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EDITORIAL

Counting Copies, Making Medicines: A Roadmap for the MSC-EV-microRNAome

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MSC-EVs have attracted significant interest as vehicles for delivering regulatory miRNAs that modulate inflammation and tissue repair (1). Vesicles are natural carriers, and miRNAs are potent post-transcriptional regulators, making their combination an appealing therapeutic platform. However, quantitative studies reveal a major constraint: most EVs carry <1 copy of any given miRNA, challenging the intuitive “one vesicle, one message” paradigm (2,3). This discrepancy compels the field to re-examine how EV-miRNAs act, whether through cumulative low-copy effects, rare high-load vesicles, or non-vesicular Argonaute-bound miRNA complexes (4,5). To advance from preclinical promise to approved medicines, investigators must adopt rigorous MISEV2023 reporting (6), employ absolute quantification, and link molecular data to functional assays. This editorial outlines the current landscape, critical translational hurdles, engineering and manufacturing considerations, and a roadmap for credible EV-miRNA therapeutics.

Current Landscape and Key Challenges

Standardization and Emerging Clinical Data

The field now has a shared methodological framework. MISEV2023 raises expectations for pre-analytics, separation, characterization (including negative markers), and functional study design with explicit controls and dose reporting (6). Despite this, adherence remains inconsistent, especially regarding isolation methods and potency assays (6,7). Clinical activity is emerging in contexts where delivery route and dose are biologically coherent. A randomized, single-blind, placebo-controlled phase I trial (ChiCTR2300075466) of nebulized hUCMSC-EVs in pulmonary fibrosis (n=24) demonstrated tolerability and improvements in lung function and patient-reported respiratory outcomes, with radiographic improvement in advanced cases (8,9). These results extend preclinical evidence for inhalation delivery.

Regulatory agencies are cautiously engaged. In December 2024, the FDA approved Ryoncil (remestemcel-L-rknd) for pediatric steroid-refractory aGVHD—the first MSC therapy licensed in the U.S.—but no EV products have yet been approved, and EV-specific guidance is lacking. Agencies apply general biologics frameworks while funding EV regulatory science (10).

Stoichiometry and Mechanistic Clarity

A pivotal quantitative study revealed that even abundant miRNAs average ≪1 copy per EV (~0.008 per vesicle), invalidating simple “one vesicle, one message” models at physiological doses (2). Independent reports confirm low occupancy and heterogeneous loading (11,12). Two explanatory models predominate: an ensemble model, in which many low-copy EVs cumulatively reprogram target cells, and a rarity model, in which a minority of high-load vesicles—or non-vesicular Argonaute-bound complexes, drive effects (4,5). Distinguishing

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between these requires absolute quantification and single-vesicle analyses before invoking mechanisms.

Potency and Dosing Standards

Particle and protein counts alone are inadequate potency surrogates. Indication-relevant bioassays such as NF-κB suppression in macrophages, M2 polarization, or T-cell proliferation should be tied to CQAs, including identity markers and mechanistic miRNA copy numbers, with analytical validation for linearity and precision (13). Dosing should progress toward a triad based on particle count, protein content, and mechanistic miRNA copies, justified by route-specific biodistribution and pharmacology. Regulators accept conventional units but prefer activity-anchored dosing when the active component is defined. Preclinical data demonstrate route- and size-dependent biodistribution: small EVs accumulate early in the liver and kidney, while larger EVs localize in the lung, with time-dependent shifts that inform dosing intervals and monitoring strategies (11).

Purity and Attribution

Contaminants such as Ago2-bound miRNAs and protein/RNP complexes complicate mechanistic claims. Over-attribution of effects to EVs can be avoided by strict adherence to MISEV guidelines: document pre-analytical variables; report recovery/depletion, include positive/negative markers; and apply RNase/protease ± detergent protection, density gradients, and Ago2 depletion. Absolute miRNA quantification combined with loss/gain-of-function studies is essential; otherwise, effects should be described as EV-associated, not EV-mediated (12).

Safety first, route matters

Route-specific risks are increasingly apparant. Large umbilical cord MSC-EVs have caused TF-dependent, dose-related lethal pulmonary embolism in mice after intravenous administration. EVs can accelerate coagulation via exposed phosphatidylserine and TF. Appropriate route selection (e.g., inhalation for pulmonary targets) and hemostasis panels (TF/CD142, thrombin generation) should be embedded in release testing and lot comparability assessments.

Engineering and Manufacturing Considerations

Preconditioning and Genetic Programming

The MSC-EV microRNAome can be intentionally modulated. Preconditioning with IL-1β enriches miR-146a in MSC-EVs, enhancing their ability to suppress NF-κB in macrophages and improve survival in murine sepsis (14). Genetic strategies include overexpressing mechanistic miRNAs (e.g., miR-424) in parental MSCs or altering sorting proteins such as YBX1 or hnRNPA2B1, which regulate miRNA loading (15–17). While these methods can generate functionally enhanced EVs, they also alter other cargos, complicating potency assays and safety evaluation.

Post-isolation loading

Electroporation remains the primary method for post-isolation miRNA loading, but it risks nucleic acid aggregation, inflated apparent loading, vesicle damage, and functional loss. Improved protocols exist, but rigorous validation is required to confirm that cargo is intravesicular, retained during storage, and functionally active (18).

Manufacturing Scale and Consistency

Tangential-flow filtration (TFF) and closed, serum-free culture systems now enable multi-liter MSC-EV production. However, variables such as cell source, medium, shear, and harvest timing affect yield and miRNA content. Manufacturing must therefore map CPPs to CQAs, use in-process controls (e.g., shear rate, transmembrane pressure, membrane cut-off, collection timing) and document recovery and depletion (19-21). Standardization across facilities remains a priority.

Stability

EV stability is frequently underestimated. Freeze–thaw cycles enlarge particles, promote aggregation, degrade RNA cargo, and reduce bioactivity. Lyophilization with protectants (e.g., trehalose, sucrose) can mitigate damage, but outcomes are analyte-specific and depend on time and temperature. Stability-indicating assays, including absolute miRNA measurements and physical readouts, should assess retention over weeks to months, not days (22-24).

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Path to Translation

Establishing credible MSC-EV-miRNA therapeutics requires aligning research practices with regulatory expectations. First, adopt the MISEV2023 checklists to define sources, pre-analytics, separation, characterization, and functional design. Vague methods are no longer acceptable for miRNA-centric claims (25). Second, quantify with precision. Particle and protein measurements must be supplemented with absolute miRNA copy numbers per dose. Third, demonstrate mechanistic sufficiency. If miR-X drives effect Y, both loss- and gain-of-function evidence with orthogonal readouts are required. Fourth, link CQAs—including mechanistic miRNA copies and identity markers—to qualified potency assays such as NF-κB reporter activity or macrophage polarization and report assay performance characteristics (25). Finally, integrate safety early. Screen lots for thrombogenic markers (TF/CD142) and match administration routes to risk; systemic intravenous and regional delivery are not interchangeable (25).

The MSC-EV-microRNAome is entering an era where it will be judged by drug standards. Translation depends on quantifying molecules rather than particles, linking molecular data to functional outcomes, dosing based on biological activity, rigorously separating EVs from contaminants, and embedding safety assessments throughout development (6, 26-28). Meeting these expectations will allow EV-miRNA therapies to earn regulatory approval on their own merits.

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