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Dilara Bulut
Üsküdar University
Ebru Cam
Üsküdar University
Ozge Demir
Üsküdar University
Joseph Borg
University of Malta
Fathi Karouia
Blue Marble Space Institute of Science, NASA Ames Research Center
Afshin Beheshti
Broad Institute of MIT and Harvard
Beyza Aydın
Üsküdar University
Cihan Tastan
cihantastan.ct@gmail.com
Üsküdar University

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Dilara Bulut[1,2], Ebru Cam [1,2], Ozge Demir [1,2], Joseph Borg[3,4,5], Fathi Karouia[6,7,8,9,10] Afshin Beheshti[11,12,13,14], Beyza Aydın [1,2,15,16], Cihan Tastan[1,15,16,17*]

1 Transgenic Cell Technologies and Epigenetic Application and Research Center (TRGENMER), Üsküdar University, Istanbul, TURKIYE

2 Molecular Biology, Institute of Science and Technology, Üsküdar University, Istanbul, TURKIYE

3 Department of Applied Biomedical Science, Faculty of Health Sciences, University of Malta, Msida, Malta

4 Center for Molecular Medicine and Biobanking, University of Malta, Msida, Malta

5 Spaceomix Ltd., Malta

Molecular Biology and Genetics Department, Faculty of Engineering and Natural Science, Üsküdar University, Istanbul, TURKIYE

6 Blue Marble Space Institute of Science, Exobiology Branch, NASA Ames Research Center, Moffett Field, CA, USA

7 Space Research Within Reach, San Francisco, CA, USA

8 Center for Space Medicine, Baylor College of Medicine, Houston, TX, USA

9 BioServe Space Technologies, Smead Aerospace Engineering Science Department, University of Colorado, Boulder, CO, USA

10 Center for Space Biomedical Science, NEXUS Institute, Kyung Hee University, Yongin-si, Republic of Korea

11 Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA, 02142, USA

12 Center for Space Biomedicine, Trivedi Institute for Space and Global Biomedicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.

13 Department of Surgery, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA.

14 Computational and Systems Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.

15 Synastra Biotechnology Inc., Istanbul, TURKIYE

16 Hidna Biotechnology Inc., Istanbul, TURKIYE

17 Molecular Biology and Genetics Department, Faculty of Engineering and Nature Science, Üsküdar University, Istanbul, TURKIYE

*** Corresponding Author: cihantastan.ct@gmail.com**

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Data Availability: TÜBİTAK UZAY (Space Technologies Research Institute) securely archives the raw and processed data generated during the MESSAGE Science Mission, including participant-level RNA-sequencing and associated molecular datasets, under the governance of the Turkish Space Agency (TUA). Because of the small, mission-specific astronaut cohort and the risk of participant re-identification, these data are not made openly available without prior review. Qualified researchers may be granted access to the underlying participant-level data upon submission of a formal scientific request and approval by TÜBİTAK UZAY and TUA, subject to compliance with the applicable NASA Institutional Review Board approval (STUDY00000620), institutional ethics requirements, participant-consent provisions, confidentiality safeguards, and any required data-use agreements. Access requests should be directed to the corresponding author, who will coordinate the institutional review process with TÜBİTAK UZAY and TUA. Aggregated and non-identifying data supporting the principal findings of this study are provided within the article and its Supplementary Information.

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Abstract

MicroRNAs (miRNAs) regulate cellular stress responses and immune homeostasis, but their temporal behavior and biogenesis machinery during human spaceflight remain poorly characterized. We performed a miRNA-focused transcriptomic analysis of peripheral blood mononuclear cells collected during the MESSAGE Science Mission, integrating terrestrial reference samples, longitudinal samples from three Axiom-3 astronauts before launch and on International Space Station Days 4, 7, and 10, and a descriptive post-flight sample from the Galactic-07 suborbital mission. Of 2,239 profiled miRNA-associated transcripts, 118 met the expression and analytical filtering criteria, and 36 were significantly altered across the replicated mission phases. Of these, 34 showed decreased expression, and 2 showed increased expression, indicating a predominantly suppressive response. Hierarchical clustering and trajectory analysis identified distinct temporal programs characterized by sustained repression, transient post-launch or early-orbital induction, and delayed changes during orbital residence. Functional enrichment associated the responsive genes with miRNA-mediated gene silencing, vascular development, angiogenesis, cell migration, extracellular organization, and metabolic regulation. Genes encoding core components of miRNA maturation and activity—including DROSHA, DGCR8, DICER1, AGO1–4, XPO1, XPO4–7, and XPOT—showed coordinated reductions from ISS Day 4 onward, with limited recovery at Days 7 and 10. Co-expression and Gene Ontology analyses further linked this network to pre-miRNA processing, nuclear export, nucleocytoplasmic transport, and RNA-induced silencing complex assembly. These findings demonstrate that orbital spaceflight is associated with coordinated remodeling of miRNA expression and suppression of transcripts required for miRNA processing, transport, and effector function in astronaut PBMCs. This regulatory signature provides a foundation for mechanistic validation and development of miRNA-based biomarkers for astronaut health monitoring during longer missions.

1.Introduction

In space, biological systems face a unique combination of adverse environmental stimuli, including microgravity, ionizing radiation, confinement, altered rhythms, and physiological isolation. Among the factors generated by the space environment, microgravity has emerged as a primary determinant of biological adaptation; it influences gene expression, cytoskeletal organization, mitochondrial function, immune response, and intracellular signal transduction pathways[1,2]. Recently, several space omics technologies have shown that microgravity exposure to human cells and tissues can mediate widespread transcriptomic and epigenetic remodeling[3,4]. A major regulatory mechanism in adaptive responses involves microRNAs (miRNAs), a conserved family of small non-coding RNAs that regulate post-transcriptional gene expression via mRNA degradation or translational inhibition[5,6]. The canonical biogenesis and functional maturation of these crucial regulatory molecules span from the nucleus to the cytoplasm, tightly coordinated by specific protein factors. The process begins with transcription of primary miRNAs (pri-miRNAs), which the Microprocessor complex in the nucleus recognizes: the DROSHA endoribonuclease and its double-stranded RNA-binding partner, DGCR8[7,8]. To maintain strict cellular homeostasis, DROSHA and DGCR8 execute a post-transcriptional cross-regulatory feedback loop, in which the Drosha-DGCR8 complex destabilizes DGCR8 mRNA through cleavage. At the same time, DGCR8 stabilizes Drosha via direct protein-protein interactions[8]. Following initial nuclear processing, the resulting precursor miRNAs (pre-miRNAs) are exported to the cytoplasm by the nuclear export receptor Exportin 5 (XPO5/XPO); however, knockout studies indicate that alternative export pathways or diffusion can partially rescue miRNA production in the complete absence of XPO5[9]. Once in the cytoplasm, pre-miRNAs are cleaved into ~22-nucleotide mature duplexes by the endoribonuclease DICER1, a process whose efficiency and cleavage rates are precisely modulated by structural determinants, such as single-nucleotide polymorphisms or the flexibility of the pre-miRNA apical loop[7,10]. In exceptional cases where DICER1 is deficient, pre-miRNAs can bypass DICER1 processing by directly loading into Argonaute (AGO) proteins, which mediate 3' to 5' trimming to generate mature functional strands[9]**.** Under physiological conditions, the processed miRNA duplexes are handed over to AGO family proteins (AGO1–4) for RNA-induced silencing complex (RISC) assembly, which involves duplex loading and the subsequent ejection of the passenger strand, a critical step that, if impaired, exposes the guide strand to anomalous shortening[7,11]. Ultimately, the fully assembled AGO-RISC utilizes the guide miRNA to recognize complementary sites within the 3' untranslated regions (3' UTR) of target mRNAs, orchestrating post-transcriptional gene silencing through translational repression or mRNA decay[12,13]. Through these intricate biogenesis and targeting mechanisms, miRNAs have been identified as regulators of cellular homeostasis and are involved in many biological processes, including apoptosis, proliferation, oxidative stress response, immune system, DNA repair, and metabolic regulation[14]. MiRNAs are highly dynamic and responsive to stress and thus serve as sensitive markers of changes in physiological functions under adverse conditions such as space flight and microgravity[15,16]. Increasing evidence indicates that both real and simulated microgravity profoundly influence the miRNA expression profile of human cells and tissues. Early transcriptomic studies indicated that microgravity treatment triggers dysregulation of miRNAs associated with cytoskeletal rearrangements, inflammation, mitochondrial damage, and survival mechanisms[17,18]. Later, integrated omics analyses also implicated a microRNA-dependent modulation of signaling pathways critical to spaceflight adaptation, including those regulated by TGF-1, as part of the mechanisms underlying spaceflight adaptation[19]. Moreover, studies on actual spacecraft indicate a tight link between changes in miRNA expression and cellular stress resistance and immune system modulation[20,21]. ISS-dependent studies have also indicated transcriptomic remodeling under microgravity conditions, affecting neuronal stem cells, fibroblasts, and extracellular vesicle miRNAs in the blood, highlighting the systemic influence of non-coding RNA-mediated regulation on adaptation to spaceflight[21-23]. To date, the comprehensive astronaut-centric multi-omics approach has dramatically increased our knowledge regarding molecular adaptation in spaceflight. Indeed, the NASA Twins study revealed that long-term spaceflight caused systemic transcriptomic, epigenetic, and physiologic

changes, including significant changes in circulatory non-coding RNA signature[2]. Furthermore, subsequent multi-omics investigations identified mitochondrial stress, immune dysregulation, and the inflammatory signaling pathway as key biological nodes of spaceflight adaptation[4,24]. More recently, miRNA signatures in plasma from astronauts exposed to spaceflight have been proposed as reliable biomarkers for monitoring astronaut health and developing space countermeasures[15]. Also, miRNAs, within circulating extracellular vesicles isolated from astronauts' plasma, have been suggested to recapitulate systemic physiological changes following spaceflight[23]. Importantly, evidence indicates that radiation-responsive miRNAs and exosomal non-coding RNAs may contribute to long-term health concerns in astronauts, including immune dysfunction and inflammatory dysregulation[25,26]. To date, much of the research has relied on simulated microgravity models or limited transcriptomic datasets from small astronaut populations. As such, duration-specific kinetics of miRNA-mediated regulation across gravitational regimes remain poorly characterized.

Human spaceflight omics studies have progressively revealed that molecular adaptation is both systemic and strongly dependent on mission phase. The NASA Twins Study provided the first longitudinal, multidimensional assessment of a one-year human spaceflight, demonstrating extensive changes in immune regulation, mitochondrial function, epigenetic state, telomere dynamics, and gene-expression networks during and after orbital residence[2]. More recently, the three-day, high-altitude Inspiration4 mission expanded human spaceflight research through the Space Omics and Medical Atlas (SOMA), integrating longitudinal multi-omics and clinical measurements from four civilian astronauts[36,37]. Single-cell profiling of Inspiration4 PBMCs identified conserved and cell-type-specific immune responses, including changes in chromatin accessibility, immune-cell states, and inflammatory signaling[38]. Collectively, these investigations show that even short-duration orbital flight can generate measurable molecular responses while also highlighting substantial variation by cell type, individual characteristics, mission duration, and sampling phase. Within this broader adaptive landscape, miRNAs have emerged as candidate regulators and biomarkers of spaceflight-associated responses. Cross-tissue analysis of NASA GeneLab datasets identified a conserved set of 13 miRNAs linked to TGF-β1 signaling, suggesting that miRNAs may act as systemic regulators of responses to microgravity and space radiation[19]. Subsequent work identified a circulating miRNA signature shared between rodents and humans and connected it to biological processes relevant to cardiovascular, skeletal, hepatic, and immune risks during spaceflight[15]. Altered miRNA profiles have also been reported in human cells exposed to real or simulated microgravity[17,18,20], neural stem cells flown in space[21], model organisms[22], and plasma-derived extracellular vesicles obtained from astronauts[23]. These observations indicate that miRNA responses are not confined to a single tissue or experimental platform but may represent a conserved regulatory layer of spaceflight adaptation. Despite these advances, the temporal organization of miRNA regulation during the transition from pre-flight conditions to early orbital residence remains insufficiently characterized in human immune cells. Most previous studies have focused on circulating miRNAs, extracellular vesicles, post-flight measurements, simulated microgravity, or integrated datasets from distinct tissues and model organisms. It also remains unclear whether coordinated alterations in the molecular machinery responsible for pri-miRNA processing, nuclear export, cytoplasmic maturation, and RISC assembly accompany spaceflight-associated changes in mature miRNA profiles. By longitudinally examining astronaut PBMCs before launch and during ISS Days 4, 7, and 10, while including a descriptive short-duration suborbital observation, the present study investigates both miRNA-associated expression trajectories and the upstream biogenesis machinery that may shape post-transcriptional adaptation during early human spaceflight.

Against this background, we performed a longitudinal, miRNA-focused transcriptomic analysis of astronaut-derived peripheral blood mononuclear cells collected during the MESSAGE Science Mission. We examined pre-launch samples and early orbital samples from ISS Days 4, 7, and 10 during the Axiom-3 mission, along with terrestrial reference samples and a descriptive post-flight observation from the Galactic-07 suborbital mission. Our objectives were to characterize temporal miRNA-associated expression programs during early orbital residence and to determine whether these changes

were accompanied by altered expression of the molecular machinery governing pri-miRNA processing, nuclear export, cytoplasmic maturation, and RISC assembly. We hypothesized that continued orbital exposure would be associated with more structured miRNA regulatory changes than those observed before flight or after brief suborbital exposure. By integrating differential-expression analysis, temporal clustering, co-expression networks, functional enrichment, and targeted assessment of DROSHA, DGCR8, DICER1, exportins, and AGO1–4, this study defines a previously underexplored post-transcriptional dimension of human spaceflight adaptation. It evaluates its potential relevance to immune monitoring and biomarker development for longer-duration missions.

2. Materials and Methods

Ethical Approval and Consent to Participate

The Microgravity Associated Genetics (MESSAGE) Science Mission proposal (Study eIRB Number: STUDY00000620) was reviewed and approved by the NASA IRB in accordance with ethical standards and the requirements of the Code of Federal Regulations for the Protection of Human Subjects (NASA 14CFR1230 and FDA 21CFR50 and 56, if applicable) (Approval Date: September 21, 2023). The MESSAGE Science Mission proposal (No: 61351342/020-275) was also approved by the Ethics Commission of Üsküdar University (Approval Date: July 03, 2023)[27]. We conducted all methods in accordance with relevant guidelines and regulations, including the applicable requirements of the NASA Institutional Review Board and the Üsküdar University Ethics Commission. All participants provided written informed consent before inclusion in the study.

Sample Collection

We obtained blood samples from astronauts who participated in an orbital spaceflight as part of the Axiom-3 mission at the ISS and the suborbital Galactic-07 Mission. We collected samples at five time points: pre-launch, post-suborbital ascent (3 hours after return), and during the ISS stay (days 4, 7, and 10 after launch). This study addresses this gap by integrating data from suborbital missions, International Space Station (ISS) experiments, and Earth-based reference samples. We compared gene expression patterns across different gravitational environments. Venous blood was collected into heparinized tubes. Additionally, we processed blood samples from healthy volunteers matched for age and gender in parallel as a control group, using the same protocol. RNA-seq analysis was performed using RNA isolated directly from astronaut-derived peripheral blood/PBMC samples collected during the MESSAGE Science Mission. We did not culture, expand, or ex vivo stimulate PBMCs before RNA isolation. After sample collection and cryopreservation, we extracted RNA, prepared libraries, sequenced, and performed downstream bioinformatic analyses on the original biological samples. We assessed RNA quality and quantity using standard quality-control procedures, and included samples that met sequencing-library preparation requirements in the transcriptomic analysis.

Transcriptome Analysis

We performed RNA-seq analysis using RNA isolated directly from uncultured peripheral blood/PBMC-derived samples collected during the MESSAGE Science Mission. We did not culture, expand, or ex vivo stimulate PBMCs before RNA isolation. SZA OMICS isolated RNA using automated QIAsymphony-based extraction with the PAXgene Blood RNA Kit, or manual extraction when required, depending on sample type and compatibility. We assessed RNA quantity and purity spectrophotometrically using NanoDrop measurements (A260/A280 and A260/A230 ratios), and further evaluated RNA concentration fluorometrically using Qubit or Victor Nivo-based assays. We assessed RNA integrity using RNA Integrity Number (RIN) values before library preparation. No samples failed at the pre-library QC step. Library preparation was performed using the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus Kit on the Hamilton NGS STAR platform. We performed paired-end sequencing using a 2 × 100 bp configuration. We evaluated sequencing quality using total read number and Q30 metrics, and removed low-quality bases and adapter sequences before alignment. **Supplementary Table S1** provides RNA quality and sequencing QC metrics for all analyzed samples**.** RNA samples that passed the sequencing provider's pre-library QC and library preparation criteria were included in the transcriptomic analysis.

Statistical Analysis:

For statistical analysis, we used the Kruskal-Wallis test because the data did not meet normality assumptions. We analyzed gene expression across the available mission phases: Pre-Launch (L−7 day), Post-Launch Suborbital (L+3 hr), ISS Day 4, ISS Day 7, and ISS Day 10. We assessed data distribution

before comparison and applied the Kruskal–Wallis test to non-normally distributed datasets, including Pre-Launch (L−7 day), ISS Day 4, ISS Day 7, and ISS Day 10. We defined statistical significance as $p < 0.05$. We performed statistical testing only for comparisons with sufficient biological replicates. The condition "Post-Launch Suborbital (L+3 hr)" with n=1, including the post-suborbital sample where applicable, was treated descriptively and was not used for inferential group testing. We used GraphPad Prism 9 for statistical analysis and basic graphical presentation. We produced heatmaps using row-wise z-score normalization to emphasize relative expression changes within each gene across mission phases. We applied hierarchical clustering to both genes and time points using Ward.D2 linkage with Euclidean distance. We reordered genes according to clustering results and annotated them by functional class where applicable. To improve figure readability, we increased heatmap contrast, enlarged font sizes, and reduced dense gene sets or organized them into biologically grouped panels. We performed Gene Ontology enrichment analysis for figure-associated gene sets using the clusterProfiler package with the org.Hs.eg.db human annotation database in R. We used Biological Process enrichment as the primary ontology and defined significantly enriched terms as adjusted $p < 0.05$. We visualized enriched terms as dot plots using ggplot2. We generated co-expression networks from the same expression matrices by calculating pairwise Pearson correlations between genes across mission phases. Network edges were defined using an absolute correlation threshold of $|r| > 0.8$. We visualized networks using igraph, ggraph, and tidygraph, annotating nodes by functional class and edges to represent strong positive or negative correlations.

Results

In this work, we conducted a comparative miRNA transcriptomic analysis of astronaut-derived biological samples following suborbital and orbital microgravity exposures of varying durations. To define microgravity-associated miRNA regulatory signatures, we compared astronaut-derived peripheral blood mononuclear cell samples collected across distinct gravitational exposure conditions. The analytical framework integrated terrestrial control samples, short-duration suborbital flight samples, and orbital spaceflight samples obtained during early residence aboard the International Space Station. Using a stepwise workflow that included RNA extraction, transcriptome profiling, quality control, read preprocessing, mapping, miRNA annotation, expression filtering, and differential expression analysis, we identified miRNA candidates associated with microgravity exposure and exposure duration. This approach enabled the detection of both statistically significant miRNA alterations and broader temporal expression patterns that may reflect regulatory adaptation to spaceflight. Overall, **Figure 1** summarizes the study design and bioinformatics strategy used to identify miRNA-centered molecular responses relevant to astronaut physiological adaptation, stress regulation, immune homeostasis, and long-duration space exploration.

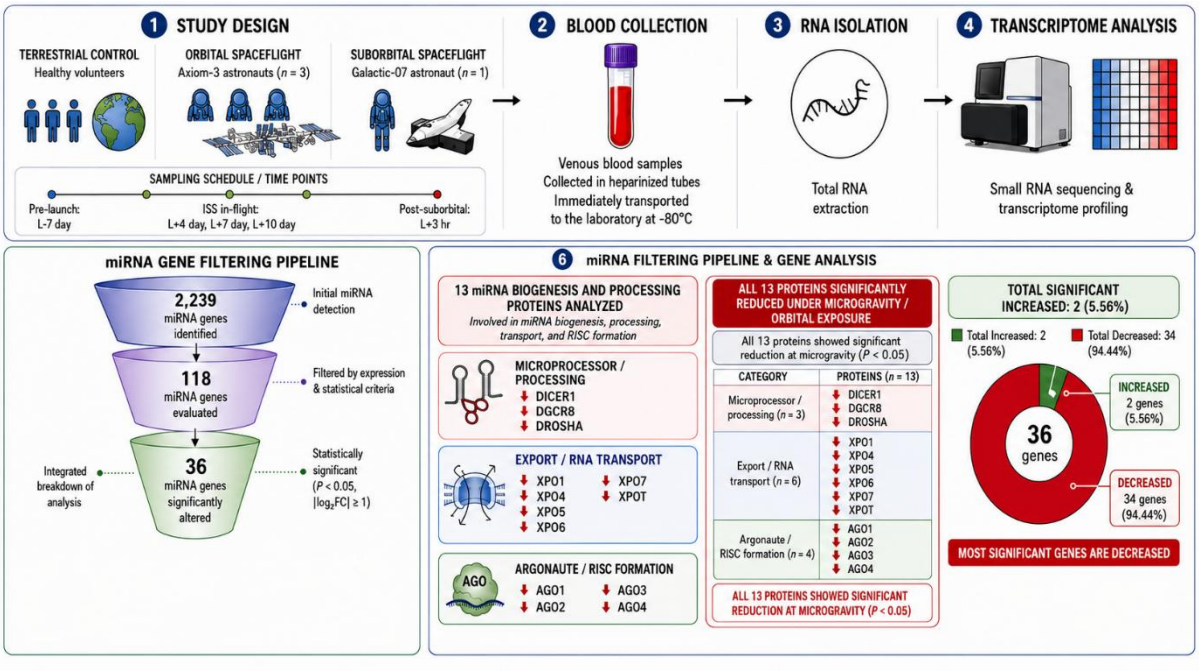


Figure 1. Integrated study design and analytical workflow for identifying microgravity-associated miRNA signatures and suppressed miRNA biogenesis machinery in astronaut-derived blood samples. Peripheral blood samples were obtained from terrestrial healthy volunteer controls, three astronauts who participated in orbital spaceflight during the Axiom-3 mission, and one astronaut who participated in suborbital spaceflight during the Galactic-07 mission. Sampling was performed before launch at L-7 days, during ISS orbital residence at L+4, L+7, and L+10 days, and after suborbital flight at L+3 hr. Venous blood samples were collected in heparinized tubes and immediately transported to the laboratory at −80°C. Total RNA was isolated from peripheral blood samples and subjected to small RNA sequencing and transcriptome profiling. The miRNA filtering pipeline identified 2,239 miRNA-associated genes; 118 met expression and statistical filtering criteria, and 36 were significantly altered under microgravity-associated conditions. In parallel, we analyzed 13 proteins involved in miRNA biogenesis, processing, RNA transport, and RISC formation, including DICER1, DGCR8, DROSHA, XPO1, XPO4, XPO5, XPO6, XPO7, XPOT, AGO1, AGO2, AGO3, and AGO4. Statistical significance was defined as $p < 0.05$ and $|\log_2 FC| \geq 1$ for differentially expressed miRNA genes.

Microgravity exposure induces coordinated temporal remodeling of miRNA-associated gene networks.

To characterize the broader regulatory response associated with spaceflight, we examined expression profiles across the pre-launch baseline, post-launch/suborbital condition, and ISS Days 4, 7, and 10. Hierarchical clustering revealed marked temporal reorganization of the analyzed miRNA-associated gene set (**Figure 2A**). A large group of genes displayed relatively high expression at baseline followed by reduced expression after launch and during orbital exposure. Other gene subsets exhibited transient increases after launch or at specific ISS time points, particularly Day 4 or Day 7. These findings indicate that the response was not uniformly upregulated or downregulated but instead comprised several temporally distinct expression programs. Functional enrichment analysis showed that the responsive gene set was associated predominantly with processes involving tissue and cellular remodeling **(Figure 2B).** The highest GeneRatios were observed for regulation of vasculature development and related biological processes. Additional enriched terms included angiogenesis, regulation of cell migration and motility, locomotion, miRNA-mediated gene silencing, epithelial differentiation, blood-vessel morphogenesis, extracellular organization, and several biosynthetic and metabolic processes. Collectively, these associations suggest that the observed expression changes may influence regulatory pathways involved in structural adaptation, cellular movement, vascular homeostasis, and stress-associated metabolic adjustment. Because the analysis is based on functional enrichment, however, these findings indicate pathway associations rather than direct evidence of altered biological activity. Correlation analysis also showed that the responsive genes formed a densely interconnected co-expression network with $|\rho| > 0.7$ **(Figure 2C).** We identified four principal clusters, with clusters 1 and 2 forming the largest and most highly connected modules. Several nodes exhibited comparatively high degree centrality, suggesting that the temporal response was organized around strongly coordinated network components rather than isolated expression changes. Although positive correlations predominated within the dense core of the network, negative associations were also detected within and between clusters, indicating potentially opposing regulatory programs. Analysis of the four cluster-specific trajectories confirmed distinct temporal response patterns **(Figure 2D).** Cluster 1 was characterized by high pre-launch expression followed by pronounced suppression after launch and persistently lower expression throughout the ISS time points. Cluster 2 showed a marked increase in the post-launch/suborbital condition, followed by a sharp decline at Day 4 and maintenance of generally reduced expression through Days 7 and 10. Cluster 3 exhibited comparatively low expression at baseline and after launch, a strong transient increase at ISS Day 4, and subsequent reduction by Days 7 and 10. Cluster 4 was more heterogeneous but showed a general tendency toward increasing expression during the later orbital phase, particularly at Days 7 and 10.

After expression filtering and statistical analysis, we identified 36 miRNAs as significantly altered across the microgravity-associated conditions analyzed. As shown in **Supplementary Figure 1**, the heatmap representation of these differentially expressed miRNAs revealed clear transcriptomic separation between terrestrial control samples and samples obtained after spaceflight exposure. Hierarchical clustering showed that microgravity-exposed samples shared more similar miRNA expression profiles than control samples, indicating that spaceflight induces coordinated remodeling of the miRNA regulatory landscape. The observed expression patterns included both upregulated and downregulated miRNAs, suggesting that microgravity does not produce a uniform directional response but rather selectively modulates distinct groups of miRNAs involved in cellular adaptation. Notably, the stronger, more coherent clustering across orbital exposure time points supports a duration-dependent regulatory response, in which sustained microgravity is associated with more structured changes in miRNA expression than short-term exposure. These findings indicate that altered miRNA expression may represent an important post-transcriptional regulatory layer contributing to stress-response stabilization, immune regulation, and maintenance of cellular homeostasis during spaceflight. Together, these findings reveal structured, time-dependent remodeling of miRNA-associated gene expression during spaceflight. The contrasting cluster trajectories suggest that acute launch-associated responses,

early orbital adaptation, and later responses to sustained microgravity involve partially distinct regulatory programs. These network-level patterns extend the differential-expression findings by indicating that microgravity-associated changes are coordinated across functionally interconnected genes and may contribute to vascular, migratory, metabolic, and cellular-homeostatic adaptation during spaceflight.

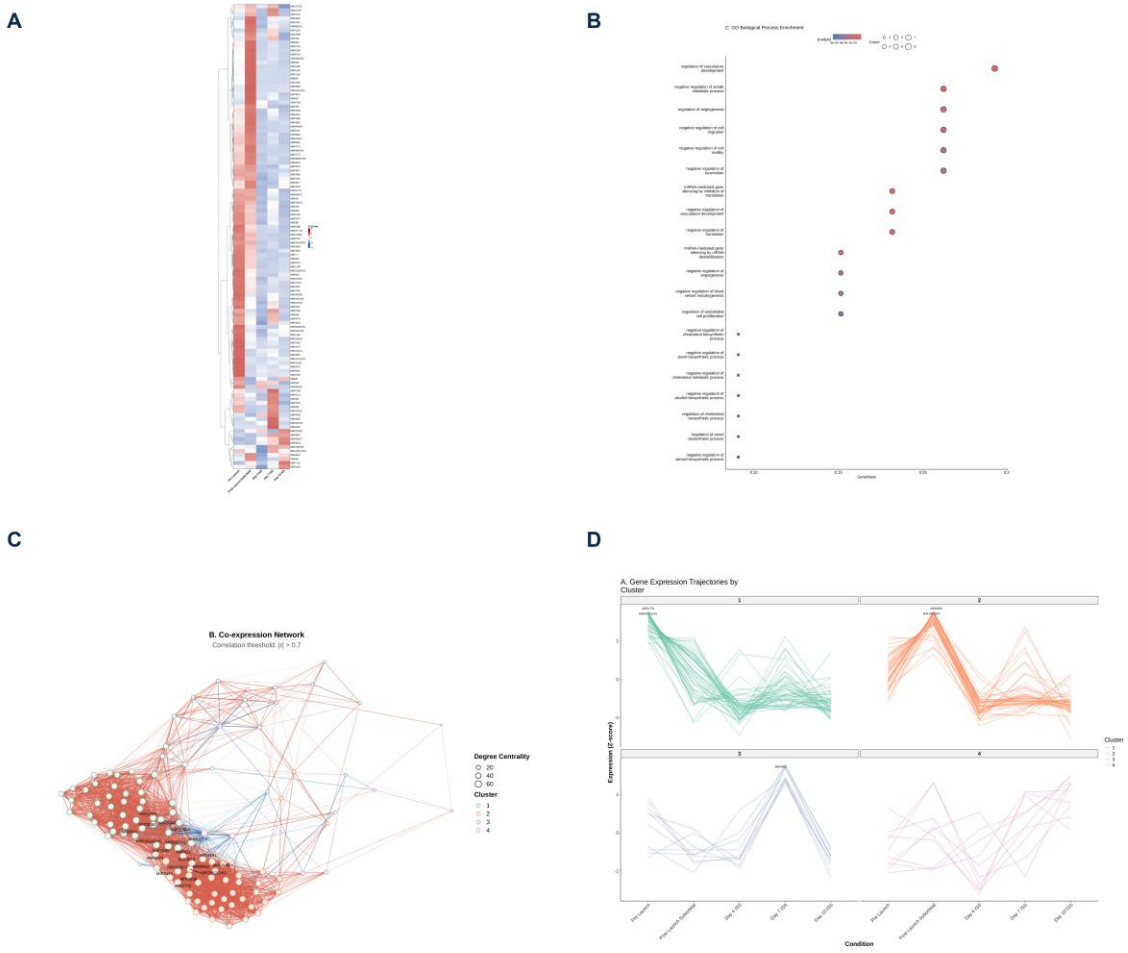


Figure 2. Temporal remodeling, functional enrichment, and co-expression architecture of microgravity-responsive miRNA-associated genes. (A) Hierarchically clustered heatmap showing standardized expression profiles of the analyzed miRNA-associated genes across pre-launch, post-launch/suborbital, and ISS Day 4, Day 7, and Day 10 conditions. Colors represent row-scaled expression values (Z-scores), with red and blue indicating relatively higher and lower expression, respectively. Distinct gene groups showed coordinated, condition-dependent changes across the spaceflight timeline. **(B)** Gene Ontology (GO) Biological Process enrichment analysis of the responsive gene set. The x-axis indicates the GeneRatio; bubble size represents the number of genes associated with each term, and bubble color denotes the adjusted p-value. Enriched processes included regulation of vasculature development, angiogenesis, cell migration and motility, locomotion, epithelial differentiation, blood-vessel morphogenesis, extracellular organization, and multiple biosynthetic and metabolic processes. **(C)** Correlation-based co-expression network constructed using an absolute correlation threshold of $|\rho| > 0.7$. Nodes represent genes, node size reflects degree centrality, and node color denotes membership in one of four co-expression clusters. Edges represent strong expression

correlations, with red and blue indicating positive and negative associations, respectively. **(D)** Standardized gene-expression trajectories for the four co-expression clusters across pre-launch, post-launch/suborbital, ISS Day 4, ISS Day 7, and ISS Day 10 conditions. Each line represents an individual gene, and expression is shown as a Z-score.

Orbital exposure coordinately suppresses miRNA biogenesis, RNA export, and RISC-associated machinery.

To determine whether spaceflight-associated miRNA remodeling was accompanied by changes in the molecular machinery governing miRNA production and activity, we examined 13 genes representing the Argonaute, Dicer, exportin, and Microprocessor functional classes. These included AGO1–AGO4, DICER1, DROSHA, DGCR8, XPO1, XPO4–XPO7, and XPOT. All examined components exhibited significant condition-dependent expression changes, with a shared transition from relatively high expression before orbital exposure to pronounced suppression during the ISS phase **(Figure 3A, B).** Hierarchical clustering revealed broadly concordant expression patterns across the functional classes **(Figure 3A).** Most genes showed their highest standardized expression at pre-launch or in the post-launch/suborbital condition, followed by a marked reduction at ISS Day 4. Expression generally remained below the pre-launch level at ISS Days 7 and 10. This pattern was evident for all four Argonaute genes (AGO1, AGO2, AGO3, and AGO4), indicating sustained attenuation of transcripts associated with miRNA loading and RISC activity during orbital exposure. The core miRNA-processing genes followed a comparable trajectory. DROSHA and DGCR8, which encode the principal components of the nuclear Microprocessor complex, were reduced at ISS Day 4 and remained comparatively suppressed through Day 10. DICER1, responsible for cytoplasmic pre-miRNA processing, likewise showed high expression before orbital exposure and a sharp decrease during the ISS phase. Although DROSHA showed a modest relative rebound at Day 7 in the standardized heatmap, its expression remained below its pre-launch level. The coordinated behavior of these genes indicates that both nuclear and cytoplasmic stages of miRNA maturation were affected. RNA transport genes also underwent substantial temporal changes. XPO1, XPO4, XPO5, XPO6, XPO7, and XPOT were all strongly reduced at ISS Day 4 relative to the earlier conditions. XPO6 exhibited the most prominent acute response, increasing from approximately 29,000 expression units at pre-launch to more than 50,000 in the post-launch/suborbital sample before falling to near-minimal levels at Day 4 **(Figure 3B).** Other highly expressed genes, including members of the exportin and Argonaute classes, also declined sharply at Day 4. Several components showed a small rebound at Day 7, but the recovery was incomplete and generally followed by persistently low expression at Day 10. Importantly, XPO5, the canonical transporter of pre-miRNAs from the nucleus to the cytoplasm, shared this orbital suppression pattern. The co-expression network supported the coordinated nature of these changes **(Figure 3C).** At a Pearson correlation threshold of $r > 0.7$, genes from all four functional classes formed a densely interconnected network rather than isolated class-specific modules. We observed extensive connections among Argonaute proteins, the DROSHA–DGCR8 Microprocessor components, DICER1, and multiple exportins. This architecture indicates that their temporal responses were strongly correlated and suggests coordinated regulation of the broader miRNA-processing and RNA-transport system during the mission. Node-size differences further showed that genes such as XPO6 displayed particularly high temporal variance, consistent with its pronounced suborbital peak and subsequent orbital decline. Gene Ontology analysis functionally confirmed this network-level pattern **(Figure 3D).** The highest GeneRatios, approximately 0.53–0.54, were observed for pre-miRNA processing, miRNA processing, regulatory ncRNA processing, ncRNA processing, and ribonucleoprotein-complex biogenesis. Nuclear export, nucleocytoplasmic transport, and nuclear transport were also significantly enriched, with GeneRatios of approximately 0.46. Additional enriched terms included RISC complex assembly, protein export from the nucleus, miRNA metabolic process, protein–RNA complex assembly and organization, RNA catabolic process, and miRNA-mediated gene silencing by inhibition of

translation. Adjusted p-values were on the order of 10^{-5}, supporting strong functional enrichment of the selected gene set. Together, these findings demonstrate that the observed miRNA remodeling was accompanied by coordinated suppression of transcripts required for primary miRNA processing, precursor export, cytoplasmic maturation, and RISC-associated silencing. The abrupt decline at ISS Day 4, followed by only partial or limited recovery, distinguishes sustained orbital exposure from the pre-launch and short-duration post-launch/suborbital conditions. These transcript-level findings suggest that orbital spaceflight may broadly restructure post-transcriptional regulatory capacity; however, confirmation of reduced processing or RISC activity will require corresponding protein-level and functional measurements.

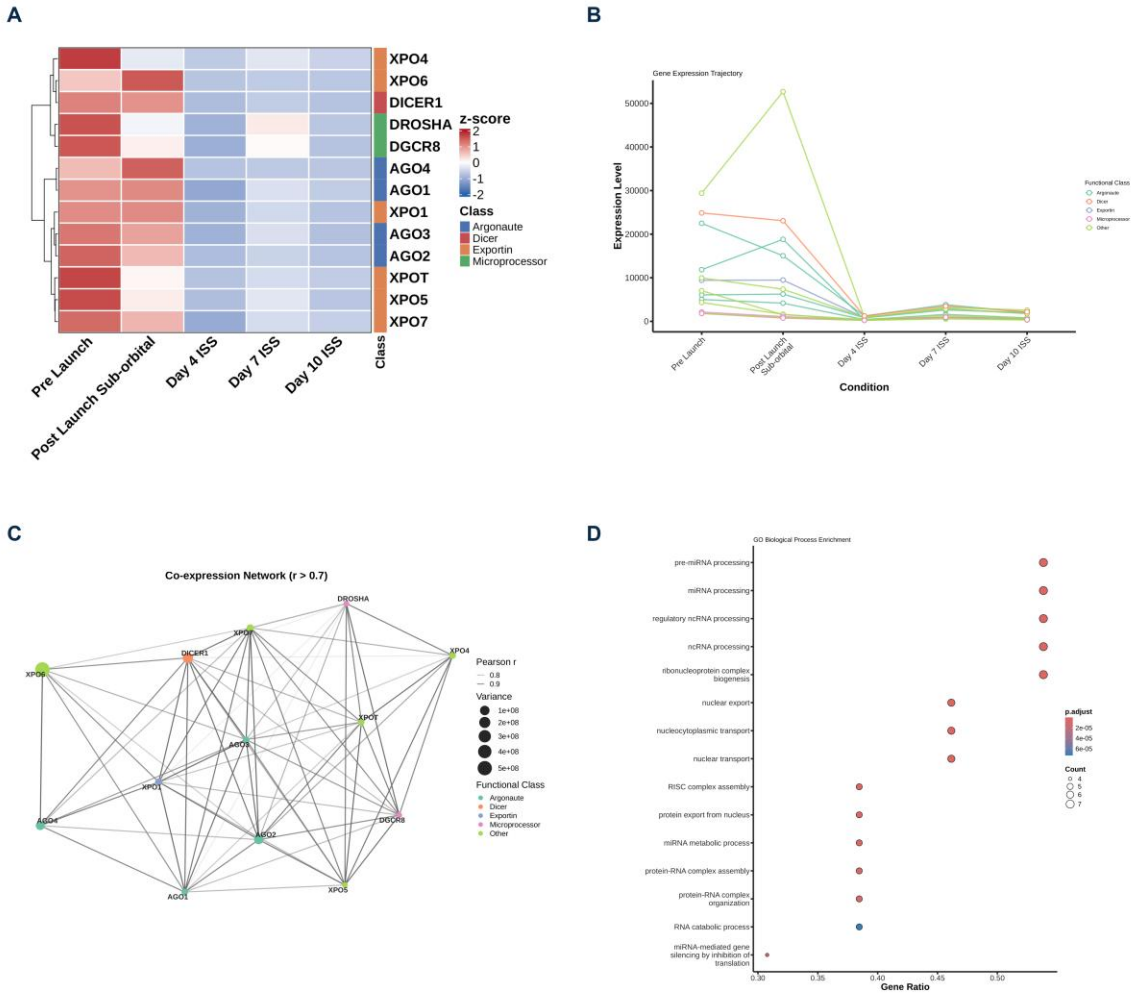


Figure 3. Coordinated suppression of miRNA biogenesis, RNA transport, and RISC-associated machinery during orbital spaceflight. (A) Hierarchically clustered heatmap of genes involved in miRNA processing, RNA export, and RNA-induced silencing complex formation across pre-launch, post-launch/suborbital, and ISS Days 4, 7, and 10. Expression values are row-standardized Z-scores; red and blue indicate relatively higher and lower expression, respectively. The annotation bar identifies Argonaute, Dicer, exportin, and Microprocessor functional classes. **(B)** Expression trajectories of the same genes across the five conditions, displayed using their measured expression levels and colored by functional class. Most genes were highly expressed before orbital exposure, whereas XPO6 exhibited a pronounced post-launch/suborbital peak. All examined genes showed a marked decrease at ISS Day 4, followed by only limited recovery at Days 7 and 10. **(C)** Co-expression network constructed using positively correlated gene pairs with Pearson's r > 0.7. Nodes represent genes, node colors indicate functional classes, and node size reflects expression variance. Edge shading represents correlation

strength. The dense network demonstrates coordinated expression among Argonaute, Dicer, exportin, and Microprocessor components. **(D)** Gene Ontology Biological Process enrichment analysis of the examined gene set. The x-axis represents GeneRatio, bubble size indicates the number of associated genes, and color denotes the Benjamini–Hochberg-adjusted p-value. Significantly enriched processes included pre-miRNA and miRNA processing, regulatory ncRNA processing, nuclear and nucleocytoplasmic transport, RISC complex assembly, miRNA metabolism, RNA catabolism, and miRNA-mediated gene silencing by translational inhibition.

Integrated pathway analysis reveals coordinated suppression of miRNA biogenesis, export, and RISC formation under microgravity

To integrate the miRNA expression findings with the upstream regulatory machinery, we generated a pathway-level model of canonical miRNA biogenesis, nuclear export, cytoplasmic processing, and RISC assembly under microgravity-associated conditions. As shown in **Figure 4**, the nuclear Microprocessor components DROSHA and DGCR8, which mediate pri-miRNA recognition and processing into pre-miRNA, were downregulated, indicating suppression of the earliest nuclear step of canonical miRNA maturation[7,8]. The XPO family, representing six RNA transport-related genes, was also reduced, suggesting that nuclear–cytoplasmic export of pre-miRNA intermediates may be broadly affected during microgravity exposure[9]. In the cytoplasm, DICER1 downregulation indicates a reduced capacity to cleave pre-miRNA into mature miRNA duplexes, a key processing step required for productive miRNA maturation[7,10]. In parallel, downregulation of the AGO family suggests suppression of the downstream effector arm of miRNA regulation, including mature miRNA loading, RISC assembly, and miRNA-guided target repression[7,11,12]. The quantitative miRNA profiling summary further showed that, among the 36 significantly altered miRNA species, 2 were increased and 34 were decreased, indicating that the dominant miRNA response under microgravity was suppressive. Together, these findings support a coordinated model in which microgravity affects multiple sequential levels of post-transcriptional regulation, including miRNA biogenesis, RNA export, cytoplasmic maturation, and functional RISC-mediated gene silencing.

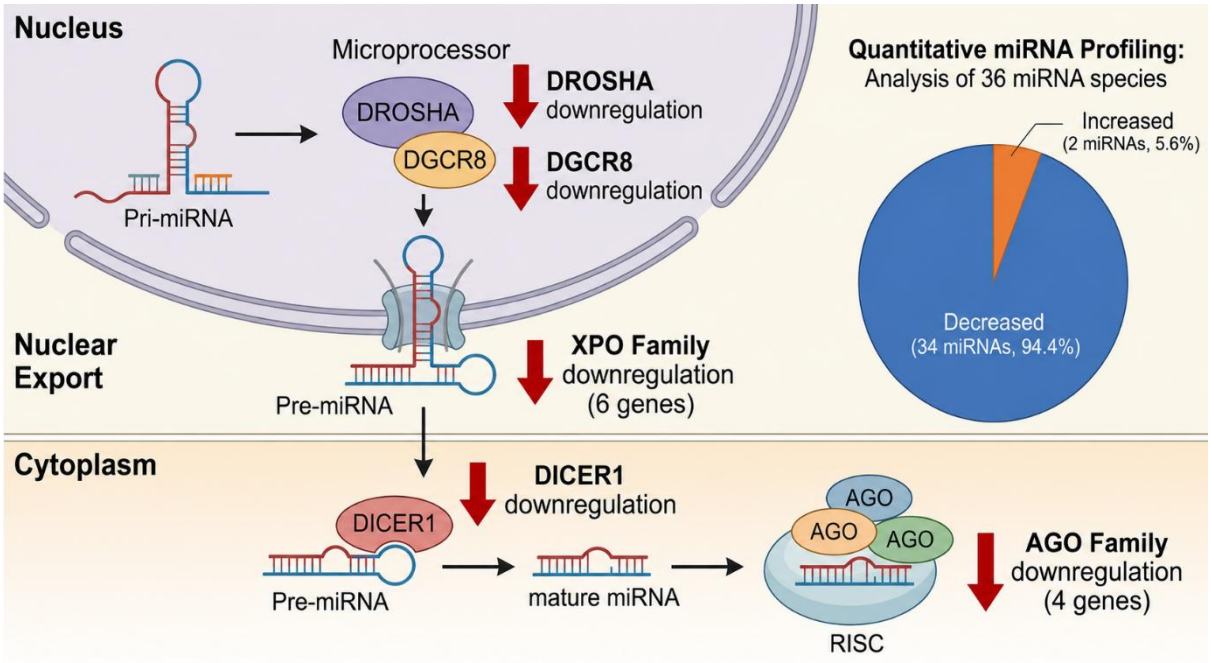


Figure 4. Integrated model of microgravity-associated suppression of miRNA biogenesis, nuclear export, cytoplasmic processing, and RISC formation. Schematic representation of the canonical miRNA biogenesis pathway under microgravity-associated conditions. In the nucleus, the Microprocessor complex processes pri-miRNA, and this process is affected by downregulation of

DROSHA and DGCR8. During nuclear export, reduced XPO family expression suggests suppressed pre-miRNA transport from the nucleus to the cytoplasm. In the cytoplasm, DICER1 downregulation indicates reduced capacity to process pre-miRNA into mature miRNA. Downregulation of the AGO family further suggests impairment of mature miRNA loading and RNA-induced silencing complex (RISC) formation. Quantitative miRNA profiling of 36 significantly altered miRNA species showed that 2 miRNAs were increased and 34 miRNAs were decreased, indicating a predominantly suppressive miRNA response under microgravity.

Discussion

This study provides a miRNA-focused comparative transcriptomic analysis of astronaut-derived peripheral blood mononuclear cells across terrestrial control, suborbital, and orbital microgravity-associated conditions. By integrating samples from short-duration suborbital exposure and early orbital residence aboard the International Space Station, this work addresses an important gap in space omics: limited understanding of the duration and context of gravitational unloading-mediated regulation of non-coding RNA in human immune-related blood cells. Among 2,239 profiled miRNA-associated transcripts, 118 miRNA candidates passed analytical filtering, and 36 miRNAs were identified as significantly altered across the analyzed microgravity-associated conditions. The major finding is that miRNA transcriptomic remodeling appears more coherent and reproducible during orbital exposure than after short-duration suborbital flight, supporting the concept that miRNA regulation is more strongly coupled to sustained physiological adaptation than to brief gravitational perturbation. This observation is consistent with the broader view that spaceflight induces layered molecular adaptation involving transcriptomic, epigenetic, immune, metabolic, mitochondrial, and stress-response pathways[1, 2, 29].

Identifying significant miRNA remodeling in astronaut PBMCs is biologically relevant because miRNAs are post-transcriptional regulators that can rapidly tune gene expression networks involved in immune regulation, oxidative stress responses, cytoskeletal dynamics, mitochondrial function, DNA repair, apoptosis, and cellular homeostasis. Previous studies show that both real and simulated microgravity can alter miRNA expression across multiple cellular systems, including human cells exposed to microgravity models, space-flown fibroblasts, neural stem cells, model organisms, and circulating extracellular vesicles from astronauts. In this context, the current findings extend prior observations by comparing suborbital and orbital exposures within an astronaut-centered framework, rather than relying solely on simulated models or single-condition spaceflight datasets. The observed duration-dependent miRNA behavior supports the hypothesis that miRNAs participate not only in acute stress sensing but also in stabilizing adaptive regulatory states during sustained microgravity exposure[5, 19, 21, 17, 23, 20]. The stronger miRNA signal observed during orbital exposure may reflect the transition from transient perturbation toward a more organized adaptive response. Suborbital flight provides a short but intense exposure to launch, altered gravity, re-entry, and recovery-related stressors. In contrast, orbital residence imposes continuous microgravity together with circadian, fluid-shift, radiation, confinement, and operational stress components. Therefore, the limited and more variable miRNA changes observed after suborbital exposure may represent acute or rapidly reversible responses, while the more structured patterns observed during ISS sampling may reflect progressive regulatory adaptation. This interpretation is supported by astronaut leukocyte transcriptome studies showing that immune-related gene expression changes occur rapidly during transition into space and often show opposite patterns upon return to Earth. In long-duration ISS missions, leukocyte transcriptomes showed downregulation of immune-related functions during early spaceflight and reactivation-like changes after return, indicating that blood-derived immune cells are highly sensitive to gravitational transitions and mission phase[30].

The present miRNA results also align with recent commercial mission multi-omics datasets showing that early spaceflight is sufficient to produce measurable molecular changes in immune, skeletal, vascular, metabolic, and blood-homeostasis pathways. Multi-omics profiling across Axiom-2 and Axiom-3 missions identified PBMC compositional changes, including reduced monocytes, dendritic cells, and megakaryocytes, together with increased naïve CD4+ and cytotoxic T-cell representation, as well as shared signatures involving immune regulation, NF-κB signaling, osteoclast differentiation, metabolic stress, coagulation, and platelet activation. These findings suggest that early spaceflight responses are systemic and involve interconnected immune, inflammatory, skeletal, and vascular processes. In this context, miRNA remodeling may represent an upstream or parallel regulatory layer contributing to these pathway-level changes, particularly because miRNAs can coordinate multiple

mRNA targets simultaneously, thereby stabilizing complex adaptive networks[29]. The relevance of the current findings to the immune system is particularly important. Spaceflight-associated immune dysregulation has been repeatedly described, including altered adaptive immunity, changes in innate immune activity, reactivation of latent herpesviruses, inflammatory imbalance, hypersensitivity reactions, and altered cytokine signaling. Since PBMCs represent a dynamic immune compartment that interacts with systemic physiology, changes in miRNA levels in these cells may provide a sensitive window into immune adaptation under microgravity. These findings therefore support the idea that miRNA profiling could complement conventional immune monitoring by capturing regulatory changes that precede or accompany overt cellular and functional immune alterations. This is especially relevant for long-duration exploration missions, where immune countermeasures may need to be personalized and guided by molecular risk indicators rather than by clinical signs alone[24, 30].

The trajectory analysis identified several expression programs rather than a uniform response to spaceflight. Genes in Cluster 1 showed high pre-launch expression followed by sustained suppression, whereas Cluster 2 displayed a transient post-launch/suborbital increase before decreasing during orbital residence. Cluster 3 showed an early orbital peak at ISS Day 4, and Cluster 4 showed more heterogeneous responses, with a tendency toward increased expression at later ISS time points. These trajectories suggest that miRNA-associated regulation may differ across gravitational transition, early orbital adaptation, and continued microgravity residence. The early changes may reflect acute responses to launch, gravitational unloading, fluid redistribution, circadian disruption, and operational stress, whereas later trajectories may represent compensatory or adaptive regulatory states. Nevertheless, these temporal patterns should be regarded as exploratory because the study design cannot isolate the individual contributions of microgravity, radiation, mission workload, or other spaceflight-associated exposures. Longer longitudinal sampling, including post-flight recovery, will be required to determine whether these clusters represent transient perturbations, stable adaptive states, or early signatures of maladaptation.

A second important implication of this work is the potential value of miRNAs as biomarkers for astronaut health monitoring. Circulating miRNA signatures have already been proposed as candidate tools for countermeasure development and health surveillance in astronauts, and extracellular vesicle miRNAs have been suggested to reflect systemic physiological changes following spaceflight. The present PBMC-based findings add a cellular immune compartment to this biomarker framework. The detection of 36 significantly altered miRNAs, together with trend-level miRNAs showing progressive expression changes toward later orbital time points, suggests that miRNA panels may help distinguish acute flight-associated perturbation from sustained adaptation. However, these candidates should be interpreted as discovery-level signatures until validated in larger astronaut cohorts, additional mission profiles, and integrated multi-omics datasets[23,15,25]. The temporal behavior of non-significant but directionally changing miRNAs is also informative. In the present study, several miRNAs did not meet the statistical threshold but still showed progressive or variable expression patterns across microgravity-associated time points. These subthreshold changes should not be overinterpreted as definitive biomarkers, but they may reflect low-amplitude, early-stage, or interindividual variability in responses. This is a recurring issue in astronaut omics, where sample sizes are necessarily small and biological variability can be high. Similar challenges have been emphasized in NASA Twins, Inspiration4, Axiom, and other space omics datasets, where longitudinal sampling and multi-modal integration are essential for extracting biologically meaningful signals from limited cohorts. Therefore, trend-level miRNA patterns may be valuable for hypothesis generation, especially when integrated with mRNA, protein, epigenetic, metabolomic, immune-cell, and clinical measurements[2, 31, 29]. The current findings may also be interpreted in the broader context of spaceflight-associated aging and frailty-like biology. Spaceflight stressors have been linked to mitochondrial dysfunction, genomic instability, inflammation, epigenomic remodeling, altered telomere dynamics, sarcopenia-related pathways, and frailty-associated molecular signatures. Because miRNAs regulate many of these pathways, duration-dependent miRNA remodeling may contribute to the molecular overlap between spaceflight adaptation and accelerated or stress-

associated aging phenotypes. This does not imply that early orbital exposure causes frailty. However, it supports the idea that miRNA signatures could help monitor biological resilience, stress recovery, and vulnerability to maladaptation during longer missions. Future studies should therefore examine whether the miRNAs identified here overlap with aging-, inflammaging-, mitochondrial stress-, and sarcopenia-associated miRNA networks[32, 2, 3].

Comparing short-duration and sustained microgravity also has implications for gravity-threshold biology. Murine studies comparing microgravity, lunar gravity, and artificial 1 g have shown that partial gravity can reduce some microgravity-induced skeletal and immune alterations. However, recovery is organ-specific and incomplete at 1/6 g. These findings suggest that different tissues and regulatory systems have distinct thresholds for maintaining homeostasis under altered gravity. Although the present study was conducted in human PBMCs and does not directly test partial gravity, the observation that miRNA responses differ between suborbital and orbital exposure supports the broader concept that exposure duration, gravitational context, and tissue-specific regulatory sensitivity shape biological adaptation. This may become increasingly relevant for future Moon and Mars missions, where partial gravity rather than complete microgravity will define much of the human biological environment[33]. The suborbital component of this study is also valuable because suborbital missions are becoming increasingly common in commercial and research settings. Recent work on muscle-derived stromal and adipocyte cells exposed to suborbital microgravity showed that even short-term exposure can influence transcriptomic programs in cell types relevant to muscle and metabolic adaptation. In the present astronaut PBMC dataset, suborbital exposure produced fewer miRNA changes than orbital exposure, suggesting that short-term flight may initiate regulatory perturbations without necessarily producing the coherent miRNA remodeling observed during sustained microgravity. Together, these findings indicate that suborbital platforms may be useful for detecting acute stress-responsive molecular events, whereas orbital missions are better suited for defining sustained adaptation and regulatory stabilization[34].

The coordinated repression of DROSHA, DGCR8, DICER1, XPO5, multiple exportin-family genes, and AGO1–4 observed in Figure 5 suggests that orbital microgravity affects the miRNA regulatory pathway at several sequential checkpoints rather than producing isolated changes in mature miRNA abundance[7]**.** Canonical miRNA biogenesis depends on DROSHA/DGCR8-mediated nuclear processing of pri-miRNAs, XPO5-dependent export of pre-miRNAs, DICER-dependent cytoplasmic maturation, and AGO-mediated RISC assembly; therefore, simultaneous downregulation of these components may reduce the efficiency, timing, or selectivity of mature miRNA generation and target-gene repression during sustained microgravity exposure[7,9]**.** Suppression of DROSHA and DGCR8 is particularly important because these proteins form a cross-regulated Microprocessor module that contributes to homeostatic control of miRNA biogenesis, suggesting that altered expression of either component could propagate through the upstream processing pipeline[8]**.** Reduced DICER1 expression may further constrain cytoplasmic pre-miRNA cleavage, as Dicer-TRBP activity is tightly influenced by RNA structural features and processing efficiency, and perturbation of this step can reshape mature miRNA output[10]**.** In parallel, downregulation of XPO5 and other export-related genes may indicate altered nuclear–cytoplasmic RNA transport capacity. However, alternative export routes can partially compensate for XPO5 loss; it remains a central pathway for pre-miRNA transport and is also linked to broader RNA export biology[9,35]**.** The broad decrease in AGO1–4 suggests that the downstream effector arm of miRNA-mediated silencing may also be weakened, since AGO proteins mediate guide-strand loading, passenger-strand removal, RISC maturation, and target mRNA repression[7,11,12]**.** Together with the significant and trend-level miRNA signatures identified in this study, these findings support a model in which sustained orbital microgravity induces multilayered remodeling of post-transcriptional regulation, involving not only changes in individual miRNAs but also suppression of the molecular machinery required for miRNA generation, transport, loading, and functional gene silencing[13]**.**

The coordinated reduction of DROSHA, DGCR8, DICER1, XPO5, other export-related genes, and AGO1–4 suggests that orbital spaceflight is associated with transcriptional suppression at multiple

stages of the canonical miRNA pathway. However, reduced expression of these components would not necessarily produce an immediate or uniform decrease in every mature miRNA. Mature miRNAs differ substantially in stability, turnover, subcellular localization, transcriptional control, and dependence on canonical processing pathways. Pre-existing mature miRNAs may persist despite reduced biogenesis, while selected miRNAs may be maintained through increased primary transcription, alternative export mechanisms, or non-canonical DICER- or DROSHA-independent processing. These factors may explain why the overall response was predominantly suppressive while a small subset of miRNAs remained increased. The results therefore support remodeling of miRNA regulatory capacity rather than complete or universal inhibition of miRNA function. Furthermore, transcript abundance alone does not establish reduced miRNA-processing activity. DROSHA, DGCR8, DICER1, exportins, and Argonaute proteins are subject to post-transcriptional regulation, protein stabilization, intracellular redistribution, and post-translational modification. Consequently, protein quantification, intracellular localization studies, precursor-to-mature miRNA ratios, AGO-loading assays, and direct measurements of RISC-mediated target repression should validate the functional implications of reduced transcripts.

This study also contributes to the expanding landscape of open, FAIR, and integrated space omics. NASA OSDR and Inspiration4 resources have shown the importance of standardized data processing, longitudinal metadata, controlled data access, and multimodal integration to enable reproducible space biology research. The MESSAGE mission adds population-specific and mission-specific molecular information to this global effort. Because much of the existing human space omics literature comes from limited astronaut cohorts and predominantly Western populations, including Turkish human spaceflight-derived data helps broaden the representation of astronaut molecular biology. Future integration of MESSAGE data with OSDR-compatible standards, AI/ML-assisted network modeling, and cross-mission meta-analysis may improve the identification of conserved versus population- or mission-specific miRNA signatures[31]. Several limitations should be acknowledged. First, astronaut cohort sizes are necessarily small, limiting statistical power and increasing sensitivity to inter-individual variation. Second, the study compares distinct mission contexts; therefore, observed differences in miRNA expression may reflect a combination of microgravity duration, mission architecture, launch and landing stress, sampling time, operational workload, radiation exposure, circadian disruption, and individual physiological background. Third, PBMC miRNA expression provides an informative but partial view of systemic adaptation; it does not directly establish causal miRNA–target relationships or tissue-level effects in muscle, bone, vasculature, central nervous system, or other organs. Fourth, although the study identifies significant and trend-level miRNA signatures, functional validation is required to determine whether these miRNAs regulate key downstream pathways involved in immune modulation, mitochondrial function, oxidative stress, inflammatory signaling, coagulation, or cellular homeostasis. Future work should therefore prioritize longitudinal validation in larger astronaut cohorts, integration with matched mRNA and protein datasets, target prediction and regulatory network reconstruction, extracellular vesicle miRNA comparison, and pathway-level validation using in vitro microgravity models and post-flight samples. Focus on miRNA–mRNA axes linked to immune regulation, NF-κB signaling, TGF-β signaling, mitochondrial stress, DNA damage response, cytoskeletal adaptation, coagulation, and aging-related resilience. In addition, integrating miRNA signatures with clinical, hematological, immune-cell composition, metabolomic, epigenetic, and proteomic readouts may allow the development of dynamic biomarker panels that distinguish adaptive remodeling from maladaptive risk. Such panels could eventually support personalized astronaut monitoring and the timing of countermeasures during long-duration missions.

The enrichment of genes associated with cell migration, motility, extracellular organization, vascular development, and angiogenesis suggests a possible relationship between miRNA remodeling and cellular responses to altered mechanical loading. Microgravity modifies cytoskeletal tension, cell morphology, mechanotransduction, nuclear architecture, and intracellular trafficking. These changes could influence miRNA regulation by altering nuclear–cytoplasmic transport, RNA-binding protein localization, or the assembly of miRNA-processing complexes. The simultaneous reduction of several

exportin-family transcripts is especially relevant because it raises the possibility that spaceflight affects broader RNA-transport dynamics, not only canonical XPO5-dependent pre-miRNA export. The vascular and migratory enrichment patterns may also be relevant to spaceflight-associated fluid redistribution, endothelial adaptation, and immune-cell trafficking. Nevertheless, these enrichment results reflect associations derived from annotated gene sets and do not directly demonstrate altered vascular function or cellular migration. Functional studies using endothelial–immune co-culture systems, simulated microgravity models, RNA-transport assays, and matched circulating vascular biomarkers will be necessary to establish whether the observed transcriptomic associations translate into physiological effects. PBMCs comprise heterogeneous populations of lymphocytes, monocytes, dendritic cells, and other immune-cell subsets, each with a distinct miRNA repertoire and miRNA-processing profile. Spaceflight can alter circulating immune-cell abundance and activation state; therefore, some of the observed expression differences may reflect changes in PBMC composition rather than uniform regulation within individual cell types. This consideration is particularly important when interpreting the coordinated decreases observed across many miRNA-associated transcripts and biogenesis genes. A reduction in a cell population with comparatively high expression of these genes could produce an apparent bulk-tissue suppression even if expression within the remaining cell populations were unchanged. Future analyses should integrate flow-cytometric cell counts, computational deconvolution, or single-cell transcriptomic and small-RNA profiling to distinguish cell-compositional effects from cell-intrinsic responses. Cell-type-resolved measurements would also clarify whether suppression of miRNA machinery is broadly shared across immune populations or concentrated in specific subsets such as monocytes, $CD4^+$ T cells, $CD8^+$ T cells, or natural killer cells.

The co-expression networks indicate that multiple miRNA-processing and regulatory genes followed similar temporal trajectories across the sampled mission phases. These correlations support coordinated behavior but should not be interpreted as direct molecular interactions, causal regulatory relationships, or stable network topology. The limited number of temporal conditions can inflate correlation coefficients, particularly when many genes decrease at the same orbital time point. Network centrality and module assignments should consequently be regarded as exploratory features requiring validation in independent astronaut cohorts. Similarly, enrichment of pre-miRNA processing, ncRNA metabolism, nuclear export, and RISC assembly is biologically consistent with the selected gene set. However, because the analysis was performed on genes preselected for their known roles in miRNA biogenesis and RNA transport, these enriched terms are partly inherent to the selection strategy. The GO analysis should therefore be interpreted as an organized functional summary of the affected machinery rather than independent evidence that these cellular processes were functionally impaired. If validated, coordinated changes in mature miRNAs and their biogenesis machinery could provide complementary information for astronaut health surveillance. Mature miRNA panels may reflect the current regulatory state, whereas expression of DROSHA, DGCR8, DICER1, XPO5, and AGO1–4 may indicate changes in the cellular capacity to generate and deploy miRNA-mediated responses. Combining these two levels could potentially distinguish an isolated miRNA fluctuation from a broader disruption of post-transcriptional regulation. Such biomarkers could be integrated with immune-cell phenotyping, inflammatory markers, hematological indices, mitochondrial measurements, and clinical data to monitor adaptation during longer missions. However, the present findings are discovery-level and do not establish clinical thresholds, predictive performance, or associations with adverse outcomes. Translation will require independent cohorts, standardized sampling, absolute miRNA quantification, assessment of within-person variability, and evaluation of specificity relative to exercise, sleep disruption, radiation exposure, infection, and psychosocial stress.

Including both suborbital and orbital samples broadens the gravitational contexts examined but does not provide a controlled comparison of exposure duration. The suborbital observation came from a single participant on a different mission, whereas the orbital data came from Axiom-3 astronauts sampled longitudinally. Differences between these conditions may therefore reflect participant characteristics, spacecraft environment, launch profile, mission procedures, sampling time, or other

exposures in addition to microgravity duration. Accordingly, the post-suborbital sample should remain descriptive and should not be used to infer statistical differences or reproducibility between mission types. The study also lacks matched in-flight 1g controls, making it impossible to isolate microgravity from the combined spaceflight environment. Future studies should incorporate larger longitudinal cohorts, matched pre- and post-flight sampling, post-flight recovery time points, standardized mission metadata, and, where feasible, artificial-gravity or ground-analog controls. Cross-mission validation through harmonized analysis of MESSAGE, Axiom, Inspiration4, and NASA Open Science Data Repository datasets will be essential for determining which miRNA signatures are conserved features of human spaceflight and which are mission- or individual-specific.

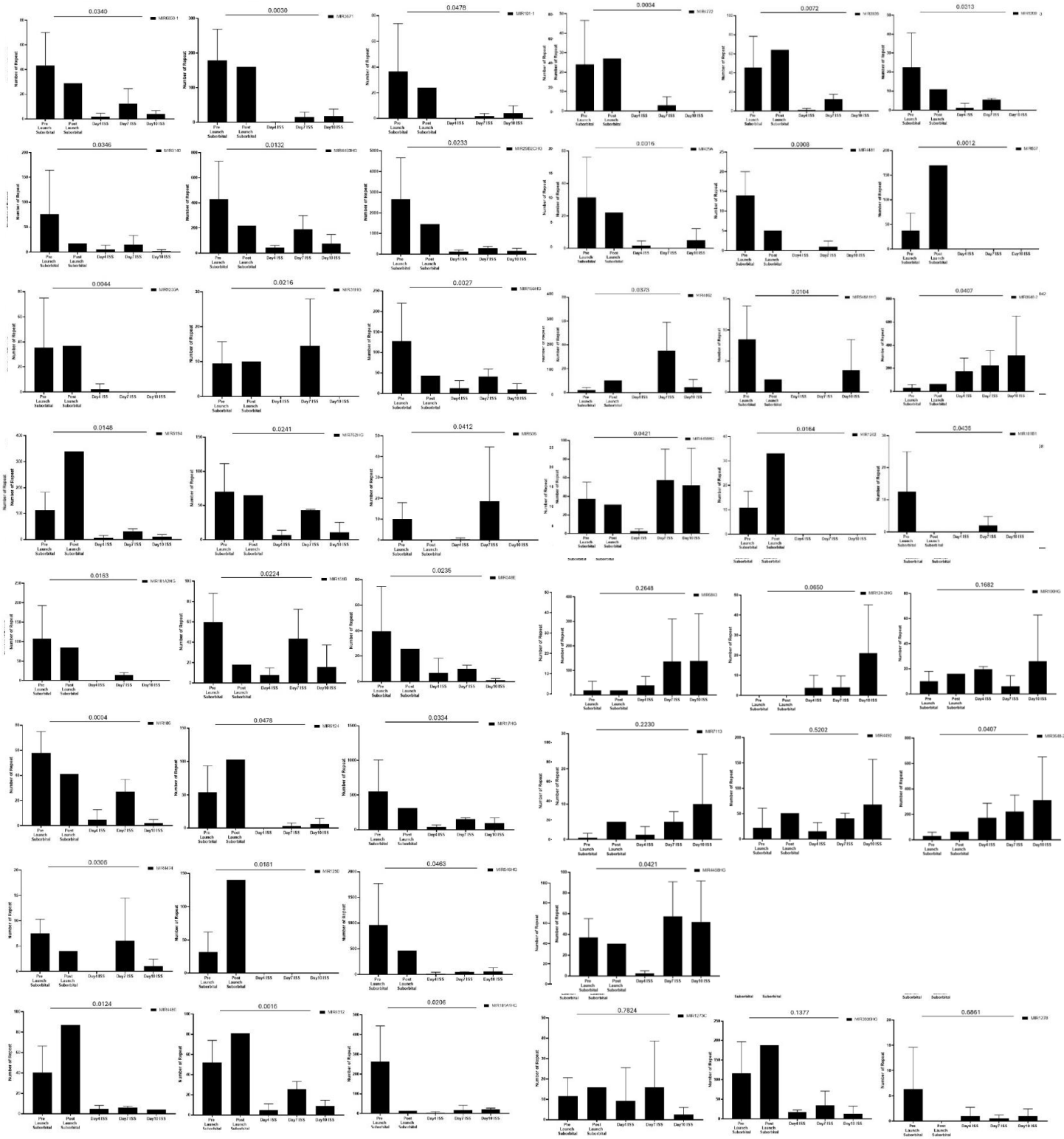
In conclusion, this study identifies coordinated remodeling of miRNA-associated expression programs in astronaut PBMCs during early orbital spaceflight, characterized by a predominantly suppressive miRNA signature and reduced transcript abundance of key components governing miRNA processing, nuclear export, cytoplasmic maturation, and RISC-mediated silencing. The distinct temporal trajectories observed across mission phases suggest that acute flight-associated responses and continued orbital residence may engage partially different post-transcriptional regulatory programs. However, these findings should be interpreted as exploratory associations because of the small astronaut cohort, the descriptive nature of the single suborbital sample, potential changes in PBMC composition, and the inability to separate microgravity from other mission-related exposures. Moreover, transcript-level suppression does not by itself establish reduced protein abundance or miRNA-processing activity. Nevertheless, integrating differential expression, temporal clustering, co-expression analysis, and functional enrichment provides convergent evidence that the miRNA regulatory system is sensitive to the orbital spaceflight environment. Validation in larger longitudinal cohorts, together with cell-type-resolved profiling, protein-level measurements, precursor-to-mature miRNA analyses, and functional assays, will be essential to determine the mechanistic and clinical significance of these responses. If confirmed, combined profiling of mature miRNAs and their biogenesis machinery may contribute to biomarker panels for monitoring immune adaptation, physiological resilience, and emerging health risks during long-duration human spaceflight.

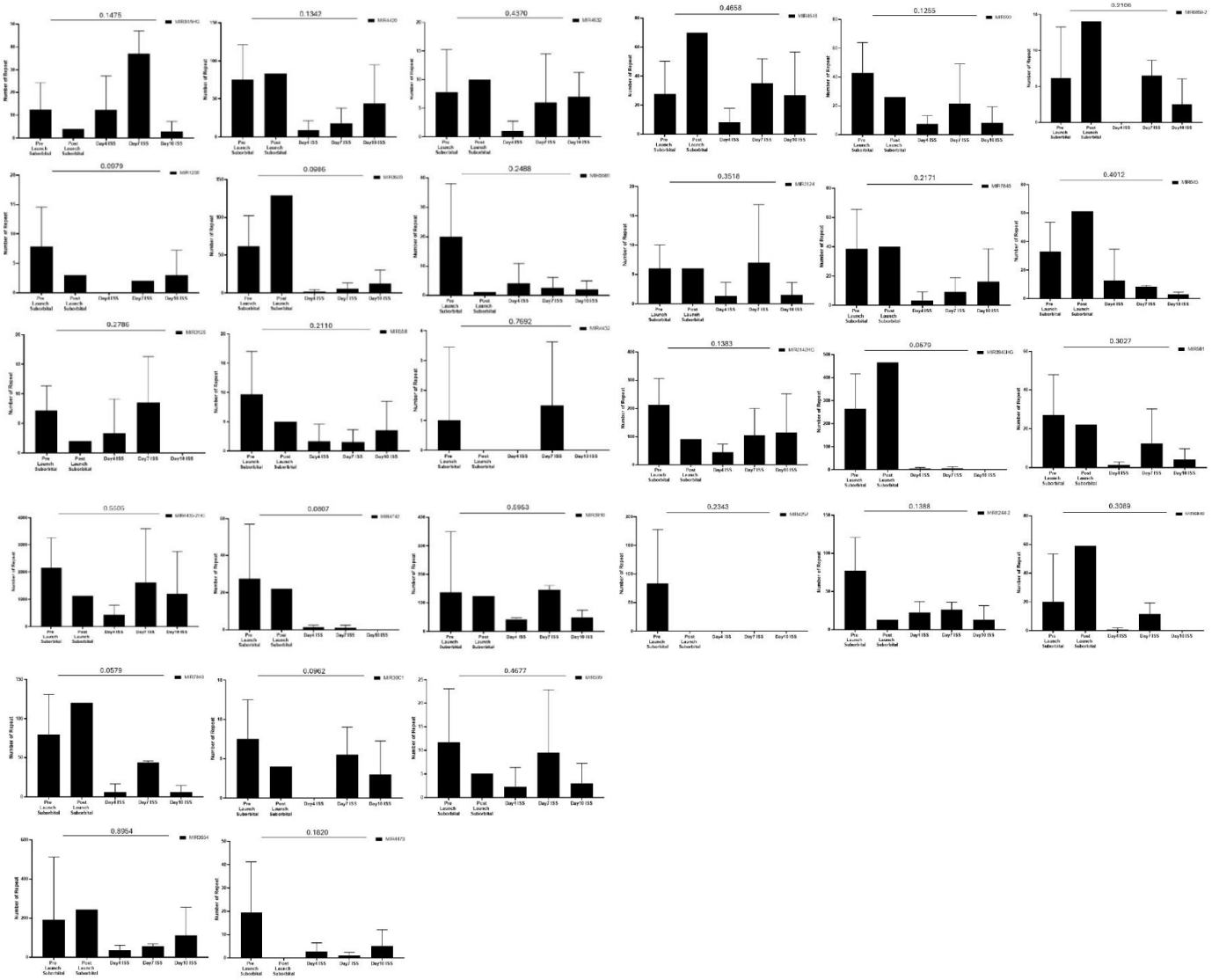
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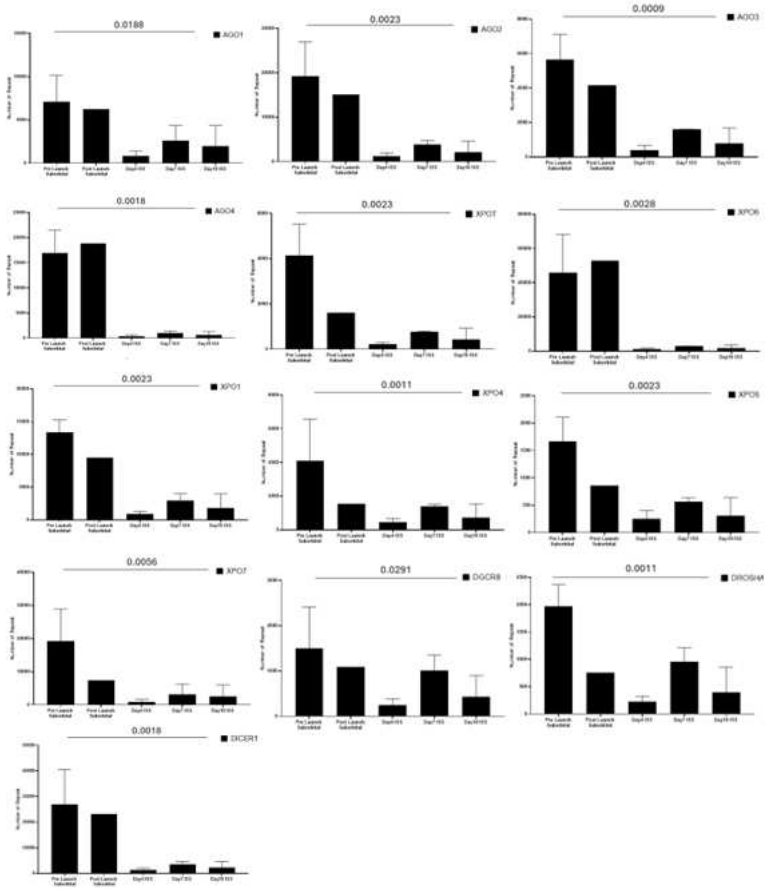
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Supplementary Figure





Supplementary Figure 1. Differential expression patterns of miRNAs under microgravity-associated conditions. Bar graphs show miRNAs with variable expression patterns that did not reach statistical significance ($p > 0.05$) following microgravity exposure. Although below the predefined significance threshold, several miRNAs exhibited modest variation and temporal responses across post-flight time points, potentially reflecting subtle microgravity-induced regulatory effects, biological variability, or early adaptive responses to spaceflight. These miRNAs are presented to provide broader context for the transcriptomic changes observed alongside miRNAs exhibiting significant alterations at later time points. The clear separation between control and microgravity-exposed samples indicates coordinated remodeling of the miRNA transcriptomic landscape in association with spaceflight.



Supplementary Figure 2. Suppression of core miRNA biogenesis, RNA export, and RISC-associated genes under orbital microgravity conditions. Bar graph analysis of 13 genes involved in miRNA processing, nuclear–cytoplasmic RNA transport, and RNA-induced silencing complex formation across pre-launch suborbital, post-launch suborbital, and ISS orbital time points. The Argonaute family genes (AGO1, AGO2, AGO3, and AGO4), exportin-related genes (XPO1, XPO4, XPO5, XPO6, XPO7, and XPOT), and core miRNA biogenesis genes (DROSHA, DGCR8, and DICER1) showed significant expression changes across the experimental groups. Statistical significance was accepted at $p < 0.05$.

Supplementary Table S1. RNA quality and sequencing quality-control metrics for samples included in transcriptomic analysis

Sample ID	Sample source	A260/A280	A260/A230	Fluorometric conc. (ng/µL)	RIN	Pre-library QC	Library prep QC	Mreads, fragment	Mreads, R1+R2	Q30 (%)
P1	Uncultured PBMC/peripheral blood-derived RNA	2.07	1.47	34.0	7.7	Pass	Pass	63.1	126.2	85.7
P2	Uncultured PBMC/peripheral blood-derived RNA	1.98	1.01	18.95	6.7	Pass	Pass	58.1	116.2	85.1
P3	Uncultured PBMC/peripheral blood-derived RNA	2.08	0.57	13.25	6.0	Pass	Pass	57.4	114.8	85.7
A12	Uncultured PBMC/peripheral blood-derived RNA	1.34	0.39	1345	5.8	Pass	Pass	85.6	171.2	91.8
A15	Uncultured PBMC/peripheral blood-derived RNA	1.12	0.23	0.87	3.4	Pass	Pass	21.0	42.0	86.4
A16	Uncultured PBMC/peripheral blood-derived RNA	1.28	0.52	4.0	4.4	Pass	Pass	65.9	131.8	90.1
A7	Uncultured PBMC/peripheral blood-derived RNA	1.81	0.62	0.88	5.2	Pass	Pass	71.5	143.0	90.9
H10	Uncultured PBMC/peripheral blood-derived RNA	2.00	0.13	2.5	2.9	Pass	Pass	78.4	156.8	88.2
H11	Uncultured PBMC/peripheral blood-derived RNA	1.95	0.55	1.5	3.1	Pass	Pass	31.5	63.0	88.3
H2	Uncultured PBMC/peripheral blood-derived RNA	2.07	1.45	35.2	7.5	Pass	Pass	91.8	183.6	91.1
H3	Uncultured PBMC/peripheral blood-derived RNA	1.97	1.60	2.43	3.4	Pass	Pass	28.7	57.4	86.4
H4	Uncultured PBMC/peripheral blood-derived RNA	1.96	0.20	19.8	2.9	Pass	Pass	28.9	57.8	87.4
H5	Uncultured PBMC/peripheral blood-derived RNA	1.85	0.15	1.9	3.4	Pass	Pass	15.5	31.0	84.2
H6	Uncultured PBMC/peripheral blood-derived RNA	1.84	0.13	3.61	3.8	Pass	Pass	6.8	13.6	87.5
H7	Uncultured PBMC/peripheral blood-derived RNA	1.90	0.23	1.4	2.8	Pass	Pass	42.8	85.6	89.6
H8	Uncultured PBMC/peripheral blood-derived RNA	1.84	0.62	1.9	3.4	Pass	Pass	32.1	64.2	88.9
H9	Uncultured PBMC/peripheral blood-derived RNA	1.92	0.25	2.9	3.5	Pass	Pass	13.2	26.4	86.8
R12	Uncultured PBMC/peripheral blood-derived RNA	2.01	0.01	0.8	2.1	Pass	Pass	7.2	14.4	81.8
R13	Uncultured PBMC/peripheral blood-derived RNA	1.40	0.02	3.06	2.8	Pass	Pass	93.4	186.8	89.2
R14	Uncultured PBMC/peripheral blood-derived RNA	2.56	0.07	0.85	4.0	Pass	Pass	27.1	54.2	89.5
R8	Uncultured PBMC/peripheral blood-derived RNA	2.05	0.02	0.935	2.3	Pass	Pass	11.8	23.6	82.9

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation.pdf