Exosomal miR-106a-5p accelerates the progression of nasopharyngeal carcinoma through FBXW7-mediated TRIM24 degradation

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Abstract

Nasopharyngeal carcinoma (NPC) is prevalent in East Asia and causes increased health burden. Elucidating the regulatory mechanism of NPC progression is important for understanding the pathogenesis of NPC and developing novel therapeutic strategies. Nasopharyngeal carcinoma and normal tissues were collected. Nasopharyngeal carcinoma cell proliferation, migration, and invasion were evaluated using CCK-8, colony formation, wound healing, and transwell assays, respectively. A xenograft mouse model of NPC was established to analyze NPC cell growth and metastasis in vivo. The expression of miR-106a-5p, FBXW7, TRIM24, and SRGN was determined with RT-qPCR and Western blot. MiR-106a-5p, TRIM24, and SRGN were upregulated, and FBXW7 was downregulated in NPC tissues and cells. Exosomal miR-106a-5p could enter NPC cells, and its overexpression promoted the proliferation, migration, invasion, and metastasis of NPC cells, which were suppressed by knockdown of exosomal miR-106a-5p. MiR-106a-5p targeted FBXW7 to regulate FBXW7-mediated degradation of TRIM24. Furthermore, TRIM24 regulated SRGN expression by binding to its promoter in NPC cells. Suppression of exosomal miR-106a-5p attenuated NPC growth and metastasis through the FBXW7-TRIM24-SRGN axis in vivo. Exosomal miR-106a-5p accelerated the progression of NPC through the FBXW7-TRIM24-SRGN axis. Our study elucidates novel regulatory mechanisms of NPC progression and provides potential exosome-based therapeutic strategies for NPC.

Keywords

exosome, FBXW7-TRIM24-SRGN axis, MiR-106a-5p, nasopharyngeal carcinoma
1 | INTRODUCTION

Nasopharyngeal carcinoma (NPC), also known as nasopharynx cancer, is the most common type of cancer that occurs in the nasopharynx. Nasopharyngeal carcinoma is a rare cancer globally, but its incidence remains high in some regions including Southern China, although the incidence has declined these years. Because of concealed localization and inconspicuous symptoms, NPC is not easy to be detected at the early stage. Therefore, many patients have advanced carcinoma at the time of initial diagnosis. Although great improvement has been made in prognosis thanks to the advance of therapies such as intensity-modulated radiation therapy, the therapeutic effects are still far from satisfactory for patients with advanced cancer. Hence, elucidating regulatory mechanisms of NPC progression is key for understanding the pathogenesis and developing novel therapeutic strategies.

Exosomes, originating from endosomes, are tiny extracellular vesicles of approximately 30 to 200 nm in diameter which are released by cells. Exosomes contain abundant cell constituents including nucleic acids, lipids, proteins, and glycoconjugates and enter recipient cells to deliver these constituents. Intriguingly, exosome-mediated intercellular communication is implicated in the pathogenesis of various diseases such as inflammatory diseases and cancers. Extracellular vesicles used in this study are exosomes according to the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018). Importantly, exosomes emerge as key regulators in the development and progression of NPC. Nasopharyngeal carcinoma–associated exosomes contribute to cancer cell apoptosis, proliferation, and immune tolerance. In addition, exosomes can promote epithelial-mesenchymal transition and tumor metastasis in NPC. In recent years, miRNAs delivered by exosomes have been well acknowledged to exert vital functions in regulating cancer progression. Intriguingly, due to the instability of miRNAs, exosome is considered an effective and safe delivery vehicle for miRNA in cancer treatment. Lin et al. reported that exosomal miRNAs accelerated the epithelial-mesenchymal transition and metastasis of hepatic cancer. MiR-10b delivered by exosomes enhanced the invasive capacity of breast cancer cells. Exosomal miR-106a-5p was reported to regulate 5-FU chemoresistance in gastric cancer. However, the role of exosomal miR-106a-5p in NPC is largely unknown.

F-box and WD repeat domain-containing 7 (FBXW7), a subunit of the E3 ubiquitin ligase complex, promotes protein ubiquitylation and degradation. FBXW7 has been widely believed to act as a tumor suppressor via targeting many key oncoproteins and promoting their ubiquitylation and degradation. Thompson and colleagues found that FBXW7 suppressed the progression of T cell acute lymphoblastic leukemia through targeting NOTCH1, c-Myc, and cyclin E and promoting their degradation. FBXW7 attenuated the metastasis of gastric cancer via promoting the degradation of Brg1. Chow et al. firstly reported Fbxw7 mutation in NPC, but its role in NPC is unclear. In recent studies, Zhang et al. found that FBP1 suppressed glycolysis through the FBXW7/mTOR axis, thus enhancing radiosensitivity of NPC cells. Nasopharyngeal carcinoma–derived extracellular vesicles promoted angiogenesis by delivering miR-144 to regulate the FBXW7/HIF-1α/VEGF-A axis. MiR-106a was found to target FBXW7 and suppress its expression to regulate the progression of hepatocellular carcinoma. However, the interaction between miR-106a-5p and FBXW in NPC has not been reported.

In summary, we sought to explore the roles of exosome-derived miR-106a-5p in NPC and underlying regulatory mechanisms. In the present study, we demonstrated that exosomal miR-106a-5p accelerated the progression of NPC by targeting FBXW7, inhibiting FBXW7-mediated TRIM24 degradation, and enhancing the expression of TRIM24 and SRGN. Our study not only elucidates novel exosomal miRNA-mediated regulation of NPC progression but also provides potential exosome-based therapeutic strategies.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens

Tumor tissues from thirty patients diagnosed with NPC by biopsy from January, 2016 to June, 2017 and thirty normal nasopharyngeal tissues were collected at the Hainan Affiliated Hospital of Hainan Medical University, which were stored at −80°C for subsequent analysis of the expression of miR-106a-5p, FBXW7, TRIM24, and SRGN. All NPC patients had primary NPC and did not receive any antitumor treatment. Clinical pathological characteristics of patients are shown in Table 1.

2.2 | Cell culture and treatment

Human NPC cell lines CNE2, HNE1, HNE2, HONE1, C666-1, SUNE-1, nasopharyngeal epithelial NP69 cells, and 293T cells provided by the National Collection of Authenticated Cell Cultures were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, HyClone) in a cell incubator. Cancer-associated fibroblasts (CAFs) and primary...
tumor cells isolated from tumor tissues were cultured in DMEM with 10% FBS. The coculture of CAFs and NPC cells was performed with 0.4 μm transwell membranes (Corning) as previously described. For GW4869 treatment, CAFs were treated with DMSO or GW4869 (Sigma) at 20 μM prior to coculture with NPC cells. For MG132 and cycloheximide (CHX) treatment, cells were treated with MG132 (CST) at 10 μM or CHX (Sigma) at 50 mg/ml.

2.3 Cell transfection

The coding region of FBXW7 was inserted into the lentiviral vector pHAGE-fEF1a (Addgene) for FBXW7 overexpression in HONE1 and SUNE-1 cells. Empty lentiviral vector was used as a negative control. TRIM24 shRNA (shTRIM24) lentiviral and negative control (NC) shRNA (shNC) particles were purchased from Sigma and transduced into HONE1 and SUNE-1 cells following the manual. miR-106a-5p mimics, mimics NC, miR-106a-5p inhibitor, and inhibitor NC were bought from RiboBio (GenePharma) and transduced into CAFs with Lipofectamine RNAiMAX (ThermoFisher).

2.4 Conditioned medium (CM) preparation

Conditioned medium was prepared as previously described with minor modification. Briefly, 3 × 10⁶ primary NPC, nasopharyngeal epithelial cells or CAFs were seeded and cultured for 24 hours, and medium was replaced with fresh DMEM without serum. After 48 hours, CM was collected and centrifuged for 10 minutes at 3000 g. For depleting exosomes in CM, CM was centrifuged at 400 g for 20 minutes, 2000 g for 20 minutes, and 11,000 g for 60 minutes successively. Conditioned medium was treated with RNase A (3 μg/ml, Sigma) or RNase A in combination with Triton X-100 (0.1%, Beyotime) for analyzing miR-106a-5p expression.

2.5 Exosome isolation and characterization

Isolation of exosomes from various CMs were performed by differential centrifugation. In brief, CMs were successively centrifuged at 300 g for 10 minutes, 2000 g for 20 minutes, 10,000 g for 30 minutes, and 100,000 g for 70 minutes. For exosome characterization,
the concentration and size distribution of exosomes were determined by dynamic light scattering (DLS) with NanoSight NS300. The size and morphology of exosomes were confirmed by transmission electron microscopy (TEM). Isolated exosomes were adsorbed on latex beads (4 µM, ThermoFisher), stained with the CD63-PE antibody (BioLegend) and analyzed by flow cytometry.

2.6 Iodixanol density gradient centrifugation

Iodixanol density gradient centrifugation was performed as previously described. In brief, OptiPrep™ iodixanol (STEMCELL) was diluted to 20%, 10%, and 5%, which were layered in centrifugation tubes to form a discontinuous gradient. Conditioned medium (2 ml) was mixed thoroughly with 1 ml of 60% iodixanol, resulting in a mixture of 40% iodixanol. Subsequently, the mixture of 40% iodixanol was layered onto the discontinuous gradient and centrifuged at 100,000 g for 18 hours. Fractions of density gradient layers were collected.

2.7 Real-time quantitative reverse-transcription PCR (RT-qPCR)

Total RNA was isolated from NPC and normal tissues and cells using TRIzol reagent (ThermoFisher). MiRNA was extracted using a mir-Premier microRNA isolation kit (Merck). After quantification, RNA and miRNA were reversely transcribed into cDNA with a SuperScript VILO cDNA synthesis kit (ThermoFisher) and a miScript II RT kit (QIAGEN), respectively. The relative expression of miR-106a-5p, FBXW7, TRIM24, and SRGN was examined by qPCR and calculated with the 2^{ΔΔCt} method. GAPDH (for FBXW7, TRIM24, and SRGN) and U6 snRNA (for miR-106a-5p) were used as normalization controls. Primers used here are listed in Table 2.

2.8 Western blot

Cells with various transfection or treatment and exosomes were lysed in SDS lysis buffer (Beyotime). Supernatants were collected after centrifugation at 4°C at 12,000 g for 20 minutes and quantified using a BCA kit from Santa Cruz. A total of 40 µg of protein was electrophoresed and transferred to polyvinylidene fluoride (PVDF) membranes (GE). Membranes were blocked and incubated with primary antibodies against TFIIB (1:1000), LaminA/C (1:800), Calnexin (1:500), CD9 (1:1000), CD63 (1:500), CD81 (1:500), Hsp70 (1:2000), TSG101 (1:1000), TRIM24 (1:2000), SRGN (1:1000), FBXW7 (1:1000), Myc (1:3000), β-actin (1:5000), or GAPDH (1:5000) for 3 hours at room temperature. Membranes were subsequently washed and incubated with HRP-labeled secondary antibodies for 1 hour. All antibodies were obtained from Abcam. Bands were visualized by ECL substrates (Bio-Rad), and the intensity was analyzed with ImageJ.

2.9 Cell Counting Kit-8 (CCK-8) assay

HONE1 and SUNE-1 cells were cultured in 96-well plates at 37°C for 0, 24, 48, or 72 hours. Then, culture medium was replaced with 100 µl of medium, and 10 µl of CCK-8 reagents (Abcam) were added per well. After incubation at 37°C for 3 hours, the absorbance at 490 nm was recorded.

2.10 Colony formation

HONE1 and SUNE-1 cells were seeded at 1 × 10^3 cells per well in six-well plates and cultured at 37°C for 2 weeks. Subsequently, cell colonies were rinsed and fixed in 4% paraformaldehyde solution, which were stained using 1% crystal violet solution (Sigma). Cell colonies were imaged and counted with ImageJ.

2.11 Wound healing assay

The wound-healing assay kit (Abcam) was used for assessing cell migration. Briefly, inserts were oriented to ensure same direction in every plate. A total of 500 µl of cell suspension (0.5 × 10^6 cells/ml) was added to each well carefully. Cells were incubated at 37°C in a cell incubator overnight. Next day, inserts were removed carefully and slowly. Cells were washed carefully, and culture medium was added. After 24 hours, the healing of wound was monitored under a light microscope (BX51, Olympus).

2.12 Transwell assay

The invasive capacity of NPC cells was evaluated by transwell assays using transwell chambers from BD. Briefly, the matrix gel was coated on the upper chamber, and HONE1 and SUNE-1 cells were seeded on it. After incubation for 24 hours, invasive cells which penetrated

| Table 2 | RT-qPCR primers in this study |
|---------|-------------------------------|
| **miR-106a-5p** | Forward: 5'-GATGCTCAAAAAGTGCTTACAGTGCA-3' |
| | Reverse: 5'-TATGGTTGTTCCTCTGTCCTC-3' |
| **FBXW7** | Forward: 5'-AAAGAGTTGTAGGCTTCTCG-3' |
| | Reverse: 5'-CCACATGGATACCATACAACTG-3' |
| **TRIM24** | Forward: 5'-TGCTGAAGACACTAGGTT-3' |
| | Reverse: 5'-GCCTGATACAGTCTTGCAG-3' |
| **SRGN** | Forward: 5'-TCCAAGAGATCCCCCTGCT-3' |
| | Reverse: 5'-TTCCGTTAGAAGGCACTCC-3' |
| **U6 snRNA** | Forward: 5'-CGCTTGGCAGCACATATACAAATGGG-3' |
| | Reverse: 5'-CGCTTCCAGAATTTCGTCA-3' |
| **GAPDH** | Forward: 5'-GTCTCCTCTGACTCAACAGCG-3' |
| | Reverse: 5'-ACACCCTGTGCTGATGGCA-3' |
to the lower surface were fixed and stained in 1% crystal violet solution (Sigma) followed by imaging using a light microscope (BX51, Olympus).

### 2.13 Ubiquitination analysis of TRIM24

TRIM24 was immunoprecipitated from FBXW7-overexpressing HONE1 and SUNE-1 cells and loaded for electrophoresis. Subsequently, TRIM24 was transferred to PVDF membranes and incubated with an anti-ubiquitin antibody (ab137025, Abcam) overnight. Next day, membranes were incubated with an HRP-labeled secondary antibody for 1 hour. Ubiquitination was visualized by ECL substrates (Bio-Rad), and the intensity was analyzed with ImageJ.

### 2.14 Dual-luciferase reporter assay

Mutant binding sites for TRIM24 in the promoter of SRGN were inserted into the pGL3 vector (Promega). Wild-type and mutant binding sites for miR-106a-5p in the 3’UTR of FBXW7 were cloned into the pmirGLO vector (Promega), and 293T cells were cotransfected with the mutant TRIM24 reporter and the FBXW7-overexpressing pcDNA3.1 vector. HUNE1 and SUNE-1 cells were cotransfected with the FBXW7 reporter and miR-106a-5p mimics. Empty vectors and miRNA mimics NC were used as negative controls. Forty-eight hours post transfection, cells were collected, and the Dual-Glo luciferase assay system (Promega) was used to analyze luciferase activity.

### 2.15 Co-Immunoprecipitation (Co-IP)

SUNE-1 cells were lysed, and supernatants were collected. A FBXW7 antibody or normal IgG isotype was precoated on magnetic beads, which were incubated with supernatants at 4°C overnight. Besides, 293T cells were transfected with flag-TRIM24 and/or the myc-FBXW7 vector. Cells were lysed, and supernatants were collected. A flag antibody was added and incubated for 16 hours at 4°C. Anti-FLAG magnetic beads (Sigma) were added and incubated for 1 hour. Proteins were recovered and the abundance of TRIM24 and FBXW7 was examined by Western blot.

### 2.16 RNA immunoprecipitation (RIP)

HONE1 and SUNE-1 cells were cotransfected with wild-type or mutant MS2-binding sequences (MS2bs)-FBXW7 3’UTR (WT/MUT) or MS2bs-Rluc mock vector and MS2-binding protein (MS2bp)-GFP constructs. After 48 hours, cells were lysed, and supernatants were collected after centrifugation. The rabbit GFP antibody (Abcam) was precoated on magnetic beads, added, and incubated at 4°C overnight. RNA was subsequently recovered with Trizol reagent (ThermoFisher) followed by quantitation using RT-qPCR.

### 2.17 Chromatin immunoprecipitation (ChIP)

Cells were cross-linked in 1% formaldehyde and lysed. DNA fragments were obtained by sonicating the cell lysates, and the length of DNA fragments was confirmed by running an agarose gel. Subsequently, DNA fragments were immunoprecipitated by a TRIM24 antibody or normal IgG isotype. DNA was recovered and quantified with RT-qPCR.

### 2.18 A xenograft mouse model of NPC

Eight- to ten-week-old male BALB/c nude mice were obtained from the Animal Center of the Hainan Affiliated Hospital of Hainan Medical University and kept in a specific pathogen-free facility. For subcutaneous inoculation, HONE1 and SUNE-1 cells were subcutaneously injected into the left flanks (1 × 10^6 cells per mouse). After 14 days, mice were sacrificed, and tumors were excised. Tumors were weighed, and tumor volume was quantified with the formula length × width^2/2. For intravenous injection, HONE1 and SUNE-1 cells were intravenously injected into mice (1 × 10^6 cells per mouse). After 21 days, mice were sacrificed, and lungs were excised for calculating the number of metastatic nodules and histopathology staining. For injecting a mixture of NPC cells and CAFs, 5 × 10^5 CAFs were mixed with NPC cells and injected into mice.

### 2.19 Statistical analysis

Results in our study were repeated at least three times and shown as mean ± SD. Student’s t test was used for analyzing the variance of two groups. The comparison of more than two groups was performed by One-Way Analysis of Variance (ANOVA); p < 0.05 was statistically significant. SPSS Statistics 24 (IBM) was used for statistical analysis.

### 3 RESULTS

#### 3.1 Abnormal expression of miR-106a-5p, FBXW7, TRIM24, and SRGN in NPC patients and cells

A recent study reported that exosomal miR-106a-5p was implicated in cisplatin resistance and tumorigenesis of NPC, but its roles and underlying mechanisms are still poorly understood. FBXW7 has been identified as a downstream target of miR-106a. In addition, our preliminary bioinformatic analysis suggested that FBXW7 might interact with TRIM24, and TRIM24 might bind to the promoter of SRGN. To explore their implication in NPC, the expression of miR-106a-5p, FBXW7, TRIM24, and SRGN in NPC and normal nasopharyngeal tissues were assessed. We found increased expression of miR-106a-5p, TRIM24, and SRGN and decreased FBXW7 expression in tumor tissues (Figure 1A).
was significantly correlated with clinical staging, recurrence, and distant metastasis rather than age, gender, smoking history, and pathological type (Table 1). We analyzed their expression in NP69 nasopharyngeal epithelial cells and NPC cells including CNE2, HNE1, HNE2, HONE1, C666-1, and SUNE-1. Compared with NP69 cells, NPC cells showed increased expression of miR-106a-5p, TRIM24, and SRGN and decreased FBXW7 expression (Figure 1B). These results suggested that miR-106a-5p, FBXW7, TRIM24, and SRGN might be implicated in NPC progression.

3.2 Exosome-derived miR-106a-5p entered NPC cells

To investigate whether miR-106a-5p is derived from exosomes, exosomes were harvested from CMs derived from NPC and normal nasopharyngeal tissues. The diameter of exosomes was approximately 30-150 nm determined by DLS (Figure 2A). Transmission electron microscopy imaging showed that exosomes were predominantly around 120 nm in diameter (Figure 2B). Moreover, fewer exosomes were isolated from normal nasopharyngeal tissues, NPC-derived exosomes exhibited high expression of CD63 (Figure 2C). In addition, Transcription factor II B (TFIIB) and Lamin A/C, which are enriched in nucleus33,34 and the endoplasmic reticulum marker calnexin, were found in NPC cell lysates but not in exosomes (Figure 2D). Conversely, exosome markers CD9, CD63, and CD8135 were found in exosomes but not in cell lysates (Figure 2D). Although Hsp70 and TSG101 were observed both in cell lysates and exosomes, exosomes showed higher expression (Figure 2D). Nasopharyngeal carcinoma-derived CAFs were transfected with Cy3-labeled miR-106a-5p mimics, and exosomes were isolated and cocultured with SUNE-1 cells. We observed that CAF exosome-derived Cy3-conjugated miR-106a-5p could enter SUNE-1 cells (Figure 2E). Moreover, RNase treatment did not change the level of miR-106a-5p, but treatment with RNase A in combination with Triton X-100 obviously reduced miR-106a-5p in NPC-derived CM (NPC-CM) and normal nasopharyngeal cells (N-CM) (Figure 2F).

**FIGURE 1** Abnormal expression of miR-106a-5p, FBXW7, TRIM24, and SRGN in nasopharyngeal carcinoma (NPC) tissues and cells. A, RT-qPCR analysis of SRGN, TRIM24, miR-106a-5p, and FBXW7 in NPC (n = 30) and normal tissues (n = 30). B, RT-qPCR analysis of miR-106a-5p and Western blotting analysis of SRGN, TRIM24, and FBXW7 in NP69, CNE2, HNE1, HNE2, HONE1, C666-1, and SUNE-1 cells (n = 3). **p < 0.01 and ***p < 0.001
Exosome-derived miR-106a-5p entered nasopharyngeal carcinoma (NPC) cells. A, The size of exosomes isolated from conditioned media (CMs) derived from NPC and normal cells was examined by dynamic light scattering (DLS). B, Transmission electron microscopy (TEM) analysis of exosomes derived from normal nasopharyngeal and NPC tissues. C, CD63 expression on exosomes was examined by flow cytometry. D, Protein levels of TFIIb, LaminA/C, CD9, CD63, Hsp70, and TSG101 in cell lysates and exosomes. E, Exosomes were isolated from CAFs transfected with Cy3-labeled miR-106a-5p mimics (red) and incubated with SUNE-1 cells. DAPI (blue) and phalloidin (green) were used for nuclear and F-actin staining (magnification, 500×; scale bar, 20 μm). F, RT-qPCR analysis of miR-106a-5p in N-CM and NPC-CM treated with RNase A or RNase A plus Triton X-100 (n = 3). G, RT-qPCR analysis of miR-106a-5p in N-CM, NPC-CM, exosomes from N-CM or NPC-CM (CM-exo), and exosome-depleted CM (CM-exo free, n = 3). H, RT-qPCR analysis of miR-106a-5p in SUNE-1 cells cultured in control N-CM (Ctrl CM), NPC-CM plus miR-106a-5p inhibitor NC (NPC-anti-miR-NC CM), NPC-CM plus miR-106a-5p inhibitor (NPC-anti-miR-106a-5p CM), NPC-CM plus miR-106a-5p inhibitor/GW4869 (NPC-anti-miR-106a-5p GW4869 CM), and NPC-CM exo free plus miR-106a-5p inhibitor (NPC-anti-miR-106a-5p CM exo free). **p < 0.01, and *** p < 0.001.
raising the possibility that miR-106a-5p might originate from exosomes. Then, we analyzed the abundance of miR-106a-5p in CM, exosomes isolated from CM (CM-exo), and exosome-depleted CM (CM-exo free). Consistently, NPC-CM contained higher level of miR-106a-5p than N-CM (Figure 2G). Conditioned medium (CM) and CM-exo showed similar levels of miR-106a-5p, but CM-exo free showed significantly reduced miR-106a-5p (Figure 2G), which suggested that miR-106a-5p in CM was mainly derived from exosomes. In addition, we found that miR-106a-5p expression was higher in the NPC-anti-miR-NC CM group than in control CM-treated SUNE-1 cells (Ctrl CM), but it was reduced in the NPC-anti-miR-106a-5p CM group (Figure 2H). Simultaneous GW4869 treatment, an inhibitor of exosome generation, further decreased miR-106a-5p expression, and NPC-anti-miR-106a-5p CM-exo free exhibited reduced miR-106a-5p expression (Figure 2H). MiR-106a-5p was highly expressed in the NPC-miR-106a-5p CM group, which was significantly reduced in the NPC-miR-106a-5p GW4869 and NPC-miR-106a-5p CM exo free groups (Figure 2H). To conclude, these observations demonstrated that exosome-derived miR-106a-5p could enter NPC cells.

3.3 | CAF-derived exosome-mediated miR-106a-5p delivery accelerated NPC progression

To explore whether exosome-derived miR-106a-5p regulates NPC progression, NPC-derived CAFs were transfected with miR-106a-5p mimics or miR-106a-5p inhibitor, and exosomes were isolated from CAF-CM and cocultured with HONE1 or SUNE-1 cells. High expression of miR-106a-5p was observed in HONE1 and SUNE-1 cells co-cultured with exosomes from CAFs transfected with miR-106a-5p mimics (Figure 3A), which suggested that miR-106a-5p expression was higher in the NPC-anti-miR-NC CM group than in control CM-treated SUNE-1 cells (Ctrl CM), but it was reduced in the NPC-anti-miR-106a-5p CM group (Figure 2H). Simultaneous GW4869 treatment, an inhibitor of exosome generation, further decreased miR-106a-5p expression, and NPC-anti-miR-106a-5p CM-exo free exhibited reduced miR-106a-5p expression (Figure 2H). MiR-106a-5p was highly expressed in the NPC-miR-106a-5p CM group, which was significantly reduced in the NPC-miR-106a-5p GW4869 and NPC-miR-106a-5p CM exo free groups (Figure 2H). To conclude, these observations demonstrated that exosome-derived miR-106a-5p could enter NPC cells.

FIGURE 3 Cancer-associated fibroblast (CAF)-derived exosomal miR-106a-5p accelerated the progression of nasopharyngeal carcinoma (NPC). Nasopharyngeal carcinoma–derived CAFs were transfected with mimics NC, miR-106a-5p mimics, inhibitor NC, or miR-106a-5p inhibitor. Exosomes were isolated and cocultured with HONE1 or SUNE-1 cells. A, RT-qPCR analysis of miR-106a-5p (n = 3). B, Cell proliferation analysis with CCK-8 (n = 3). C, Colony formation of HONE1 and SUNE-1 cells (magnification, 1×; n = 3). D, The migration of HONE1 and SUNE-1 cells was assessed with wound-healing assays (magnification, 20×; scale bar, 500 µm; n = 3). E, The invasion of HONE1 and SUNE-1 cells was evaluated by transwell assays (magnification, 50×; scale bar, 200 µm; n = 3). F, HONE1 and SUNE-1 cells were subcutaneously inoculated into nude mice. Excised tumors were imaged, measured, and weighed (n = 4). G, HONE1 and SUNE-1 cells were intravenously injected into nude mice. Lungs were excised and imaged. Metastatic nodules were calculated, and H&E staining was performed for evaluating lung damage (magnification, 200×; scale bar, 50 µm; n = 4). H, Protein levels of FBXW7, TRIM24, and SRGN. *p < 0.05, **p < 0.01, and ***p < 0.001.
of miR-106a-5p (Figure 3D and E). HONE1 and SUNE-1 cells were cocultured with miR-106a-5p-overexpressing or knockdown CAF-derived exosomes and subcutaneously inoculated into nude mice. Overexpression of miR-106a-5p promoted tumor volume and weight, but knockdown of miR-106a-5p dramatically reduced tumor volume and weight, suggesting that exosome-derived miR-106a-5p accelerated NPC growth (Figure 3F). Aforementioned HONE1 and SUNE-1 cells were intravenously injected into nude mice. We observed that overexpression of miR-106a-5p significantly increased pulmonary metastatic nodules and exacerbated lung damage, but miR-106a-5p knockdown reduced pulmonary metastatic nodules and attenuated lung injury (Figure 3G), which implied that miR-106a-5p enhanced NPC metastasis. Taken together, these data demonstrated that exosome-mediated miR-106a-5p transfer accelerated NPC progression. Compared with controls, miR-106a-5p-overexpressing CAF-derived exosomes enhanced the expression of TRIM24 and SRGN and inhibited FBXW7 expression, whereas MiR-106a-5p-knockdown CAF-derived exosomes suppressed the expression of TRIM24 and SRGN and promoted FBXW7 expression (Figure 3H), indicating that these molecules might be involved in exosomal miR-106a-5p-mediated regulation of NPC. We also examined the role of exosomal miR-106a-5p in NP69 cells. The expression of miR-106a-5p was increased in NP69 cells cocultured with exosomes from CAFs transfected with miR-106a-5p mimics but decreased in NP69 cells cocultured with exosomes from CAFs transfected with miR-106a-5p inhibitor (Figure S1A). Similarly, exosomal miR-106a-5p enhanced the proliferation, colony formation, migration, and invasion in NP69 cells, and these effects were suppressed by knockdown of exosomal miR-106a-5p (Figure S1B-E). Moreover, exosomal miR-106a-5p transferred tumorigenic phenotypes to NP69 cells, but no obvious tumorigenic phenotypes were observed in NP69 cells cocultured with exosomes from CAFs transfected with miR-106a-5p inhibitor (Figure S1F-G). TRIM24 and SRGN were upregulated and FBXW7 was downregulated in NP69 cells cocultured with miR-106a-5p-overexpressing CAF-derived exosomes, whereas miR-106a-5p-knockdown CAF-derived exosomes reduced the expression of TRIM24 and SRGN and promoted FBXW7 expression (Figure S1H). These results revealed that exosomal miR-106a-5p from CAFs could transfer tumorigenic phenotypes to NP69 cells.
3.4 | Exosomal miR-106a-5p reversed TRIM24 knockdown–mediated suppression of NPC progression

To investigate whether TRIM24 is involved in exosomal miR-106a-5p–mediated regulation of NPC, TRIM24 was silenced in HONE1 and SUNE-1 cells. TRIM24 expression was markedly reduced by shTRIM24 transfection, which was restored by incubation with exosomes derived from miR-106a-5p–overexpressing CAFs. Cells transfected with shNC showed higher expression of TRIM24 after incubation with miR-106a-5p–overexpressing CAF-derived exosomes, suggesting that exosomal miR-106a-5p enhanced TRIM24 expression (Figure 4A). Knockdown of TRIM24 suppressed the proliferation, colony formation, migration, and invasive capacity of HONE1 and SUNE-1 cells, but these effects were abrogated by miR-106a-5p–overexpressing CAF-derived exosomes (Figure 4B-E). Exosomes apparently promoted the proliferation, colony formation, migration, and invasion in shNC-transfected cells (Figure 4B-E). Additionally, the size, volume, and weight of subcutaneous tumors, pulmonary metastatic nodules, and lung damage were obviously decreased by knockdown of TRIM24, which were reversed by incubation with miR-106a-5p–overexpressing CAF-derived exosomes (Figure 4F-G). Protein levels of TRIM24 and SRGN were reduced in HONE1 and SUNE-1 cells by knockdown of TRIM24 but enhanced after incubation with exosomes (Figure 4H). Knockdown of TRIM24-mediated suppression was abolished by miR-106a-5p–overexpressing CAF-derived exosomes (Figure 4H). These findings demonstrated that exosomal miR-106a-5p accelerated NPC progression by promoting TRIM24 expression.

3.5 | TRIM24 regulated SRGN expression by binding to its promoter in NPC cells

To explore whether TRIM24 targets SRGN in NPC cells, three potential binding sites (BS1-3) for TRIM24 in the promoter of SRGN were predicted using JASPAR (http://jaspar.genereg.net/, Figure 5A). Chromatin immunoprecipitation assays showed that TRIM24 was recruited to BS1 but not BS2 and BS3 (Figure 5B). Moreover, luciferase activity was fully restrained in cells cotransfected with TRIM24 and mutated BS2 and BS3 reporters, but unaffected in cells transfected with mutated BS1 (Figure 5C). These data indicated that TRIM24 bound to the promoter of SRGN and promoted its expression in NPC cells.

3.6 | Exosomal miR-106a-5p reversed FBXW7-mediated alleviation of NPC progression

As FBXW7 was downregulated in NPC tumor tissues, FBXW7 was overexpressed in HONE1 and SUNE-1 cells to investigate its role in NPC. FBXW7 was overexpressed by transfection of the FBXW7-overexpressing vector, but it was reversed by exosome-derived CAFs with miR-106a-5p overexpression (Figure 6A). CAF-derived exosomes inhibited FBXW7 expression in HONE1 and SUNE-1 cells (Figure 6A). Overexpression of FBXW7 restrained the proliferation, colony formation, migration, and invasion of HONE1 and SUNE-1 cells (Figure 6B-E). MiR-106a-5p–overexpressing CAF-derived exosomes reversed FBXW7-mediated suppression and promoted NPC cell proliferation, colony formation, migration, and invasion (Figure 6B-E). Besides, overexpression of FBXW7 reduced the size,
volume, and weight of subcutaneous tumors and attenuated pulmonary metastasis and lung damage (Figure 6F-G). MiR-106a-5p-overexpressing CAF-derived exosomes facilitated tumor growth and pulmonary metastasis and abolished FBXW7-mediated suppressive effects on NPC progression (Figure 6F-G). The expression of TRIM24 and SRGN were decreased in FBXW7-overexpressing HONE1 and SUNE-1 cells, and CAF-derived exosomes reversed and enhanced their expression (Figure 6H). Collectively, exosomal miR-106a-5p repressed FBXW7 expression to regulate NPC progression and TRIM24/SRGN might be downstream targets of FBXW7 in NPC.

3.7 MiR-106a-5p targeted FBXW7 and regulated FBXW7-mediated degradation of TRIM24

To explore the relationship among miR-106a-5p, FBXW7, and TRIM24, we predicted a potential binding site for miR-106a-5p in the 3′UTR of FBXW7 using StarBase (http://starbase.sysu.edu.cn/, Figure 7A). The luciferase activity was suppressed in HONE1 and SUNE-1 cells cotransfected with miR-106a-5p mimics and wild-type FBXW7 luciferase reporters, but it was unchanged in cells transfected with mutant reporters (Figure 7B). We also found that the enrichment of miR-106a-5p to wild-type 3′UTR of FBXW7 was dramatically increased, but no obvious enrichment to mutant 3′UTR of FBXW7 was observed (Figure 7C), implying that miR-106a-5p targeted FBXW7 to regulate its expression in NPC cells. Subsequently, we performed Co-IP assays to investigate the interaction of FBXW7 and TRIM24 and found that TRIM24 was immunoprecipitated by FBXW7 in SUNE-1 cells (Figure 7D). In turn, FBXW7 was also immunoprecipitated by TRIM24 in 293T cells (Figure 7D), indicating the direct interaction between FBXW7 and TRIM24. As FBXW7 plays an important role in mediating protein ubiquitination and degradation, 37 293T cells were transfected with Myc-TRIM24 and treated with MG132. MG132 treatment suppressed TRIM24 degradation (Figure 7E), suggesting that TRIM24 degradation might be ubiquitination dependent. TRIM24 degradation was slowed down in cells treated with
MG132 in the presence of CHX (Figure 7F). Overexpression of FBXW7 accelerated TRIM24 degradation in the presence of CHX in SUNE-1 cells (Figure 7G). Besides, immunoprecipitated TRIM24 from FBXW7-overexpressing HONE1 and SUNE1 cells exhibited increased ubiquitination (Figure 7H). Thus, miR-106a-5p targeted FBXW7 to suppress its expression, and FBXW7 interacted with TRIM24 to mediate TRIM24 ubiquitination and degradation in NPC.
FIGURE 8 Inhibition of exosomal miR-106a-5p attenuated nasopharyngeal carcinoma (NPC) growth and metastasis through the FBXW7-TRIM24-SRGN axis in vivo. HONE1 or SUNE-1 cells were transfected with NC mimics or miR-106a-5p mimics, or cancer-associated fibroblasts (CAFs) were transfected with NC inhibitor or miR-106a-5p inhibitor or treated with GW4689 and subsequently mixed with HONE1 or SUNE-1 cells. HONE1 or SUNE-1 cells or the mixture of CAFs and HONE1 or SUNE-1 cells were subcutaneously or intravenously inoculated into nude mice. A, Excised tumors were imaged, measured, and weighed (n = 4). B, Lungs were excised and imaged. Metastatic nodules were calculated, and H&E staining was performed for evaluating lung damage (magnification, 200×; scale bar, 50 µm; n = 4). C, Protein levels of FBXW7, TRIM24, and SRGN in tumor tissues. *p < 0.05, **p < 0.01, and ***p < 0.001.
Inhibition of exosomal miR-106a-5p attenuated NPC growth and metastasis through the FBXW7-TRIM24-SRGN axis in vivo

HONE1 or SUNE-1 cells were transfected with miR-106a-5p mimics, and CAFs were transfected with miR-106a-5p inhibitor or treated with GW4689. Subsequently, CAFs were mixed with HONE1 or SUNE-1 cells prior to subcutaneous and intravenous injection into nude mice. The tumors formed by HONE1 or SUNE-1 cells with miR-106a-5p overexpression or exosome-derived CAFs with NC inhibitor grew faster, and tumor growth was significantly suppressed in mice injected with NPC cells plus CAFs transfected with miR-106a-5p inhibitor or treated with GW4689 (Figure 8A). In addition, miR-106a-5p overexpression or CAFs with NC inhibitor exacerbated pulmonary metastasis and damage, but CAFs with miR-106a-5p knockdown or GW4689 treatment reduced pulmonary metastasis and damage (Figure 8B). FBXW7 was downregulated and TRIM24 and SRGN were upregulated in tumors formed by NPC cells or miR-106a-5p overexpression (Figure 8C). Conversely, CAFs with miR-106a-5p knockdown or GW4689 treatment enhanced FBXW7 expression and suppressed the expression of TRIM24 and SRGN (Figure 8C). To conclude, inhibition of exosomal miR-106a-5p restrained NPC growth and metastasis through the FBXW7-TRIM24-SRGN axis in vivo.

4 | DISCUSSION

Nasopharyngeal carcinoma shows obvious regional distribution, and Southern China has the highest incidence. Thanks to the development of radiotherapy and chemoradiotherapy, the prognosis of patients has been greatly improved in recent years.38,39 However, curative effects for patients with distant metastasis are still poor.40 Understanding the pathogenesis of NPC and...
regulatory mechanism is essential for seeking novel therapies. In this study, we found that the expression of miR-106a-5p, TRIM24, and SRGN were increased and FBXW7 was downregulated in NPC tissues and cells. Furthermore, exosomal miR-106a-5p promoted NPC progression and reversed TRIM24 knockdown and FBXW7 overexpression–mediated suppression of NPC progression. Suppression of exosomal miR-106a-5p attenuated NPC growth and metastasis in vivo. We firstly demonstrated that exosomal transfer of miR-106a-5p accelerated NPC progression via targeting FBXW7 to reduce its abundance, thus inhibiting FBXW7-mediated degradation of TRIM24 and subsequently enhancing SRGN expression (Figure 9).

MiR-106a-5p exerts antitumor function or acts as an oncogene in various human cancers.41 MiR-106a-5p was downregulated in renal cell carcinoma (RCC), and it restrained the migration and invasion of RCC cells by binding to the 3′-UTR of PAK5 and reducing its abundance.42 Paradoxically, miR-106a-5p was highly expressed in glioblastoma tissues and cells, and it accelerated the proliferation and invasion of glioblastoma cells via targeting APC and activating the Wnt/β-catenin signaling.43 These apparent paradoxical functions, tumor suppressor and oncogene, might be ascribed to the imperfect binding of miRNA to its targets and various tumor microenvironment. Zheng et al. reported that miR-106a-5p could be sponged by SMAD5–AS1, and its overexpression suppressed tumorigenesis by targeting SMAD5 in NPC,44 suggesting antitumor function of miR-106a-5p in NPC. In contrast, Zhu et al. reported that miR-106a-5p was highly expressed in NPC tissues and cell lines including CNE-1, CNE-2, and 5-8F, and miR-106a-5p inhibited autophagy and accelerated malignant phenotypes in NPC. These opposing results suggested further exploration on the role of endogenous miR-106a-5p in NPC.45 Intriguingly, we firstly found that exosome-derived miR-106a-5p functioned as an oncogene to promote NPC progression in this study, identifying a novel role of exosomal miR-106a-5p in NPC.

Exosomes carry various components, such as RNA, lipid, and protein, and deliver them to target cells, which plays essential roles in intercellular communication and regulating physiological and pathological processes. In recent years, exosome-mediated miRNA delivery has attracted much attention in regulating cancer progression. Lung cancer–derived exosomes were loaded with miR-23a and targeted ZO-1, thus promoting vascular permeability and cancer cell migration.48 Exosomal miR-103 derived from hepatoma cells enhanced vascular permeability and accelerated cancer metastasis through suppressing the expression of junction proteins.48 Therefore, exosomes released by cancer cells have become an important regulator in tumor cell invasion, metastasis, and tumor microenvironment remodeling via delivering miRNAs. Importantly, exosomes have become a rising star in drug delivery for cancer treatment thanks to excellent biocompatibility and low immunogenicity.49 Exosome-mediated delivery of drug has been shown to significantly suppress cancer progression.50 Based on our data in this study, exosome-mediated miR-106a-5p inhibitor might be potentially developed as a novel therapy for NPC treatment in future.

MiRNA negatively regulates gene expression via targeting downstream mRNAs through base pairing.54 MiR-106a was reported to target FBXW7 in hepatocellular carcinoma.55 Increased miR-106a-5p expression and decreased FBXW7 expression were observed in NPC tissues from patients in this study, suggesting the potential interaction between miR-106a-5p and FBXW7 in NPC. Luciferase activity and RIP assays confirmed that miR-106a-5p targeted FBXW7 to inhibit its expression in NPC. FBXW7 is an important tumor suppressor in many cancers via mediating the ubiquitylation and degradation of oncogenic proteins. In NPC, FBXW7 enhanced cisplatin chemosensitivity via reducing MRP expression.55 TRIM24 is identified as an oncogenic transcription cofactor in various cancers including prostate cancer and breast cancer.56,57 However, there is no direct evidence supporting that FBXW7 targets TRIM24. For the first time, we proved that FBXW7 targeted TRIM24 to induce its degradation in NPC. Serglycin was overexpressed in NPC and exerted vital function in regulating NPC metastasis.58 Consistently, we observed high expression of SRGN in NPC tissues and cells and identified SRGN as a novel target of TRIM24 in NPC. Collectively, we demonstrated that exosomal miR-106a-5p regulated NPC progression through the FBXW7–TRIM24–SRGN axis.

To summarize, we firstly demonstrated that exosomal miR-106a-5p accelerated NPC progression through suppressing FBXW7 expression and FBXW7-mediated TRIM24 degradation and increasing the expression of TRIM24 and SRGN. Our study not only highlights exosomal miR-106a-5p–mediated regulation of NPC progression, but also provides potential therapeutic strategies and targets for NPC treatment. In order to do this, further investigations are needed for elucidating the regulation in detail. Moreover, further studies are ongoing for evaluating whether plasma exosomal miR-106a-5p predicts prognosis in NPC.

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DISCLOSURE

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

The study was approved by the Ethics Committee of the Hainan Affiliated Hospital of Hainan Medical University. All patients were informed and provided written consent. All animal procedures were in accordance with National Institutes of Health guidelines and approved by the Animal Care and Use Committee of Hainan Affiliated Hospital of Hainan Medical University.

CONSENT FOR PUBLICATION

Informed consent was obtained from study participants.
DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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