Title

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Journal

Stem cells (Dayton, Ohio), 38(1)

ISSN

1066-5099

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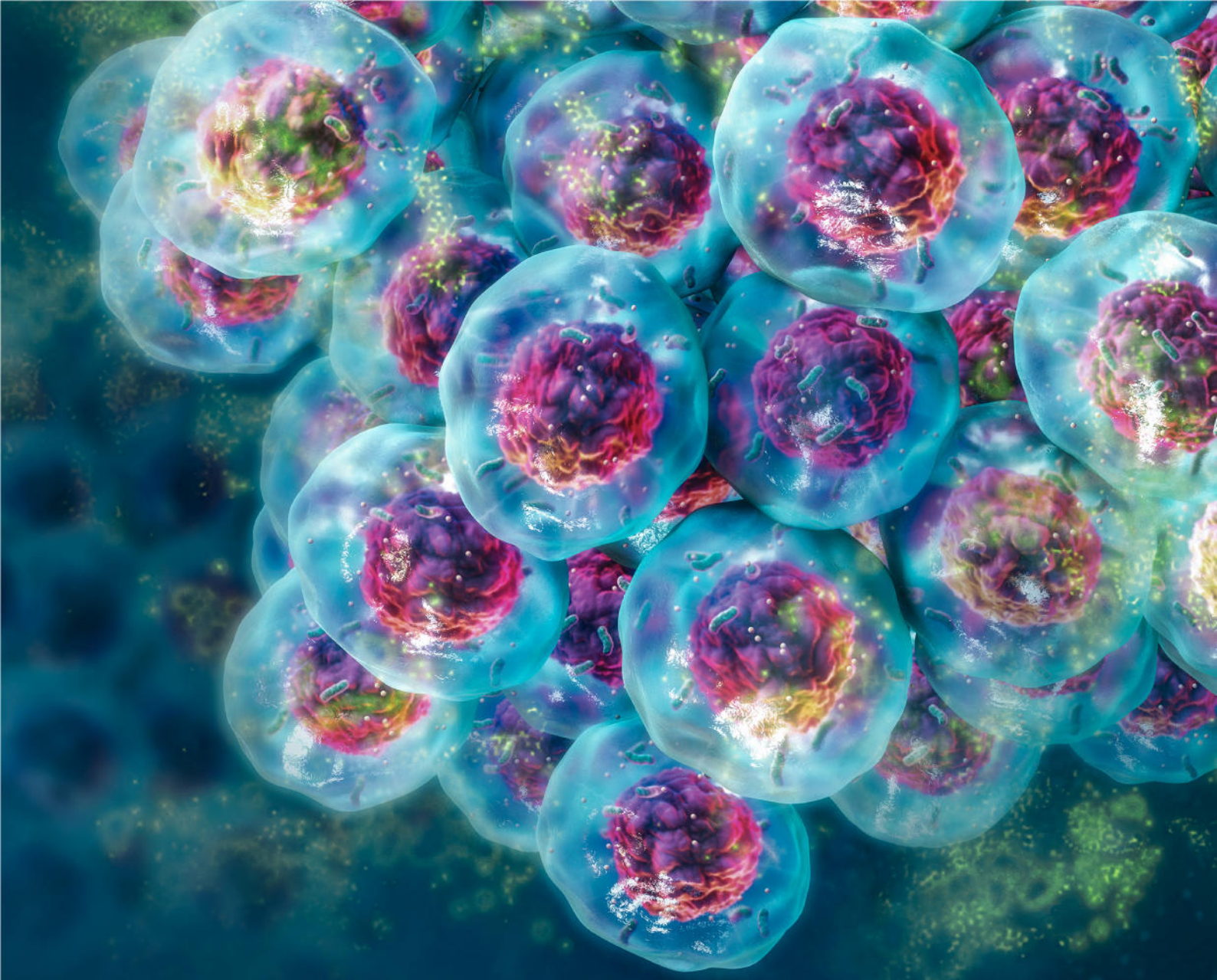
Publication Date

2020

DOI

10.1002/stem.3061

Peer reviewed



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Received: 21 March 2019 | Revised: 7 June 2019 | Accepted: 3 July 2019

DOI: 10.1002/stem.3061

CONCISE REVIEW

STEM CELLS

Preclinical translation of exosomes derived from mesenchymal stem/stromal cells

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Funding information
Swedish Research Council; National Institutes of Health, Grant/Award Numbers: KL2 TR001859, UL1 TR001860; National Center for Advancing Translational Sciences

Abstract

Exosomes are nanovesicles secreted by virtually all cells. Exosomes mediate the horizonal transfer of various macromolecules previously believed to be cell-autonomous in nature, including nonsecretory proteins, various classes of RNA, metabolites, and lipid membrane-associated factors. Exosomes derived from mesenchymal stem/stromal cells (MSCs) appear to be particularly beneficial for enhancing recovery in various models of disease. To date, there are over 200 preclinical studies of exosome-based therapies in a number of different animal models. Despite a growing number of studies reporting the therapeutic properties of MSC-derived exosomes, their underlying mechanism of action, pharmacokinetics, and scalable manufacturing remain largely outstanding questions. Here, we review the global trends associated with preclinical development of MSC-derived exosome-based therapies, including immunogenicity, source of exosomes, isolation methods, biodistribution, and disease categories tested to date. Although the in vivo data assessing the therapeutic properties of MSC-exosomes published to date are promising, several outstanding questions remain to be answered that warrant further preclinical investigation.

KEYWORDS
exosomes, extracellular vesicles, mesenchymal stem cells, mesenchymal stromal cells, microvesicles

1 | INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) have been the subject of clinical trials since they were first tested as a putative therapeutic in human subjects in 1995 by Lazarus.[1] MSCs have shown very encouraging results in preclinical studies investigating their therapeutic application in a wide array of disease models and benefit from a stellar record of safety to date.[2,3] Indeed, Mesoblast recently demonstrated efficacy in their primary outcomes in a recent phase III trial for pediatric graft versus host disease (NCT02336230). SanBio has reported promising results from a phase II trial of chronic stroke patients (NCT02448641). However, there have been more late-stage clinical trials that have fallen short of expectations than there have been successes to date.[4] There are likely several reasons for such differences observed between MSC preclinical and clinical studies such as potency, consistency and scale-up manufacturing issues which have been reviewed elsewhere.[4,5]

MSCs' therapeutic effects are generally thought to be mediated through the secretion of a variety of factors including canonical secretory proteins such as cytokines and growth factors, as well as exosomes[6-10] (Figure 1). MSCs act as a localized delivery system by secreting such factors, which then in turn affect the physiology of both adjacent and distant responder cells.[11-13] As MSCs are sensitive to their microenvironment, the profile of the therapeutic factors they secrete can be highly context dependent and may potentially vary from patient to patient.[14] Currently, there is much interest in the

potential application of the regenerative and immunomodulatory factors found in MSC conditioned media, especially exosome-enriched fractions, which are now understood to be key active pharmaceutical ingredients of MSC-based therapies.[15] Exosome-based approaches may hold some advantages over the administration of some adult stem cell-based therapies, including increased consistency, enhanced potency, and greater scalability of manufacturing.[15] However, much more work is needed to establish whether such potential advantages are reproducible for exosome-based therapies. As such, there is currently interest in investigating the therapeutic capacity and safety profile of exosomes derived from MSCs (MEX).[16] Published reports of MEX's therapeutic properties in various animal models have significantly increased in recent years, which we review here.[17] These recent studies may portend coming future exosome trials. Indeed, the first U.S. clinical trial investigating an exosome-based therapy (NCT03608631) was recently listed in the clinical trials database (clinicalTrials.gov) by Dr. Gauri Varadhachary at MD Anderson.

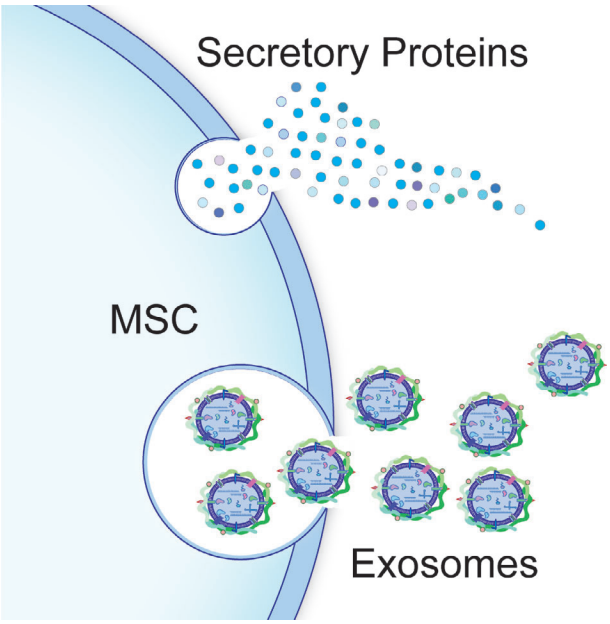

FIGURE 1 Mesenchymal stem/stromal cells secrete immunomodulatory and regenerative factors, including canonical secretory protein monomers as well as exosomes. The latter of which has been the subject of increasing preclinical investigation in recent years

Significance statement

Mesenchymal stem/stromal cells (MSCs) are under clinical development for the treatment of numerous disease indications. There is growing interest surrounding the therapeutic application of purified and concentrated regenerative factors secreted by MSCs, particularly exosome-enriched fractions (MEX), which are now understood to be key active pharmaceutical ingredients of MSC-based therapies. The present study summarizes the current state of preclinical development of MEX parsed from over 200 peer-reviewed reports utilizing various animal models. It also discusses opportunities that may be addressed which would help strategically advance the field of MEX-based therapeutic development.

2 | EXOSOMES

Exosomes are nanosized, cellularly secreted vesicles, which transport a variety of classes of proteins, RNA, metabolite, and lipid membrane components to neighboring and distal cell subpopulations (Figure 2).[18-20] Although the term "exosomes" is most commonly used to identify such vesicles, the terms "microvesicles" and "extracellular vesicles" (EVs) are also frequently reported.[21] The term "exosomes"

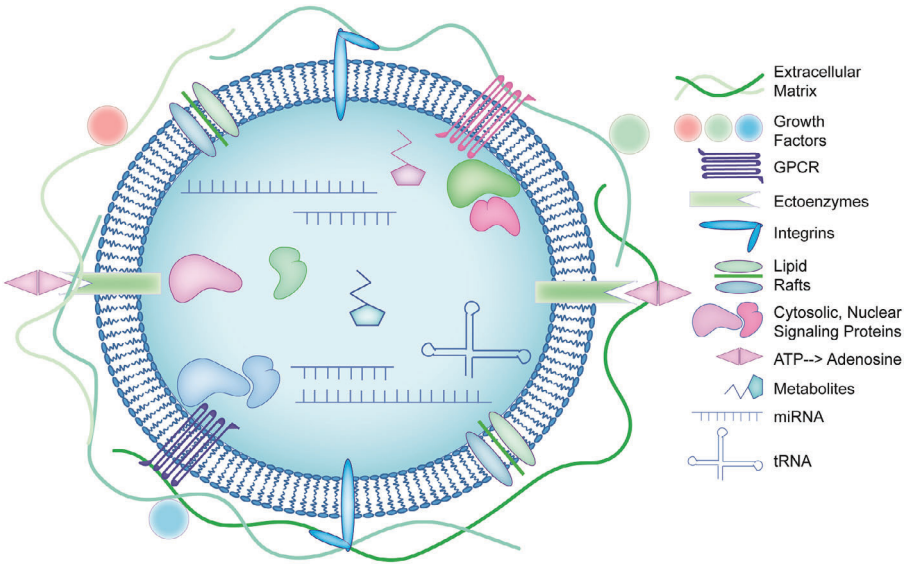

FIGURE 2 Mesenchymal stem cell-derived exosomes are packaged with a diverse profile of macromolecules, including extracellular, membrane-bound, cytosolic, and nuclear associated factors

applies only to the fraction of extracellular vesicles generated via the endosomal pathway.[22] Most reported MSC-derived exosome preparations contain species other than exosomes, such as vesicles derived from the budding of the plasma membrane budding (ectosomes, microvesicles) or through apoptotic cellular disintegration. Due to the various isolations reported to date, it is feasible that some of these so-called exosomal fractions may actually represent a minority of the isolated EVs. As there is currently no accepted manner in which to prove beyond a shadow of doubt the origin of EVs isolated with current technologies, we shall use the term "exosome" here as a proxy for preparations comprised by extracellular vesicles of diverse origins.

Historically, such factors have been thought to be cell autonomous, but numerous studies over the past decade have established that exosomes mediate a highly evolutionarily conserved intercellular communication system.[23] Indeed, many of the proteins and mechanisms associated with exosome biogenesis are conserved down to Gram-negative bacteria.[24] The term "exosome" was coined in 1981, by Johnstone[25] and Stahl groups.[26] Exosome biogenesis has been shown to be associated with distinct intracellular complexes including, the endosomal sorting complex, tetraspanins, sphingolipid ceramide, and Rab proteins, which comprise the largest part of the Ras-like small GTPase.[27] The multiplicity of the pathways involved in exosome biogenesis may contribute to their inherent heterogeneity in any given population.[28]

Exosomes possess notable physiological properties and originate via the inward budding of endosome membranes, called multivesicular bodies (MVBs).[29] MVBs fuse with the plasma membrane and exosomes are released into the extracellular milieu, either to be taken up by target cells residing in the local microenvironment or carried to distal sites via biological fluids.[30] Exosome membranes are enriched in cholesterol, sphingomyelin, ceramide, and lipid raft components, in addition to their protein, RNA, and metabolite constituents.[31] Exosomes are packaged with an evolutionary conserved set of proteins including tetraspanins (CD9, CD63, CD81), heat-shock proteins (HSP60, HSP70, HSP90), numerous annexins, and programmed cell death 6-interacting protein.[31] However, exosomes are also packaged with specific proteins that are representative of their parental cell source and reflective of their microenvironmental niche.[32]

Mesenchymal stem cell-derived exosomes (MEX) are generally isolated and purified from media conditioned by MSCs. However, there is evidence that suggests that the therapeutic effects of MEX batches are derived from a cacophony of billions of vesicles with both overlapping factors and distinct factors encompassing their composition.[33] The reported pleiotropic therapeutic effects, therefore, are due to the complex interactions of a variety of factors packaged across MEX subpopulations.[34] Consequently, precisely controlled manufacturing of MEX is needed to ensure interbatch consistency of the resulting product.[34]

It has yet to be determined whether the protein, RNA, lipid, or metabolite contents packaged into MEX mediate their observed therapeutic effects. To date, several studies have focused on the miRNA content of exosomes as potentially key regulators of their functional properties.[35] However, recent studies have shed light on the relatively low abundance of exosomal miRNA, with at least one report indicating that MEX contain several orders of magnitude more total protein than total RNA.[35,36] In addition, MEX are highly enriched for extracellular proteins.[7] Therefore, an increased focus on the proteins packaged into MEX is warranted. However, the critical and essential factors that are packaged into MEX that mediate their immunomodulatory and tissue healing properties have yet to be robustly characterized. It is likely that the culture conditions under which MEX are manufactured greatly influence the proteins packaged into them, just as MSCs respond by modifying the growth factors and cytokines they secrete in response to various priming conditions.[37] However, to date few studies have robustly explored such lines of investigation, and it remains unclear as to whether proteins detected in most exosome preparations are contained within or attached to the outside of the vesicles.[38,39] Here, we review the common trends reported in over 200 peer-reviewed preclinical studies of MSC-derived exosomes/microvesicles/extracellular-vesicles listed in the PubMed database (Supporting Information Table S1).

3 | MEX IMMUNOGENICITY

Numerous studies have established the low immunogenicity of MEX administered as both a single bolus as well as with repeated doses. MEX have also been observed to have similar hypoimmunogenic

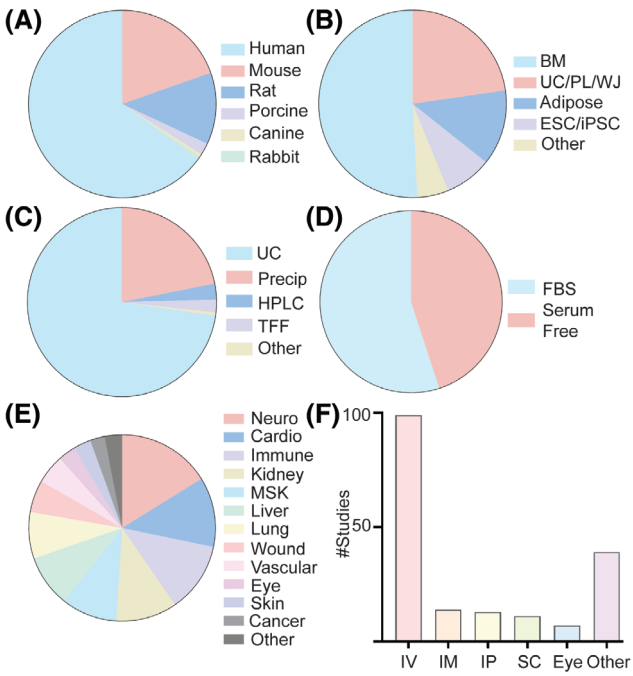

FIGURE 3 The diversity of, A, species, B, tissue source, C, isolation technique, D, culturing methods, E, disease indication, and F, route of administration represented from over 200 preclinical reports on exosomes derived from mesenchymal stem cells. Abbreviations: BM, bone marrow; ESC, embryonic stem cell; FBS, fetal bovine serum; HPLC, high pressure liquid chromatography; IM, intramuscular; IP, intraperitoneal; iPSC, induced pluripotent stem cell; IV, intravenous; MSK, musculoskeletal; PL, placenta; precip, precipitation; SC, subcutaneous; TFF, tangential flow filtration; UC, ultracentrifugation; UC, umbilical cord; WJ, Wharton's jelly

properties as well. Comprehensive proteomic analysis of MEX has not detected major histocompatibility complex (MHC) I or MHC II complex to date.[6] Indeed, 65% of the >200 in vivo studies published to date have administered MEX derived from human sources into a wide variety of animal models of disease, mostly in mice (Figure 3A). No explicit immunogenicity has been reported in any of these species crossing studies. Therefore, MEX may be considered hypoimmunogenic, in a similar fashion to their cells of origin, MSCs. Current evidence for the hypoimmunogenic nature of MEX includes at least one study that investigated repeated doses of MEX in mice, which did not observe any overt toxicity according to hematologic and blood chemistry analyses, as well as in-depth histopathological evaluation of several different tissues.[40] Similarly, toxicity has not been observed with repeated dosing of exosomes derived from other fibroblasts and Hek293 cells.[40,41] However, further studies are required to validate that the apparent hypoimmunogenicity properties of MEX are reproducible across different disease models and dosing regimens.

4 | MEX SOURCES AND PURIFICATION METHODS

The tissue source for parental MSCs for MEX studies have been isolated from a variety of tissues, including bone marrow (51%), umbilical/placental tissues (23%), and adipose tissue (13%), derived from embryonic or induced pluripotent stem cells (8%), or others (5%; Figure 3B). The most common exosome isolation method used to purify MEX from conditioned media to date has been ultracentrifugation (72%), followed by precipitation methods (23%; Figure 3C). Unfortunately, ultracentrifugation does not scale easily and also causes processing associated damage to MEX due to the extreme forces involved, thereby limiting the value of such an approach for preclinical studies.[42] Precipitation methods are even more problematic as it is widely accepted that such methods coisolate many contaminants, especially when used in conjunction with serum containing media, as most such studies report.[43] Such differences in the tissue source and exosome isolation techniques undoubtedly affect both the packaging and observed functional properties of the resulting MEX isolates. Consequently, substantial caution is warranted when interpreting and comparing such preclinical MEX studies and their reported outcomes. To increase the translatability of future MEX preclinical studies investigators should give strong consideration to these issues. For example, industry-appropriate manufacturing and isolation methods such as ultrafiltration and the use of serum-free isolation media would greatly enhance the value of preclinical studies assessing MEX therapeutic properties, mechanisms of action (MoA), and safety profile.

Approximately 55% of MEX studies used precleared fetal bovine serum (FBS)-containing media, while 45% of published reports used serum-free or chemically defined media (Figure 3D). Due to the potential for coisolation of residual FBS exosomes, as well as FBS-derived protein aggregates, it may be advantageous to use serum-free isolation media to diminish the possibility of introducing bovine-derived artifacts.[7] However, the optimum media constituents required to manufacture MEX with maximum potency has yet to be determined, and may vary according to the target disease.

5 | MEX BIODISTRIBUTION

The vast majority of preclinical animal studies of MEX's therapeutic effects have used systemic routes of administrations. Consequently, establishing the pharmacokinetics of EV systemic administration is required for their successful progression through preclinical development. To date, there is a dearth of studies that have investigated the biodistribution patterns and kinetics, especially within the context of relevant pathophysiology. Several studies have investigated the biodistribution patterns of fluorescently labeled MEX.[40,44-46] Based on these published reports, systemically administered MEX appear to be cleared within a few hours and generally ultimately accumulate within the liver and spleen. However, these studies have largely focused on biodistribution associated with healthy, wild-type animals, which does not take into account the distinct underlying pathophysiology associated with individual diseases. For example, several published reports have demonstrated that exosomes are capable of crossing the blood-brain barrier (BBB) when active neuroinflammation is present.[47-49]

Neuroinflammatory cascades often result in the compromised integrity of the BBB, thereby allowing for large macromolecules and even cells to enter from the periphery.[50,51] In addition, some methods of manufacturing and labeling MEX use extended processing times, which may decrease their resulting functional properties. This may be a key point if it is determined that MEX uptake by specific cellular populations is mediated by receptor mediated endocytosis, as some proteins are more labile than others. Special consideration should also be given to the methods chosen for labeling MEX for biodistribution studies.

To date only a few studies have investigated the biodistribution patterns and kinetics of systemically administered exosomes. These studies have often relied on lipid-incorporating fluorescent dyes together with in vivo optical imaging (eg, IVIS).[40,45,46,52] This approach is based on the assumption that lipid incorporating dyes remain embedded in EV membranes for the duration of the study. However, several studies have demonstrated that up to 75% of such dyes dissociated from vesicles, when incubated in plasma.[53,54] In addition, these commonly used dyes can spontaneously form EV-like particles.[54] Radiolabeling of exosomes presents an alternative imaging strategy; however, few published reports have investigated the biodistribution of postinserted radiolabeled EVs.[55,56] An alternative labeling method of engineering MEX with Cre-recombinase in Lox reporter mice has been reported, but it remains unclear whether such engineering methods modulate the functions of the resulting vesicles.[48,57,58] Taken together, the field could benefit from continued investigation of the MEX pharmacokinetics that take these factors into account. Finally, given that the labeling procedures themselves often require substantial manipulation of EVs, the

validation of the resulting labeled exosomes would be an insightful control. The continued investigation of MEX's therapeutic targets in vivo is likely critical to successful translation of this technology to the clinic.

6 | DISEASE CATEGORIES OF MEX PRECLINICAL STUDIES

The pleiotropic nature of both MSCs and MEX-based therapies allows for the feasibility of their assessment in a wide range of disease models. There are now over 200 published reports of MEX therapeutic properties in vivo, which span numerous disease categories. Neurological (16%), cardiovascular (12%), immunological (12%), and kidney (10%) diseases represent the four most investigated areas of disease, respectively (Figure 3E). A significant portion of studies used animal models of musculoskeletal (10%), liver (9%), and pulmonary (8%) diseases (Figure 3E). However, it would greatly benefit the MEX field to continue to establish their putative MoA.

The robust characterization of MEX's MoA would allow for shrewdly designed release criteria, relevant potency assays, and open future avenues of research investigating the biological underpinnings of responders versus nonresponders.[59,60] Such determinations are likely highly dependent of the tissue source of parental MSCs, donor-to-donor variation, manufacturing, and isolation methods used, as well as the specific pathophysiology involved in a particular disease.[4] However, there also lies the potential for there to be broad overlapping observations across these variables. For example, many of these disorders involve a substantial inflammatory component, which may be ameliorated by MEX-based therapy. MSCs and MEX have been reported to possess anti-inflammatory properties in both preclinical and clinical studies. Increasing the granularity of our understanding of the molecular underpinnings of these affects would increase the rationale for the preclinical investigation of MEX as a putative therapeutic platform technology.

There exists a substantial degree of heterogeneity in dosing regimens applied across these published reports the bridge both small and large animal models (route of administration, number of doses, quantity of dose, how dose is calculated, etc.; Supporting Information Table S1). Consequently, the optimization of dosing regimens (eg, repeated dosing, dose range finding, disease specific routes of administration, etc.) should be investigated in greater depth (Figure 3F).

7 | DISCUSSION

It is now well established that exosomes are biological agents central to intercellular communication and possess therapeutic potential. Although it is of interest to the field, there exists a paucity of studies that have attempted to directly compare MSC efficacy to that of exosomes purified from MSC-conditioned media. Indeed, the methods used for such studies comparison studies require some consideration, such as the requisite thawing of cryopreserved product immediately prior to administration, which is not common in preclinical studies, but a necessary supply chain aspect of cell-based therapies. In addition, some contemplation on what constitutes an equivalent dose is appropriate in light of the fact that methods for the purification of exosomes isolated from conditioned media are not 100% efficient. Ultimately, such comparisons may be best viewed in terms of the cost of goods for each prospective approach necessary to reach an equivalent clinical outcome.

Another confounding factor is the fact there are reports, which describe inconsistent results. Such inconsistencies are likely the result of the different culturing methods used prior to and during exosome harvests, as well as variances in the purification techniques used. Therefore, further process development of exosome-based therapies utilizing scalable production methods, and standardized operating procedures are needed to advance the field forward. Such methodologies likely affect the cargo and downstream functional properties of the resulting exosomes in significant ways. In addition, the development of appropriate release criteria and relevant, potency assays would benefit from robust follow-up studies elucidating putative, disease-specific MoA. Lessons learned from the MSC field about the critical need to develop robust potency assays and release criteria provide valuable insight to MEX researchers. There exists the potential that release criteria developed for exosome-based therapies may be more robust as they are not dynamic living medicines, but rather EVs packaged with a static payload of therapeutic factors. Taken together, the preclinical development of MEX-based therapies has advanced considerably in the last few years, as interest in this therapeutic platform technology continues to grow. It is feasible that the MEX field may use the considerable insights to be gained from both the clinical successes and barriers to commercialization experienced by MSC-based drug developers.

ACKNOWLEDGMENTS

This work was supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through grant number UL1 TR001860 and linked award KL2 TR001859. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. As well as, the UC Office of the President's Multi-campus Research Program Grant (MRP-17-454909), STAIR Grant, STAIR-Plus Grant, CTSC Rapid Translational Grant (UL1-TR001860), U2C ES030158, T32 Cardio NIH T32-HL086350, Denny & Jeanene Dickenson Fellowship, NIH Transformative R01GM099688, NSF GROW 201111600, NIH T32-GM008799, and NSF GRFP 2011116000, SELA is supported by the Swedish Research Council (VR-Med and EuroNanoMedII).

CONFLICT OF INTEREST

D.G.F. declared research funding from NIH RO1 with Intuitive surgical as a co-PI. J.D.A. declared leadership position, stock and intellectual property rights ownership in Somos Therapeutics, Inc.

AUTHOR CONTRIBUTIONS

F.M.E. and J.D.A.: conception and design, financial support, collection and/or assembly of data, interpretation, manuscript writing, final approval of manuscript; D.G.F. and J.A.N.: interpretation, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Elahi FM, Farwell DG, Nolta JA, Anderson JD. Preclinical translation of exosomes derived from mesenchymal stem/stromal cells. *Stem Cells*. 2019;1–7. https://doi.org/10.1002/stem.3061