for intellectual property protection. In the absence of consistency and reproducibility and without a clear consensus on the definition of an MSC, it is difficult to obtain a composition-of-matter patent, and given the variability, even if a patent were obtained, it would likely be easy to circumvent. This puts significant and undue competitive pressure on companies and scientists isolating, purifying, and expanding a rare subpopulation that they believe is unique but may appear to be superficially similar to a ''generic cell''; it is, therefore, difficult to distinguish and differentiate a product in the absence of product consistency. Without intellectual property protection, the product cannot be defined as unique and better in some fashion, thereby making it easier to produce any approved cell that is less expensive and a perceived equivalent but really is inferior or at least not equivalent. Therefore, a third important reason for establishing a reference material is to maintain a consistency in prescription for the product, and to stabilize product price. A reference material enables users to distinguish between products of different quality.

Similarly, without consistency, it is difficult to extend the use of a product for new indications. Off-label or new uses that initiate new directions are important aspects of increasing the value of a product and amortizing the development costs. However, without product consistency, it might be impossible to extend the use of a product that is readily available but difficult to define.

Table 2. Suggested Definitions of an Mesenchymal Stromal Cells

Criteria	*Reference*
Plastic adherence	[18]
$CD105^{+}$, $CD73^{+}$ and $CD90^{+}$, $CD45^{-}$, $CD34^{-}$, $CD14^{-}$ or $CD11b^{-}$, $CD79a^{-}$ or $CD19^{-}$ and HLA class II^{-}	[18]
Anti-STRO-1, anti-CD146, anti-CD271, anti-nestin positive, CD45-negative cells	[22,24–27]
In vitro trilineage differentiation	[18]
In vitro immunoplasticity assay of MSCs activated by IFN-$\gamma \pm$ TNF-α	[21]
IDO activation in primed MSCs	[21,26,28–30]
In vitro clonal CFU-F formation efficiency	[31]
In vivo ossicle formation	[22]

IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; IDO, indoleamine 2,3,-dioxygenase; iNOS, inducible nitric oxide synthase; CFU-F, colony-forming unit-fibroblast.

Obtaining Consistency

In addition to pre-clinical mechanistic studies, reference material is of value during a process or clinical manufacturing, in clinical trials or when considering patenting, licensing, and off-label issues. Thus, such a reference could enable comparing two samples from different private entities without those samples being physically present in the same laboratory. It also enables independent verification of claims and meta-analyses of clinical data and pooled multicenter trials. Indeed, physical standards are routinely used in many systems. For gene transfer vectors, recombinant adeno-associated virus 2 (AAV2) has been produced to provide a standard of reference for particle, vector genome, and infectious titer of AAV2 vectors [9]. In flow cytometry cell sorting or analysis, there are fluorescent beads to standardize the performance of the lasers and machines. Protein or DNA ladders are routinely run on gels to determine the molecular size of unknown molecules.

It is important to emphasize that the proposed reference material is not meant to be a gold standard. A gold standard implies a bar of quality that needs to be met, while a reference material is more akin to a ruler. A ruler simply provides a measure of the cells and is an easy, standardized way to compare two populations with each other by comparing them to a readily available, inexpensive third population. This is an important distinction in determining what can function as a reference material. For example, bone mass is measured by assessing optical density of radiographic images of bone and aluminum wedges, which act as a reference material; the mass of the aluminum wedge with equal optical density as the bone provides a measure of bone mass. No one would argue that an aluminum wedge is a standard for bone, but it serves as a useful calibrator for measuring bone mass. Similarly, a strong argument can be made to use an immortalized cell line or a mixed population of cells as a reference, but no one would suggest that it is a gold standard for applied use or that it defines the full set of characteristics which MSCs have.

A ruler is impartial and using it does not mandate attaining a certain standard. Standards are generally set by government bodies; a ruler simply provides a mechanism to measure a standard or an unknown. Moreover, a ruler does not need to have identical attributes to the product under consideration. Indeed, beads used in flow cytometry are a good ruler to standardize measurements, but differ from labeled cells in fundamental ways. These differences, however, do not detract from the utility for a particular measure. An important extension of this logic, however, is that one may use different reference materials which are appropriate for different measurements; this may be particularly relevant for MSCs which are considered as having multiple mechanisms of actions. While feasible, this imposes the additional cost of maintaining and testing different reference materials. One way of addressing this may be by simply providing access to a virtual data set generated from characterizing multiple reference materials in different readouts, in addition or in some cases, in lieu of making physical reference material(s) available.

We believe that a ruler type of reference material as opposed to a gold quality standard may be a suitable option in the MSC field. This is likely preferable to other possible alternatives such as predicting or mandating standards in this rapidly evolving field or arbitrarily defining some gold standard that most cells may not meet or which may not be relevant to specific uses. We propose a model in which a ruler cell or reference cell material is offered at a low cost, is made widely available without restrictions and that has a well-characterized profile.

Requirements for Establishing a Reference Material

In general, a standard is perceived as impartial and is established through high-level international standardization programs [eg, World Health Organization (WHO), Joint Committee for Traceability in Laboratory Medicine (JCTLM)]. Written standards depending on their scope may require the backing of government agencies, standards laboratories, standards development organizations, or industry-wide associations. For example, the National Institute of Standards and Technology (NIST) and Food and Drug Administration (FDA) offer standards for testing; the US Pharmacopeial Convention (USP) and the National Institute for Biological Standards and Control (NIBSC) manufacture WHO-approved international reference materials for biological medicines. The Laboratory of Government Chemists (LGC, based in the UK) produces and distributes chemical, molecular, and cell biology (certified) reference materials and quality control materials. The Institute of Reference Materials and Measurements (IRMM) produces reference materials for environmental importance (eg, genetically modified seed), and the Institute of Electrical and Electronics Engineers (IEEE) provides chip standards.

At present, no standard is available for MSCs. As such, what we propose is the development of a ruler type of reference cell material, similar to what has been done in the field of gene vectors. A recombinant AAV2 Reference Standard Material [9] was developed over several years as a consortium effort. A similar consortium of groups, including governmental agencies such as the National Institutes of Health, (NIH), and FDA, or foundation, industry, or other groups including, for example, the California Institute of Regenerative Medicine (CIRM), the International Society for Stem Cell Research (ISSCR), the International Society for Cellular Therapy (ISCT), and nonprofit organizations such as the Drug Information Association (DIA) could take the lead in developing and validating such a ruler type of reference cell material. Such an operation would need to be developed as a partnership with members who are capable of delivering key competencies, relevant experience, and/or facilities to provide a standardization program. The consortium would require coordinated efforts to develop a consensus on MSC features that will need to be tested and standardized, at least initially, to identify sources of MSCs that may be considered and pilot-evaluated to form a suitable reference cell material, and to manage the roll-out of reference cell bank [working cell banks (WCBs)] preparation, characterization, distribution, and re-testing among global participating labs. Importantly, this endeavor will require sustained sponsorship from industries and other groups, especially if more than one reference cell material is to be developed.

In addition to having the reference cell material widely endorsed, it needs to be widely and conveniently available,

and the data resulting from in-depth analyses of the reference cell materials should also be widely and freely available. There should be a voluntary means of maintaining datasets of comparisons from individual laboratories. This requires developing and distributing an initial, well-tested, and characterized dataset, identifying the key minimum qualities, and agreeing that the dataset will be made available. Users can verify the performance of their MSCs by querying the dataset, akin to the PluriTest dataset effort for pluripotent stem cells (PSCs) led by Jeanne Loring and colleagues (Scripp's Research Institute) that provides an assessment of in-house PSC lines against 450 genome-wide transcriptional profiles associated with pluripotency [10]. The same consortium could conceivably define consensus low- and high-acceptance limits for testing MSCs against the reference cell material(s); the reference cell material(s) simply serving to provide the dataset to make such decisions, and not a critical bar against which to judge therapeutic value.

More than one reference cell bank(s) may also be developed in parallel to be able to calibrate different MSC functions, or reference cell bank(s) may be displaced by newer or better reference cell materials. In these cases, interconvertibility options, including data and agreements, could be put in place. In addition, consistent and optimized media composition and recommended culture conditions could also be tested and provided.

Cell Populations Serving as Potential Reference Cell Bank(s)

Sufficient amounts of reference cell materials will be needed to generate baseline characterization data (phenotype; karyotype; gene expression, profile; cytokine secretion profile and functional read-outs) and to form a master cell bank (MCB) of 500–1,000 vials [although a larger number will be needed for global use, thus necessitating the creation of multiple WCBs] of between 1 and 5×10^6 MSCs per vial (these can be thawed and analyzed without cell expansion although a brief recovery time in culture may be recommended; standard protocols for various assays could be provided) that can be distributed globally. This will serve as the basis for an evolving reference cell bank(s) that can be used by investigators to benchmark their own research studies and/or clinical investigations.

We propose four potential types of candidates that could serve as reference material; readers are invited to provide feedback on these candidates or other options at www.surveymonkey.com/s/3DH3S2W:

(i) Mixed pooled populations—for example, MSCs from BM but can be applied to adipose or other tissue collection sources.

In brief, this would consist of pooling MSCs from at least 20 individual healthy consenting and screened donors (for safety considerations), aged 18–35 years. A reasonable estimate is that $\sim 240 \times 10^6$ MSCs can be obtained from an individual BM aspirate of 10–30 mL by Passage 3. Twenty donors would, thus, provide $\sim 500 \times 10^7$ MSCs, which is sufficient to generate an initial MCB, from which several WCBs can be created at different labs or biorepositories around the world; additional donors may be needed to generate larger or subsequent MCBs. It is unclear at this stage whether the BM aspirates would be cryopreserved or whether the BM cells would be passaged (to one passage after plastic adherence), pooled, and then cryopreserved. The latter option has the advantage of eliminating most hematopoietic cells, and potential lymphocyte reactions between donor populations, but subjects the cells to additional manipulations and repeat freeze-thaw cycles. Pilot studies should be performed to determine the more effective method.

The major advantage of using pooled BM-derived (or other tissue-sourced) MSCs as a reference cell material is how well it represents the current field of MSCs, although it may be argued that heterogeneity arising from a pooled cell bank(s) may mask some characteristic of the cells under certain test conditions. Feasibility, especially with regard to the mixed lymphocyte reaction (MLR) and other functional assays, will likely be a key determinant.

(ii) Single donor banks isolated using next-generation MSC isolation and expansion conditions

In the last decade, optimization of classic MSC culture expansion conditions has been developed that enables extended ex vivo expansion and delayed entry into replicative senescence. Maintaining a hypoxic environment during culture, supplementing with growth factors including basic fibroblast growth factor, epidermal growth factor, and platelet-derived growth factor, and maintaining cells at subconfluent levels are important parameters that are associated with these improvements [11,12]. These conditions are known to influence retention of telomerase activity and maintenance of signaling pathways that are associated with pluripotency in other environments [13]. Using these or other conditions, a routine expansion to 60–70 population doublings can be achieved.

These expansion strategies enable clinical manufacturing through MCBs, with cells from an individual donor that are sufficient to create an MCB of 200 vials, from which each vial can produce a WCB of 500 vials at 1 to 10 million cells per vial in each expansion run. This would provide 100,000 research material vials from a single donor at population doublings below 30. While higher population doublings may be achievable, it may be useful to limit them to about 20 population doublings, at which point some MSC preparations begin to lose subpopulations of precursor-like cells [14].

At present, these nonclassical MSC growth and expansion conditions are not routinely accepted; however, the approach yields sufficient manufacturing depth to provide a reference cell bank(s) with the arguments made earlier, distinguishing a ruler type reference material from a gold standard.

Alternatively, a commercial source of an expanded clonal population that is already in multiple late-stage clinical trials and has supporting documentation on its manufacturing, safety, and efficacy profile can be used as a reference cell bank(s) provided that the owners agree to such use. A disadvantage to this approach is that a single arbitrary donor may not be representative, although this could be addressed by the generation of multiple MCBs from different individual donors. In addition, using a commercial cell product as a reference cell material may be viewed as an endorsement of that cell product and result in hesitancy in adoption.

(iii) An immortalized population

In this option, MSCs may be immortalized to generate a sustained cell line using molecular methods. MSC lines generated using a combination of siRNA against p53 and overexpression of human telomerase reverse transcriptase retain similar proliferation, colony formation, differentiation, and gene expression profiles as do primary MSCs [15]. Other approaches for immortalization, including the use of conditionally induced oncogenes, may also be considered [16]. It could also be argued, however, that disruption of key regulatory control pathways by immortalization and genetic manipulation may cause reluctance to accept such reference cell bank(s) as a standard, although it is important to emphasize that such cell bank(s) would serve as reference standards, not gold standards. It is important to weigh the value of an appropriate manufacturing capability providing sufficient ruler inventory against perturbations of biology associated with the immortalization event.

(iv) A self-renewing population

A fourth option would consist of generating mesodermal cells from induced pluripotent stem cell (iPSC) lines or another PSC source. Mesodermal cells from iPSCs and human embryonic stem cells have phenotypic and differentiation properties akin to traditional MSCs [17], but lack the epigenetic imprinting of MSCs maturing in vivo in an organ/tissue environment. Nonetheless, the fundamental properties (phenotype, in vitro mesenchymal potential, and production of anticipated trophic factors) are maintained and could serve as a ruler for standard in vitro assays and comparability, and this, combined with the essentially unlimited expansion potential, makes this approach very attractive.

The generation of renewable quantities of mesodermal derivatives from iPSC lines transduced with reporter genes such as green fluorescent protein (GFP) or luciferase (provided by Dr. Rao, CRM, NIH) would, therefore, be feasible with this approach (without additional manipulation of the cells), in the context of validating manufacturing requirements and serving as a pilot cell line for characterization assays.

The advantages and disadvantages of these four candidate reference cell materials are summarized in Table 3. The MSC Reference Material Working Group created a website poll (www.surveymonkey.com/s/3DH3S2W) and encourages readers to provide feedback on these reference cell materials. The survey polls readers on six questions ranging from potential candidates, to how readers would use such a reference, and how much they would be willing to pay for a vial of MSC reference cell material. In addition to this feedback, the Working Group recommends conducting small-scale pilot experiments to evaluate these candidate reference materials. The MSC Reference Material Working Group will forward the input from the community of MSC investigators and the outcome of pilot investigations to the entity(ies), such as ISCT, that will lead the creation of the reference cell bank(s), and put out a recommendation/position paper on MSC reference cell materials.

Characterization of the Reference Material

Two sets of tests are important for the reference material. The first is a set of quality control and quality assurance tests to ensure batch-to-batch consistency of the reference cell bank(s), and between manufacturers of the WCBs. The second is a more detailed characterization of the activity and identity of the ruler.

As a baseline, standard sterility, mycoplasma, endotoxin, viability, and karyotype testing will be done on the ruler. Stability testing of the reference cell bank(s) should also be performed beyond a consensus population doubling or

Table 3. Advantages and Disadvantages of Different Mesenchymal Stromal Cell Bank(s)

Donor origin	*Expansion capacity (vials/donor)*	*Biological properties compared to classical MSC experience*	*Comments*
Pooled BM donors using classical MSC conditions	250 vials of MCB from 20 donors	Strongest predicted correlation to classical MSC properties	Mixed donor pool may contribute to heterogeneity or dominance across donors
Donor isolation using enhanced expansion conditions	100,000 to 1 million vials/donor	Equivalent to classical MSC; limited experience base in community for cells produced under nonclassical conditions	Unfamiliarity with expansion conditions may bring reluctance to adopt
Immortalized from MSC cultures	Unlimited (millions of vials/donor)	Disruption of key regulatory control pathways by immortalization may cause uncertainity on validity of reference material	Disruption of key regulatory control pathways by immortalization may cause uncertainity on validity of reference material
Pluripotent stem cells	Unlimited donor material	Relatively little experience to date regarding validation of cells from iPS/ESC source; limited in vivo data	Concerns about conserved biology for nonadult-derived cell population may bring reluctance to adopt

MCB, master cell bank; ESC, embryonic stem cell; iPS, induced pluripotent stem.

passage number. Additional quality control testing, including genome stability testing, could also be conducted. The choice of assays of genome stability could, in addition, include more sensitive read-outs such as comparative chromosomal hybridization. Identity testing will include immunophenotypic detection of cell surface antigens, including CD271, CD105, CD146, CD13, CD73, CD90, CD44, CD45, CD34, CD14, CD11b, CD79a, CD19, HLA Class II, CD36, and CD106 (AT), based on recommendations from ISCT [18] and IFATS [19]. Additional array- or bead-based gene expression profiling could be conducted to enable a quick global comparison between different WCB lots to ensure consistency. The PluriTest for pluripotent cells [10] could serve as an important model of analysis of microarray data from MSCs, but will require the identification of a cluster of MSC-specific genes similar in concept to the 450 genes used to identify pluripotent cells. New technologies for epigenetic analyses of cells could provide important information about MSCs [20]. The large amount of data generated are often difficult to interpret given the variability between different MSC preparations, thus underscoring the importance of developing standardized methods to test the reference cells.

For each assay, the protocol for preparation of the MSCs could be documented by cell density in cells/cm^2 at plating and at harvest, and by population doublings per passage. Quantitative data could be provided whenever possible, that is, the percent of cells positive for each cell surface epitope.

The second set of assays will include functional assays. As a baseline, we suggest that the reference cell bank(s) undergo functional characterization with a spectrum of assays; for example, those that measure immune suppression by the MLR as suggested by ISCT [21], those that measure stem cell characteristics by clonal assays and the promotion of bone formation in vivo [22], and those that measure secretion of trophic factors by analyzing the secretome and/or exosome profiles. At a minimum, protocols for these baseline functional characterizations will be circulated to investigators wishing to use these assays. Wherever possible, standard curves for assays should be developed for the quantification of the data so that test MSCs can be compared with the reference cell bank(s). This does not preclude individual investigators from using additional tests to assess their MSCs and/or the reference cell bank(s). Individual investigators wishing to make comparison data publicly available may choose to do so, adding to the annotation of the reference material. Other investigators can then compare outcomes of these assays for individual MSCs against the reference cell bank(s) or other MSCs. Standardized templates for entering characterization data and centralizing data repositories could be developed by the entity providing the cells and will be a critical component of the data collection and analyses.

It should be emphasized that many tests are available and most likely each laboratory investigator will have a preference for particular assays; hence, there is little merit in defining the assay(s). Importantly, an assay performed in any given laboratory can readily be compared with results from another laboratory that ran their own set of samples with the same reference cell bank(s), even if they used a slightly different protocol for the assay. The reference material will, therefore, enable a comparison to be made of different methods and samples, obviating the need to transport many samples for multiple pair-wise comparisons.

A natural extension of providing both reference material and standardized test protocols will be the generation of data sets from multiple labs. This, in turn, can lead to additional efforts of method comparisons, and monitoring measurements between laboratories as a part of a scheme for proficiency testing, which could potentially be implemented by a group such as LGC. In addition, a comparison of results will enable the development of consensus approaches to, and values for, relevant attributes of MSCs that may contribute to potency in any given disease. A reference cell bank(s) and its associated data base will provide a critical forum for developing these approaches.

Experienced repositories and Good Manufacturing Practice (GMP) facilities could be engaged to produce the reference cell bank(s). They would use standardized methods to source and collect tissues, GMP-grade reagents, substrate, and dissociation materials. They would generate appropriate Certificates of Analyses. In addition, appropriate limits on passage number or population doublings, standard methods to freeze and thaw would be distributed with the cells as a ''package insert'' to provide a frame of reference. It is important here to re-emphasize that ''standardized'' does not mean the same everywhere or that this is the method which needs to be used to collect cells. This is simply to ensure that the cells provided by the repository are similar each time—enabling the reference material to stay constant in the hands of individual investigators. One method that would help further in controlling this would be to specify that the reference cells should not be expanded before use; they may require some time in culture to recover functional properties [23], but not be expanded. This is important, as there is no intention to mandate how laboratories collect and process their own MSCs. The purpose is simply to help process and use the reference cell bank(s) and its derivatives (eg, DNA or RNA collected from the reference cells) according to standard operating procedures, thereby enabling a valid comparison to be made to a pre-existing dataset.

Thus, we propose that reference cells be characterized similar to any material manufactured and distributed by a biorepository (such as ES lines provided by ATCC), with careful documentation of lot-to-lot variability and functional characterization performed by defined, and publicly accessible, protocols. This is accompanied by a dataset enabling reference cell users to upload their results to a common data set, akin to currently established microarray databases. This will enable investigators to showcase results and enable the repository to modify its protocols should other methods prove popular or more accurate. This will also facilitate proficiency testing of the methods themselves, and could lead to potential standardization of assays to characterize MSCs.

Implementation

We believe the ruler approach to be feasible but it requires the key decision to select the cell(s), cell line(s), or cell type(s) that will serve as reference cell material. The choices are under discussion and after input from the community, and clarification via pilot experiments, a

recommendation will be forthcoming, likely from the ISCT MSC subcommittee which is also involved in the efforts of this Working Group.

Several possible candidates exist as potential repositories and manufacturing cores for the creation of a central MCB (of limited passage of reference cells), and the creation of subsequent WCBs (extended by a few more passages) (Fig. 3). These include the NIH Production Assistance for Cellular Therapies (PACT) centers that manufacture cells on a large scale, the NIH Clinical Center cGMP facility, the Canadian Center for Commercialization of Regenerative Medicine (CCRM), or Darwin Prockop's NIH-funded center, which has been supplying MSCs widely to investigators. Biorepositories such as the ATCC, Coriell Cell Repositories, Wicell, Rutgers, and the UK Stem Cell Bank (NIBSC) could also readily undertake such a function. Indeed, the ATCC serves as a repository for many reference materials and characterized lines, and NIBSC releases reference cell banks of MRC-5 for vaccine manufacture, and 3T3 cells as feeder cells in certain cell therapies.

Costs for generating such reference line(s) depends on the source of the MSCs, and whether the MSCs are generated in serum-supplemented or serum-free medium, and on the size of the MCBs and subsequent WCBs. Using serum-supplemented media and generating about 500–1,000 vials (additional vials and/or MCBs may be required) of between 1 and 5 million cells each would cost ~US$10,000–20,000 in reagent costs with another US$10,000-$15,000 in baseline standard and functional characterization [this does not include donor screening or labor costs which would need to be subsidized by the entity(ies)]. This equates to cost of production of ~US$100 per vial. Using serum-free media and generating about 500–1,000 vials of 1 to 5 million cells each vial could cost up to 10 times more—US$100,000–$120,000 for each MCB. This may become prohibitively costly but has the advantage of eliminating serum variability, especially important as global serum inventories have become depleted and fetal bovine serum prices are significantly rising. Vials could be provided for US$150–$500 to recover the initial outlay, with emphasis on reaching a cost neutral manufacturing practice.

This is affordable to funded investigators; furthermore, the private sector and individual agencies or foundations could subsidize costs for their group of funded investigators. We estimate that a set-up cost will be required for the entity(ies), and will require some combination of grant funding, industry sponsorship, and funding from nonprofit organizations. The additional cost of developing and maintaining a database could be covered through well-established mechanisms such as for the human reference genome or, alternatively, adding to an existing database may be a cost effective option.

We recommend that this model become self-sustaining by recovering ongoing costs for the manufacture and testing of cells from a modest fee-per-vial processing fee. At present, we do not suggest a free model given the start-up and manufacturing and validation costs involved.

Potential Suggestions for Implementation of Reference Cell Material(s)

- Creation of reference cell bank(s) meant to act as a ''ruler'' to measure and compare MSC preparations rather than as a gold standard with definitive attributes
- A consortium of academic and industry groups (entity) to collaboratively develop and distribute reference cell materials via WCBs
- Reference cell bank(s) will be freely available on a royalty-free basis or some other basis to ensure that ownership is unrestricted

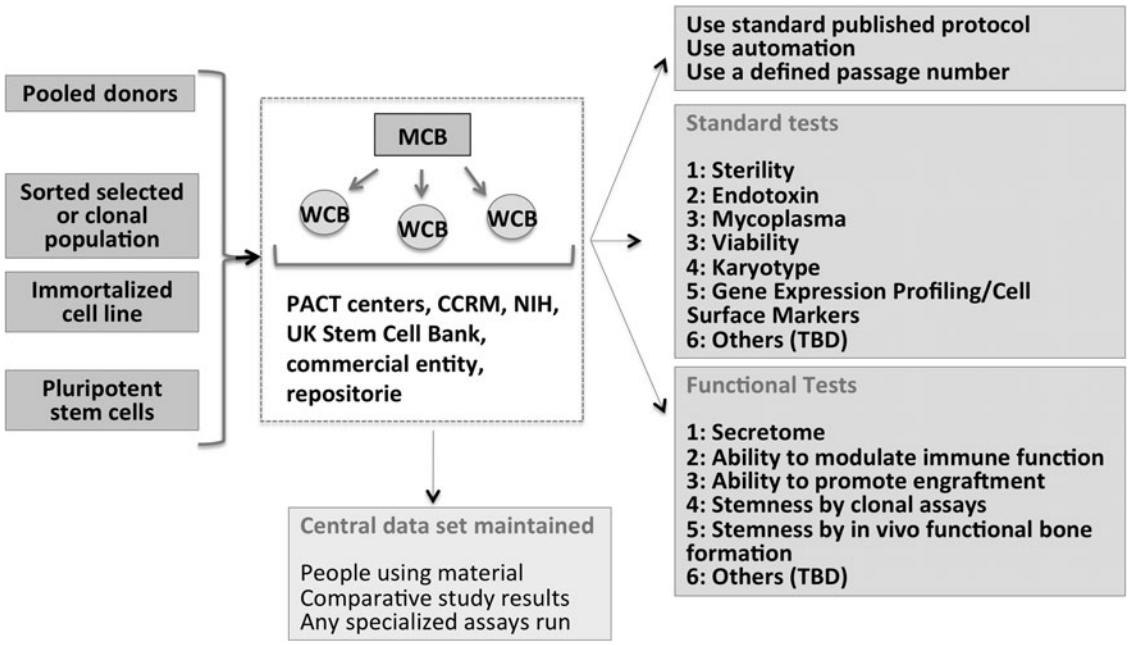

FIG. 3. Flowchart on how one might use such a reference material. iPSCs, induced pluripotent stem cells; PACT, production assistance for cellular therapies; CCRM, Center for Commercialization of Regenerative Medicine; NIH, National Institutes of Health; TBD, to be determined.

- Clarification and assurance that the materials used to generate the reference cell bank(s) are not entangled in patent issues, and there is freedom to operate
- To ensure wide spread distribution, the entity that creates the MCB could aim at working with multiple distributors internationally and, in addition, deposit WCBs in nonprofit agencies such as ATCC or Developmental Studies Hybridoma Bank (DSHB)
- The entity should oversee the testing of samples from different distributors to ensure consistency and provide standardized protocols for cell culturing and testing
- The entity should provide data sheet inserts with baseline characterization and will also maintain a freely accessible evolving data set to provide virtual references
- The entity could be responsible for appropriate nomenclature of the reference cell bank(s); a formalized, codified naming system will likely need to be developed and maintained to differentiate multiple reference cell bank(s) and their variants to avoid confusion.
- The entity could seek endorsement of the reference cell bank(s) by industry associations and other standards groups

Importantly, there is no mandate to use the reference cell material; uptake can be measured by metrics such as number of vials distributed and references cited. Further, if there is sufficient uptake, key journals may eventually require authors to submit comparison information akin to the microarray field. These activities will ensure that a reference cell bank(s) is widely available at a reasonable cost, from multiple vendors with a well-characterized data sheet and peer-reviewed publications, and backed by unbiased interested parties in the field.

Summary

A significant first step in obtaining consistency in the MSC field is to provide a well-characterized reference cell material to investigators through a biorepository or other nonprofit distribution entities. The proposed MSC reference material(s) will be generated at a defined early passage according to a published protocol and manufactured in MCB lot sizes of 500–1,000 vials at a time. Each lot will be analyzed within the limits of the latest available technologies by phenotype characterization, gene expression profiling, and functional assays and compared with previous lots and other WCBs. This dataset will be stored in a publicly accessible database for download by any purchase of the reference cells from the non-for-profit biorepositories. The biorepositories could work with stakeholders to generate additional datasets of samples grown in different commercially available media, and the information could be made available to investigators using the same public database. The investigators could deposit comparative datasets of their samples compared with the reference cell bank(s) and could offer to make the comparative data available. We believe that such a model could enable regulatory and patent agencies to develop their own criteria for similarity, and for academic investigators to define cells more precisely or to determine whether a population of cells is homogenous or not.

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The opinion reflected in this article is the consensus opinion of the authors and individual investigators on the MSC Reference Materials Working Group and does not represent the official view of their employers, societies, or a policy recommendation. This opinion piece is provided to solicit input from stakeholders in order to develop a plan, to obtain consistency in the field, and to pave the way for a wider position paper.

We have used the term MSCs to represent mesenchymal stromal cells (which may include a population of cells that qualify as mesenchymal stem cells by functional definitions) in deference to common usage until a consensus is reached for a nomenclature for cells that fall under the definition of mesenchymal stromal cells.

We have used the term reference material to refer to MSCs with consensus-defined biological activity. This is not intended to be the same as the reference material definition established by the World Health Organization (WHO) [1], but will likely be self-implemented through a consortium effort rather than by government standards.

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Author Disclosure Statement

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