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Vps4A Functions as a Tumor Suppressor by Regulating the Secretion and Uptake of Exosomal MicroRNAs in Human Hepatoma Cells

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Supporting Information

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Abstract

The deregulation of microRNAs (miRNAs) plays an important role in human hepatocarcinogenesis. In this study, we highlight exosomes as mediators involved in modulating miRNA profiles in hepatocellular carcinoma (HCC) cells. First, we examined the different miRNA expression profiles in HCC cells and HCC cell-derived exosomes. Next, coculture experiments indicated that HCC cell-derived exosomes promoted the cell growth, migration, and invasion of HCC cells and had the ability to shuttle miRNAs to recipient cells. Further, our data showed that Vps4A, a key regulator of exosome biogenesis, was frequently down-regulated in HCC tissues. The reduction of Vps4A in HCC tissues was associated with tumor progression and metastasis. In vitro studies revealed that Vps4A repressed the growth, colony formation, migration, and invasion of HCC cells. We further investigated the role and involvement of Vps4A in suppressing the bioactivity of exosomes and characterized its ability to weaken the cell response to exosomes. By small RNA sequencing, we demonstrated that Vps4A facilitated the secretion of oncogenic miRNAs in exosomes as well as accumulation and uptake of tumor suppressor miRNAs in cells. A subset of Vps4A-associated miRNAs was identified. Kyoto Encyclopedia of Genes and Genomes pathway analysis indicated that the phosphatidylinositol-3-kinase/Akt signaling pathway was the most likely candidate pathway for modulation by these miRNAs. Indeed, we proved that the phosphatidylinositol-3-kinase/Akt pathway was inactivated by Vps4A overexpression.

Conclusion—Exosome-mediated miRNA transfer is an important mechanism of self-modulation of the miRNA expression profiles in HCC cells, and Vps4A may function as a tumor suppressor, which utilizes exosomes as mediators to regulate the secretion and uptake of miRNAs in hepatoma cells; these observations provide new insights into the development of HCC.

The incidence of hepatocellular carcinoma (HCC), the second most frequent cause of cancer-related death worldwide, accounting for 90% of all primary liver neoplasias, is increasing.¹ Recent studies have demonstrated that microRNAs (miRNAs) can act as oncogenes or tumor suppressors, and miRNA dysregulation may play an important role in cancer onset and progression.^{2, 3} Transcriptional and epigenetic regulations may play a major role in miRNA expression.⁴ However, the molecular mechanisms involved in disorders associated with miRNAs remain unclear.

In recent years, miRNAs within exosomes have gained significant attention due to their potential role in delineating the molecular pathogenesis of cancer.^{5, 6} Exosomes are small (40-100 nm) cell-derived extracellular vesicles that initially form with the maturation of multivesicular bodies as intraluminal vesicles (ILVs).⁷ These ILVs are released in the extracellular medium by fusion of the limiting membrane with the plasma membrane and are thereafter known as “exosomes.”⁷ Cancer cells secrete exosomes that function in an autocrine or paracrine manner to modulate the tumor microenvironment.⁸⁻¹⁰ Accumulating evidence indicates that exosomes are involved in cell-cell communication by delivering proteins and nucleic acids to recipient cells, thereby impacting the biological processes in recipient cells.^{7, 11} However, little is known about whether exosomes can function as mediators by self-regulation of miRNA expression profiles.

Cargo sorting of ILVs (exosomes) depends on the endosomal sorting complex required for transport (ESCRT) machinery.¹² Loss-of-function mutations in ESCRT components lead to

disorders of cargoes within ILVs, which could lead to alterations in several signaling pathways.^{13, 14} Membrane neck cleavage mediated by ESCRT-III is crucial for the formation of ILVs, which is regulated by Vps4.¹⁵ In humans, there are two paralogs of the yeast Vps4, Vps4A and Vps4B, both of which exhibit functions that are similar to that of the protein in yeast. Recent studies have shown that Vps4 is responsible for hepatitis B virus budding and the release of infectious hepatitis C virus particles.^{16, 17} Rodahl et al. reported that disruption of Vps4 and c-Jun N-terminal kinase function could cause tumor growth in *Drosophila*.¹⁸ Until now, the role of Vps4 in human cancer development has been poorly understood.

In this study, we demonstrated that Vps4A is frequently down-regulated in HCC tissues. Ectopic expression of Vps4A led to significant repression of HCC growth and metastasis. Our study provides mechanistic evidence that Vps4A interferes with the biological activity of exosomes and the cell response to exosomes through affecting exosomal miRNA secretion and uptake. Overexpression of Vps4A can lead to inactivation of the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. These results implicate Vps4A as a novel tumor suppressor for HCC.

Materials and Methods

Cell Lines and Culture

The human HCC cell lines Hep3B and Huh-7 were obtained from the American Type Culture Collection (Manassas, VA) and the Japanese Collection of Research Bioresources (Tokyo, Japan), respectively. GP2-293 cells were purchased from Clontech (BD Biosciences, San Jose, CA). SMMC-7721 cells were purchased from the China Center for Type Culture Collection (Wuhan, China). These cell lines were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum and 1% antibiotic-antimycotic (Beyotime, Haimen, China), at 37°C with 5% CO₂. For all studies, vesicle-depleted medium was prepared by centrifuging cell-culture medium at 110,000g overnight to spin down any preexisting vesicular content.⁹

Isolation of Exosomes

The HCC cells (1×10^6) were plated in 12 mL of vesicle-depleted medium on 10-cm dishes.⁹ After 48-72 hours, the medium was collected and sequential centrifugation was performed.¹⁹ The medium was first centrifuged at 300g for 10 minutes and at 2000g for 20 minutes at 4°C to remove cells. The supernatant was then centrifuged at 10,000g for 70 minutes at 4°C. The supernatant was further treated with ExoQuick-TC (System Biosciences, Mountain View, CA) for final exosome isolation according to the manufacturer's protocol. The exosome pellet was dissolved in 500 μ L of 1 $\times$ phosphate-buffered saline.

Electron Microscopy

The morphology of exosomes was examined by transmission electron microscopy as described.²⁰ In brief, exosomes were fixed in 1% glutaraldehyde. A 2- μ L drop of the suspension was loaded onto Formvar carbon-coated electron microscopy copper grids and

allowed to stand for 2 minutes at room temperature. Excess fluid was drawn off with a piece of Whatman filter paper. The sample was then negatively stained on ice with a 2- μ L drop of 1% uranyl acetate for 2 minutes and allowed to dry under an electric incandescent lamp for 10 minutes before viewing in a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) operated at 120 kV.

Details regarding 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[lissamine rhodamine B sulfonyl] (N-Rh-PE) labeling exosomes, imaging of miRNA transfer, western blot, RNA isolation, small RNA library preparation and sequencing, bioinformatics analysis, patients and specimens, generation of plasmid constructs, stably expressing Vps4A clones, cell proliferation, colony formation, migration, invasion, *in vivo* tumorigenicity, immunohistochemical staining, and statistical analysis are provided in the Supporting Information.

Results

Exosome Isolation and Identification

We isolated tumor cell–derived nanoparticles from culture supernatant of HCC cells (SMMC-7721, Hep3B, and Huh-7) in culture by multistep ultracentrifugation. Transmission electron microscopy showed membrane-limited particles that were homogeneous in appearance and ranged 50–100 nm in size (Fig. 1A). Based on western blot analysis, the isolated particles expressed markers associated with exosomes (Alix, Tsg101, CD63, Hsp90 α/β , and Hsp60) (Fig. 1B). Thus, these nanovesicles have the characteristics of exosomes and could be isolated in a consistent manner.

The miRNA Expression Profiles in HCC Cells and Exosomes Are Different

To investigate the difference in miRNA expression profiles between HCC cells and exosomes, we conducted small RNA (<40 nt) sequencing for SMMC-7721 cells and exosomes derived from these cells. An overview of miRNA expression profiles is provided in Fig. 1C. The miRNAs occurring in SMMC-7721 cells and the exosomes released by these cells were ranked based on the clean reads data, and the 10 most prevalent miRNAs in each population were compared (Table 1). Certain miRNAs, such as miR-423-5p and miR-21-5p, were abundant in both cellular and exosomal RNAs. However, several highly abundant cellular miRNAs, such as let-7d-5p, let-7b-5p, and let-7c-5p, occurred only in very low abundance in exosomal RNAs. Conversely, the highly abundant exosomal miRNAs (miR-486-5p and miR-10b-5p) were less abundant in cellular RNAs. These data indicated that the packaging of miRNAs into the exosomes was a selective process.

HCC Cell–Derived Exosomes Promote Proliferation and Metastasis of the Parental HCC Cells

To investigate the “bioactive” effect of exosomes in HCC cells, we conducted cell growth and transwell assays. Incubation of SMMC-7721 cells with self-derived exosomes (10 μ g/mL) caused a notable increase in cell growth (Fig. 2A), migration (Fig. 2B, Supporting Fig. S1A), and invasion (Fig. 2C, Supporting Fig. S1B). Similar results were observed in Hep3B cells and the corresponding exosomes (Fig. 2A–C, Supporting Fig. S1A,B). These

data indicate that HCC cell–derived exosomes can promote the biological behavior of the parental HCC cells.

HCC Cell–Derived Exosomes Can Shuttle miRNAs to Recipient Cells

To test whether HCC cell–derived exosomes had the ability to shuttle miRNAs to recipient cells, we conducted imaging experiments to visualize the transfer of labeled miRNAs between cells. First, SMMC-7721 cells were cotransfected with fluorescein amidite–labeled miR-21-5p and Cy5-labeled miR-122-5p. After 24 hours, N-Rh-PE, a fluorescent phosphatidyl ethanolamine analogue, was used to label exosomes. After 24 hours, the culture supernatant was collected for exosome isolation. Then, a new dish of SMMC-7721 cells was incubated with 10 μ g of isolated exosomes for 24 hours. We found that N-Rh-PE-labeled exosomes had been endocytosed and that the two fluorescent miRNAs colocalized in exosomes, as visualized by fluorescence microscopy (Fig. 2D). This result indicated that exosomes derived from HCC cells can transport miRNAs to the recipient cells.

Down-Regulation of Vps4A Is Significantly Associated With Tumor Progression, Metastasis, and Worse Prognosis

Using immunohistochemistry, we examined the protein expression of Vps4A in tumor and adjacent nontumor tissues of 113 primary HCC patients and normal liver tissues of 22 patients with benign liver diseases. We found that Vps4A protein expression was significantly down-regulated in primary HCCs compared with the paired adjacent nontumor tissues ($P < 0.01$) and normal liver tissues ($P < 0.01$) (Fig. 3A,B). Low Vps4A expression was correlated with hepatitis B viral infection ($P < 0.05$), TNM stage ($P < 0.05$), tumor size ($P < 0.05$), tumor capsule integrity ($P < 0.05$), and regional lymph node metastasis ($P < 0.05$) (Supporting Table S1). Kaplan-Meier plots revealed an association of lower Vps4A expression with shorter recurrence-free survival ($P = 0.001$) (Fig. 3C). Multivariate Cox regression analysis further confirmed Vps4A down-regulation as an independent risk factor for recurrence-free survival (hazard ratio = 3.054, $P < 0.001$) (Supporting Table S2).

Ectopic Expression of Vps4A Represses HCC Cell Growth, Colony Formation, Migration, and Invasion

To confirm whether Vps4A has a tumorsuppressive function, we asked whether overexpression of Vps4A in SMMC-7721 and Hep3B cells can suppress tumor growth. Overexpression of Vps4A in SMMC-7721 and Hep3B cells was confirmed by western blot analysis (Fig. 4A). Overexpression of Vps4A significantly decreased HCC cell growth and colony formation in SMMC-7721 and Hep3B cells (Fig. 4B,C; Supporting Fig. S2A). Additionally, overexpression of Vps4A in SMMC-7721 and Hep3B cells significantly suppressed the migration (Fig. 4D, Supporting Fig. S2B) and invasion ability (Fig. 4E, Supporting Fig. S2C) of the tumor cells.

Vps4A Inhibits Tumor Formation in BALB/c Nude Mice

The subcutaneous tumor growth of SMMC-7721 or Hep3B cells stably transfected with Vps4A or vector control in BALB/c nude mice is shown in Fig. 5A. The tumor volume was

significantly smaller in Vps4A-transfected BALB/c nude mice than in vector control mice ($P < 0.05$) (Fig. 5B). After 3 weeks, the tumors were taken out and weighed. The mean tumor weight was significantly lower in Vps4A-transfected BALB/c nude mice than in vector control mice ($P < 0.05$) (Fig. 5C).

Vps4A Inhibits the Biological Activity of HCC Cell-Derived Exosomes and the Cellular Response to Exosomes

To explore whether Vps4A interferes with the biological activity of HCC cell-derived exosomes, we isolated exosomes derived from SMMC-7721 and Hep3B cells stably expressing Vps4A (SMMC-Vps4Aexo and Hep3B-Vps4A-exo, respectively) using exosomes from vector control cells (SMMC-ctrl-exo and Hep3B-ctrl-exo, respectively) as controls. Our main aim was to examine the difference in bioactivity between Vps4A exosomes and control exosomes when cocultured with HCC cells. SMMC-7721 cells transfected with vector control (SMMC-ctrl) cocultured with SMMC-Vps4A-exo (10 $\mu\text{g/mL}$) showed decreased cell growth (Fig. 6A), migration (Fig. 6B, Supporting Fig. S3A), and invasion ability (Fig. 6C, Supporting Fig. S3B) compared to SMMC-ctrl cocultured with SMMC-ctrl-exo (10 $\mu\text{g/mL}$). Similar results were observed in Hep3B cells transfected with vector control (Hep3B-ctrl) when incubated with Hep3B-Vps4Aexo (10 $\mu\text{g/mL}$), using Hep3B-ctrl cocultured with Hep3B-ctrl-exo (10 $\mu\text{g/mL}$) as a control (Fig. 6A-C, Supporting Fig. S3A,B).

To further explore whether Vps4A modulates the cellular response to exosomes in HCC cells, we used SMMC-7721 and Hep3B cells stably expressing Vps4A (SMMC-Vps4A and Hep3B-Vps4A, respectively) in the research, with vector-transfected cells (SMMC-ctrl and Hep3B-ctrl, respectively) serving as controls. We aimed to investigate the diverse cellular response between Vps4A cells and control cells, when cocultured with HCC cell-derived exosomes. SMMC-Vps4A cocultured with SMMC-ctrl-exo (10 $\mu\text{g/mL}$) showed a notable reduction in cell growth (Fig. 6D), migration (Fig. 6E, Supporting Fig. S3C), and invasion ability (Fig. 6F, Supporting Fig. S3D) compared with SMMC-ctrl cocultured with SMMC-ctrl-exo (10 $\mu\text{g/mL}$). Similar results were found in Hep3B-Vps4A incubated with Hep3B-ctrl-exo (10 $\mu\text{g/mL}$) using Hep3B-ctrl cocultured with Hep3B-ctrl-exo (10 $\mu\text{g/mL}$) as a control (Fig. 6D-F, Supporting Fig. S3E,F).

Vps4A Overexpression Affects the Secretion and Uptake of Exosomal miRNAs in HCC Cells

To further explore whether Vps4A also interferes with miRNA profiles in HCC cells and corresponding exosomes, we examined the miRNA profiles of SMMCctrl, SMMC-Vps4A, SMMC-ctrl-exo, and SMMC-Vps4A-exo through small RNA sequencing. Two comparisons, SMMC-Vps4A versus SMMC-ctrl and SMMC-Vps4A-exo versus SMMC-ctrl-exo, were established. Based on the sequencing data, we selected two sets of differentially expressed miRNAs (number of reads >200 , false discovery rate <0.05 , fold change >1.5): exosomal and cellular (Supporting Table S3 and S4, respectively). In SMMC-Vps4A-exo versus SMMC-ctrl-exo, two oncogenic miRNAs (miR-27b-3p and miR-92a-3p) were up-regulated in SMMC-Vps4A-exo (Fig. 7A). In SMMC-Vps4A versus SMMC-ctrl, we observed the accumulation of three highly enriched tumor suppressor miRNAs

(miR-193a-3p, miR-320a, and miR-132-3p) in SMMC-Vps4A (Fig. 7B). These results indicated that overexpression of Vps4A facilitated not only the secretion of oncogenic miRNAs by exosomes but also the accumulation of tumor suppressor miRNAs in cells.

To further investigate the influence of Vps4A on the uptake of exosomal miRNAs by HCC cells, we cocultured SMMC-Vps4A with 10 $\mu\text{g/mL}$ SMMC-ctrl-exo (SMMC-Vps4A + SMMC-ctrl-exo) using SMMC-ctrl cocultured with 10 $\mu\text{g/mL}$ SMMC-ctrl-exo (SMMC-ctrl + SMMC-ctrl-exo) as a control. After an incubation period of 24 hours, the supernatant was depleted, and the cells were harvested for small RNA sequencing. In SMMC-Vps4A + SMMC-ctrl-exo versus SMMC-ctrl + SMMC-ctrl-exo, nine miRNAs were differentially expressed (number of reads >200, false discovery rate <0.05, fold change >1.5) according to the sequencing data (Supporting Table S5). Among them, six tumor suppressor miRNAs (miR-122-5p, miR-33a-5p, miR-34a-5p, miR-193a-3p, miR-16-5p, and miR-29b-3p) were significantly up-regulated in SMMC-Vps4A + SMMC-ctrl-exo compared with SMMC-ctrl + SMMC-ctrl-exo (Fig. 7C). In addition to miR-193a-3p, which was previously up-regulated in SMMC-Vps4A, another five miRNAs were newly up-regulated in SMMC-Vps4A + SMMC-ctrl-exo, indicating that Vps4A increased the uptake of tumor suppressor miRNAs from exosomes in HCC cells. These data suggest that Vps4A may be a key regulator in the secretion and uptake of exosomal miRNAs and that Vps4A exerted a tumor-suppressive effect.

Network Analysis of Vps4A-Correlated miRNAs

According to the above three sets of differentially expressed miRNAs, we selected 14 important cancer-related miRNAs (Supporting Table S6) for network analysis. The regulation networks among these miRNAs and their targets are shown in Fig. 7D.

The PI3K/Akt Signaling Pathway Appears to Be the Most Likely Candidate Pathway Modulated by Vps4A-Correlated miRNAs

We used the 14 selected miRNAs for Kyoto Encyclopedia of Genes and Genomes pathway analysis. The five most significant pathways were acquired (Supporting Table S7). The PI3K/Akt signaling pathway was believed to be the primary pathway regulated by Vps4A-correlated miRNAs. Targets and relevant miRNAs in the PI3K/Akt pathway are shown in Supporting Fig. S4.

Vps4A Overexpression Causes Inhibition of the PI3K/Akt Signaling Pathway in HCC Cells

To determine whether Vps4A could inhibit the PI3K/Akt signaling pathway, we tested the expression of some key members in this pathway in HCC cells by western blot. As we predicted, the results showed that overexpression of Vps4A led to increased expression of PTEN and down-regulation of PDK1, total Akt, phospho-Akt (Thr308), and phospho-Akt (Ser473) compared with control cells (Fig. 7E). In addition, we found that TSC2 was up-regulated and mTOR was down-regulated, which were the downstream molecules of Akt (Fig. 7E). These results prove that the PI3K/Akt signaling pathway, indicated as the most likely target pathway of Vps4A-associated miRNAs, is inactivated by overexpression of Vps4A in HCC cells.

Discussion

Over the past 10 years, Vps4 has been shown to regulate endosomal transport function in cell biology.^{21, 22} As a member of the AAA adenosine triphosphatase family, the activity of Vps4 appears to be an important point of regulation of the ESCRT machinery, which regulates the formation of ILVs (known as exosomes when released).^{22, 23} In addition, recent studies have shown that Vps4 is required for the release of hepatitis B and C viruses.^{16, 17} However, the function of Vps4 in HCC has not yet been examined. In this study, we found that Vps4A was frequently downregulated in primary HCC tissues, which was correlated with tumor progression, metastasis, and poor prognosis. Furthermore, we demonstrated that Vps4A, a potential tumor suppressor, could inhibit the growth, migration, and invasion of HCC cells in vitro and the tumorigenicity in vivo. However, the mechanism by which Vps4A exerts tumor-suppressive effects remains unclear. We also tested the expression of Vps4B in tumor and adjacent tissues from 61 HCC patients by real-time quantitative polymerase chain reaction. We found that Vps4B also had lower expression in HCC tissues (data not shown). The function of Vps4B in HCC is currently under investigation.

Previous reports have demonstrated that tumor-derived exosomes contribute to tumor progression and tumor cell migration in an autocrine or paracrine manner.^{8, 10} Indeed, our data showed that exosomes derived from HCC cells could boost the growth, migration, and invasion of parental cells. Considering the essential role of Vps4 in exosome biogenesis, we hypothesized that Vps4A interferes with the activity of exosomes. Nickerson et al. reported that regulators of Vps4 adenosine triphosphatase activity influenced the size and rate of formation of ILVs.²³ Interestingly, we found that Vps4A suppresses the biological activity of HCC cell-derived exosomes. Additionally, the recipient cell response to exosomes may be involved in the function of bioactive exosomes. As we assumed, HCC cells transfected with Vps4A were less sensitive to exosomes, which led to a decrease in the stimulation of cell growth, migration, and invasion. However, the mechanism by which Vps4A modulates exosome activity and the recipient cell response to exosomes require further investigation.

Exosomes have emerged as important mediators of intercellular communication that can shuttle miRNAs to recipient cells.^{6, 9, 24, 25} In recent years, accumulating evidence has demonstrated that the packaging of miRNAs into exosomes is selective.^{9, 26} We found that exosomal miRNA profiles in SMMC-7721, an HCC cell line, differed from those of parental cells, which suggested that exosomes serve as mediators in the regulation of intracellular miRNAs. Based on this hypothesis, we assume that Vps4A utilizes exosomes as mediators to modulate miRNA profiles in HCC cells. Gibbings et al. reported that altering ESCRT integrity generally compromised miRNA functions, most likely by interfering with the sorting of GW182 (a main component of the RNA-induced silencing complex) into multivesicular bodies.²⁷ In the current study, we surveyed the changes in miRNA profiles associated with Vps4A via small RNA sequencing. Two oncogenic miRNAs (miR-27b-3p and miR-92a-3p) were up-regulated in SMMC-Vps4A-exo (exosomes from SMMC-7721 stably expressing Vps4A). MiR-27b-3p has been reported as an oncogenic miRNA in gastric cancer,²⁸ glioma,²⁹ and ovarian cancer.³⁰ And miR-92a-3p, a member of the miR-17-92 cluster, was upregulated in various solid tumors.³¹⁻³³ In addition, three highly enriched

tumor suppressor miRNAs (miR-193a-3p, miR-320a, and miR-132-3p) accumulated in SMMC-Vps4A (SMMC-7721 stably expressing Vps4A). MiR-193a-3p was identified as a tumor suppressor in breast cancer.³⁴ MiR-320a was frequently down-regulated in colorectal cancer, and miR-132-3p exhibited a tumor-suppressive effect in osteosarcoma³⁵ and pancreatic carcinoma.³⁶ These results were consistent with the hypothesis mentioned above.

In this study, we confirmed that miRNAs contained within exosomes could be functionally delivered to target cells. Considering the selective packaging of miRNAs into exosomes, we hypothesize that the uptake of exosomal miRNAs is also a selective process. Fabbri et al. reported that secreted miRNAs within exosomes could bind to Toll-like receptors to induce a prometastatic inflammatory response.³⁷ However, the mechanisms and the signaling events involved in the uptake of exosomal miRNAs remain ill-defined. In accordance with our data, six tumor suppressor miRNAs (miR-122-5p, miR-33a-5p, miR-34a-5p, miR-193a-3p, miR-16-5p, and miR-29b-3p) were significantly upregulated in SMMC-Vps4A incubated with SMMC-ctrl-exo compared with the control. Recent studies have shown that miR-122-5p, miR-33a-5p, miR-34a-5p, miR-16-5p, and miR-29b-3p are potential tumor suppressor miRNAs in HCC,³ suggesting that Vps4A facilitates the uptake of tumor suppressor miRNAs within exosomes by SMMC-7721 cells. Moreover, Kyoto Encyclopedia of Genes and Genomes pathway analysis indicated that the PI3K/Akt signaling pathway may be the most likely candidate pathway modulated by Vps4A-associated miRNAs, which plays an important role in cancer development, progression, and therapeutic resistance.^{38, 39} By western blot, we confirmed that overexpression of Vps4A could cause inactivation of the PI3K/Akt pathway, which was a comprehensive effect by the associated miRNAs. Recent study showed that VPS4 was a key effector of the abscission checkpoint through Aurora B kinase, which was miRNA-independent.⁴⁰ In this study, Vps4A functioned as a tumor suppressor in an miRNA-dependent manner. We will explore the correlation of Vps4A in an miRNA-independent manner in a later study.

In conclusion, our data indicate that Vps4A functions as an important tumor suppressor during the development of HCC. Regulated by Vps4A, exosomes may serve as mediators by maintaining aberrant miRNA expression in HCC cells. These findings may lead to the identification of new therapeutic targets and the development of treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ESCRT	endosomal sorting complex responsible for transport
HCC	hepatocellular carcinoma
Hep3B-ctrl	Hep3B transfected with vector control
Hep3B-ctrl-exo	exosomes from Hep3B transfected with vector control
Hep3B-Vps4A	Hep3B stably expressing Vps4A
Hep3B-Vps4A-exo	exosomes derived from Hep3B stably expressing Vps4A
ILV	intraluminal vesicle
miRNA	microRNA
N-Rh-PE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl]
PI3K	phosphatidylinositol-3-kinase
SMMC-ctrl	SMMC-7721 transfected with vector control
SMMC-ctrl-exo	exosomes from SMMC-7721 transfected with vector control
SMMC-Vps4A	SMMC-7721 stably expressing Vps4A
SMMC-Vps4A-exo	exosomes derived from SMMC-7721 stably expressing Vps4A

Fig. 1. Characterization of isolated vesicles and miRNA expression profiles of HCC cells and exosomes. (A) Electron microscopy was used to image resuspended particles isolated from SMMC-7721 cells. (B) Equal amounts of proteins from exosomes and cells were analyzed by western blotting to identify proteins enriched in exosomes, including Alix, Tsg101, CD63, Hsp90 α/β , and Hsp60. (C) Heat map of cellular and exosomal miRNA expression. Abbreviations: S, SMMC-7721 cells; Se, SMMC-7721 cell-derived exosomes.

Fig. 2.

Incubation of exosomes promotes HCC cell growth, migration, and invasion. (A) Cell growth (CCK-8) assays were performed using SMMC-7721 and Hep3B cells cocultured with self-derived exosomes (10 μ g/mL). (B,C) Transwell migration (B) and invasion (C) assays were performed in SMMC-7721 and Hep3B cells cocultured with self-derived exosomes (10 μ g/mL). (D) Visualization of exosomes shuttling FAM-labeled miR-21-5p and Cy5-labeled miR-122-5p to recipient cells (SMMC-7721). * $P < 0.05$. Abbreviations: 10 μ g exo, 10 μ g/mL exosomes; Cy5-miR-122, Cy5-labeled miR-122-5p; DAPI, 4',6-diamidino-2-phenylindole; FAM-miR-21, fluorescein amidite-labeled miR-21-5p; OD, optical density.

Fig. 3.

Frequent down-regulation of Vps4A in HCC tissues. (A) Expression of Vps4A was calculated according to the integrated optical density. Levels of Vps4A protein were lower in HCC tissues than in adjacent tissues ($P < 0.01$) and normal liver tissues ($P < 0.01$). The results are plotted as a box plot in which the box spans the 25th and 75th percentiles, the line marks the median integrated optical density, and the whiskers mark the minimum and maximum data points. $**P < 0.01$. (B) Expression pattern of Vps4A in tumor tissues, adjacent tissues, and normal liver tissues as revealed by immunohistochemical staining. (C) Kaplan-Meier plots revealed an association of lower Vps4A levels with a shorter recurrence-free survival. Expression of Vps4A was examined by immunohistochemical staining. According to the integrated optical density, the median value of all 113 cases was chosen as the cutoff point for separating Vps4A low-expression tumors from Vps4A high-expression cases. Abbreviations: IOD, integrated optical density.

Fig. 4.

Ectopic expression of Vps4A represses HCC cell growth, colony formation, migration, and invasion. (A) Ectopic Vps4A expression was established and is shown in different HCC cell lines as indicated by western blotting. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control. (B,C) Cell proliferation (CCK-8) (B) and colony formation (C) assays were performed in SMMC-7721 and Hep3B cells expressing Vps4A. (D,E) Transwell migration (D) and invasion (E) assays were performed in SMMC-7721 and Hep3B cells expressing Vps4A. Note that the overexpression of Vps4A significantly inhibited the cell growth, colony formation, migration, and invasion of HCC cell lines. $*P < 0.05$. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Fig. 5.

In BALB/c nude mice Vps4A inhibits tumor formation. Cells (1×10^7 , SMMC-7721 and Hep3B cells) transfected with vector control or Vps4A were injected into the right flank of BALB/c nude mice. (A,B) The tumor growth curve of Vps4A-expressing cells was compared with that of vector-only cells. Points represent the mean tumor volumes of three independent experiments ($n = 5$); bars represent the standard error. (C) Tumor weight from the Vps4A and vector control groups. The results were obtained from three independent experiments ($n = 5$); bars represent the standard error. Significant differences were determined using the Mann-Whitney U test. $*P < 0.05$.

Fig. 6.

The biological activity of exosomes and the cell response to exosomes is inhibited by Vps4A. (A-C) SMMC-ctrl cells were incubated with 10 μ g/mL SMMC-Vps4A-exo and SMMC-ctrl-exo. Incubation of SMMC-Vps4A-exo repressed the growth (A), migration (B), and invasion (C) of SMMC-ctrl cells compared with SMMC-ctrl-exo; thus, Vps4A reduced exosome bioactivity. Similar results were found in Hep3B-ctrl cells cocultured with Hep3B-Vps4A-exo compared to those cultured with Hep3B-ctrl-exo (A-C). (D,E) SMMC-ctrl-exo (10 μ g/mL) was incubated with SMMC-Vps4A or SMMC-ctrl cells. Incubation of SMMC-Vps4A with SMMC-ctrl-exo led to a notable reduction in cell growth (D), migration (E), and invasion ability (F) compared with SMMC-ctrl cells. Similar results were observed in Hep3B-Vps4A cells cocultured with Hep3B-ctrl-exo compared to Hep3B-ctrl cells (D-F). Increased cell viability (%) = $[\text{OD}_{450}^{\text{(treatment group)} - \text{OD}_{450}^{\text{control group}}} / \text{OD}_{450}^{\text{control group}}] \times 100\%$. Increased cell migration (%) = $[\text{migrating cells}^{\text{(treatment group)} - \text{migrating cells}^{\text{control group}}} / \text{migrating cells}^{\text{control group}}] \times 100\%$. Increased cell invasion (%) = $[\text{invading cells}^{\text{(treatment group)} - \text{invading cells}^{\text{control group}}} / \text{invading cells}^{\text{control group}}] \times 100\%$. * $P < 0.05$.

Abbreviations: OD, optical density.

Fig. 7.

In SMMC-7721 cells Vps4A regulates exosomal miRNA secretion and uptake as determined by small RNA sequencing. (A,B) Differential miRNA expression caused by Vps4A in SMMC-7721 cell-derived exosomes (A) and SMMC-7721 cells (B). (C) Influence of Vps4A on the uptake of exosomal miRNAs by SMMC-7721 cells. (D) Network analysis of 14 selected miRNAs and their targets. (E) Molecules involved in the PI3K-Akt signaling after Vps4A overexpression in SMMC-7721 and Hep3B cells were determined by western blot analysis. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; PDK1, pyruvate dehydrogenase kinase 1; PTEN, phosphatase and tensin homolog; TSC2, tuberous sclerosis 2.

Table 1

Enriched miRNAs in Cellular and Exosomal RNAs

miRNA	Rank in Cells	Rank in Exosomes
Abundant cellular miRNAs		
let-7a-5p	1	26
let-7f-5p	2	23
let-7b-5p	3	66
let-7c-5p	4	60
miR-320a	5	13
miR-21-5p	6	12
let-7e-5p	7	45
miR-423-5p	8	3
let-7d-5p	9	123
miR-103a-3p	10	27
Abundant exosomal miRNAs		
miR-486-5p	421	1
miR-10b-5p	115	2
miR-423-5p	8	3
miR-10a-5p	32	4
miR-30a-5p	22	5
miR-99b-5p	45	6
miR-25-3p	15	7
miR-92b-3p	33	8
miR-92a-3p	27	9
miR-191-5p	19	10

The miRNAs were ranked according to the frequency of their occurrence (number of clean reads). Indicated are the 10 most prevalent miRNAs in cells or exosomal RNA and the corresponding ranking in the other sample.