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Original Articles

Vps4A mediates the localization and exosome release of β-catenin to inhibit epithelial-mesenchymal transition in hepatocellular carcinoma

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A B S T R A C T

We previously reported that Vps4A acted as a tumor suppressor by influencing the microRNA profiles of exosomes and their parental cells in hepatocellular carcinoma (HCC). However, the underlying mechanism and if Vps4A contributes to sorting proteins into exosomes are not well known. Here, we performed mass spectrometry analysis of the immunoprecipitated Vps4A complex and confirmed that Vps4A was associated with β-catenin and CHMP4B. Through this interaction, Vps4A promoted the plasma membrane (PM) localization and exosome release of β-catenin. Silencing Vps4A or CHMP4B decreased the PM localization and exosome sorting of β-catenin. Vps4A overexpression decreased β-catenin signaling pathway and inhibited epithelial-mesenchymal transition (EMT) and motility of HCC cells. And, silencing Vps4A or CHMP4B promoted EMT in HCC. Furthermore, the expression of Vps4A was significantly related to that of several EMT markers in HCC tissues and the level of exosomal β-catenin in patients with metastatic HCC was significantly lower compared to that of control patients. In conclusion, through the interaction with CHMP4B and β-catenin, Vps4A regulates the PM localization and exosome sorting of β-catenin, consequently decreases β-catenin signaling, and thereby inhibits EMT and metastasis in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related deaths worldwide [1]. Although many therapeutic options are available, the long-term prognosis of patients diagnosed with HCC remains poor. Tumor metastasis is the main cause of death in HCC patients, emphasizing the need for an improved mechanistic understanding of HCC metastasis [2,3].

The endosomal sorting complex required for transport (ESCRTs, which contain ESCRT-0 to -III and Vps4 subunits) are well known for their role in membrane remolding and scission, through which they participate in the formation of multivesicular bodies (MVBs) and exosomes, membrane repair, cytokinesis and budding of viruses [4–6]. The AAA + ATPase Vps4 consisting of Vps4A and Vps4B in human contributes to the disassembly of ESCRT-III [7]. The ESCRT machinery plays a crucial role in sorting proteins into endosomes, centrosomes and the plasma membrane (PM) [8–10]. Although not all complexes are required, ESCRT-III and Vps4 are thought to be necessary for these biological processes mentioned above [5,8]. However, the function of Vsp4A in cancers including HCC is not well studied. We previously reported that Vps4A functioned as a tumor suppressor by influencing the microRNA profiles of exosomes and their parental cells in HCC [11].

Abbreviations: EMT, epithelial-mesenchymal transition; MVB, multivesicular body; HCC, hepatocellular carcinoma; ZEB, zinc-finger E-box-binding; ESCRT, Endosomal Sorting Complex Required for Transport; DAPI, 4′,6-diamidino-2-phenylindole; HE staining, hematoxylin-eosin staining

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Nevertheless, more detailed knowledge of the underlying mechanism is needed.

Epithelial-mesenchymal transition (EMT) is considered a critical step in cancer cell metastasis [12,13]. During the conversion process, epithelial markers such as E-cadherin decrease, while mesenchymal proteins such as N-cadherin and Vimentin increase [14]. Several transcription factors, such as Snail, Twist1 and zinc-finger E-box-binding (ZEB) control the switch of these markers. The expression profiles of these genes vary depending on the signals that mediate EMT [15].

β-catenin acts as the central component and transcriptional coactivator of the Wnt signaling pathway, which is aberrantly activated in many cancers including HCC, and is implicated in the regulation of the tumorigenesis and EMT of cancers [16–19]. Once the Wnt signaling cascade is activated, β-catenin translocates from the cytosol to the nucleus and promotes EMT directly or indirectly by regulating the expression of target genes. β-catenin also contributes to the formation of adherens junction by directly interacting with E-cadherin on the PM. Therefore, β-catenin plays a dual role in regulating EMT depending on its localization [20,21]. However, the mechanism that finely coordinates these two different roles of β-catenin is not well known.

Exosomes are small extracellular membrane vesicles (40–200 nm) that are released by the fusion of MVB with the PM [22]. Proteins, lipids and various RNAs have been detected in exosomes. Cancer cells can communicate with other cells by transferring exosomes to the recipient cells [23,24]. Alternatively, by regulating secretion of exosomes containing cytosolic materials, cells can maintain their homeostasis or change their activity of cellular signaling cascades [25–27]. Recently, increasing evidence indicates that exosomes are involved in cancer invasion, metastasis and EMT, and that exosomes can be used as biomarker for the diagnosis of cancer [28–31]. However, the mechanisms involved in the regulating of the selective sorting of target cargos to exosomes and the role of exosomes in EMT of cancer have not been fully elucidated [32].

In this report, we found that Vps4A interacted with both CHMP4B and β-catenin, and that Vps4A played a crucial role in mediating the PM localization and exosome sorting of β-catenin. Our study uncovers a novel mechanism of Vps4A in regulating the EMT and motility of HCC cells, and these findings may lead to the development of biomarkers and treatment strategies.

2. Materials and methods

2.1. Cell culture

SMMC-7721 was purchased from the China Center for Type Culture Collection (China). MHCC97H cells were purchased from CELLCOOK Biological Technology (China), Huh-7 cells were obtained from the Japanese Collection of Research Bioresources (Japan). All cell lines were cultured in Dulbecco's modified Eagle's medium (Corning, USA) containing 10% fetal bovine serum (FBS) (Corning, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% CO_2.

2.2. Antibodies

Commercial antibodies were purchased as follows: N-cadherin (Cat. #13116), Vimentin, Snail, ZEB1, β-catenin (for Western blot), DAPI, HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG, Anti-rabbit IgG (Alexa Fluor 594 Conjugate) and Anti-mouse IgG (Alexa Fluor 647 Conjugate) were purchased from Cell Signaling Technology (USA); Vps4A, GAPDH, β-actin, E-cadherin (for IHC), N-cadherin (for IHC), β-catenin (for IHC and immunogold labeling) and Anti-Mouse IgG–Gold (5 nm) antibody were purchased from Sigma (USA); E-cadherin (for Western blot), and Alix were purchased from Santa Cruz (USA); CD63 was purchased from ABGENT (USA); Vps4A produced in rabbit, Twist1 and CHMP4B were purchased from Proteintech (USA); Tsg101 was purchased from Epitomics (USA).

2.3. Plasmid construction, transfection and establishment of stable cell lines

Recombinant Lentivirus expressing luciferase or expressing both Vps4A and luciferase were purchased from Genechem (China). The construction of the retroviral expression vectors for Vps4A (pLNCX2-Vps4A) and the stable cell lines was described previously [11]. An identical procedure was used to construct the expression plasmid for HA-tagged β-catenin and GFP-Vps4A the vector of which was pLVX-AcGFP-N1(Clontech, USA). Plasmids expressing β-catenin(S33Y) and Rab5Q79L were purchased from Genecreate (China). We purchased negative control (NC) RNA and siRNA for Vps4A, CHMP4B and E-cadherin from Sangon Biotech (China). The target sequences were reported by other studies [9,33]. Transfection of plasmids and siRNA was performed using Lipofectamine LTX with Plus Reagent and Lipofectamine RNAiMAX Reagent (Invitrogen, USA), respectively, according to the manufacturer's instructions.

2.4. Co-immunoprecipitation assay

For immunoprecipitation, cells were lysed with Western and IP lysis buffer (Beyotime, China) with protease inhibitor and phosphatase inhibitor (Sigma). The primary antibody or control IgG was added to the lysates and incubated at 4 °C overnight. Then, Protein A/G Agarose (Pierce, USA) was added and incubated for 2–4 h. The agarose was washed with lysis buffer 3 times before immunoblotting.

2.5. Mass spectrometry analysis

Co-immunoprecipitation was performed with anti-β-catenin monoclonal antibody as described above and the coprecipitates were electrophoresed by SDS-PAGE. The gel was stained with Coomassie Blue. The gel with the sample was cut off, digested and analyzed by LC-MS/MS by Bioclouds (Shanghai, China).

2.6. Exosome isolation and labeling

HCC cells were cultured on 10-cm dishes with vesicle-depleted medium for 48–72 h. The culture medium was collected and centrifuged at 300 g for 10 min and then 3000 g for 30 min. Next, the supernatant was further centrifuged at 11,000 g for 30 min at 4 °C. The supernatant was then ultracentrifuged at 100,000 g for 2 h or transferred to a sterile vessel and appropriate volume of ExoQuick-TC Exosome Precipitation Solution (System Biosciences, USA) was added to isolate exosomes according to the manufacturer's instructions. The exosome pellet was dissolved in 1000 μl 1 × PBS for NanoSight (Malvern, UK) or lysed in 100 μl of 1 × RIPA lysis buffer (Beyotime, China) for western blot analysis or used for transmission electron microscopy and immunogold labeling as follows.

Circulating exosomes were isolated from the plasma using ExoQuick Plasma prep and Exosome precipitation kit (System Biosciences, USA) according to the manufacturer's instructions.

2.7. Transmission electron microscopy and immunogold labeling

Exosomes isolated by the two methods mentioned above were examined using a JEM-1400 transmission electron microscope (JEOL, Japan) operated at 120 kV. The detailed procedure was described previously [11].

For immunogold labeling, exosomes isolated by ultracentrifugation were fixed by adding an equal volume of 4% paraformaldehyde. The fixed exosomes were placed on formvar-carbon coated electron microscopy grids (100 meshes). After permeabilized with 0.2% Triton for 5 min, the exosomes were blocked with 5% bovine serum albumin (BSA) and incubated with anti-β-catenin monoclonal antibody overnight under 4 °C. The grids were washed with PBS-0.1% BSA and incubated with IgG–gold (5 nm) antibody for 30 min. The grids were fixed

with 1% glutaraldehyde for another 5 min. Finally, the grids were stained with 2% uranyl acetate before the examination by transmission electron microscopy.

2.8. ELISA

The exosomes isolated from equal volume of plasma were lysed in non-denatured protein solubilization reagent (Invent, USA) supplemented with proteinase inhibitors. The concentration of β-catenin in exosomes was examined by using a commercial ELISA kit (CUSABIO, China) according to the manufacturer's instruction.

2.9. Total and nuclear protein extraction

Total protein was extracted using a standard protocol. A nuclear extract kit purchased from Active Motif (USA) was used to prepare nuclear and cytoplasmic extracts according to the manufacturer's protocol.

2.10. RNA isolation and quantitative RT-PCR

Total RNA was extracted from HCC cells using TRIzol (Invitrogen, USA) according to a standard protocol. Complementary DNA was obtained after reverse transcription of 2 μg RNA using Prime Script RT Master Mix (TaKaRa, Japan) according to the instructions. SYBR Premix EX Taq TMII (TaKaRa, Japan) was used for quantitative PCR. The primer sequences used for RT-PCR are provided in Table S3.

2.11. Immunofluorescence

After the cells were washed with PBS, they were fixed for 15 min in 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Next, cells were blocked with 10% goat serum in PBS for 1 h at room temperature, followed by overnight incubation with primary antibodies at 4 °C. After being washed with PBS, the cells were incubated with Alexa-conjugated secondary antibody and then incubated with DAPI for 5 min. The cells were visualized and photographed on a confocal microscope (Olympus LV3000, Japan).

2.12. Wound-healing assay

Cells were plated in a 6-well plate and cultured in DMEM containing 10% FBS until a confluent monolayer formed. Then, the monolayer was scraped with a 10-μl pipette tip. After the plate was washed with PBS, DMEM without FBS was added. The migration distance was visualized and photographed every 24 h.

2.13. Migration and invasion assay

Migration and invasion assays were performed to determine the motility of HCC cells. After trypsinization, the cells were centrifuged and resuspended in DMEM containing 0.1% FBS. Appropriate numbers of cells (3-5 × 10^5 for migration, 0.5-2 × 10^5 for invasion) were added to the upper chamber of a transwell chamber (pore size 8 μm, BD, USA) with or without Matrigel (BD, USA). The bottom chamber was loaded with 600 μl DMEM containing 15% FBS. After incubation for 12–24 h, the cells on the upper side were scraped with a cotton swab, and the filter was then fixed with 4% paraformaldehyde for 15 min at room temperature and stained with crystal violet for 10 min. After being washed with PBS, the cells in three randomly selected fields were counted under a microscope.

2.14. In vivo metastasis assays

All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals and in accordance with the institutional ethical guidelines for animal experiments. For lung metastasis assays, 3 × 10^6 MHCC97H luciferase-positive cells (control or Vps4A-expressing cells) resuspended in 100 μl PBS were injected via tail vein into 6–8-week-old male NOD/SCID mice. Six weeks later, lung metastatic signals were detected using an IVIS Lumina XR (PerkinElmer, USA) bioluminescence imaging system and D-luciferin (PerkinElmer, USA). Then, the mice were sacrificed and the lungs were removed and fixed in 4% paraformaldehyde. The lungs were embedded in paraffin for sectioning and stained with hematoxylin-eosin (HE).

2.15. Patients' samples and immunohistochemistry (IHC) analysis

A total of 61 tissue samples were obtained from patients who were diagnosed with primary HCC and underwent hepatectomy at Sun Yat-sen Memorial Hospital, Sun Yat-sen University (Guangzhou, China), between December 2009 and September 2012. Peripheral blood specimens were collected from 23 control patients who were diagnosed with benign disease such as hemangioma, and 45 HCC patients at Sun Yat-sen Memorial Hospital between 2016 and 2018. The metastatic HCC patients were those who had multifocal HCC or lymphatic metastasis. Ethical approval was obtained from the Ethics Committee of Sun Yat-sen Memorial Hospital (Guangzhou, China), and informed consent was obtained from each patient. IHC assays were performed as previously described [11].

2.16. Statistical analysis

The data were analyzed using Student's *t*-test except for Pearson's correlation, which was analyzed by Pearson's $\chi 2$ test. The results represent a total of at least three independent experiments performed in duplicate. All statistical analyses and graph construction were performed using SPSS statistic 19 (USA) and GraphPad Prism 6 (USA) and were presented as $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ and $^{\star\star\star}P < 0.001$ and ns = non-significant ($P \geq 0.05$). The results were expressed as the mean ± SD.

3. Results

3.1. Vps4A interacts with both β-catenin and CHMP4B

To explore the direct role of Vps4A in HCC, we performed endogenous Vps4A immunoprecipitation experiments combined with mass spectrometry using the Huh7 cell line. Several proteins that were reported to interact with Vps4A were identified, such as CHMP4B (a member of ESCRT-III) and ANCHR [34,35] (Fig. 1A). Also, hnRNPA2B1 and YBX1, which were reported to play a direct role in sorting microRNAs into exosomes, were identified [36,37]. Surprisingly, we found that β-catenin was associated with Vps4A (Fig. 1A and B). This interaction was further validated by co-immunoprecipitation analyses. Our results showed that Vps4A bound to endogenous β-catenin in both Huh7 and SMMC7721 cells (Fig. 1C and D). To further confirm the interaction between Vps4A and β-catenin, we exogenously expressed β-catenin with a HA-tag in Huh7 cells, and immunoprecipitation assays using an anti-HA-tag antibody showed that β-catenin interacted with not only E-cadherin but also Vps4A and CHMP4B (Fig. 1E). However, the interaction between β-catenin and Vps4A was E-cadherin independent (Fig. S1). We also performed immunofluorescence assays and observed the colocalization of Vps4A and β-catenin in the membrane-bounded compartments (Fig. 1F). These results indicated that β-catenin interacted with Vps4A and CHMP4B, implying the possible role of Vps4A in the regulation of the β-catenin signaling.

3.2. Vps4A promotes the PM localization of β-catenin

As the localization of β-catenin determines its function, we examined the influence of Vps4A on the localization of β-catenin by

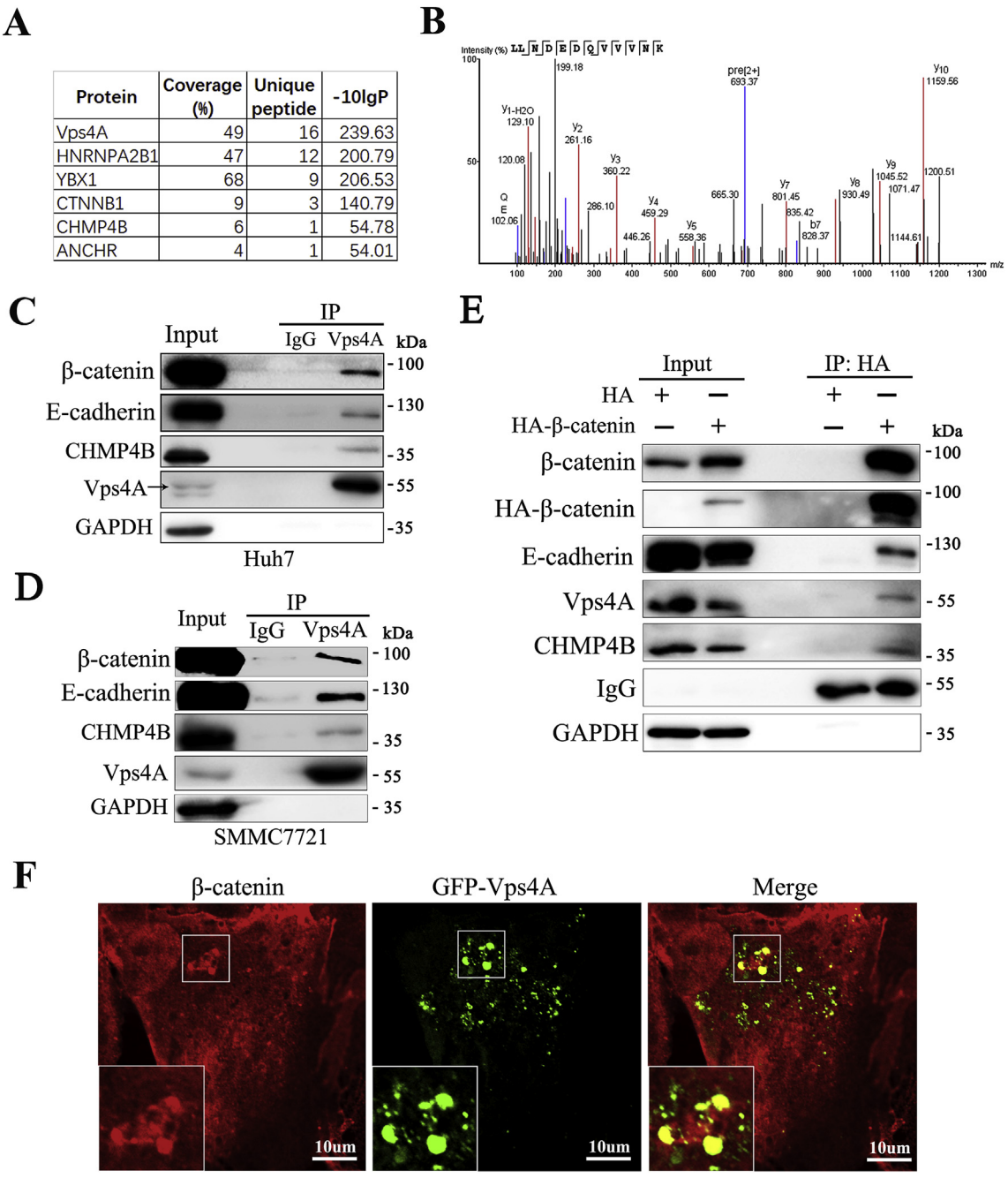

Fig. 1. Vps4A interacts with both β-catenin and CHMP4B. **A.** Immunoprecipitation assays were performed using a monoclonal anti-Vps4A antibody. The coprecipitates were analyzed by LC-MS/MS. Several identified Vps4A-associated proteins are shown in the table. **B.** One of the identified unique peptides of β-catenin is shown. **C** and **D.** Vps4A interacts with endogenous β-catenin. Immunoprecipitation assays using a monoclonal anti-Vps4A antibody were performed in Huh7 (**C**) and SMMC7721(**D**) cells, and immunoblotting analyses were carried out using the indicated antibodies. **E.** β-catenin interacts with Vps4A. HA-tagged β-catenin was transiently expressed in Huh7 cells. Immunoprecipitation was performed with an antibody against the HA tag, and immunoblotting analyses were performed using the indicated antibodies. **F.** Vps4A colocalizes with β-catenin. Huh7 cells transiently transfected with GFP-Vps4A were stained with an anti-β-catenin antibody and were then analyzed by confocal microscopy.

immunofluorescence. Stable cell lines overexpressing Vps4A were built (Fig. 2A). Surprisingly, we found that Vps4A overexpression promoted PM localization of β-catenin and reduced the nuclear β-catenin level (Fig. 2B). To confirm this effect of Vps4A, we knocked down Vps4A in Huh7 in which β-catenin mainly localized in the PM. As Vps4A is thought to coordinate with ESCRT-III to exert its function under some circumstances, we also knocked down CHMP4B to determine if it had the same effect as that of Vps4A (Fig. 2C). Our results showed that silencing Vps4A or CHMP4B both decreased the PM localization of β-catenin while the level of nuclear β-catenin was unchanged (Fig. 2D). Taken together, these results showed that Vps4A participated in regulating the PM localization of β-catenin.

3.3. *Vps4A promotes exosome sorting of β-catenin*

Considering the interaction between Vps4A and β-catenin, the effects of Vps4A on localization of β-catenin and the important role of ESCRTs in the formation of MVB and exosomes, we further investigated if Vps4A played a role in mediating the exosome sorting of β-catenin. First, exosomes were isolated from culture medium of control cells and

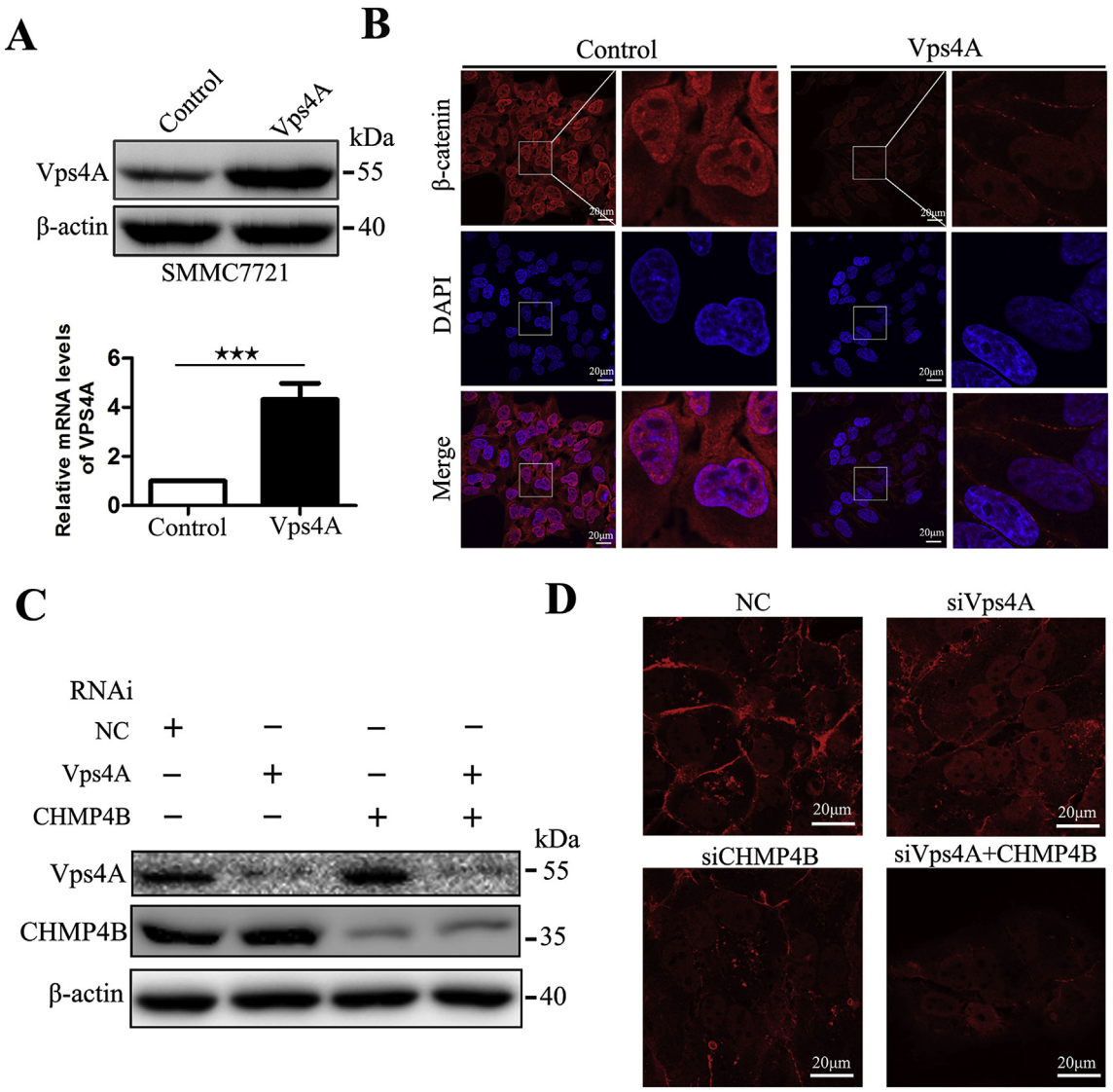

Fig. 2. Vps4A promotes the PM localization of β-catenin. **A.** Stable cell lines expressing Vps4A and control cell lines were built in SMMC7721. The expression levels of Vp4A in these cell lines were examined by western blot and RT-PCR analyses. ★★★$P < 0.001$. **B.** SMMC7721 stable cell lines expressing Vps4A and control cell lines were immunostained with anti-β-catenin antibody and analyzed by confocal microscopy. **C.** Huh7 cells were transfected with negative control (NC) RNA or siRNA as indicated and the protein levels of Vps4A and CHMP4B after transfection were analyzed by western blot. **D.** Silencing Vps4A or CHMP4B decreased the PM localization of β-catenin.

cell lines overexpressing Vps4A by ultracentrifugation or precipitation reagent. Then, the morphology and size of the exosomes were identified by transmission electron microscopy and NanoSight. Several markers of exosomes, such as CD63, Alix and Tsg101, were examined by western blot analysis. Our results showed that both methods could effectively isolate the exosomes (Fig. S2A, Fig. 3A and B). Overexpressing Vps4A had no effect on the number or size of the exosomes released into the culture medium (Fig. S2B). To confirm that β-catenin was released into exosomes, we performed immunogold labeling assays using an anti-β-catenin antibody. As expected, β-catenin could be detected in the exosomes (Fig. 3B). As MVBs were formed mainly in endosome membranes, we investigated whether β-catenin colocalized with Vps4A in the membranes of endosome by transiently expressing GFP-Vps4A and mCherry-Rab5Q79L. We found that both β-catenin and GFP-Vps4A localized in the membranes of enlarged endosomes marked by Rab5(Fig. 3C). Furthermore, we examined the protein level of β-catenin in the exosomes in stable cell lines overexpressing Vps4A (Fig. S3). Our results showed that overexpressing Vps4A promoted exosome release of β-catenin without changes in CD63 for both SMMC7721 and MHCC97H compared to the control cell lines (Fig. 3D). Also, we found that overexpressing Vps4A slightly increased the exosomal level of E-cadherin, though the signal was very weak (Fig. S2C). Additionally, silencing Vps4A or CHMP4B reduced the release of β-catenin into exosomes, which might partially be caused by the decreased exosome release, considering the important role of Vps4A on the formation of exosomes and the reduction of CD63 (Fig. 3E and Fig. S4). Taken together, we confirmed that β-catenin was present in the exosomes secreted by HCC cells and that Vps4A coordinating with CHMP4B played a role in selectively sorting β-catenin into exosomes.

3.4. Overexpressing Vps4A inhibits β-catenin activity

The effects of Vps4A on the PM localization and exosome release of β-catenin lead us to further explore the role of Vps4A in regulating the activity of β-catenin signaling. We firstly investigated the protein level of β-catenin and, as expected, the results showed that total β-catenin was markedly decreased in cell lines ectopically expressing Vps4A, while Vps4A had no influence on the mRNA level of β-catenin (Fig. 4A

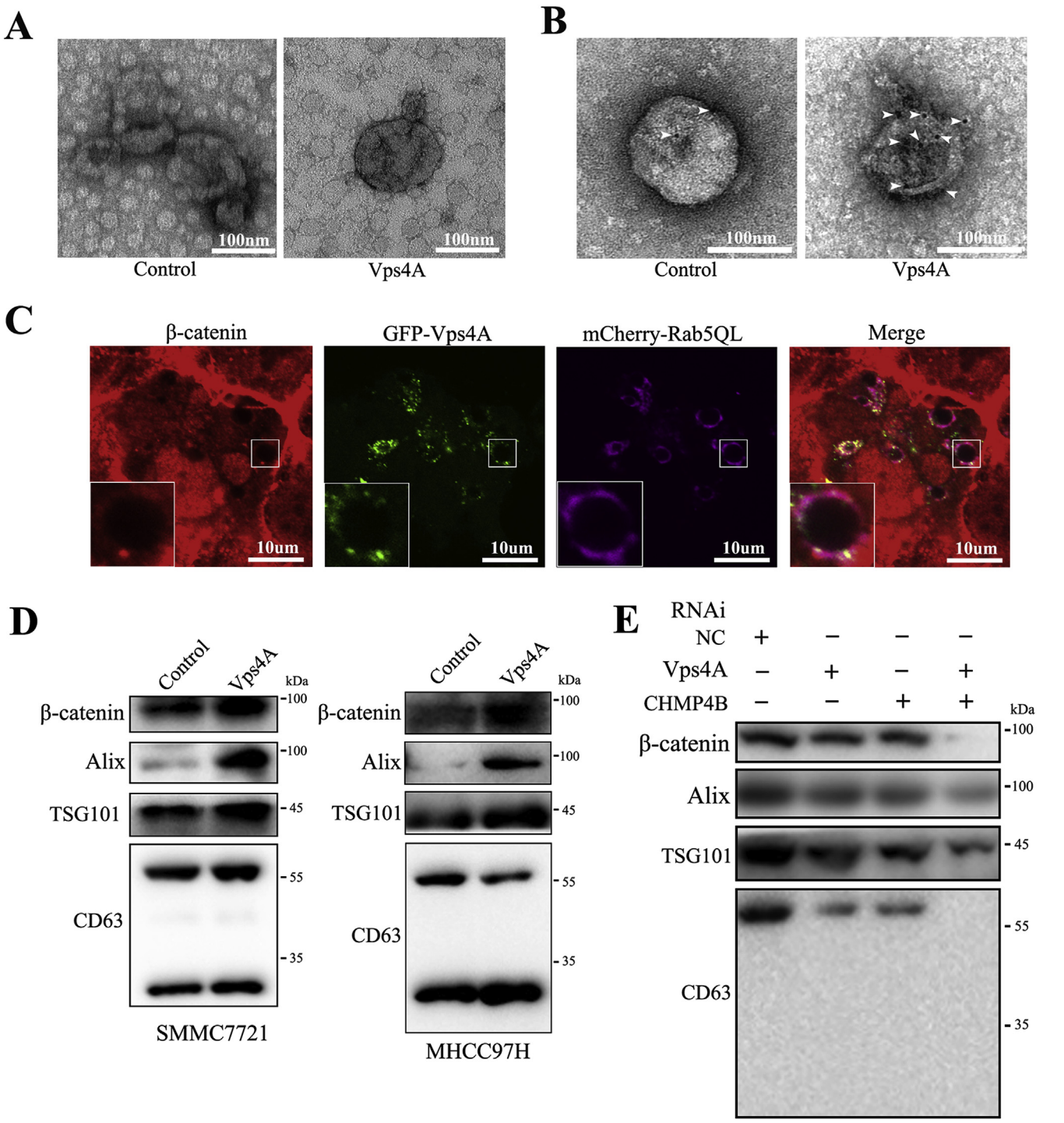

Fig. 3. Vps4A promotes exosome sorting of β-catenin. **A.** Exosomes isolated from the culture medium of SMMC7721 control cells and Vps4A-overexpressing cells by precipitation reagent were identified by transmission electron microscopy. **B.** Immunogold labeling with an anti-β-catenin antibody was performed for exosomes isolated from the cells mentioned above by ultracentrifugation. Arrowheads indicate the gold particles (5 nm of gold). **C.** Huh7 cells co-transfected with GFP-Vps4A and mCherry-Rab5Q79L (pseudocolor as purple) were immunostained with an anti-β-catenin antibody (pseudocolor as red) and analyzed using confocal microscopy. **D.** The effects of Vps4A on sorting β-catenin into exosomes were analyzed by western blot analysis using the exosomes isolated from the stable cell lines of both SMMC7721 and MHCC97H. **E.** Huh7 cells were transfected with NC or siRNA as indicated. The protein levels of β-catenin released into exosomes were analyzed by western blots.

and B). Because the activity of β-catenin depends on its nuclear distribution, nuclear proteins were extracted and examined. We found that the protein level of β-catenin was down-regulated in both the cytosol and nucleus in both SMMC7721 and MHCC97H cell lines overexpressing Vps4A (Fig. 4C). Moreover, overexpression of Vps4A markedly reduced the mRNA levels of c-Myc and cyclin D1, which are specific target genes of β-catenin signaling (Fig. 4D). Consistent with the results of immunofluorescence, silencing Vps4A or CHMP4B decreased the total protein level of β-catenin, while the activity of β-catenin was unchanged (Figs. 4E and 5E). So, Vps4A and CHMP4B might not directly participate in regulating the translocation of β-catenin into the nucleus. Collectively, these results suggested that overexpressing

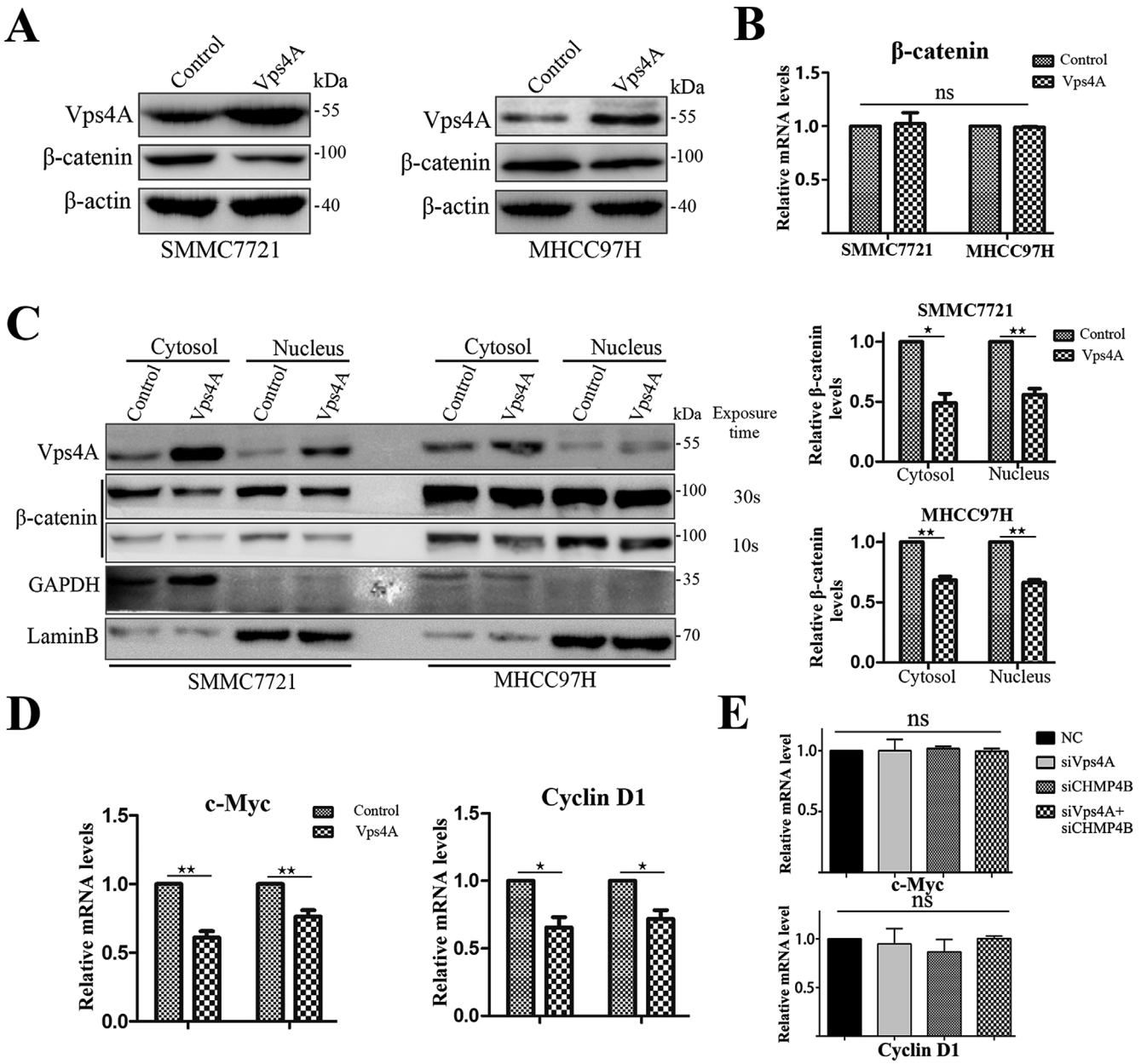

Fig. 4. Vps4A inhibits the activity of β-catenin. **A.** Overexpressing Vps4A reduced the total protein level of β-catenin. **B.** RT-PCR assays were performed to show the mRNA level of β-catenin. ns = non-significant ($P > 0.05$). **C.** The nuclear and cytosolic distribution of β-catenin in the control cells and cells overexpressing Vps4A for both SMMC7721 (left) and MHCC97H (right). $^{\star}P < 0.05$, $^{\star\star}P < 0.01$. **D.** The mRNA levels of c-Myc and Cyclin D1, both of which are the direct target genes of β-catenin, were analyzed by RT-PCR. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$. **E.** Huh7 cells were transfected with NC or siRNA as indicated. The mRNA levels of c-Myc and Cyclin D1 were analyzed by RT-PCR. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$.

Vps4A inhibited β-catenin activity implying a crucial role of Vps4A on regulating the localization of β-catenin.

3.5. Vps4A inhibits EMT in HCC cells

Considering that β-catenin plays dual roles in regulating EMT depending on its localization, we further investigated the influence of Vps4A on EMT in HCC cells. We examined the expression patterns of EMT biomarkers, including E-cadherin, N-cadherin and Vimentin. As expected, ectopic expression of Vps4A enhanced the protein level of E-cadherin, while the expression levels of N-cadherin and Vimentin were down-regulated (Fig. 5A). These results were further confirmed by RT-PCR (Fig. 5B) and immunofluorescence (Fig. 5C). Additionally, the expression patterns of EMT-associated transcription factors were also examined. We found that both the protein and mRNA levels of Snail and Twist1 were clearly decreased in Vps4A-overexpressing cells. And, a slight but significant decrease in ZEB1 level was detected (Fig. 5A and B). To determine if the decreased activity of β-catenin was responsible for the inhibitory effect of Vps4A on EMT, we performed the rescue assays. A constitutively active form of β-catenin(S33Y) was transiently overexpressed in SMMC7721 cells overexpressing Vps4A, and hallmarks of EMT were detected 48–72 h later by western blot analyses. As shown in Fig. 5D, ectopical expression of β-catenin(S33Y) decreased the protein level of E-cadherin and dramatically up-regulated the protein level of N-cadherin and Snail. As silencing Vps4A or CHMP4B decreased the level of β-catenin on the PM, which participated in the formation of adhesions junction, we furthermore determined the change of the markers of EMT after knocking down Vps4A or CHMP4B. The results showed that silencing Vps4A or CHMP4B decreased the protein level of E-cadherin and increased the levels of N-cadherin and Twist1, which

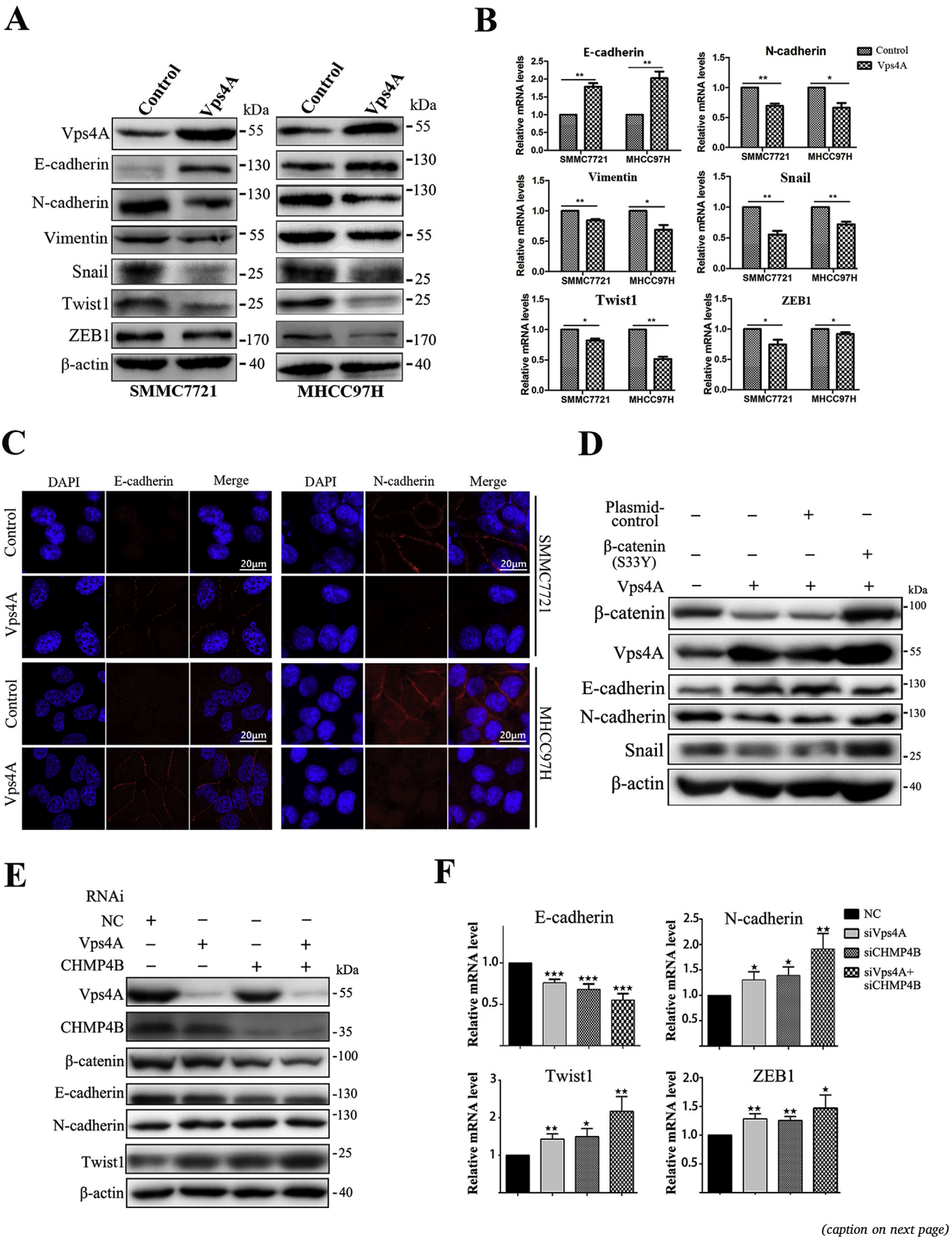

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Fig. 5. Vps4A inhibits EMT in HCC cells. **A.** Western blot assays were performed to examine the protein levels of several EMT markers and EMT-associated transcriptional markers in SMMC7721 and MHCC97H stable cells. **B.** RT-PCR assays were performed to determine the mRNA levels of the genes in (**A**). β-actin was used as the normalization control in all RT-PCR assays. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$. **C.** The changes in EMT markers, including E-cadherin and N-cadherin, were verified by immunofluorescence assays. DAPI was used to visualize the nuclei. **D.** Transiently overexpressing β-catenin (S33Y) in SMMC7721 cells ectopically expressing Vps4A restored the reversion of EMT induced by Vps4A. **E** and **F.** Silencing Vps4A or CHMP4B promoted the EMT in HCC. The changes in the mRNA levels of several EMT markers were tested by western blot (**E**) and RT-PCR (**F**) after Huh7 cells were transfected with indicated siRNA. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ and $^{\star\star\star}P < 0.001$.

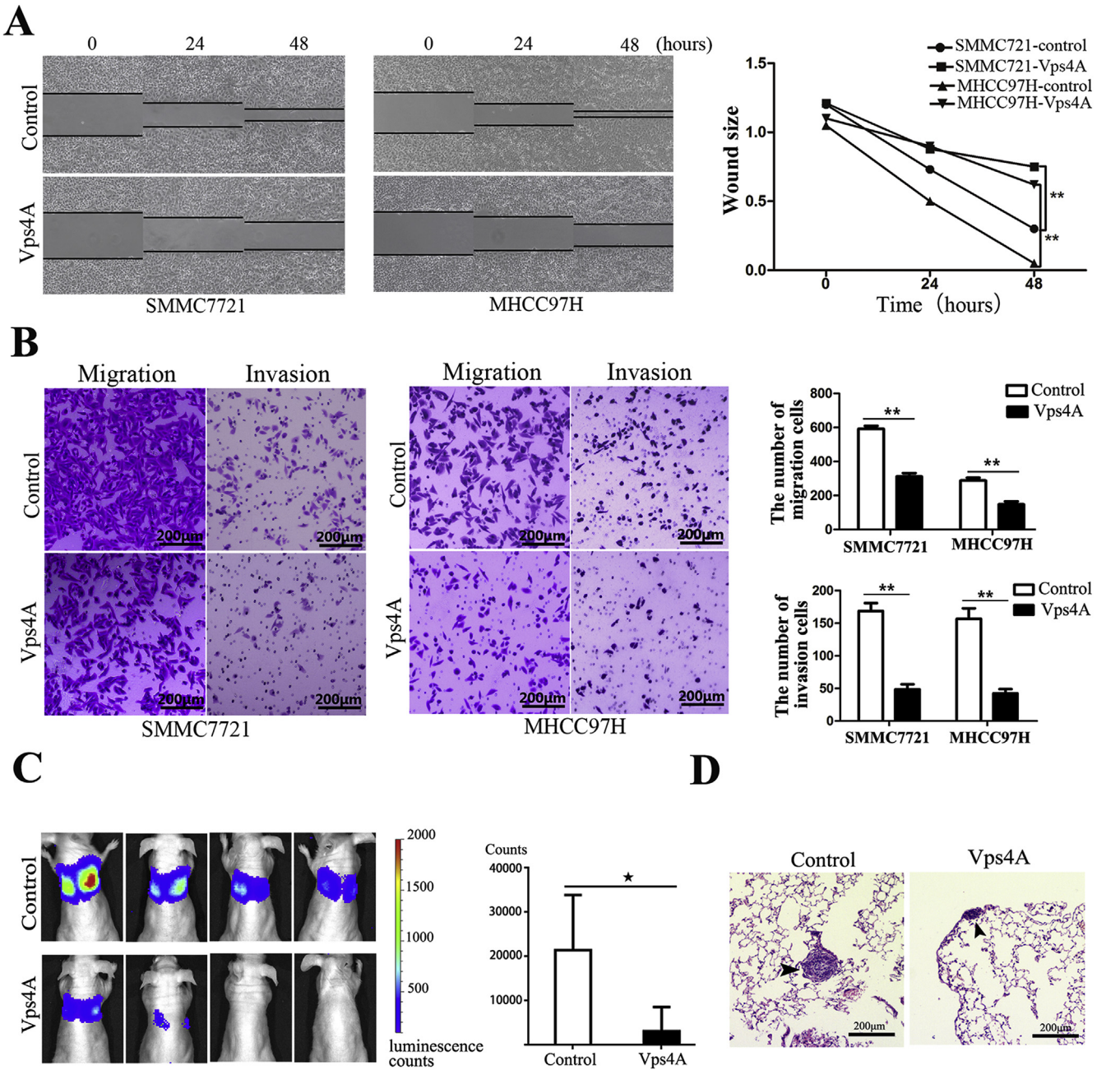


Fig. 6. Vps4A suppresses the migration, invasion and metastasis of HCC cells. **A.** Wound-healing assays using SMMC7721 and MHCC97H stable cell lines. Wound sizes were visualized (200 ×) and photographed at the indicated time points after the wounds were created. The distances between wound edges were analyzed. $^{\star\star}P < 0.01$. **B.** Transwell assays. Appropriate numbers of SMMC7721 and MHCC97H stable cells were added into the upper chamber with (for invasion) or without Matrigel (for migration) separately. The numbers of migrating and invasive cells were photographed and quantified in 12–24 h later. $^{\star\star}P < 0.01$. **C.** In vivo detection of lung metastasis. MHCC97H-luc-control or MHCC97H-luc-Vps4A cells were injected into nude mice through the tail vein. Tumor metastasis was examined by bioluminescent images. $^{\star}P < 0.05$. **D.** HE staining of lung for (**C**). Arrowheads indicate lung metastasis.

was further confirmed by RT-PCR (Fig. 5E and F). Collectively, these data suggested that Vps4A played a crucial role in EMT by regulating the localization and activity of β-catenin in HCC cells.

3.6. Vps4A inhibits cell migration, invasion and metastasis of HCC cells

Because EMT plays a crucial role in cancer invasion, migration and metastasis, we further validated the effects of Vps4A on the motility of HCC cells. As expected, our results showed that overexpressing Vps4A significantly inhibited wound closure compared with that of control cells by performing wound-healing assays (Fig. 6A). Additionally, overexpressing Vps4A significantly decreased both the migration and invasion of SMMC7721 and MHCC97H cells, as shown by transwell assays with or without Matrigel (Fig. 6B). In the in vivo study, control cells expressing luciferase (MHCC97H-luc-control) and cells expressing both luciferase and Vps4A (MHCC97H-luc-Vps4A) were injected into nude mice through the tail vein. Tumor metastasis was observed by bioluminescent images and was verified by HE staining. We found that overexpression of Vps4A suppressed the lung metastasis of MHCC97H (Fig. 6C and D). Taken together, these results suggested that overexpression of Vps4A inhibited metastasis of HCC cells both in vitro and in vivo.

3.7. Correlated expression of Vps4A and EMT markers in cancer tissues from HCC patients, and the level of β-catenin in circulating exosomes

To determine the clinical relevance of Vps4A and EMT, we further analyzed the expression pattern of Vps4A and two frequently used EMT markers, including E-cadherin and N-cadherin, by performing immunohistochemistry (IHC) analysis in tumor tissues from 61 HCC patients. Our results showed that the enhanced expression of Vps4A was correlated with increased protein level of E-cadherin and was negatively correlated with the expression of N-cadherin (Fig. 7A and B). The correlation between the expression of Vps4A and the plasma membrane (PM) localized β-catenin was also determined by performing IHC. Consistently, we found that there was a positive correlation between them (Fig. 7A and B). We further analyzed the correlation between the mRNA level of Vps4A and that of several EMT-associated markers in both HCC and other types of cancer using the data from TCGA downloaded from ChIPBase v2.0 (http://rna.sysu.edu.cn/chipbase/) [38]. Although there was no significant correlation between the mRNA levels of Vps4A and E-cadherin (CDH1), the mRNA level of Vps4A was significantly correlated with that of several EMT markers, including N-cadherin (CDH2), in HCC (Fig. 7C and Fig. S5). Additionally, we found that there was also a significant correlation between Vps4A mRNA level and that of E-cadherin or N-cadherin in several other cancer types (Tables S1 and S2).

We previously reported that Vps4A was down-regulated in HCC tissues. As Vps4A plays a direct role in regulating the exosome release of β-catenin, we further examined the level of β-catenin on circulating exosomes in HCC patients and control patients. Consistently, we found that β-catenin was existed in the circulating exosomes and that the protein level of β-catenin was significantly lower ($P < 0.05$) in exosomes derived from metastatic HCC patients compared to those from control patients by performing ELISA, though no significant difference was seen for β-catenin in exosomes isolated from control patients and HCC patients without metastases (Fig. 7D and E).

We concluded that the correlation between the expression of Vps4A and the EMT-related markers highlighted the crucial role of Vps4A in regulating the metastasis of HCC in clinical patients. And, lower circulating exosomal β-catenin could be used to predict the outcome of HCC patients.

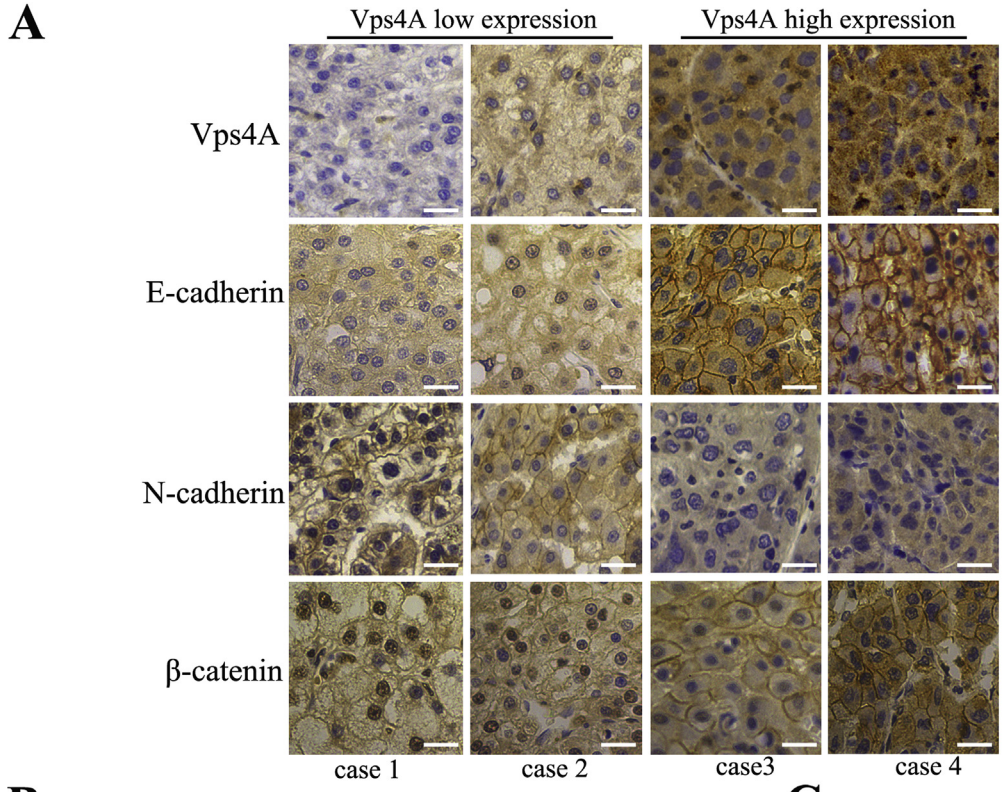
4. Discussion

Tumor metastasis is the main cause of poor prognosis of hepatocellular carcinoma (HCC) patients and β-catenin plays dual roles in tumor metastasis depending on its localization. Though Vps4A plays multiple roles in biological processes, its role in cancer cells is unclear. We have previously reported that Vps4A was frequently down-regulated and acted as a tumor suppressor through influencing the microRNA profiles of exosomes and their parental cells in HCC [11]. However, the precise mechanism underlying its tumor-suppressive effect and whether Vps4A plays a role in sorting proteins into exosome are not well known. Here, we performed mass spectrometry analysis of the immunoprecipitated Vps4A complex and found that β-catenin and CHMP4B were associated with Vps4A, which was validated by series of co-immunoprecipitation assays. In addition, we found that both Vps4A and CHMP4B were co-immunoprecipitated with β-catenin. So, Vps4A could form a complex with CHMP4B and β-catenin implying its role in regulating β-catenin signaling. Our mass spectrometry analysis also identified several RNA binding proteins, such as hnRNPA2B1 and YBX1, which played a direct role in sorting microRNA into exosomes [36,37]. So, it is possible that Vsp4A could influence the microRNA profiles of exosomes and their parental cells through this interaction, which need to be further validated.

β-catenin plays dual roles in cell invasion and migration depending on its localization [21]. It can participate in cadherin-based adhesions junction or act as a coactivator of the Wnt signaling pathway. However, the mechanism underlying the coordination of these two functions is not well known. It was reported that Vps4A contributed to recycling some proteins to the PM. For example, together with ESCRT-III, Vps4A contributed to sorting Ras or EGFR to the PM [10,39]. Our results showed that Vps4A could regulate the PM localization of β-catenin. Overexpression of Vps4A promoted the PM localization of β-catenin and reduced nuclear β-catenin. Moreover, silencing Vps4A or CHMP4B decreased the PM localization of β-catenin. Therefore, our study highlighted a novel mechanism of Vps4A in regulating the localization of β-catenin.

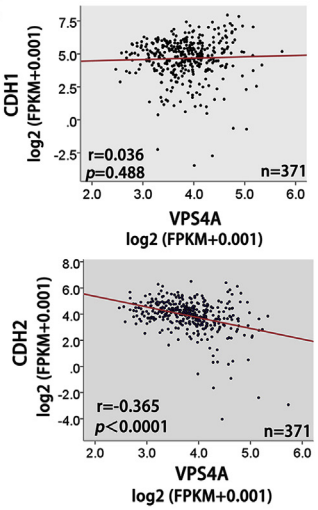
The PM-associated β-catenin can be degraded by endocytosis, during which MVBs would form [40]. It was reported that cancer cells could release β-catenin into exosomes, although the underlying mechanism was unclear [27]. As ESCRTs play a crucial role in the formation of MVBs and exosomes, and Vps4A interacts with β-catenin, we explored the influence of Vps4A on sorting β-catenin into exosomes. We confirmed that β-catenin was present in exosomes secreted by HCC cells using immunogold labeling. We found that Vps4A overexpression enhanced the protein level of β-catenin detected in exosomes without influencing the size or the number of exosomes. And, this was consistent with the decrease of total protein level of β-catenin after overexpressing Vps4A. Additionally, silencing Vps4A or CHMP4B reduced exosome secretion of β-catenin, suggesting that both Vps4A and CHMP4B could participate in sorting β-catenin into exosomes. It was reported that Syndecan–Syntenin–ALIX contributed to the formation of exosomes [6]. Considering that ALIX interacted with CHMP4, it was possible that Vps4A together with CHMP4B could directly participate in sorting β-catenin into exosomes and thereby decrease the β-catenin signaling pathway. Notably, increasing numbers of studies have shown that cells could maintain their homeostasis or change their activity of cellular signaling cascades by regulating exosome secretion of cytosolic materials [25–27]. We previously reported that Vps4A might function as a tumor suppressor by facilitating the secretion of oncogenic miRNAs into exosomes [11]. So, Vps4A might play a direct role in sorting oncogenic materials into exosomes besides its role in the formation of exosomes.

Through regulating the PM localization and exosomal sorting of β-catenin, overexpressing Vps4A reduced the activity of β-catenin and consequently inhibited EMT of HCC cells. Additionally, silencing Vps4A or CHMP4B inhibited EMT in HCC cells, which might be caused by the decreased PM localization of β-catenin. However, silencing Vps4A or CHMP4B had no influence on the nuclear localization and activity of β-catenin. Therefore, Vps4A and CHMP4B might not directly participate



B

		Vps4A		*p* value	Correlation
		Low	High		
E-cadherin	Low	16 (26.2%)	6 (9.8%)	$p=0.001$	$R=0.457$
	High	10 (16.4%)	29 (47.5%)		
N-cadherin	Low	10 (16.4%)	30 (49.2%)	$p < 0.001$	$R=-0.492$
	High	16 (26.2%)	5 (8.2%)		
β-catenin (PM)	Low	21 (34.4%)	18 (29.5%)	$p < 0.05$	$R=0.302$
	High	5 (8.2%)	17 (27.9%)		



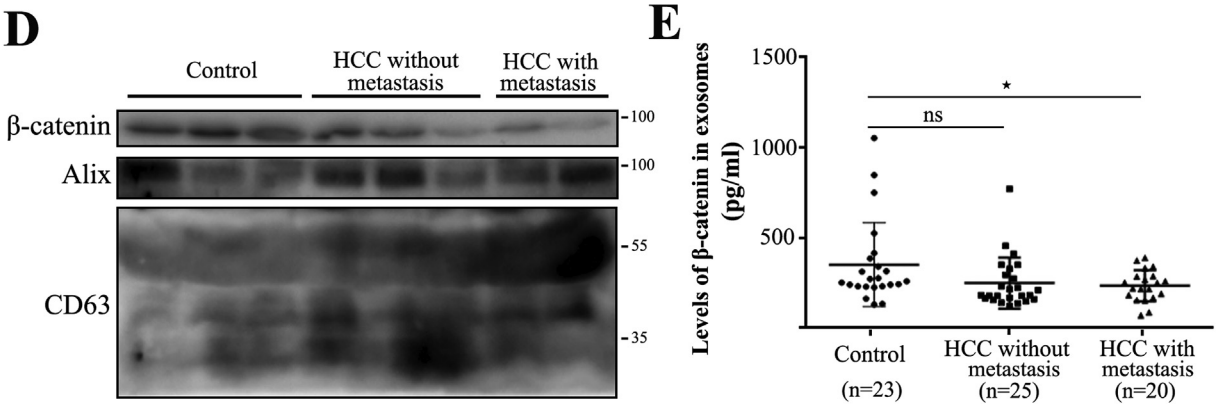


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Fig. 7. Correlation between Vps4A expression and that of EMT markers in HCC patient samples, and the level of β-catenin in circulating exosomes. **A.** Representative IHC images of Vps4A, E-cadherin, N-cadherin and β-catenin staining with HCC tissues. Scale bars, 20 μm. **B.** The association between Vps4A expression and E-cadherin, N-cadherin or β-catenin localized on the plasma membrane (PM). The number of cases, percentage of positive staining and *P* value based on Pearson's $\chi 2$ test and Pearson's correlations are shown in the tables. **C.** The correlation between the mRNA level of Vps4A and that of EMT markers such as CDH1 (E-cadherin) and CDH2 (N-cadherin) in HCC tissues. The data originated from TCGA were downloaded from ChIPBase v2.0 (http://rna.sysu.edu.cn/chipbase/). Pearson's correlations and *P* values were analyzed and are shown. **D and E.** Immunoblot (D) and ELISA (E) assays were performed to analyze the protein level of β-catenin in exosomes isolated from plasma of the control and HCC patients. ns = non-significant ($P > 0.05$) and $^{\star}P < 0.05$.

in regulating the translocation of β-catenin into the nucleus, which was predominantly influenced by the activity of upstream Wnt signaling. As the mechanism that regulates the transportation, localization and degradation of β-catenin is complex and not well elucidated, it is possible that there are some other factors that influence the activity of β-catenin [21,40–42]. And, if Vps4A plays other roles in this process will also be further studied.

As EMT plays a crucial role in cancer invasion, migration and metastasis, we further validated the effects of Vps4A on the motility of HCC cells in vitro and in vivo. We also determined the clinical relevance of Vps4A and EMT, and found there were significant correlations between the expression of Vps4A and that of EMT markers. Recently, increasing numbers of studies showed that exosomes could be used as biomarker for the diagnosis of cancer and predicting the outcome of the cancer patients. We found that the level of β-catenin was significantly lower in exosomes derived from metastatic HCC patients compared to those from control patients. So, circulating exosomal β-catenin could be used a biomarker to predict the outcome of HCC patients.

Taken together, this study demonstrated that Vps4A could mediate the PM localization and exosome release of β-catenin by interacting with both CHMP4B and β-catenin and consequently regulated EMT in HCC cell lines. These results reveal an important role of Vps4A in the selective transportation of target proteins and identify a novel mechanism underlying the function of Vps4A in HCC, which may lead to the development of biomarkers and treatment strategies.

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Conflicts of interest

No conflict of interest exits in the submission of this manuscript, and the manuscript is approved by all authors for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.035.

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