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Exosome-delivered miR-221/222 exacerbates tumor liver metastasis by targeting SPINT1 in colorectal cancer

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Abstract

MicroRNAs (miRNAs) are involved in the progression of many cancers through largely unelucidated mechanisms. The results of our present study identified a gene cluster, miR-221/222, that is constitutively upregulated in serum exosome samples of patients with colorectal carcinoma (CRC) with liver metastasis (LM); this upregulation predicts a poor overall survival rate. Using an in vitro cell coculture model, we demonstrated that CRC exosomes harboring miR-221/222 activate liver hepatocyte growth factor (HGF) by suppressing SPINT1 expression. Importantly, miR-221/222 plays a key role in forming a favorable premetastatic niche (PMN) that leads to the aggressive nature of CRC, which was further shown through in vivo studies. Overall, our results show that exosomal miR-221/222 promotes CRC progression and may serve as a novel prognostic marker and therapeutic target for CRC with LM.

KEYWORDS

colorectal cancer, exosome, liver metastasis, miR-221, miR-222

Abbreviations: CRC, colorectal carcinoma; EVs, extracellular vesicles; HAI1, hepatocyte growth factor activation inhibitor type 1; HGF, hepatocyte growth factor; LM, liver metastasis; MET, mesenchymal-epithelial transition factor; miRNAs, microRNAs; NTA, nanoparticle tracking analysis; PMN, premetastatic niche; SPINT1, serine protease inhibitor Kunitz type 1; TEM, transmission electron microscopy.

Tian, Wang, Lin, and Dai equally contributed to this study.
Zhu and Kong are joint corresponding authors.

1 | INTRODUCTION

Colorectal carcinoma (CRC) is the third most common cancer type and the leading cause of cancer-related deaths worldwide.[1] The most frequent site of CRC metastasis is the liver. Approximately 30% of all CRC patients are diagnosed with liver metastasis (LM), which causes at least two-thirds of CRC deaths.[2] Emerging evidence suggests that in addition to local signals in the primary tumor microenvironment, the tumors also send signals to future metastatic sites from a long distance to foster the emergence of the hospitable premetastatic niche (PMN) which can promote the proliferation of spreading tumor cells.[3] Although many reports have described a large number of molecular abnormalities of coding proteins or noncoding RNAs associated with the pathogenesis of LM in CRC that play crucial roles in this process,[4,5] the exact regulatory mechanism remains unclear.

Tumor-secreted extracellular vesicles (EVs) are important messengers for intercellular crosstalk from tumor to mesenchymal cells in local and remote microenvironments.[6-8] Exosomes, which are EVs released by cells, range in size from 30 to 100 nm and carry proteins, lipids, and various types of nucleic acids.[9] Exosomes are important cancer cell–derived factors that initiate the formation of a niche before distant-organ metastasis.[10-12] The results of recent studies have indicated that exosomes act as mediators to regulate the tumor microenvironment and support tumor metastasis and progression.[13] However, the precise physiological function of exosomes in promoting tumor metastasis needs to be further elucidated.

MicroRNAs (miRNAs) are ~22-nucleotide (nt) noncoding RNAs whose primary function is to suppress the translation of the target messenger RNA.[14] A large amount of evidence have suggested that exosomes contain high levels of miRNAs that exert crucial effects on the immune regulation, chemotherapy resistance, and metastasis of many tumor types.[15-] miR-221 and miR-222 (miR-221/222) are located within a 1-kb region of the X chromosome, have identical seed sequences, and form a gene cluster. Recently, the role of miR-221/222 in tumorigenesis, whether as an oncogene or as a tumor suppressor gene, has been reported.[17-19] Studies have shown that miR-221 and miR-222 overexpression in the cancer stroma is associated with the malignant potential of colorectal cancer.[20,21] However, to date, the discovery of tumor-derived exosomal miR-221/222 dysregulation, their specific biological features, and potential regulatory mechanism in the development of LM require further investigation in CRC.

Serine protease inhibitor Kunitz type 1 (SPINT1), which is named hepatocyte growth factor activation inhibitor type 1 (HAI1), is a type I transmembrane serine protease inhibitor that is frequently found on the surface of epithelial cells.[22] The abnormal expression of SPINT1 has been confirmed in many cancers and may be a significant factor in the mechanism of HGF/MET activation.[23-25]

In the present study, we demonstrate that exosomal miR-221/222 is frequently overexpressed in colorectal cancer patients with LM and predicts poor disease survival. We further demonstrated that c-met is overexpressed in LM of CRC, while HGF is not, suggesting that CRC metastases primarily bind HGF secreted by the liver. Using an in vitro cell coculture model, we showed that CRC exosomes harboring miR-221/222 activate liver HGF by suppressing SPINT1 expression. Importantly, in vivo studies have shown that miR-221/222 has a key role in forming hospitable PMNs, which promotes the invasiveness of CRC cells. In summary, tumor-derived exosomal miR-221/222 regulates the liver microenvironment and exacerbates LM, while upregulated paracrine HGF creates favorable conditions for incoming metastatic CRC cells.

2 | MATERIALS AND METHODS

2.1 | Human samples

Human CRC LMs and adjacent noncancerous tissues were obtained from patients undergoing surgery at the Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). This study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Informed consent was signed from each patient before collecting samples. During the surgery, tissue samples were promptly placed into liquid nitrogen.

2.2 | Animals

Female BALB/c-nu mice (6 weeks of age) were housed in a special pathogen-free animal facility. The experiments involving mice were performed with the approval of the Institutional Animal Care and Research Advisory Committee of Tianjin Medical University Cancer Institute and Hospital.

2.3 | Cell culture

The human CRC cell line SW480 was acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SW480 cells were cultured in 1640 medium (Gibco). Primary mouse liver cells were acquired from the livers of C57BL/6J mice (6 weeks) and maintained in RPMI 1640 culture medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco).

2.4 | Isolation of exosomes from serum and cell culture media

Serum exosomes (sr-exosomes) were extracted using an exosome isolation kit (Invitrogen). Exosomes were extracted from cell by gradient centrifugation, with all isolation processes performed at 4°C. First, the cells and other debris were removed by centrifugation at 3000 g, followed by centrifugation at 10 000 g for 30 minutes. Next, the cell culture medium was harvested and centrifuged at 110 000 g for 70 minutes. Finally, the pellet was resuspended in PBS to collect exosomes.

2.5 | Protein extraction and western blot analysis

Western blotting was performed to assess protein levels, which were normalized to that of GAPDH. Total proteins form lysates were separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Roche). Then, membranes were blocked with 5% bovine serum albumin, followed by an incubation with the primary antibody at 4°C overnight. The proteins were visualized with an enhanced chemiluminescence (ECL) reagent after incubation with a suitable HRP-conjugated secondary antibody at room temperature for 1 hour.

2.6 | RNA isolation and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using avian myeloblastoma virus (AMV) reverse transcriptase (TaKaRa). U6 small nuclear RNA was used as an internal reference for miR-221/222, and GAPDH was used as the internal reference for mRNA. All reactions were assayed in triplicate. The sequences of the primers used for target gene amplification were as follows:

5′-CCTGGTGCTACACGGGAAAT-3′ (HGF, sense)
5′-CACATCCACGACCAGGAACA-3′ (HGF, antisense)
5′-CAGCAGTGCCTCGAGTCTTGTC-3′ (SPINT1, sense)
5′-GATGGCTACCACCACCACAATG-3′ (SPINT1, antisense)
5′-AGAAGGCTGGGGCTCATTTG-3′ (GAPDH, sense)
5′-AGGGGCCATCCACAGTCTTC-3′ (GAPDH, antisense).

2.7 | ELISA

An ELISA kit was used to assess HGF secretion according to the manufacturer's protocol (Sigma).

2.8 | Immunohistochemistry

Tissue sections were fixed in 4% paraformaldehyde and embedded in paraffin. Then, after blocking the activation of endogenous peroxidase, the tissue sections were incubated with anti-HGF antibody (1:200, Abcam) before being stained with DAB substrate and counterstained with hematoxylin for the same amount of time. Five fields of each section were analyzed under a microscope.

2.9 | miRNA target analysis and luciferase reporter assays

The PicTar (http://pictar.mdcberlin.de/), miRanda (http://www.microrna.org/), and TargetScan (http://www.targetscan.org/) algorithms were used to predict and analyze the miRNA binding sites. The reporter plasmids were designed by Genscript (Nanjing, China). Briefly, cells were transfected with firefly luciferase reporter plasmids, β-galactosidase expression vectors, and the same amount of mimics, inhibitors, or scrambled negative control (NC) RNA for luciferase reporter assays with the β-galactosidase vector (Ambion) used as an internal control. A dual luciferase assay kit (Promega) was used to analyze the luciferase activity 24 hours after transfection.

2.10 | CCK-8 cell viability assay

A cell counting kit (CCK-8) (Biosharp) was used to assess cell proliferation. The cells were seeded into 96-well plates, and after treatment or coincubation with exosomes for 48 hours, 10 μL of CCK-8 reagent was mixed into each well. Then, the 96-well plates were incubated in an incubator for 2 hours. Finally, the optical density (OD) value at 450 nm was analyzed on a microplate reader (Thermo) to calculate the cell viability. The assay was carried out at least in triplicate.

2.11 | Cell migration assay

The migratory ability of cells was determined using 24-well Transwell chambers (Costar) covered by 8.0-mm-pore polycarbonate membranes. Approximately 1×10^5 pretreated cells were seeded into the upper chamber in 200 μL of serum-free culture medium, while 600 μL of culture medium supplemented with 20% fetal bovine serum was added to the lower chamber for chemotaxis assays. After 12 hours, the migrated cells were fixed with methanol for 15 minutes and then stained with a 0.1% crystal violet solution for 15 minutes.

2.12 | Establishment of in vivo tumor-bearing mouse models

SW480 cells were transfected with lentiviruses to overexpress miR-221, miR-222, or miR-221/222, and untreated SW480 cells were used as a control. Transfected SW480 cells were injected into BALB/c-nu mice to establish in vivo models of tumor growth and metastasis.

2.13 | Statistical analyses

The data were obtained from three independent experiments and are presented as the mean ± SE. Differences between groups were measured using Student's *t*-test for comparisons between two groups or one-way ANOVA for multiple comparisons. Differences were considered significant at $P < .05$ ($^*P < .05$; $^{**}P < .01$; and $^{***}P < .001$).

3 | RESULTS

3.1 | Expression of exosomal miR-221/222 and HGF in CRC LMs

Sr-exosomes were isolated from CRC patients and normal subjects, and transmission electron microscopy (TEM) observations showed that isolated sr-exosomes were approximately 100 nm in size and had a round morphology (Figure 1A).

In addition, the exosome markers CD9, Alix, and TSG101 and the exosome negative marker GM130 were used to validate the isolated exosomes (Figure 1B). As shown in Figure 1C, most of the isolated exosomes were approximately 100 nm in size. To assess the expression of exosomal miR-221/222 in CRC accompanied by LM, we measured exosomal miR-221/222 expression by qRT-PCR. Our data showed that miR-221/222 are highly expressed in sr-exosomes of CRC patients but underexpressed in those of normal subjects. Moreover, the exosomal miR-221/222 contents were enriched in the serum of patients with LM (Figure 1D). These results showed that CRC sr-exosomes harbor miR-221/222, which may have a significant role in the process of CRC. Kaplan-Meier analysis revealed that the progression-free interval rate in the low–miR-221/222 group was consistently higher than that in the high–miR-221/222 group (Figure 1E).

Among all the miR-221/222–related mRNAs predicted with bioinformatics methods, SPINT1 was selected for analysis because of its upregulated expression in many cancers (Figure 1F). HGF is frequently expressed in the liver and has cancer-promoting effects. Accordingly, we evaluated the effect of CRC exosomes on HGF. The serum HGF levels of CRC patients were higher than those of normal subjects and were significantly increased in patients with LM (Figure 1G). The immunohistochemistry results showed high HGF expression in primary tumor and liver tissues and low HGF expression in CRC metastases (Figure 1H). These findings were supported by the Western blot results. However, the HGF receptor c-MET was highly expressed in CRC LMs (Figure 1I). These results indicate that metastatic cancer cells bind HGF and that HGF is secreted into the liver microenvironment in a paracrine manner. In addition, the Western blot results showed that HGF was highly expressed in CRC primary tumor tissues, whereas SPINT1 was downregulated (Figure 1J). In addition, the enzyme-linked immunosorbent assay (ELISA) results indicated that exosomal miR-221/222 significantly promoted liver HGF release (Figure 1K).

3.2 | miR-221/222 directly targets SPINT1 in the liver

Stromal cell and hepatocyte markers were used to characterize the types of primary hepatocytes. Alpha smooth muscle actin (α-SMA), desmin (markers of hepatic stellate cells), and F4/80 (a marker of Kupffer cells) were enriched in primary cells (Figure 2A).

Primary liver cells were treated with NC RNA or miR-221/222 mimics or inhibitors. As expected, the expression of miR-221/222 in primary liver cells treated with miR-221/222 mimics was greatly decreased compared that that observed in the NC group (Figure 2B). To further investigate the regulatory mechanism of miR-221/222 and SPINT1, luciferase reporter plasmids with a wild-type or mutant 3′-UTR of SPINT1 mRNA were obtained, with the predicted interactions between miR-221/222-3p and the 3′-UTR of SPINT1 shown in Figure 2C. The results showed marked attenuated luciferase activity in the miR-221– and miR-222–overexpressing groups, while miR-221/222 inhibition caused enhanced luciferase activity (Figure 2D). When the binding site of miR-221-3p or miR-222-3p was mutated, miR-221-3p/miR-222-3p had no effect on luciferase activity (Figure 2D). Subsequently, mRNA and protein levels were assessed in primary liver cells. SPINT1 mRNA levels remained essentially unchanged upon miR-221/222 overexpression or underexpression (Figure 2E). The Western blot results indicated that SPINT1 expression was relatively downregulated when miR-221 or miR-222 was upregulated, while treatment with miR-221/222 strongly enhanced SPINT1 protein expression (Figure 2F,G). The above results were consistent with these miRNAs inhibiting mRNA expression at the post-transcriptional level. In short, miR-221 and miR-222 were shown to regulate SPINT1 expression in primary hepatocytes by binding directly to the 3'-UTR of SPINT1 mRNA.

3.3 | SW480 exosome–delivered miR-221/222 regulates the biological behavior of primary liver cells in vitro

Exosomes secreted from SW480 cells (SW480-exos) were isolated (Figure 3A), and Western blot assays verified that the isolated exosomes expressed exosomal marker proteins but not a negative exosomal marker protein (Figure 3B). As shown in Figure 3C, miR-221/222 expression in liver stromal cells was greatly reduced upon transfection with miR-221/222 inhibitors, and SW480 cells transfected with miR-221/222 inhibitors were used to prepare exosomes removing miR-221/222 (SW480 exos miR-221/222 del). SW480 exosomes significantly suppressed SPINT1 and promoted HGF protein expression in liver stromal cells, while the enhanced HGF protein expression was blocked when miR-221/222 was depleted from the exosomes (Figure 3E). However, both SW480 and SW480 exos miR-221/222 del exosomes induced no obvious changes in SPINT1 mRNA levels (Figure 3D) indicating that exosomal miR-221/222 activates liver HGF by suppressing SPINT1. Subsequently, we assessed the impact of SW480 exosome–delivered miR-221/222 on liver stromal cell invasion and migration. As shown in the schematic diagram presented in Figure 3F, primary hepatocytes were indirectly cocultured with SW480 cells using a 0.4-mm polyester membrane, which allowed HGF to pass between the chambers (Figure 3F). CCK-8 and Transwell assays were used to evaluate the effect of SW480 exosomes on the biological function of liver stromal cells. As HGF levels increased upon exosomal miR-221/222 treatment,

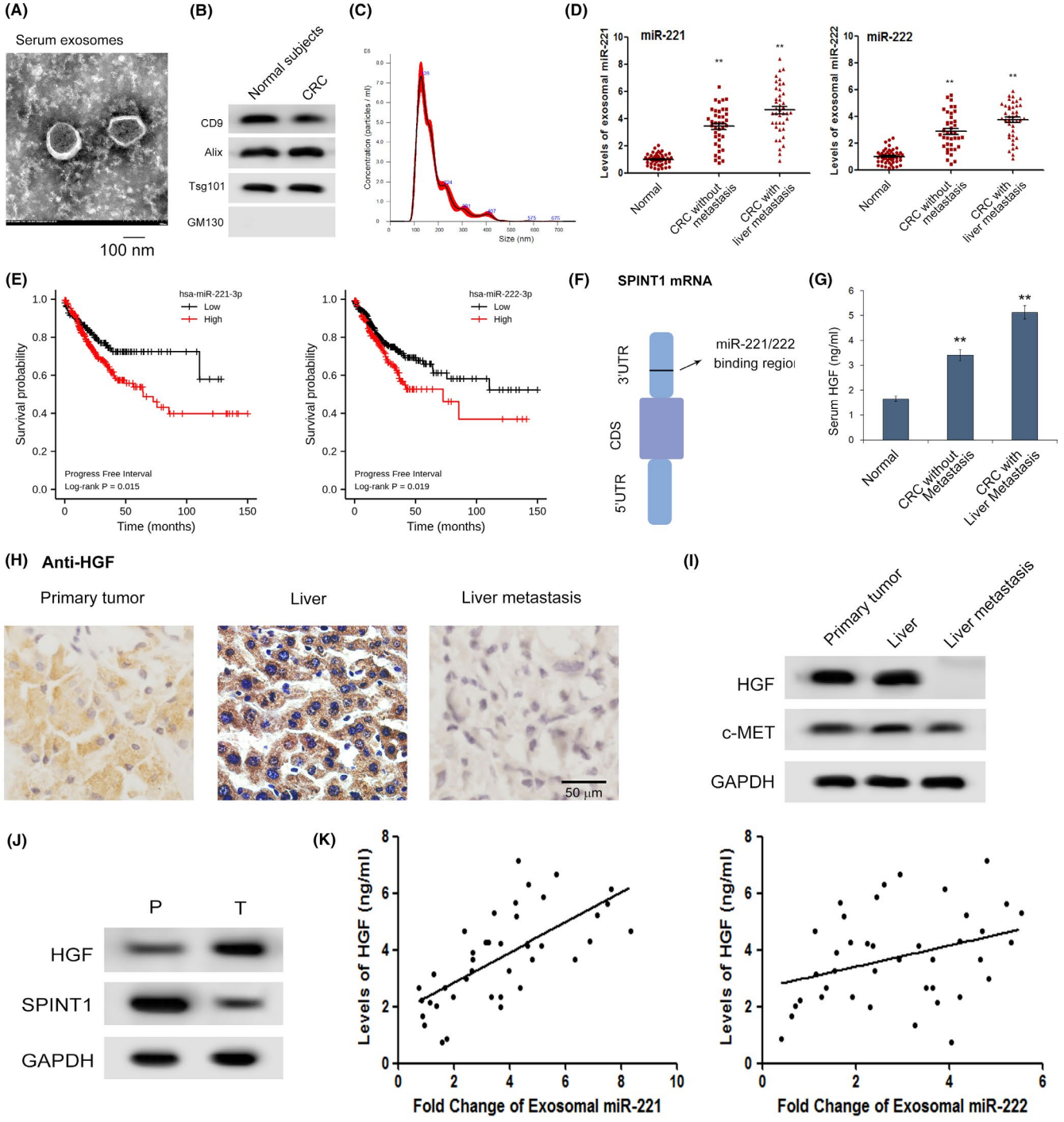


FIGURE 1 Correlation between exo-miR-221/222 and hepatocyte growth factor (HGF) in colorectal carcinoma (CRC) liver metastasis (LM). A, Scanning electron microscopy of human sr-exosomes (scale bar, 100 nm). B, Western blot (WB) of the exosome markers CD9, Alix, and TSG101 and of the negative exosome marker GM130. C, Nanoparticle tracking analysis (NTA) of isolated exosomes. D, Sr-exosomal miR-221/222 is associated with CRC LM. Exosomes were isolated from the serum of normal subjects (Normal), CRC patients without metastasis, and CRC patients with LM (n = 40). E, miR-221 and miR-222 expression was closely linked with survival in CRC. F, The potential binding positions of miR-221/222 in SPINT1 mRNA. G, ELISA analysis of serum HGF secretion in healthy donors (Normal), CRC patients without metastasis, and CRC patients with LM (n = 3). H, Immunohistochemistry (IHC) analysis of HGF (scale bar, 50 μm). I, WB analysis of HGF and c-mesenchymal-epithelial transition factor (MET) expression. J, HGF and SPINT1 expression in CRC primary tumor and CRC paracarcinoma tissues. The cancer tissue is represented by T, and the paracarcinoma tissue is represented by P. K, The clinical relevance between plasma exo-miR-221/222 and HGF expression levels (n = 42). $^{**}P < .01$

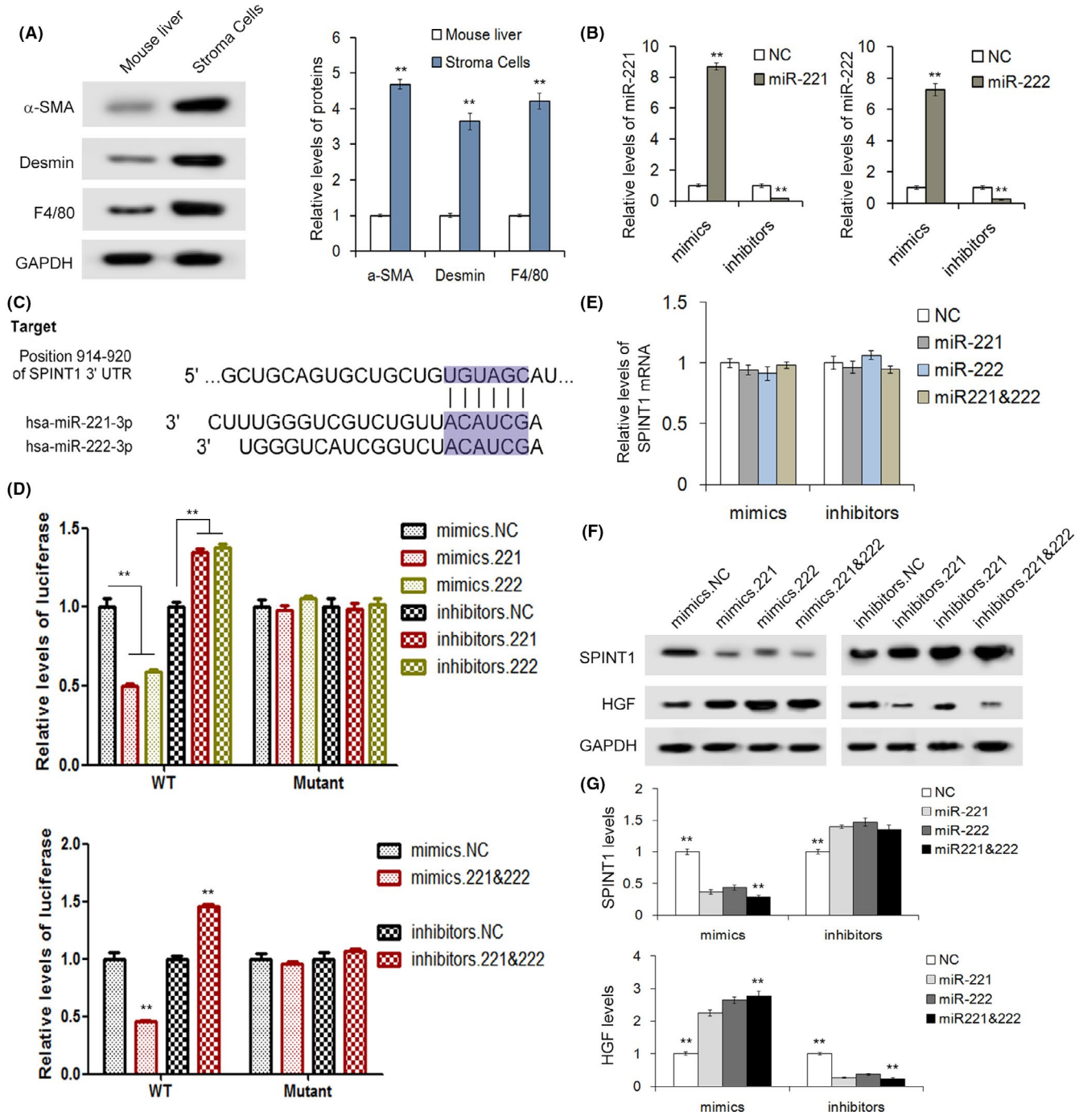
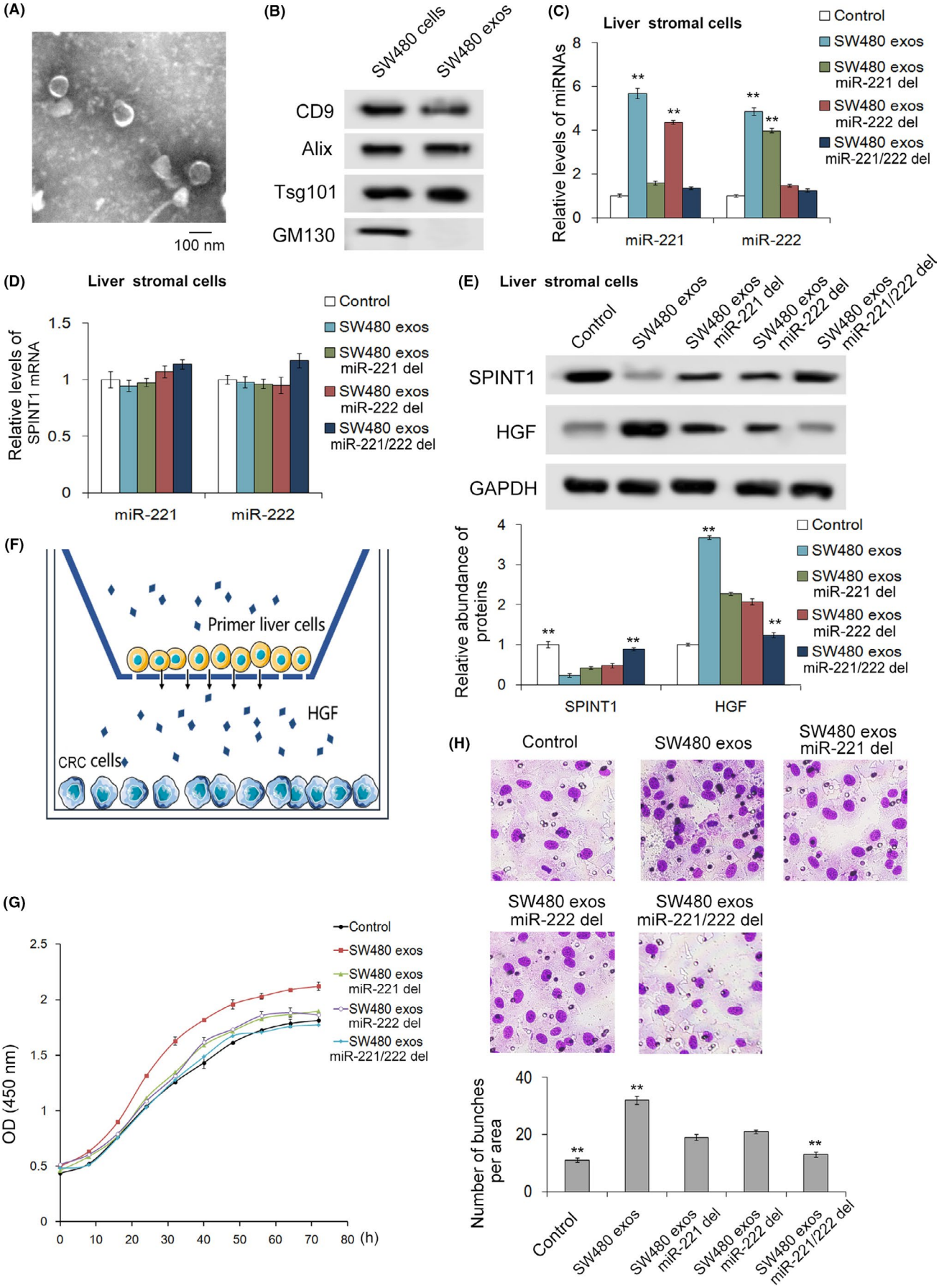


FIGURE 2 Identification of SPINT1 as the direct target of miR-221/222. A, Western blot (WB) analysis and quantification of alpha smooth muscle actin (α-SMA), desmin, and F4/80 to identify liver cells (n = 3). B, RT-qPCR quantification of miR-221 and miR-222 in samples treated with miR-221/222 mimics or miR-221/222 inhibitors (n = 3). C, The putative binding sites for miR-221/222 and SPINT1. D, Direct recognition of SPINT1 by miR-221/222 (n = 5). The relative luciferase levels were detected using a luciferase kit after transfection (n = 5). E, Relative levels of SPINT1 mRNA in liver cells transfected with miR-221/222 mimics or inhibitors (n = 4). F, WB analysis of hepatocyte growth factor (HGF) and SPINT1 levels in primary liver cells with overexpressed or suppressed miR-221 or miR-222 (n = 4). G, Quantification analysis of F. $^{**}P < .01$

FIGURE 3 Colorectal carcinoma (CRC) exosomes harboring miR-221/222 facilitate liver hepatocyte growth factor (HGF) by blocking SPINT1 expression. A, Transmission electron microscopy (TEM) images of SW480 cell exosomes (scale bar, 100 nm). B, Western blot (WB) analysis of positive and negative exosomal markers in SW480 cell exosomes. C, SW480 exos miR-221/222 del decreased liver miR-221/222 levels (n = 5). D, Effects of SW480 exosomes delivering miR-221/222 on SPINT1 mRNA levels in liver stromal cells (n = 5). E, Effects of SW480 exosomes delivering miR-221/222 on SPINT1 and HGF protein levels in mixed primary liver cells (n = 5). F, Schematic diagram of an in vitro model of cell coculture. G, The influence of SW480 exosome–delivered miR-221/222 on primary liver cell viability (n = 5). H, The influences of SW480 exosome–delivered miR-221/222 on cell invasion (n = 5). $^{**}P < .01$


(A)
100 nm
(B)
SW480 cells
SW480 exos
CD9
Alix
Tsg101
GM130
(C)
Liver stromal cells
Relative levels of miRNAs
Control
SW480 exos
SW480 exos miR-221 del
SW480 exos miR-222 del
SW480 exos miR-221/222 del
miR-221
miR-222
(D)
Liver stromal cells
Relative levels of SPINT1 mRNA
(E)
Liver stromal cells
Control
SW480 exos
SW480 exos miR-221 del
SW480 exos miR-222 del
SW480 exos miR-221/222 del
SPINT1
HGF
GAPDH
Relative abundance of proteins
(F)
Primer liver cells
HGF
CRC cells
(G)
OD (450 nm)
(h)
(H)
Control
SW480 exos
SW480 exos miR-221 del
SW480 exos miR-222 del
SW480 exos miR-221/222 del
Number of bunches per area

SW480 exos miR-221/222 del was used to reduce the HGF levels. Primary hepatocytes treated with SW480 exosomes promoted the proliferation and migration of liver stromal cells, while hepatocytes treated with SW480 exos miR-221/222 del showed the opposite effect (Figure 3G,H). In summary, these results support that SW480 exosome–mediated production of HGF plays a crucial role in influencing the biological behavior of liver stromal cells.

3.4 | SPINT1 regulates the biological behavior of liver stromal cells in vitro

As shown in Figure 4A,B, the mRNA and protein expression of HGF in liver stromal cells was highly decreased by treatment with the small interfering RNA of SPINT1 (si-SPINT1). Primary liver cells were directly treated with miR-221/222 mimics or inhibitors and cotransfected with miR-221/222 inhibitors and si-SPINT1 or miR-221/222 mimics and SPINT1-overexpressing lentivirus (OE.SPINT1). Primary liver cells overexpressing miR-221/222 notably inhibited the levels of SPINT1, whereas cotransfection with miR-221/222 mimics and OE.SPINT1 rescued this phenotype (Figure 4C,D). Then, we evaluated the influence of SPINT1 on the suppression of primary liver cell proliferation and migration. As expected, si-SPINT1 notably promoted the proliferation of primary liver cells. However, cotreatment with miR-221/222 inhibitors and si-SPINT1 reversed the effect of miR-221/222 inhibitors on the proliferation of primary liver cells (Figure 4E). Similarly, OE.SPINT1 significantly blocked the migration of primary liver cells (Figure 4F), while cotransfection with miR-221/222 mimics and OE.SPINT1 rescued this phenotype (Figure 4G). The above in vitro results supported the conclusion that miR-221/222 has a great influence on the malignant biological properties of liver stromal cells by regulating SPINT1.

3.5 | Exosomal miR-221/222 regulates liver HGF by suppressing SPINT1 in vivo

We generated tumor xenograft models to verify the effect of miR-221/222 on tumor growth. SW480 cells were treated with lentiviruses to overexpress miR-221, miR-222, or miR-221/222, with untreated SW480 cells used as a control (miR-221 OE, miR-222 OE, miR-221/222 OE, and untreated, respectively). The diameters and weights of the tumors were recorded over time, as shown in Figure 5A. The diameters and weight of tumors were markedly increased in the miR-221 OE, miR-222 OE, and miR-221/222 OE groups compared with the untreated group (Figure 5B,C). The overexpression of miR-221 or miR-222 promoted the growth of metastatic foci, increasing the liver metastatic ratio and diameters of metastatic foci (Figure 5D,E,F). Detailed data on the number/proportion of LMs in mice and the diameters of LMs are provided in the Supplementary Material. Then, the miR-221/222 contents in mouse plasma exosomes were measured by RT-qPCR. Compared with the untreated group, miR-221 or miR-222 levels were upregulated in the plasma exosomes in the miR-221 OE and miR-222 OE groups and were markedly increased in the miR-221/222 OE group (Figure 5G). The overexpression of miR-221 or miR-222 caused a dramatic decrease in SPINT1 levels in tumor tissue but had no effect on SPINT1 mRNA levels. As expected, miR-221 or miR-222 overexpression greatly increased the RNA and protein levels of HGF (Figure 5H,I,J). These results obtained using tumor-implanted mouse models further demonstrated that CRC exosomes harboring miR-221/222 activate liver HGF by suppressing SPINT1 expression.

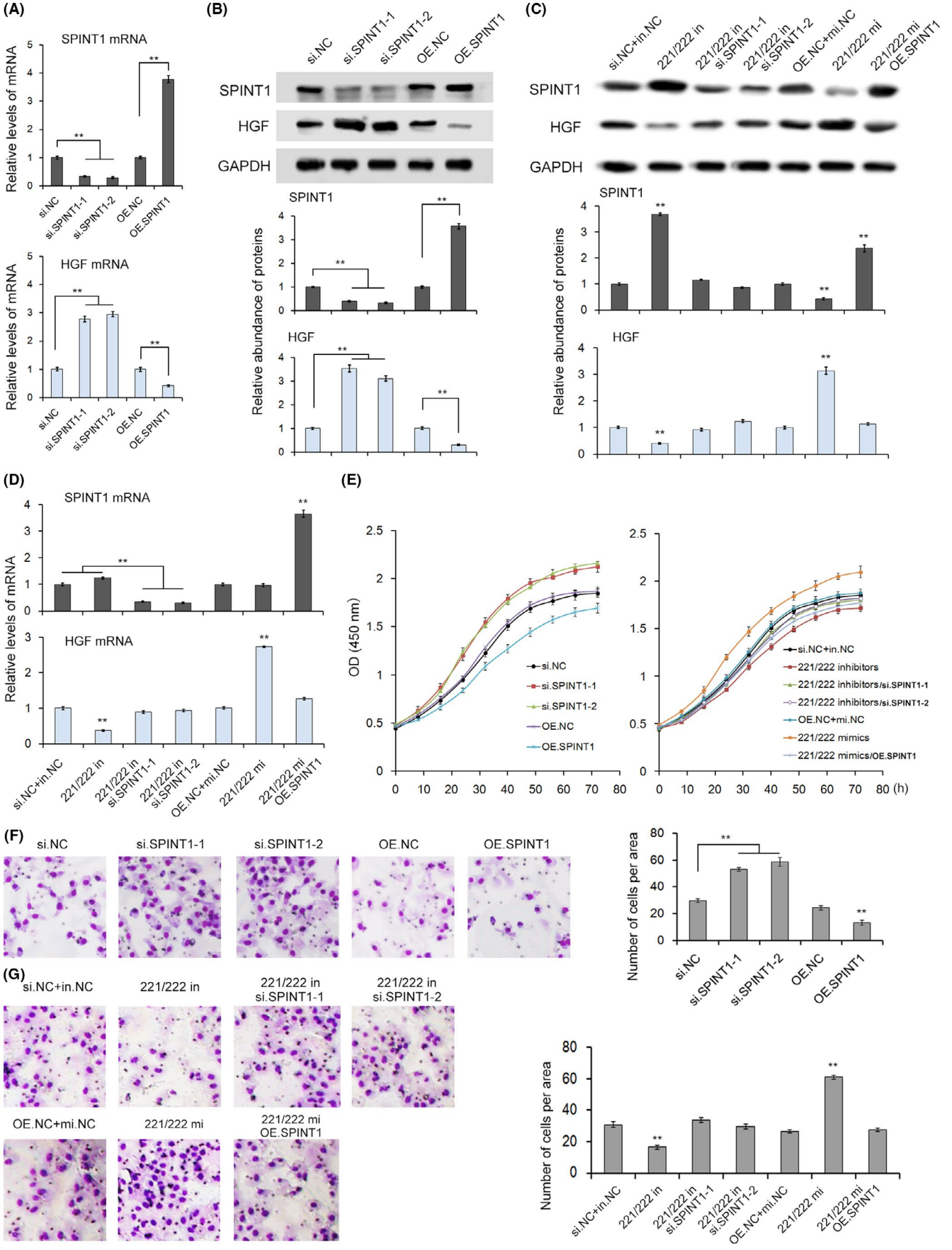
4 | DISCUSSION

The tumor microenvironment can be fine-tuned, is responsible for tumorigenesis and development, and coordinates communication between cells.[26] Kaplan and Lyden initially showed that the adaptability of the PMN prior to the arrival of incoming tumor cells is a crucial means by which tumors promote their natural behavior.[27] For instance, exosomes of melanoma are located in the sentinel lymph node and exert synchronous molecular signals that affect cell recruitment, remodeling of the extracellular matrix, and lymph node angiogenesis.[28] Similarly, recent findings have emphasized the importance of other tumor secretory elements in the establishment of a niche before tumor metastasis.[29-31] In the present study, we showed the importance of miR-221/222 in the crosstalk between primary tumors and metastases.

In the present study, we revealed that miR-221/222 secreted by CRC can be transported to liver stromal cells via exosomes, inducing the formation of a hospitable metastatic environment, providing an appropriate colonization environment for incoming metastatic tumor cells, and consequently contributing to CRC metastasis. Moreover, our data show that tumor-derived exosomal miR-221/222-3p can induce hepatic stromal cells to secrete HGF in the PMN (Figure 6).

Exosomes secreted from tumors play an important role in the construction of the tumor microenvironment to accelerate tumor occurrence, development, and migration.[32-35] Exosomes are known to mediate tumor immune escape and drug resistance.[36,37] Nevertheless, the impact of exosomes on the relationship between primary tumors and metastatic foci needs to be further investigated.

FIGURE 4 Liver SPINT1 downregulation promotes the proliferation and invasion of primary liver cells. A, RT-qPCR quantification of SPINT1 and hepatocyte growth factor (HGF) in samples treated with si-SPINT1 and SPINT1-overexpressing lentivirus (OE.SPINT1) (n = 5). B, SPINT1 and HGF protein levels in liver cells transfected with SPINT1 siRNA and OE.SPINT1 (n = 5). C, Liver stromal cells were directly treated with miR-221/222 mimics or inhibitors and cotransfected with miR-221/222 inhibitors and si-SPINT1 or miR-221/222 mimics and OE.SPINT1. Western blot (WB) analysis of SPINT1 and HGF expression in primary liver cells. (n = 7). D, Relative mRNA levels of SPINT1 and HGF in liver cells. E, Growth curves of liver cells after transfection. F, Liver SPINT1 downregulation promoted the migration of liver cells (n = 5). G, Transwell assays of liver stromal cells after transfection, as described above (n = 7). $^{**}P < .01$


(A)
SPINT1 mRNA
Relative levels of mRNA
si.NC
si.SPINT1-1
si.SPINT1-2
OE.NC
OE.SPINT1
HGF mRNA
(B)
SPINT1
HGF
GAPDH
Relative abundance of proteins
(C)
si.NC+in.NC
221/222 in
221/222 in si.SPINT1-1
221/222 in si.SPINT1-2
OE.NC+mi.NC
221/222 mi
221/222 mi OE.SPINT1
(D)
si.NC+in.NC
221/222 in
221/222 in si.SPINT1-1
221/222 in si.SPINT1-2
OE.NC+mi.NC
221/222 mi
221/222 mi OE.SPINT1
(E)
OD (450 nm)
si.NC
si.SPINT1-1
si.SPINT1-2
OE.NC
OE.SPINT1
221/222 inhibitors
221/222 inhibitors/si.SPINT1-1
221/222 inhibitors/si.SPINT1-2
OE.NC+mi.NC
221/222 mimics
221/222 mimics/OE.SPINT1
80 (h)
(F)
Number of cells per area
(G)

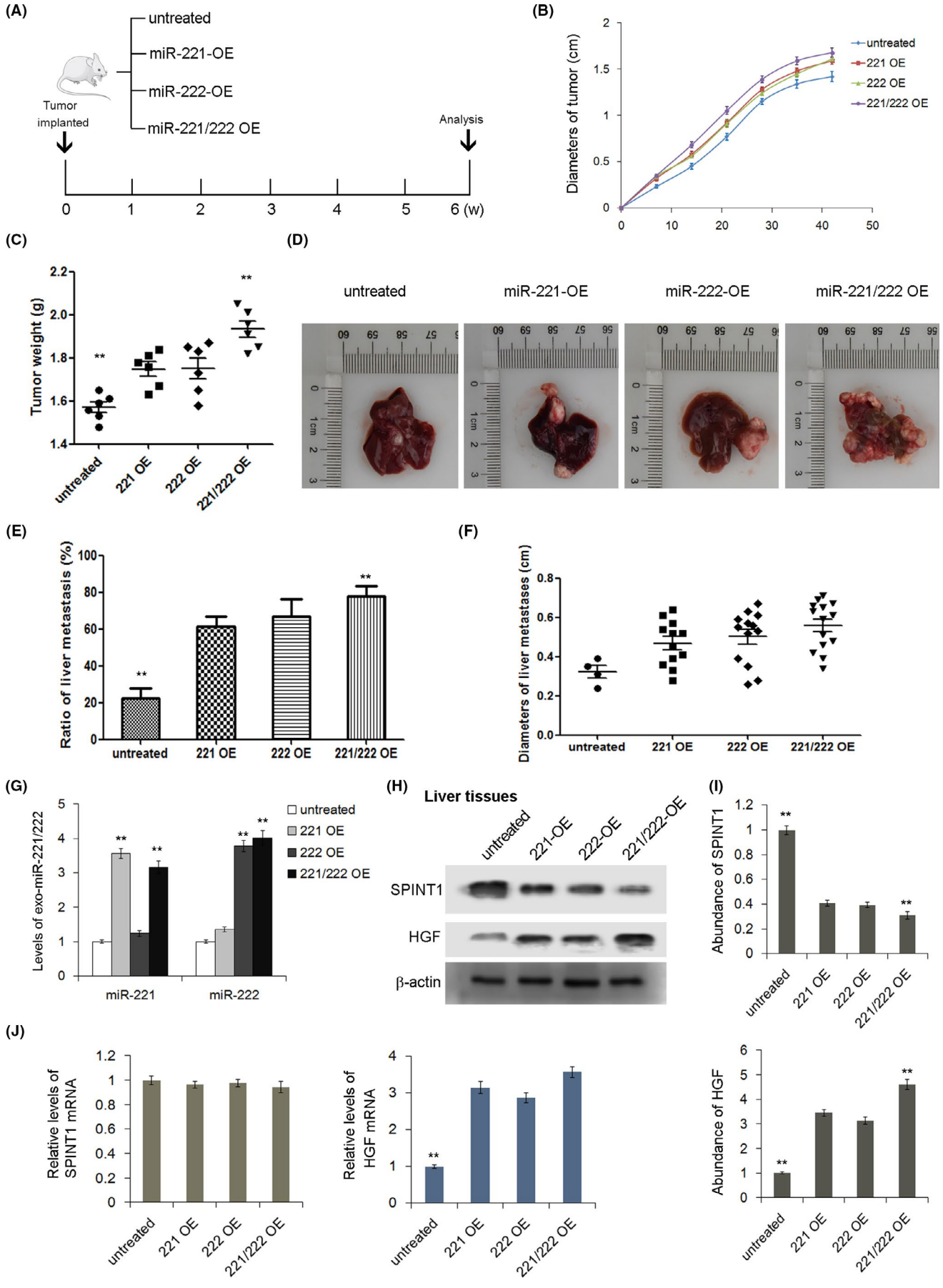

(A)
untreated
miR-221-OE
miR-222-OE
miR-221/222 OE
Tumor implanted
Analysis
0 1 2 3 4 5 6 (w)
(B)
Diameters of tumor (cm)
untreated
221 OE
222 OE
221/222 OE
(C)
Tumor weight (g)
untreated
221 OE
222 OE
221/222 OE
(D)
untreated
miR-221-OE
miR-222-OE
miR-221/222 OE
(E)
Ratio of liver metastasis (%)
untreated
221 OE
222 OE
221/222 OE
(F)
Diameters of liver metastases (cm)
untreated
221 OE
222 OE
221/222 OE
(G)
Levels of exo-miR-221/222
untreated
221 OE
222 OE
221/222 OE
miR-221
miR-222
(H)
Liver tissues
untreated
221-OE
222-OE
221/222-OE
SPINT1
HGF
β-actin
(I)
Abundance of SPINT1
untreated
221 OE
222 OE
221/222 OE
(J)
Relative levels of SPINT1 mRNA
Relative levels of HGF mRNA
Abundance of HGF
untreated
221 OE
222 OE
221/222 OE

FIGURE 5 In vivo validation showing that exosomal miR-221/222 activates liver hepatocyte growth factor (HGF) by inhibiting SPINT1. A, Effects of exosomal miR-221/222 on colorectal carcinoma (CRC) in a murine model. Schematic diagram of the experimental design used to establish the animal models. B, The diameters of the tumors (n = 6). C, Alterations in weight per group (n = 6). D, Representative images of tumor liver metastasis in nude mice in different groups (n = 6). E, Rate of liver metastasis in nude mice (n = 6). F, The diameters of mouse liver metastases (n = 6). G, RT-qPCR quantification of sr-exosomal miR-221/222 in mice (n = 6). H, Levels of SPINT1 and HGF in liver tissues (n = 6) and I, quantification. J, Relative levels of SPINT1 and HGF mRNA in mouse liver tissues (n = 6). $^{**}P < .01$

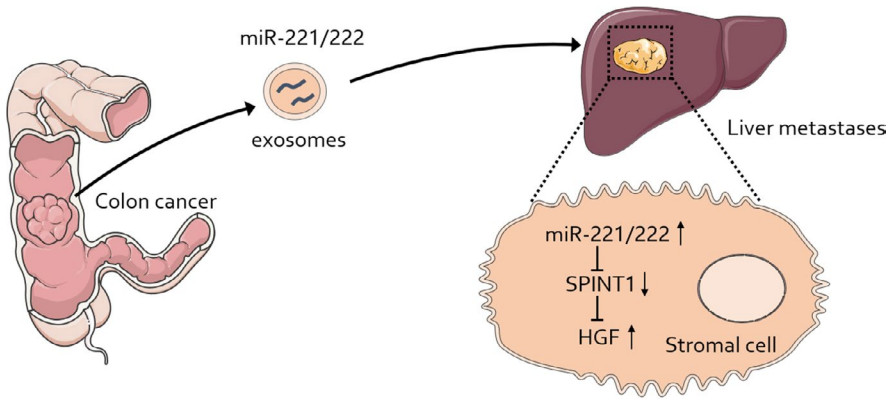


FIGURE 6 Schematic representation of the regulatory landscape in which the miR-221/222/SPINT1/hepatocyte growth factor (HGF) signaling axis contributes to the pathogenesis of liver metastasis (LM) in colorectal carcinoma (CRC)

Thus, it is necessary to study the interactions between the tumor and stroma through exosomes. MiR-221/222-3p is an onco-promoting miRNA that is closely related to poor prognosis in CRC patients.[38,39] However, the unique mechanisms by which miR-221/222-3p regulates CRC metastasis have been largely unelucidated. In the present study, we report a new signaling pathway between tumors and metastatic cells. As a result of this intercellular communication, miRNAs secreted by tumor exosomes exert an important influence on tumor metastasis by regulating the premetastatic microenvironment, which in turn accelerates primary tumor metastasis.

As a serine protease inhibitor, SPINT1 is involved in many biological behaviors in cells and, more importantly, affects the process of tumorigenesis.[40,41] SPINT1 has been reported to effectively inhibit the activity of a variety of trypsin-like serine proteases, such as hepatocyte growth factor activator (HGFA) and matrix metalloproteinases, which participate in tumor processes.[42] However, the regulatory mechanism that leads to abnormal SPINT1 expression under different circumstances has remained unclear. A lack of SPINT1 promotes tumor transformation and regulates signaling between tumor cells and the immune microenvironment, which is involved in the invasiveness of skin cutaneous melanoma.[43] In our present study, we demonstrated that SPINT1 regulates the malignant biological behavior of CRC cells in vitro.

Taken together, our results show that CRC exosomes deliver miR-221/222 to exert a crucial effect in promoting liver-specific metastasis through remodeling of the liver microenvironment. Our data support the importance of the PMN and reveal a new mechanism of CRC LM. Whether miR-221/222 secreted by tumor exosomes affects the process of tumorigenesis and metastasis through other signaling pathways needs to be further studied. In future research, we will continue to assess how tumor exosomes affect the tumor microenvironment to provide novel directions for the treatment of tumor metastasis.

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DISCLOSURE

The authors have no conflict of interest.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424.
2. Kopetz S, Chang GJ, Overman MJ, et al. Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. *J Clin Oncol*. 2009;27(22):3677-3683.
3. Brodt P. Role of the microenvironment in liver metastasis: from pre- to prometastatic niches. *Clin Cancer Res*. 2016;22(24):5971-5982.
4. Nguyen A, Loo JM, Mital R, et al. PKLR promotes colorectal cancer liver colonization through induction of glutathione synthesis. *J Clin Invest*. 2016;126(2):681-694.
5. Villalba M, Evans SR, Vidal-Vanaclocha F, Calvo A. Role of TGF-β in metastatic colon cancer: it is finally time for targeted therapy. *Cell Tissue Res*. 2017;370(1):29-39.
6. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. *Cancer Cell*. 2016;30(6):836-848.

7. Feng W, Dean DC, Hornicek FJ, Shi H, Duan Z. Exosomes promote pre-metastatic niche formation in ovarian cancer. *Mol Cancer*. 2019;18(1):124.
8. Guo Y, Ji X, Liu J, et al. Effects of exosomes on pre-metastatic niche formation in tumors. *Mol Cancer*. 2019;18(1):39.
9. Liu Y, Gu Y, Cao X. The exosomes in tumor immunity. *Oncoimmunology*. 2015;4(9):e1027472.
10. Peinado H, Alečković M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;18(6):883-891.
11. Costa-Silva B, Aiello NM, Ocean AJ, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol*. 2015;17(6):816-826.
12. Liu Y, Gu Y, Han Y, et al. Tumor exosomal RNAs promote lung pre-metastatic niche formation by activating alveolar epithelial TLR3 to recruit neutrophils. *Cancer Cell*. 2016;30(2):243-256.
13. Taylor DD, Gercel-Taylor C. Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. *Semin Immunopathol*. 2011;33(5):441-454.
14. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19(1):92-105.
15. Zhao S, Mi Y, Guan B, et al. Tumor-derived exosomal miR-934 induces macrophage M2 polarization to promote liver metastasis of colorectal cancer. *J Hematol Oncol*. 2020;13(1):156.
16. Fang JH, Zhang ZJ, Shang LR, et al. Hepatoma cell-secreted exosomal microRNA-103 increases vascular permeability and promotes metastasis by targeting junction proteins. *Hepatology*. 2018;68(4):1459-1475.
17. Garofalo M, Quintavalle C, Romano G, Croce CM, Condorelli G. miR221/222 in Cancer: their role in tumor progression and response to therapy. *Curr Mol Med*. 2012;12(1):27-33.
18. Xu J, Su Y, Xu A, et al. miR-221/222-Mediated inhibition of autophagy promotes dexamethasone resistance in multiple myeloma. *Mol Ther*. 2019;27(3):559-570.
19. Liu S, Wang Z, Liu Z, et al. miR-221/222 Activate the Wnt/beta-catenin signaling to promote triple-negative breast cancer. *J Mol Cell Biol*. 2018;10(4):302-315.
20. Iida M, Hazama S, Tsunedomi R, et al. Overexpression of miR221 and miR222 in the cancer stroma is associated with malignant potential in colorectal cancer. *Oncol Rep*. 2018;40(3):1621-1631.
21. Yuan K, Xie K, Fox J, et al. Decreased levels of miR-224 and the passenger strand of miR-221 increase MBD2, suppressing maspin and promoting colorectal tumor growth and metastasis in mice. *Gastroenterology*. 2013;145(4):853-864.e9.
22. Hoshiko S, Kawaguchi M, Fukushima T, et al. Hepatocyte growth factor activator inhibitor type 1 is a suppressor of intestinal tumorigenesis. *Cancer Res*. 2013;73(8):2659-2670.
23. Liu CL, Yang PS, Chien MN, Chang YC, Lin CH, Cheng SP. Expression of serine peptidase inhibitor Kunitz type 1 in differentiated thyroid cancer. *Histochem Cell Biol*. 2018;149(6):635-644.
24. Ishikawa T, Kimura Y, Hirano H, Higashi S. Matrix metalloproteinase-7 induces homotypic tumor cell aggregation via proteolytic cleavage of the membrane-bound Kunitz-type inhibitor HAI-1. *J Biol Chem*. 2017;292(50):20769-20784.
25. Oberst MD, Johnson MD, Dickson RB, et al. Expression of the serine protease matriptase and its inhibitor HAI-1 in epithelial ovarian cancer: correlation with clinical outcome and tumor clinicopathological parameters. *Clin Cancer Res*. 2002;8(4):1101-1107.
26. Leonardi GC, Candido S, Cervello M, et al. The tumor microenvironment in hepatocellular carcinoma (review). *Int J Oncol*. 2012;40(6):1733-1747.
27. Sethi N, Kang Y. Unravelling the complexity of metastasis - molecular understanding and targeted therapies. *Nat Rev Cancer*. 2011;11(10):735-748.
28. Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. *Cancer Res*. 2011;71(11):3792-3801.
29. Hiratsuka S, Ishibashi S, Tomita T, et al. Primary tumours modulate innate immune signalling to create pre-metastatic vascular hyperpermeability foci. *Nat Commun*. 2013;4:1853.
30. Hiratsuka S, Watanabe A, Aburatani H, Maru Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol*. 2006;8(12):1369-1375.
31. Fong MY, Zhou W, Liu L, et al. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. *Nat Cell Biol*. 2015;17(2):183-194.
32. Kurywchak P, Kalluri R. An evolving function of DNA-containing exosomes in chemotherapy-induced immune response. *Cell Res*. 2017;27(6):722-723.
33. Tian X, Shen H, Li Z, Wang T, Wang S. Tumor-derived exosomes, myeloid-derived suppressor cells, and tumor microenvironment. *J Hematol Oncol*. 2019;12(1):84.
34. Sun Z, Yang S, Zhou Q, et al. Emerging role of exosome-derived long non-coding RNAs in tumor microenvironment. *Mol Cancer*. 2018;17(1):82.
35. Wortzel I, Dror S, Kenific CM, Lyden D. Exosome-mediated metastasis: communication from a distance. *Dev Cell*. 2019;49(3):347-360.
36. Yin Y, Cai X, Chen X, et al. Tumor-secreted miR-214 induces regulatory T cells: a major link between immune evasion and tumor growth. *Cell Res*. 2014;24(10):1164-1180.
37. Pink RC, Samuel P, Massa D, Caley DP, Brooks SA, Carter DR. The passenger strand, miR-21-3p, plays a role in mediating cisplatin resistance in ovarian cancer cells. *Gynecol Oncol*. 2015;137(1):143-151.
38. Liu S, Sun X, Wang M, et al. A microRNA 221- and 222-mediated feedback loop maintains constitutive activation of NFκB and STAT3 in colorectal cancer cells. *Gastroenterology*. 2014;147(4):847-859.e11.
39. Yau TO, Wu CW, Dong Y, et al. microRNA-221 and microRNA-18a identification in stool as potential biomarkers for the non-invasive diagnosis of colorectal carcinoma. *Br J Cancer*. 2014;111(9):1765-1771.
40. Nakamura K, Hongo A, Kodama J, Hiramatsu Y. The role of hepatocyte growth factor activator inhibitor (HAI)-1 and HAI-2 in endometrial cancer. *Int J Cancer*. 2011;128(11):2613-2624.
41. Nakamura K, Abarzua F, Hongo A, et al. The role of hepatocyte growth factor activator inhibitor-1 (HAI-1) as a prognostic indicator in cervical cancer. *Int J Oncol*. 2009;35(2):239-248.
42. Cheng H, Fukushima T, Takahashi N, Tanaka H, Kataoka H. Hepatocyte growth factor activator inhibitor type 1 regulates epithelial to mesenchymal transition through membrane-bound serine proteinases. *Cancer Res*. 2009;69(5):1828-1835.
43. Gómez-Abenza E, Ibáñez-Molero S, García-Moreno D, et al. Zebrafish modeling reveals that SPINT1 regulates the aggressiveness of skin cutaneous melanoma and its crosstalk with tumor immune microenvironment. *J Exp Clin Cancer Res*. 2019;38(1):405.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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