The Unsettling Ambiguity of Therapeutic Extracellular Vesicles from Mesenchymal Stromal Cells

The regenerative and immunomodulatory properties of mesenchymal stromal cells (MSCs) have been long recognized, and their effectiveness in preclinical models of disease has led to numerous clinical trials investigating MSC transplantation as a therapy for diverse pathologies. To date, although safety has been demonstrated, outcomes represent a rather mixed bag, probably owing to the challenge of translating logistics and protocols involving live cells from benchtop to bedside. It is now accepted that the observed beneficial effects of MSCs in most (but not necessarily all) disease models are not due to engraftment and transdifferentiation, but instead occur through paracrine mechanisms (1). Studies such as the pioneer work of Bruno and colleagues in an acute kidney injury model (2) and the comprehensive work of Lai and colleagues in a myocardial ischemia/reperfusion model (3) led to the recognition that extracellular vesicles (EVs) are the main therapeutic vector in the MSC secretome. In the pulmonary field, Lee and colleagues established that MSC-EV administration is effective in treating hypoxia-induced pulmonary hypertension (PH) in the mouse (4), and this finding was subsequently paralleled in studies by Aliotta and colleagues using a monocrotaline-induced model of the disease (5).

As reported in this issue of the *Journal*, Klinger and colleagues (pp. 577–587) contribute significantly to the field by using MSC-EVs to treat a more robust and arguably more physiologically relevant model of PH, induced in the rat by the combined actions of hypoxia and Sugen 5416 (semaxanib) (6). Their study complements the report of Hogan and colleagues on the effectiveness of MSC-EV treatment in the rat Sugen/hypoxia PH model (7), and expands on it by demonstrating an impressive reversal of the pathology. In addition, the authors systematically investigate the effects of multiple dosing and timing protocols on the treatment's efficacy in preventing and reversing PH characteristics. One intriguing observation is the absence of a dose dependence of MSC-EVs' action, indicating a threshold dose effect that presumably initiates a cascade of events leading to the reversion of pathology. This characteristic, together with observations that a single bolus dose of MSC-EVs can prevent pulmonary pathology and confer long-lasting protection (8, 9), may provide clues to decipher the molecular mechanisms involved and the impact of treatment on the target host cell(s). Significantly, such information will be pertinent in designing protocols for clinical trials of MSC-EV–based therapies.

Although it is generally accepted that MSC-EVs can be effective in preventing and, in certain cases, even reversing pulmonary pathologies, we are plagued by many ambiguities in the field of MSC-EV–based therapies. We are confronted with vague definitions and an absence of widely acceptable standards, and we have no reliable potency assays to serve as proxies for *in vivo* MSC-EV efficacy. These ambiguities extend even to MSCs, originally designated as mesenchymal stem cells, which display significant intraclone heterogeneity and are indistinguishable from freshly isolated fibroblasts using the routine surface-marker criteria. Their designation has been downgraded to "mesenchymal stromal cells," and even the variants "mesenchymal signaling cells" and "medicinal signaling cells" have been suggested to highlight our lack of a robust definition (10). Ambiguities become paramount when we attempt to define MSC-EVs. The International Society for Extracellular Vesicles has suggested the minimal information required for studies on EVs (11), but unfortunately this has not been adhered to in all publications. Significantly, a dialogue was recently initiated to identify specific criteria for defining MSC-EVs for therapeutic applications (12). The focus is on the subpopulation of smaller vesicles, between 30 and 150 nm in diameter, that can be harvested from MSC-conditioned media after apoptotic body derivatives and diverse types of cell detritus have been cleared by low-speed centrifugation (typically 10–15k $\times g$). This fraction, commonly called small EVs (sEVs), includes the vesicles that are generated in multivesicular bodies through the endosomal pathway and are subsequently released as exosomes. Here, Klinger and colleagues used a cruder EV preparation, and this may be construed as a limitation of the study, especially considering that Hogan and colleagues obtained comparable results from the same model using the MSC-EV sEV fraction (7). Clearly, the better we define the MSC-EV preparation, the closer we will be to understanding the molecular mechanism of the effects we observe (13).

MSC-EVs can exert their therapeutic effects in the lung through immunomodulation, as evidenced in preclinical models with an underlying inflammatory insult, such as bronchopulmonary dysplasia, idiopathic fibrosis, and PH. The target cell type appears to be of the myeloid lineage, and indeed, adoptive transfer of macrophages or monocytes treated *in vitro* with MSC-EVs can protect the lung from injury (9, 14). Nevertheless, we face considerable ambiguity regarding the specific molecular mechanisms of action. The established pleiotropic efficacy of MSC-EVs in ameliorating widely diverse diseases, as well as the inherent heterogeneity of vesicular preparations, may indicate that the molecular mechanism of action is not the same in all contexts. As an example, although

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the transfer of intact mitochondria to macrophages by MSC-EVs has been reported (14, 15), it is difficult to visualize this as relevant to the mechanism of action of sEVs, given the obvious size limitations. Similarly, although there is ample evidence that MSC-EVs can transfer microRNA (miR) cargo to target cells, it has been argued that the expected stoichiometry of miR molecules to target cells required by that mechanism simply does not exist in certain *in vivo* models. Indeed, Toh and colleagues advanced a well-documented and carefully constructed thesis proposing that the therapeutic effects of MSC-EVs on the injured myocardium are independent of miR cargo and occur exclusively though vesicle-associated enzymatic activities that restore energy homeostasis (16).

There is also a question, one that *prima facie* appears teleological, whose answer could help define parameters for increased medicinal sEV yield and potency: Why do MSCs produce these highly efficacious sEVs? It is accepted that the main purpose of EVs (especially exosomes) is to enable the cell to jettison unwanted moieties, akin to a cellular garbage disposal apparatus. The high evolutionary conservation of the genes involved indicates that this apparatus was in place before the emergence of multicellular organisms. Later in evolution, parts of the apparatus were recruited or coopted into other functions, such as virus assembly, and arguably, in MSCs, the apparatus was recruited for the production of a specialized sEV, the "signaling EV." This is the "purposeful vector" hypothesis, which holds that MSCs respond to environmental cues by releasing sEVs, putatively to restore homeostasis in neighboring cell targets. Of course, MSCs, like any cell, would also keep using EVs for routine garbage disposal, and any signaling sEVs would represent a subset of the total MSC-EV population. This situation can also be interpreted according to a second hypothesis: MSCs jettison unwanted moieties packaged in EVs to optimize their survival under certain conditions, and, fortuitously, a subset of these packages exerts beneficial effects on certain other cell types. This is called the "flea market" hypothesis, as one cell's junk is another cell's treasure.

Despite the ambiguities, the promise of EV-based therapeutics has led to a growing number of clinical trials using autologous, allogeneic, or modified EVs from diverse sources that target diverse pathologies. This year represents an exciting milestone in the pulmonary field, with the launching of the first clinical trial of an MSC-EV–based pharmaceutical, a multicenter, dose escalation, safety, and tolerability study in premature infants at high risk for bronchopulmonary dysplasia (ClinicalTrials.gov Identifier: NCT03857841). Favorable outcomes in this trial and anticipated future studies will pave the way for the development of the next generation of MSC-EV–based pharmaceuticals to treat lung disease, permitting us to harvest the regenerative and homeostatic power of MSCs without the inherently complicated logistics and putative hazards associated with live cell transplantation. To achieve this goal faster, it is crucial to emphasize the importance of disambiguation. ■

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