JOURNAL OF EXTRACELLULAR VESICLES
2019, VOL. 8, 1609206
https://doi.org/10.1080/20013078.2019.1609206

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Defining mesenchymal stromal cell (MSC)-derived small extracellular vesicles for therapeutic applications

Kenneth W. Witwer[§a,b], Bas W.M. Van Balkom[§c], Stefania Bruno[d], Andre Choo[*e], Massimo Dominici[‡f,g], Mario Gimona[§h], Andrew F. Hill [§i], Dominique De Kleijn[*§j], Mickey Koh[¶k,l], Ruenn Chai Lai[*m], S. Alex Mitsialis[§n], Luis A. Ortiz[‡§o], Eva Rohde[§h], Takashi Asada[p], Wei Seong Toh[*q], Daniel J. Weiss[‡r], Lei Zheng[¶s], Bernd Giebel[‡§t] and Sai Kiang Lim[‡*§m,u]

[a]Department of Molecular and Comparative Pathobiology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA; [b]Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA; [c]Division Internal Medicine and Dermatology, Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, The Netherlands; [d]Department of Medical Sciences and Molecular Biotechnology Center, University of Torino, Torino, Italy; [e]Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore, Singapore; [f]MAB Laboratory, TPM of Mirandola, Mirandola, Italy; [g]Division of Oncology, University of Modena and Reggio Emilia, Modena, Italy; [h]GMP Laboratory, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI-TReCS), Research Program Nanovesicular Therapies, Department of Transfusion Medicine and Celericon Therapeutics G.m.b.H., Paracelsus Medical University (PMU), Salzburg, Austria; [i]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Australia; [j]Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands; [k]Department of Haematology, St George's University Hospital NHS Trust, London, UK; [l]Cell Therapy Facility, Blood Services Group Health Sciences Authority, Singapore, Singapore; [m]Institute of Medical Biology, Agency for Science, Technology and Research, Singapore, Singapore; [n]Department of Pediatrics, Harvard Medical School & Division of Newborn Medicine, Boston Children's Hospital, Boston, MA, USA; [o]Division of Environmental and Occupational Medicine, Department of Environmental and Occupational Health, Graduate School of Public Health at the University of Pittsburgh, Pittsburgh, PA, USA; [p]Department of Tissue Regeneration Science and Engineering, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan; [q]Faculty of Dentistry, National University of Singapore, Singapore, Singapore; [r]Health Sciences Research Facility, University of Vermont College of Medicine, Burlington, VT, USA; [s]Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China; [t]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; [u]Department of Surgery, YLL School of Medicine, National University of Singapore, Singapore, Singapore

ABSTRACT

Small extracellular vesicles (sEVs) from mesenchymal stromal/stem cells (MSCs) are transiting rapidly towards clinical applications. However, discrepancies and controversies about the biology, functions, and potency of MSC-sEVs have arisen due to several factors: the diversity of MSCs and their preparation; various methods of sEV production and separation; a lack of standardized quality assurance assays; and limited reproducibility of *in vitro* and *in vivo* functional assays. To address these issues, members of four societies (SOCRATES, ISEV, ISCT and ISBT) propose specific harmonization criteria for MSC-sEVs to facilitate data sharing and comparison, which should help to advance the field towards clinical applications. Specifically, MSC-sEVs should be defined by quantifiable metrics to identify the cellular origin of the sEVs in a preparation, presence of lipid-membrane vesicles, and the degree of physical and biochemical integrity of the vesicles. For practical purposes, new MSC-sEV preparations might also be measured against a well-characterized MSC-sEV biological reference. The ultimate goal of developing these metrics is to map aspects of MSC-sEV biology and therapeutic potency onto quantifiable features of each preparation.

ARTICLE HISTORY
Received 14 January 2019
Revised 9 April 2019
Accepted 14 April 2019

KEYWORDS
Please check whether the inserted Keywords are correct; MSC; sEV; Therapeutics; Definition

Introduction

Mesenchymal stromal cells (MSCs) have widely documented therapeutic efficacy in many pre-clinical models of immunological and degenerative diseases and a record of safety in human patients [1–11]. Although MSCs were initially called "mesenchymal stem cells" and thought to act therapeutically as stem cells via cellular differentiation and cell replacement, it is now apparent that effects of MSCs are mediated mainly by paracrine factors. Consequently, the stem cell nature of MSCs has been challenged, and they may be more appropriately labelled mesenchymal stromal cells or even "medicinal signalling cells" [12]. Furthermore, increasing and compelling evidence suggests that

CONTACT Bernd Giebel Bernd.Giebel@uk-essen.de Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; Sai Kiang Lim saikiang.lim@imb.a-star.edu.sg Institute of Medical Biology, Agency for Science, Technology and Research, Singapore
*Authors who are members of the Society for Clinical Research and Translation of Extracellular Vesicles Singapore (SOCRATES). †Authors who are members of the International Society for Cell and Gene Therapy (ISCT) §Authors who are members of the International Society for Extracellular Vesicles (ISEV) ¶Author who is member of the International Society of Blood Transfusion (ISBT)

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MSCs exert many if not most of their paracrine effects through the release of extracellular vesicles (EVs), vesicles of roughly 50–1000 nm in diameter that are secreted by all cell types [13]. In particular, small EVs (sEVs, 50 to 200 nm diameter), harvested using different protocols from cell culture supernatants of MSCs grown under diverse culture conditions, have been reported to be therapeutically efficacious in various preclinical models [13,14]. Accordingly, MSC-sEVs have emerged as promising therapeutic agents that are proposed for testing in clinical trials [13,15]. In light of the diversity of sources and separation methods for MSC-sEVs, validated metrics and functional analyses are required for better characterization of MSC-sEV preparations to facilitate comparisons across different preparations.

A key to the rapid translation of MSC-sEVs from bench to bedside is their derivation from MSCs, which have already been tested extensively in the clinic and proven to be generally safe. To date, almost 1000 clinical trials[1] have been registered to assess administration of MSCs derived from a variety of sources, including bone marrow (BM), adipose tissue (AD), cord blood (CB), and others. In general, MSCs have a well-established safety profile in patients. Nevertheless, to date, only a few MSC products have been approved for market. This might be related to the lack of therapeutic efficacy observed in some clinical trials as well as to the heterogeneity of MSC products prepared by different laboratories. Diverse strategies of MSC isolation and expansion from different tissues are used, and standardization across groups is largely absent. To facilitate the development of MSCs as therapies, the *International Society for Cell and Gene Therapy* (ISCT), formerly known as the *International Society for Cellular Therapy*, distilled basic features of human MSCs into a set of minimal suggested criteria [16]. Specifically, MSCs are plastic adherent fibroblastic cells with the "trilineage potential" of osteogenic, chondrogenic and adipogenic differentiation capabilities. Furthermore, they express the cell surface markers CD73, CD90, and CD105, and do not express haematopoietic and endothelial antigens (CD14 or CD11b, CD19 or CD79α, CD34, CD45, HLA-DR) [16]. Notwithstanding the lack of defining complex functional properties, the minimal ISCT criteria help to exclude cell preparations that are not MSCs or are contaminated with non-MSCs.

To enable the development of MSC-sEVs as therapeutics, it is critical that similar standardized criteria be developed to define and qualify the human "MSC-sEV preparation" (In this document, we use the term "preparation" because absolute separation of EVs from other cell culture supernatant components is likely unachievable with current techniques. Furthermore, co-isolated non-sEV components may contribute to observed therapeutic effects.) Currently, most MSC-EV preparations are characterized according to the *Minimal Information for Studies of EVs* (MISEV2014), published by the International Society for Extracellular Vesicles (ISEV) in 2014 [17]. MISEV2014 recommends specific criteria for definition and classification of EVs including presence of several characteristic markers and depletion of presumed non-EV markers, quantitation, and single-vesicle visualization. MISEV2014 was recently updated and expanded to MISEV2018 [18], with broad input from members of ISEV, including the ISCT Exosome Working Group.

Thus, the ISCT minimal criteria provide guidance for MSCs, and the MISEV recommendations are a framework for defining and characterizing EVs of all sizes and morphologies, and from many cell types and biological sources, some of them with great heterogeneity. For example, EVs in biological fluids originate from many distinct cell types and display a wide range of phenotypes. Although both criteria are helpful for characterizing MSC-sEV preparations, they are insufficient to define MSC-sEVs as a unique entity that is distinguishable from non-MSC-sEVs. Furthermore, the existing criteria do not provide guidance on functional testing of the biological activities of MSC-sEVs.

We suggest that the MSC-sEV field should therefore build on the ISCT suggested definition of MSCs and the broad MISEV criteria for EVs to define MSC-sEV preparation-specific criteria for therapeutic applications. These new criteria must encompass the potential diversity of independent MSC-sEV preparations, arising from the heterogeneity of MSCs by culture and origin as well as different EV preparation and separation protocols. Possibly, each manufacturing procedure will generate unique MSC-EV preparations [14].

Since regimented global standardization of MSC and MSC-sEV production is unlikely, defining the final product by physical, biochemical, and functional attributes will be necessary. Clear guidelines must be balanced with flexibility in the choice of the manufacturing process and facilitate data sharing and comparison between independently generated MSC-sEV products. **The overall need, then, is to define MSC-sEV preparations physically, biochemically, and functionally by quantifiable features and using reproducible and standardized assays.**

Major questions to answer:

(1) What is an MSC? Are all ISCT criteria relevant?
(2) What are the key features of MSCs that are likely to also define an sEV preparation as originating from MSCs?

(3) Do MSC-sEV fractions contain non-MSC-sEVs? What are the likely sources of such non-MSC-EVs? How can non-MSC-EVs in MSC-sEV preparations be discriminated from MSC-EVs, and must they be removed?
(4) What is an MSC-sEV preparation as opposed to an MSC-origin protein, RNA or lipid particle preparation?
(5) What is the purity of the MSC-sEV preparations?
(6) How to measure integrity of MSC-sEVs in a preparation?
(7) Which functions do MSC-sEV preparation need to fulfil to be considered biologically and therapeutically active?

Mesenchymal stromal/stem cells: definitions and considerations

The definition of MSC-sEV preparations begins with the MSCs, which are highly heterogeneous cells. Here, we adopt as the starting point of discussion the minimal defining criteria of human MSCs established by the Mesenchymal and Tissue Stem Cell Committee of the ISCT [16]. These criteria state that:

> First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro.

In general, the ISCT definition is extremely useful in providing a common reference for defining MSCs; however, the criteria represent a rudimentary definition that serves more to eliminate cells that are not MSCs rather than to define cells that are MSCs. However, these basic parameters were established in 2006 and no longer represent the totality of knowledge about biological activities and mechanisms of action that correlate with therapeutic potency of MSCs, such as immunomodulation, inhibition of fibrosis, or facilitating the proliferation of neighbouring cells or release of EVs. Based on the original criteria, it is difficult to predict the therapeutic potency of an MSC preparation or the reproducibility of potency in independent preparations. More investigations are needed to identify additional MSC-defining criteria, keeping in mind that increasing awareness of MSC complexity may make a single definition elusive. To enhance therapeutic reproducibility of MSCs, different aspects of the manufacturing process should be considered (Table 1).

Improved robustness of the MSC criteria may be achieved by re-examining the necessity of certain MSC surface markers, the necessity of the differentiation potential into all three lineages, and also further definition and clarification of biological activities and reproducibility and reliability. There is a growing recognition that biological activities will be different for different clinical applications, and that MSCs respond to and are shaped by local inflammatory conditions. As such, it may be desirable to focus more on biological activities and less on the traditional suggested criteria, particularly the necessity for osteogenic, chondrogenic and adipogenic differentiation potential. Defining MSCs as a cell type is different from defining MSCs as the source of sEVs. For example, it is possible that prolonged culture of MSCs might compromise trilineage potential but not the characteristics and potential therapeutic efficacy of derived sEVs.

Table 1. Factors to be considered during the MSC manufacturing process.

1.	Tissue source of the MSCs
2.	Age of donor and age of the MSCs (passage number or perhaps doubling time)
3.	Donor-to-donor variability and previous pathological conditions
4.	Allogeneic versus autologous sources
5.	Procedures of MSC isolation
6.	Heterogeneity within the MSC culture
7.	Preconditioning via addition of proinflammatory cytokines and/or cultivation under hypoxic conditions.
8.	Genetic modification or immortalization of MSCs

Based on the points discussed above, we propose that the minimal information required of MSCs as the cell source of MSC-sEVs should include:

(1) Specification of the tissue source from which MSCs are isolated (i.e. CB, AD, BM);
(2) Confirmation of at least one of the three lineage potentials, which should be sufficient to discriminate MSCs from fibroblasts, which share many MSC features but seem to lack therapeutic activities, and to verify the source of MSC-sEVs as a therapeutic product;
(3) Definition of source cells as fresh or primary MSCs or as modified (e.g. immortalized) MSCs; and
(4) Functional testing of the final MSC-sEV preparation to ascertain if the MSC is capable of producing functional sEVs.

MSC and MSC-EV production

The release of sEVs is now considered to be one of the mediators of the therapeutic activities of MSCs [13]. sEVs prepared from *in vitro* MSC cultures are widely reported to display therapeutic activities that recapitulate those of MSCs in various *in vitro* functional assays and in related pre-clinical disease models [19,20].

However, it is possible that sEVs produced from static MSC culture may act differently from sEVs released by MSCs after administration *in vivo*. MSCs that produce therapeutically active sEVs *in vitro* may not produce active sEVs *in vivo*, and vice versa. In addition, although the current view is that most therapeutic effects of MSCs occur through paracrine mechanisms, mainly sEVs, non-paracrine actions of MSCs *in vivo* cannot be excluded. Therefore, features that qualify MSCs as therapeutically active *in vivo* may not necessarily qualify MSCs as producers of active sEVs *in vitro*. Defining better those attributes of sEVs produced by MSCs in any given condition will allow development of tools, for example engineered MSCs, that reliably and reproducibly provide the desired sEV products under appropriate conditions. For example, MSCs identified as producers of functional sEVs *in vitro* could be immortalized as a monoclonal stable EV-producing cell line to improve batch-to-batch reproducibility of sEVs. However, immortalization may have potential safety concerns that would require mitigation. Unlike primary cells with limited lifespan, immortalized cell lines are highly amenable to extensive and intensive "omics" characterization such as genomic, transcriptomic, proteomic, etc. Additionally, it has been shown that functionality of MSC-EVs may be retained after immortalization. For example, after myc-mediated immortalization, MSCs continued to produce cardioprotective sEVs [21]. Importantly, MYC protein was not detected in the sEVs. The myc-immortalized MSCs were karyotypically stable, expressed the typical MSC surface antigens and retained two of the trilineage potentials.

Extracellular vesicles: definitions and considerations

For definition of EVs, MISEV2014 [17] and the updated MISEV2018 [18] are the consensus and basis for discussion. As vesicles released by cells, EVs are delimited by a lipid bilayer membrane and are incapable of self-replication. MISEV2014 recommends that studies of EVs should include general characterization, including detection of transmembrane and luminal proteins and depletion of presumed cellular or extracellular non-EV proteins. Quantitation and single-particle characterization should be performed by methods including but not limited to sizing and counting by particle tracking techniques, imaging by electron microscopy, and advanced flow cytometry. MISEV2018 provides additional guidance in six major areas: i) nomenclature, ii) collection and pre-processing of fluids for EV extraction, iii) EV preparation and concentration, iv) EV characterization, v) functional studies and vi) reporting.

Nomenclature

The term "sEVs" is the best descriptor for the target population of therapeutic MSC-EVs that we discuss here. "Small" denotes a population ranging in diameter from around 50 nm to around 200 nm. EVs are frequently classified generically as "exosomes," "microvesicles," or by a variety of other terms without strict consideration of definitions. However, the term "exosome" commonly refers to a specific class of sEV formed by the endosomal system [22,23], in contrast with "ectosomes" (microvesicles, microparticles) that bud from the plasma membrane [24,25] or other similarly sized EVs with unknown biogenesis [26]. Specifically, intraluminal vesicles (ILVs) are released into the extracellular space as exosomes when the multivesicular body (MVB) fuses with the plasma membrane. While exosomes are generally thought to be smaller than microvesicles, the two EV types cannot be differentiated by size alone, as their respective size ranges overlap. To classify sEVs as "exosomes" requires evidence of an endosomal biogenesis pathway. Similarly, to prepare a pure MSC-exosome population, separation, and characterization protocols would be required to deplete non-endosome-origin vesicles from a heterogeneous population, and to verify this removal. These requirements remain experimentally challenging to fulfil at this time and is of academic rather than of clinical interest. In the clinical setting, it is more important that the MSC-sEV preparations can be manufactured reproducibly and confer therapeutic activities that can be confirmed by robust and adequate *in vitro* and *in vivo* models while avoiding significant side effects [15]. Since physical separation of EVs by biogenesis is unrealistic, the term "sEV" is recommended, which is agnostic to the site of subcellular origin. This term also derives from recent recommendations that EVs be classified by physical characteristics or isolation method [18,27]. For example, EVs that pass through a 0.22 μm filter or are pelleted at 100,000 x g are generally smaller than 200 nm in diameter and could be classified as sEVs or a 100k fraction, respectively. While such classification provides little information on the biology of EVs, it is practical and enabling as it defines an EV population that could be universally prepared.

For clarity and to align with the recent recommendations, we use the term "MSC small EVs (sEVs)" to describe bilipid membrane vesicles of roughly 50–200 nm in diameter and that are released by MSCs.

Collection and pre-processing of cell culture conditions media for EV preparation

The process of producing MSC-sEVs is a major consideration in developing standardized criteria to define and qualify human MSC-sEV preparations for clinical applications. To apply and test MSC-sEVs in clinical studies, a scalable and GMP-compatible manufacturing process is necessary. The choice of production steps depends on the quantity needed (batch size), and an acceptable degree of reproducible purity, identity, safety, and stability of the final product must be confirmed by quality testing. Influence of storage conditions should also be considered.

Cell production

Cell density and 2D vs bioreactor culture

Cell density in 2D culture is well known to affect cell behaviour and the nature of the secretome. Cells seeded at lower density have been reported to produce more sEVs per cell, and this release seems to decline as confluence is reached [28] although it is not clear if this is a universal observation. Replicative ageing of the culture (senescence) may also modulate sEV production or alter sEV efficacy. To scale MSC-sEV production, bioreactors may be required. Three main bioreactor types are currently available, each uniquely affecting sEV-producing MSCs: bioreactors with expanded 2D surfaces, hollow fibre-based bioreactors, and stirred-tank bioreactors (in which MSCs must be grown on appropriate microcarriers) [29]. Although each has been used for MSC expansion, their impact on sEV production and function requires further investigation. Well-established growth conditions may not be transferable from classical static cultures to bioreactors, necessitating optimization of culture conditions. However, 3D bioreactors are advantageous as they are more amenable to monitoring of cell number, viability, morphology and proliferation, and in providing for a more uniform distribution of nutrients and oxygen [30].

Cell culture medium components

MSCs, like other mammalian cells, are easily grown in culture with media supplemented with fetal bovine serum (FBS) as an energy source. However, to facilitate approval of therapeutic applications, xenogenic components should be eliminated at least during the sEV production and harvest phase. Hence, human serum or platelet lysate (hPL) has been substituted for FBS [31–33]. However, like FBS, both serum and hPL are themselves rich sources of EVs or EV-like particles that will co-purify with sEVs produced by the MSCs. These exogenous EVs are likely to be safe, as evidenced by the well-established safety of EV-rich transfusion products, and could even contribute to therapeutic effects directly or acting as co-factors. Indeed, MSC-sEVs used for successful treatment of a graft *versus* host disease (GvHD) patient [34] contained a large population of hPL vesicles. Since identically prepared, hPL vesicle-containing MSC-sEV preparations exerted the same therapeutic effects in a murine ischaemic stroke model as MSCs [35], there is currently no evidence that hPL vesicles negatively affect therapeutic activities of MSC-EV preparations. As hPL is generally safe, a subset of hPL such as hPL-vesicles should be considered to be safe as well [36]. Still, exogenous sEVs or sEV-like particles could conceivably dilute or block some effects of MSC-sEVs. It is thus necessary to establish whether exogenous vesicles support or counteract specific MSC-sEVs therapeutic functions, or whether they can be considered functionally neutral.

To eliminate exogenous EVs

MSCs could be grown in serum-free (defined) medium or with serum or hPL that has been depleted of EVs. Several protocols for EV depletion have been developed, achieving variable degrees of depletion. Standard centrifugation and ultrafiltration protocols for serum depletion have been used [37,38], as well as tangential flow filtration (TFF) [3]. Importantly, fibrinogen/fibrin depletion is an apparent prerequisite for hPL-EV depletion using filtration-based methods (including tangential flow filtration), as clotted hPL components such as fibrin polymers can block filter pores. To this end, an efficient depletion method for fibrinogen and fibrin aggregates has been described [39]. Some culture systems that involve circulation of fluids through molecular weight cut-off filters may also permit culture with non-EV-depleted components, since EVs and large macromolecules in culture media reservoirs will have little or no access to the cell culture compartment [40].

Both serum-free and EV-depleted culture options should be critically evaluated while addressing at least three important considerations. First, components of serum or hPL may persist for some time in the culture after changing to defined conditions, as evidenced by studies of extracellular RNAs [41]. Second, the stress of a switch to serum-free or depleted medium may alter cellular programs [42]. Cells may require gradual acclimatization to the new conditions. Third, energy source starvation is also known to stimulate EV production by some cells, at least in the short term, and may alter the molecular content of released EVs or EV-like particles. Due to these impacts of the culture conditions, MSC-sEVs produced under different culture conditions may have different

biological features. To identify a scaled MSC-sEV production strategy, the composition and functionality of obtained MSC-sEVs must be investigated carefully to ensure the quality and biological activities of MSC-sEV samples.

EV separation and concentration

Drawbacks of legacy methods

Variations on differential ultracentrifugation have been the most widely used EV separation and concentration method [43]. However, ultracentrifugation has several drawbacks that make it an unlikely choice for large-scale MSC-sEV purifications, especially for therapeutic applications. Ultracentrifugation does not result in highly pure EVs. Although serial washes can improve purity, they also reduce yield [44]. sEV aggregation and poor resuspension have been reported after ultracentrifugation [45], and disruption may also occur. Finally, ultracentrifugation is not scalable: it is time-consuming for the volumes that can be processed.

Commercial "kit-based" EV separation methods

Commercial products advertised as specific for EVs or even EV subtypes such as exosomes have proliferated in recent years as the EV research field has grown. While some of these products are well described and even potentially GMP-compliant, for many, the exact principle of the separation method is not stated, and the identity of proprietary matrices and other reagents is unknown. It is thus difficult to assess safety or how components of the systems might contribute to possible EV actions. Unless GMP compliance has been demonstrated, and proprietary additives are revealed by suppliers of commercial kits to the pharmaceutical manufacturers to assess clinical usability and safety of ingredients, the use of such kits to produce EVs for clinical use is presently unrealistic.

Polyethylene glycol/polymer-based EV enrichment

Precipitation by "salting out" with polyethylene glycol (PEG) or other polymers is an effective way to reduce volume and thus enrich EVs in a reproducible and scalable manner [46]. While ultracentrifugation can be performed for only up to approximately 500 mL culture medium per run (depending on rotor and buckets), the lower-speed centrifugation required to pellet PEG precipitates can be done for up to several litres per run. Each run is also shorter for PEG precipitates. Because of abundant co-precipitates, however, PEG-precipitated EVs should not be considered pure preparations [46]. Also, removal of PEG and other contaminants by wash steps and UC re-pelleting may be necessary for some applications. Nevertheless, PEG-precipitated MSC-sEVs have already been used in a clinical investigation [34,46], and MSC-sEVs concentrated by PEG exerted the same effects in an ischaemic stroke model as corresponding cells. Thus, the procedure does not appear to interfere with MSC-sEV activity, and co-isolated materials do not appear to negatively affect sEV function [35].

Size-based fractionation

Size-based fractionation methods such as size exclusion chromatography (SEC) and tangential flow filtration (TFF) have gained increasing recognition and adoption as GMP-compatible and highly scalable technologies by researchers [47–49]. These methods are faster and easier to implement than legacy methods, while at the same time producing EVs of comparable or superior purity and/or functional activity [46,50]. Conditioned media with high protein content (e.g. serum- or hPL-supplemented media) may clog pores, especially in the case of fibrin formation from concentrated, unprocessed hPL. In this case, clotting can be induced in advance and clotted components removed [39]; however, pre-processing serum or hPL may also change MSC-supportive properties. Apart from these considerations, these methods are considered scalable and time efficient, allowing a high degree of process standardization.

For additional information on EV separation, we refer the reader to the MISEV2018 criteria and references cited therein [18]

Characterization

For objective evaluation of MSC-sEV preparations independent of the manufacturing process, quantifiable metrics are needed to measure the key defining and biologically important parameters of MSC-sEVs. Several questions arise.

When can an sEV preparation be described as an MSC-sEV preparation?

As a minimum prerequisite, MSC-sEV preparations must derive from supernatants of MSCs that largely fulfil the ISCT minimal criteria, as discussed above. The use of different MSC sources, culture conditions and media, and EV-harvesting strategies, however, could introduce significant variations in MSC-sEV preparations. Therefore, using a single definition to describe all MSC-sEV preparations would classify many different products under one

umbrella. It is rather unlikely that all MSC-sEV preparations display the same therapeutic activities in different disease models. To avoid underclassification, MSC-sEV preparations could be classified according to their manufacturing process. However, since most laboratories use unique protocols, such an approach could lead to overclassification. With no elegant solution and the unlikelihood of a standard MSC-EV production protocol, the terms "MSC-EVs" or "MSC-sEVs" will for now continue to cover EV-containing products derived from MSCs. However, MSC-sEV preparations from MSCs that are grown in media supplemented with human or non-human serum or hPL, may contain non-MSC-EVs. As discussed above, these non-MSC-sEVs may confer functional properties on MSC-sEV preparations, acting additively, synergistically, or even antagonistically to MSC-sEVs. Ideally, the ratio of non-MSC-EVs to MSC-EVs should be documented and the biological activities of the non-MSC-EVs defined. To do so, MSC-EVs must be discriminated from non-MSC-EVs. Among the three features of MSCs in the ISCT suggested minimal criteria for MSCs, the surface antigens are logically the most immediately detectable features that could be transferred to EVs. Thus, antigens like CD73 and CD105 might be used to identify at least a subset of MSC-sEVs. In contrast, serum or hPL-derived sEVs are expected to display platelet or haematopoietic cell markers that are not present on MSCs, such as CD34 and CD45, the endothelial marker CD31, the red blood cell marker Glycophorin A, or the platelet markers CD41, CD42, and P-selectin. Very recently, we could discriminate hPL-EVs and MSC-EVs with anti-CD9 and anti-CD81 antibodies [51].

To identify such MSC-sEV-specific antigens, the presence of MSC surface antigens in published MSC-(s)EV proteome databases was recently investigated. These analyses revealed three MSC surface antigens from the ISCT minimal criteria (CD73, CD90 and CD105), were found in at least 7 of 10 published MSC-EV proteomics datasets, while 3 non-MSC surface antigens from the ISCT minimal criteria (CD14, CD34 and CD11b), were not found in any of the 10 datasets [52]. Although the data are mostly from BM-derived MSCs (5 of 10), they also include MSCs derived from ESCs, placenta chorionic villi, CB, and AD tissues. These findings support using the MSC positive and negative surface antigens listed above to assess the identity and purity of the cellular source of an MSC-sEV preparation. Technically, the presence of MSC- and non-MSC-sEV markers can be analysed by methods including Western blotting, enzyme-linked immunosorbent assays, classical flow cytometry of bead-captured EVs, or advanced flow cytometry at the single EV level.

In summary, the MSC cellular origin of an sEV preparation could be identified by the presence of MSC markers, CD73, CD90 and CD105, and the absence of CD14, CD34 and CD11b. It is, however, recognized that it may not be practical for all MSC-sEV preparations to be devoid of non-MSC markers, especially if the MSCs were cultured in the presence of supplements such as hPL or serum. In the final analysis, the relative abundance of MSC *versus* non-MSC markers in an MSC-sEV population helps to assess the relative ratio of MSC-sEV to non-MSC-sEVs and will be useful in calibrating comparison between different MSC-sEV preparations.

When is a biological preparation an sEV, and not a protein, RNA or lipid preparation?

Particle quantitation methods such as nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and resistive pulse sensing (RPS) [53–57] are not specific to EVs. There is increasing evidence that most particles in many EV preparations are not in fact EVs. Thus, novel EV identification and quantitation methods are required. Since EVs are lipid membrane vesicles, an sEV preparation will be distinguished from more homogeneous protein, RNA, or lipid preparations by having membrane lipids that are associated with proteins and/or RNAs. Since the structure and size of sEVs are physically defined by the lipid bilayer, the amount of RNA and/or protein that can be accommodated within each EV is limited by the amount of membrane lipids. Therefore, for specific sEVs from a particular cell type, cultured under specified conditions and separated by a specified protocol, the ratio of membrane lipids to protein or RNA should be a definitive, more quantifiable feature of the sEVs than the numbers obtained by conventional particle analysis.

A comparison of sEV preparations and the producing cell is also instructive. Relative amounts of certain lipids are different in MSC-sEV membranes *versus* the bulk cellular membranes of the parent MSCs [reviewed by [58]]. For example, the major plasma membrane phospholipids, cholesterol, sphingomyelin and phosphatidylcholine, are enriched in the sEV membrane. The proportion of sphingomyelin to phosphatidylcholine in sEV preparations was also reportedly twice as high as in the corresponding cells [59]. It was also observed that sEV preparations generally have an 8.4-fold enrichment of lipids per mg of protein compared with cells [60].

To distinguish an sEV preparation from other biological nanoparticle preparations, the following quantifiable metrics could be used:

a. ratio of specific membrane lipids to proteins
b. ratio of sphingomyelin to phosphatidylcholine

What is the concentration of lipid membrane vesicles in an sEV preparation?

In addition to the ratiometric approaches above, absolute quantitation of sEVs might also be useful. Unfortunately, with the exception of volumetric cryoelectron microscopy (which also unambiguously reveals lipid membrane vesicles), identifying particles as EVs is difficult. Nevertheless, progress in single EV analysis by methods such as fluorescence-augmented NTA or nano-flow is promising to the extent that lipid and protein labelling can be included [51,61–64].

What is the purity of the sEV preparation?

A pure sEV preparation might be defined as one in which all proteins and RNA are anchored in or encapsulated within a lipid bilayer membrane. For sEVs with a size range of 50–200 nm and a specific membrane lipid to protein ratio, the number of sEVs would be limited by the amount of membrane lipids or proteins. Hence, if the number of sEVs is known and measured as lipid membrane vesicles as discussed in question 3, the number of sEVs per unit membrane lipids or proteins would reflect the degree of purity. A caveat is that a completely "pure" MSC-sEV preparation is not likely to exist. Also, highly pure and concentrated EVs without "carrier" materials might conceivably be lost by binding to the surfaces of their containers. Furthermore, loosely associated factors that contribute to MSC-sEV biological activities might be removed by stringent purification, resulting in a reduction or even loss of therapeutic activities in the disease models of interest. As there are presently no metrics to evaluate the purity of an MSC-sEV preparation, a practical alternative is to benchmark sEV preparations to a universal MSC-sEV preparation. This universal MSC-sEV preparation should be one that is manufactured reproducibly on a large scale and with long-term stability.

How to measure integrity of sEVs in a preparation?

EV integrity is synonymous with lipid membrane integrity. Electron microscopy provides irrefutable evidence for the presence of intact membrane vesicles but is not high-throughput. Instead, membrane integrity may be assayed using proteins that are tethered to a membrane lipid, such as GM1 ganglioside. GM1 gangliosides are highly enriched in MSC-sEVs and are bound with high affinity by cholera toxin B chain (CTB) [65]. CTB-binding sEVs are also enriched in CD81, and this association can be readily assayed by ELISA. Disruption of vesicles by homogenization disrupts the association between CD81 and CTB binding, paralleled by loss of function [e.g. cardioprotective activity [66]] and reduced CD81 in CTB-bound sEVs (unpublished results, SKL). Thus, the level of CTB-associated CD81 in a preparation provides a global quantitative assessment of membrane integrity in an sEV preparation [65] provided that the level of CTB-CD81 for intact MSC-sEV can be established. In lieu of this, the level of CTB-CD81 could be benchmarked against a universally accepted MSC-sEV preparation. $CD81^+$ EVs might also be quantified by imaging flow cytometry, plasmon resonance-based technologies, or by novel, more sensitive fluorescence NTA instruments.

What surrogates of cargo biological activity can be measured?

Small EVs consist of and carry a diverse load of proteins, lipids and RNA. The general consensus is that upon reaching a target, the proteins and/or RNAs in sEV preparations act as effector molecules either outside or inside the cell. Therefore, the protein and RNA cargo in or on sEVs in general, or the MSC-sEVs considered here, should be intact and not be degraded or denatured if sEVs are to exert biological effects. To assess if the cargo is generally intact, we propose measuring the enzyme activity of a surface protein as a surrogate of the integrity of the cargo. The enzymatic activity of a protein is a function of its primary, secondary, tertiary and quaternary structures, and is proportional to the integrity of these structures. Thus, the biological integrity of an MSC-sEV preparation could be assessed via surrogate proteins. One possible example would be CD73, an ecto-5-prime-nucleotidase (5-prime-ribonucleotide phosphohydrolase; EC 3.1.3.5) that converts AMP to adenosine, and this enzymatic activity can be easily assayed using commercially available assay materials [65]. CD73 is also an MSC-associated surface marker and one of the key MSC markers of the ISCT minimal criteria. As a surface protein, CD73 may be vulnerable to protein denaturation and loss of enzyme activity. Thus, it might serve as a sentinel to monitor the preservation of sEV cargo functionality during preparation or storage. However, cargo functionality may not depend on the integrity of sEVs and may not be indicative of vesicle integrity. Cargo functionality may not always be sufficient to predict therapeutic activity, as therapeutic activity may depend on many other factors, such as delivery to the appropriate cell type, interaction of the cargo with the appropriate subcellular compartments, and the kinetics of cargo delivery.

Conceivably, RNA cargo and composition might also be used to predict biological activity of an MSC-sEV preparation. MicroRNAs in particular have been

the focus of many EV RNA studies, with evidence that EV miRNAs are highly stable and can be transferred into and function in recipient cells [67]. However, detection of a mature miRNA alone might not equate with canonical, RNA-induced silencing complex (RISC)-associated function, as the miRNA (like an siRNA) must be incorporated into the RISC machinery of the recipient cell to be functional [68]. This loading occurs during processing of the pre-miRNA form, and dissociation of mature miRNA from RISC components during the sEV harvest or purification process might render the miRNA non-functional. Therefore, a miRNA-based surrogate assay for MSC-sEV activity should assay mature miRNA association with RISC components (e.g. Argonaute) or examine the presence and activity of pre-miRNA. However, there may be non-canonical actions of miRNAs that such assays would not reflect.

Conclusions and action plan

A key confounding factor in MSC-sEV research and translation is the wide variability in MSC-sEV preparations that permeates the entire process from the starting producer cell source through the production and purification to the final product. This, coupled with the lack of standardized quality assurance assays and *in vitro* and *in vivo* functional assays, has led to discrepancies and controversies about the biology, functions, and therapeutic potency of MSC-sEVs. While defining a biological product by its manufacturing process is an accepted practice in early stage clinical research, defining MSC-sEV products by the process is not recommended for advancing the science and later-stage clinical applications of MSC-sEVs. This approach could lead to overclassification of MSC-sEVs and hinder data sharing or comparison among different research groups. Furthermore, there is presently no consensus on the best way to produce therapeutically active MSC-sEVs, particularly as these have not yet been strictly defined and tested for clinical indications.

A more practical approach would be to develop a set of minimal quantifiable metrics to harmonize the definition of MSC-sEVs and provide a denominator for comparative manufacturing and functional testing of different preparations (please refer to checklist). This will facilitate data sharing or comparison among MSC-sEV preparations as differences in biology or therapeutic applications could be mapped to quantifiable differences in the defining features.

Here, we have identified potential metrics of MSC-sEVs, namely the ratio of MSC to non-MSC surface antigens, ratio of membrane lipids to protein, ratio of specific lipids, concentration of membrane lipid vesicles, vesicle integrity, and biological activity. The next step will be to quantify and validate each metric. One practical approach is to use a well-characterized MSC-sEV preparation as reference for each metric and to have each metric assessed by several independent laboratories to ensure the robustness and reproducibility of the metric assays. In assessing the metrics, reagents for a phenotypic and a functional assay, if not commercially available, should be shared or bought from the same supplier for distribution to all. The evaluating teams will compare their own MSC-sEVs with the reference sample utilizing their *in-house* assays as well as the shared assays. Results will be collected, analysed, and discussed. If positively evaluated, strategies for producing reference samples and phenotypic/functional assays for the community of MSC-EV researchers will be developed. This spirit of collaboration is necessary and desirable in promoting MSC-sEV research and applications.

Future perspectives

In this exercise, we have focused on identifying the key defining physical and biological characteristics of MSC-sEVs, and on developing assays to measure these characteristics. The underlying assumption is that these characteristics are ultimately crucial to the therapeutic potency of the sEV preparations. However, these characteristics convey only the physical and biological integrity of EVs and may not predict therapeutic potency. Therefore, the immediate next challenge will be to develop assays that predict the therapeutic potency of MSC-EVs in quantifiable, robust, and reproducible parameters. Based on the complexity of MSC-sEV preparations and the wide spectrum of diseases against which they have been reported to be efficacious, the therapeutic mode of action will likely be different and specific for each disease condition. In addition to the mode of action, the therapeutic potency of MSC-sEV will also be influenced by an agonistic or antagonistic disease microenvironment, delivery route, and time window for therapeutic intervention for a particular disease. Establishing appropriate functional assays to measure therapeutic activity will also require a deep understanding of MSC-sEV biology, e.g. the half-life and *in vivo* biodistribution of MSC-sEVs in a normal and diseased state and knowledge of direct and indirect target cells in different tissues. Therefore, defining the therapeutic potency of MSC-sEV preparations will require another level of discussion and will be the topic of future activities of SOCRATES, ISCT, ISEV, and ISBT.

Note

1. (https://clinicaltrials.gov/ct2/results?cond=&term=mesenchymal+cells&cntry=&state=&city=&dist=&Search=Search Search conducted 2018–12-05, results = 964).

Acknowledgments

SKL and BG initiated the project through SOCRATES. Representative members from ISCT, ISEV, ISBT and SOCRATES discussed, drafted and edited the paper. Funding was provided by The Silent Foundation, Singapore. SKL was funded by ICP I1801E0019 by ASTAR.

Disclosure

The authors report that they have no conflicts of interest in the authorship and publication of this article. SKL holds founder shares in Paracrine Therapeutics Pte Ltd.

ORCID

Andrew F. Hill http://orcid.org/0000-0001-5581-2354

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