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Original research

S100A10 promotes HCC development and progression via transfer in extracellular vesicles and regulating their protein cargos

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ABSTRACT

Objective Growing evidence indicates that tumour cells exhibit characteristics similar to their lineage progenitor cells. We found that S100 calcium binding protein A10 (S100A10) exhibited an expression pattern similar to that of liver progenitor genes. However, the role of S100A10 in hepatocellular carcinoma (HCC) progression is unclear. Furthermore, extracellular vesicles (EVs) are critical mediators of tumourigenesis and metastasis, but the extracellular functions of S100A10, particularly those related to EVs (EV-S100A10), are unknown.

Design The functions and mechanisms of S100A10 and EV-S100A10 in HCC progression were investigated in vitro and in vivo. Neutralising antibody (NA) to S100A10 was used to evaluate the significance of EV-S100A10.

Results Functionally, S100A10 promoted HCC initiation, self-renewal, chemoresistance and metastasis in vitro and in vivo. Of significance, we found that S100A10 was secreted by HCC cells into EVs both in vitro and in the plasma of patients with HCC. S100A10-enriched EVs enhanced the stemness and metastatic ability of HCC cells, upregulated epidermal growth factor receptor (EGFR), AKT and ERK signalling, and promoted epithelial–mesenchymal transition. EV-S100A10 also functioned as a chemoattractant in HCC cell motility. Of significance, S100A10 governed the protein cargos in EVs and mediated the binding of MMP2, fibronectin and EGF to EV membranes through physical binding with integrin αV. Importantly, blockage of EV-S100A10 with S100A10-NA significantly abrogated these enhancing effects.

Conclusion Altogether, our results uncovered that S100A10 promotes HCC progression significantly via its transfer in EVs and regulating the protein cargoes of EVs. EV-S100A10 may be a potential therapeutic target and biomarker for HCC progression.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The key factors governing hepatic differentiation in liver stem/progenitor cells are of critical importance in the tumourigenicity and progression of hepatocellular carcinoma (HCC).
⇒ S100A10 is a plasminogen receptor which mediates the plasminogen activation by plasminogen activators.
⇒ S100A10 was found to promote HCC cell proliferation, migration and invasion in vitro.

WHAT THIS STUDY ADDS

⇒ S100A10 is a liver progenitor-specific gene that enhances HCC stemness.
⇒ S100A10 is carried in extracellular vesicles (EVs) and EV-S100A10 promotes HCC stemness.
⇒ S100A10 mediates the targeting of specific secretory proteins to the membranes of EVs.
⇒ S100A10 in EVs also functions as a chemoattractant in HCC cell motility.
⇒ S100A10-neutralising antibody significantly abrogates the promoting effects of EV-S100A10.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ EV-S100A10 may be a potential therapeutic target against HCC progression.

INTRODUCTION

A large body of evidence has shown that the key factors governing hepatic differentiation in liver stem/progenitor cells are of critical importance in the tumourigenicity and progression of hepatocellular carcinoma (HCC). To this end, our previous studies have shown that, using an in vitro liver differentiation model derived from human embryonic stem cells and comparing the gene expression signatures between different liver developmental stages and HCC clinical samples, HCCs with oncofetal properties have been found to be associated with a poor prognosis of patients, and the gene signatures of liver progenitor cells may be involved in regulating the stemness of HCC.[1 2]

In the present study, we found that, in the in vitro liver differentiation model, S100A10 showed an expression pattern similar to those stemness-related markers that were highly expressed in the liver progenitor stage and may play a critical role in HCC stemness and progression. S100A10 belongs to the S100 family that is clustered on chromosome 1q21, the gain of which is commonly identified in

multiple types of cancer.[3–5] Aberrant expression of S100A10 was associated with fibrinolysis in acute promyelocytic leukaemia,[6] migration of tumour-promoting macrophages into tumour sites via extracellular matrix (ECM) remodelling,[7 8] migration and invasion of cancer cells in colonic cancer[9] and angiogenesis in renal cell carcinoma.[10] Recently, S100A10 was found to promote HCC proliferation, migration and invasion in vitro; however, further functional studies, especially in vivo study, and underlying mechanism have not been investigated.[11] To this end, we aimed to investigate the role of S100A10 in HCC stemness-related signatures including tumour initiation, metastasis and chemoresistance in vitro and in vivo, especially its extracellular function, and explore the underlying mechanism.

Some members of the S100 protein family have been reported to be secreted extracellularly.[12] For instance, S100A9 protein, a member of the S100 family, is secreted into extracellular vesicles (EVs) and promotes disease progression in cancers.[13 14] Although S100A10 is also predicted to be secreted into extracellular space,[15] there are few studies confirming whether S100A10 could be secreted into EVs and the extracellular roles of S100A10 in cancers are unknown. To the best of our knowledge, there are no reports that S100A10 is secreted and carried in EVs in HCC. EVs are small secreted vesicles (30–160 nm), also called exosomes, containing functional biomolecules (including proteins, lipids and RNA, etc) that can be secreted extracellularly into blood or body fluids and transferred to target cells.[16–18] Tumour-secreted EVs are emerging as critical messengers in tumour progression and metastasis.[19 20] Here, we found that S100A10 was present and carried in HCC-derived EVs and played a pivotal role in EV-mediated HCC progression. Furthermore, we also found that S100A10 mediated the targeting of extracellular proteins including MMP2, fibronectin and EGF to the membrane of EVs through binding integrin αV (ITGAV). Blockage of S100A10 in EV with S100A10-neutralising antibody (NA) significantly abrogated these enhancing effects. Taken together, the present study demonstrates the pivotal role of S100A10 in HCC and uncovers the importance of S100A10 in HCC-derived EVs. The results also suggest that S100A10 protein in EV is a potential biomarker for HCC detection and a potential therapeutic target in HCC treatment.

MATERIALS AND METHODS

Detailed experimental procedures are provided in the online supplemental materials.

RESULTS

Clinical significance of S100A10 in HCC

In this study, when we analysed our in vitro liver differentiation model derived from human embryonic stem cells reported previously,[1 2] we found that S100A10 was highly expressed in the liver progenitor and premature hepatocyte stages when compared with mature hepatocytes (figure 1A). The expression of S100A10 was then validated by real-time reverse transcription-PCR (qRT-PCR) in a cohort of 86 paired HCC clinical samples. S100A10 was highly expressed in HCC tumours as compared with the non-tumorous livers (figure 1B), with an upregulation by ≥2 folds in 38.4% (33/86) of tumours. In addition, high expression of S100A10 was associated with poorer prognosis with significantly shorter overall survival rates (figure 1C); similar results were observed in The Cancer Genome Atlas (TCGA) database (figure 1D). On clinicopathologic correlation, higher expression of S100A10 was significantly associated with more aggressive tumour behaviour including more frequent venous invasion (p=0.044) and poorer cellular differentiation (p=0.047) (figure 1E). From TCGA database, high expression of S100A10 in HCCs is significantly associated with hepatitis B/C infection (online supplemental figure 1A). As S100A10 is located on chromosome 1q21, which is frequently amplified in multiple types of cancer,[3–5 21] we analysed the copy number variation (CNV) of S100A10 from the genomic DNA of 80 HCC samples from this cohort. Most (63.75%, 51/80) of the tumours showed CNV of ≥3 copies, with 27.5% showing 4 or more copies (figure 1F and online supplemental figure 1B). Furthermore, the S100A10 expression levels in patients' tumours with copy number amplification were significantly higher than those without, and the relative CNV and S100A10 expression were significantly and positively associated (figure 1G).

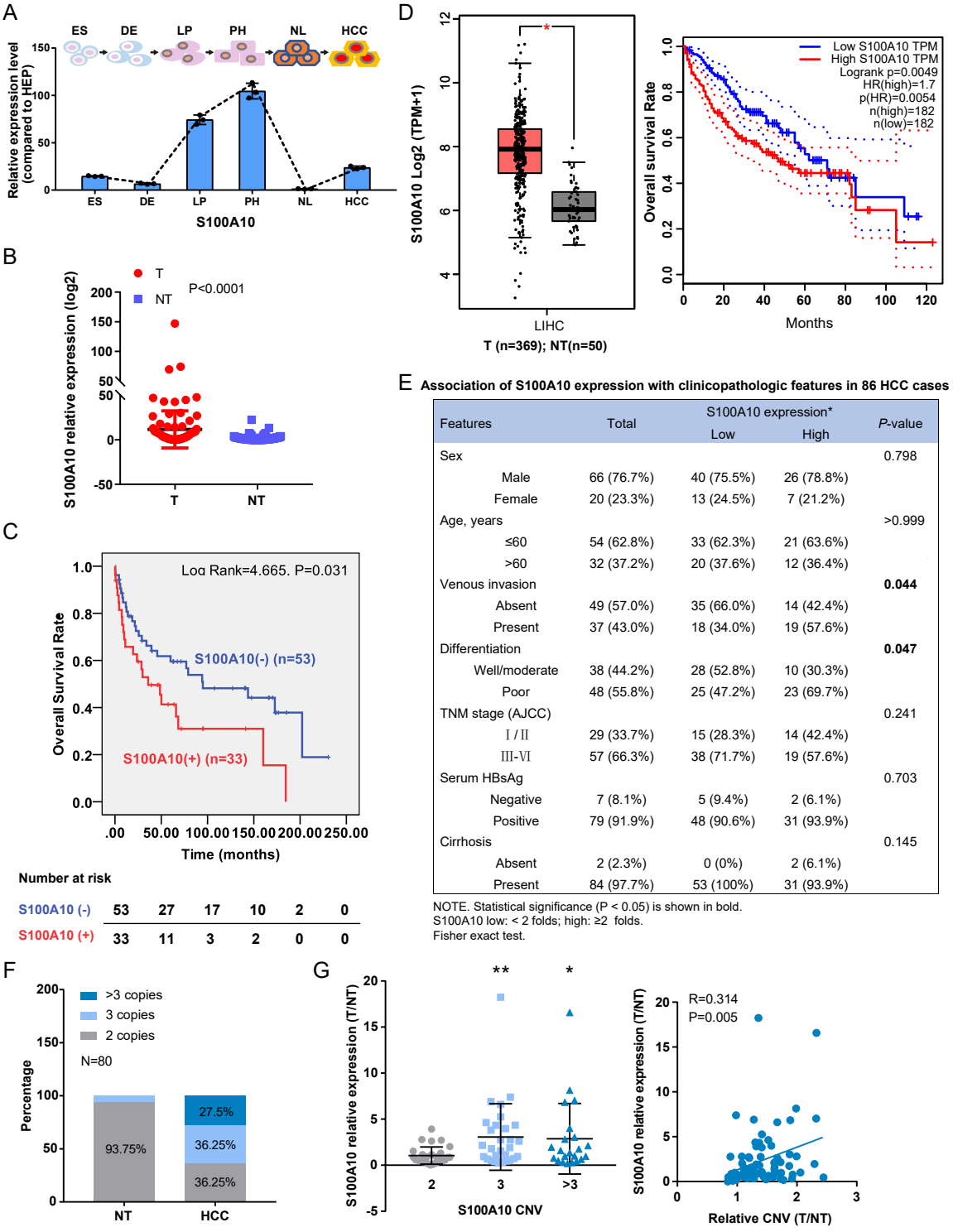
S100A10 enhances the stemness characteristics of HCC

As S100A10 is specifically expressed in liver progenitor cells and premature hepatocytes stages, we hypothesised that S100A10 might regulate HCC stemness. To investigate the functions of S100A10, S100A10 was stably overexpressed in an immortalised normal liver cell line MIHA and two HCC cell lines PLC/PRF/5 (PLC) and MHCC97L (97L) (authenticated to have no contamination; see online supplemental information and figure 2), knocked down in Huh7 cells, and knocked out in MHCC97H (97H) cells using the CRISPR/Cas9 system (figure 2A). Overexpression of S100A10 increased the expression of stemness-related genes including CD24, CD44, LGR5, SOX2 and C-MYC in both HCC cell lines PLC and 97L (figure 2B and online supplemental figure 3A). Focus formation ability was upregulated when S100A10 was upregulated, and was downregulated when S100A10 was knocked out or knocked down (online supplemental figure 3B). Overexpression of S100A10 promoted the sphere-forming ability, while S100A10 knockout (KO) significantly suppressed the sphere formation ability of HCC and MIHA cells (figure 2C and online supplemental figure 3C), indicating that S100A10 enhances self-renewal ability of HCC. To further investigate the in vivo tumourigenic ability of S100A10-overexpressing (OE) cells, limiting dilution assays performed in nude mice showed significantly higher tumour incidence and tumour growth rate in S100A10-OE cells in both PLC and 97L cell lines (figure 2D and online supplemental figure 3D). In addition, S100A10 knockdown (KD) Huh7 and S100A10 KO 97 H cells were subcutaneously injected into dorsal flanks of nude mice, and the tumour volume was significantly smaller compared with non-target control (NTC) cells (online supplemental figure 3E). These results indicate that S100A10 promotes the tumourigenicity of HCC cells both in vitro and in vivo.

S100A10 enhances the chemoresistance of HCC in vitro and in vivo

Chemoresistance is one of the important stemness-related characteristics of tumour cells. On treatment with chemotherapy drug sorafenib (alternative first-line drug for advanced HCC), cisplatin or 5-flurouracil (5-FU), the cell viability of S100A10-OE cells (PLC and 97L) was significantly higher, and the apoptotic indices were lower than those of the controls (online supplemental figure 4A,B). Consistently, opposite results were observed in S100A10-KO 97 H cells (online supplemental figure 4A,B).

We also confirmed that S100A10 enhanced the chemoresistance of HCC in vivo in nude mice with xenograft tumours induced by PLC-S100A10 cells. When treated with sorafenib, cisplatin or 5-FU, the xenograft tumours in the PLC-S100A10



Features	Total	S100A10 expression*		*P*-value
		Low	High	
Sex				0.798
Male	66 (76.7%)	40 (75.5%)	26 (78.8%)	
Female	20 (23.3%)	13 (24.5%)	7 (21.2%)	
Age, years				>0.999
≤60	54 (62.8%)	33 (62.3%)	21 (63.6%)	
>60	32 (37.2%)	20 (37.6%)	12 (36.4%)	
Venous invasion				**0.044**
Absent	49 (57.0%)	35 (66.0%)	14 (42.4%)	
Present	37 (43.0%)	18 (34.0%)	19 (57.6%)	
Differentiation				**0.047**
Well/moderate	38 (44.2%)	28 (52.8%)	10 (30.3%)	
Poor	48 (55.8%)	25 (47.2%)	23 (69.7%)	
TNM stage (AJCC)				0.241
I / II	29 (33.7%)	15 (28.3%)	14 (42.4%)	
III-VI	57 (66.3%)	38 (71.7%)	19 (57.6%)	
Serum HBsAg				0.703
Negative	7 (8.1%)	5 (9.4%)	2 (6.1%)	
Positive	79 (91.9%)	48 (90.6%)	31 (93.9%)	
Cirrhosis				0.145
Absent	2 (2.3%)	0 (0%)	2 (6.1%)	
Present	84 (97.7%)	53 (100%)	31 (93.9%)	

NOTE. Statistical significance (P < 0.05) is shown in bold.
S100A10 low: < 2 folds; high: ≥2 folds.
Fisher exact test.

Figure 1 Clinical significance of S100A10. (A) Human ES cells were induced to differentiate along hepatic lineages into adult hepatocytes. Cells from different developmental stages, including ES cells, DE, LP cells and PHs, as well as normal liver (NL) and HCC tissues, were used for transcriptomic sequencing. The expression pattern of S100A10 was confirmed using qRT-PCR. (B) Relative expression of S100A10 in HCC and corresponding non-tumorous livers from 86 pairs of samples of patients with HCC. Paired t-test. (C) Kaplan-Meier overall survival curve and numbers at risk for these 86 patients with HCC. (D) Relative S100A10 expression and Kaplan-Meier curve for overall survival of TCGA cohort (analysed by GEPIA website). (E) Correlation between S100A10 expression and clinicopathological features in 86 HCC cases. Fisher exact test. (F) CNV of S100A10 in 80 pairs of samples of patients with HCC. (G) S100A10 expression stratified according to S100A10 CNV (left panel), non-parametric Mann-Whitney test. *P<0.05, **P<0.01. Correlation between relative S100A10 expression and relative CNV (right panel). AJCC, American Joint Committee on Cancer; CNV, copy number variation; DE, definitive endoderm; ES, embryonic stem; HCC, hepatocellular carcinoma; LIHC, liver hepatocellular carcinoma; LP, liver progenitor; NT, non-tumorous liver tissues; TCGA, The Cancer Genome Atlas; TNM, TNM classification of malignant tumours.

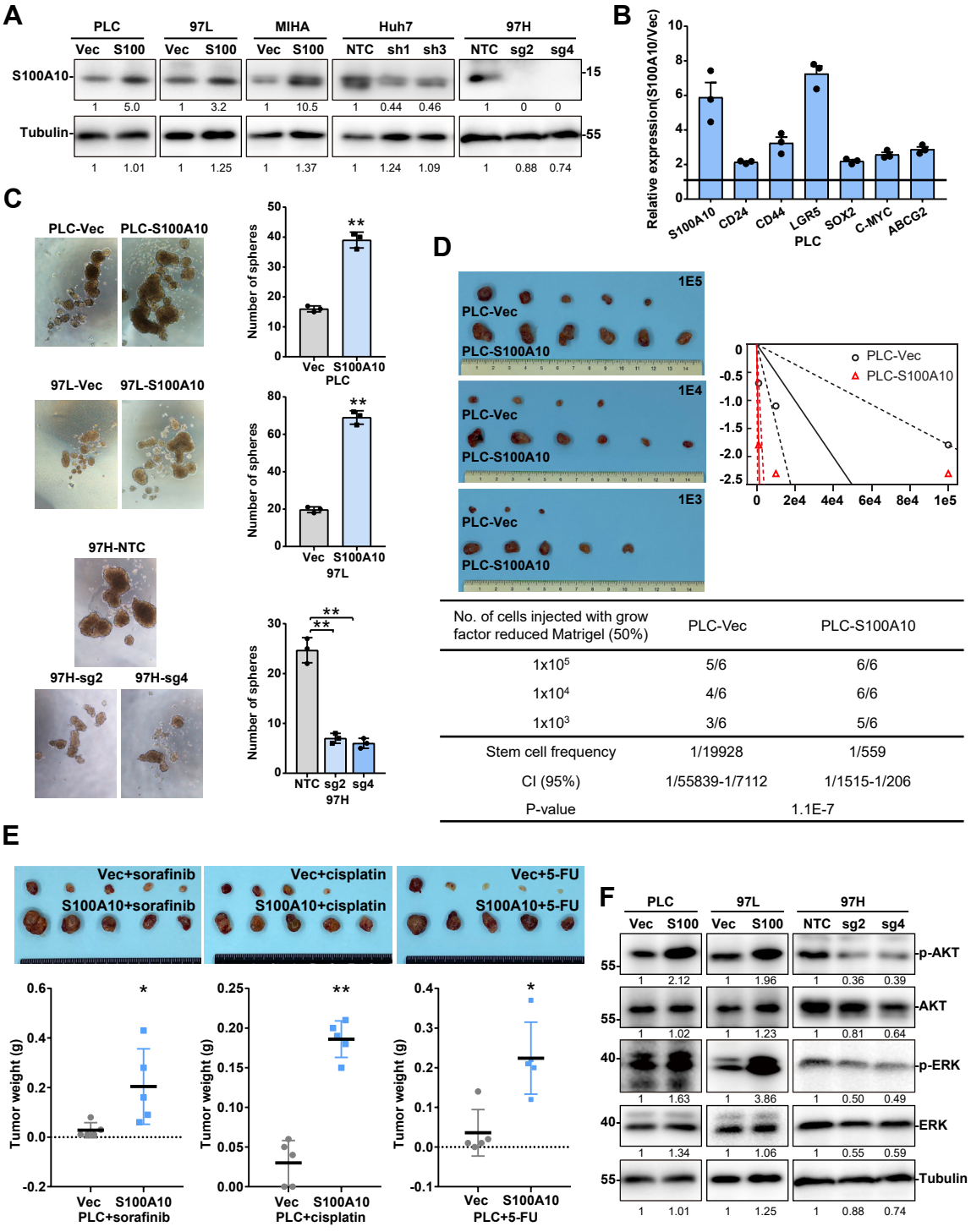


Figure 2 S100A10 enhances HCC stemness. (A) Western blot showing efficiency of ectopic S100A10 expression, and S100A10 silencing with shRNA against S100A10 (shS100A10-1: sh1, shS100A10-3: sh3) and with sgRNA against S100A10 (sgS100A10-2: sg2, sgS100A10-4: sg4). S100, S100A10; NTC, non-target control. Tubulin was used as a loading control. Band intensities were quantitated by ImageJ. (B) Relative expression of HCC stemness-related markers with S100A10-OE compared with Vec plasmid by qRT-PCR. (C) Sphere formation assay of PLC-Vec/S100A10, 97L-Vec/S100A10 and 97H-NTC/sgS100A10s, respectively. (D) Limiting dilution assays of PLC-Vec/S100A10. The tumour-initiating frequency is summarised in chart and table form. (E) Chemoresistance assay in nude mice by subcutaneous injection. Representative images of PLC-Vec/S100A10 tumours treated with indicated drugs. Tumour weights expressed as mean±SD of five mice in each group, Mann-Whitney test (lower panel). (F) Western blot showing the expression of p-AKT, AKT, p-ERK and ERK in PLC-Vec/S100A10, 97L-Vec/S100A10 and 97H-NTC/sgS100A10s, respectively. Tubulin was used as a loading control. HCC, hepatocellular carcinoma; NTC, non-target control; p-AKT, phosphorylated AKT; p-ERK, phosphorylated ERK; Vec, vector control.

group grew significantly larger than the control group (figure 2E). These results were consistent with those of previous report that S100A10 was related to multidrug resistance.[15] Our qRT-PCR results also showed that S100A10 upregulated the expression of ABCG2, which is known to exert multidrug resistance (figure 2B and online supplemental figure 3A).

In addition, activation of AKT and ERK has been implicated in stemness and sorafenib resistance in HCC.[22–25] To this end, western blotting showed that the activation of AKT and ERK was significantly enhanced in S100A10-OE cells and reduced in S100A10-KO 97H cells (figure 2F). Previously, S100A10 was found to be upregulated on chemotherapy treatment in breast cancer via HIF-1.[26] To investigate if this also held true in HCC, we treated HCC cells with chemotherapeutic agents and found that sorafenib, cisplatin and 5-FU all upregulated the expression of S100A10 and HIF-1α, indicating that S100A10 can also be upregulated by chemotherapeutic agents in relation to HIF-1 in HCC (online supplemental figure 4C).

S100A10 promotes HCC metastasis in vitro and in vivo, enhancing epithelial–mesenchymal transition (EMT)

Cell migration and invasion assays revealed that S100A10-OE in HCC cell lines significantly enhanced cell migration and invasion (figure 3A), whereas silencing S100A10 significantly suppressed these abilities (figure 3A). We used two mouse models to assess metastasis: (1) liver metastasis model by intrasplenic injection and (2) lung metastasis model by tail vein injection. In the liver metastasis (intrasplenic injection) model, 10 weeks after intrasplenic injection, all mice injected with S100A10-OE 97L cells had metastatic nodules on their liver surfaces, whereas much fewer and smaller metastatic nodules were observed in three of six mice in the control group (figure 3B). Along the same direction, S100A10 KO in 97H cells significantly inhibited its metastasis to the liver in this intrasplenic injection model (figure 3C). In the lung metastasis (tail vein injection) model, 8 weeks after tail vein injection, significantly higher luciferase signals were detected in S100A10-OE 97L cells as compared with control cells (figure 3D). Histology further confirmed the liver and lung metastatic lesions, respectively (figure 3B–D).

As EMT is well known to contribute to tumour metastasis, with western blotting, we found that S100A10 upregulated the expression of mesenchymal markers (N-cadherin, fibronectin and vimentin) and downregulated the expression of epithelial marker E-cadherin (figure 3E). Taken together, these findings showed that S100A10 promotes HCC liver and lung metastases, likely via the promotion of EMT.

S100A10 is present in HCC-derived EVs

Although S100A10 has been predicted to be secreted into extracellular space, there have been no reports of such secretion in HCC. Furthermore, there are no reports of its transport into EVs. To investigate these queries, we isolated and characterised the EVs extracted from the plasma samples of 25 patients with HCC and 15 healthy subjects and HCC cell lines. The EVs were enriched by sequential centrifugation of plasma or supernatant through increasing gravitational forces to remove cellular debris and apoptotic bodies, before finally pelleting at 100 000 gravitational forces. Nanoparticle tracking analysis confirmed the extracted EVs had a size with peak around 120–140 nm (online supplemental figure 5A); western blotting also confirmed the presence of EVs with EV-specific markers (including CD81, CD63, HSP70, CD9, TSG101 and Alix) and EV-negative markers (GM130 and p62) (figure 4A,B and online supplemental figure 5B). In addition, on transmission electronic microscopy, we identified the EVs with a cup-shaped morphology and a diameter around 30–160 nm (figure 4C and online supplemental figure 5C). Immunogold labelling further revealed the presence of S100A10 on the surface of CD63-positive EVs derived from both plasma and HCC cells of patients with HCC (figure 4C).

In the S100A10-OE HCC cells, the level of S100A10 carried in EVs (termed EV-S100A10 and thereafter) was also upregulated, whereas in S100A10-KD and S100A10-KO HCC cells, the EV-S100A10 level was consistently downregulated or not detectable (figure 4B). Western blotting also suggested that EVs derived from plasma samples of patients with HCC exhibited a higher level of S100A10 as compared with those from healthy subject plasma (figure 4A and online supplemental figure 5B). All these data indicate that S100A10 is secreted and carried in EVs.

S100A10-enriched EVs promote HCC metastasis

To investigate whether S100A10-enriched EVs promoted HCC cell motility, different concentrations of EVs derived from S100A10-OE cells (S100A10 EVs) were used to pretreat HCC cells for 48 hours. From the migratory assay, treatment with 2 μg/mL S100A10 EVs was already potent enough to significantly promote PLC cell migration (online supplemental figure 5D). Furthermore, compared with phosphate buffered saline (PBS)-treated cells, both S100A10 EVs and extracellular vesicle from vector control cell (Vec EVs) (EVs derived from vector control (Vec) cells) promoted HCC cell migration and invasion, but S100A10 EVs exhibited a stronger promoting ability than the Vec EVs (figure 4D and online supplemental figure 6A). On the other hand, we queried whether EVs derived from different HCC cell lines might exert similar effects on HCC cells; EVs derived from S100A10-KO 97H (sgS100 EVs) or NTC control (NTC EVs) were used to treat PLC or 97L cells, and the cell migration and invasion assays were performed. The results showed that NTC EVs significantly promoted the migratory and invasive abilities, while KO of S100A10 abrogated these effects (figure 4D and online supplemental figure 6A). These findings indicate EVs with S100A10 exhibit remarkable effects on HCC cell motility.

Furthermore, we employed two EV education mouse models to further evaluate the effects of S100A10 EVs in facilitating HCC liver or lung metastasis (liver metastasis model by intrasplenic injection and lung metastasis model by tail vein injection). First, nude mice intrasplenically injected with 97L or 97H HCC cells were given intravenous injection of EVs derived from S100A10-OE or S100A10-KO HCC cells every 4 days for 4 weeks to study the metastatic ability to the livers (figure 4E, left panel). On treatment with 97L-S100A10-EVs, the 97L liver metastatic nodules were markedly increased in both size and number when compared with treatment with 97L-Vec-EVs or PBS (figure 4F). In contrast, in mice treated with EVs derived from S100A10-KO 97H cells, there was no increase in the liver metastasis, whereas the 97H-NTC-EVs enhanced the liver metastatic ability compared with PBS control (online supplemental figure 6B).

Second, NOD SCID mice intravenously injected (via tail veins) with luciferase-labelled 97L HCC cells were similarly treated with S100A10 EVs to evaluate the metastatic ability to the lungs (figure 4E, right panel). Similarly, in the lung metastasis model using tail vein injection, the luciferase signal was significantly enhanced by EVs derived from S100A10-OE cells, and the luciferase signal was much stronger in S100A10 EV-treated

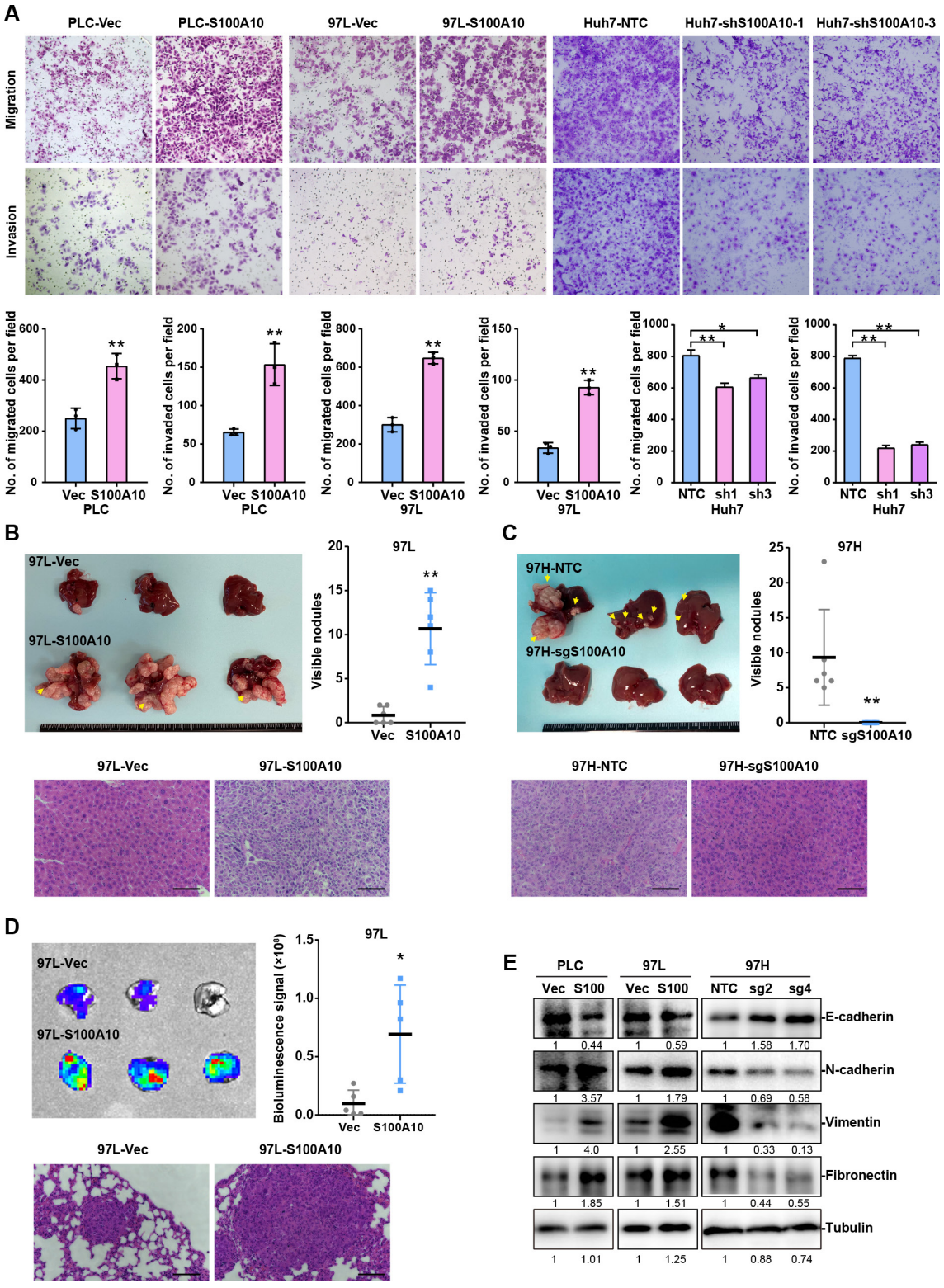


Figure 3 S100A10 promotes HCC migratory, invasive and metastatic abilities in vitro and in vivo. (A) The migratory and invasive abilities of PLC-Vec/S100A10, 97L-Vec/S100A10 and Huh7-NTC/shS100A10 were assessed by transwell migration and matrigel invasion assays. (B,C) Representative image of livers from nude mice after intrasplenic injection of indicated cells and representative histology of the corresponding liver sections (lower panel). Scale bar, 100 µm. Bar charts show the numbers of metastatic nodules on the liver surface (yellow arrows) in six mice in each group. (D) Representative image of lungs from NOD SCID mice after tail vein injection of 97L-Vec/S100A10 and representative histology of the corresponding lung sections (lower panel). Scale bar=100 µm. The bioluminescence signals represent mean±SD. *P<0.05, **P<0.01. Mann-Whitney test. (E) Western blot showing the expression of E-cadherin, N-cadherin, vimentin and fibronectin of HCC cells transfected with Vec and S100A10, or NTC and sgS100A10s. HCC, hepatocellular carcinoma; NTC, non-target control; Vec, vector control.

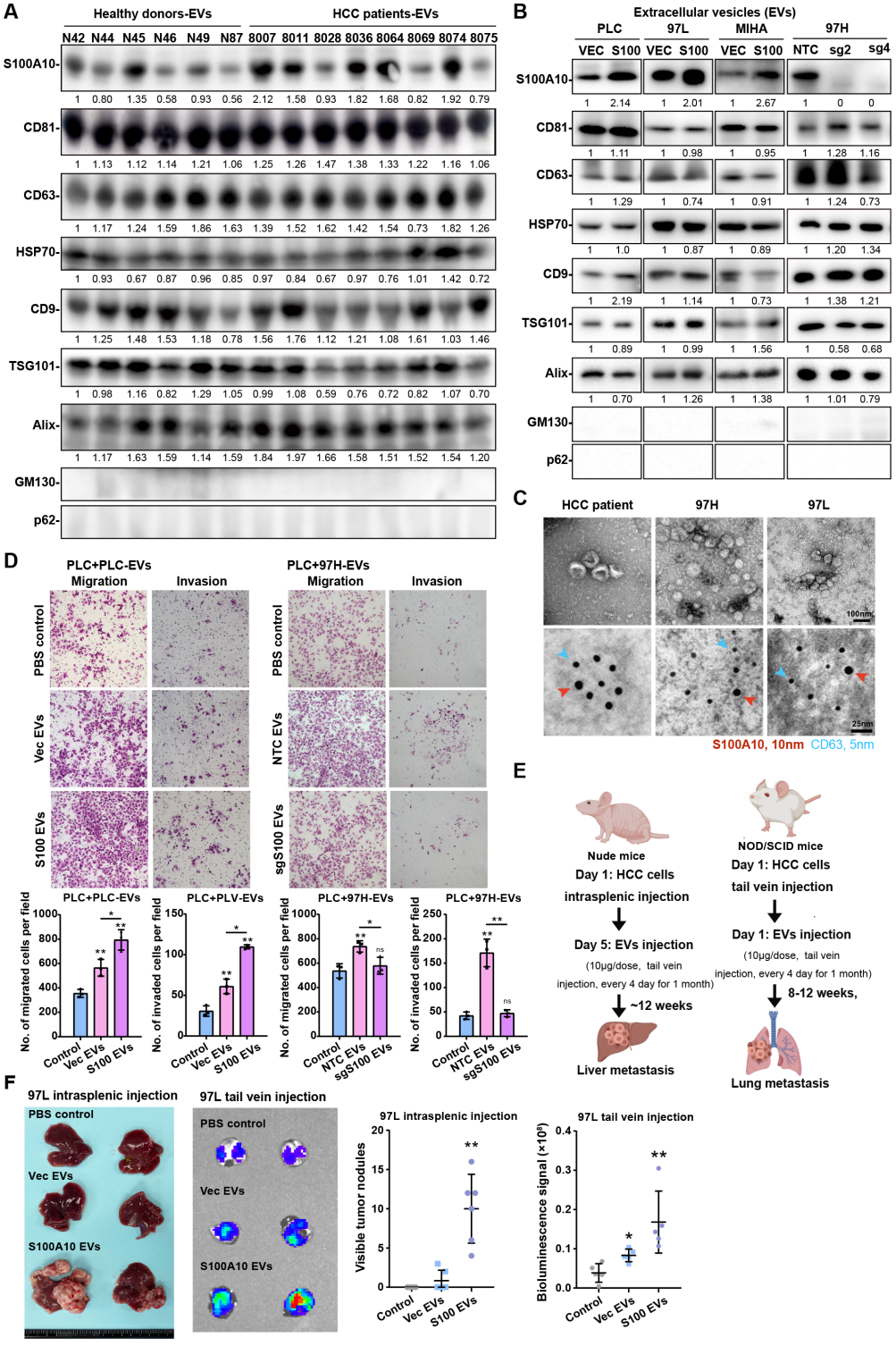


Figure 4 S100A10 is present in HCC-derived EVs and promotes HCC motility and metastasis. (A) Western blot showing detection of S100A10 and EV markers including CD81, CD63, HSP70, CD9, TSG101, Alix, Golgi marker GM130 and nucleoporin p62 in EVs from the plasma of patients with HCC. Each lane represents either a healthy donor or a patient with HCC. (B) Western blot showing detection of S100A10 and EV markers including CD81, CD63, HSP70, CD9, TSG101, Alix and Golgi marker GM130 and nucleoporin p62 in EVs from HCC cells. (C) Representative electron-micrographs of EVs derived from the plasma of patients with HCC, 97H and 97L cells (upper panel). Immunogold labelling of EVs using anti-CD63 and anti-S100A10 antibodies followed by secondary antibodies conjugated to 5 and 10 nm gold particles, respectively (lower panel). (D) Cell migration and invasion assays of PLC cells treated with Vec EVs or S100A10-OE cells (S100 EVs), or with EVs from 97H-NTC (NTC EVs) or -sgS100A10#4 (sgS100 EVs) cells. (E) Schematic diagram of the two EV education mouse models: liver metastasis model (left panel) and lung metastasis model (right panel). (F) Representative images of liver from nude mice of intrasplenic 97L cell injection model educated with PBS or indicated EVs, and bioluminescence signals of lungs from NOD SCID mice after tail vein injection of 97L educated with PBS or indicated EVs (left panel). The numbers of metastatic nodules on liver surface and bioluminescence signals of lung are summarised in the bar charts (right panel). EV, extracellular vesicle; HCC, hepatocellular carcinoma; NTC, non-target control; Vec, vector control; Vec EV, extracellular vesicle from vector control cell.

mice (figure 4F). The histology also supported the observations (online supplemental figure 6C).

EV-S100A10 is functional and promotes HCC stemness, which is abrogated with NA

To investigate the underlying mechanism by which S100A10-enriched EVs enhanced HCC development, we employed NA against S100A10 to see if it was able to block the effects of S100A10 EVs in promoting cancer stemness. When PLC-S100A10-EVs were preincubated with the NA and then applied to HCC cells as pretreatment for migration and invasion assays, the enhanced migratory and invasive abilities of PLC cells were markedly abrogated (figure 5A). Furthermore, the in vivo metastatic ability of 97L cells induced by S100A10 EVs was markedly abrogated by coinjection with S100A10 NA in both liver metastasis and lung metastasis mouse models (figure 5B,C). The histology confirmed the observations (online supplemental figure 7A). In addition, cotreatment with S100A10 NA abolished the focus formation, sphere formation and chemoresistance enhanced by S100A10 EVs (figure 5D,E, and online supplemental figure 7B,C). Altogether, these findings demonstrated that S100A10 in EVs plays a key role in promoting HCC stemness features and indicate that S100A10 NA is a potential therapeutic option for HCC.

EV-S100A10 affects the distribution of EVs and promotes pulmonary leakiness

Various studies revealed that EVs can prime a supportive microenvironment to facilitate metastasis. To investigate the distribution and uptake of EVs in different organs, EVs derived from 97L-Vec or S100A10-OE were labelled with PKH26 and injected intravenously into mice. The results showed that EVs predominantly localised in the lungs and livers, and more S100A10 EVs were observed compared with Vec EVs in these organ tissues (online supplemental figure 7D). As destabilisation and increased vascular permeability in the lungs are early events in premetastatic niche formation,[20] we then performed pulmonary leakiness assay. The result showed that S100A10 EVs enhanced the pulmonary endothelial permeability in mice when compared with PBS control and Vec EVs, as indicated by the larger area of dextran staining (figure 5F). When S100A10 EVs were injected together with S100A10 NA, the effect was significantly abolished (figure 5F).

S100A10 alters the protein cargos of EVs

Mass spectrometry was performed to compare the different proteins in 97L-Vec-EVs and 97L-S100A10-EVs. 364 proteins were found to be significantly upregulated more than 1.5 folds in S100A10-enriched EVs. Gene ontology (GO) analysis revealed that most of the upregulated proteins were involved in the protein binding and ECM constituent (online supplemental figure 8A). KEGG enrichment analysis showed that the upregulated proteins mainly participated in ECM receptor interaction, endocytosis and pathways in cancer (online supplemental figure 8A).

From the results of GO and KEGG analyses, MMP2 and fibronectin were enriched in more than one of these processes. As a plasminogen receptor, S100A10 is important in accelerating the degradation of ECM such as fibronectin, activation and secretion of MMPs and growth factors. Therefore, we focused mainly on MMP2 and fibronectin, and determined if growth factors such as EGF and HGF in EVs were affected by S100A10. Western blotting verified that OE of S100A10 increased the MMP2, fibronectin and EGF levels in the EVs (figure 6A), although EGF was not detected in the mass spectrometry results, whereas KO of S100A10 decreased the levels of the MMP2, fibronectin and EGF in the EVs (figure 6B). Recent study has shown that cytokines in the tumour microenvironment could bind to the surface of EVs secreted by cancer cells.[27] We next examined whether MMP2, fibronectin and EGF were localised within EVs or present on the surface of EVs. First, our immunogold labelling indicated that MMP2, fibronectin and EGF were present on the membrane of EVs together with S100A10 (figure 6C). Then, we treated 97H-EVs with proteinase K to degrade proteins at a concentration that led to the degradation of the outer membrane proteins such as CD81, but not the intravesicular HSP70. The findings showed that the distribution of MMP2, fibronectin and EGF was limited to the EV surface, while S100A10 was localised both within and on the membrane of EVs (figure 6D). To assess if MMP2 carried by EVs was biologically activated, we performed gelatin zymography assay and found significant MMP2 activity of EVs derived from HCC cells (online supplemental figure 8B). On the other hand, S100A10 EVs treated with MMP2 inhibitor abrogated the enhanced migratory ability of HCC cells by EVs (online supplemental figure 8C).[28]

S100A10 mediates the targeting of MMP2, EGF and fibronectin to EV membranes through integrin

To evaluate the role of S100A10 in regulating the binding of MMP2, fibronectin and EGF to the surface of EVs, we treated the 97H cells with S100A10 NA before isolation of EVs and assessed the levels of MMP2, fibronectin and EGF of EVs. The results showed that, similar to S100A10-KO, when S100A10 was blocked, MMP2, fibronectin and EGF levels on EVs were significantly decreased, while that of S100A10 remained relatively unchanged (figure 6B). The expression levels of MMP2, fibronectin and EGF in 97H-S100A10-KO cells and 97H cells treated with S100A10 NA or IgG were not reduced as significantly as those in the EVs (online supplemental figure 8D). These results suggest that S100A10 may mainly affect the packaging of MMP2, fibronectin and EGF into EVs but not through affecting these proteins level in cells.

ITGAV has been reported to bind MMP2, EGF and fibronectin, and to be present on the surface on EVs. Using immunogold labelling and proteinase K treatment, we confirmed that ITGAV was present on the surface of EVs (figure 6C,D). Previous study has shown that targeting of fibronectin to EVs depends on binding to integrins.[29] To test whether S100A10 mediated the targeting of MMP2, fibronectin and EGF to EVs by binding to integrins, we inhibited the possible interaction with ITGAV by treating S100A10-OE cells with integrin ligand-mimetic peptide GRGDSP peptides (RGD). On treatment of PLC-S100A10 cells with the integrin-binding RGD peptide for 48 hours, the levels of MMP2, fibronectin and EGF on EVs were greatly reduced, but no changes were observed in S100A10, as compared with cells treated with the control GRADSP peptides (RAD) (figure 6E). Likewise, KD of ITGAV in S100A10-OE cells also decreased the MMP2, fibronectin and EGF levels in EVs (figure 6E). These data indicate that S100A10 mediates MMP2, EGF and fibronectin targeting to EVs through binding to integrin and then secreted in an adhesive form in EVs to promote the cell motility of recipient cells. Coimmunoprecipitation assay also revealed that S100A10 physically bound with ITGAV, MMP2, fibronectin and EGF (figure 6F). These findings suggest that S100A10 is an important mediator between integrin and specific secretory proteins targeting to EV, as represented by the schematic diagram (figure 6G).

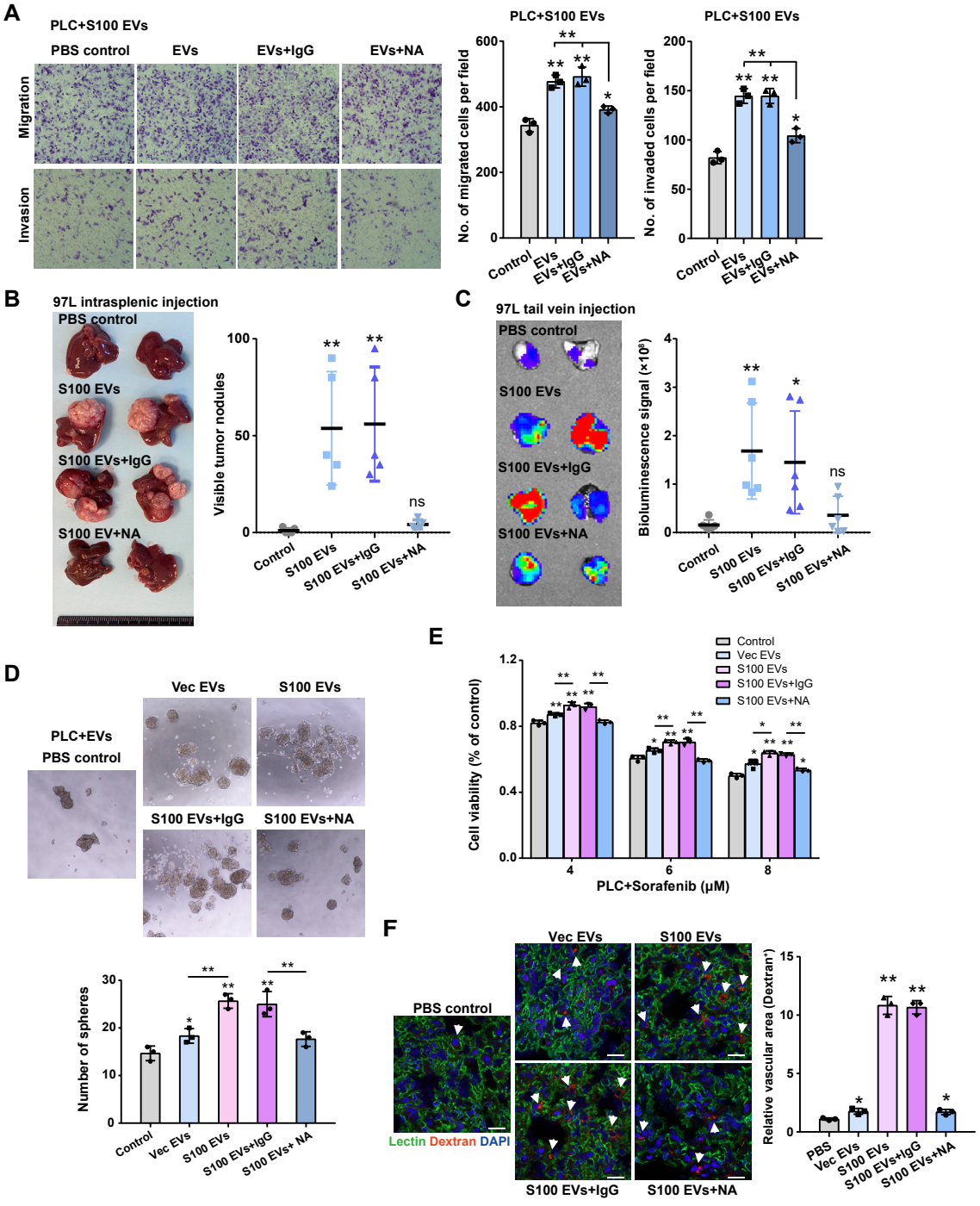


Figure 5 S100A10-NA abrogates the functions of S100A10 EVs in HCC stemness. (A) Migration and invasion assays on PLC cells treated with PBS or EVs derived from S100A10-OE cells (S100 EVs). The EVs were preincubated and added together with S100A10 NA or IgG control antibody. (B) Intrasplenic injection of HCC cells. EVs were injected together with S100A10 NA or IgG. (C) Bioluminescence signals of lungs from NOD SCID mice after tail vein injection of luciferase labelled 97L, educated with PBS or indicated EVs together with S100A10 NA or IgG. Sphere formation (D) and chemoresistance (E) were assessed on PLC cells treated with PBS, Vec EVs, or EVs from S100A10-OE cells (S100 EVs). The EVs were preincubated and treated together with S100A10 NA or IgG control antibody. (F) Representative images for lung vascular leakiness after tail vein injection of EVs, Texas Red-Dextran and FITC-lectin. Arrowheads indicate the area of endothelial leakiness. Scale bar=20 μm. *P<0.05, **P<0.01. EV, extracellular vesicle; NA, neutralising antibody; ns, not significant; Vec, vector control; Vec EV, extracellular vesicle from vector control cell.

S100A10 enhances HCC progression through EGFR activation

To further investigate the underlying mechanism how EV-S100A10 enhanced HCC metastasis, self-renewal and chemoresistance, we examined the possible signalling pathways involved. PLC-S100A10 EVs upregulated the expression of the mesenchymal markers (N-cadherin, fibronectin and vimentin) and downregulated that of epithelial marker E-cadherin (figure 7A), and S100A10 EVs

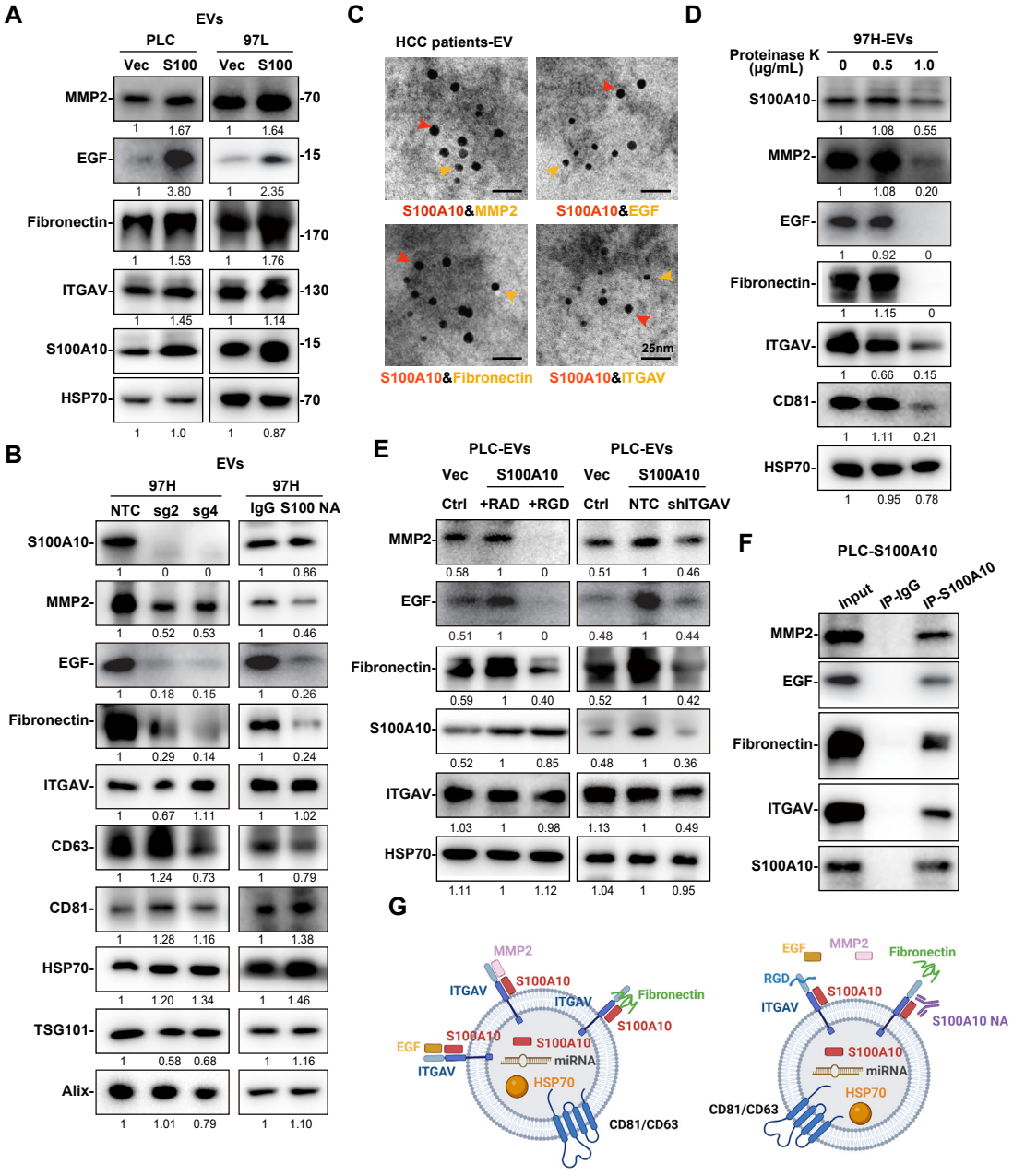


Figure 6 S100A10 alters the protein contents of EVs. (A) Western blot showing the levels of MMP2, EGF, fibronectin and ITGAV in EVs derived from PLC-Vec/S100A10 and 97L-Vec/S100A10. (B) Western blot showing the expression levels of S100A10, MMP2, EGF, fibronectin and ITGAV in EVs derived from 97H-NTC/sgS100A10s or 97 H cells treated with S100A10 NA or IgG 48 hours before EV isolation. The EV markers, including CD63, CD81, HSP70, TSG101 and Alix, were used as loading control. (C) Representative electron micrograph of EVs from the plasma of patients with HCC subjected to immunogold labelling using anti-S100A10 together with anti-MMP2, anti-EGF, anti-fibronectin or anti-ITGAV antibodies, followed by secondary antibodies conjugated to 10 and 5 nm gold particles, respectively. Scale bar=25 nm. (D) Western blot showing the expression of S100A10, MMP2, EGF, fibronectin, surface marker CD81 and intra-EV marker HSP70 in 97H-derived EVs treated with increasing concentrations of proteinase K. Untreated EVs (first lane from left) served as control. (E) Western blot showing MMP2, EGF, fibronectin, S100A10 and ITGAV in EVs. GRADSP peptide (control peptide RAD as control) or GRGDSP peptide (RGD which is integrin-binding) were added to PLC-S100A10 cells for 48 hours before EV isolation. HSP70 was used as a loading control. (F) Coimmunoprecipitation assay showing the interaction between S100A10 and MMP2, EGF, fibronectin and ITGAV, using anti-S100A10 (IP-S100A10) or IgG (IP-IgG) in S100A10-OE PLC cells. Total cell lysate (input) used as positive control. (G) A schematic diagram illustrating the proposed S100A10-mediated binding of MMP2, fibronectin and EGF to the surface of EVs through ITGAV. EV, extracellular vesicle; HCC, hepatocellular carcinoma; ITGAV, integrin αV; NA, neutralising antibody; NTC, non-target control.

showed a stronger effect than the PLC-Vec-EVs. Furthermore, S100A10 EVs were able to activate the AKT/ERK pathway (figure 7A), and again, S100A10 EVs exhibited a stronger effect than the Vec EVs. Interestingly, when the HCC cells were treated with S100A10 NA together with S100A10 EVs, these promoting effects were significantly abolished (figure 7A). Furthermore, the activation of EGFR was upregulated by S100A10 EVs, indicating that S100A10

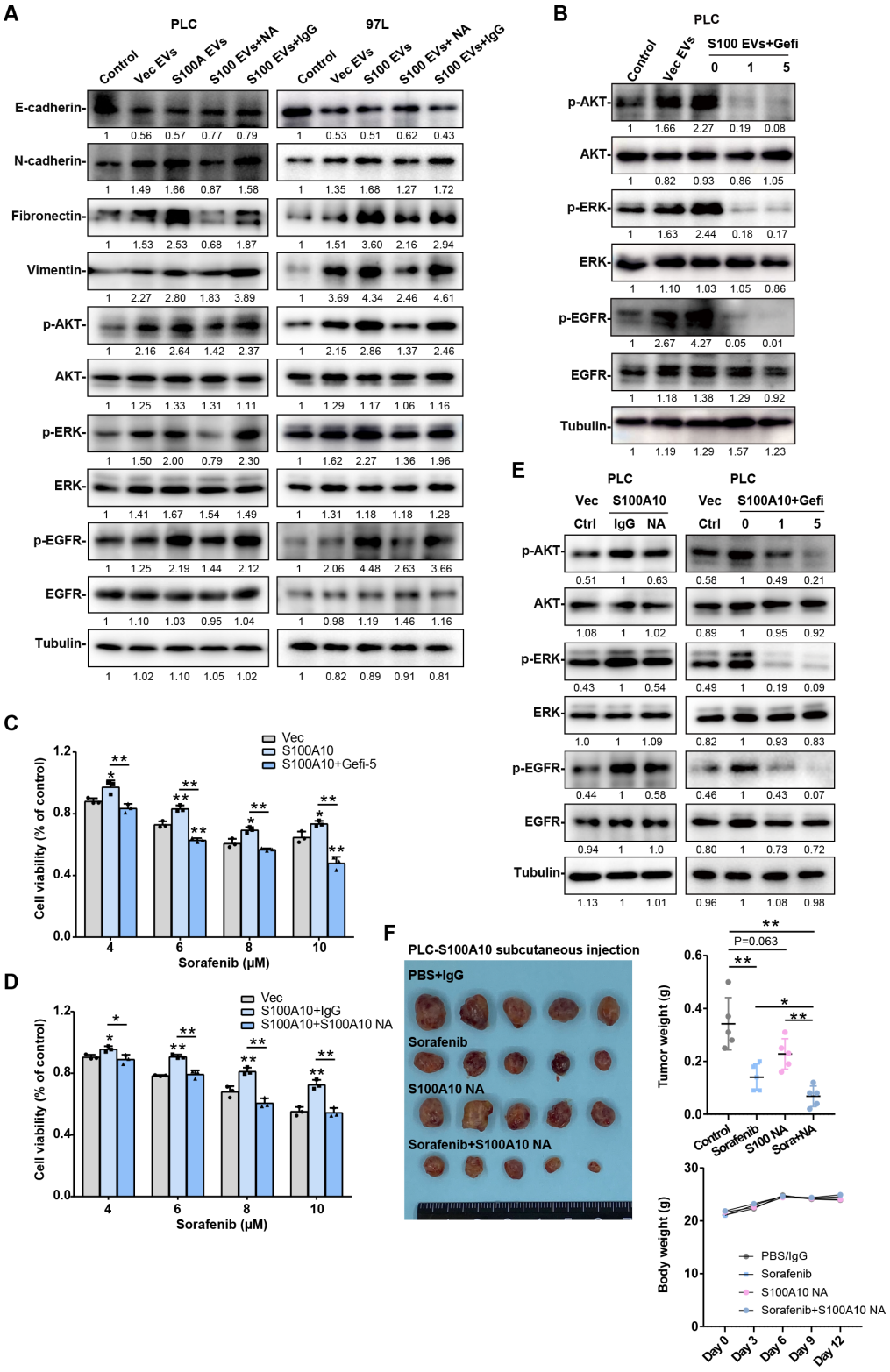


Figure 7 EV-S100A10 and S100A10 enhance HCC progression through EGFR activation. (A) Western blot showing the expression levels of EMT-related markers and EGFR, AKT and ERK signalling in cells treated with PBS, Vec EVs, S100 EVs, or S100 EVs, together with S100A10 NA or IgG. (B) Western blot showing the activation of EGFR, AKT and ERK signalling in cells treated with PBS, Vec EVs, S100 EVs, or S100 EVs together with different concentrations of EGFR inhibitor-Gefi (μM). (C, D) Chemoresistance to sorafenib of PLC-Vec, S100A10-OE cells, or S100A10-OE cells treated with Gefi, S100A10 NA or IgG control antibody. (E) Western blot showing the activation of EGFR, AKT and ERK signalling in Vec or S100A10-OE cells treated with S100A10 NA, IgG or different concentrations of Gefi (μM). (F) Nude mice subcutaneously injected with S100A10-OE PLC cells were administered with vehicle and IgG, sorafenib, S100A10 NA, or a combination of sorafenib and S100A10 NA. Sorafenib was administered daily via oral gavage. Control IgG and S100A10 NA were injected peritoneally once every 3 days. the tumour weight and body weight of mice were measured and plotted (right panel). *P<0.05, **P<0.01. EV, extracellular vesicle; Gefi, gefitinib; Gefi-5, gefitinib at 5 μM; p-AKT, phosphorylated AKT; p-ERK, phosphorylated ERK; Vec, vector control.

might activate AKT and ERK with associated activation of EGFR (figure 7A). To further validate the importance of EGFR activation in this process, we used an EGFR inhibitor, gefitinib, together with S100A10 EVs, to treat HCC cells, and the results showed that gefitinib significantly inhibited the activation of EGFR, AKT and ERK induced by EV-S100A10 (figure 7B).

To further determine if the EGFR inhibitor gefitinib could decrease the oncogenic effects of S100A10, gefitinib was used to treat PLC S100A10-OE cells. First, XTT assay for cell proliferation showed that S100A10-OE cells were more sensitive to gefitinib than the Vec cells (online supplemental figure 9A). Furthermore, gefitinib significantly suppressed focus formation, sphere formation, chemoresistance, migration and invasion ability of S100A10-OE cells (figure 7C and online supplemental figure 8B–D). Moreover, when S100A10 NA was added to the culture medium of S100A10-OE cells, the NA treatment significantly decreased the chemoresistance to sorafenib (figure 7D). The treatment with both S100A10 NA and gefitinib suppressed the activation of EGFR, AKT and ERK, indicating that the oncogenic effect of S100A10 is dependent on the EGFR activation (figure 7E). With subcutaneous injection of the mouse model, we also found that S100A10 NA suppressed tumour growth and enhanced the antitumour efficiency of sorafenib in vivo (figure 7F). By monitoring the body weights of the mice that undergo different treatment, no significant changes were observed, and this implies no significant adverse effects of S100A10 NA in body organs in mice (figure 7F).

EV-S100A10 exerts chemotaxis on HCC cells as a potential ligand of EGFR

EVs have been reported to contain multiple cytokines/chemokines to exert chemotaxis on cancer cells.[27 30–32] Extracellular S100 proteins have been reported to function in a cytokine-like manner via interaction with cell surface receptors.[12] However, whether EV-S100A10 has chemoattractive function is unknown. To this end, we applied condition medium (CM) from S100A10-OE or Vec cells in the lower compartment of the transwell chambers. The CM from S100A10-OE cells significantly enhanced the migratory and invasive abilities of the HCC cells (online supplemental figure 10A). On the other hand, we queried whether CM derived from different cell lines might exert similar effects on HCC cells. We applied the CM derived from S100A10-KO 97H to PLC or 97L cells and performed the cell migration and invasion assays. The migratory and invasive abilities of PLC and 97L cells were significantly decreased with S100A10-KO 97H CM compared with the NTC-97H CM (online supplemental figure 10A). These results suggest that the secreted biomolecules in CM regulated by S100A10 exert chemotactic effects on HCC cells.

Furthermore, we added EVs, instead of CM, to the lower compartment of the transwell chambers. Addition of S100A10 EVs promoted the migratory and invasive abilities of HCC cells (figure 8A and online supplemental figure 10B), and S100A10 EVs exhibited a stronger effect as compared with Vec EVs. While 97H-NTC derived EVs promoted migration and invasion of HCC cells, S100A10-KO abolished these effects (figure 8B and online supplemental figure 10B). To evaluate the importance of S100A10 in this process, S100A10 NA or IgG was added together with S100A10 EVs to the lower chambers. The results showed that S100A10 NA completely abolished such promoting effect (figure 8C). These findings indicate that S100A10 in EVs is a critical functional component in the chemotaxis of HCC cells.

To this end, we explored the potential receptor of S100A10. As S100A10 was found to increase the activation of EGFR, and EGFR is an important receptor in chemotactic responses,[33 34] we queried if EGFR is a receptor for S100A10. When we applied EGFR NA to PLC cells in the migration assay, the migratory ability was significantly decreased. Furthermore, EGFR NA treatment abolished the differences between S100A10 EVs treated with S100A10 NA and IgG (figure 8D). On other hand, EGFR abolished the suppression of cell migration exerted by S100A10 NA (figure 8E). Furthermore, coimmunoprecipitation assay showed that S100A10 bound to EGFR, which further supports that EGFR is a potential receptor in S100A10 chemoattracting process (figure 8F).

DISCUSSION

The identification and characterisation of specific gene expression signatures and molecular phenotypes of liver progenitor cells in the process of HCC development allows a better understanding of the molecular pathogenesis and progression of HCC. In the present study, we investigated S100A10 as it was highly expressed in the liver progenitor stage.[1 2 35] Here our results showed that S100A10 expression was highly upregulated in patients with HCCs and was significantly associated with more aggressive tumour behaviour including more frequent vascular invasion, poorer cellular differentiation and poorer prognosis. S100A10 also promoted stemness properties of HCC, including self-renewal, tumourigenicity, tumour initiation and chemoresistance. S100A10 enhanced HCC metastasis through the EMT process. These findings indicate that S100A10 has a critical role in HCC progression.

Although the extracellular function of S100 family proteins at large has been reported to promote disease progression in cancers,[12–14] there have been few studies investigating the extracellular roles of S100A10. To the best of our knowledge, there are no reports on whether S100A10 are secreted into EVs in cancers. In this study, we clearly demonstrated that S100A10 was secreted into EVs, and in this form, it played a key functional role in HCC. EVs derived from S100A10-OE HCC cells promoted the metastasis and stemness features of HCC. In addition, we found that EV-S100A10 promoted the EGFR, AKT and ERK signalling pathways and enhanced the EMT. Of note, all these effects were effectively abrogated with S100A10 NA. Moreover, treatment with S100A10 NA in xenograft mice suppressed HCC growth and enhanced the antitumour efficiency of sorafenib, indicating that S100A10 NA is a potential therapeutic option for HCC treatment. Furthermore, we found that, while EVs could function as a chemoattractant, S100A10 is a key component of EVs in chemoattracting HCC cell migration. Interestingly, S100A10 was able to interact with EGFR and function as a potential ligand of EGFR to activate its signalling pathways and promote chemotaxis of HCC cells.

S100A10 is well accepted as a receptor for plasminogen and plasminogen activator and facilitates the conversion of plasminogen to plasmin, accelerating the degradation of ECM such as fibronectin, active MMPs and growth factors.[36–39] To this end, combining the results of mass spectrometry, we found that S100A10 increased the MMP2, fibronectin and EGF levels in EVs. Furthermore, we found that MMP2, fibronectin and EGF were limited to the EV surface, while S100A10 localised both within and on the EV membranes. Of note, S100A10 NA was able to inhibit the binding of MMP2, fibronectin and EGF to the surface of EVs. ITGAV has been reported to bind MMP2, fibronectin and EGF.[40–42] In addition, it has been reported that

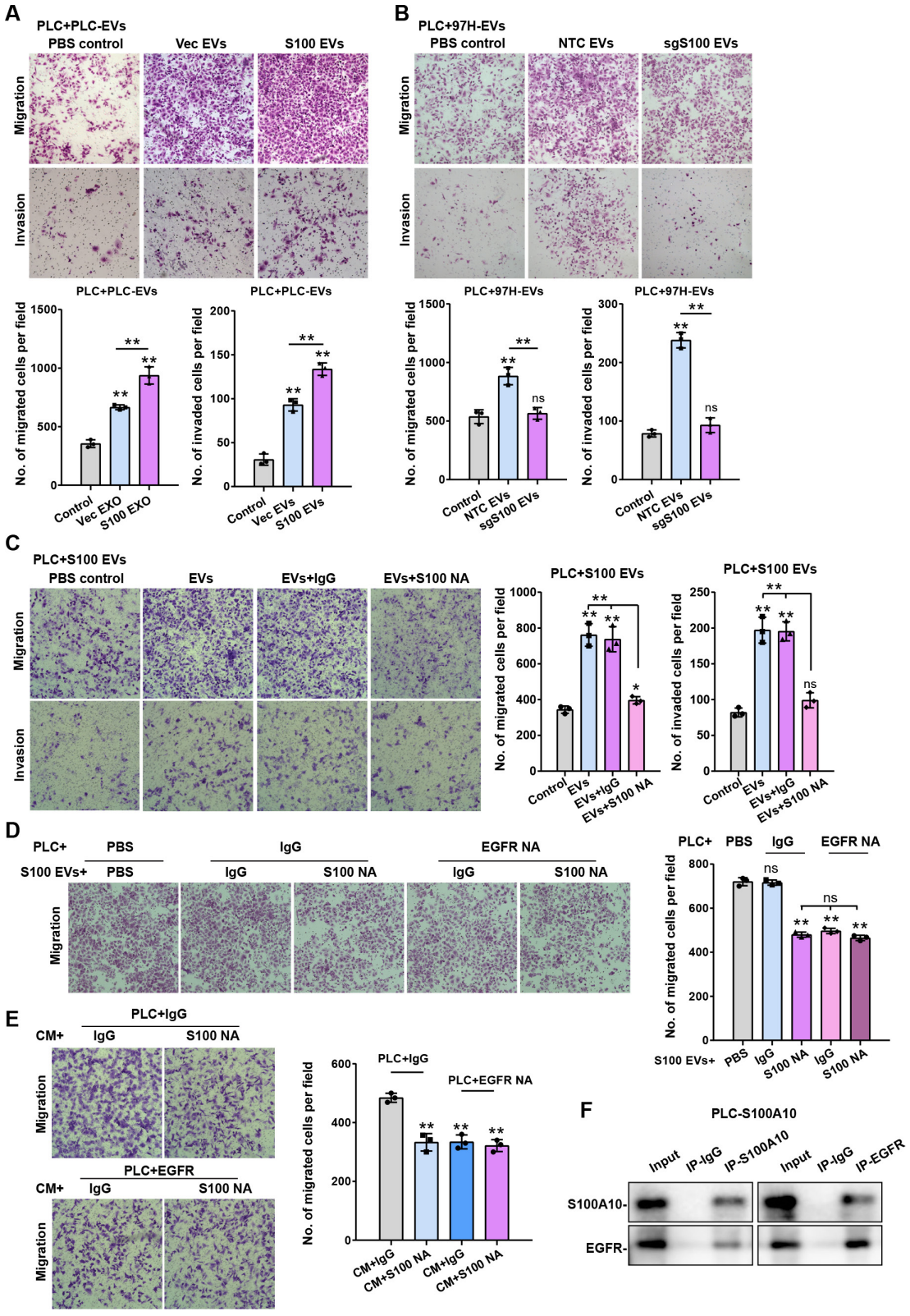


Figure 8 EV-S100A10 promotes chemotaxis of HCC cells. (A,B) Cell migration and invasion assays. Vec EVs, or S100A10-OE cells (S100 EVs), or 97H-NTC (NTC EVs) or -sgS100A10#4 (sgS100 EVs) were added to the lower compartment of transwell chambers as indicated. (C) Migratory and invasive abilities of PLC cells assessed with PBS or S100 EVs added to the lower compartment of transwell chambers. EVs were preincubated and added together with S100A10 NA or IgG antibody. (D) Migratory ability of PLC treated with IgG or EGFR NA, with S100A10 EVs and IgG or S100A10 NA were added to the lower compartment of transwell chambers. (E) Migratory ability of PLC treated with IgG or EGFR NA, with CM and IgG or S100A10 NA were added to the lower compartment of transwell chambers. (F) Coimmunoprecipitation assay showing interaction between S100A10 and EGFR using anti-S100A10 (IP-S100A10) or anti-EGFR antibody (IP-EGFR) or IgG (IP-IgG) in S100A10-OE PLC cells. Total cell lysate (input) used as positive control. EV, extracellular vesicle; NTC, non-target control; Vec, vector control; Vec EV, extracellular vesicle from vector control cell.

targeting of fibronectin to the surface of EVs is dependent on the binding to integrins.[29] Along this line, treatment with integrin-binding RGD peptide to inhibit the interaction, we were able to demonstrate that the S100A10-mediated binding of MMP2, EGF and fibronectin to EVs was dependent on the interaction with integrins. Our coimmunoprecipitation results also confirmed that S100A10 physically interacted with ITGAV, MMP2, EGF and fibronectin. All these findings suggest that S100A10 plays a crucial role in mediating targeting of MMP2, EGF and fibronectin to EVs through binding to integrin receptors.

In summary, we demonstrated that S100A10 is secreted into EVs and plays a pivotal role in mediating HCC stemness-related properties and activating EGFR signalling pathway to promote EMT. S100A10 also mediates the targeting of specific secretory proteins to EV membranes through physical interaction with integrins. Moreover, EV-S100A10 also functions as a chemoattractant. Importantly, S100A10 NA is able to block the functions of EV-S100A10, and it may be a potential therapeutic strategy for HCC. Recently, a bioinformatics analysis study revealed the potential usage of S100 protein family as a biomarker for HCC including S100A10.[43] As our data also suggest that the EV-S100A10 level was relatively higher in the plasma of patients with HCC than in the plasma of healthy subjects, more work is warranted to test the use of S100A10 or EV-S100A10 as a potential diagnostic marker for HCC.

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Contributors XW and IO-LN provided the study concept and design, interpreted and analysed the data, andwrote the manuscript. XW, HH, KM-FS, JW, LT, JL, Y-MT, H-TM, EL, AC and YW performed the experiments. T-TC and JM-FL collected the patients' samples. JW-PY advised on extracellular vesicle-related experiments. XG and IO-LN provided financial support. IO-LN is responsible for the overall content as the guarantor and supervised the project. All authors approved the final version of the manuscript.

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Competing interests IOLN is Loke Yew professor in pathology.

Patient and public involvement Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants (tissues and blood samples) and was approved by institutional review board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW-19-667 and UW 17-056). Participants gave informed consent to participate in the study before taking part. Research ethics approval was issued by Committee on the Use of Liver Animals in Teaching and Research, The University of Hong Kong (reference number CULATR 5393–20).

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