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RESEARCH ARTICLE

Liver cancer cells with nuclear MET overexpression release translation regulatory protein-enriched extracellular vesicles exhibit metastasis promoting activity

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Abstract

MET receptor tyrosine kinase is a cell surface receptor that plays important role in embryonic development and tissue regeneration. Aberrant MET activation has been widely reported in different human cancers, making MET an attractive therapeutic target. The presence of truncated MET within the nucleus (nMET) with potential novel functions poses a great challenge to the current therapeutic strategies against MET surface receptor. Previous work has demonstrated the promoting effect of nMET in aggressive properties of hepatocellular carcinoma (HCC) cells by activating TAK1/NF-κB signalling pathway. Herein, we report the role of nMET in modulating tumour microenvironment and tumour metastasis mediated by extracellular vesicles (EVs). EVs released by nMET overexpressing cells enhanced cell motility and provoked metastasis. Proteomic profiling revealed the enrichment of translational regulatory proteins in EVs derived from nMET overexpressing cells. These proteins include eukaryotic initiation factor (EIF), ribosomal protein small subunit (RPS) and ribosomal protein larger subunit (RPL) gene families. Knockdown of EIF3I, RPS3A and RPL10 diminished the promoting effect of EVs in cell migration invasiveness and metastasis. In conclusion, the findings reveal an unrecognized capacity of nMET to augment HCC through the release of EVs with oncogenic effect. Targeting these translation-related proteins may serve as an alternative treatment for patients with nMET overexpression.

KEYWORDS
eukaryotic initiation factor, hepatocellular carcinoma, MET receptor tyrosine kinase, ribosomal protein, translation

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and accounts for nearly 90% of cases. Currently, it is the fourth leading cause of cancer-related death worldwide (Llovet et al., 2021; Villanueva, 2019). HCC at early stage is often asymptomatic, resulting in late diagnosis that limits late-stage patient to curative surgical resection. Understanding of molecular mechanism that regulates HCC development and metastasis is pivotal to overhaul the current diagnosis and treatment of HCC.

Extracellular vesicles (EVs) are membrane-derived vesicles in nanometre size. Emerging evidence has revealed the unequivocal role of EVs in mediating intercellular communication through transferring donor cell-derived bioactive molecules into recipient

cells. Uptake of bioactive molecules, including nucleic acids, lipids and proteins, triggers signalling cascades that regulate various physiology and pathophysiology processes in the recipient cells. In tumour microenvironment, cancer cell-derived EVs function as critical messengers in connecting tumour cells and their local and distant microenvironment to facilitate cancer progression (Han et al., 2019).

MET receptor tyrosine kinase is a cell surface receptor that binds to its ligand, hepatocyte growth factor (HGF). HGF-MET axis plays indispensable role in the coordination of embryonic development and tissue regeneration under normal circumstances. However, aberrant HGF-MET axis activation promotes tumorigenesis and metastasis in many carcinomas, making it an attractive therapeutic target (Comoglio et al., 2018; Recondo et al., 2020). In HCC, inappropriate MET activation promotes cancer cell proliferation, mobility, metastasis, drug resistance as well as antiapoptosis features (Giordano & Columbano, 2014).

Intriguingly, localization of MET is not restricted to the cell membrane and cytoplasm but also the nucleus. Nuclear MET (nMET) was first reported in melanoma tumours and cell lines independent of HGF activation (Saitoh et al., 1994). However, the presence of MET in the nuclei of uveal melanomas and metastatic breast cancer cells are found to be influenced by HGF stimulation (Previdi et al., 2010; Ye et al., 2008). In addition, nMET has been also reported in various cancer cell lines and cancerous tissues (Brusevold et al., 2010; Edakuni et al., 2001; Matteucci et al., 2009). Our previous work revealed that nMET accelerated HCC tumorigenesis and metastasis (Tey et al., 2017). Although the identification of nMET/TAK1/NF-κB axis provided mechanistic insight into the intracellular nMET-induced cancer cell aggressiveness, the role of nMET in modulating tumour microenvironment and tumour metastasis remained unknown. The current study revealed EVs released by nMET overexpressing cells are enriched with proteins that regulate translation. Functionally, EVs of nMET overexpressing cells exhibited augmenting promoting capacity in cell migration, invasiveness and metastasis.

2 | MATERIAL AND METHODS

2.1 | Cell culture

Human HCC cell line PLC/PRF/5 and human embryonal kidney cells 293FT were purchased from American Type Culture Collection. For other HCC cell lines, Huh7 and MHCC97L, were obtained from Japanese Collection of Research Bioresources and Cancer Institute, Fudan University, China, respectively. Human immortalized normal liver cell line MIHA was provided by Jayanta Roy-Chowdhury, Albert Einstein College of Medicine, New York (Brown et al., 2000). Mouse p53-/-;Myc hepatoblast was provided by Scott Lowe, Memorial Sloan Kettering Cancer Center, New York (Zender et al., 2005, 2006). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco) and 100 U/ml penicillin-streptomycin (Gibco) in a 5% CO_2 humidified incubator at 37°C. Mycoplasma checking was regularly performed by PCR screening (oligos listed in Supplementary Table S1).

2.2 | Establishment of stable cell lines

nMET overexpressing MHCC97L cells were established as described previously (Tey et al., 2017). In brief, N-terminally truncated form of MET (D972) and a myc tag sequence was subcloned into the expression vector pLVX-Tight-Puro (Clontech). Parental MHCC97L cells transfected with empty vector was used as control cells. Doxycycline (2 μg/ml, Clontech) was added into culture media for 24 h to induce vector expression in both control and nMET cells. To establish EIF3I, EIF4G2, RPS3A and RPL10 knockdown stable cells, short-hairpin RNA (shRNA) sequences were sourced from Genetic Perturbation Platform Web Portal (https://portals.broadinstitute.org/gpp/public/) (Yang et al., 2011). The oligos of shRNA were listed in Supplementary Table S1. The complimentary oligos of shRNA sequence were annealed before ligating into pLKO.1 Hygro (#24150, Addgene) through AgeI and EcoRI sites. The knockdown plasmids were transfected into 293FT cells using Lenti-Pac™ HIV Expression Packaging Kit (#LT001, GeneCopoeia) according to manufacturer's protocol. The viral supernatant was collected, centrifuged and filtered before used for transducing nMET overexpressing cells. Polybrene (8 μg/ml, Sigma-Aldrich) was added as transduction enhancer and transfected cells were selected by hygromycin (100 μg/ml, Gibco).

2.3 | Isolation and validation of EVs

EV-depleted FBS was collected after overnight centrifugation at 100,000*g* at 4°C (Beckman Coulter, Optima™ XPN-100). For conditioned-media collection, cells were cultured in media supplemented with 10% EV-depleted FBS and doxycycline (2 μg/ml) for 72 h. Conditioned-media were subjected to serial ultracentrifugation (Beckman Coulter, Optima™ XPN-100) to isolate EVs as described elsewhere (Liu et al., 2021; Tey et al., 2021). EVs were resuspended in PBS and stored at -20°C for further use. Lysed EV proteins were subjected to immunoblotting using anti-Alix (#2171, Cell Signalling Technology), anti-CD9 (#ab92726, Abcam),

anti-TSG101 (#612696, BD Biosciences), anti-GM130 (#ab52149, Abcam) and anti-α-tubulin (#2144, Cell Signalling Technology) antibodies. The particle size and concentration of EVs were measured by ZetaView® TWIN-NTA PMX-220 (Particles Metrix GmbH) according to manufacturer's protocol. We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV220069) (Van Deun et al., 2017).

2.4 | Immunogold labelling of EVs

EVs in PBS were incubated on formvar carbon-coated nickel grids for 20 mins at room temperature before washing thrice with PBS. The grids were blocked with 1% BSA/PBS for 10 mins before incubating with anti-CD63 (#ab134045, Abcam; 1:25 ratio in 1% BSA) for 30 mins. After washing with 1% BSA for three times, the grids were incubated with secondary antibodies conjugated with 5 nm gold particles (#ab272351, Abcam; 1:1 ratio in 1% BSA) for 20 mins. The grids were then counterstained with Reynold's lead citrate after washing with PBS. The grids were visualized under Tecnai T12 Transmission Electron Microscope (FEI Company).

2.5 | Functional assay

Cells were seeded at a density of 1×10^5 in 60 mm culture plate and pretreated with 25 μg/ml of EVs for 72 hr before functional assays. The concentration of EVs were measured by BSA assay (Bio-Rad Corporation). For colony formation assay, cells were seeded at 1×10^3 per well in six-well plate and incubated until colonies formed could be visualized. Cells were fixed with methanol and stained with crystal violet. The number of colonies was counted. For migration and invasion assays, Transwell® Permeable Supports (#3433, Corning) were used. For invasion assay, Corning® Matrige® Basement Membrane Matrix (#354234, Corning) was used to coat the transwell inserts before use. For both assays, 4×10^4 cells were seeded in serum-free medium at the upper chamber of transwells. At the bottom of chamber, medium with 10% FBS was added as chemoattractant. After 16 h, cells were fixed with methanol and stained with crystal violet. Four fields were randomly selected and the number of migrated and invaded cells was counted.

2.6 | Experimental metastasis assay

For lung colonization model, 1×10^5 mouse p53-/-; Myc hepatoblasts together with 5 μg EVs or PBS were coinjected into 6-week-old male BALB/cAnN-nu mice. The mice were monitored by weekly bioluminescence imaging by IVIS spectrum imaging system (Perkin Elmer). At the end of experiment, *ex vivo* bioluminescence imaging of lungs was performed, and dissected lungs were subjected to histological analysis. All animal studies were performed under the research protocol CULATR 2229-10 and 3783-15 approved by the Committee of the Use of Live Animals in Teaching and Research (CULATR), The University of Hong Kong. All animal work and procedures were followed strictly according to the Animals (Control of Experiments) Ordinance (Hong Kong) and the Institute's guidance from Centre for Comparative Medical Research (CCMR), Li Ka Shing Faculty of Medicine, The University of Hong Kong. All mice were provided by and housed in specific pathogen free area in the CCMR building.

2.7 | EV protein extraction, in-solution digestion and mass spectrometry

Extracted EVs were lysed in urea lysis buffer (8 M urea in 100 mM Tris, adjusted to pH 8.5) followed by centrifugation at 14000*g* for 30 min. The supernatant fraction was collected for protein quantitation using Bradford assay (Bio-Rad) using bovine serum albumin as standard. Briefly, 10 μg of protein was subjected to reduction and alkylation using 10 mM tris(2-carboxyethyl)phosphine and 100 mM iodoacetamide, respectively. Protein digestion was performed by incubating with trypsin (Promega) overnight at 37°C. The tryptic peptide was dried using Concentrator Plus (Eppendorf). Peptides were desalted using Pierce™ C18 Tips (Thermo Scientific) prior to LC-MS/MS analysis. Eluted peptides were analyzed using Orbitrap Fusion™ Lumos™ Tribid™ mass spectrometer (Thermo Scientific). Peptides were separated on commercial C18 column (75 μm i.d. × 50 cm length) with 1.9 μm particle size (Thermo Scientific). Separation was attained using a linear gradient of increasing buffer B (80% ACN and 0.1% formic acid) and declining buffer (0.1% formic acid) at 300 nL/min. Buffer B was increased to 30% B in 150 min and ramped to 40% B in 10 min followed by a quick ramp to 95% B, where it was held for 5 min before a quick ramp back to 5% B, where it was held and the column was re-equilibrated. Mass spectrometer was operated in positive polarity mode with capillary temperature of 300°C. Full MS survey scan resolution was set to 120,000 with an automatic gain control (AGC) target value of 2×10^6, maximum ion injection time (IT) of 50 ms, and for a scan range of 350–1500 m/z. A data-dependent top 10 method was operated during which higher-energy collisional dissociation (HCD). Spectra were obtained at 30000 MS2 resolution with AGC target of

1×10^5 and maximum ion IT of 100 ms, 1.6 m/z isolation width, and normalized collisional energy of 30. Preceding precursor ions targeted for HCD were dynamically excluded of 40 s.

2.8 | Label-free protein quantification

Raw mass spectrometry data were processed using Maxquant version 2.0.1.0, wherein the MS data analysed in triplicate for each condition were searched using the Andromeda algorithm against the Human UniProt protein database (UP000005640_9606) using the following settings: oxidized methionine (M), acetylation (Protein N-term) as dynamic modifications, and carbamidomethyl (C) as fixed modifications, with a minimum peptide length of seven amino acids. The parameters were set appropriately to obtain the peptide and protein data using 0.1% FDR at both the peptide and protein level. The EV proteins identified were quantified using the peptide LFQ intensities. Data visualization and analysis were performed using Perseus software version 1.6.15.0 with ratio ≥2-fold as cut-off for candidate protein identification. Gene Ontology aspect (molecular function, biological process and cellular compartment) enrichment analysis were performed using DAVID platform (https://david.ncifcrf.gov/home.jsp) (Huang et al., 2009a, 2009b). The EV proteins identified were also compared to protein listed in online database Vesiclepedia (http://microvesicles.org/) (Kalra et al., 2012). The mass spectrometry proteomics data had been deposited to the ProteomeXchange Consortium (Deutsch et al., 2020) via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD029838.

2.9 | Quantitative real-time PCR analysis (qPCR)

Total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN) and reverse transcription was performed using SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on LightCycler® 480 System (Roche Life Science). Oligos used in qPCR is listed in Supplementary Table S1.

2.10 | Western blot analysis

Cells were lysed with RIPA lysis buffer (Thermo Scientific), supplemented with 10% cOmplete™ protease inhibitor cocktail and 10% PhosStop phosphatase inhibitor cocktail (Roche Applied Science). Protein amount was quantified by Bradford assay (Bio-Rad) using BSA as standard. A total of 30 μg of protein per lane was resolved by 10% SDS–PAGE (Bio-Rad), followed by transferring to PVDF membranes (Amersham) using Trans–Blot Turbo System (Bio-Rad). Chemiluminescent signals were detected by ECL™ Western Blotting Detection Reagents (GE Healthcare). Anti-c-Myc (#sc-40), anti-Met (#sc-161) were purchased from Santa Cruz Biotechnology. Anti-β-actin (#A5316) was purchased from Sigma-Aldrich. Anti-EIF4G2 (#5169) and anti-RPL10 (#72912) was purchased from Cell Signalling Technology. Anti-RPS3A (#ab40745) and anti-EIF3I (#ab40745) was purchased from Abcam. Quantification of band intensity was performed using ImageJ software.

2.11 | Statistical analysis

The readings of all assays were calculated as the mean ± standard error of mean (SEM). Student's *t*-test was performed using Prism (Version 8.0.1, GraphPad) were used for statistical analysis. A *p*-value of less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | nMET overexpressing cell-derived EVs promote aggressiveness of HCC cells

Growing evidence has revealed the unequivocal role of HCC cell line- and patient-derived EVs in enhancing HCC development (Liu et al., 2021; Mao, Tey et al., 2020; Mao, Zhou et al., 2020; Tey et al., 2021). Our previous study emphasized mainly on the intrinsic activation of signaling cascades by nMET that contribute to HCC tumorigenesis and metastasis (Tey et al., 2017). To explore the involvement of nMET in modulating tumour microenvironment, EVs were collected from doxycycline inducible vector control (Vec-EV) and nMET stable cells established in MHCC97L cell line (nMET-EV). MHCC97L-nMET cells were treated with doxycycline to induce the expression of nMET that is under tight regulation of Tet-on responsive system (Figure 1a). According to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines (Théry et al., 2018), the isolated EVs were validated by nanoparticle tracking analysis in terms of their concentration and size (Figure 1b). These EVs were also validated by

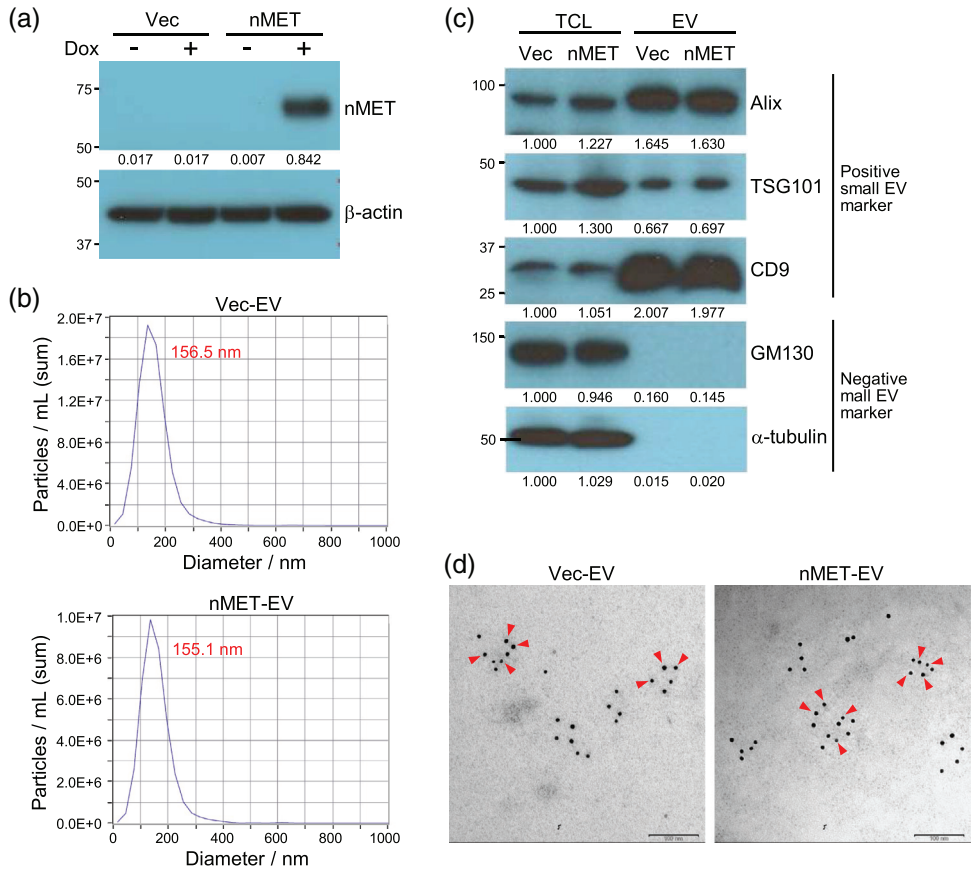


FIGURE 1 Characterization of EVs derived from MHCC97L nMET overexpressing cells. (a) EVs were isolated from the conditioned media of vector control (Vec) and nMET overexpressing (nMET) cells established in MHCC97L cells. (a) Expression of nMET in nMET overexpressing cells after 2 μg/ml of doxycycline (Dox) treatment for 24 h was shown by western blotting. Quantification of nMet band intensity normalized to band of β-actin is shown. (b) Size distribution of EVs collected from Vec and nMET cells was measured by ZetaView® TWIN-NTA PMX-220. The mode size of EV is indicated. (c) Positive small EV markers (Alix, TSG101 and CD9) and negative small EV markers (GM130 and α-tubulin) in total cell lysate (TCL) and EV were validated by western blotting. Band intensity of small EV markers relative to respective TCL Vec is shown. (d) Electron micrograph of the indicated EVs labelled with anti-CD63 antibody followed by secondary antibody coupled with gold particles indicated by arrowheads. Representative images are shown. Scale bar, 100 nm

their presence of positive markers and absence of negative markers of small EVs (Figure 1c) by immunoblotting as to ascertain the purity of isolated EVs (Jeppesen et al., 2019; Willms et al., 2016). The integrity of EVs was revealed by immunogold staining by anti-CD63 antibody under electron microscopy (Figure 1d). Functionally, MHCC97L Vec-EVs significantly promoted colony formation, cell migration and invasiveness of normal human hepatocyte MIHA and two nonmetastatic HCC cell lines Huh7 and PLC/PRF/5. Further enhancement was observed when all cells were treated with nMET-EVs (Figure 2a–c). Experimental metastasis mouse model performed by the intravenous coinjection of EVs and murine p53-/-;Myc-transduced hepatoblasts revealed that nMET-EV displayed a more potent effect than Vec-EV in provoking metastasis (Figure 2d–g). These findings suggest that the MHCC97L-nMET-EVs is capable of modulating and enhancing HCC cell aggressive properties.

3.2 | Enrichment of proteins that regulate translation in MHCC97L nMET-EVs

To ascertain the enhancement effect of MHCC97L nMET-EVs, we performed mass spectrometry to compare the proteomic profiles of Vec-EV and nMET-EV. In total, 1226 proteins were identified in EVs of Vec and nMET cells. Among these proteins, 1202 were commonly identified in EVs reported in Vesiclepedia database (Figure 3a). Volcano plot revealed a total of 67 EV proteins of nMET cells were upregulated by at least 2-fold compared to Vec-EV and with p-value <0.05 (Figure 3b). Downregulated EV proteins with significant p-value were not observed in our proteomic profiling. Further Gene Ontology (GO) analysis of all upregulated genes (≥2-fold, p-value < 0.05) using DAVID platform showed a significant enrichment of proteins that are involved in ribosomal constitution and translational regulation, in terms of biological process, molecular functions and cellular component (Figure 3c). In order to identify proteins that were involved in these processes, eukaryotic initiation factors gene family (EIF) and ribosomal protein gene family that include both ribosomal protein large subunit (RPL) and ribosomal protein

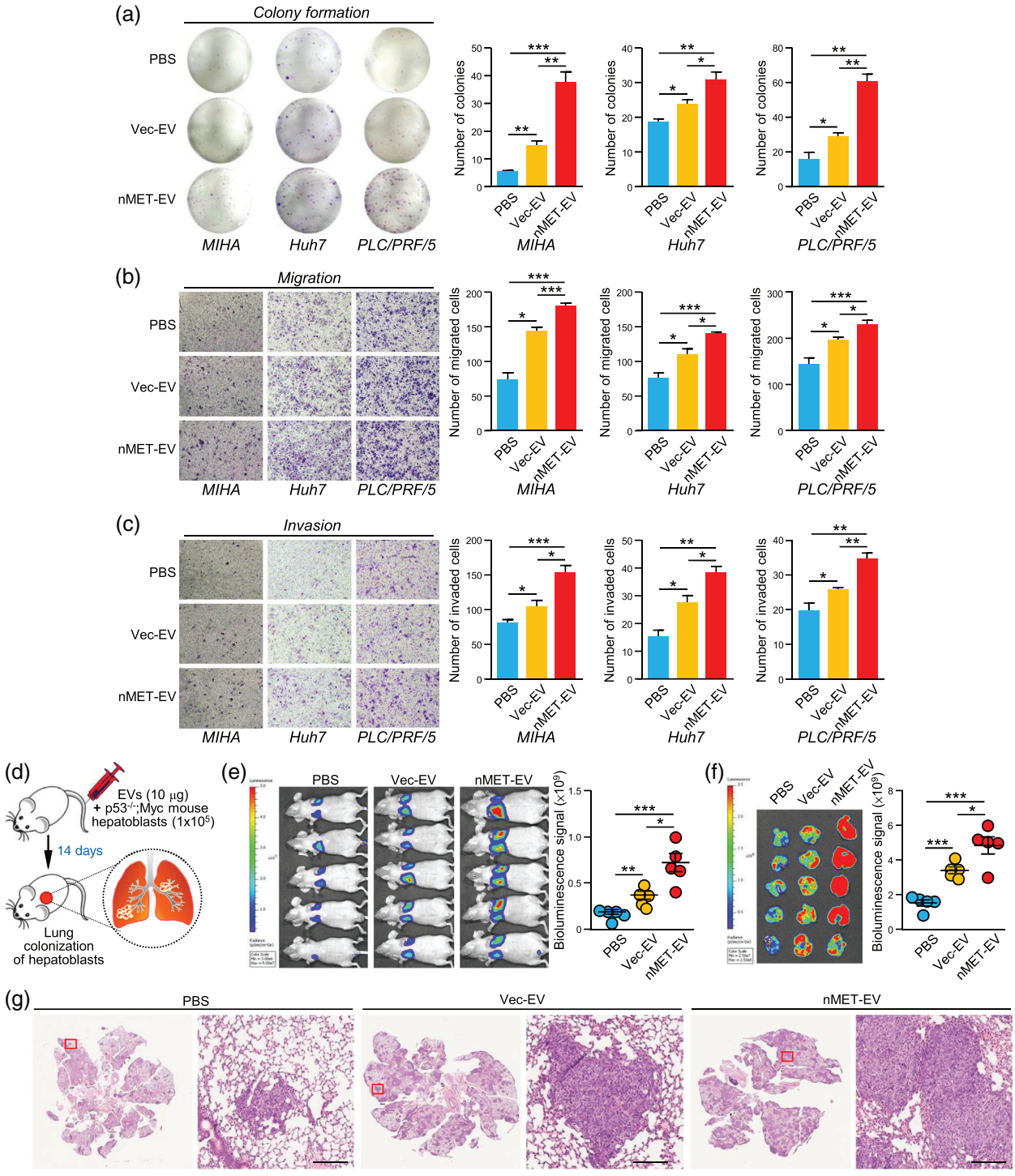
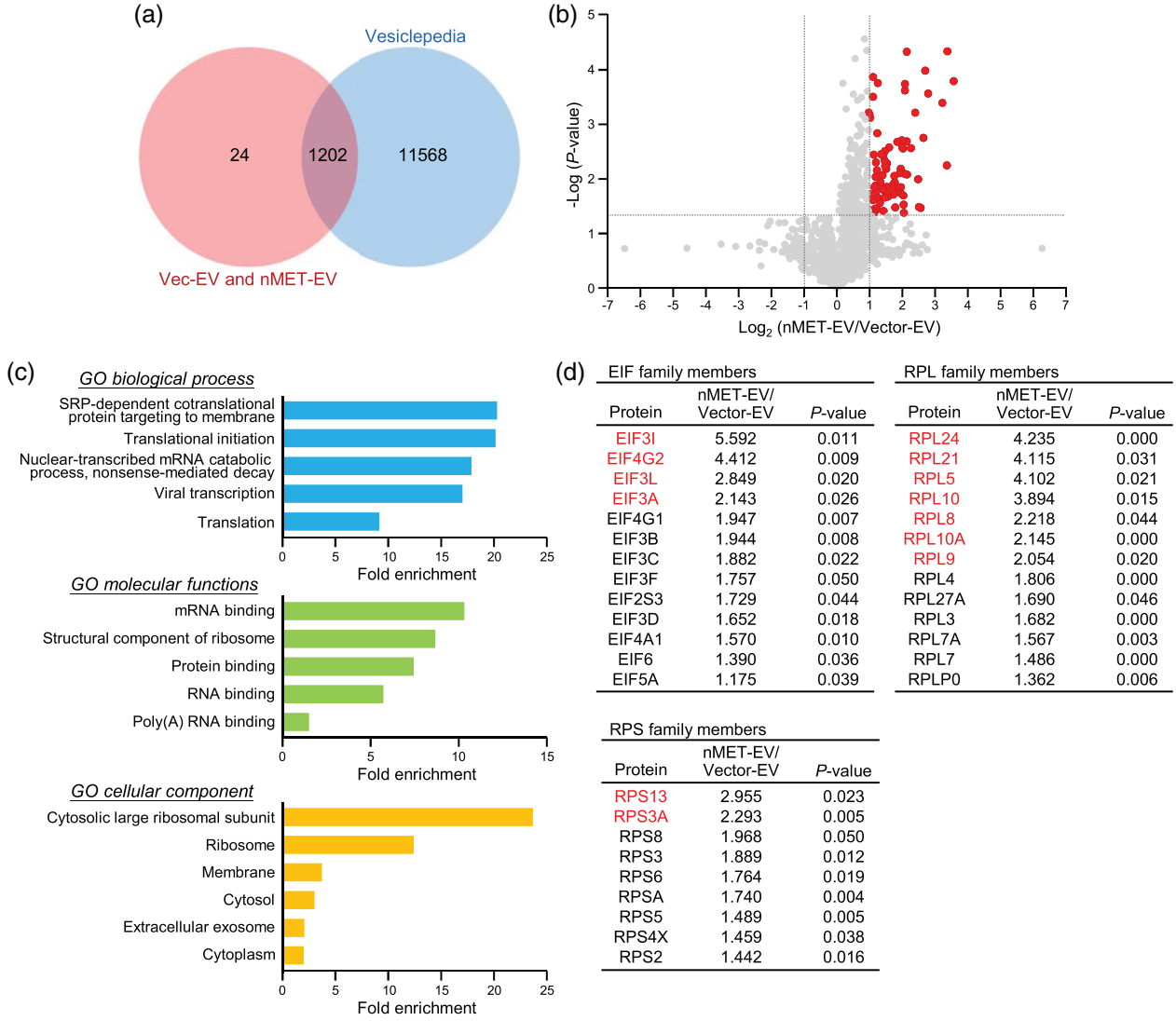


FIGURE 2 EVs of MHCC97L nMET cells promote HCC cell migration, invasiveness and metastasis. Colony formation (a), migration (b), and invasion (c) assays of immortalized human hepatocyte MIHA, and HCC cell lines Huh7 and PLC/PRF/5 pretreated with Vec-EVs and nMET-EVs were performed. PBS was included as control. (D) Schematic diagram of experimental metastasis assay. Luciferase-labelled murine p53-/-;Myc hepatoblasts (1×10^5) were coinjected with the indicated EVs (10 μg) into mice through tail vein ($n = 5$). (e) Bioluminescence imaging of animals was taken 14 days post injection and luciferase signal was quantified. (f) Bioluminescence imaging of excised lungs and luciferase signal was quantified. (g) Representative image of H&E staining of lung tissues. Scale bar, 200 μm. Data are represented as the mean ± SEM. Student's t-test for two groups was used. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$



(d)

EIF family members

Protein	nMET-EV/ Vector-EV	*P*-value
EIF3I	5.592	0.011
EIF4G2	4.412	0.009
EIF3L	2.849	0.020
EIF3A	2.143	0.026
EIF4G1	1.947	0.007
EIF3B	1.944	0.008
EIF3C	1.882	0.022
EIF3F	1.757	0.050
EIF2S3	1.729	0.044
EIF3D	1.652	0.018
EIF4A1	1.570	0.010
EIF6	1.390	0.036
EIF5A	1.175	0.039

RPL family members

Protein	nMET-EV/ Vector-EV	*P*-value
RPL24	4.235	0.000
RPL21	4.115	0.031
RPL5	4.102	0.021
RPL10	3.894	0.015
RPL8	2.218	0.044
RPL10A	2.145	0.000
RPL9	2.054	0.020
RPL4	1.806	0.000
RPL27A	1.690	0.046
RPL3	1.682	0.000
RPL7A	1.567	0.003
RPL7	1.486	0.000
RPLP0	1.362	0.006

RPS family members

Protein	nMET-EV/ Vector-EV	*P*-value
RPS13	2.955	0.023
RPS3A	2.293	0.005
RPS8	1.968	0.050
RPS3	1.889	0.012
RPS6	1.764	0.019
RPSA	1.740	0.004
RPS5	1.489	0.005
RPS4X	1.459	0.038
RPS2	1.442	0.016

FIGURE 3 Translation related proteins were enriched in EVs of nMET overexpressing cells. Proteins were extracted from EVs derived from Vec and nMET cells and were subjected to mass spectrometry analysis (technical triplicate/sample). (a) Venn diagram illustrating the number of proteins identified by mass spectrometry and number of proteins that were previously reported and deposited in Vesiclepedia. (b) Volcano plots of proteins that were significantly modulated by at least 2-fold in nMET-EVs. (c) Gene Ontology (GO) enrichment analysis was performed by DAVID in terms of biological process (*Top*), molecular functions (*Middle*) and cellular components (*Bottom*). (d) Significantly upregulated proteins ($p < 0.05$) in families of eukaryotic initiation factor (EIF), ribosomal protein large subunit (RPL) and ribosomal protein small subunit (RPS) were listed

small subunit (RPS), in which *p*-value is less than 0.05, were identified in the proteomic profiles of nMET-EV (Figure 3d). A total of 13 proteins including EIF3I, EIF4G2, EIF3L, EIF3A, RPL24, RPL21, RPL5, RPL10, RPL8, RPL10A, RPL9, RPS13, and RPS3A were upregulated by at least 2-fold in nMET-EVs compared to Vec-EV. The full list of proteins that belong to these three families is shown in Supplementary Table S2. The enrichment of proteins that regulate translation suggests the potential role of these proteins in promoting cancer aggressiveness upon transferring to the recipient cells.

3.3 | Translation related proteins are functionally responsible for the promoting effect of MHCC97L nMET-EVs

qPCR analysis was first conducted to examine the upregulation of the 13 identified proteins in transcript level in nMET cells. As shown in Figure 4a, all 13 genes were upregulated in nMET cells compared to Vec cells, suggesting the enrichment of these proteins in EVs was resulted from transcriptional upregulation modulated by nMET overexpression. To delineate the involvement of translation related proteins within EVs of nMET cells in promoting HCC aggressiveness, EIF3I, EIF4G2, RPL10 and RPS3A, based on their obscure functional role in HCC, were chosen for further investigation. However, EIF4G2 could not be detected at

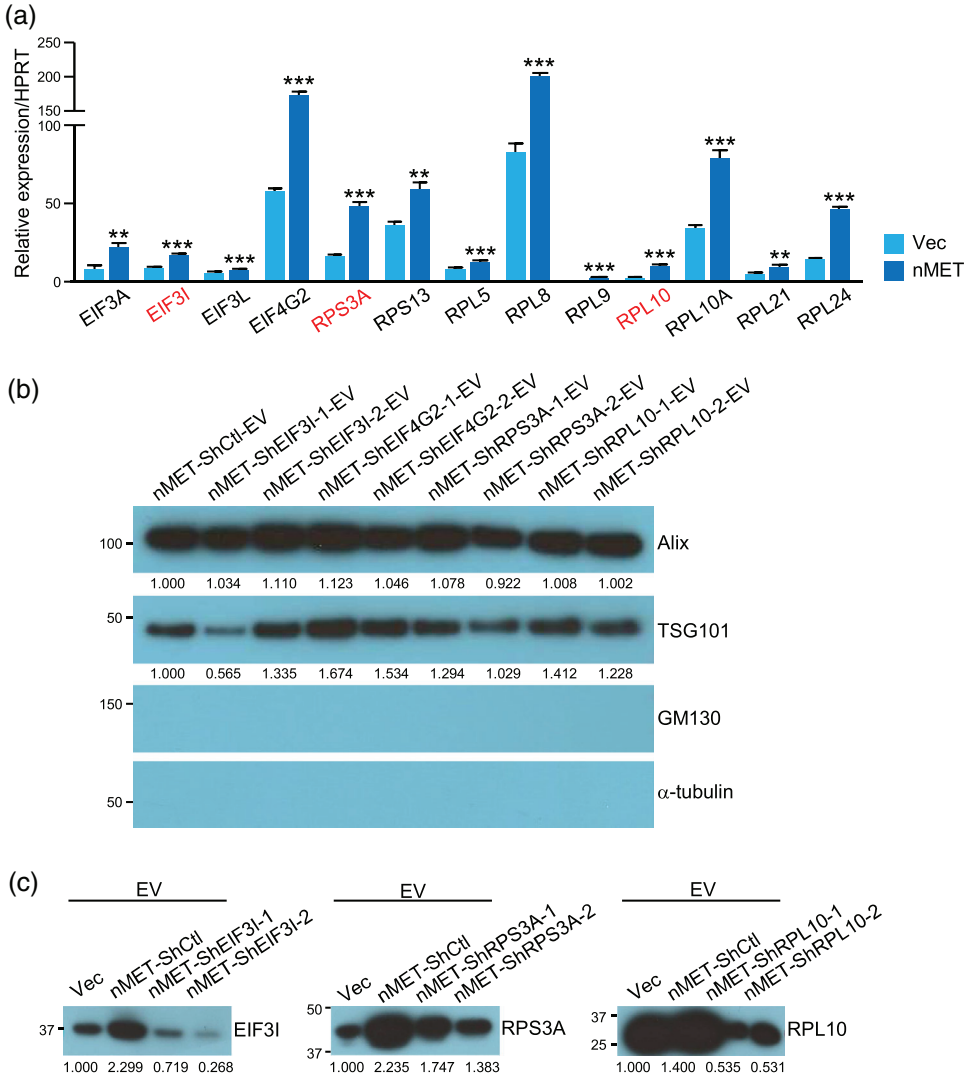


FIGURE 4 Expression of EIF3I, RPS3A and RPL10 in EVs derived from stable knockdown clones established in MHCC97L nMET cells. (a) qPCR analysis of transcript level of shortlisted translation related proteins in Vec and nMET cells. (b) Immunoblotting of positive small EV markers (Alix and TSG101) and negative small EV markers (GM130 and α-tubulin) in EVs derived from stable knockdown cells established in MHCC97L nMET cells. (c) Expression of EIF3I, RPS3A and RPL10 was validated in EVs derived from respective stable knockdown cells by western blotting. Relative band intensity of EIF3I, RPS3A, and RPL10 relative to the respective Vec is shown

the protein level in the total cell lysates and EVs of nMET cells (data not shown). Stable knockdown cells for the remaining three chosen target, EIF3I, RPL10 and RPS3A, were established by transfecting two ShRNA sequences (Sh01 and Sh02) into MHCC97L nMET cells. EVs derived from the nontarget control cells (nMET-ShCtl) and knockdown cells of EIF3I, RPL10 and RPS3A (nMET-EIF3I, nMET-RPL10 and nMET-RPS3A) were isolated from the conditioned media and subjected to immunoblotting of small EV markers, nanoparticle tracking analysis and immunogold staining of CD63 for validation (Figures 4b and S1). Consistent with the results obtained from mass spectrometry, upregulation of these chosen targets was observed in EVs of nMET-ShCtl cells compared to Vec cells (Figure 4c). Reduced expressions of EIF3I, RPL10 and RPS3A were detected in EVs collected from the respective knockdown cells. To examine whether reduced expression of EIF3I, RPL10 or RPS3A affected the promoting capacity of EVs of nMET cells, the motility of HCC cells treated with EVs of nontarget control and knockdown cells were functionally characterized. Parental Huh7 and PLC/PRF/5 cells were treated with EVs and subjected to migration and invasion assays. Compared to untreated cells, cell migration and invasion were significantly promoted by MHCC97L-EV and further significantly provoked by nMET-ShCtl-EV. The promoting effect of nMET-ShCtl-EV was compromised when the expression of EIF3I, RPS3A or RPL10 was suppressed (Figures 5a,b). In animal model of metastasis, coinjection of murine p53-/-;Myc-transduced hepatoblasts and EVs derived from ShEIF3I-2, ShRPS3A-2 and ShRPL10-2 showed reduced *ex vivo* bioluminescence signal compared to mice injected with ShCtl-EVs (Figures 6a,b). Histological examination of the lungs revealed the extent of metastasis correlated to

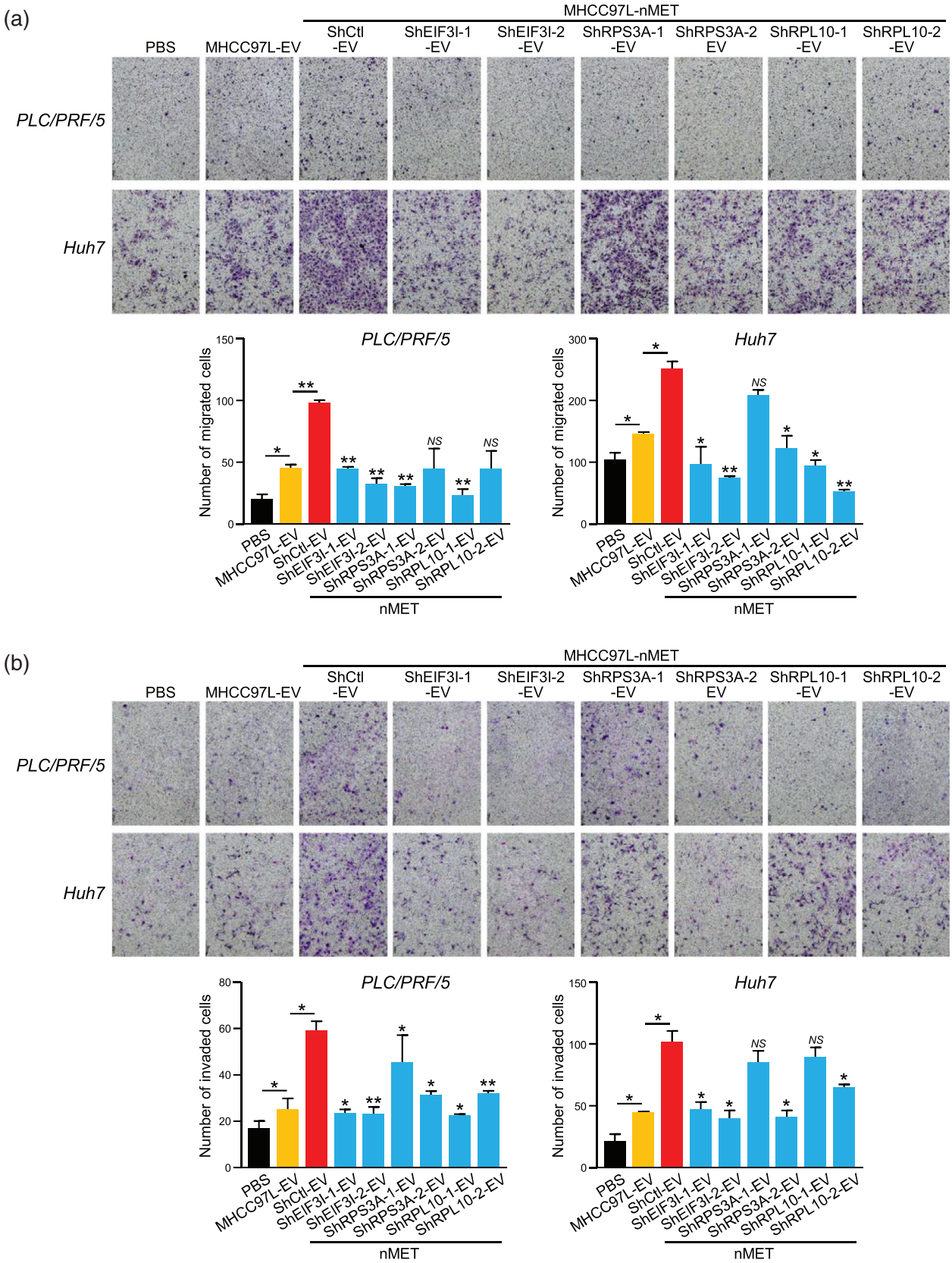


FIGURE 5 EVs derived from nMET overexpressing cells with knockdown of translation regulators displayed compromised promoting activity in cell migration and invasion. Migration (a) and invasion (b) assays were performed on PLC/PRF/5 and Huh7 cells pretreated with indicated EVs for 72 h. Cells were fixed and stained with crystal violet after 16 h of incubation. Representative images of migrated and invaded cells are shown. The number of cells were quantified and plotted. Data are represented as the mean ± SEM. Student's *t*-test for two groups was used. $^{*}p < 0.05$; $^{**}p < 0.01$. *NS*, not significant

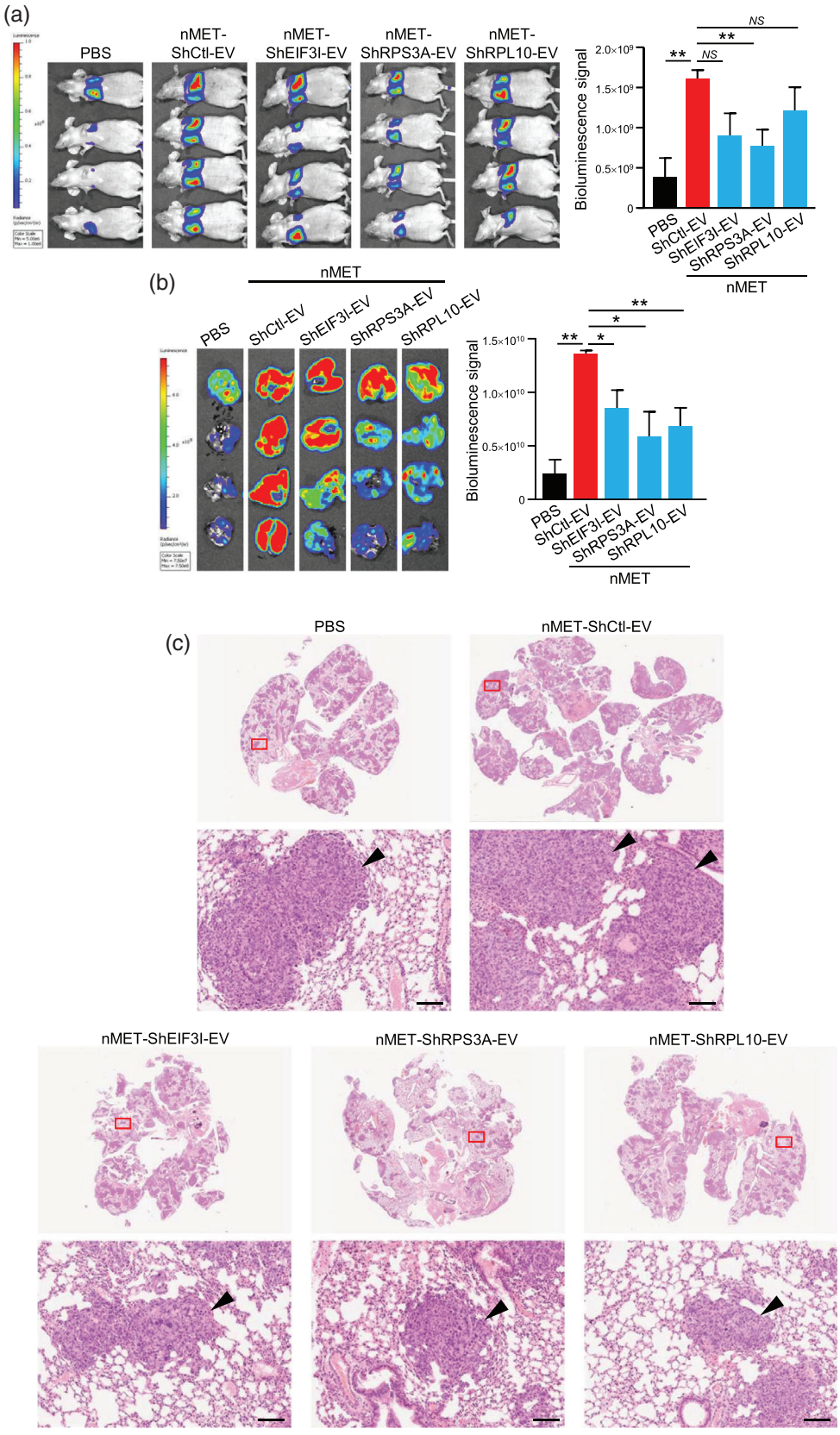


FIGURE 6 EVs from nMET overexpressing cells with reduced translation regulators exhibited diminished activity in promoting metastasis. Analysis of lung colonization of murine p53-/-;Myc hepatoblasts after intravenously coinjected with EVs (10 μg) into mice ($n = 4$). (a) Bioluminescence imaging of mice 14 days after injection. Quantification of the luciferase signal is shown. (b) Bioluminescence signal of dissected lung tissues. Quantification of the luciferase signal is shown. (c) Representative image of H&E staining of lung tissues after fixation. Inlets showing the enlarged area of the metastatic lesions that are indicated by arrowheads. Scale bar, 100 μm. Data are represented as the mean ± SEM. Student's *t*-test for two groups was used. *$p < 0.05$; **$p < 0.01$. *NS*, not significant

the luciferase signal of dissected lung tissues (Figure 6c). These findings highlight the importance of translation related proteins that were enriched within nMET overexpressing cell-derived EVs in modulating HCC metastasis.

4 | DISCUSSION

Emerging evidence has reported the presence of MET in the nucleus, either in full-length or truncated cytoplasmic domain form, with or without influence from HGF. Despite the presence of nMET has been widely reported, its functions remain obscure. The existence and potential functions of MET in the nucleus possess a great challenge to the current therapeutic strategies. Indeed, nMET was reported to reduce the treatment efficacy of olaparib and gemcitabine in PDAC (Gao et al., 2021). Recent study has revealed that nMET is stabilized by ARF and activates β-catenin signalling for transcriptional regulation of downstream signalling cascades in prostate cancer (Xie et al., 2019). In other context, nMET interacts with SOX9/β-catenin axis and SMC-1 (Schaaf et al., 2005; Xie et al., 2014). Our previous study also reported that nMET elevates TAK1 transcript expression through activation of its promoter (Tey et al., 2017). Herein, we reported a novel mechanism by which nMET mediates the upregulation of translational-related proteins and these proteins were further packaged into EVs and secreted into tumour microenvironment to promote HCC aggressiveness. Similar to many other studies in the past, the detailed mechanism of how nMET functions and activates their downstream targets, whether it takes place in a direct or indirect fashion, remains unanswered and warrants further investigations.

Gene expression is regulated primarily at levels of transcription and translation. Deregulation of mRNA translation, especially at the rate-limiting step of translational initiation, results in a global increase of protein synthesis or selective enhanced translation of oncogenic mRNAs, which may play an important role in cancer development and progression (Bracic Tomazic et al., 2021; Robichaud et al., 2019; Silvera et al., 2010). In HCC, previous studies have reported the enhanced expression of translation factor mRNAs including both EIFs and ribosomal proteins (RPs) (Kondoh et al., 2001; Shuda et al., 2000). Indeed, majority of EIF members were up-regulated and facilitate tumour progression of multiple cancer types. However, detail mechanisms of how the aberrant expression of individual EIFs directly involved in the tumorigenic signalling pathways rather than simply accompanied with aberrant protein synthesis remain critical issues for elucidation. Our study demonstrated the upregulation of EIFs when nMET is overexpressed in HCC where EIF3I was further studied and shown to play important roles in tumour metastasis. EIF3I is one of the important components of the mammalian EIF3 complex which associated with the 40S ribosome and played role in the formation of 43S preinitiation complex (Kolupaeva et al., 2005). This is supported by depletion of EIF3I results in diminished of all other EIF3 subunits via destabilization and deregulation, leading to reduction of global mRNA translation initiation and protein synthesis (Naranda et al., 1997). EIF3I plays important role in various cellular functions, both embryonic and disease-related (Ma et al., 2021). In HCC, EIF3I-mediated constitutive activation of Akt1 signaling through the inhibition of PP2A functions serve as an oncogenic mechanism of tumorigenesis (Y.-W. Wang et al., 2013). Upregulation of EIF3I by cluster in was also shown to activate Akt pathway, which in turn promotes MMP13 expression and facilitates HCC metastasis (C. Wang et al., 2015). Under hypoxia, EIF3I also activates VEGFA and promotes angiogenesis in HCC (Yuan et al., 2014). Upregulation of EIF3I expression in endothelial cells promotes angiogenesis by selectively up-regulating ERK and VEGFR2 translation (Zhang et al., 2017). Besides that, overexpression of EIF3I upregulates the cyclooxygenase-2 protein synthesis and activates β-catenin signalling pathway to promote colon oncogenesis (Qi et al., 2014). All these findings provide hints on how EV-EIF3I might modulates recipient cell's signalling cascades upon uptake, which required in-depth study to elucidate the detail mechanism.

It is noteworthy that besides the importance of ribosome to the growth and proliferation of cancer cells, individual free RPs could also play a role in tumorigenesis (Pelletier et al., 2018; X. Zhou et al., 2015). The eukaryotic ribosome is the cellular translational machinery primarily responsible for protein synthesis that consists of four ribosomal RNA (rRNA) species and 79 RPs. Study on differential expressed gene profiles in HBV-induced HCC revealed a number of ribosomal subunit components that were overexpressed (Kim et al., 2001). Besides that, RPL4, RPL9, RPL10, and RPL13A were shown to be upregulated in HCC cell lines and tissues (Yoon et al., 2006). Recently, overexpression of polymeric immunoglobulin receptor (PIGR) was reported to upregulate 15 RPs to exert oncogenic function in HCC (Zhang et al., 2021). Overexpression of nMET upregulates both RPSs and large subunit (RPLs). We showed that elevated expression of RPS3A and RPL10 impact in HCC aggressiveness. In other study, RPS3A overexpression significantly enhanced NF-κB activity through a novel chaperoning function for HBx protein in HCC (Lim et al., 2011). High RPS3A expression are predisposed to a low immune infiltration status and to worse prognosis in HCC patients (C. Zhou et al., 2020). Dual inhibition of MET and EFGR has been shown to downregulate RPS6 in breast cancer and reduce cell proliferation, providing first evidence on connection between MET and RPs (Yi et al., 2015).

To date, the study of functions and impact of translational-related proteins within EVs is limited. Previous studies have showed that intracellular EIF3I can be packaged and exported to the self-assembled collagen matrix in the ECM via EVs to orchestra the formation of a mineralized matrix of osteoblasts (Ramachandran et al., 2016). Another study revealed that EIF4E was highly expressed in EVs of NSCLC patients and could serve as a biomarker for survival prediction (Dong et al., 2020). Importantly, ribosomal proteins were reported as distinct "passengers" of EVs that mediated horizontal transfer between different niche of mesenchymal stem cells and myeloma cells in a systemic way to bestow procancer advantages (Dabbah et al., 2021). Our study

unrevealed the role of translational-related proteins-enriched EVs derived from nMET overexpressing cells in enhancing cancer metastasis. However, further insight into the downstream signalling pathways triggered within tumour cells and other recipient cells within tumour microenvironment required detail investigation.

In conclusion, current study provides evidences implicating EVs with enhanced translational-related proteins as mediators of HCC aggressiveness. Importantly, the expanding knowledge on the signalling networks and actions of nMET will provide critical evident into the development of novel therapeutic strategies against aberrant MET in human cancers. Nevertheless, focusing the efficacy study of newly developed translation-targeting drugs on the tumour microenvironment will help to identify useful therapeutic combinations.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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