

Article

m6A RNA Methylation Promotes the Melanoma Metastasis Mediated by Extracellular Vesicle *miR-23a-5p*

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Simple Summary

Melanoma is an aggressive skin cancer with a high tendency to metastasize. This study reveals that highly metastatic melanoma cells can enhance the metastatic ability of less aggressive tumor cells by sending extracellular vesicles (EVs) containing a molecule called *miR-23a-5p*. Once inside the recipient cells, *miR-23a-5p* reduces levels of the protein METTL14, which alters m6A modifications on RNA and silences two tumor suppressor genes, *Mtus1* and *Prrg4*. This process ultimately increases the invasive behavior of melanoma cells. These findings provide new insights into how tumor cells communicate to drive metastasis and suggest potential targets for future diagnosis and therapy.

Abstract

Background/Objectives: Melanoma, characterized by high rates of metastasis and recurrence, is a particularly aggressive malignant tumor. The underlying mechanisms driving its progression remain enigmatic. The close interplay between tumor and non-tumor cells is pivotal, significantly shaping the tumor microenvironment. Extracellular vesicles emerge as a crucial vector influencing this environment, as they can modulate cellular mechanisms and biological functions—marking a key frontier in tumor mechanism research. However, the potential impact of intercellular communication on tumor cell biology remains largely unexplored. **Methods:** In the study, we employed a pair of cell lines derived from melanoma M14 cells, designated as highly metastatic cells (POL cells) and the low metastatic cells (OL cells), and elucidate their characteristics. **Results:** Our findings revealed that POL cells can potentiate the metastatic potential of OL cells through the transfer of extracellular vesicles, which harbor functional microRNAs, specifically *miR-23a-5p* in this context. Upon entering OL cells, the EV-*miR-23a-5p* orchestrates changes in the m6A modification levels of the mRNA of tumor suppressor genes *Mtus1* and *Prrg4*. **Conclusions:** This modulation subsequently influences the expression of these genes and, in turn, the invasive behavior of OL cells.



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Keywords: melanoma; extracellular vesicle; metastatic microenvironment; miRNA; m6A; *Mtus1*; *Prrg4*

1. Introduction

Melanoma has high incidence and mortality rates due to its metastatic propensity and frequent recurrence [1], yet its etiology remains elusive. The field of melanoma research is extensive, covering areas such as immunotherapy [2], BRAF/NRAS mutations [3] and checkpoint blockade [4]. Currently, researchers are increasingly focusing on histone lactylation, a modification that regulates methylation processes and influences tumor initiation and progression [5]. The regulation of autophagy also plays a role in the advancement of melanoma [6]. Additionally, alterations in the tumor microenvironment are a pivotal area of investigation [7], particularly concerning the mechanisms behind these changes [8–13]. Research into melanoma's tumor microenvironment currently encompasses the impact of tumor cells on growth and development through interactions with normal cells via extracellular vesicles [14]; adoptive cell therapy, utilizing in vitro-amplified tumor-infiltrating lymphocytes, can help eliminate or reduce metastatic melanoma [15]; and miRNAs derived from highly invasive melanoma cells can enhance the invasive capacity of less invasive melanoma cells, at least in part via EV-mediated *miR-411-5p* transfer [16].

Investigations into the complex interactions between neoplastic cells and their healthy somatic microenvironment are central to delineating the intricate mechanisms that drive the initiation and progression of tumors. While the study of inter-tumor dynamics remains in its nascent stages, compelling research has identified a significant role for extracellular vesicles. These EVs, originating from melanoma primary lesions, have been shown to stimulate lymphangiogenesis, thus establishing a favorable landscape for the metastasis and adhesion of tumor cells [17]. Our laboratory has successfully established melanoma cell lines exhibiting high and low invasive and metastatic tendency to investigate the mechanisms of inter-tumor communication (POL cells and OL cells). Our studies reveal that POL cells can transduce high invasive and metastatic characteristics to OL cells via microRNA-enriched EVs. This transmission modulates tumor-related signaling pathways, furthering cancer progression. This exemplifies a common pattern of extracellular vesicle-mediated intercellular communication observed across different cancer types. For instance, we noted a correlation with findings from other research where highly invasive and metastatic liver cancer cells were reported to have a pronounced effect on transforming normal fibroblasts into cancer-associated fibroblasts (CAFs). These transformative effects are mediated through microRNAs in secreted EVs, which specifically target the β 1-integrin-NF- κ B signaling pathway [18].

Despite the underexplored domain of m6A in investigating the crosstalk between POL cells and OL cells, it is a critical post-transcriptional modification of mRNA in eukaryotic organisms. m6A regulates the expression of oncogenes and tumor suppressor genes. This regulation can influence the proliferation and metastatic abilities of tumor cells, thereby significantly impacting the progression of tumorigenesis [19]. Malignant tumors are characterized by metabolic reprogramming, with m6A modification influencing nearly all stages of RNA metabolism. Consequently, m6A plays a pivotal role in tumor metabolism [20,21]. The impact of m6A on RNA metabolism is inevitably linked to the alterations in key factors that govern tumor initiation and progression, underscoring the significant influence of m6A on both tumor onset and development [22,23].

To address this gap, we established melanoma cell lines with differing invasive capacities (POL and OL). We then investigated whether POL-derived, microRNA-enriched

EVs regulate m6A modification in recipient tumor cells and thereby modulate the stability and turnover of tumor suppressor mRNAs, ultimately promoting melanoma migration and invasion. Therefore, this study aimed to determine whether EV *miR-23a-5p* promotes melanoma metastasis via m6A-dependent regulation of tumor suppressor *Mtus1* and *Prrg4* mRNAs.

2. Materials and Methods

2.1. Cell Culture

Green Fluorescent Protein (GFP)-tagged M14 cells were human melanoma sublines provided by Dr. Robert Hoffman (University of California San Diego) through a generous contribution to our research [24,25]. Derived from the M14 cell line, OL, POL, and their various engineered variants (POL-sh*Rab27a*, OL-mimics-*miR-23a-5p*, POL-inhibitor-*miR-23a-5p*, OL-agomir-*miR-23a-5p*, OL-OEMettl14 and OL-shMettl14) exhibit distinct molecular characteristics vital for our investigation into melanoma cell behavior. These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) high-glucose medium enriched with 10% Fetal Bovine Serum (FBS) to maintain optimal growth conditions. The cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2. Animal Experiments

All animal experiments were approved by the Ethics Committee of Chongqing Medical University and conducted in accordance with international and national guidelines for laboratory animal welfare. The study was conducted in an SPF facility using IVC cages, with controlled environmental parameters (temperature, humidity, light cycle). Eight-week-old female NOD/SCID mice were randomly assigned to comparative groups, each comprising 5 animals, to elucidate the effects of *miR-23a-5p* on melanoma cells. The groups were as follows: 1. OL-agomir-NC vs. OL-agomir-*miR-23a-5p*; 2. OL-agomir-*miR-23a-5p*-OENC vs. OL-agomir-*miR-23a-5p*-OEMettl14. In compliance with the approved experimental protocol, each of the eight-week-old female NOD/SCID mice was administered a controlled volume of 5×10^5 tumor cells via intravenous injection through the tail vein, with precision dosing to allow for systematic observation and monitoring of the disease progression. On the 25th day post-inoculation, the mice were euthanized by cervical dislocation following appropriate anesthesia, in accordance with institutional animal care guidelines. Lung metastases in the mice were observed using Sellstrom Z87 fluorescent goggles, equipped with an LDP 470 nm light source for exciting fluorescent proteins.

2.3. Cell Transfection

The *miR-23a-5p* mimics, agomir of *miR-23a-5p*, Cy5-labeled *miR-23a-5p* mimics, and inhibitor of *miR-23a-5p*, essential for the experimental components of this study, were procured from GenePharma (Shanghai, China). Melanoma cells were seeded in 6-well plates and cultured until they reached 70–80% confluency. For each well, 50 pmol of miRNA mimics, agomir, or Cy5-labeled mimics, and 100 pmol of miRNA inhibitor, were used. These RNA oligonucleotides were diluted in 250 µL of Opti-MEM[®] reduced serum medium (Gibco, Waltham, MA, USA). Separately, 5 µL of Lipofectamine[™] 2000 (Invitrogen, Carlsbad, CA, USA) was diluted in 250 µL of Opti-MEM[®]. After 5 min incubation at room temperature, the diluted RNA and diluted Lipofectamine[™] 2000 were combined, mixed gently, and incubated for another 20 min at room temperature to allow complex formation. The entire mixture was then added dropwise to each well containing complete culture medium without antibiotics. The final concentrations were 25 nM miRNA mimics, 25 nM agomir and 25 nM Cy5-labeled mimics, and 50 nM miRNA inhibitor. Following

the 48 h incubation period post-transfection, the efficiency of the transfection process was quantitatively assessed.

After confirming transfection efficiency, subsequent experiments were performed. The lentiviral particles for both *Mettl14* overexpression and knockdown were obtained from GeneChem (Shanghai, China). Cells were infected with a multiplicity of infection (MOI) of 10 according to the manufacturer's protocol. Cells in logarithmic growth phase were seeded at a density of 5×10^4 cells/well in 24-well plates. Subsequently, each well received an inoculation of 5 μ L of the corresponding lentiviral particle reagent. After a 3-day incubation period, cells were prepared for quantitative real-time polymerase chain reaction (qRT-PCR) to evaluate the efficiency of *Mettl14* modulation.

2.4. EV Isolation

Cells (OL and POL) were maintained in DMEM supplemented with 10% FBS for 48 h. The conditioned medium was centrifuged at $800 \times g$ for 5 min and then at $2000 \times g$ to clear cellular fragments. The ultracentrifuge we used was the Beckman Coulter Optima L-100XP, equipped with a fixed-angle rotor. The k factor for this rotor is 951. The supernatant was further processed with Beckman Coulter's Optima L-100XP at $10,000 \times g$ for 30 min followed by ultracentrifugation at $100,000 \times g$ for 70 min. Precipitates were washed in PBS, centrifuged at $100,000 \times g$ for 70 min at 4 °C, resuspended in PBS, filtered (0.22 micron), and stored at -80 °C.

2.5. TEM

EVs were prepared for transmission electron microscopy (TEM) by fixation with 2.5% glutaraldehyde at 4 °C overnight. Post-washing in deionized water, they were deposited on formvar/carbon-coated grids and stained with phosphotungstic acid for 1 min. For TEM analysis, approximately 6–8 μ g of total protein (based on BCA assay) in a volume of 20 μ L was loaded onto each grid. The TEM analysis was conducted using a JEM-1400PLUS microscope from JEOL.

2.6. Nanoparticle Tracking Analysis

EV concentrations and sizes were assessed via Nanoparticle Tracking Analysis (NTA) by following the ZetaVIEW S/N 17-310's user manual. Samples were suspended in 1 mL PBS and analyzed with the ZetaVIEW instrument (Particle Metrix GmbH, Munich, Germany). Their sizes and Brownian motion were determined, with ZetaView 8.04.02 software processing the results.

2.7. miRNA Expression Profiling

RNA was extracted, examined for quality, and processed for library construction and sequencing by BGI in Wuhan, China. ReadCount values were obtained, and sample uniformity between OL-EVs and POL-EVs was assessed using the Hmisc package in R. A correlation heatmap was generated with the pheatmap package. Differential expression analysis was performed using the limma package to calculate log fold changes (logFC) and *p*-values, enabling the identification of differentially expressed miRNAs. To investigate how POL cells may promote OL cell metastasis through cancer-promoting miRNAs carried by EVs, we selected miRNAs with $\text{Log}_2(\text{POL-EVs/OL-EVs}) > 1$ as candidates highly enriched in POL-EVs.

2.8. Western Blot

Cell lysates were prepared by incubating in 1% PMSF-treated RIPA buffer on ice. A total of 5–20 μ g of protein was added to each well on 12% polyacrylamide gels (Beyotime, Shanghai, China). The gels were first electrophoresed at 80 V for 30 min, followed by

120 V for 1 h, and then transferred to PVDF membranes by wet transfer at a constant current of 300 mA for 1 h. The membrane was blocked for 1 h at room temperature with 5% milk in Tris-buffered saline, followed by overnight incubation with primary antibodies (anti-ALIX, CD81, CD63, 1:1000, ALBUMIN 1:5000, from Abcam; goat anti-rabbit IgG, goat anti-mouse IgG, both at 1:5000, from Proteintech Group, Inc., Rosemont, IL, USA) at 4 °C and a subsequent 1 h incubation with secondary antibodies at room temperature. Band analysis was conducted using ImageJ 1.50e software (National Institutes of Health, Bethesda, MD, USA), and the process was repeated for three independent trials to ensure statistical reliability.

2.9. qRT-PCR Analysis

RNA was isolated using TRIzol Reagent from Takara Biotechnology. mRNA levels were quantified after reverse transcription of 1 µg total RNA with PrimeScript RT Master Mix. Quantitative RT-PCR (qRT-PCR) was conducted using the SYBR Green Real-Time PCR Master Mix with a program consisting of pre-incubation at 95 °C for 30 s, followed by 39 cycles at 95 °C for 5 s and 60 °C for 30 s. We used the Agilent AriaMx Real-Time PCR System. The mRNA expression levels were determined via the $2^{-\Delta\Delta CT}$ method. For miRNA analysis, the Mir-X miRNA First-Strand Synthesis Kit facilitated cDNA synthesis, and subsequent qRT-PCR was similarly performed with SYBR Green. The expression of U6 served as an endogenous control for normalization. The oligonucleotide sequences used are detailed in Supplementary Materials: Primers used in qRT-PCR.

2.10. Transwell Migration and Invasion Assays

Cell invasion was assessed using Transwell inserts equipped with 8-micrometer polycarbonate filters. For invasion assays, Matrigel diluted to 1:8 in serum-free medium (30 µL, supplied by BD Biosciences) was applied to the filters. A control migration assay was set up without Matrigel. A volume of 500 µL serum-free medium supplemented with 3×10^4 cells was placed in the upper chamber, while 800 µL culture medium enriched with 20% FBS was added to the lower chamber. The apparatus was incubated for 20 h. Post-incubation, cells adhering to the membrane's lower surface were fixed with 70% ethanol for 20 min at 4 °C and then stained with crystal violet for 10 min. Any cells remaining on the upper surface were gently wiped off with a cotton swab. Images of the cells that had migrated and invaded were captured using light microscopy. For subsequent statistical analysis, we randomly selected nine fields of view in the Transwell chambers and counted the number of migrated and invaded cells. Three independent replicates were performed under consistent conditions.

2.11. EV Labelling and Visualization of Cellular Uptake

We transfected POL cells with Cy5-tagged *miR-23a-5p* mimics and cultured them for three days, allowing them to secrete fluorescent EVs. These EVs, marked with Sigma's PKH26 dye, were collected and added to OL cells. The EVs' interaction with OL cells was explored through steps of 6 h co-culture periods. The cells were fixed with 4% paraformaldehyde, stained with DAPI to visualize the nuclei, and then observed under a TCS SP2 LEICA confocal microscope. Distinct interactions between cells and fluorescently labeled EVs were documented to assess the *miR-23a-5p* mimics' mobility and influence on OL cells.

2.12. Bioinformatics Analysis

miRDB (miRDB—MicroRNA Target Prediction Database) and miRWalk (Home—miRWalk (uni-heidelberg.de)) were deployed to forecast the potential targets of *miR-23a-5p*. We applied the DAVID (<https://david.ncifcrf.gov/> (accessed on 15 October 2025)) platform to conduct Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathway analyses. RMVar (database of functional variants involved in RNA modification (renlab.org)) and GEO (GSE154952 and GSE134388, Home—GEO—NCBI (nih.gov)) datasets were used to find the genes related to m6A and Mettl14 ($\log_{2}FC > 1$, $p < 0.05$).

2.13. m6A Methylation-Level Detection

The experiment utilized the EpiQuik™ m6A RNA Methylation Quantification Kit, where fresh cells were sourced for RNA extraction. For each reaction, 200 ng of total RNA was added per well. RNA was immobilized and purified within wells before staining. Relative quantification was achieved through spectral analysis at 400 nm, comparing RNA samples to both negative and positive controls.

2.14. RNA-Binding Protein Immunoprecipitation (RIP)

Harvest cells into centrifuge tubes for lysing, then prepare the resulting protein solution for future use. Magnetic beads should be pre-cleaned with three washes in RIPA buffer. Following this, co-incubate the protein solution with magnetic beads at 4 °C with gentle stirring at 10 r/min for 2 h. Subsequently, clear unbound proteins from the beads, and proceed to an overnight m6A antibody (Proteintech, Cat No. 68055-1-Ig) incubation under the same temperature and rotation settings. Once the proteins have been specifically tagged with antibodies, clean the beads again and resuspend the content in RIPA buffer. Split the mixture into two: one aliquot goes towards the isolation of RNA for subsequent qRT-PCR analysis, and the other towards the acetone-induced precipitation aimed for protein fractionation.

2.15. Co-Culture Experiments

Seed OL cells at 60% density in a 6-well plate and allow 2 h for adhesion. Then, position a 0.4 µm pore-sized (Corning) cross well co-culture chamber on the plate, populating it with 5×10^4 POL cells. Cultivate the POL cells in DMEM supplemented with 10% FBS. After a 24 h co-culture period, detach the chamber and apply trypsin to OL cells to facilitate Transwell migration/invasion and qRT-PCR studies.

2.16. Ethical Approval

The in vivo research was conducted with a high standard of animal care and ethics, following the stringent protocols reviewed and approved by Chongqing Medical University (Ethics Approval ID: 2024055; Date Approved: 20 March 2024). Their guidelines ensured that all animal work was ethically sound and conformed to the best practices in animal welfare.

2.17. Statistical Analysis

All experiments were independently repeated at least three times, and the data were subjected to statistical analysis. Quantitative results are expressed as mean $\pm$ standard error of the mean (SEM). Data analysis was performed using the two-tailed Student's *t*-test in GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, USA). Significance levels are denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

2.18. MISEV2023 Compliance Statement

All EV-related experiments in this study were conducted in accordance with the Minimal Information for Studies of Extracellular Vesicles 2023 (MISEV2023) guidelines [26]. This includes adherence to recommended nomenclature, isolation protocols, orthogonal characterization methods (TEM, NTA, immunoblotting) and functional study controls to ensure rigor and reproducibility. Detailed experimental parameters are provided in the relevant sections to align with MISEV2023 principles.

3. Results

3.1. EVs from POL Cells Can Promote the Migration and Invasion of OL Cells

OL and POL cells derived from the M14 melanoma cell line represented low metastasis ability and high metastasis ability, respectively [24,25]. At first, we carried out characterization of the isolated EVs, including transmission electron microscopy (TEM) analysis, which revealed their typical double-layered membrane vesicles; nanoparticle tracking analysis, which confirmed their nanometer scale (100–200 nm); and immunoblotting to identify associated proteins such as ALIX, CD81, CD63, and ALBUMIN (Figure 1A–C, Document S1). To assess the influence of EVs from POL cells on OL cells, we adopted two experimental approaches. Directly adding POL-derived EVs to OL cells resulted in a noticeable elevation in both in vitro migration and invasion capabilities of the latter, an effect that was statistically significant (Figure 1D, $p < 0.01$). Co-culturing these cells yielded similar results, with OL cells' migration and invasion abilities being noticeably improved (Figure 1E, $p < 0.05$). To ascertain whether the observed effects were mediated by POL EVs, we knocked down the *Rab27A*, which could promote EV secretion in POL cells. Consequently, the reduction in secretion was mirrored by a decrease in the aggressive phenotype of OL cells, succinctly proving the catalytic role of POL EVs in cell migration (Figure 1F, $p < 0.001$) and invasion (Figure 1F, $p < 0.01$).

All of these results indicated that POL cell-derived EVs potentiate the metastatic potential of OL cells in vitro.

3.2. EV *miR-23a-5p* Is a Key Regulatory Factor That Promotes the Migration and Invasion Ability of OL Cells

Our hypothesis posits that microRNAs (miRNAs), specifically those residing within POL cell EVs, hold a pivotal role in modulating the metastatic potential of OL cells, as evidenced by previous studies [16,17]. To validate this, we conducted exhaustive sequencing of EVs, revealing distinct miRNA expression profiles between high-metastatic POL and low-metastatic OL cells (Figure 2A). Notably, *miR-23a-5p* stood out for its marked upregulation in POL EVs, a finding that aligns with its documented association with cancer progression. To confirm this observation, we employed quantitative reverse transcription PCR (qRT-PCR), which corroborated the elevated expression of *miR-23a-5p* in POL cells (Figure 2B, $p < 0.001$) and further demonstrated its enhanced expression in OL cells upon exposure to POL-derived EVs (Figure 2C, $p < 0.001$), underscoring its pivotal function. To delve deeper into *miR-23a-5p*'s modus operandi within OL cells, we utilized confocal microscopy. By tagging *miR-23a-5p* with a CY5 green fluorescence marker and staining EVs with PKH26 red fluorescence, we observed its packaging into EVs in POL cells and subsequent transfer into OL cells during a 6 h co-culture period (Figure 2D). This finding positions *miR-23a-5p* as a prime candidate for functional exploration. To assess the functional implications of *miR-23a-5p* in cancer cell migration and invasion, we conducted a battery of functional assays. Overexpression of *miR-23a-5p* in OL cells significantly augmented their migratory (Figure 3A, $p < 0.01$) and invasive (Figure 3A, $p < 0.001$) capabilities in vitro, hinting at a pro-metastatic role. Conversely, when *miR-23a-5p* was knocked down in POL cells and their EVs were subsequently administered to OL cells, we observed a notable decrease in OL cell migration (Figure 3B, $p < 0.05$) and invasion (Figure 3B, $p < 0.01$), emphasizing the influence of *miR-23a-5p*-depleted EVs. To extend our findings to an in vivo setting, we injected *miR-23a-5p*-overexpressing OL cells into immunodeficient NOD/SCID mice via tail vein injection. Given that OL cells typically induce pulmonary oligometastasis, whereas POL cells elicit multi-organ metastasis [18], we aimed to discern the effect of *miR-23a-5p* upregulation on OL cell metastasis. After 25 days of monitoring, we performed cervical dislocation and lung dissection, followed by histological analysis using Hema-

toxylin and Eosin (H&E) staining. Our findings revealed a striking enhancement in the metastatic potential and colonization of OL cells (Figure 3C,D), conclusively demonstrating that *miR-23a-5p* overexpression promotes OL cell metastasis in vivo.

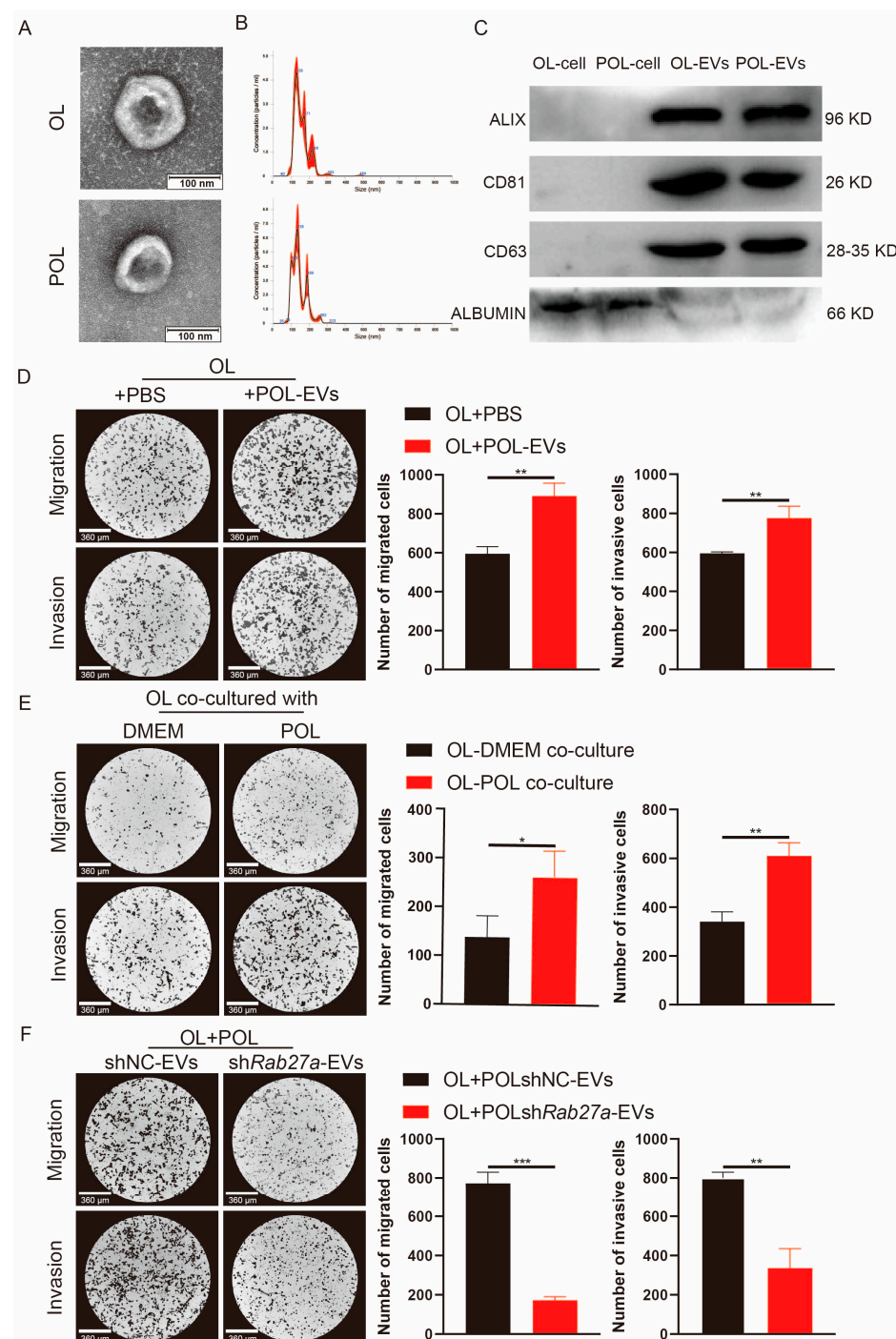


Figure 1. Extracellular vesicles originating from POL cells facilitated the metastatic process in OL cells. (A) Electron microscopy images showcasing extracellular vesicles isolated from both OL cells and POL cells, bar = 100 nm; (B) the NTA technique was utilized for assessing the size distribution and abundance of extracellular vesicles; (C) WB analysis was conducted to evaluate the expression levels of extracellular vesicle markers, including ALIX, CD81, and CD63, as well as the cellular marker ALBUMIN; (D,E) the Transwell assay, both with and without Matrigel, was employed to investigate the migratory impact of POL-EVs (bar = 360 μ m, * $p < 0.05$, ** $p < 0.01$); (F) effect of POL-shRab27a-EVs on the migration and invasion of OL cells (bar = 360 μ m, ** $p < 0.01$, *** $p < 0.001$). Data are shown in means $\pm$ SEM. Student's *t*-test was used to determine statistical significance.

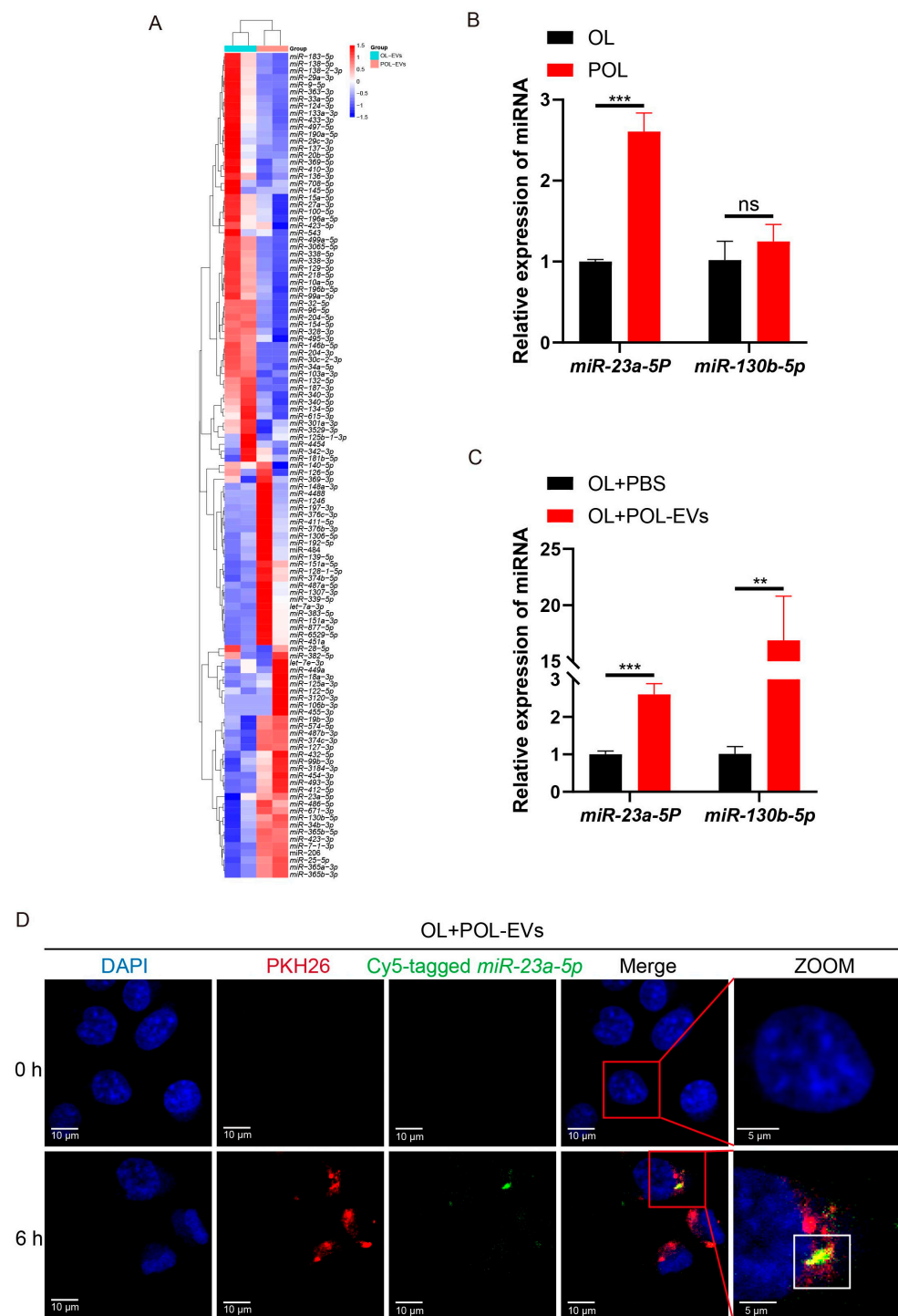


Figure 2. *miR-23a-5p* is significantly upregulated in POL cells and is implicated in the mediation of EVs' uptake by OL cells. **(A)** The heat map visually depicts the expression profile of miRNAs across POL and OL cells; **(B)** the expression levels of *miR-23a-5p* and *miR-130b-5p* in both OL and POL cells were confirmed using quantitative real-time PCR (qRT-PCR) analysis (** $p < 0.001$); **(C)** qRT-PCR validation of *miR-23a-5p* and *miR-130b-5p* expression in OL cells with or without POL-EV treatment (** $p < 0.01$, *** $p < 0.001$). Data are shown in means $\pm$ SEM. Student's *t*-test was used to determine statistical significance; **(D)** time-course staining and imaging were performed to visualize the uptake kinetics of extracellular vesicles secreted by POL-*miR-23a-5p*-expressing cells in OL cells (scale bar: 10 μ m, 5 μ m (ZOOM)).

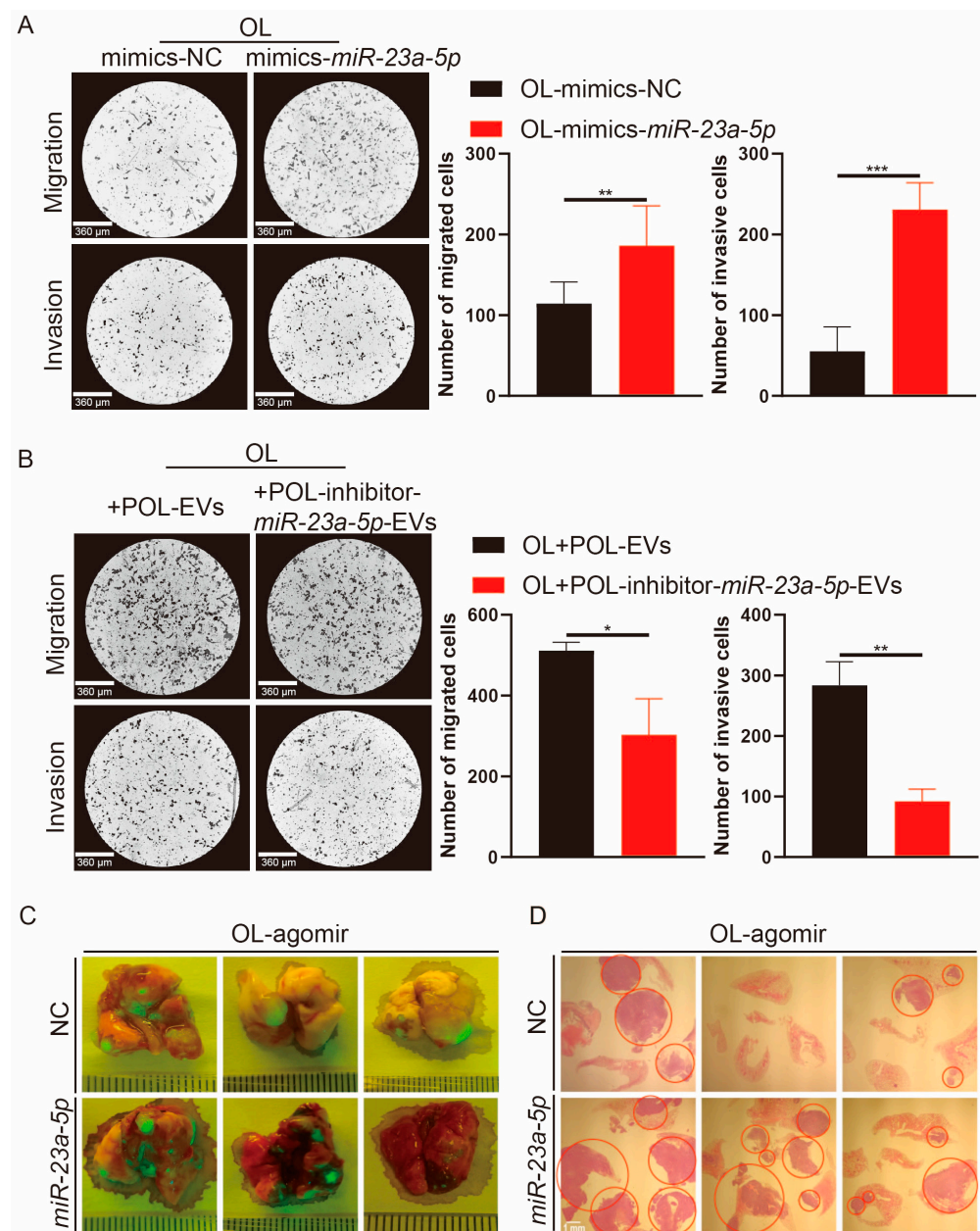


Figure 3. *miR-23a-5p* accelerates both the initiation and progression of melanoma tumors both in vitro and in vivo. **(A)** The Transwell assay was employed to examine the migration and invasion abilities of OL cells that have been genetically modified to overexpress *miR-23-5p* (bar = 360 μ m, ** $p < 0.01$, *** $p < 0.001$). **(B)** Transwell assays show the migration and invasion of OL cells between two groups: one group treated with EVs from *miR-23a-5p*-inhibitor POL cells, and the other treated with control EVs (bar = 360 μ m, * $p < 0.05$, ** $p < 0.01$). Data are shown in means $\pm$ SEM. Student's *t*-test was used to determine statistical significance. **(C)** Visualization analysis was conducted to elucidate the impact of overexpressing *miR-23a-5p* on the metastatic colonization process in vivo (scale bar: The black interval lines are scale reference marks; the distance between two lines is 1 mm). **(D)** H&E staining was performed to examine and document the characteristics of metastatic foci (marked by red circles) within the lung tissue (scale bar, 1 mm).

In conclusion, our study underscores the ability of highly metastatic POL cells to bolster the metastatic prowess of low-metastatic OL cells through the extracellular vesicle transfer of the oncogenic *miR-23a-5p* both in vitro and in vivo.

3.3. *miR-23a-5p Promotes Transfer Properties Through Mettl14*

Through miRDB and miRWalk, we pinpointed potential targets of *miR-23a-5p*, as depicted in Figure 4A. Subsequently, we harnessed the power of KEGG pathway analysis through DAVID, zeroing in on methylation-related pathways that exhibited a notable connection to *miR-23a-5p*'s actions. Recognizing the pivotal role of m6A methylation amidst transcriptome dynamics, we decided to delve deeper into its mechanisms. To unravel the pivotal regulatory player in the interplay between m6A genes and *miR-23a-5p*, we embarked on a study using a univariate approach, analyzing sequencing data from OL and POL cells. *Mettl14* emerged as a central figure in this narrative. Subsequent qRT-PCR assays uncovered a marked downregulation of *Mettl14* in POL cells (Figure 4C), with a similar trend observed in OL cells upon *miR-23a-5p* overexpression (Figure 4D). By cross-referencing with the NCBI database, we precisely located the *miR-23a-5p* binding site on *Mettl14* and introduced a mutation for a dual-luciferase reporter assay. Our findings unequivocally showed that *miR-23a-5p* significantly repressed the expression of *Mettl14* with the wild-type sequence, as opposed to the mutated version (Figure 4E–G).

To evaluate *Mettl14*'s contribution to tumor progression, we knocked down its expression in OL cells and then conducted both in vitro Transwell assays and in vivo metastasis studies. Notably, *Mettl14* knockdown significantly enhanced the metastatic potential of OL cells under ex vivo conditions (Figure 5A). Furthermore, tail vein injection of OL sh-*Mettl14* cells in mice facilitated lung tumor colonization, reinforcing its crucial in vivo role (Figure 5B,C). To further explore the relationship between *miR-23a-5p* and *Mettl14*, we concurrently manipulated the expression levels of in OL cells. Our results indicated that elevating *Mettl14* levels could effectively modulate the oncogenic effects elicited by *miR-23a-5p* both in vitro and in vivo, as evidenced in Figure 5D–F.

3.4. *Mtus1 and Prrg4 Are the Main Pathways for Inhibiting Tumor Metastasis*

The N6-methylation of adenosine (m6A) stands as the predominant internal modification within eukaryotic mRNA, playing a pivotal role in regulating cellular processes such as differentiation, invasion, and apoptosis by modulating mRNA expression profiles [19–21]. Our investigation uncovered that POL cancer cells harbored notably lower m6A levels compared to OL cells, with *miR-23a-5p* overexpression augmenting these levels, potentially fostering cancer progression (Figure 6A). Then, we leveraged the RMVar and GEO resource database datasets, specifically GSE154952 and GSE134388, focusing on melanoma and *Mettl14*. This comprehensive analysis enabled us to identify and correlate differentially expressed genes regulated by m6A with *Mettl14*, ultimately narrowing down our focus to 19 positively correlated genes (Figure 6B–D). Notably, *Mtus1* and *Prrg4* emerged as key genes from our correlation studies, exhibiting a favorable correlation with patient prognosis. Intriguingly, our experiments utilizing the transcription inhibitor actinomycin D revealed that m6A modification stabilized the mRNA of *Mtus1* and *Prrg4*. This finding was further underscored by a subsequent decrease in their expression levels upon *miR-23a-5p* overexpression, highlighting the intricate interplay between m6A and these genes (Figure 6E,F). To confirm the regulation of these genes by m6A modification, we conducted RIP assays employing an m6A-specific antibody. Our results unequivocally demonstrated that both *Mettl14* suppression and *miR-23a-5p* overexpression led to reduced expression of *Mtus1* and *Prrg4*, reinforcing the pivotal role of m6A in their regulation (Figure 6G).

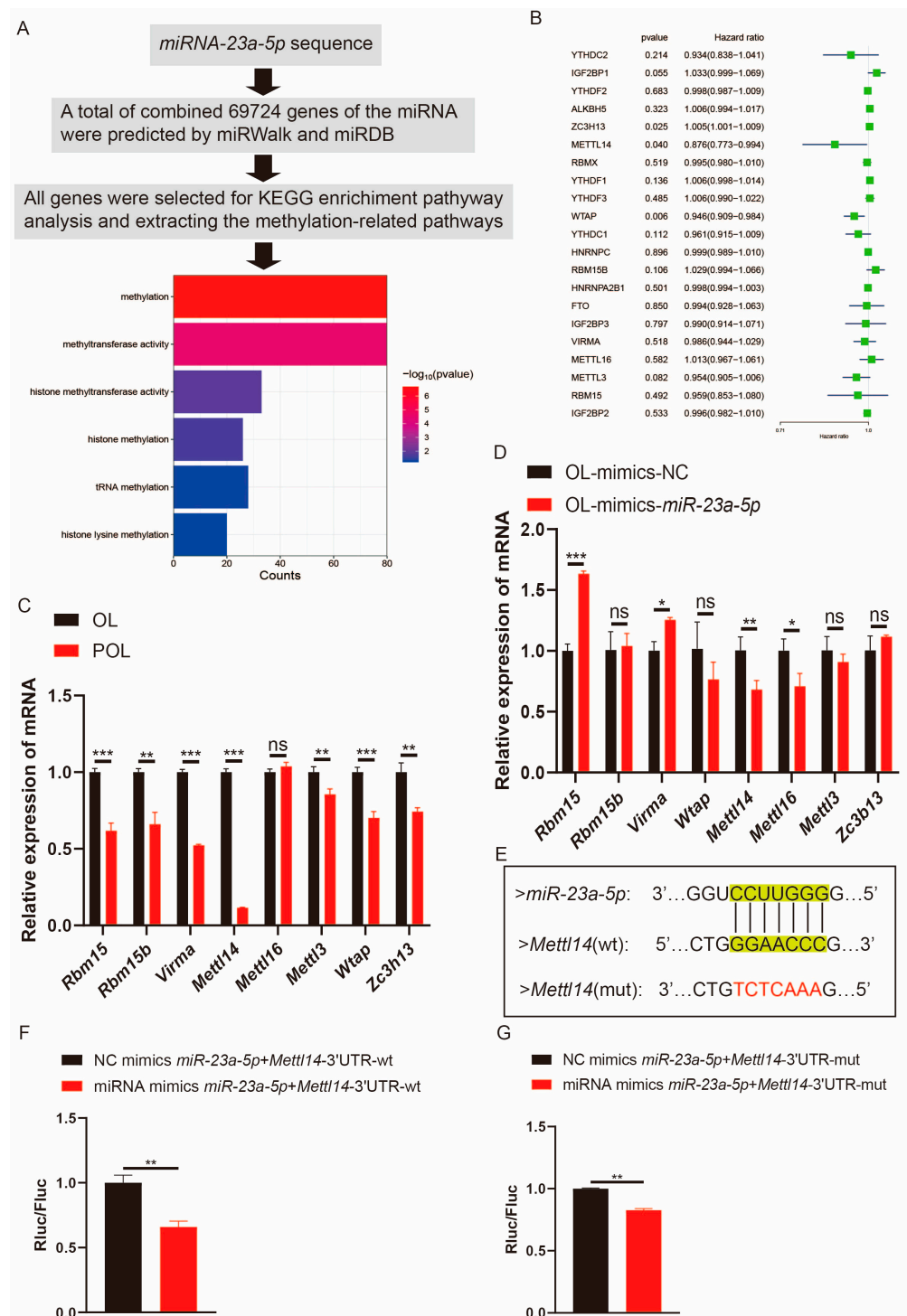


Figure 4. *miR-23a-5p* influences the expression levels of m6A-regulatory genes within OL cells. (A) Employing the sequence-binding characteristics of *miR-23a-5p* and data from databases, a schematic representation was deduced to illustrate the methylation research; (B) an analysis was conducted to ascertain the differential expression patterns of m6A-related genes in OL and POL cells, utilizing sequencing data for insights; (C) qRT-PCR experiments were performed to validate the differential expression profiles of m6A-related genes between OL and POL cells (D) and the impact of *miR-23a-5p* on the expression of m6A-related genes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); (E) sequence alignment of *miR-23a-5p*, *Mettl14*-wt, and *Mettl14*-mut genes. Yellow highlights indicate the predicted *miR-23a-5p* binding site on *Mettl14*(wt), and red nucleotides denote the mutated bases in *Mettl14*(mut); (F,G) dual luciferase assay of *miR-23a-5p* with *Mettl14*-wt (F) and *Mettl14*-mut (G). Data are shown in means $\pm$ SEM. Student's *t*-test was used to determine statistical significance.

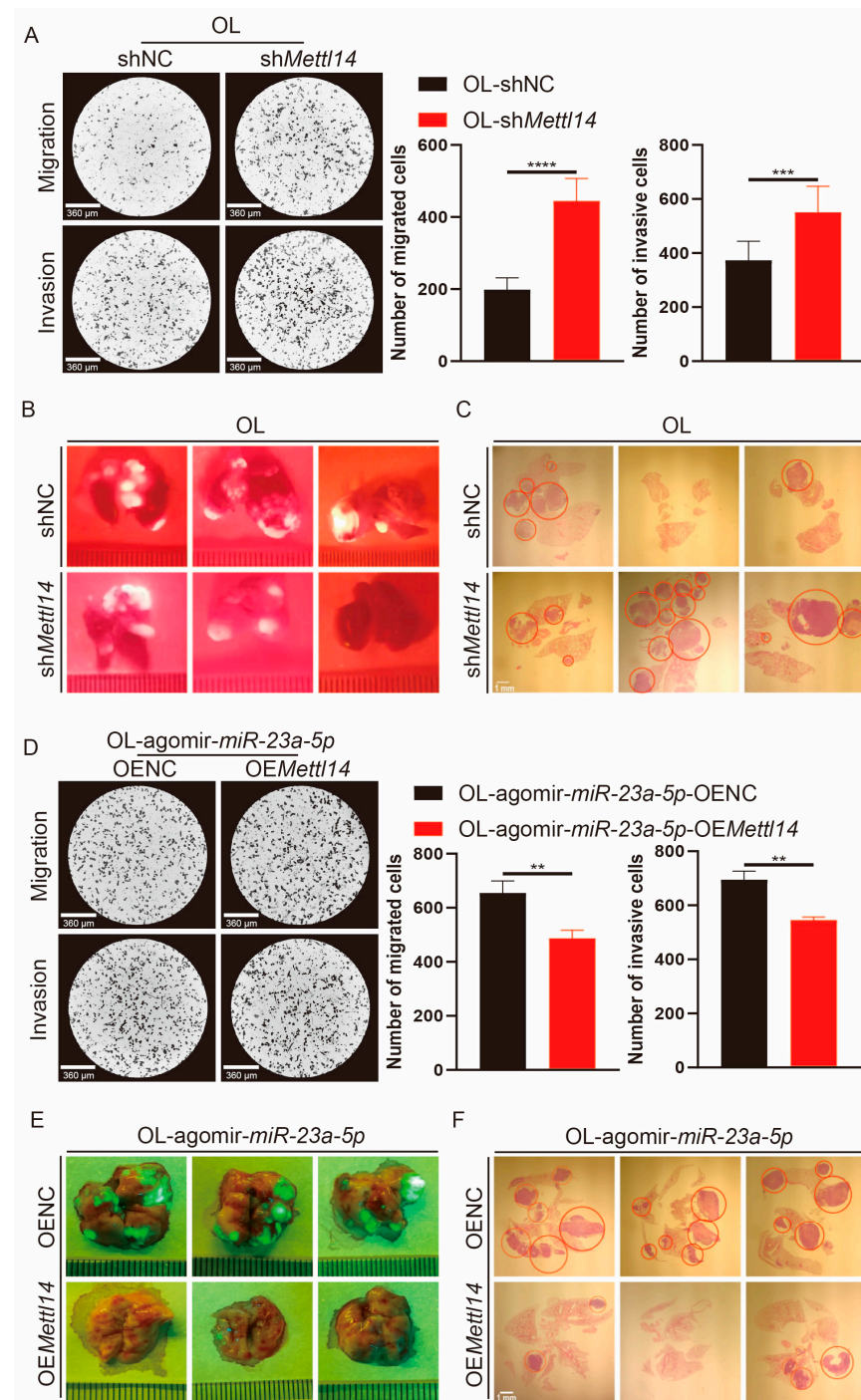


Figure 5. *Mettl14* inhibits the occurrence and progression of melanoma tumors in vitro and in vivo. (A) The Transwell assay was employed to assess the impact of *Mettl14* knockdown on cell migration and invasion capabilities (bar = 360 μ m, *** $p < 0.001$, **** $p < 0.0001$). (B,C) H&E staining was conducted to meticulously examine and meticulously document the distinguishing features of metastatic foci within the lung tissue (scale bar: The black interval lines are scale reference marks; the distance between two lines is 1 mm (B), 1 mm (C)). (D) The Transwell assay was employed to assess the impact of the concomitant overexpression of *miR-23a-5p* and *Mettl14* on the migratory and invasive capacities of the cells (bar = 360 μ m, ** $p < 0.01$). Data are shown in means $\pm$ SEM. Student's *t*-test was used to determine statistical significance. (E,F) H&E staining of lung metastatic lesions (scale bar: The black interval lines are scale reference marks; the distance between two lines is 1 mm (E), 1 mm (F)).

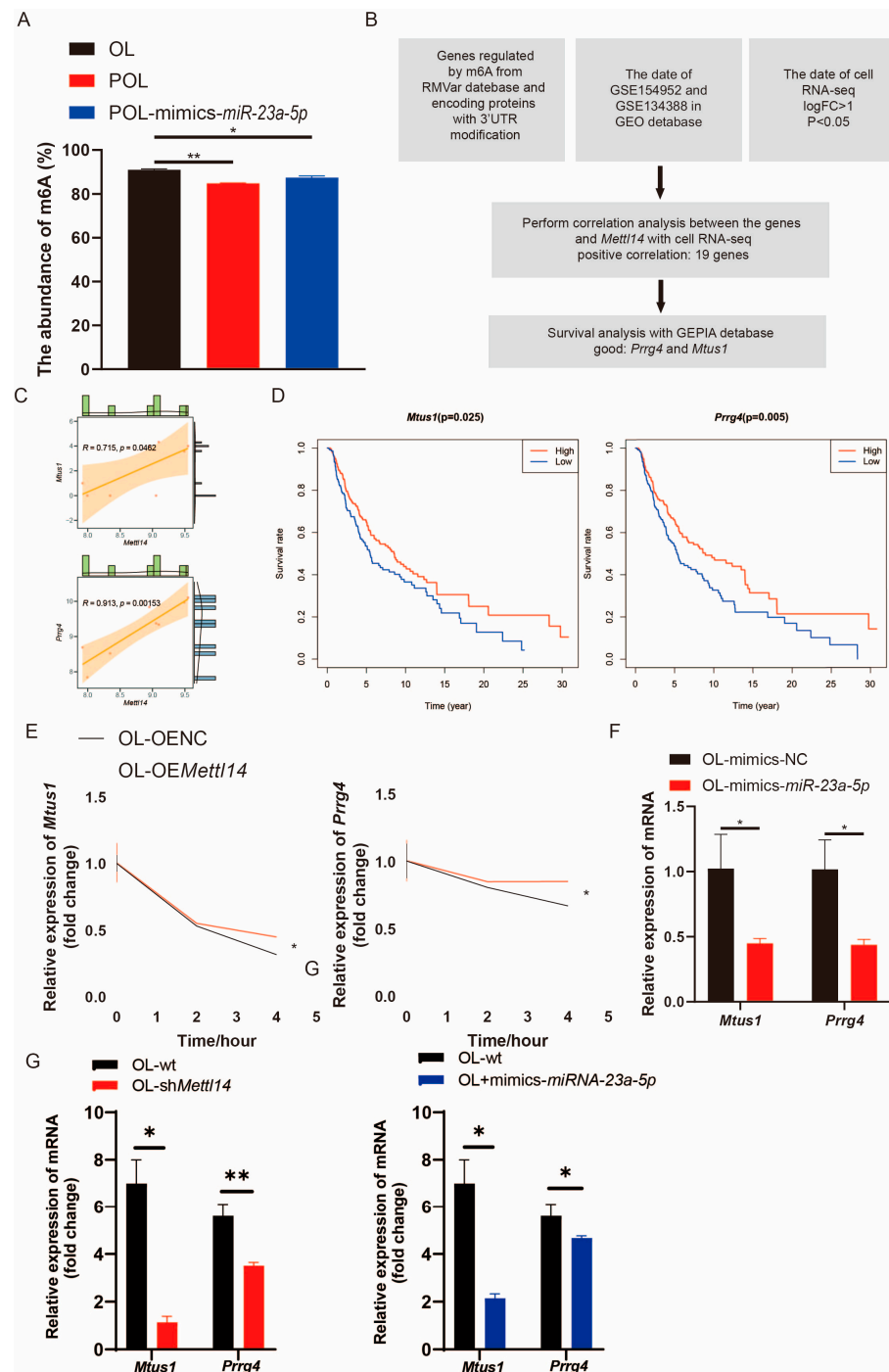


Figure 6. *Mettl14* inhibits the occurrence and progression of melanoma tumors in vitro and in vivo. (A) The m6A modification enrichment levels in OL cells, POL cells, and cells with overexpressed miRNA in the POL context were assessed. (B) Illustrative flowchart for identifying m6A target genes. (C) The expression correlation between *Mettl14*, *Mtus1*, and *Prrg4* in cellular sequencing data. The yellow areas represent the 95% confidence intervals. (D) Evaluation of survival prognosis in melanoma patients via the markers *Mtus1* and *Prrg4*. (E) Investigation of the influence of *Mettl14* on mRNA stability in *Mtus1* and *Prrg4* via actinomycin D experiments. Data are shown in means $\pm$ SEM. Student's *t*-test was determined statistical significance. (F) qRT-PCR technology was employed to validate the impact of *Mettl14* on the expression levels of *Mtus1* and *Prrg4* (* $p < 0.05$). (G) RIP assay was performed in OL-wt, OL-shMettl14, and OL cells transfected with miR-23a-5p mimics to assess the interaction of m6A with *Mtus1* and *Prrg4* under the modulation of *Mettl14* and miR-23a-5p (* $p < 0.05$, ** $p < 0.01$). Data are shown in means $\pm$ SEM. Student's *t*-test was used to determine statistical significance.

4. Discussion

Metastatic melanoma's rising incidence and variable prognosis are largely influenced by EVs, which disseminate factors like miRNAs that foster tumor progression by altering the recipient cell's microenvironment. While earlier studies targeted tumor impacts on angiogenesis and cell inhibition, the role of EV-mediated intercellular tumor communication remains underexplored [22–24]. This study illuminates EVs' influence on the metastatic potential of tumor cells, particularly how *miR-23a-5p* released from POL cells affects m6A levels and inhibits tumor suppressors *Mtus1* and *Prrg4* in OL cells, boosting their metastatic and invasive properties. The findings suggest therapeutic potentials in melanoma via *miR-23a-5p* or methylation interventions.

Research indicates that tumor EVs facilitate signal transduction between tumor and non-tumor cells, thereby driving the reconfiguration of the tumor microenvironment [27]. Stem cells can modulate the tumor microenvironment through EVs, contributing to drug resistance in cancer therapy [28]. Furthermore, EVs from tumor cells can induce M2 polarization in macrophages, thereby enhancing tumor invasion by altering the tumor microenvironment [29,30]. However, this article delves into the relationship between highly invasive and low invasive cells within tumor cells. It underscores that while the tumor microenvironment's significance lies in interactions between tumor and normal cells, it also holds a crucial role within the tumor itself.

m6A modulation exhibits a bi-directional effect in tumors [31], acting to either promote cancer progression by influencing the translation of specific proteins or inhibit tumor growth through the regulation of distinct mechanisms [32–35]. This dual functionality of m6A is evident in its varied impact on different types of cancer, as it governs the expression of downstream genes. For instance, research has demonstrated that m6A can suppress melanoma development by modulating the expression levels of certain key proteins [36]. In our study, we investigate how m6A regulates the expression of downstream genes to inhibit the progression of melanoma.

Our studies on *miR-23a-5p* in EVs found that it significantly advances melanoma metastasis. Due to this role, *miR-23a-5p* holds promise as a biomarker for diagnosis, with miRNA mimics offering potential for extracellular vesicle diagnostics. Most notably, targeting *miR-23a-5p* could offer a therapeutic intervention for treating melanoma. Restoring *Mettl14*-dependent m6A on *Mtus1* and *Prrg4* may impede melanoma progression. If *miR-23a-5p* and reduced methylation promote tumor growth, further methylation modulation of *Mtus1* and *Prrg4* could amplify therapeutic efficacy.

While our study elucidates a novel EV–miRNA–m6A axis in melanoma metastasis, several limitations should be acknowledged. First, the experiments were conducted primarily in a single genetic background using M14-derived cell lines, which may not fully capture the heterogeneity of melanoma. Second, the reliance on immunodeficient tail vein metastasis models focuses on late metastatic stages and does not recapitulate the early steps of the metastatic cascade within an intact immune microenvironment. Third, the absence of patient-derived samples or clinical validation limits the direct translation of our findings to human disease. Finally, our mechanistic insights are limited by the reliance on total RNA m6A measurements, as we did not directly assess the m6A status of the two downstream tumor suppressor gene transcripts. Consequently, the observed correlations between global m6A modification levels and target gene stability remain indirect and require further locus-specific validation to establish causality. Addressing these limitations in future studies will strengthen the biological and clinical relevance of the proposed pathway.

5. Conclusions

In summary, this study demonstrates that highly metastatic melanoma cells enhance the aggressiveness of low metastatic cells by delivering *miR-23a-5p* via extracellular vesicles. Mechanistically, *miR-23a-5p* downregulates the m6A methyltransferase METTL14, leading to reduced m6A modification of the tumor suppressors *Mtus1* and *Prrg4*. This results in destabilization and increased degradation of *Mtus1* and *Prrg4* mRNA, thereby downregulating their expression and ultimately contributing to the cancer-promoting effects of *miR-23a-5p*. These findings reveal a new EV–miRNA–m6A axis that promotes melanoma metastasis through intercellular communication, highlighting *miR-23a-5p* and m6A modulation as potential targets for diagnosis and therapy.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers18050792/s1>, Document S1: Original blot images.

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Abbreviations

The following abbreviations are used in this manuscript:

m6A	N6-methyladenosine
RNA	Ribonucleic acid
miR	microRNA
EVs	Extracellular vesicles
OL cells	Low metastatic cells
POL cells	Highly metastatic cells
mRNA	Message RNA
CAFs	Cancer-associated fibroblasts
TEM	Transmission electron microscopy
qRT-PCR	Quantitative reverse transcription PCR
FISH	Fluorescence in situ hybridization
H&E	Hematoxylin and Eosin
NCBI	National Center for Biotechnology Information

GEO	Gene Expression Omnibus
GO	Gene Ontology
RIP	RNA Immunoprecipitation
GFP	Green Fluorescent Protein
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PBS	Phosphate buffer saline
NTA	Nanoparticle Tracking Analysis
PVDF	Polyvinylidene fluoride
RIPA buffer	Radio Immunoprecipitation Assay

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