Defining Potency-Relevant & Tissue-Specific microRNA Cargo in RoosterBio hMSC-Exosomes Characterized Using TAmiRNA's miND® Workflow

Romana Bischl[1], Matthias Hackl[1], Trey Picou[2], and Elie Zakhem[2]

[1] TAmiRNA GmbH, Vienna, Austria
[2] RoosterBio, Inc., Frederick, Maryland, USA

Introduction

About Extracellular Vesicles

Human mesenchymal stem/stromal cell–derived extracellular vesicles (hMSC-EVs) have emerged as a leading modality for regenerative medicine, immunomodulation, and next-generation biologics. These nanoscale vesicles carry lipids, proteins, and nucleic acids reflective of their hMSC tissue of origin, enabling potent signaling and therapeutic activity while avoiding many of the challenges associated with cell-based therapies. As interest in EV-based products accelerates, developers require highly characterized, reproducible, and scalable EV raw materials that can seamlessly bridge research, translational, and clinical manufacturing environments.

To fulfill this market need, RoosterBio has established a fully integrated, xeno-free hMSC bioprocessing platform specifically engineered to deliver high-volume, high-quality cells and EVs that seamlessly transition from research grade products to clinical-grade GMP materials. These process-ready solutions have been validated through multiple industry collaborations demonstrating reliable cell attachment, healthy metabolism, stable phenotype, and reproducible EV yields across donors and tissue sources.

As EV-based therapeutics make their way into clinical trials, robust analytical characterization is a foundation of the Chemistry, Manufacturing, and Controls (CMC) section of regulatory submissions. Rigorous analytics enable developers to establish product consistency, ensure safety and efficacy, and satisfy regulatory expectations throughout development and scale-up. CMC planning starts early: developers should think about it from day one to ensure a smooth path to commercialization. By helping developers define and categorize Critical Quality Attributes (CQAs) of EV preparations, RoosterBio allows developers to translate complex biological materials into reproducible, manufacturable products. One potentially applicable analytical method is RNA sequencing.

About Exosome RNA Sequencing

High-resolution RNA profiling has become an essential analytical tool for characterizing EV preparations and defining their biological activity. TAmiRNA has developed a specialized small RNA-sequencing workflow optimized for low-input, EV-derived RNA, enabling precise and reproducible quantification of microRNA (miRNA) and other small RNA species across sample types and manufacturing processes. TAmiRNA's platform integrates standardized EV RNA isolation, spike-in controlled library preparation and next-generation sequencing to deliver absolute and relative quantification at unparalleled sensitivity.

At the core of this workflow is TAmiRNA's miND® spike-in technology, which provides internal calibration standards to ensure experiment-to-experiment comparability, verify sequencing linearity, and enable absolute quantification of miRNA copies per sample. Combined with TAmiRNA's validated small RNA library preparation protocols, this approach ensures robust detection even from challenging EV inputs with low RNA abundance.

TAmiRNA's end-to-end analytical pipeline includes NGS data quality control, RNA species classification, miRNA quantification, differential expression analysis, and customizable data visualization tailored to EV product characterization. This enables developers to define miRNA-based CQAs, assess batch-to-batch reproducibility, identify cell source-specific miRNA signatures, and monitor manufacturing consistency.

With extensive experience in EV analytics and industrial collaborations, TAmiRNA provides a regulatory-aligned, scalable solution for EV RNA sequencing that supports research, process development, and CMC documentation. TAmiRNA's platform is designed to help developers translate complex small-RNA signals into valuable insights for product quality, mechanism-of-action studies, and comparability assessments across donors, lots, and manufacturing platforms.

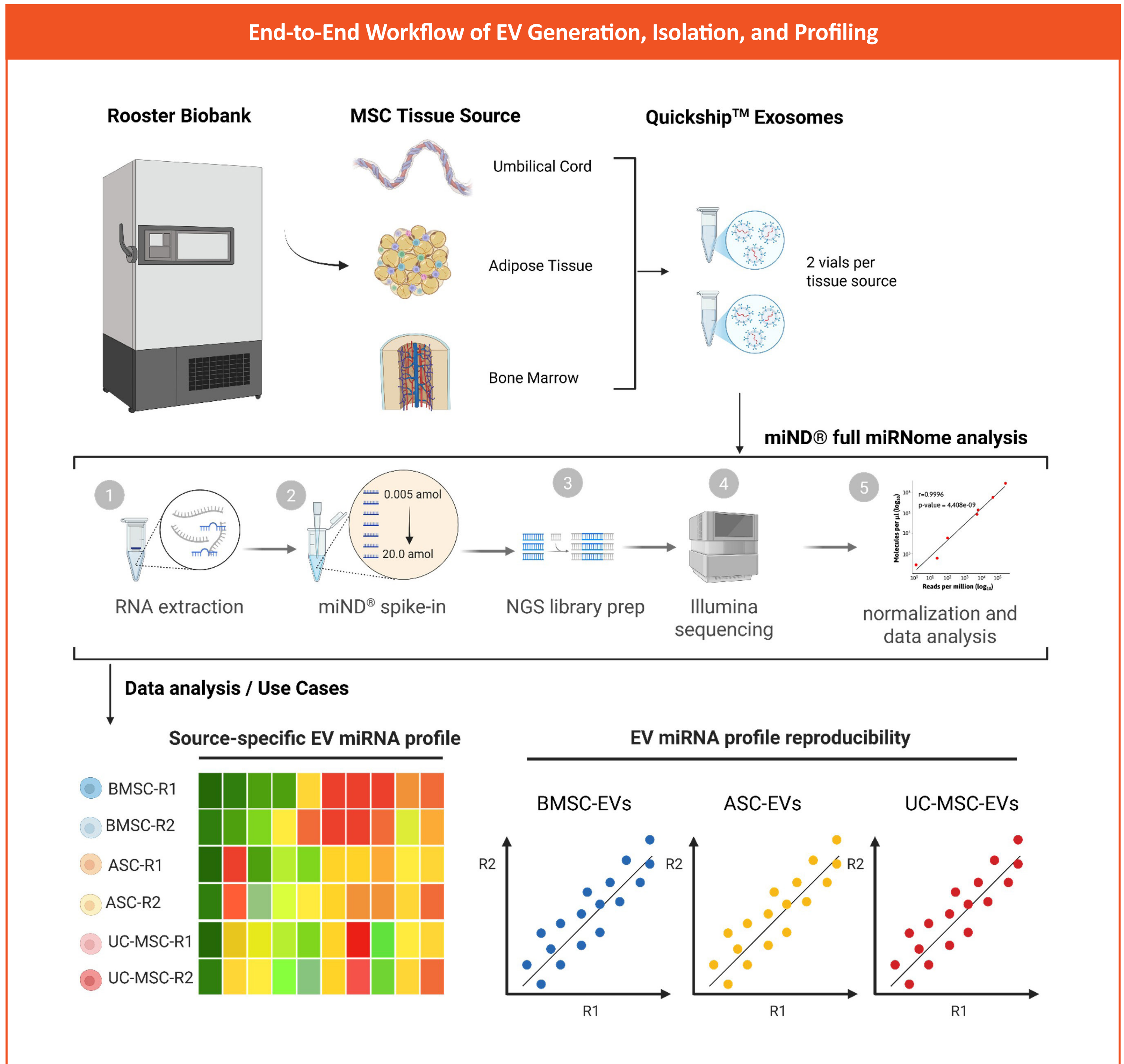
Scope

In this whitepaper, RoosterBio and TAmiRNA partnered to perform comprehensive small RNA-sequencing of RoosterVial Exosome products using the miND® (miRNA NGS Discovery) workflow. RoosterBio's RoosterVial Exosome products provide standardized, tissue-specific hMSC-EV preparations from umbilical cord (hUC), bone marrow (hBM), and adipose (hAD) MSCs. These EV products are manufactured using RoosterBio's optimized xeno-free workflow and are designed to accelerate advanced therapy development by offering a consistent, well-characterized EV input material suitable for discovery biology, potency assessment, process development, and emerging translational applications. For the purpose of this collaboration, RoosterVial Exosomes' consistent manufacturing process and well-characterized nature allow them to serve as a material analog for extracellular vesicle products manufactured using a therapeutic developer's

clinical program. This collaboration establishes a high-resolution view of the microRNA cargo carried by hMSC-EVs from distinct tissue sources, evaluates both biological and analytical reproducibility, and demonstrates the feasibility of quantitative miRNA profiling directly from RoosterBio's raw materials. The resulting data provides a molecular foundation for linking MSC tissue origin to EV mechanism of action, supporting potency-relevant biomarker discovery and future CMC-aligned analytical development.

High-quality Small RNA-sequencing Data Confirm Feasibility of miRNA Profiling from MSC-derived EVs

RoosterBio RoosterVial™ Exosome vials from umbilical-cord (hUC), bone-marrow (hBM), and adipose-tissue (hAD) MSCs were analysed (termed "MSC-EV source"). From each MSC-EV source two independent vials were processed

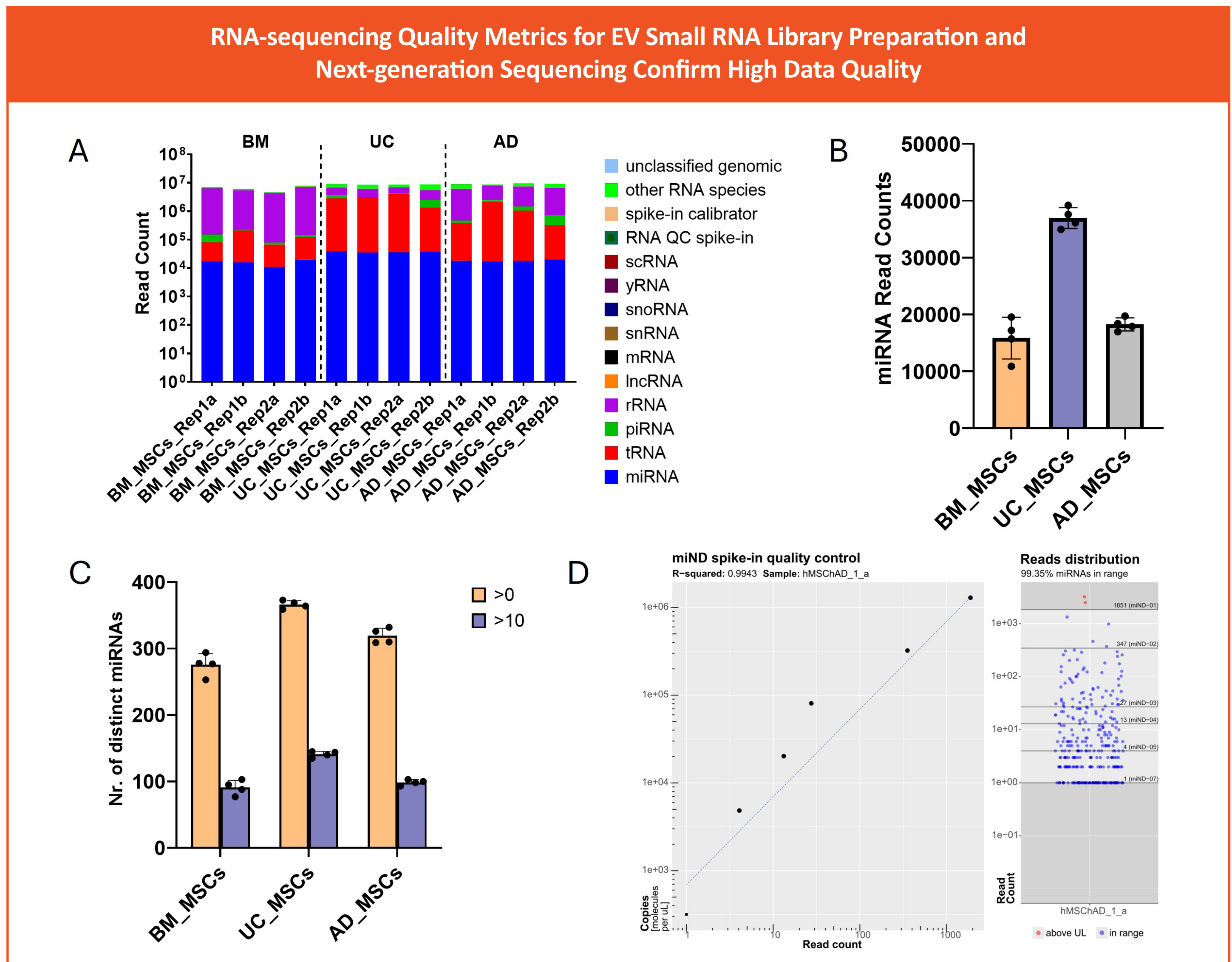


▲ **Figure 1** (*above*) Experimental design.

in two replicates, resulting in four measurements per MSC-EV source. This design enabled measurement of the biological reproducibility between vials and the analytical reproducibility between technical replicates (Figure 1).

Total RNA was isolated from EVs using the Maxwell RSC miRNA Tissue Kit (Promega). A total of 8.5 µl total RNA were mixed with 1 µl miND® spike-ins (TAmiRNA) and the mixture was used for small RNA library preparation using the RealSeq small RNA kit (RealSeq Biosciences) based on the laboratory workflow described by Khamina et al (Khamina et al., 2022) and bioinformatic workflow described by Diendorfer et al. (Diendorfer et al., 2022).

All libraries passed quality control and produced high sequencing depth between 4 and 8 million reads (Figure 2A). Most reads mapped to ribosomal RNA (rRNA) fragments, which is a typical feature of EV-derived RNA samples. MicroRNAs were found to account for 10,000 to 40,000 miRNA reads per sample. EVs derived from hUC-MSCs showed the highest miRNA read count (Figure 2B). Approximately 300 distinct miRNAs were detected per sample, of which approximately one third gave more than 10 reads. This demonstrates a good coverage of the expected miRNA diversity in MSC-EVs (Figure 2C). The inclusion of miND® spike-in controls enabled quantitative calibration and dynamic-range assessment. The calibration curves

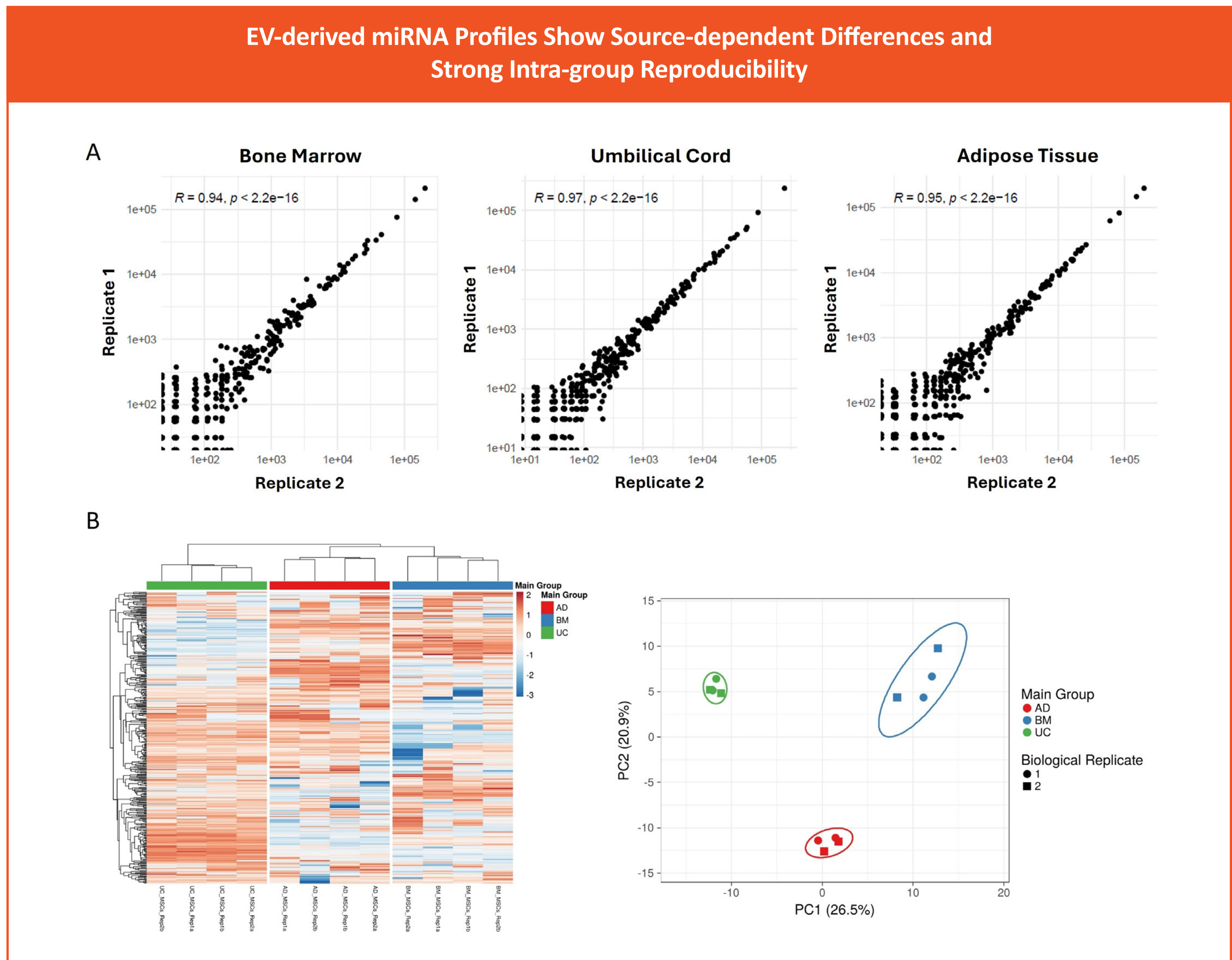


▲ **Figure 2** (*above*) **EV RNA-sequencing quality metrics.** (A) Stacked bar chart showing read-type composition for all MSC EV samples grouped by tissue source (hBM, hUC, hAD); sample names (e.g., BM_MSCs_Rep1a) indicate biological (Rep1/Rep2) and technical (a/b) replicates. (B) Total miRNA read counts summarized per EV type with individual sample points. (C) Numbers of detected miRNAs per source, comparing all (> 0 reads) and stably detected (> 10 reads) miRNAs. (D) Representative miND® spike-in calibration curve (copies µL^{-1} vs reads) showing excellent linearity (R^2 = 0.995); ~98.8 % of detected miRNAs fall within the spike-in dynamic range, confirming robust quantification.

showed consistently high linearity (R^2 = 0.95-0.99) across all libraries (example shown in Figure 2D), confirming high accuracy and reproducibility of the workflow.

Excellent Reproducibility and Clear Source-specific miRNA Signatures

Technical replicates demonstrated very high correlation (average R ~ 0.95) across all MSC-EV types, confirming both the stability of RoosterBio's EV preparations as well as the reproducibility of TAmiRNA's small RNA-sequencing workflow (Figure 3A). Unsupervised analysis was preformed using hierarchical clustering together with visualization as heatmap of 365 microRNAs filtered for expression robustness (RPM > 5 in ≥ 50 % of samples) and principal component analysis (Figure 3B). Unsupervised analysis revealed that MSC-EV miRNA profiles are consistent within each cell source but show clear differences between cell sources. These observations confirm that the tissue source is a main factor influencing the miRNA profile in MSC-EVs, while the variation between technical replicates (at the



▲ Figure 3 (*above*) **Analysis of robustness and cell-source specific EV microRNA cargo.** (A) Correlation plots comparing biological replicates (Rep1/2) for each MSC EV source. Axes show log-scaled RPM values; Spearman correlation coefficients (R) and significance levels (p-val) are indicated above each plot, demonstrating excellent reproducibility (average R ≈ 0.95). (B) Heatmap and principal component analysis (PCA) of RPM-normalized miRNA expression profiles. Heatmap rows represent individual miRNAs and columns represent samples labelled by tissue source (hBM, hUC, hAD). Data were scaled by unit variance, and clustering was performed using correlation-based distance and Ward.D2 linkage. The accompanying PCA shows clear separation by EV source along PC1 and PC2, indicating distinct miRNA signatures for each MSC type.

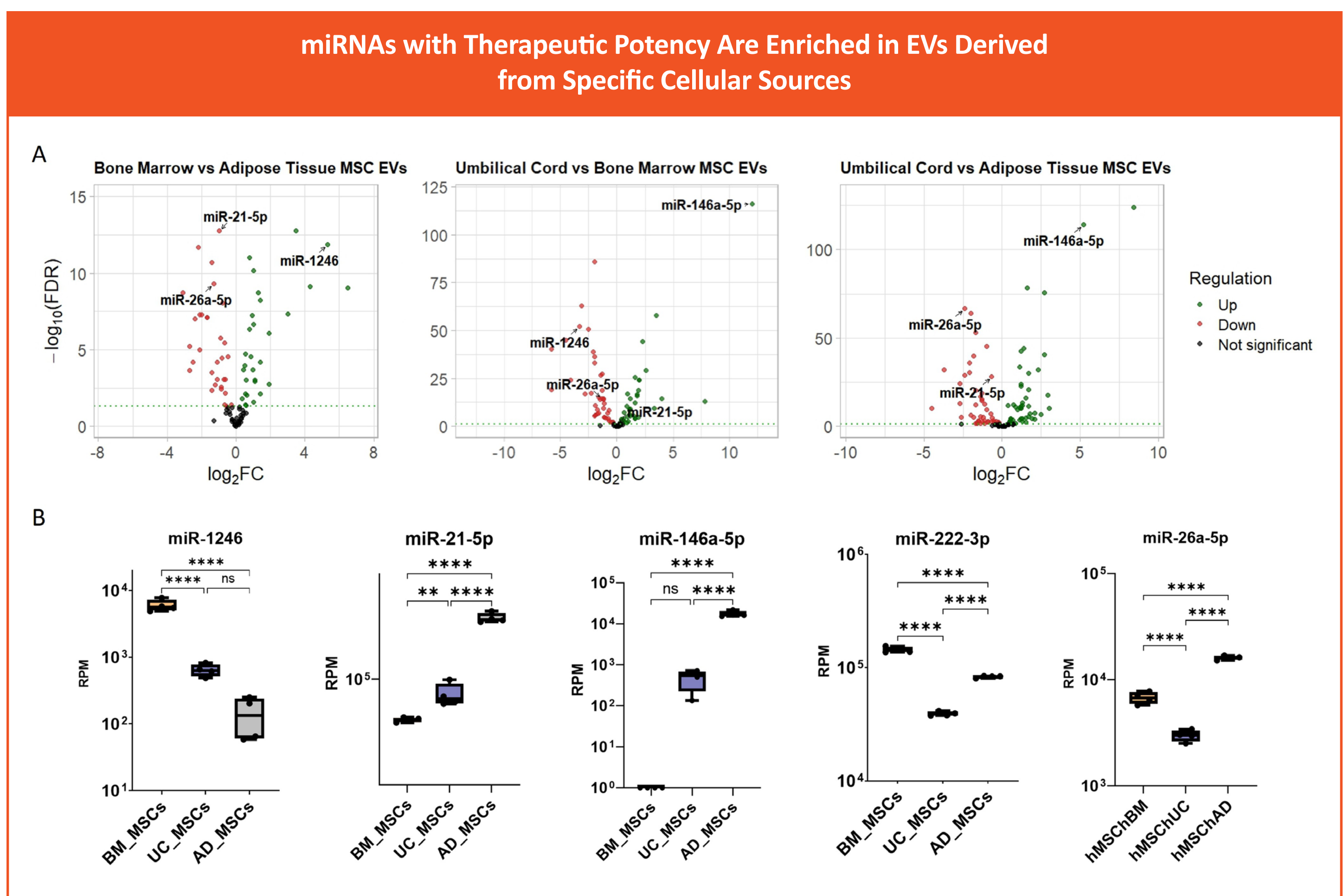
level of NGS analysis) and biological replicates (at the level of EV aliquots of the same source) is low.

Distinct and Therapeutically Relevant microRNA Patterns Between EV Sources

We applied differential expression analysis to identify miRNAs with significant (FDR < 0.05) enrichment in RoosterBio MSC-derived EVs of different origin and visualized the enrichment using Volcano Plots (Figure 4A). Each type of MSC-EV showed a distinct set of enriched miRNAs (Figure 4B):

miR-1246 showed the highest abundance in hBM-EVs. This well-established EV-associated miRNA has been identified as a key functional component of BM-MSC-derived extracellular vesicles, with recent work demonstrating immunoregulatory and therapeutic effects of miR-1246-enriched BM-MSC EVs (Xia et al., 2023). miR-1246 has also been implicated in MSC differentiation processes, including chondrogenic commitment (Breulmann et al., 2025), further supporting its relevance as a biologically active and source-specific cargo molecule. Its elevated levels in hBM-EVs therefore likely reflect the characteristic communication and regulatory functions associated with bone marrow-derived MSCs.

miR-21-5p showed the highest enrichment in adipose-derived EVs, reflecting its involvement in adipogenic



▲ **Figure 4** (*above*) **Extracellular vesicles display enrichment with therapeutically active microRNAs depending on their cellular origin.** (A) Volcano plots showing differential expression of all detected miRNAs between MSC-EV sources. Dotted line marks the false-discovery-rate (FDR) threshold of 0.05; miRNAs above are significantly regulated (green = up-regulated, red = down-regulated, black = not significant). Differential expression was calculated using the edgeR toolkit (Robinson et al., 2009). The five biologically relevant and significantly regulated miRNAs highlighted are displayed in B. (B) Boxplots showing RPM of the five selected miRNAs across EVs derived from hBM, hUC and hAD MSCs. Boxes indicate the interquartile range with median lines, whiskers show data range, and individual points represent replicate samples. Statistical significance between groups was tested using one-way ANOVA (GraphPad Prism).

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differentiation and pro-repair signalling (Kim et al., 2019).

miR-146a-5p was found to be highly enriched in umbilical cord and adipose tissue derived MSC-EVs compared to bone marrow MSCs. This miRNA is a potent regulator of inflammatory response, and its enrichment in adipose and umbilical cord EVs may contribute to the strong immunomodulatory and anti-inflammatory phenotype of MSC EVs (Pei et al., 2023). Vice-versa miR-222-3p, which is part of the miR-221/222 cluster and contributing to the stress response machinery of mesenchymal cells (Zhang et al., 2020), showed lower levels in hUC-EVs compared to both hBM-EVs and hAD-EVs.

Finally, miR-26a-5p, a differentiation-related miRNA involved in adipogenic and metabolic pathways, was reduced in hUC-EVs compared with hAD-EVs (Karbiener et al., 2014).

Conclusion

Together, this exploratory analysis of microRNA profiles RoosterBio's RoosterVial™ Exosome products demonstrate robust cell-type-specific molecular signatures in extracellular vesicles, which may be linked to a cell-source specific potency and mode-of-action of MSC-EVs. TAmiRNA's miND® small RNA-sequencing workflow is capable of highly reproducible parallel analysis of hundreds of miRNAs in EVs and thereby resolves biologically relevant and therapeutically important differences in EV microRNA cargo.

In summary, these data show that:

1. Total RNA extraction and small RNA-sequencing from RoosterBio MSC-EVs is feasible and highly reproducible.
2. RoosterBio´s RoosterVial™ Exosomes show microRNA profiles that are highly reproducible between different vials.
3. Distinct, biologically meaningful miRNA profiles are observed in MSC-EVs depending on the original tissue source, supporting the notion that EV origin determines miRNA cargo composition and biological activity.

RoosterBio®

www.roosterbio.com
info@RoosterBio.com
1.301.200.5366

5295 Westview Drive, Suite 275
Frederick, MD, USA 21703

TAmiRNA
stability for life.

www.tamirna.com
office@tamirna.com
+43 1 391 33 22

TAmiRNA GmbH
Leberstrasse 20, 1110 Vienna, Austria

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Authored by

Romana Bischl[1]
Matthias Hackl[1]
Trey Picou[2]
Elie Zakhem[2]

1. TAmiRNA GmbH, Vienna, Austria
2. RoosterBio, Inc., Frederick, Maryland, USA

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