Title: Critical considerations for the development of potency tests for therapeutic applications of mesenchymal stromal cell (MSC)-derived small extracellular vesicles

Mario Gimona[#,1], Maria Felice Brizzi[2], Andre Boon Hwa Choo[3], Massimo Dominici[4,5], Sean M. Davidson[6¶], Johannes Grillari[7,8,¶], Dirk M. Hermann[9], Andrew F. Hill[10,¶], Dominique de Kleijn[11,&,¶], Ruenn Chai Lai[12,&], Charles Lai[13,¶], Rebecca Lim[14,¶], Marta Monguió-Tortajada[15,¶],, Maurizio Muraca[16], Takahiro Ochiya[17], Luis A. Ortiz[18,#,¶], Wei Seong Toh[19,&,¶], Yong Weon Yi[20,¶], Kenneth W. Witwer[21,22,¶], Bernd Giebel[23,#,¶,∗] and Sai Kiang Lim[12,24,&,#,¶,∗]

[1]GMP Laboratory, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI-TReCS) and Research Program "Nanovesicular Therapies", Paracelsus Medical University (PMU), Salzburg, Austria

[2]Department of Medical Sciences and Molecular Biotechnology Center, University of Torino, Torino, Italy

[3]Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore

[4]TPM of Mirandola, Mirandola, Italy

[5]Division of Oncology, University of Modena and Reggio Emilia, Modena, Italy

[6]The Hatter Cardiovascular Institute, University College London, United Kingdom

[7] Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria

[8]Christian Doppler Laboratory on Biotchnology of Skin Aging, Institute for Molecular Biotechnology, Department of Biotechnology, BOKU – University of Natural Resources and Life Sciences, Vienna, Austria

[9]Department of Neurology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[10]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Australia

[11]Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands

[12]Institute of Medical Biology, and Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore

[13]Institute of Atomic and Molecular Sciences, Academia Sinica, Taipei, Taiwan

[14]The Ritchie Centre, Hudson Institute of Medical Research; Department of Obstetrics and Gynaecology, Monash University, Victoria, Australia 14

[15]ICREC Research Program and REMAR-IVECAT group, Health Science Research Institute Germans Trias i Pujol (IGTP), Can Ruti Campus, and Cardiology Service, Germans Trias i Pujol University Hospital, Badalona, Spain

[16]Department of Women's and Children's Health, University of Padova, Padova, Italy

[17]Department of Molecular and Cellular Medicine, Tokyo Medical University, Tokyo, Japan

[18]Division of Environmental and Occupational Medicine, Department of Environmental and Occupational Health, Graduate School of Public Health at the University of Pittsburgh, Pittsburgh, USA

[19]Faculty of Dentistry, National University of Singapore, Singapore

[20]ExoCoBio Exosome Institute (EEI), ExoCoBio Inc., Seoul, Korea

[21]Department of Molecular and Comparative Pathobiology,

[22]Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, USA

[23]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[24]Department of Surgery, YLL School of Medicine, National University of Singapore, 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)

***Co-corresponding authors**

Author email addresses:

mario.gimona@pmu.ac.at; mariafelice.brizzi@unito.it; andre_choo@bti.a-star.edu.sg; massimo.dominici@unimore.it; s.davidson@ucl.ac.uk; Johannes.Grillari@trauma.lbg.ac.at; dirk.hermann@uk-essen.de; Andrew.Hill@latrobe.edu.au; dkleijn@umcutrecht.nl; ruennchai.lai@imb.a-star.edu.sg; laicharles@sinica.edu.tw; rebecca.lim@monash.edu; mmonguio@igtp.cat; maurizio.muraca@aopd.veneto.it; tochiya@tokyo-med.ac.jp; lao1@pitt.edu; dentohws@nus.edu.sg; yongweon.yi@exocobio.com; kwitwer1@jhmi.edu; Bernd.Giebel@uk-essen.de; lim_sai_kiang@imcb.a-star.edu.sg

Abstract

Mesenchymal stromal/stem cells (MSCs) have been widely tested against many diseases, with about 1000 registered clinical trials. Despite many setbacks, MSCs have been approved for the treatment of Graft-versus-Host Disease (GvHD) and Crohn's disease. However, it is increasingly clear that MSCs exert their therapeutic functions in a paracrine manner through the secretion of small extracellular vesicles (sEVs) of 50 to 200 nm in diameter. Unlike living cells that could persist long-term, sEVs are non-living, non-replicative and have a transient presence in the body. Their small size also renders sEV preparations highly amenable to sterilization by filtration. Together, acellular MSC-sEV preparations are potentially safer and easier to translate into the clinic than cellular MSC products. Nevertheless, there are inherent challenges in the development of MSC-sEV drug products.

MSC-sEVs are products of living cells and living cells are sensitive to changes in the external microenvironment. Consequently, quality control metrics to measure key identity and potency features of MSC-sEV preparations have to be specified during development of MSC-sEV therapeutics.

We have previously described quantifiable assays to define the identity of MSC-sEVs. Here, we discuss requirements for prospective potency assays to predict the therapeutic effectiveness of the drug substance in accordance with the *International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use* (ICH) guidelines. Although potency assays should ideally reflect the mechanism of action (MoA), this is challenging because the MoA for the reported efficacy of MSC-sEV preparations against multiple diseases of diverse underlying pathology is likely to be complex and different for each disease, and also difficult to be fully elucidated. Nevertheless, robust potency assays could be developed by identifying the EV attribute most relevant to the intended biological activity in EV-mediated therapy and quantifying the EV attribute. Specifically, we highlight the challenges and mitigation measures to enhance manufacture of consistent and reproducibly potent sEV preparations, to identify and select the appropriate EV attribute for potency assays despite a complex "work-in-progress" MoA, and to develop assays likely to be compliant with regulatory guidance for assay validation.

Abbreviations

EMA: European Medicines Agency

ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH)

ISBT - International Society of Blood Transfusion

ISCT - International Society for Cell and Gene Therapy

ISEV - International Society for Extracellular Vesicles

MISEV - Minimal Information for Studies of EVs

MoA - Mechanism of action

MSCs - mesenchymal stromal/stem cells

NIH – National Institute of Health

sEVs - small extracellular vesicles

SoA - Site of action

SOCRATES - Society for Clinical Research and Translation of Extracellular Vesicles Singapore

US FDA: U.S. Food and Drug Administration

Introduction

Among cell-based therapies that include stem and immune cells, mesenchymal stromal/stem cells (MSCs) are presently the most clinically investigated non-hematopoietic cell types with more than 1,000 registered clinical trials worldwide (https://clinicaltrials.gov/). MSCs are reportedly efficacious against a wide spectrum of diseases in animal models and have an established record of safety in human patients. However, in contrast to the large number of clinical trials, very few MSC products have successfully completed rigorous clinical testing and received market approval [1].

Transplanted MSCs were initially postulated to exert their therapeutic effects by migrating to injured or diseased tissues where they engraft and differentiate to form new replacement tissues, or by cell-cell interactions to modulate cellular and immune responses. However, this view has been challenged over the years by observations that despite functional improvements, MSCs or differentiated MSCs were not detected in biologically significant numbers in affected tissues. As MSCs were observed to secrete many bioactive molecular species [2], Caplan and Dennis postulated in 2006 that MSCs mediate their therapeutic activity via secretion rather than by direct cellular interactions [3]. Until 2007, the prevailing view was that the bioactive agents in MSC secretion were small soluble molecules such as the cytokines and chemokines.

In 2007, however, size fractionation of MSC conditioned media revealed, in the example of a myocardial infarction model, that the ingredients of the cardioprotective fraction had molecular weights (MW) larger than 1,000 kDa [4]. In a follow up study, this fraction was found to be highly enriched for small extracellular vesicles (sEVs) with diameters between 110 and 130 nm, which at that time were termed exosomes [5]. In parallel, MSC conditioned media were shown to improve acute kidney injury in a mouse model and the activity was recovered in a fraction highly enriched for EVs (80 nm to 1 μm), originally termed microvesicles [6]. In head-to-head comparisons in different pre-clinical models, EV-enriched preparations were shown to be as therapeutically effective as their parental cells [6-8]. Today, it is widely accepted that the therapeutic effects of MSCs are mediated significantly by sEVs with diameters between 50 and 200 nm [9]. Consequently, there is a growing interest in MSC-EVs as exemplified by the exponentially increasing yearly number of PubMed publications on MSC-EVs (Figure 1).

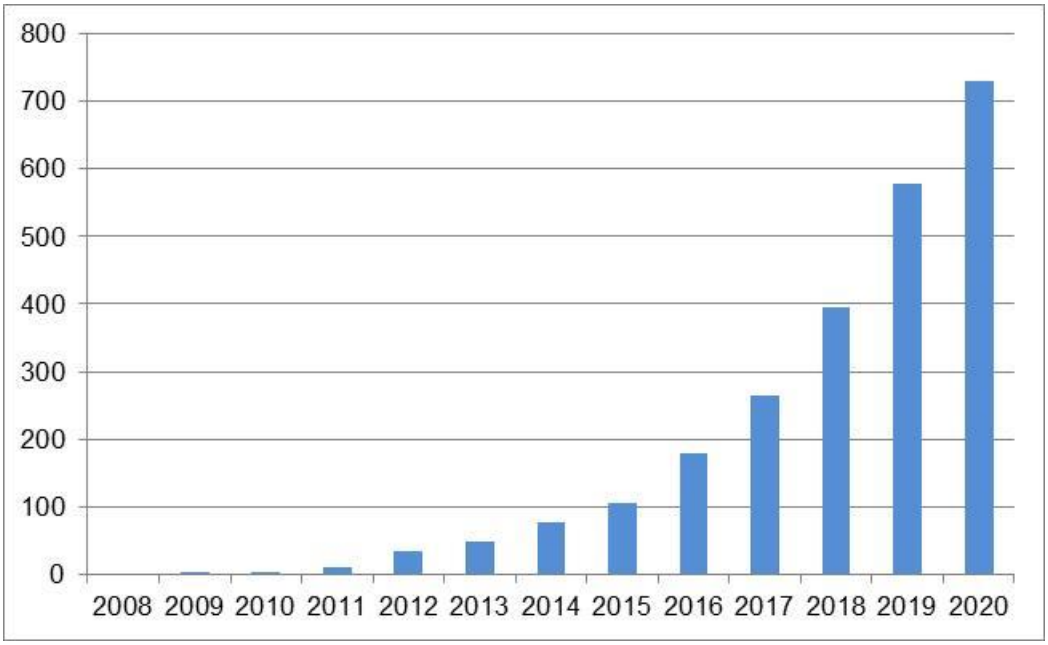

Figure 1: *Number of PubMed entries applying the search phrase* - ("mesenchymal stem cells" and "exosomes") or ("mesenchymal stem cells" and "microvesicles") or ("mesenchymal stem cells" and "extracellular vesicles") or ("mesenchymal stromal cells" and "exosomes") or ("mesenchymal stromal cells" and "microvesicles") or ("mesenchymal stromal cells" and "extracellular vesicles"), *search performed on December 9th 2020.*

The promising therapeutic potential of MSC-sEV preparations is complemented by an apparent lack of adverse side effects. This feature has been widely reported in various preclinical animal models and more recently in humans, including a human GvHD patient [10], a cochlea implant patient [11] and in non-registered clinical application in 20 chronic kidney disease patients [12] and severe COVID19 [13].

To successfully translate MSC-sEV products into clinical applications, it is essential that the bioactivity of MSC-sEV products are reproducible and meet pre-defined quantitative criteria of identity and potency before the products are released for clinical testing or use.

Notably, as we have discussed previously in detail, the identity and potency of MSC-sEV products depend on the MSC source and on the MSC culture condition [14]. The choice of basal medium and additives, including serum supplements such as human platelet lysate and proinflammatory cytokines for MSC licensing, the cell culture vessels including 2D vs. 3D MSC culture types, and the environmental conditions, such as the oxygen concentration will affect the

product characteristics. EV enrichment technologies can also affect product characteristics [14]. These different manufacturing parameters may enhance the therapeutic efficacy of the MSC-sEVs for some and diminish it for other diseases. Therefore, the desired MSC-sEV product for an intended disease target may require different manufacturing parameters, i.e. different manufacturing processes. Different manufacturing processes will generate different MSC-sEV products with qualitative and quantitative differences in identity and potency. Thus, it is imperative to optimize the manufacturing of MSC-sEV products with the desired characteristics in treating its intended disease target prior to the development of identity and potency criteria for their release as drug products for clinical trials or therapies.

We had previously discussed and proposed several metrics to quantify the identity of MSC-sEV products (see Box 1) [14]. Here, we address the issue of potency testing, where potency is defined by the US FDA in its 2011 Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result" (https://www.fda.gov/media/79856/download). Some of the intrinsic features in MSC-sEV manufacture and enrichment that could cause batch variations in the potency of the product and possible mitigation controls to minimize such effects will be discussed. We will also discuss the current state of the art in elucidating the potential MoA of MSC-sEV preparations, the identification of MSC-sEV attributes for potency assay development, and alignment of assay development to relevant regulatory guidelines.

Mitigating challenges posed by the intrinsic features in the manufacture of MSC-sEV preparations

As a cellular product, the cell source poses the first major challenge to reproducibly manufacture MSC-sEV products. MSCs can be isolated from many different tissues using a wide range of protocols, and while they share many key characteristics, MSCs from different tissues have been observed to differ in their functional properties [15-18]. As the agent of MSC therapeutic functions, MSC-sEVs would presumably be similarly affected. The biological properties of MSCs and their released components can also vary with different donors [10, 18].

Therefore, a long-term strategic plan to mitigate the impact of tissue sources and donors on the reproducibility of therapeutic potency in MSC-sEV preparations will be critical to the clinical translation of MSC-sEV products. The extensive clinical trials of MSC-based cell therapy led many to an apparent and intuitively logical conclusion: MSCs that were efficacious in clinical trials would produce therapeutically potent MSC-sEVs. However, the premise to support this intuition is weak. It is well documented that the composition of EV preparations is highly dependent on the cell culture system and EV enrichment processes [19]. Therefore, the composition of sEVs released from transplanted MSCs *in vivo* is likely to be different from that of sEVs released from the same cells *in vitro*. Hence, MSCs that are therapeutically active in transplantation studies may not necessarily generate therapeutically potent sEV preparation *in vitro*. Conversely, MSCs that were not beneficial in transplantation studies may still generate potent sEV preparations *in vitro*.

When appropriate cell sources of therapeutically active sEVs are identified, we must next address the issue of maintaining consistency of the cell source for all subsequent batches of sEV preparations. One approach is to adopt the standard industry practice of establishing master and working cell banks such as banks of cell pools from the same tissue source of different donors or of different tissue sources from the same donor. A combination of both might ensure that identical populations of producer cells are available for production of multiple sEV batches, mitigating the challenges of multiple tissue sources or donors. Such banks will help to normalize donor- or tissue-specific differences in sEV qualities. However, such banks are finite, and replication of these banks to re-create the composition of cell pools may not be possible or might alter the potency of sEV products. A more practical alternative to banks of pooled primary cells is to use immortalized monoclonal cell lines. This is done widely in the biologics industry, e.g. for the production of monoclonal antibodies. However, effects of immortalization will have to be carefully investigated to ensure that the resulting cells are stable and continue to produce sEV products that are bioequivalent to those from non-immortalized parental cells. Notably, proof of concept for this approach is furnished by studies showing that therapeutic function of sEV preparations was not compromised when MSCs derived from human embryonic stem cells [20] and umbilical cord derived MSCs [21] were immortalized by over-expression of c-myc.

Beyond cell-intrinsic factors, cell culture conditions and sEV enrichment technologies present many additional parameters that could potentially affect the quality and batch reproducibility of

the final MSC-sEV product. However, such parameters are not unique to the manufacture of MSC-sEV products, and are present in any manufacturing process of biologics and cell therapeutics. By appropriate process controls such as the *Good Manufacturing Practices* (GMP) for Biologics and Cell Therapy industries, many of the parameters can be effectively controlled. In terms of separation, MSC-sEVs cannot be homogeneously purified; rather, they are partially separated or enriched, resulting in the presence of certain co-isolates in the final preparation [22]. As such, MSC-sEV products are preparations that are highly enriched for nanoparticles within a given size range, inevitably containing non-EV-associated molecules. These components may even contribute to therapeutic effects [14, 23]. Thus, as discussed previously, it may be more accurate to use the term "preparation" for MSC-EV products to reflect the current state of the art in MSC-sEV production [14]. In addition to the presence of non-EV molecules, sEV preparations that are fractionated by size-based techniques are also not homogeneous collections of sEVs, but may contain EV subtypes such as endosome-origin exosomes, plasma membrane-derived microvesicles/ectosomes, apoptotic vesicles and others with diameters in the 50-200 nm range. Indeed, MSC-sEV preparations have been shown to contain more than one sEV type that differ in their membrane lipids and cargo of proteins and RNAs [24]. In summary, the quality of an MSC-sEV preparation is dependent on the source of the MSCs, the culture conditions of MSCs and the sEV enrichment protocols. Therefore, MSC-sEV preparations manufactured using different protocols may have different disease-relevant potencies.

Box 1: Summary of Identity Metrics for MSC-sEV preparations [14]

The term “MSC” and “sEVs” were aligned to the definition of “MSC” and “sEVs” in the minimal defining criteria of human MSCs, as established by the *Mesenchymal and Tissue Stem Cell Committee* of the *International Society of Gene and Cell Therap*y (ISCT) and the *Minimal Information for Studies of EVs* (MISEV2014 and MISEV2018) published by the *International Society for Extracellular Vesicles* (ISEV) [25, 26], respectively. For the purpose of developing MSC-EV identity metrics, MSCs were defined as cells that are “plastic-adherent when maintained in standard culture conditions, express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR cell surface molecules, and must have at least one lineage differentiation potential to form osteoblasts, adipocytes or chondroblasts *in vitro*.” MSC-sEVs refer to vesicles of 50-200 nm in diameter that are

surrounded by a lipid membrane bilayer and that are released by MSCs; the term is independent of the site/s of their subcellular origin (e.g. their endosomal or plasma membrane origin).

We previously proposed that the quantifiable metrics to define the identity of MSC-sEV preparations should reflect 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 [14]. The combination of these metrics could quantify the identity of MSC-sEVs and facilitate stratification and comparison of different MSC-sEV preparations.

The metrics are:

1. the relative ratio of MSC to non-MSC surface antigens to measure the relative concentration of MSC-sEVs (CD73, CD90 and CD105) vs. non-MSC-sEVs (CD14, CD34 and CD11b) in the sEV preparation. The rationale for the surface antigens is consistent with the ISCT minimal criteria for MSCs. Their prevalence in MSC-EV preparations has been confirmed by published MSC-EV proteomic datasets [27].
2. the number of 50-200 nm particles per unit weight protein/membrane lipids and the ratio of protein to membrane lipids e.g. cholesterol, phosphatidylcholine to establish that the preparation is an sEV preparation, and not a mixture of lipids, proteins and nucleic acids.
3. the presence of biochemically active proteins or integrity of RNA complexes to assess the biological competence of the sEV preparation e.g. the enzymatic activity of CD73, one of the characteristic MSC cell surface antigens.

Definition of potency for a drug substance

As discussed in the introduction, drugs have to meet pre-defined quantitative criteria of potency before being released for clinical testing or use, and potency could potentially be established by appropriate laboratory tests or adequately controlled clinical studies. Practically, potency must be determined before release using appropriate laboratory tests that measure potency or potency assays. According to ICH guideline for the Specifications: Test procedures and acceptance criteria for biotechnological/biological products (https://database.ich.org/sites/default/files/Q6B Guideline.pdf), a potency assay or bioassay is a suitably quantitative biological assay that measures the biological activity of the product attribute linked to the relevant biological

properties. In this context, “biological activity” is defined as “the specific ability or capacity of a product to achieve a defined biological effect”. The US FDA further recommends that potency assays should ideally represent the product’s MoA (www.fda.gov/media/79856/download).

Challenges in elucidating the mechanism of action for MSC-sEV preparations

Based on pre-clinical models, MSC-sEV preparations are reportedly efficacious against more than 30 different diseases. Many of them are characterized by complex pathologies such as myocardial reperfusion injury, ischemic stroke, acute kidney injury and acute GvHD. The disease pathology is orchestrated by a combination of different underlying pathological processes that can include hypoxia-ischemia, oxidative stress, apoptosis and inflammation. Alleviating the pathology of such diseases may require interference at more than one pathological process. MSC-sEV preparations are ideal therapeutics for these complex diseases, as their diverse cargo have the capacity to mount a multi-faceted MoA to elicit biological effects against different pathological processes.

Elucidating the MoAs of MSC-sEV preparations against a disease presents several unique challenges. In elucidating a multi-faceted MoA where multiple pathological processes are modulated, only processes mediated directly by MSC-sEV preparations should be mapped to attributes in the sEV preparations. The manifestation of these processes should minimally be aligned with the spatiotemporal biodistribution of MSC-sEVs and their components at the tissue, cellular, subcellular and molecular levels. A cause-and-effect relation between biological activity (as induced by the attribute) and disease outcomes should also be demonstrated.

Challenges in establishing a spatiotemporal site of action for MSC-sEVs

The key challenge in identifying the spatiotemporal site of action (SoA) of MSC-sEVs is the lack of sensitive and specific labels to track their spatiotemporal biodistribution. MSC-sEVs must be physically present in the right place and at the right time to exert their effects on target cells. There is a prevailing assumption that the primary target of sEVs is the injured/diseased tissue/cell. While intuitively logical, MSC-sEV preparations have also been demonstrated to act indirectly by modulating immune cells, attenuating inflammatory states and thereby alleviating inflammation in injured cells/tissues [7, 28]. As such, the therapeutic targets of MSC-sEVs may involve cells or tissues beyond the actual site of injury or functional decay. Experiments intended to identify EV target cells frequently use lipophilic dyes to label EVs fluorescently, and

introduce labeled EV preparations into animal models. Cells that take up the label are assumed to be the target cells of applied EVs. However, this interpretation may be incorrect or misleading. First, intracellular uptake of MSC-sEVs by target cells may be dispensable for sEV-mediated activity. sEVs are rich in membrane proteins and display various other molecules, allowing interactions with the cell membrane, its receptors, and extracellular matrix. Internalization and fusion may not be required for these interactions. Second, observation of dye distributions may not equate with EV distribution. One of the challenges in the EV field is to identify methods that specifically label EVs and not other components in the EV preparation. Most of the technologies currently used for EV labelling are adopted from those used for the labelling of cells and other biologics such as antibodies. For example, lipophilic dyes that are widely used for the labelling of cells for biodistribution studies are less useful in EV studies as these dyes can form sEV-sized micelles in aqueous solution that cannot be separated from EVs. They also bind avidly to lipoproteins and soluble proteins, which are often present in EV preparations [29]. The presence of free-dye micelles, and dye-labeled lipoproteins and proteins could lead to extensive non-EV-associated labeling of cells in various tissues *in vivo* and of cells *in vitro* [30]. Although the use of cell permeant pro-dyes such as calcein and carboxyfluorescein succinimidyl ester (CFSE) that could be cleaved to form impermeant fluorescent molecules [31] would, in principle circumvent non-specific labelling, such labelling will be limited to those EVs that have the relevant enzymes.

To reduce potential labelling artifacts, genetic labelling technologies have been applied. For example, cells engineered to express green fluorescent protein (GFP) fused to an exosomal protein such as CD63 have been shown to release labelled EVs that can be taken up by their target cells [32]. MISEV2018 has cautioned, though, that such genetic engineering may alter the biology of the EV-producing cells and the functionality of respective EVs and therefore should be appropriately controlled [19]. Furthermore, labelling may be biased if some subsets of sEVs are labeled while others remain unlabeled. Radioactive labelling may be less biased towards a specific EV type but would require a specialized handling facility and equipment [33]. In summary, there are many strategies to label and track EVs, with each having its own strengths and weaknesses [34]. It may be necessary to use complementary strategies to understand the complete spatiotemporal distribution of administered EVs.

Challenges in establishing a cause-and-effect link between attribute biological activity and disease resolution

A general approach to the elucidation of the MoA by MSC-sEV preparations in eliciting a therapeutic effect is to analyze the RNA or protein cargo for candidates that might mediate the therapeutic effect. To validate the candidate in eliciting the therapeutic effect, a common strategy is to demonstrate a direct cause-and-effect of a candidate attribute and the therapeutic effect by "knock-out" or "knock-down" experiments. In such experiments, the candidate in the EV is eliminated or reduced by "knock-out" or "knock-down" of the gene encoding for the candidate in the EV-producing cells. A direct cause and effect is frequently considered to be validated if the efficacy of the modified EVs is attenuated or lost. However, MISEV2018 specifically cautioned that such a "*conclusion may or may not be valid*" because the manipulation of the EV donor cells may lead to major alterations resulting in changes in the quantity and quality of their released EVs [19].

Fully characterized MoA

In summary, identifying the spatiotemporal SoA of MSC-sEVs and establishing the cause and effect of a candidate EV attribute in mediating an EV effect remain challenging and may require substantial technological development and advancement in the field. Consequently, it is unrealistic to expect that the MoA of MSC-sEV products can be fully characterized prior to clinical testing.

Strategies in identifying MSC-sEV attributes for potency assays

The MoA of many biologics and cell products is complex and for the most part not fully characterised. For such products, US FDA has recommended the use of an array matrix consisting of several potency assays, as one assay alone will not sufficiently capture the complexity of the MoA (https://www.fda.gov/media/79856/download). An array matrix is appropriate for MSC-sEV preparations, as their multi-faceted MoA involves modulation of several biological, physiological and pathological processes.

Before a potency test can be developed, it is necessary to first determine the pathological processes that are modulated by the MSC-sEV preparation in a pre-clinical animal model and

then map each modulation to a biological activity that could be elicited by an attribute in the MSC-sEV preparation. We illustrate this strategy by the two examples below.

In the first report of therapeutic MSC-EVs, Bruno et al. reported that MSC EV preparation of 80 nm to 1 µm protect against glycerol-induced acute kidney injury [6]. One of the associated pathological processes was tissue injury that leads to cell death, and the biological activities induced by this MSC EV preparation were enhanced cell proliferation and apoptosis resistance. The attributes in this preparation that elicited these activities were identified as the mRNAs of *CCNB1*, *CDK8* and *CDC6*; protein growth factors such as the insulin growth factor (IGF)-1 and hepatocyte growth factor (HGF); and miRNAs such as miR-486-5p, miR-126 and miR-34 [35]. Therefore, the candidate potency assays to predict the potency of this MSC-EV preparation against acute kidney injury could be quantitative RT-PCR assays to measure the mRNAs levels of *CCNB1*, *CDK8*, and *CDC6* or of miRNAs such as miR-486-5p, miR-126 and miR-34. In addition, antibody-based assays such as enzyme-linked immunosorbent assay (ELISA) to measure growth factors and proteins in the IGF-1 and HGF signaling pathways appear appropriate.

In the second report, Lai et al. 2010 published that MSC-sEV preparation of 110-130 nm reduce myocardial ischemia/reperfusion injury [5] by alleviating reperfusion-associated pathological processes such as depleted ATP levels, increased oxidative stress and apoptosis by inducing biological activities such as increasing ATP synthesis, reducing anti-oxidative stress through the degradation of denatured proteins and activating survival signalling via AKT activity. These biological activities could be mapped to ATP-generating enzymes such as pyruvate kinase, 20S proteasome and CD73/ecto-5'-nucleotidase present in MSC-sEV preparations, respectively [36-38]. Therefore, probable candidate potency assays to predict the potency of this MSC-sEV preparation against myocardial ischemia/reperfusion injury could be enzymatic assays for pyruvate kinase, 20S proteasome and CD73.

As illustrated by the two examples, the potency assays for MSC-sEV preparations are likely to vary for different diseases and may also be different for similar pathological processes, e.g. apoptosis. This could be due to differences in the manufacturing scheme of MSC-sEV preparations resulting in enrichment of different components, as discussed above, functional redundancies among the EV cargo, where more than one attribute exerts similar biological

activity, or multiple effects, where one attribute exerts more than one biological activity. Therefore, each MSC-sEV preparation that improves disease in an animal model should have demonstrable effects on one or more pathological process/es relating to the disease. If the biological activities modulating these processes can be mapped to attributes in the MSC-sEV preparation, then quantification of each attribute could be developed into a potency assay to determine the potency of that MSC-sEV preparation for that specific disease.

Generic technical considerations in developing potency assays

Potency assays could be animal-, organoid, tissue or cell-based biological assays or biochemical assays. In animal-based biological assays, the response of the organism to the product is measured. An example is the Lethal Dose 50 (LD50) test. Of note in view of the efforts towards replacement, reduction, and refinement - the 3R principle - animal testing as potency assay should be used as a measure of last resort only, if no other bioassay is available [39]. Cell-based assays essentially measure the cellular responses to a product, e.g., proliferation (for example T cell proliferation evaluated by a mixed lymphocyte reaction assay); cell death, cytokine release, signal transduction, gene transcription or migration (for example using transmigration assays). Biochemical assays measure biochemical activity such as enzyme activities or ion flux.

Independent of the type of assay, potency assays for MSC-sEV preparations should be qualified and validated for their quantitativeness, sensitivity, accuracy, precision and robustness. Clearly, not all assays will perform well in all of these parameters. Animal-based assays are less robust quantitatively but reflect complex biology better. Tissue or cell-based assays using animal or human cell/tissue materials help to reduce the usage of animal models, however, due to donor variabilities they may not help to increase quantitative robustness. Quantitative robustness can be increased upon using cell line cells within the functional assay. Notably, neither primary tissues/cells nor cell line cells will reflect the complex biology as well as animal-based assays but will reflect a much broader spectrum of biological functions than biochemical assays. Biochemical tests such as enzyme assays can be quantitative but measure a very narrow spectrum of biological functions. As described above, these disadvantages can be readily mitigated by the use of multiple assays (array matrix).

As potency assays are one of the gatekeepers for the release of drug products, these assays will have to be stringently validated where possible. According to the ICH guidance for validation of

analytical procedures (https://database.ich.org/sites/default/files/Q2_R1__Guideline.pdf), potency assays should be validated on the following key parameters: **analytical procedure,** which describes the steps to perform the assay in its entirety; **specificity,** to assess unequivocally the analyte in the presence of other components that may be expected to be present; **accuracy,** or ability to measure close to the true or reference value; and **precision,** where measures from multiple sampling of the same sample under the prescribed conditions are in close agreement**.** Other parameters include the **detection limit, quantitation limit, linearity range and robustness.**

Considerations in designing potency assays for a MSC-sEV preparation

MSC-sEV preparations, being biological preparations, impose some constraints on the design of potency assays such as:

Time Unlike small molecules, complex biological products such as MSC-sEV preparations may have much shorter shelf-life that can impose a time constraint on the testing for product release. Assays, e.g. animal testing, that require a lengthy period of testing (up to months) may significantly consume the shelf-life of the product and thus reduce the commercial viability upon release.

Availability of reagents Production of MSC-sEV preparations is currently expensive and of limited scalability. As such, the amount of material needed for an assay should be considered early in assay choice and design so that minimal material is consumed during testing. For assays requiring cells or other biological reagents such as antibodies, the availability or supply of these assay reagents with lot-to-lot consistency is critical and will be a decisive element in the choice and design of the assay. For cell-based assays, this limitation could be mitigated by using immortalized cell lines and establishing standard operating procedures for preparing, culturing and passaging cells, and by banking validated cells in a standard two-tier banking system of Master Cell Bank (MCB) and Working Cell Bank (WCB). The risk posed by the uncertainty of consistent availability or supply of biological reagents such as antibodies and recombinant proteins could also be mitigated by documenting cell source, e.g. hybridoma clone ID, the immunogen/plasmid used and the separation process so that alternative supply sources could be developed if necessary.

Reference materials A key aspect of a potency assay is the measurement readout, which "*should be expressed in units of activity calibrated against an international or national reference standard, when available and appropriate for the assay utilized.*" [39]. As the potency test for MSC-sEV preparations is likely to be an array matrix consisting of multiple assays, each individual assay will require its own reference standard. For established biochemical assays such as enzyme activity assays, validated international or national reference standards, usually in the form of the chemical substrates or products, are generally available. In the case of cell-based assays such as mixed lymphocyte reaction or proliferation assays, which are generally comparative assays, the ideal comparator will be a validated MSC-sEV preparation but this is currently not available. Thus, there should be parallel efforts to develop comparators such as reference banks of stable lyophilized MSC-sEV preparations, liposomes or EV mimetics.

Conclusions

MSC-sEVs are now widely accepted as the major mediator of the therapeutic efficacy of MSCs against a wide spectrum of diseases. As non-living cellular products, MSC-sEVs merge the therapeutic potency of relatively large, living MSCs with the safety of nano-sized, non-living vesicles. A major impediment to the translation of MSC-sEV preparations into therapeutic products is in defining potency metrics, i.e. the measure of the specific ability or capacity of a product to achieve a defined biological effect [39]. The challenge arises in part from the biological complexity of MSC-sEV preparations. As nano-sized lipid membrane vesicles with the capacity to carry relatively large cargo of hundreds to thousands of proteins, nucleic acids and other biomolecules, MSC-sEVs are intrinsically complex and can potentially influence diverse biological activities. This complexity is further complicated by variables associated with the broad variety of MSC sources, culture systems, and sEV enrichment processes.

The complexity of MSC-sEV preparations, combined with the wide spectrum of diseases against which they act, suggest that the therapeutic MoA will likely involve a disease-specific permutation of the diverse attributes present in MSC-sEV cargo. We anticipate that a full characterization of all aspects of the MoA cannot be expected before the start of clinical testing, and that an established potency assay may not be needed for phase I/II clinical trials. Investigators should nevertheless prepare for and envision an array of potency assays that will

correlate with the clinical outcomes. Despite the multiple challenges that we have highlighted, the development of good potency assays for MSC-sEV products is highly tractable through reference to guidance issued by ICH, EMEA and FDA (Figure 2). A fully characterized MoA would be ideal for the development of a potency test, but may not always be available for complex biological products such as MSC-EV preparations. However, this could be mitigated by the use of multiple assays (matrix array) to measure multiple MSC-sEV attributes that correlate with the multiple biological activities of a complex MoA in the intended use. As with all potency assays, MSC-sEV potency assays will have to be validated to demonstrate that the results are specific, accurate, precise and quantitative over reasonably large dynamic ranges.

In conclusion, we believe that based on the current state of the art, the development of rigorous potency assays for MSC-sEV preparations in compliance with guidance from major regulatory bodies is tractable with the challenging caveat that the specific components in the MSC-sEV preparation that modulate key pathological processes in the target disease can be determined (Figure 2). These assays will ensure that only MSC-sEV products of consistent potency are released for clinical testing or therapeutic use. This will promote rigorous, robust clinical testing of MSC-sEV-based drug products and accelerate clinical translation of medicinal MSC-sEV products.

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Disclosures:

MG: consulting and advisory role in MDimune

JG is co-founder and scientific advisor of Evercyte GmbH

YWY: co-founder, share-holder and employee of ExoCoBio Inc.

KWW: consulting role in MycoMed Technologies, Mosaic Ventures, Guidepoint, NeuroTrauma Sciences.

BG: scientific advisory board member of Evox Therapeutics and Innovex Therapeutics SL.

SKL: founder of Paracrine Therapeutics; scientific advisory role in Ilias Biologics and ExoCoBio Inc.

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