

Tumor-derived miR-9-5p-loaded EVs regulate cholesterol homeostasis to promote breast cancer liver metastasis in mice

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Cancer cells secrete extracellular vesicles (EV) encapsulating bioactive cargoes to facilitate inter-organ communication in vivo and are emerging as critical mediators of tumor progression and metastasis, a condition which is often accompanied by a dysregulated cholesterol metabolism. Whether EVs are involved in the control of cholesterol homeostasis during tumor metastasis is still undefined and warrant further investigation. Here, we find that breast cancer-derived exosomal miR-9-5p induces the expression of HMGCR and CH25H, two enzymes involved in cholesterol synthesis and the conversion of 25-hydroxycholesterol from cholesterol by targeting INSIG1, INSIG2 and ATF3 genes in the liver. Notably, in vivo miR-9-5p antagomir treatment and genetic CH25H ablation prevents tumor metastasis in a mouse model of breast cancer. Thus, our findings reveal the regulatory mechanism of tumor-derived miR-9-5p in liver metastasis by linking oxysterol metabolism and Kupffer cell polarization, shedding light on future applications for cancer diagnosis and treatment.

Breast cancer (BC) is the most commonly diagnosed cancer type worldwide^{1,2}. Most breast cancer-related deaths result from metastasis to distant organs, such as the brain, bone, lung and liver^{3,4}. There are distinct alterations in gene expression, immune signatures or metabolic profiling underlie metastasis to the specific metastatic sites⁵. Before the formation of micro- and macro-metastasis, disseminated cancer cells need to overcome sequential inhibitory signals, survival in circulation, arrest at the distant site and extravasation^{6,7}. Complicated factors residing in tumor cells or tumor microenvironment mediate tumor invasion and progressive

outgrowth at a distant site⁸. For instance, immune cells and their mediators are known to facilitate BC metastasis and cancer cell dissemination is highly correlated with the capability to evade immunosurveillance^{8–10}. Besides, liver-metastatic BC cells exhibit a unique metabolic program compared to the bone- or lung-metastatic cells, characterized by an increased glycolysis rate but a concomitant reduction in mitochondrial metabolism¹¹. Therefore, to cope with complicated challenges during the metastatic process, tumor cells dynamically take multiple strategies to induce vascular leakage, recruitment of pro-angiogenic immune cells, or influence

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of organotropism to extend the effect of the intrinsic factors, such as the secretion of nanoparticles^{12,13}.

Cancer exerts systemic effects partially through secreting extracellular vesicles (EV) that travel throughout the body via blood circulation and deliver bioactive cargo including miRNAs, lipids and proteins to various organs^{14–16}. The biological function of those circulating EVs was involved in reprogramming cells in the tumor micro-environment or in a metastatic niche to promote tumor growth, immune evasion, and metastasis^{15,17–19}. Indeed, cancer patients-secreted-circulating EVs (exosomes and micro-vesicles) have been associated with metastasis or relapse, serving as important diagnostic and prognostic markers as well as therapeutic targets²⁰. Moreover, tumor-secreted EVs are major mediators for cancer cells to adapt and modify foreign microenvironments²¹. Accumulating evidence demonstrates that microRNA as well as proteins could be transferred to local or distant cells in these secretory forms to modulate cell function, emerging as a new group of messengers and effectors between inter-cellular communication²².

Dysregulated lipid metabolism has been reported to regulate cancer incidence and metastasis^{23,24}. Among the environmental factors believed to be responsible for this phenomenon, cholesterol has recently received considerable attention. Cholesterol is a precursor of bile acids, steroid hormones and vitamin D, and a component of cell membranes, which plays a crucial role in cell growth and differentiation^{25,26}. Upregulated cholesterol biosynthesis has been discovered in several types of cancers, where it supports the growth, metastasis, stemness, and therapeutic resistance of tumors²⁷. In BC, cholesterol and its metabolites have been found to promote tumor progression both preclinically and clinically²⁸. Compared to other BC subtypes, TNBC displays elevated cholesterol biosynthesis, which could have profound biological functions and indicate potential therapeutic strategies²⁹. However, the regulatory mechanism and biological function of BC-derived EVs in cholesterol homeostasis during tumor metastasis remain poorly defined. Here, we show that the miRNA cargo loaded by cancer-secreted extracellular vesicles, functioning as “tri-brakes” (targeting three genes on the cholesterol homeostasis pathway), promotes cholesterol conversion into 25-hydroxycholesterol in liver. This onco-metabolite thereby induces breast cancer liver metastasis. This important result provides a new understanding of the dynamic interaction between cancer and host at the systemic level, providing valuable insights into how cancer alters specific metabolite level, which we consider to be highly innovative. The study also reveals the possibility of using circulating miR-9-5p and 25-hydroxycholesterol as novel blood-based biomarkers to identify breast cancer patients with high risk of developing metastasis to guide preventive screenings and early intervention.

Results

Breast cancer-derived EV enhanced cholesterol biosynthesis in liver

To explore the systemic effect of breast cancer-derived EVs in vivo, both human triple-negative breast cancer MDA-MB-231 cells and MDA-MB-231/Rab27a KD cells with stable expression of a membrane-targeted Lck-GFP were used to establish orthotopic xenograft tumors (231/Ctrl and 231/Rab27a KD) through injection into the mammary fat pads (Fig. 1a). Notably, a previous report showed *Rab27a* was involved in EV secretion and cells with *Rab27a* knockdown displayed impaired EV secretion³⁰. Both MDA-MB-231 EV and 231/Rab27a KD EV displayed classic exosome size distribution (Supplementary Fig. 1a). In agreement with our previous finding¹⁸, decreased *Rab27a* expression in MDA-MB-231 cells leads to significantly reduced EV secretion (Supplementary Fig. 1b, c). We performed nanoflow cytometry analysis to further confirm the presence of CD63 and GFP proteins (~20% expression) on the EV membrane (Supplementary Fig. 1d). We detected GFP signals in the liver of 231/Ctrl mice rather than tumor

free mice or 231/Rab27a KD mice, indicating uptake of GFP-labeled EVs by the liver from circulation in vivo (Fig. 1a). We thereby performed RNA-sequencing for the liver tissues, and the following GSEA enrichment analysis revealed the cholesterol biosynthesis pathway was the only significantly upregulated pathway in 231/Ctrl mice than either tumor free or 231/Rab27a KD mice (Fig. 1b). Furthermore, the total cholesterol level (TC) was increased in both serum and liver tissues from 231/Ctrl mice than the control groups (Fig. 1c). Consistently, there was more lipid accumulation in the liver of 231/Ctrl mice than those from either tumor free mice or 231/Rab27a KD mice (Fig. 1d), suggesting an elevation of cholesterol metabolism in the liver of 231/Ctrl mice mediated by EVs. Next, we examined the expression levels of the enzymes in the cholesterol biosynthesis pathway in both human (231/Ctrl) and murine breast cancer cell xenograft (4T1/Ctrl) mouse models (Fig. 1e). In the 4T1 mouse model, we also xenografted 4T1 cells with *Rab27a* gene knockout (4T1/Rab27a KO) cells, which displayed impaired exosome secretion capability when compared to wild type 4T1 cells (Supplementary Fig. 1c–e). Both the RNA and protein abundance of HMGCR, FDFT1, LSS and DHCR7 were highly induced while INSIG1 and INSIG2, negative regulators of HMGCR²⁶, were significantly suppressed in liver tissues from 231/Ctrl mice than its relative control groups (Fig. 1f, g). Accordingly, reduced expression of INSIG1 and INSIG2 but elevated HMGCR levels were observed in immunohistochemistry-stained liver tissues from 231/Ctrl mice (Fig. 1h). A similar pattern was detected in the 4T1 mouse model (Supplementary Fig. 1f, g), suggesting the interorgan communication between the primary tumor and liver in vivo.

To further verify whether EV resulted in this phenomenon, the GFP-labeled EVs (normal human breast epithelial cell MCF-10A derived EV and MDA-MB-231 EV) were intravenously (i.v.) injected into female NOD/SCID/IL2Rγ-null (NSG) mice semiweekly-for-5 weeks, with liver and lungs collected for downstream bioluminescence imaging (Fig. 1i). We found there were sufficient GFP signals captured in both liver and lung, further confirming that cancer-derived EV could be taken up in vivo (Supplementary Fig. 1h, i). GSEA analysis also enriched the cholesterol biosynthesis pathway in the liver of mice receiving MDA-MB-231 EV compared to the control groups (Fig. 1j). We next performed ELISA quantification and oil Red O staining for the liver from EV-injected mice. We found MDA-MB-231 EV-educated liver exhibited more lipid accumulation than liver from either MCF-10A EV or PBS-injected mice (Fig. 1k and 1l). Similarly, this effect from cancer-derived EV on lipid accumulation was also detected in BALB/c mice receiving 4T1 EV (Supplementary Fig. 1j). Mechanistically, both RNA and protein abundance of cholesterol biosynthesis-related enzymes were upregulated in MDA-MB-231 EV-educated liver from both human (Fig. 1m, n) and murine BC mouse models (Supplementary Fig. 1k, l). Moreover, primary hepatocyte cells showed sufficient uptake of GFP-labeled EVs in vitro (Supplementary Fig. 1m), and MDA-MB-231 EV reduced liver INSIG1 and INSIG2 expression, along with remarkable induction of HMGCR, FDFT1, LSS and DHCR24 levels (Fig. 1o and Supplementary Fig. 1n). Collectively, these results demonstrated breast cancer-derived EVs enhanced cholesterol biosynthesis both in vitro and in vivo.

Circulating miR-9-5p induced cholesterol biosynthesis by targeting INSIG1 and INSIG2

To further explore the underlying mechanism of how cancer EVs regulated cholesterol biosynthesis, we thoroughly searched the potential miRNA pools predicted targeting both INSIG1 and INSIG2, and compared with miRNAs previously reported with higher expression in MDA-MB-231 EV compared to MCF-10A EV¹³ (see Supplementary Table 1), we found only miR-9-5p co-existed in both databases (Fig. 2a). There was fivefold upregulation of miR-9-5p level in BC serum than in non-cancer patients and threefold enrichment in MDA-MB-231 EV in comparison with MCF-10A EV (Fig. 2b and Supplementary Fig. 2a). Moreover, there were ~40-fold and ~10-fold of miR-9-5p higher

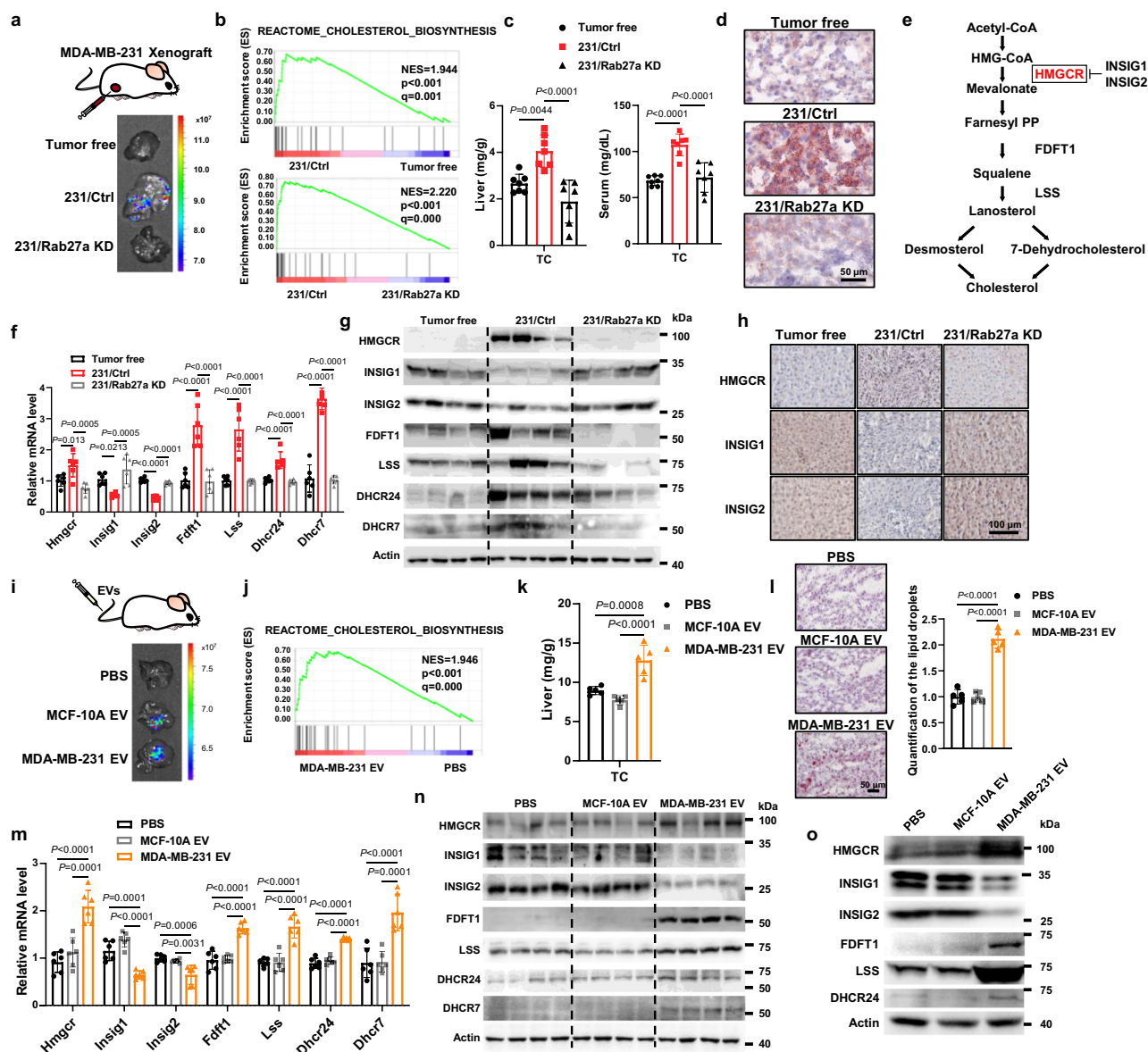


Fig. 1 | Breast cancer derived EV induced cholesterol biosynthesis in liver.

a Representative images for GFP signals in liver from mice xenografted MDA-MB-231/Lck-GFP cells (231/Ctrl) or MDA-MB-231/Rab27a KD/Lck-GFP cells (231/Rab27a KD) and tumor free mice. **b** Liver tissue collected from 231/Ctrl mice and 231/Rab27a KD mice for 50 days and tumor free mice were subjected to RNA-seq and GSEA, showing enrichment of genes related to indicated pathways (Permutation test, $n = 3$ mice per group). **c** Total cholesterol (TC) in liver and serum in indicated group (one-way ANOVA, $n = 7$ mice per group). **d** Liver sections were stained with oil red O. Scale bars, 50 μm . **e** Schematic of cholesterol synthesis pathway. **f** The qRT-PCR showing relative mRNA levels in the liver in indicated group (one-way ANOVA, $n = 6$ mice per group). **g** Western blots showing proteins related to cholesterol synthesis in the liver in indicated group. **h** IHC of indicated protein in liver sections. Representative images are shown. Scale bars, 100 μm . **i** GFP signals in liver from mice receiving tail vein injections of Lck-GFP-labeled EVs. **j** Liver tissue

collected from mice that had received 5 weeks of i.v. injections of EVs or control mice were subjected to RNA-seq and GSEA, showing enrichment of genes set related to cholesterol biosynthesis pathway (Permutation test, $n = 3$ mice per group). **k** Total cholesterol (TC) in liver in indicated group (one-way ANOVA, $n = 5$ mice per group). **l** Left: Liver sections were stained with oil red O in NSG mice receiving tail vein injections of EVs. Scale bars, 50 μm . Right: Quantification of the lipid droplets of the liver (one-way ANOVA, $n = 5$ biological replicates). **m** Quantification of gene expression of several markers in cholesterol synthesis pathway using qRT-PCR in NSG mice receiving indicated EVs (one-way ANOVA, $n = 6$ mice per group). **n** Immunoblot analysis of liver sample collected from mice that had received MCF-10A or MDA-MB-231-derived EVs for 5 weeks. **o** Immunoblot analysis of proteins related to cholesterol synthesis in primary hepatocytes treated with indicated EVs, with PBS as control. Data are presented as mean $\pm$ s.d. Source data and exact P value are provided as a Source data file.

expression levels in liver and serum, respectively, from 231/Ctrl mice than the relative control groups (Fig. 2c). In the liver of mice receiving MDA-MB-231 EV, there was a fourfold miR-9-5p higher abundance than those mice receiving either PBS or MCF-10A EV (Fig. 2d). Further in situ hybridization staining evidenced more miR-9-5p expression in the liver from 231/Ctrl mice (Fig. 2e). To examine the biological role of miR-9-5p in EVs, we thereby constructed miR-9-5p overexpressed MCF-10A cells (MCF-10A/miR-9) and demonstrated 15-fold and sixfold of miR-9-5p

expression higher within intracellular and EV fraction from MCF-10A/miR-9-5p cells than MCF-10A cells (Supplementary Fig. 2b). Intriguingly, interrogation of sequences near the 3'UTR of human and mouse *INSIG* genes identified there was each miR-9-5p binding site for human *Insig1* and *Insig2* while two miR-9-5p binding sites for mouse *insig1* and *insig2*, respectively (Fig. 2f). Luciferase reporter assays comparing miR-9-5p responsiveness of wild-type and miR-9-site-mutated constructs confirmed direct targeting of *INSIGs* by miR-9-5p through the

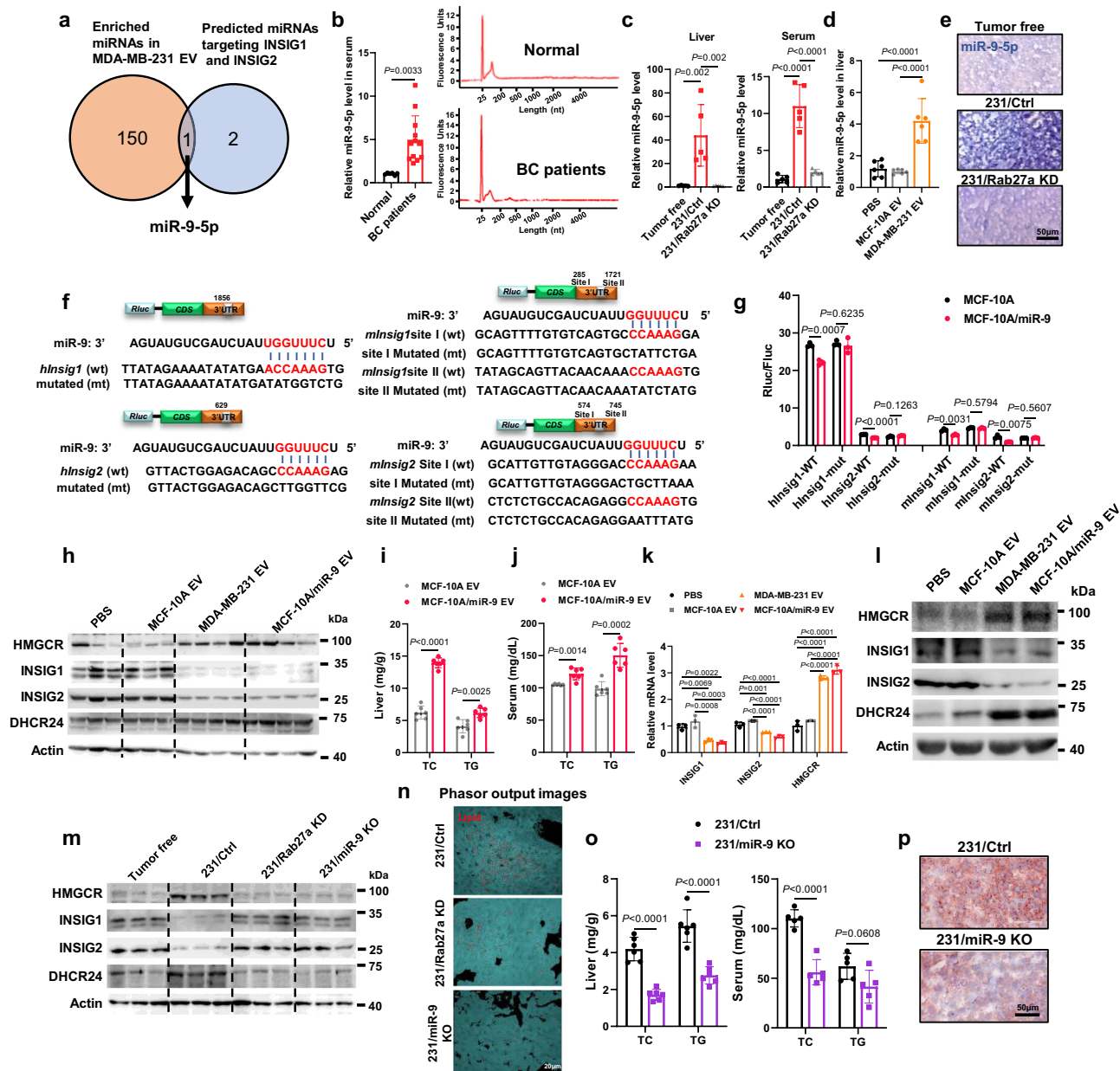


Fig. 2 | Exosomal miR-9-5p induced cholesterol biosynthesis by targeting INSIG1/2. **a** The enriched miRNAs from MDA-MB-231 EVs versus MCF-10A EVs overlapping with miRNA pools targeting both *Insig1* and *Insig2* from TargetsScan database in cholesterol pathway. **b** Left: qRT-PCR-determined miR-9-5p levels in an equal amount of EVs derived from serum of normal (n=6) and breast cancer patients (n=12) (unpaired two-tailed *t* test). Right: The RNA quality of serum EVs was demonstrated. **c** RT-qPCR-determined miR-9-5p levels in liver (n=5) and serum (n=5) in indicated tumor-bearing or tumor-free mice (one-way ANOVA). **d** RT-qPCR-determined miR-9-5p levels in liver from mice receiving tail vein injections of indicated EVs (one-way ANOVA, n=6 mice per group). **e** In situ hybridization for miR-9-5p in liver of mice from tumor free, 231/Ctrl and 231/Rab27a KD. Scale bar, 50 μ m. **f** Predicted miR-9-5p binding sites in human and mouse *Insigs* genes (*Insig1* and *Insig2*, respectively). The corresponding sequences in WT and mutated reporters are shown. **g** Luciferase reporter assay showing responsiveness of the reporters to transfected human and mouse *Insig1* or *Insig2* in MCF-10A and MCF-

10A/miR-9 cells (unpaired two-tailed *t* test, n=3 independent batches). **h** Immunoblot analysis of liver sample collected from indicated mice received EVs injection. **i, j** Total cholesterol (TC) and triglycerides (TG) level in liver and serum in indicated mice received EVs (unpaired two-tailed *t* test, n=6 mice per group). **k** Quantification of gene expression of HL7702 cell after EVs/PBS treatment for 48 h using qRT-PCR (one-way ANOVA, n=3 independent batches). **l** Immunoblot analysis of liver sample collected from indicated tumor bearing mice. **m** According to the gates in phasor plot, LD region was segmented in livers of indicated groups. Scale bars, 20 μ m. **n** Total cholesterol (TC) and triglycerides (TG) level in liver (n=6) and serum (n=5) in indicated tumor bearing mice (unpaired two-tailed *t* test). **o** Liver sections were stained with oil red O in indicated groups. Scale bars, 50 μ m. Data are presented as mean $\pm$ s.d. Source data and exact *P* value are provided as a Source data file.

upstream site in both human and mice (Fig. 2g). Consistently, remarkable reductions of *INSIG1* or *INSIG2* levels were observed in intracellular MCF-10A/miR-9-5p cells versus MCF-10A cells (Supplementary Fig. 2c), further proving miR-9-5p could target both *INSIG1* and *INSIG2*.

To investigate the in vivo effect of circulating miR-9-5p on cholesterol metabolism in the liver, we performed miR-9-5p EV injection through tail vein after a series of characterizations. Electron microscopy demonstrated MCF-10A EV, MDA-MB-231 EV and MCF-10A/miR-9-5p EV with classic exosome morphology and under 200 nm diameter

(Supplementary Fig. 2d). Further nanoflow cytometry showed MCF-10A/miR-9 EV with GFP and CD63 expression on the EV membrane (Supplementary Fig. 2e). Additional density ultracentrifugation confirmed the classical exosome distribution of MCF-10A/miR-9 EV (Supplementary Fig. 2f, g). Mice receiving MCF-10A/miR-9 EVs and MDA-MB-231 EVs (EVs loading high amount of miR-9-5p) showed more abundance of HMGCR but lower INSIG1 and INSIG2 expression in the liver (Fig. 2h). Similarly, there was increased TC and total glyceride (TG) in both liver and sera of mice receiving high miR-9-5p carrying EVs (Fig. 2i, j). Oil Red O staining and quantification further supported more lipids accumulation in the liver of mice receiving high miR-9-5p carrying EVs (Supplementary Fig. 2h). Suppressed INSIG1 and INSIG2 expression were observed in the liver from mice receiving MCF-10A/miR-9 EVs (Supplementary Fig. 2i). Moreover, in vitro cultured primary hepatocytes incubated with MDA-MB-231 EVs or MCF-10A/miR-9 EVs also showed increased HMGCR and DHCR24 expression but lower INSIG1 and INSIG2 levels than PBS or MCF-10A EV treated cells (Fig. 2k, l). To further confirm the effect of miR-9-5p on the regulation of cholesterol synthesis, we knocked out 2 of 3 copies of the miR-9-5p gene in MDA-MB-231 cells (231/miR-9 KO) and 4T1 cell (4T1/miR-9 KO) by employing CRISPR-Cas9 technology (Supplementary Fig. 2j). A series of validation confirmed ~80–90% reduced miR-9-5p abundance in intracellular and EV fractions of 231/miR-9 KO cells and 4T1/miR-9 KO cells (Supplementary Fig. 2k, l). We next xenografted both 231/miR-9 KO cells and 4T1/miR-9 KO cells into mice to validate cholesterol related gene expression in 231/miR-9 KO mice and 4T1/miR-9 KO mice, respectively. There was less HMGCR expression in the liver of the 231/miR-9 KO group compared with 231/Ctrl mice (Fig. 2m). Raman imaging also demonstrated lipids were highly enriched in the liver of 231/Ctrl mice rather than either 231/miR-9 KO or 231/Rab27a KD mice (Fig. 2n and Supplementary Fig. 2m). Consistently, mice xenografted with 231/miR-9 KO cells showed decreased total cholesterol (TC) concentration in both liver and serum, along with decreased TG abundance in liver rather than sera (Fig. 2o). Indeed, oil red O (ORO) staining further strengthened the lipid accumulation was blocked in the liver from 231/miR-9 KO mice when compared to 231/Ctrl mice (Fig. 2p). However, there was no noticeable change in body weight detected between 231/Ctrl mice and 231/miR-9 KO mice (Supplementary Fig. 2n). In summary, our results demonstrated BC-derived miR-9-5p induced HMGCR expression to promote cholesterol synthesis by targeting both INSIG1 and INSIG2 in liver tissue.

Circulating miR-9-5p promoted cholesterol to 25-HC conversion by targeting ATF3

Cholesterol is converted into bile acids in the liver through oxysterols²⁵. GSEA enrichment analysis also highlighted the upregulation of oxysterol derived from cholesterol pathway in the liver from mice receiving MDA-MB-231 EV in comparison with MCF-10A EV (Supplementary Fig. 3a). Intriguingly, the same pathway was highly enriched in the liver from 231/Ctrl mice when compared to either 231/miR-9 KO or 231/Rab27a KD mice (Fig. 3a and Supplementary Fig. 3b), suggesting circulating miR-9-5p involved in oxysterol production from cholesterol. Considering cholesterol could be converted into several kinds of oxysterols including 25-hydroxycholesterol (25-HC), 22(R)-HC, 24S-HC, 27-HC, 7 β -HC, 7 α -HC, and 4 β -HC (Fig. 3b), we performed targeted LC-MS/MS for the liver of mice from both tumor xenograft model and EV injected mice model. We found 25-HC was the only oxysterol enriched in the liver from both 231/Ctrl mice and high miR-9-5p EV-educated mice than their respective control groups (Fig. 3c, d). Elevated 25-HC abundance was also detected in lung from 231/Ctrl mice than 231/miR-9 KO mice (Supplementary Fig. 3c). Subsequent qRT-PCR quantification further validated Ch25h, an enzyme converting cholesterol into 25-HC, was highly induced in the liver of 231/Ctrl mice and mice receiving high miR-9-5p EV than their corresponding control groups in both NSG and BALB/c mice (Figs. 3e, f,

Supplementary Fig. 3d). Additionally, we also measured other enzymes catalyzing oxysterol conversion, while no significant changes for either Cyp11a1 or Cyp46a1 gene expressions between liver from corresponding mouse models were detected (Supplementary Fig. 3e, f). These results demonstrated cholesterol was mainly converted into 25-HC in liver under the context of tumor.

To explore the regulation mechanism between miR-9-5p and CH25H, we searched again for all the potential targets for miR-9-5p (see Supplementary Table 2). Both activating transcription factor 1 (ATF1) and ATF2 were predicted by the “Targetscan” database. Considering ATF3 had been shown to suppress CH25H expression in macrophages³¹, miR-9-5p overexpression significantly suppressed ATF3 expression rather than ATF1 or ATF2 genes (Supplementary Fig. 3g). Interrogation of sequences near the 3'UTR of human and mouse ATF3 genes identified three miR-9-5p binding sites in humans but only one site in mice (Fig. 3g). Luciferase reporter assays comparing miR-9-5p responsiveness of wild-type and miR-9-5p site-mutated constructs confirmed direct targeting of ATF3 by miR-9-5p through the upstream sites in human (Site 1) and mice (Fig. 3h). Transfection of primary hepatocytes with miR-9-5p mimic significantly suppressed ATF3 expression rather than ATF1 or ATF2, leading to increasing expression of CH25H (Fig. 3i, j). There was suppressed ATF3 expression but significant upregulation of CH25H in primary hepatocytes incubation with MCF-10A/miR-9 EVs and MDA-MB-231 EVs (high miR-9-5p containing EVs) (Fig. 3k, l). Besides, higher CH25H but lower ATF3 protein abundance was observed in the liver of 231/Ctrl mice (Fig. 3m, n) and mice receiving high miR-9-5p containing EV than their respective control groups (Fig. 3o, p). Consistently, inverse RNA levels of *Atf3* were detected in the liver of 231/Ctrl mice and mice receiving high miR-9-5p containing EV than their respective control groups (Supplementary Fig. 3h). Conclusively, the above findings showed BC-derived exosomal miR-9-5p induced CH25H expression to promote cholesterol conversion into 25-HC by targeting ATF3.

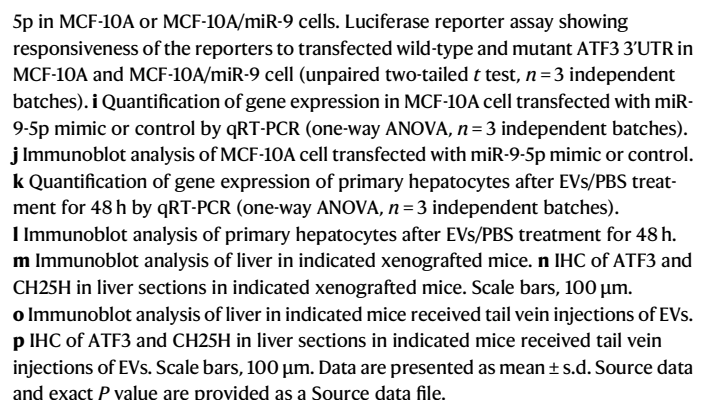
Oncometabolite 25-HC promoted BC liver and lung metastasis

We next investigated the biological effect of miR-9-5p-mediated 25-HC accumulation on tumor progression in vivo. Mice xenografted 231/miR-9 KO cells displayed similar tumor growth rates with 231/Ctrl mice (Fig. 4a), suggesting the energy fuel-independent effect of 25-HC induced by circulating miR-9-5p in vivo. Likewise, miR-9-5p knockout showed a mild impact on the proliferation of MDA-MB-231 cells in vitro (Supplementary Fig. 4a). However, we observed 231/miR-9 KO mice displayed less tumor liver and lung metastasis than 231/Ctrl mice (Fig. 4b, c, Supplementary Fig. 4b). Additionally, there was less Ki67 (proliferation marker of cancer cell) expression in the liver of 231/miR-9 KO mice and 231/Rab27a KD mice, indicating the connection between miR-9-5p secretion and BC metastasis (Fig. 4d, e, Supplementary Fig. 4c). To further investigate the role of 25-HC in vivo, we intraperitoneally injected exogenous 25-HC into 231/Ctrl and 231/miR-9 KO mice (Fig. 4f). We found 25-HC had no obvious effect on tumor growth rate (Supplementary Fig. 4d). Indeed, 25-HC significantly enhanced liver metastasis (Fig. 4g, h), accompanied by more tumor metastatic nodules in the liver (Fig. 4i). The levels of 25-HC in 231/Ctrl mice with 25-HC supplementary was much higher than the other groups (Fig. 4j). Meanwhile, Ki67 expression was further enhanced in 25-HC treated liver tissue (Supplementary Fig. 4e, f). Besides, we found 25-HC promoted both migration and invasion of MDA-MB-231 cells in vitro (Supplementary Fig. 4g, h). Correspondingly, the wound healing assay also indicated 25-HC promoted the migration of cancer cells, simultaneously (Supplementary Fig. 4i, j).

Ch25h blockage inhibited BC liver and lung metastasis

To further confirm the metastasis-related function of 25-HC in vivo, we knocked down the CH25H expression by injection of AAV8 virus encoding shRNA against CH25H mRNA through the tail vein. There was

genetic depletion of CH25H, we also employed Ch25h liver-conditional knockout mice (*Ch25h^{L-/-}*) and validated the decreased plasma 25-HC level in *Ch25h^{L-/-}* mice than control mice (Fig. 5a). The TC/TG levels and lipids accumulation were also decreased in *Ch25h^{L-/-}* mice serum and



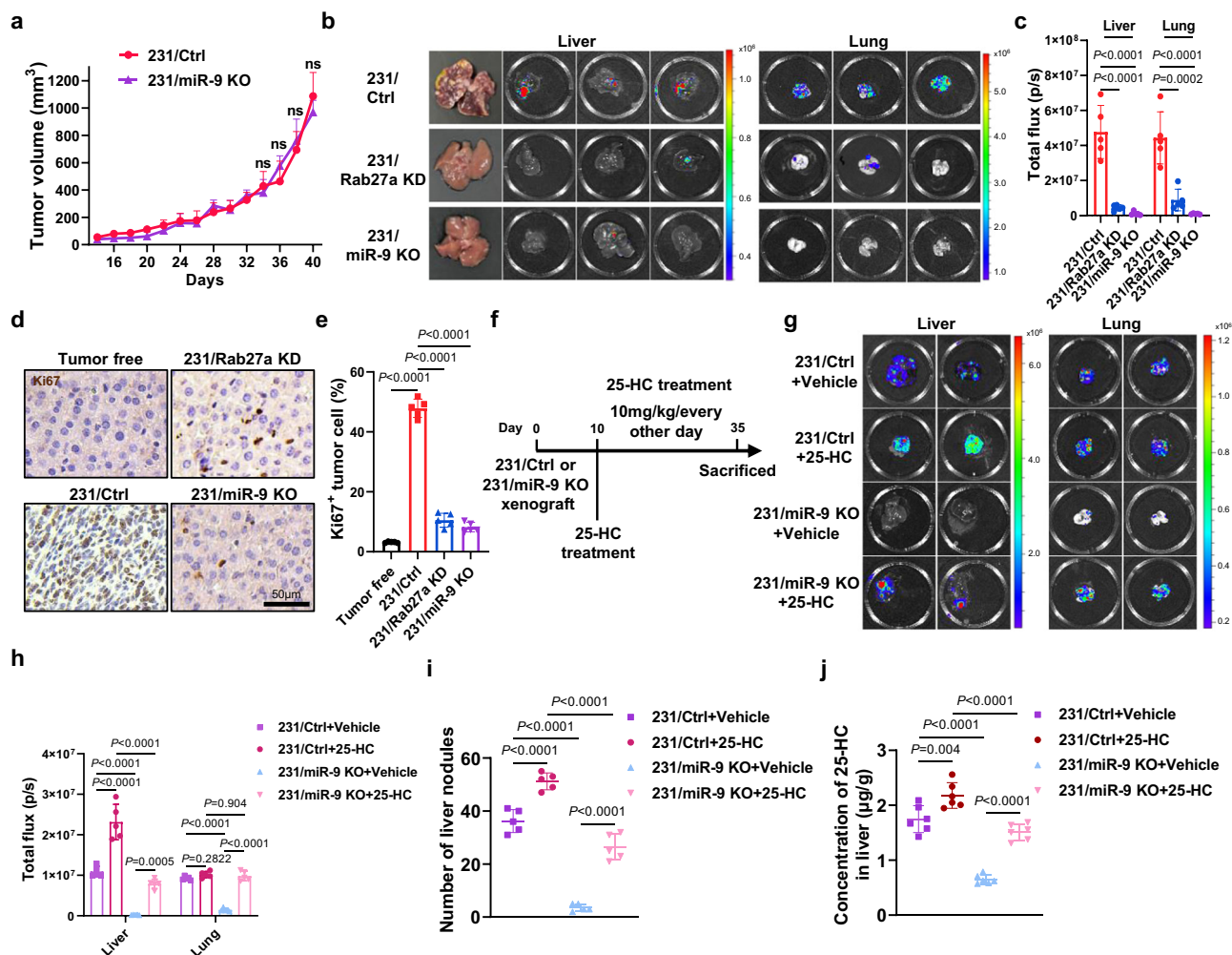


Fig. 4 | Oncometabolite 25-HC promoted breast cancer liver and lung metastasis. **a** Tumor volume followed in indicated xenografted mice (two-way ANOVA, $n = 5$ mice per group). **b, c** Representative bioluminescence images and quantification of mice inoculated with indicated group (one-way ANOVA, $n = 5$ mice per group). **d, e** Representative IHC images showing Ki67 staining and the overall percentage of Ki67⁺ tumor cells (one-way ANOVA, $n = 5$ mice per group). Scale bar, 50 μm . **f** Schematic diagram of 25-HC treated mouse model. 231/Ctrl or 231/miR-9 KO cells injected at day 0. On day 10, mice were treated with vehicle or 25-HC

(10 mg/kg) every other day. At day 35, tissue was harvested, imaged and fluorescence quantified. Experimental timeline outlined above quantified data.

g, h Representative bioluminescence images and quantification of mice inoculated with indicated groups (one-way ANOVA, $n = 5$ mice per group). **i** Quantification of liver nodules (one-way ANOVA, $n = 5$ mice per group). **j** 25-HC levels of livers in each group by ELISA kit. (one-way ANOVA, $n = 6$ mice per group). Data are presented as mean $\pm$ s.d. Source data and exact P value are provided as a Source data file.

liver (Fig. 5b, c). Positron Emission Tomography-Computed Tomography (PET-CT) is particularly useful for tumor imaging. The results of PET-CT mainly compared the metastases of liver and lung under different treatments, which mainly proved the metastases, just like the previous bioluminescence imaging and Ki67 staining. PET-CT confirmed less metastasis in both the liver and lung of *Ch25h*^{-/-} mice (Fig. 5d). Moreover, bioluminescence imaging data showed there were less metastasis sites in both liver and lung from 4T1/shCH25H group (Fig. 5e). We also found reduced number of Ki67-positive cells (Fig. 5f and Supplementary Fig. 5c), along with decreased metastatic nodules in the liver and lung from 231/shCH25H mice (Supplementary Fig. 5d). To further blocked the activity of CH25H, we treated mice with desmosterol, a potent inhibitor of CH25H as a previous report^{32,33} and observed the same reduced expression of CH25H on metastasis (Fig. 5g). Likewise, bioluminescence imaging data showed there were fewer metastasis sites in both liver and lung from 4T1/desmosterol group (Fig. 5h). Similar effects were further confirmed by Ki67 staining (Fig. 5i). PET-CT also confirmed less metastasis in both the liver and lung of mice treated with desmosterol than the vehicle group (Fig. 5j). The toxicity of desmosterol to hepatocytes was weak (Supplementary

Fig. 5e). Compared with the 4T1 control group, mice injected with desmosterol showed no effect on tumor growth rate but successfully maintained body weight especially at a later stage (Supplementary Fig. 5f, g). Besides, desmosterol suppressed both CH25H expression and the proliferation of hepatocytes in vitro (Supplementary Fig. 5h, i). Collectively, 25-HC promoted both liver and lung metastasis while either miR-9-5p knockout or blocking CH25H expression totally abolished the effect of 25-HC on tumor metastasis.

25-HC induced Kupffer cell polarization into M2 type by elevating MAFK level to promote liver metastasis

Emerging evidence highlights the fundamental role of immune determinants in the pre-metastatic niche formation³⁴, and our gene enrichment analysis for transcriptome profiling demonstrated that both “macrophage M1 versus M2 down” and “tolerant macrophage up” pathways were upregulated in 25-HC treated macrophages when compared with Vehicle treatment (Fig. 6a). To verify whether liver macrophage-mediated tumor metastasis in the context of the dysregulated cholesterol metabolism, we injected mice with clodronate liposomes to deplete macrophages and accordingly, the macrophage

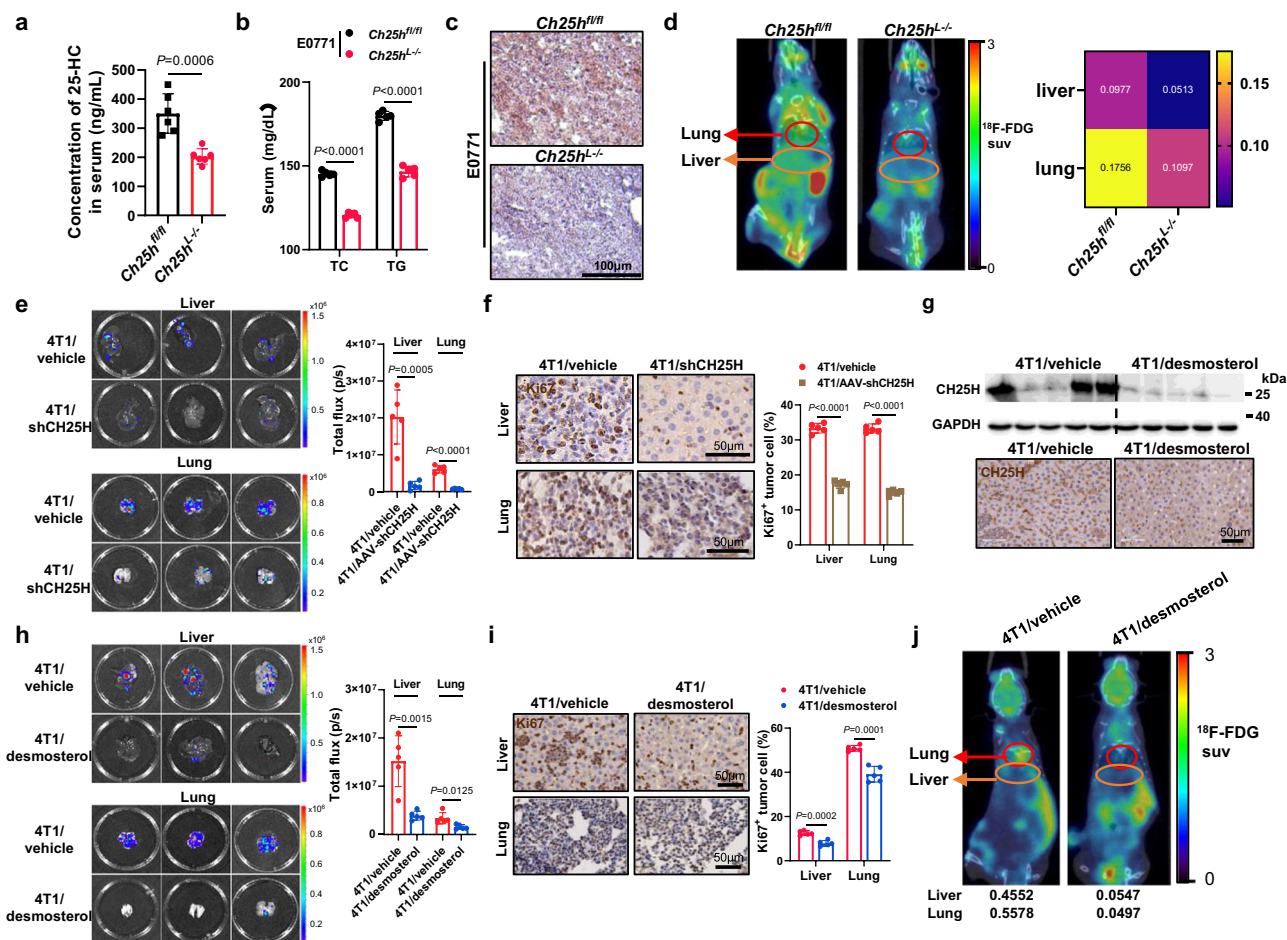


Fig. 5 | Ch25h blockage inhibited BC liver and lung metastasis. a LC-MS/MS analysis of 25-HC in serum in indicated CH25H liver conditional knock out (*Ch25h^{-/-}*) and control (*Ch25h^{fl/fl}*) mice (unpaired two-tailed *t* test, *n* = 6 mice per group). **b** Total cholesterol (TC) and triglyceride (TG) levels were examined from CH25H liver conditional knock out (*Ch25h^{-/-}*) and control (*Ch25h^{fl/fl}*) mice. (unpaired two-tailed *t* test, *n* = 5 mice per group). **c** Liver sections were stained with oil red O in *Ch25h^{-/-}* and *Ch25h^{fl/fl}* mice with E0771 xenograft. Scale bars, 100 μ m. **d** Representative PET/CT imaging with ¹⁸F-FDG for accessing liver and lung uptake and quantification in indicated CH25H liver specific KO and WT mice. **e** Representative bioluminescence images of metastasis site in liver and lung and quantification of mice inoculated with indicated groups (unpaired two-tailed *t* test, *n* = 5 mice per group). **f** Representative

IHC images showing Ki67 staining and the overall percentage of Ki67⁺ tumor cells in indicated groups (unpaired two-tailed *t* test, *n* = 5 per group). Scale bar, 50 μ m. **g** Immunoblot analysis of CH25H in liver in indicated groups (top), IHC and of CH25H in liver sections in indicated groups (bottom). Scale bar, 50 μ m. **h** Representative bioluminescence images of metastasis site in liver and lung and quantification of mice inoculated with indicated groups (unpaired two-tailed *t* test, *n* = 5 per group). **i** Representative IHC images showing Ki67 staining and the overall percentage of Ki67⁺ tumor cells of liver and lung from indicated groups (unpaired two-tailed *t* test, *n* = 5 per group). Scale bar, 50 μ m. **j** Representative PET/CT imaging with ¹⁸F-FDG for accessing liver and lung uptake and quantification in indicated mice. Data are presented as mean $\pm$ s.d. Source data and exact *P* value are provided as a Source data file.

population was largely diminished upon clodronate treatment (4T1/clodronate) when compared to the control treatment (4T1/vehicle) in the liver of tumor bearing mice (Fig. 6b). Indeed, we detected lower F4/80 abundance in both lung and liver tissue upon clodronate treatment (Fig. 6c). Intriguingly, both bioluminescence imaging and immunohistology data demonstrated sharp decrease of tumor metastasis, as well as the reduction of Ki67 in both liver and lung tissue from 4T1/clodronate mice when compared to 4T1/vehicle mice (Fig. 6d, e). Considering Kupffer cells as the major immune cell type in the liver, we subsequently determined whether 25-HC induced liver metastasis by regulation of macrophage polarization. Then, we isolated Kupffer cells from the liver and performed flow cytometry to analyze the polarization signature. We found there was much lower CD163 abundance in Kupffer cells residing in the liver of 4T1/miR-9 KO mice rather than 4T1/Ctrl mice, while 25-HC treatment significantly induced CD163 level, along with the elevation of M2/M1 ratio, indicating M2 macrophage polarization attributed to 25-HC treatment in Kupffer cells (Fig. 6f, g). Beyond, 25-HC treatment also induced other M2 genes including *Arg1* and *Mrc1* expression in both lung and liver tissues of 231/miR-9-5p KO

mice, suggesting the contribution of 25-HC on genetic induction of M2 polarization of macrophages (Fig. 6h and Supplementary Fig. 6a).

Mechanistically, although “Cistrome Data Browser” predicted MAFK, FOXA1 and RAD21 to activate the transcription of both *Arg1* and *Mrc1* genes (see Supplementary Table 3), only MAFK was screened out from the enriched tolerant macrophage pathway (Fig. 6i). We found both RNA and protein expression of MAFK were induced by supplementary 25-HC in the liver of 231/miR-9 KO mice (Fig. 6j and Supplementary Fig. 6b). Moreover, MAFK was induced by 25-HC with a dose-dependent manner (Fig. 6k). We detected significantly reduced MAFK protein expression when we knocked down *Mafr* expression with small interfering RNAs in BMDM cells (Supplementary Fig. 6c, d). Accordingly, *Arg1* or *Mrc1* were remarkably inhibited due to the MAFK reduction (Supplementary Fig. 6e). M2 macrophages produced growth factors, extracellular matrix-remodeling molecules, and cytokines to regulate cancer migration and metastasis³⁵. Indeed, when we treated RAW 264.7 cells with 25-HC and analyzed the levels of chemokines using a mouse chemokine screening test kit and observed a significant elevation of C-X-C motif chemokine ligand 2 (CXCL2), CXCL10 and

CXCL16 secretion into the medium (Fig. 6l). 25-HC also induced the secretion of anti-inflammatory cytokines IL-10 and TGF- β (Fig. 6m). Of note, we also detected the induction of M2 polarization in alveolar macrophages in tumor-bearing mice (231/Ctrl) when compared to

tumor free mice (Supplementary Fig. 6f). However, we failed to detect any alteration of key enzymes of cholesterol metabolism in lung tissue, suggesting the possibility of uptake of 25-HC from serum by lung tissue from tumor bearing mice (Supplementary Fig. 6g, h). Collectively,

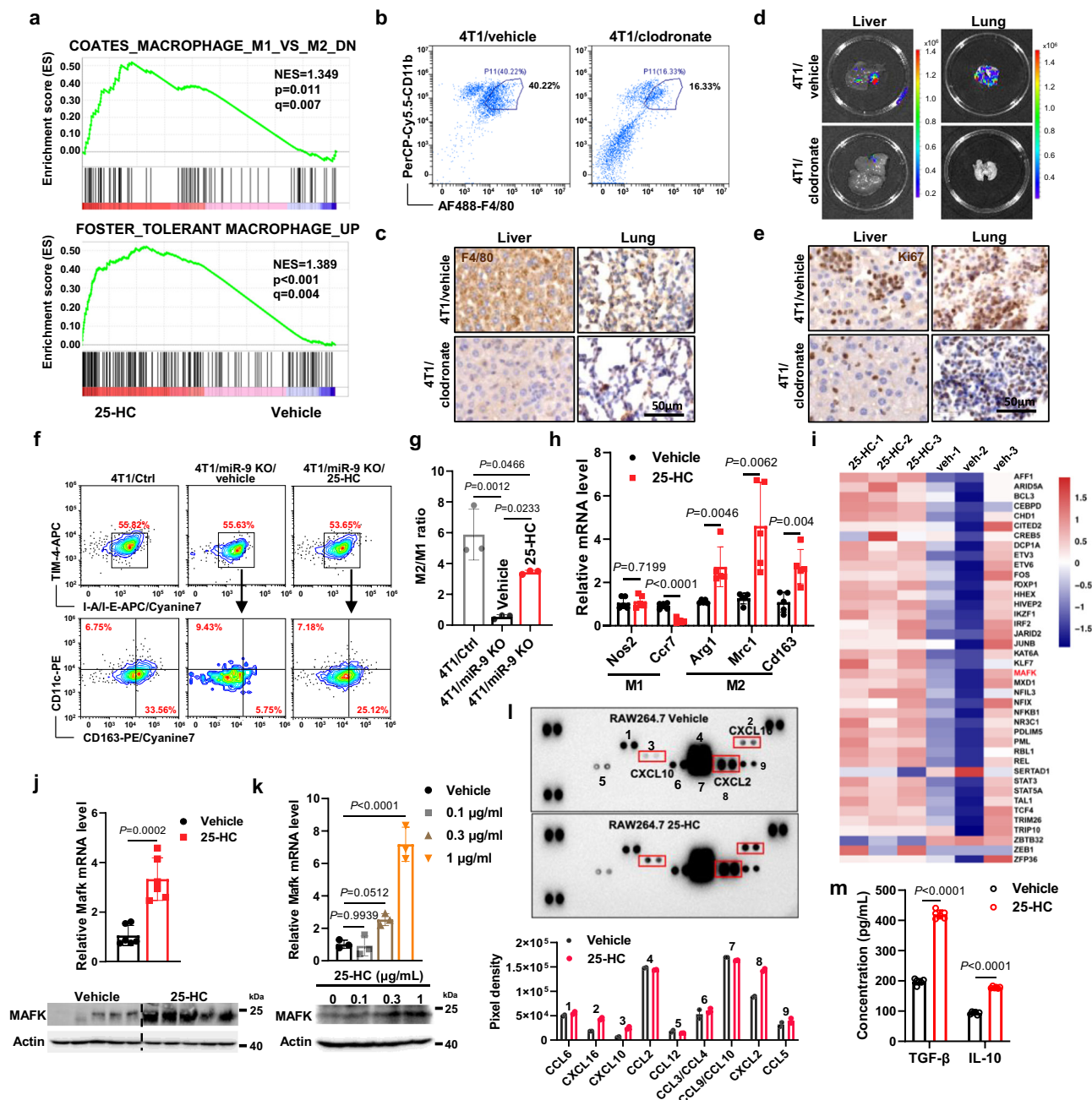


Fig. 6 | 25-HC induced Kupffer cells polarization into M2 type to promote liver metastasis. **a** RAW264.7 cells treated with 25-HC and vehicle were subjected to RNA-seq and GSEA, showing enrichment of genes set related to macrophage pathway (Permutation test, $n = 3$ independent batches per group). **b** Quantification of purified Kupffer cells with CD11b⁺ and F4/80⁺ expression by flow cytometry in indicated groups. **c** IHC of F4/80 in liver and lung sections in indicated groups. Scale bar, 50 μ m. **d** Representative bioluminescence images of liver and lung metastasis sites in indicated groups. **e** IHC of Ki67 in liver and lung sections in indicated groups. Scale bar, 50 μ m. **f** Purified Kupffer cells were selected with TIM4⁺, I-A/I-E^{int} expression and stained and further analyzed for CD163 and CD11c expression. Representative flow cytometry results were shown. **g** Quantification of the M2/M1 ratio in flow cytometry results (one-way ANOVA, $n = 3$ independent batches). **h** RNA level in liver tissue of mice from indicated groups by qRT-PCR (unpaired two-tailed t test, $n = 5$ mice per group). **i** Heatmap showing the relative

levels of selected genes related to tolerant macrophage pathway based on the RNA-seq data. **j** Relative mRNA level of Mafk in liver from indicated mice (unpaired two-tailed t test, $n = 6$ mice per group) (top); immunoblot analysis of liver in indicated mice (bottom). **k** The qRT-PCR of Mafk in the RAW264.7 cells after 25-HC treatment with a dose dependent for 48 h (unpaired two-tailed t test, $n = 3$ independent batches) (top); immunoblot analysis of Raw264.7 cells after 25-HC treatment with a dose dependent for 48 h (bottom). **l** Different expression and quantification of various chemokines in media derived from 25-HC treated or control-RAW264.7 cells was detected by a mouse chemokine array kit ($n = 2$ independent batches). Red boxes designate the chemokines whose expression was altered by 25-HC treatment. **m** TGF- β and IL-10 secreted from BMDM cells treated with vehicle and 25-HC were detected by ELISA kit (unpaired two-tailed t test, $n = 6$ independent batches). Data are presented as mean $\pm$ s.d. Source data and exact P value are provided as a Source data file.

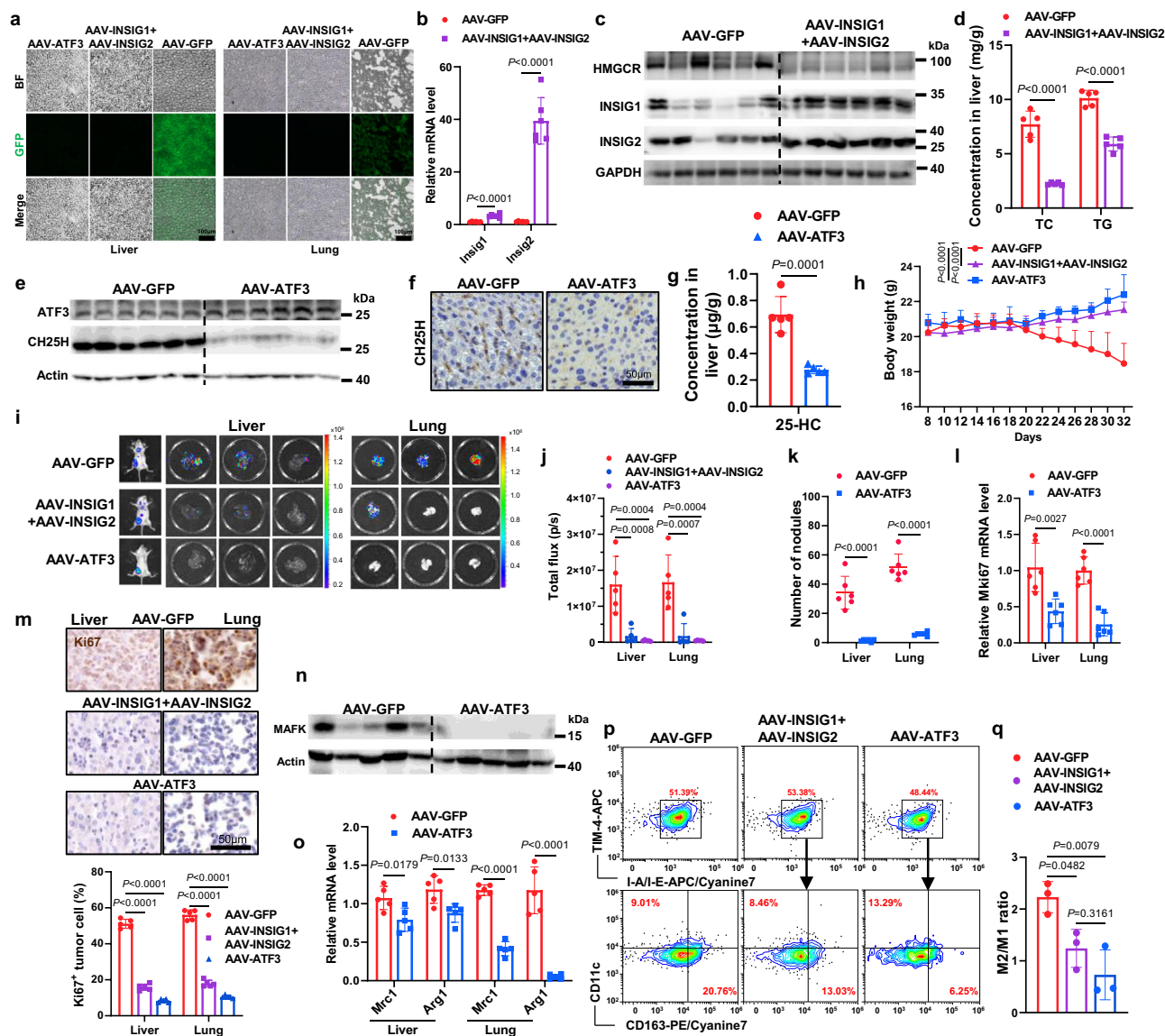


Fig. 7 | Exogenous INSIGs and ATF3 blocked miR-9-5p-induced tumor metastasis. **a** GFP signals in liver and lung from mice receiving tail vein injections of AAV-GFP, AAV-ATF3 and AAV-INSIG1 + AAV-INSIG2 virus. Scale bar, 100 μ m. **b** Quantification of gene expression in liver from mice receiving tail vein injections of AAV-GFP, AAV-INSIG1 and AAV-INSIG2 virus. (unpaired two-tailed *t* test, *n* = 6 mice per group). **c** Western blots showing the expression of key proteins related to cholesterol synthesis in liver from mice receiving tail vein injections of AAV-GFP, AAV-INSIG1 and AAV-INSIG2 virus. **d** Total cholesterol (TC) and triglycerides (TG) in liver in indicated mice (unpaired two-tailed *t* test, *n* = 5 mice per group). **e** Western blots showing protein expression in liver from mice receiving tail vein injections of AAV-GFP and AAV-ATF3 virus. **f** Representative IHC images showing CH25H staining. Scale bar, 50 μ m. **g** LC-MS/MS analysis of 25-HC level in liver in indicated groups (unpaired two-tailed *t* test, *n* = 5 mice per group). **h** Body weight was monitored (two-way ANOVA, *n* = 6 mice per group). **i, j** Representative bioluminescence

images and quantification of mice inoculated with indicated groups (one-way ANOVA, *n* = 5 mice per group). **k** Quantification of liver and lung metastatic nodules (unpaired two-tailed *t* test, *n* = 6 mice per group). **l** RNA level of Mki67 in liver and lung in indicated mice (unpaired two-tailed *t* test, *n* = 6 mice per group). **m** Representative IHC images showing Ki67 staining and the overall percentage of Ki67⁺ tumor cells (one-way ANOVA, *n* = 5 per group). Scale bar, 50 μ m. **n** Western blots showing proteins in liver from mice receiving tail vein injections of AAV-GFP and AAV-ATF3 virus. **o** RNA level of M1 and M2 marker gene in liver and lung in indicated mice (unpaired two-tailed *t* test, *n* = 6 mice per group). **p** Purified Kupffer cells were selected with TIM4⁺, I-A/I-E^{int} expression and stained and further analyzed for CD163 and CD11c expression. Representative flow cytometry results were shown. **q** Quantification of the M2/M1 ratio in flow cytometry results (one-way ANOVA, *n* = 3 independent batches). Data are presented as mean $\pm$ s.d. Source data and exact *P* value are provided as a Source data file.

all the above results showed that 25-HC induced the polarization of macrophage into M2 type to promote both lung and liver metastasis.

Exogenous expression of INSIGs and ATF3 blocked miR-9-induced tumor metastasis

We next overexpressed the three targets of miR-9-5p individually or with combination mode following transduction with adeno-associated virus 8 (AAV8) expressing mouse INSIG1, INSIG2 and ATF3 after tumor implantation, with AAV-GFP serving as a control upon additional verification in vitro (Supplementary Fig. 7a). As we

replaced the GFP coding sequences with the ATF3 or INSIG1/2 coding sequences on the backbone of AAV plasmid, the green fluorescence signals were hardly detectable in AAV-ATF3 injected mice (Fig. 7a). GFP signals were mainly detected in the liver but with little signal in lung and kidneys from AAV-GFP-injected mice (Supplementary Fig. 7b). Both RNA and protein levels of INSIG1 and INSIG2 were highly expressed in the liver (Fig. 7b, c), accompanied by a significant reduction of TC and TG levels in the mice liver with exogenous expression of INSIG1 and INSIG2 (Fig. 7d and Supplementary Fig. 7c).

Meanwhile, AAV-ATF3 restoration effectively inhibited CH25H in the liver from tumor-bearing mice (Supplementary Fig. 7d). Exogenous expression of ATF3 suppressed the protein expression of CH25H (Fig. 7e, f), confirming the central regulatory role of the ATF3/CH25H axis in cholesterol homeostasis. However, there was not too much change for TC and TG concentration in serum and liver under ATF3 overexpression except ~1.5-fold increase of TG accumulation in the liver (Supplementary Fig. 7e, f). 25-HC level was also decreased along with ATF3 overexpression (Fig. 7g). Collectively, we measured the body weight (BW) from mice with INSIG1, INSIG2 and ATF3 overexpression, and observed restored BW but no altered effect on tumor growth rate in AAV-INSIG1 + AAV-INSIG2 or AAV-ATF3 mice than AAV-GFP control mice (Fig. 7h and Supplementary Fig. 7g). Those results further indicated the effect of miR-9-5p mildly contributed to primary tumor growth. Bioluminescence imaging data showed both liver and lung metastasis were blocked with exogenous those tri-targets (Fig. 7i, j). Consistently, there were fewer metastatic sites in both organs from mice treated with the AAV-ATF3 virus (Fig. 7k). Less Ki67 positive cells with high Ki67 expression were detected in both liver and lung from AAV-INSIG1 + AAV-INSIG2 and AAV-ATF3 mice (Fig. 7l, m). Moreover, ATF3 overexpression successfully reduced MAFK abundance in the liver of mice receiving AAV-ATF3 virus (Fig. 7n). Additionally, decreased expression of M2 markers including Arg1, Mrc1 and CD163 were detected in the livers of those mice as well (Fig. 7o, p), along with a reduced M2/M1 ratio (Fig. 7q). Either ATF3 overexpression or INSIG1 or INSIG2 overexpression showed no interference with other targets' expression (Supplementary Fig. 7h, i). Altogether, by employing a gain-of-function approach to express the three targets exogenously, we demonstrate a significantly restored regulation of cholesterol homeostasis mediated by miR-9-5p.

Silencing of miR-9-5p attenuated 25-HC-mediated tumor metastasis

To verify to what degree the primary tumor regulated the metastasis through circulating miR-9-5p, we injected miR-9-5p antagomir into the tail vein of both human (MDA-MB-231) and murine (4T1) cells xenografted mice (Fig. 8a). The miR-9-5p antagomir treatment remarkably decreased in vivo miR-9-5p levels in the serum, tumor, liver and lung tissue of mice (Fig. 8b). Besides, we failed to detect any significant alterations of either tumor size among miR-9-5p antagomir and the control group or miR-9 KO and Rab27a KD, which further confirmed that miR-9-5p displayed mere regulatory effect on tumor growth (Supplementary Fig. 8a). However, PET-CT captured significantly reduced metastasis niches in both liver and lung from the mice receiving antagomir miR-9-5p treatment (Fig. 8c). Moreover, bioluminescence imaging data further strengthened that miR-9-5p antagomir blocked the metastasis in both BC xenograft models (Fig. 8d, e, and Supplementary Fig. 8b, c). Mechanistically, miR-9-5p antagomir relieved the inhibitory expression of tri-targets (*Insig1*, *Insig2* and *Atf3*), and subsequently reduced the RNA levels of *Hmgcr* and *Ch25h* (Fig. 8f). Besides, miR-9-5p antagomir treatment resulted in a reduction of the M2 marker genes abundance but an elevation of M1 marker genes level (Fig. 8g). Simultaneously, miR-9-5p antagomir also restored the expression of ATF3 and blocked the expression of CH25H and HMGCR (Fig. 8h). The TC and TG levels in liver were also decreased by miR-9-5p antagomir (Supplementary Fig. 8d). Collectively, silencing of miR-9-5p attenuated 25-HC-mediated tumor metastasis by switching macrophage polarization, suggesting the potential therapeutic applications for targeting both circulating miR-9-5p and 25-HC biosynthesis.

Circulating miR-9-5p was correlated with BC patients with metastasis

To investigate whether the mechanism of miR-9-5p-mediated cholesterol metabolism and tumor metastasis widely exists in breast cancer,

we examined the miR-9-5p expression in BC cell lines under different subtypes. We found a relatively higher level of miR-9-5p detected in intracellular MCF-7, BT474, SK-BR-3, 4T1 and E0771 cells and their derived EV fraction than the level of intracellular and EV fraction from MCF-10A cells (Fig. 9a, Supplementary Fig. 9a). We also expanded our current mouse model to investigate the role of miR-9-5p in the mice xenografted with distinct TNBC cell line (MDA-MB-468), MDA-MB-231-Lung Metastasis-2 (231-LM2), which displayed higher capability of lung metastasis, as well as ER positive BC cell line (MCF-7). Intriguingly, we observed that miR-9-5p knockout also significantly inhibited the liver and lung metastasis, especially blocking their liver macrophage polarization into M2 type in 468/miR-9 KO mice when compared to 468/Ctrl mice (Supplementary Fig. 9b, c). However, there was no effect on tumor growth due to miR-9-5p knockout (Supplementary Fig. 9d). Similar pattern was detected in mice xenografted with 231-LM2/miR-9 KO tumor when compared to their wild type tumors (Supplementary Fig. 9e–g) as well as MCF-7/miR-9 KO tumor versus MCF-7 tumor (Supplementary Fig. 9h, i). All the above data showed miR-9-5p was generally correlated with liver metastasis of mice xenografted with distinct BC types. Beyond, more circulating miR-9-5p levels were also detected in mice xenografted with lung cancer (A549), hepatocarcinoma (Hep3B) and colon cancer (SW480) models (Fig. 9b).

In situ hybridization (ISH) demonstrated higher miR-9-5p levels in the liver tissue of BC patients with liver metastasis (Fig. 9c). BC patients with metastasis also have higher serum miR-9-5p levels than healthy individuals or patients without metastasis (Fig. 9d). Interestingly, according to the TCGA database, miR-9-5p was highly expressed in primary BC tumors than in adjacent normal tissues (Fig. 9e). Of note, miR-9-5p was highly expressed in patients with both triple-negative BC (TNBC) tumors (GSE45498) and lymph node metastatic lesion tissues (Fig. 9f). Moreover, higher miR-9-5p level was detected in patients of Stage I to Stage IV BC patients (Fig. 9g). We also examined the expression of cholesterol biosynthesis-related enzymes in BC patients with high metastasis (high Ki67 level) or low metastasis (low Ki67 level) and non-BC individuals. IHC demonstrated both HMGCR and CH25H were highly expressed but INSIG1, INSIG2 or ATF3 displayed much lower abundance in BC patients with liver metastasis when compared to non-BC or patients with low liver metastasis (Fig. 9h). Both TC and TG concentrations were significantly elevated in BC patients (Supplementary Fig. 9j). Clinically, significantly elevated plasma 25-HC levels from BC patients than in non-cancer individuals (Fig. 9i). MiR-9-5p was highly expressed in primary BC tumors than in adjacent normal tissues among different types of breast cancer (Supplementary Fig. 9k). With more aggressive liver metastasis, the expression of MRC1 and M2 marker, was also detected with high expression simultaneously (Fig. 9j and Supplementary Fig. 9l). Collectively, our finding reveals the circulating miR-9-5p functions as “tri-brakes” to regulate cholesterol homeostasis and 25-hydroxycholesterol as an oncometabolite to induce BC metastasis by regulating Kupffer cell polarization in the liver (Fig. 9k).

Discussion

Here, we identify the biological function of circulating miR-9-5p encapsulated by EVs and 25-hydroxycholesterol as oncometabolite in liver and lung metastasis of BC. Functioning as “tri-brakes”, miR-9-5p is endowed with three key targets in this study. The first two targets are insulin-induced genes including *Insig-1* and *Insig-2*, widely involved in cholesterol metabolism, lipogenesis, and glucose homeostasis. Following the third target is *Atf3*, functioning as the repressive transcription factor of CH25H expression. A recent report further supports the fundamental role of the ATF3-CH25H axis in cancer, primarily working on the attenuation of anti-tumor immunity, stimulation of tumor growth, and impeding the efficacy of chimeric antigen receptor (CAR) T cell adoptive therapy³⁶. Therefore, miR-9-5p partially erases the inhibitory regulation of

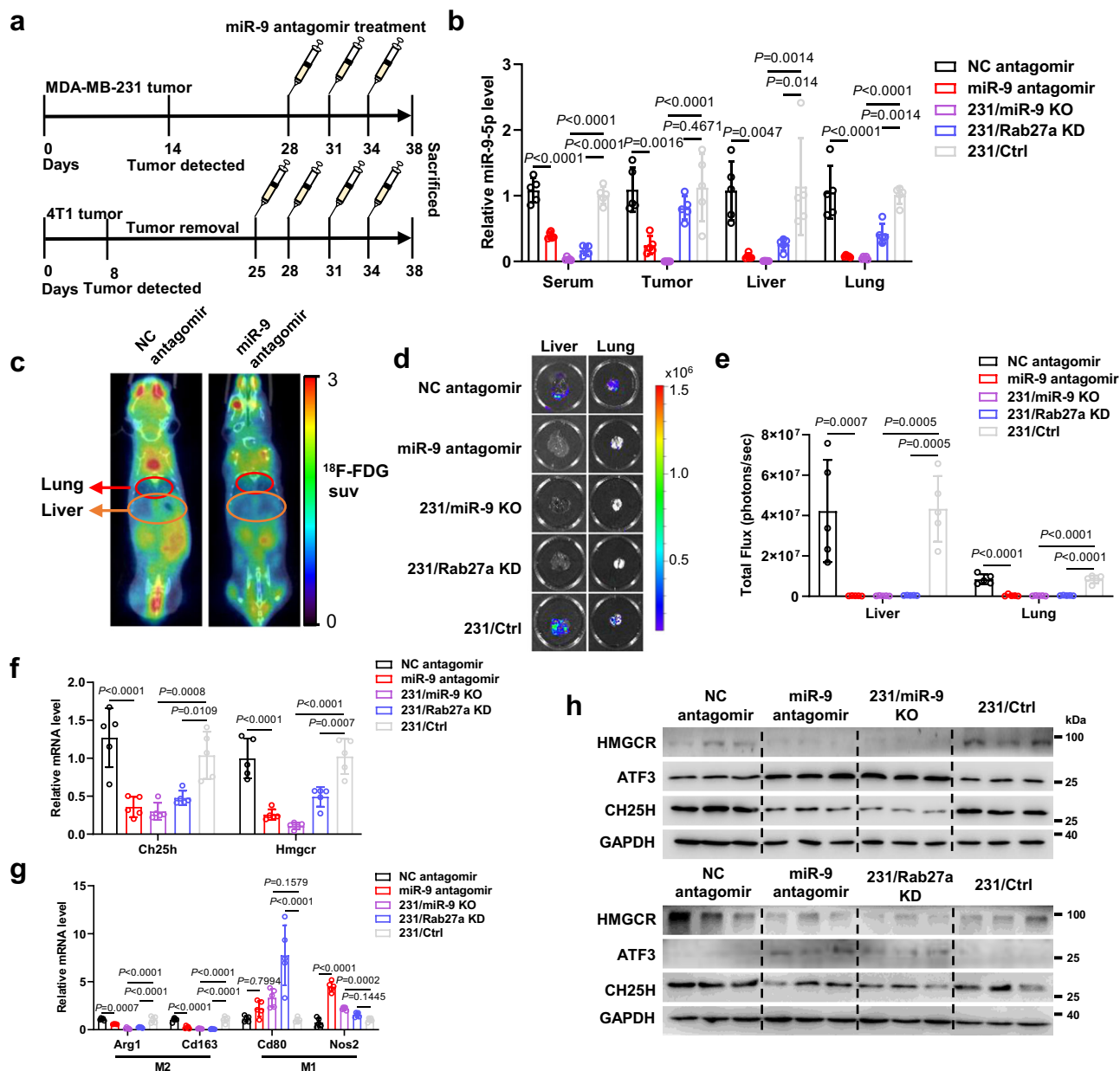


Fig. 8 | Silencing of miR-9-5p attenuated 25-HC-mediated tumor metastasis.

a Schematic diagram of antagomir treated mouse model. 231/Luc cells injected at day 0 in NSG mice. On day 14, tumor was detected. At day 28, mice were treated with antagomir (10 nmol) every 3 days until euthanized. 4T1/Luc cells injected at day 0 in BALB/c mice. On day 8, tumor was detected. At day 25, mice were treated with antagomir (10 nmol) every 3 days until euthanized, during which time the tumor was removed on day 28. **b** RT-qPCR-determined miR-9-5p levels in serum, tumor, liver and lung in 231/Luc cells xenografted mice treated with indicated antagomir and with 231/Ctrl, 231/Rab27a KD and 231/miR-9 KO mice (one-way ANOVA, $n = 5$ mice per group). **c** Representative PET/CT imaging with ^{18}F -FDG for accessing liver and lung uptake and quantification in 4T1/Luc cells xenografted mice treated with indicated antagomir. **d** Representative bioluminescence images

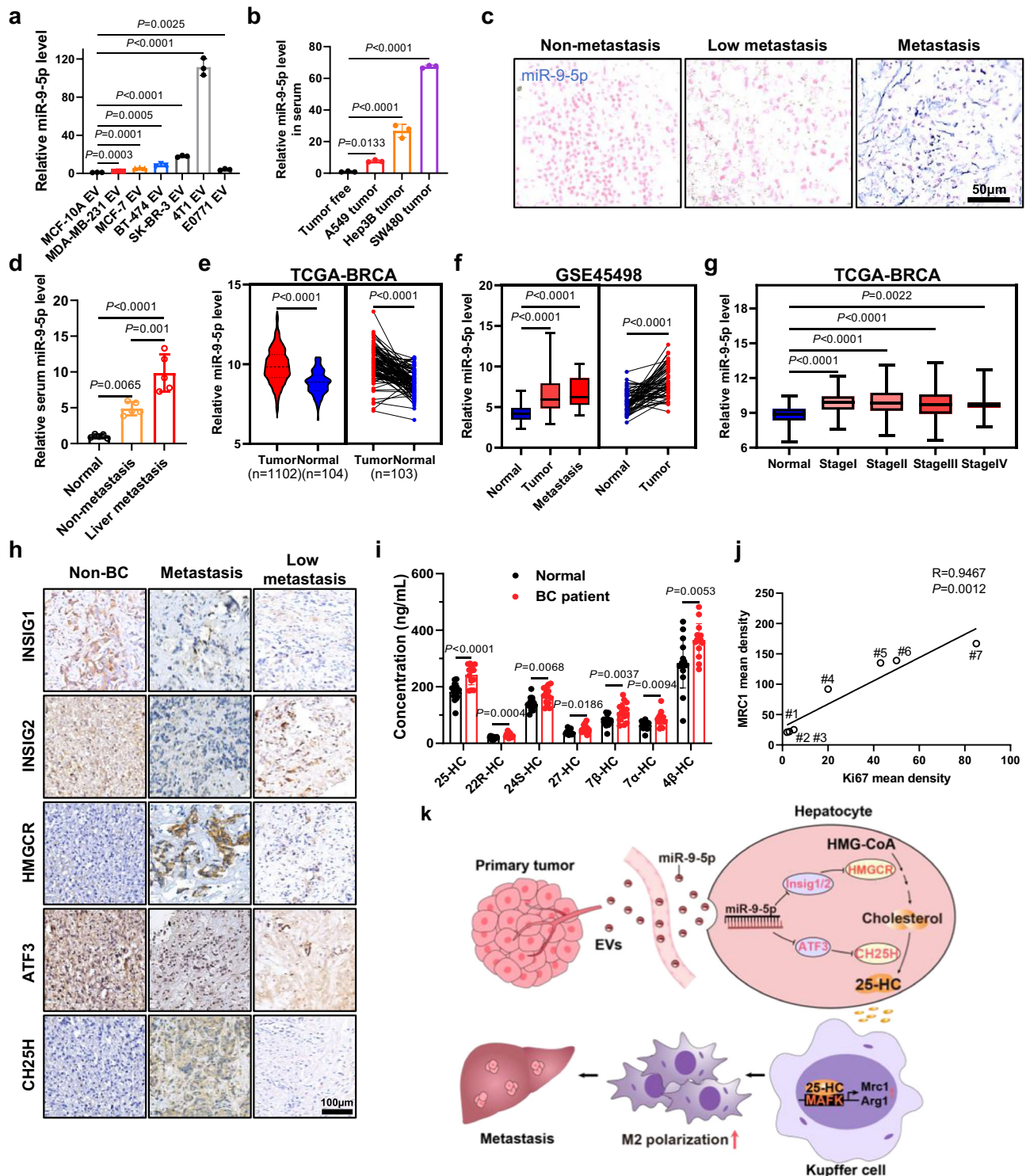
of metastasis site in liver and lung of xenografted mice treated with indicated antagomir and with 231/Ctrl, 231/Rab27a KD and 231/miR-9 KO mice.

e Quantification of liver and lung metastasis of xenografted mice treated with indicated antagomir and with 231/Ctrl, 231/Rab27a KD and 231/miR-9 KO mice (one-way ANOVA, $n = 5$ mice per group). **f** Quantification of gene expression in liver from xenografted mice treated with indicated antagomir and with 231/Ctrl, 231/Rab27a KD and 231/miR-9 KO mice (one-way ANOVA, $n = 5$ mice per group).

g Quantification of gene expression in liver from xenografted mice (one-way ANOVA, $n = 5$ mice per group). **h** Immunoblotting analysis of liver sample collected from xenografted mice treated with indicated antagomir and with 231/Ctrl, 231/Rab27a KD and 231/miR-9 KO mice. Data are presented as mean $\pm$ s.d. Source data and exact P value are provided as a Source data file.

cholesterol biosynthesis and enhances cholesterol to 25-HC conversion by targeting Insig1, Insig2 and Atf3. Additionally, miR-9-5p has also been reported with other targets in the cholesterol metabolism pathway including acyl-coenzyme A: cholesterol acyltransferase (ACAT), a key enzyme that catalyzes the formation of cholesteryl esters (CE) from cholesterol and long-chain fatty acyl-coenzyme A³⁷. Those studies further strengthen the effect of miR-9-5p as a master regulator on cholesterol homeostasis in vivo.

In our study, we demonstrate the effect of 25-HC on the induction of liver Kupffer cells polarization into M2 type, which further facilitates metastatic BC cells to escape from immune surveillance to promote liver metastasis. We found that lung metastasis of breast cancer may be caused by the accumulation of 25-HC synthesized by the liver in the lungs through blood transport, leading to M2 polarization of alveolar macrophages. Indeed, the 25-HC level has been evidenced with a remarkable elevation in



lipopolysaccharide (LPS)-induced acute lung inflammation and its sulfated metabolite, 25-hydroxycholesterol-3-sulfate, exerts a strong suppression effect on neutrophil³⁸. More reports show tumor cells induce neutrophils to form neutrophil extracellular traps, thereby supporting lung colonization of metastasis-initiating BC cells³⁹. The recent finding also reveals nicotine promotes BC metastasis by stimulating N2 neutrophils and generating a pre-metastatic niche in the lung⁴⁰. Besides, lipid-laden lung mesenchymal cells reshape the local immune landscape via metabolic reprogramming of lung-resident fibroblasts to promote BC metastasis^{41,42}.

Identifying secreted regulators in cancer incidence and metastasis determinants provides potential prognostic markers and therapeutic targets for cancer intervention⁴³. Our study reveals the possibility of employing miR-9-5p as a novel blood-based biomarker to identify BC patients with a high risk of developing metastasis to guide preventive screenings and early intervention. Considering CH25H is the key enzyme in our model to regulate tumor metastasis, targeting this expression or blocking its activity might be a powerful strategy for clinical applications. Proteolysis-targeting chimeras (PROTAC) are becoming an emerging therapeutic modality with the potential to tackle disease-causing proteins that have historically been highly

Fig. 9 | Circulating miR-9-5p was correlated with BC patients with metastasis. **a** RT-qPCR-determined miR-9-5p levels in an equal amount of EVs derived from indicated cells (normalized to miR-16) (unpaired two-tailed *t* test, *n* = 3 independent batches). **b** RT-qPCR-determined miR-9-5p levels of EVs in serum from indicated xenografted mice. (one-way ANOVA, *n* = 3 independent batches). **c** Representative images of miRNA in situ hybridization (miRNA-ISH) in liver metastases from breast cancer patients. Scale bar, 50 μ m. **d** RT-qPCR-determined miR-9-5p levels in serum from normal or BC patient with or without metastasis (unpaired two-tailed *t* test, *n* = 5). **e** Expression levels of miR-9-5p in BRCA tumor samples from the TCGA database. Statistical significance was assessed using two-tailed Mann–Whitney test (left, *n* = 1102 for Tumor and *n* = 104 for Normal) and paired two-tailed Student's *t* test (right, *n* = 103). **f** Expression levels of miR-9-5p in BRCA tumor samples from the GEO database. Box plots show minima, maxima, median bounds of box and

quartiles (one-way ANOVA for left, *n* = 59 for Normal, *n* = 169 for Tumor, *n* = 54 for Metastasis; paired two-tailed *t* test for right, *n* = 59). **g** Expression levels of miR-9-5p in different stage of BRCA tumor samples. Box plots show minima, maxima, median bounds of box and quartiles (one-way ANOVA, *n* = 104 for normal, *n* = 182 for stage I, *n* = 609 for stage II, *n* = 244 for stage III, *n* = 20 for stage IV). **h** IHC analysis of HMGCR, CH25H, INSIG1, INSIG2 and ATF3 expression in liver metastases from breast cancer and non-BC individuals. Scale bar, 100 μ m. **i** LC-MS/MS analysis of oxysterol derived from cholesterol in serum from normal and BC patients (unpaired two-tailed *t* test, *n* = 15 per group). **j** Correlation analysis of MRC1 and Ki67 expression IHC from BC patients metastatic liver. The two-tailed Pearson's *r* was indicated (*n* = 7). **k** Schematic summary of this study. Data are presented as mean $\pm$ s.d. Source data and exact *P* value are provided as a Source data file.

challenging to target with conventional small molecules^{44,45}. Therefore, the investigation of targeting CH25H with PROTACs would be a powerful strategy for downstream clinical applications to treat cancer.

Methods

Cells and constructs

Cell lines applied in this study were obtained from American Type Culture Collection (ATCC) and cultured in the recommended medium or as indicated. These include MDA-MB-231 (HTB-26), 4T1 (CRL-2539), SK-BR-3 (HTB-30), MCF-7 (HTB-22), E0771 (CRL-3461), MCF-10A (CRL-10317), BT474 (HTB-20), HEK 293 T (ACS-4500), RAW264.7 (TIB-71). The HL-7702 human hepatocyte cell line was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. MDA-MB-468 (HTB-132) and MDA-MB-231-Lung Metastasis-2 (MDA-MB-231-LM2) cell lines were generous gifts from Ke Gong, Wuhan University and Qian Zhao, Shanghai Jiao Tong University. Primary hepatocytes were obtained from mice livers based on the classic two-step collagenase perfusion technique⁴⁶. Kupffer cells were prepared by two-step liver collagenase digestion and fractionation on a two-step percoll density gradient, as previously described⁴⁷. All cell lines were cultured in an atmosphere of 5% CO₂ incubator at 37 °C. MCF-10A cells were engineered to stably overexpress miR-9-5p (MCF-10A/miR-9) or the empty PLVX-IRES-zsGREEN vector (MCF-10A/vec). MDA-MB-231/Lck-GFP and MCF-10A/Lck-GFP cells were constructed by transfection of an Lck-GFP expression plasmid (Addgene #61099) into MDA-MB-231 and MCF-10A cells followed by selection in G418 and sorting of GFP⁺ cells by Flow Cytometry. MDA-MB-231/Luciferase and 4T1/Luciferase were constructed by infected MDA-MB-231 and 4T1 cells with packaged plenti-CMV-Puro-Luc virus (Addgene #17477). To construct 231/Rab27a KD cells, short hairpin RNA targeting Rab27a sequence CCAGTGTACTTTACCAATATA (231/Rab27a KD) were designed by BLOCK-iT RNAi Designer (Thermo Fisher Scientific) and the corresponding oligonucleotides were cloned into pLKO.1 vector (Addgene #10878). Cells were transduced by the lentivirus and selected based on puromycin resistance.

To knock out *hsa-miR-9-5p* gene by employing CRISPR-Cas9 genomic editing system, two single guide RNAs (sgRNAs; DNA sequences ACTCCTACCTGTGCCGACTG and GGGACACGAGTGAGATTGAG for *hsa-miR-9-1*; DNA sequences TCATCCACCTTTAATCATA and AAAAGGAGGAATCTTAAGCG for *hsa-miR-9-2*) predicted by the sgRNA Designer (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgRNA-design>) were synthesized in DNA form and annealed into double strands, treated with T4 polynucleotide kinase and inserted into the BbsI-digested Lenti-crispr-V2 vector. The two constructs, psPAX2 and pMD2.G were co-transfected into 293 T cells. Virus was harvested at 72 h post transfection and filtered through a 0.45 μ m filter. MDA-MB-231, MDA-MB-468, MCF-7 and MDA-MB-231-LM2 cells were infected with collected lentiviruses with the addition of polybrene, selected by puromycin resistance and seeded on 96-well plates. Monoclonal cell was screened by genotyping PCR and confirmed by sequencing. A similar strategy was applied to generate 4T1/miR-9 KO cells by using two

sgRNAs (DNA sequences GCTCCGAGCTACCGACTGCA and AGTCCG-GAGAAGCCGCTCCC) and 4T1/Rab27a KO cells using sgRNA (DNA sequence AGCGTCCCTGAAGAATGCAG). To construct AAV-mINSIG-1, AAV-mINSIG-2 and AAV-mATF3, the enhanced GFP (eGFP) region in pscAAV-CMV-eGFP plasmid⁴⁸ was replaced with the DNA fragment containing mouse *Insig1*, *Insig2* and *Atf3* cDNAs. The resulting construct pscAAV-CMV-mouse *Insig1*, pscAAV-CMV-mouse *Insig2* and pscAAV-CMV-mouse *Atf3* were confirmed by sequencing. AAV serotype 8 was used to achieve robust transgene expression in liver. Viruses were produced and titer was determined by qPCR. For mouse injection, 5×10^{11} genome copy resuspended in 100 μ L PBS was used per mouse.

To construct reporters for miR-9-5p-mediated regulation of *INSIG1*, *INSIG2* and *ATF3*, 3' UTR of human or mouse *INSIG1* (primers 5'-gaaaaaattccaatgaagatgtcagca and 5'-gtaaaaatgaatgcacaaagaaca caatg for human; primers 5'-gggacggtttcttaagtcttctgg and 5'-gcagcatt gtgtcttttgcattct for mouse), *INSIG2* (primers 5'-aaagcatatggg gagctgtgattt and 5'-tgcatgatgacagcaaatatcatttgc for human; primers 5'-gccaaactgtgtactgacacaga and 5'-aggccctctatagctgctcagta for mouse) and *ATF3* (primers 5'-atgccaccgttaggattcag and 5'-accatg tcttggtccaatattattattctg for human; primers 5'-cttgataggtgggtgggac and 5'-gttcgatattattattgttgacagctag for mouse) gene were cloned by PCR and inserted into the psiCHECK-2 reporter vector (Promega) downstream of the Renilla luciferase (R-Luc) gene. Site-directed mutagenesis was used to mutate the following putative miR-9-5p binding sites with corresponding primer sets: 5'-cagcgtttgat ttgagttgttaaaatgtaaaa and 5'-tcatatattttctataaactattggcaaaaaatcc for human *INSIG1*; 5'-aactttgagggaggttgatggtgcttaa and 5'-gcactgacacaaa actgctagttgg for mouse *Insig1* site I; 5'-taactcttgactgagttgtacaatggta and 5'-ttgtttgtaactgctataactattggcaaaagc for mouse *Insig1* site II; 5'-ttggtctgtaattttctgtttgacagg and 5'-gctgtctccagtaacgcaggtttgaa for human *INSIG1*; 5'-ttcgtatatacttcccgaattttctcagaag and 5'-gtccctacaa caatgcagactttaaacc for mouse *Insig1* site I; 5'-aatctatgatgctaattgt- cactggctact and 5'-cctctgtggcagagagagggtcga for mouse *Insig1* site II; 5'-ctcagttctctatcacaggaagaaagcagaaa and 5'-tctctgtataggagaactgagtttg caacaga for human *ATF3* site I; 5'-ttcaactctcgtaggttaggactctccactcaa and 5'-tcttaacctacgagaagttgaactttctgtttctt for human *ATF3* site II; 5'-ctggaagagctctcaaatattccattttctttcc and 5'-tggaatattgagagctctccag cctagacacaact for human *ATF3* site III; 5'-aggaagagatgctaataaccacag ccacagctgt and 5'-tggttattagcatctcttctgcccctgctactga for mouse *Atf3* site III.

EV purification and characterization

EVs were purified by ultracentrifugation of conditioned medium as previously described^{13,49,50}. Briefly, conditioned medium was collected from cells grown in medium containing EV-depleted serum for 48 h, and pre-cleared by centrifugation at 500 $\times g$ for 15 min and then at 10,000 $\times g$ for 20 min. EVs were pelleted by ultracentrifugation at 110,000 $\times g$ for 70 min, and washed in PBS following the same ultracentrifugation conditions. The EV pellet was suspended in PBS and used in various experiments. For cell treatment, 2 μ g of EVs (equivalent to those derived from $\sim 5 \times 10^6$ producer cells) based on protein

measurement by BCA protein assay kit (Thermo Fisher Scientific) was added to 2×10^5 recipient cells in a six-well plate. The characterization of EVs was confirmed by measuring expression of exosome-specific markers TSG101, ALIX and EV-associated protein markers CD63, and CD9 by western blot analysis and nano-flow cytometer (CytoFlex S, Beckman Coulter). In addition, we further examined the characteristics of EVs using iodixanol gradient centrifugation as reported by Kowal et al.⁵¹. EVs size distribution and particle concentration were analyzed using Flow NanoAnalyzer U30 (NanoFCM, Xiamen, China) according to the manufacturer's instructions. Briefly, the instrument was calibrated for particle concentration to 10^7 – 10^9 particles/mL using 250 nm fluorescent silica nanospheres (NanoFCM Inc., QS2503) and for size distribution using Silica Nanospheres Cocktail (NanoFCM Inc., S16M-Exo). EVs were incubated with APC-CD63 antibody at room temperature for 30 min for APC positive detection. The report for size distribution and particle concentration was generated directly from the NF Profession 2.0 software, with the particles from PBS subtracted. Flow cytometer verified the EVs quantity of Rab27a knock out or knock down outcomes in 4T1 and MDA-MB-231 cells. The instrument calibrated for gating EVs with 100 nm and 200 nm Latex Beads (PCS Control Mixed Kit, Beckman Coulter, 6602336). The report for size distribution and particle concentration was generated directly from CytoFLEX 2.4 software.

RNA extraction and quantitative PCR with reverse transcription

Total RNA was harvested from cultured cells or tissues using the TRIzol reagent (Invitrogen, 15596026) followed by cDNA synthesis using the MonScript RTIII Super Mix with dsDNase kit (Monad Biotech, MR05201). A CFX Connect real-time PCR system (Bio-Rad Laboratories) was used to perform quantitative real-time PCR (RT-qPCR) of cDNA samples using MonAmp ChemoHS qPCR Mix (Monad Biotech, MQ00401). The exosomal RNAs were extracted by TRIzol LS reagent (Invitrogen, 10296028). EVs resuspended in 1 mL TRIzol LS and then incubated at room temperature for 10 min. 200 μ L of chloroform was added, gently mixed, and incubated for 10 min on ice. After incubation, microcentrifuge tubes were centrifuged for 10 min at $12,000 \times g$ at 4 °C. The aqueous phase was transferred to new tubes, an equal volume of isopropanol and 1 μ L glycogen (Roche, 10901393001) were added and mixed with incubation at –20 °C overnight. Samples were centrifuged at $12,000 \times g$ for 15 min at 4 °C. Aspirated off liquid and added 500 μ L of cold 75% ethanol to wash the pellet. The RNA pellet was air dried for a few minutes at room temperature and subsequently resuspended in 20 μ L of RNase-free water. The cellular or exosomal miRNA was reverse transcribed according to the miScript II RT Kit (QIAGEN, 218161) and measured using miScript SYBR Green PCR Kit (QIAGEN, 218073). The primers for miR-9-5p, miR-16 and U6 were purchased from Tiangen (Cat# CD201-0142, CD201-0235 and CD201-0145). All the other primers were seen in Supplementary Table 4.

Western blot analysis

Cells or tissues were homogenized in lysis buffer containing 50 mM Tris (pH 7.4), 500 mM NaCl, 1% NP40, 20% glycerol, 5 mM EDTA and 1 mM PMSF, 1 mM DTT (Roche), supplemented with protease and phosphatase inhibitor cocktails (Roche). Proteins were separated by electrophoresis on a 10% or 12% SDS polyacrylamide gel. Protein detection was performed using the antibodies described in Supplementary Table 5.

Immunohistochemistry (IHC), Immunofluorescence (IF) and in situ hybridization (ISH)

Mouse liver and lung were fixed in 10% neutral buffered formalin and embedded in paraffin for sectioning. Immunohistochemistry (IHC) and immunofluorescence (IF) were performed as previously described⁵⁰. In situ hybridization (ISH) analysis following previously reported protocol²². Ki67 IHC was used to calculate Ki67⁺ cells against total cell number from >4 views in each mouse. The antibodies used were listed

in Supplementary Table 5. The hsa-miR-9-5p probe with '5' DIG and 3'DIG (5' UCAUACAGCUAGAUACCAAAGA3') was purchased from Sangon Biotech (Order number: R11929).

Oil red O staining

Snap frozen liver specimens were sectioned at 6 μ m and fixed with 4% paraformaldehyde (Sigma-Aldrich, P6148) in PBS after being restored to room temperature and then washed twice with PBS. Once completely air-dried at room temperature, cells were further incubated with Oil Red O working solution at room temperature for 1 h. Cells were washed 3 times with PBS before images were acquired for downstream analysis.

Plasmid transfection and siRNA treatment

HEK293T cells were transfected using Lipofectamine 2000 (Life Technologies) with packaging plasmid: psPAX2 (Addgene, #12260) and pMD2.G (Addgene, #12259). Forty-eight hours after transfection, lentiviral supernatant was harvested and filtered through a 0.45- μ m filter, and utilized for subsequent infection of MCF-10A, MDA-MB-231 and 4T1 cells (48 h incubation) together with 10 μ g/mL polybrene in the medium. Cells were selected based on puromycin selection or sorted by GFP⁺ cells with FACS. For MAFK knockdown, siRNAs designed for MAFK (siG150120091935 and siG150120091948) and control (siN000000-1-1-5) were purchased from RiboBio (Guangzhou, China).

RNA-seq and gene set enrichment analysis (GSEA)

RNA-seq and data analysis were carried out by Shanghai Majorbio Bio-pharm Technology company. RNA was extracted according to the manufacturer's instructions (Invitrogen) and genomic DNA was removed using DNase I (TaKara). Then RNA quality was determined by 2100 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). RNA-seq transcriptome library was prepared following TruSeq RNA sample preparation Kit from Illumina (San Diego, CA) using 1 μ g of total RNA. Each library was sequenced on the Illumina HiSeq xten/NovaSeq 6000 platform using paired-end, non-strand-specific sequencing strategy. The raw paired end reads were trimmed and quality controlled by SeqPrep (<https://github.com/jstjohn/SeqPrep>) and Sickle (<https://github.com/najoshi/sickle>) with default parameters. Then clean reads were separately aligned to reference genome with orientation mode using HISAT2⁵² software. The mapped reads of each sample were assembled by StringTie (<https://ccb.jhu.edu/software/stringtie/index.shtml?t=example>) in a reference-based approach⁵³. To identify DEGs (differential expression genes) between two different samples, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method. RSEM⁵⁴ was used to quantify gene abundances. Essentially, differential expression analysis was performed using the DESeq2 /DEGseq⁵⁵ /EdgeR⁵⁶ with Q value ≤ 0.05 , DEGs with $|\log_2 FC| > 1$ and Q value ≤ 0.05 (DESeq2 or EdgeR) /Q value ≤ 0.001 (DEGseq) were considered to be significantly different expressed genes. For gene set enrichment analysis (GSEA), fragments per kilobase million was uploaded to GSEA 4.2.0 and enrichment of gene sets was interrogated with 1000 random permutations to obtain the normalized enrichment score (NES), nominal P value and false discovery rate (FDR) q value.

Measurement of total cholesterol (TC) and triglycerides (TG)

Mice blood was collected by retro-orbital bleeding. The livers were homogenized and supernatants were collected for lipid extraction. Serum from breast cancer patients and healthy individuals were collected from Renmin Hospital of Wuhan University. Total cholesterol and triglyceride levels were determined by the total cholesterol kit and triglyceride kit according to the manufacturer's instructions (Kehua Bioengineering).

LC-MS/MS analysis of oxysterols

Analysis of sterols were performed on an Agilent 1290 ultrahigh performance liquid chromatography system connected to an Agilent 6460 triple-quadrupole mass spectrometer (UHPLC-QQQ-MS) equipped with a dual AJS electrospray ionization source (Agilent Technologies, Inc.). Sample preparation was followed a previously reported method⁵⁷ with some modifications. In brief, coprostanol as an internal standard was added to serum and tissue samples. Saponification was carried out in 1 N ethanolic KOH at 37 °C for 1 h. After addition of distilled water, sterols were extracted twice with n-hexane and evaporated to dryness. The reagent mixture for derivatization consisted of picolinic acid (PA), 2-methyl-6-nitrobenzoic anhydride (MNBA), 4-dimethylaminopyridine (DMAP) with molar ratio 2:2:1 (PA: MNBA: DMAP) in anhydrous pyridine. The freshly prepared reagent mixture was added to the dried serum and tissue extraction, and the reaction mixture was allowed to stand at 60 °C for 10 min. After evaporation to dryness, the residue was redissolved in acetonitrile for UHPLC-QQQ-MS analysis. The samples (2 µL) were individually injected on an UHPLC column (Agilent Zorbax Eclipse plus C18, 2.1 mm × 100 mm, 1.8 µm) with temperature at 40 °C. Ultrapure water containing formic acid (0.1%, v/v) and acetonitrile were used as respective mobile phase A and B with flow rate of 0.6 mL/min. The gradient elution scheme was set as follows: 0 min (80% B), 2–4 min (90% B), 7 min (100% B). Mass spectra were recorded with positive MRM mode (530–146 and 635–146 for oxysterols, 516–146 for coprostanol). MassHunter Workstation software (Agilent Technologies, Inc.) was used for data analysis. All the oxysterols examined were summarized in Supplementary Data 1.

Cell migration and invasion assay

To evaluate the migration and invasion capacity of cells, 24-well plates equipped with cell culture inserts containing 8.0 µm pore size membrane were used. Briefly, cells were suspended in 100 µL of serum-free medium and placed in the upper compartment chambers. The lower chamber was filled with 10% FBS as the chemoattractant. For invasion assay, diluted extracellular matrix gel coated the inserts preincubated for 30 min. In all, 5×10^4 cells incubated for the migration assay and 1×10^5 cells incubated for the invasion assay. At the end of the experiments, cells on the upper surface of the filters were wiped, and migrated cells on the lower surface were fixed in 4% paraformaldehyde and stained by 0.1% crystal violet. For wound healing assay. Briefly, cells were seeded into wells of 6-well plates and cultured for 48 h to reach 95%–100% confluence. The cells were then vertically scratched using a sterile pipette tip. Fresh culture medium was added to the cells. Cell migration (wound/scratch closure) was determined based on the images obtained with inverted optical microscopy every few hours.

Flow cytometry

For liver macrophage analysis, NPCs were prepared by two-step liver collagenase digestion and fractionation on a two-step percoll density gradient, as previously described⁴⁷. TruStain Fc is specific to the common epitope of CD16/CD32 (Biolegend, Cat# 101319). It is useful for blocking non-specific binding of immunoglobulin to the Fc receptors. Zombie Aqua™ is an amine-reactive fluorescent dye (Biolegend, Cat#423101). The antibodies for surface staining were CD45 (Biolegend, Cat#103145), Ly-6G (Biolegend, Cat#127611), CD3 (Biolegend, Cat# 155611), CD19 (Biolegend, Cat# 115526), F4/80 (Biolegend, Cat#123119), CD11b (Biolegend, Cat#101227), CD11c (Biolegend, Cat# 117307), CD163 (Biolegend, Cat#155319), Tim4 (Biolegend, Cat#130021) and I-A/I-E (Biolegend, Cat#107627). Numbers obtained were subsequently represented as the percentage of the highest subsets. Un-stained, single-stained, and the FMO (fluorescence minus one) control were used for setting compensation and gates.

Electron microscopy (EM)

For electron microscopy, EVs were loaded on formvar and carbon-coated copper grids for 1 min. Then the grids were rinsed with 1x PBS before being left for 30 s on 2% (wt/vol) uranyl acetate (Ladd Research Industries, Williston, VT). Excess solution was removed with Whatman 3MM blotting paper, and grids were left to dry for a few minutes before viewing. Grids were examined using a JEOL JEM-1400 Plus transmission electron microscope operating at 80 kV. Images were recorded using a GATAN OneView 4 K digital camera.

Stimulated Raman scattering (SRS) imaging of liver tissue

The liver tissue samples were sliced using a cryostat at 20 µm thickness. Before SRS imaging, the liver tissue samples were washed with PBS solution for 5 min. Subsequently, the liver tissue samples were fixed by 10% neutral formalin (HT501128, Sigma-Aldrich) for 15 min. Finally, the liver tissue samples were washed with PBS solution and immersed in PBS solution for SRS imaging. SRS imaging was performed on a home-built picosecond SRS microscope. An ultrafast laser system with dual output at 80 MHz (picoEmerald, Applied Physics & Electronics) provided both pump (tunable wavelength 700–990 nm) and Stokes beams (fixed wavelength 1031 nm). The Stokes beam was modulated at 20 MHz by an electro-optic modulator. A 60× water objective (numerical aperture 1.2, Olympus) was used to focus the light into the sample. Then, an oil condenser (numerical aperture 1.4, Olympus) was used to collect the forward-going pump and Stokes beams through the specimen. A short-pass filter (1000 nm SP, Thorlabs) was used to block the Stokes beam and transmit only the pump beam onto a large area photodiode (10 × 10 mm) to detect the stimulated Raman loss signal. The powers of the pump and the Stokes beams at the specimen were maintained at ~45 mW and ~45 mW, respectively. The images were acquired at 10 µs pixel dwell time. For hyperspectral SRS (hSRS) imaging of the C-H stretching vibration modes, the pump beam was tuned between 784.4 and 800.0 nm while the Stokes beam was fixed at 1031 nm to obtain 50 image stacks within 3050–2800 cm⁻¹.

In situ LD analysis of hSRS image stack

Phasor analysis was used to segment LDs from the tissue samples based on the SRS spectral profile⁵⁸. In previous report, the spectrum in the 2750–3050 cm⁻¹ is well fitted as a sum of seven Lorentzian bands^{59,60}. We apply those seven Lorentzian bands and an additional C=C-H stretching vibration band to multi-peak fitting. The Lorentz peak function ($y = y_0 + (2A/\pi) * (w / (4*(x - x_c)^2 + w^2))$) were used for multi-peak fitting in OriginPro 2021 according to fitting parameter in Supplementary Table 6. The max height of Lorentz peak in ~3015 cm⁻¹ is used to quantify the unsaturation level.

Animals

Animal experiments in this study were approved by and performed in accordance with the institutional animal care and use committee (IACUC) at the College of Life Science, Wuhan University (WDSKY0202008). All mice used in this study were purchased from Shanghai Model Organisms Center, Inc. (NSG mice, Cat.no. NM-NSG-001), Nanjing GemPharmatech (BALB/c-Nude mice, Cat.no. D000521) and Center for Disease Control (CDC; Hubei, China). 6–8 weeks old female NSG mice (for EV injection and MDA-MB-231 tumor models) or female BALB/c mice (for 4T1 tumor models) and female BALB/c-Nude mice were used in all studies and at least 5 mice per group. Mice were bred under specific pathogen free (SPF) condition, under 12 h light/12 h dark cycle (on at 6:00/off at 18:00 pm) with temperature of 72 ± 2 °F and 30–70% humidity. The experimental group and control mice were bred separately. For EV treatment, EVs were injected into the tail vein semiweekly for 5 weeks (~10 µg EVs per injection per mouse). For in vivo tracking of GFP- labeled EVs, EVs derived from MCF-10A/Lck-GFP, MDA-MB-231/Lck-GFP were injected into the tail vein for three consecutive days; mice were euthanized 6 h after the last

injection and liver were collected for sectioning. Xenograft tumors were established by injecting 2×10^5 of MDA-MB-231 or 4T1 cells mixed with Matrigel (Corning) into the No.4 mammary fat pad. MDA-MB-468, MCF-7 and MDA-MB-231-LM2 were orthotopically injected into the No.4 mammary fat pad with 10^6 cells and Matrigel in nude mice, respectively. Before establishing the MCF-7 tumor, mice were subcutaneously injected with 40 μ L of estradiol valerate (1 mg/mL) for two days. Mice received estradiol at 2, 5, 9, 13, 17, 21 and 25 days after cell injection. Tumor growth was monitored starting from 1 to 2 weeks after tumor cell injection, and tumor volume was calculated every other day using the formula $(\text{length} \times \text{width}^2)/2$. The maximum diameter of the tumor does not exceed 1.5 cm according to the IACUC. For restoration assay in liver, 5×10^{11} genomic copy of AAV8-*mInsig1*, AAV8-*mInsig2* and AAV8-mATF3 virus or control virus were injected through the tail vein 1 week before implantation of MDA-MB-231 cells.

For 25-HC treatment, 231/Ctrl or 231/miR-9 KO mice were established by injecting 2×10^5 of wild type or miR-9-5p KO cells mixed with Matrigel into the number 4 mammary fat pad. When the volumes of xenograft tumors reached an average of 100 mm³, mice were randomly divided into control group and 25-HC group. For 25-HC group, mice were intraperitoneally injected with 25-HC (10 mg/kg) every other day until euthanized. For control group, mice were received the same volume of NaCl. For CH25H knockdown in liver, 5×10^{11} genomic copy of AAV-shCH25H virus or control virus were injected through the tail vein. One week after AAV injection, 4T1 cells were injected into the mammary fat pad to establish xenograft tumors. For desmoterol treatment, 4T1 cells were established into the number 4 mammary fat pad. Desmoterol (3 μ g per mouse) was injected intraperitoneally every 2 days after tumor formation. For macrophage depletion in liver, MDA-MB-231 or 4T1 cells tumor bearing mouse were injected intravenously with clodronate liposomes 150 μ L (SunLipo NanoTech) per mouse firstly, followed by 100 μ L every 10 days. The mice were then euthanized and liver was collected for imaging and analysis of Kupffer cells. Oxygen consumption (VO₂), carbon dioxide production (VCO₂), heat and respiratory exchange ratio (RER) were measured concurrently using a 12 station Comprehensive Lab Animal Monitoring System (Columbus Instrument). Mice were placed in the metabolic cages 24 h before starting the experiment. At the start of the recording, measurements were taken at 12 min intervals. Ch25h-floxed mice were generated by Shanghai Model Organisms Center, Inc. using the CRISPR-cas9 system. Cas9 mRNA, single guide RNAs (sgRNAs) and donor were co-injected into zygotes. sgRNAs directed cas9 endonuclease cleavage in both sides of the Ch25h gene target exon 1, resulting in LoxP sites inserted, respectively, by homologous recombination. To obtain liver-conditional knockout mice (*Ch25h*^{fl/fl}), the floxed Ch25h mice (C57BL/6Smoc-*Ch25h*^{em1(flox)Smoc}; Shanghai Model Organisms Center, Inc. Cat.no. NM-CKO-215043) were crossed with albumin-cre transgenic mice (B6.Cg-*Spear6-psi*^{Tg(Alb-cre)21Mgn}/J; Jackson Laboratory. Cat.no. 003574). Xenograft tumors were established by injecting 1×10^6 of E0771 cells mixed with Matrigel into the No.4 mammary fat pad. Before taking PET/CT, tumor cells (5×10^5 cells per injection) were re-suspended in 100 μ L PBS and infused into the recipient mice by tail vein injection, followed by imaging. For miR-9-5pantagomir treatment, MDA-MB-231/Luc cells or 4T1/Luc cells were established into the number 4 mammary fat pad in NSG or BALB/c mice respectively. After tumor formation, NSG or BALB/c mice were randomly divided into two groups. MiR-9-5p antagomir or control (RiboBio) was directly injected into the implanted tumor at a dose of 10 nmol (dissolved in 50 μ L PBS) every 3 days in NSG mice at the later stage of tumor growth or BALB/c mice at the later stage of tumor removal by tail vein injection. The mice were euthanized after 38 days and were collected for subsequent analysis. For lung, liver and colon cancer models, 1×10^6 of A549 cells, Hep3B cells and SW480 cells suspended in 100 μ L PBS were injected subcutaneously into the right flank of nude mice. In all experiments, mice were euthanized by cervical dislocation.

Micro-PET/CT

Before 18F-FDG administration, mice were deprived of food for 8–12 h. 18F-FDG (5.5 MBq (150 μ Ci) in 0.1 mL) was injected through the tail vein. Micro-PET/CT imaging was started 1 h after 18F-FDG injection. Micro-PET/CT images was performed on a PET/CT scanner InViView-3000B (Novel Medical, Beijing, China). For micro-PET/CT imaging, all mice were subjected to isoflurane anesthesia (1–2% in 100% oxygen) on a temperature self-adjust tube to maintain body temperature throughout the procedure. Mice were visually monitored for breathing and any other signs of distress throughout the full imaging period. The images were reconstructed using a three-dimensional ordered subsets expectation maximum algorithm (OSEM) without attenuation correction. For data analysis, the region of interest (ROI) was manually drawn to cover the whole liver and lung on fused images.

Bioluminescence imaging

Bioluminescence imaging (BLI) of the luciferase activity in tumor cells was used to monitor the tumor metastasis progression. Before performing the BLI, the mice were anesthetized under sodium pentobarbital (100 mg/kg) and administered with D-luciferin (150 mg/kg) by intraperitoneal (i.p.) injection 10 min before the imaging. At the end time point, the liver and lungs were harvested in a 60 mm dish plate in 15 mg/ml D-luciferin diluted in PBS for bioluminescence imaging. Image exposure times were between 10 s and 1 min, depending on the signal strength. Light emission from the region of interest was quantified as photons s⁻¹ cm⁻² steradian⁻¹.

Proteome profiler mouse chemokines array

The protein array was carried out using a proteome profiler mouse chemokines array kit (ARY020, R&D Systems, Minneapolis, USA). Two identical membranes precoated with antibodies against 25 different chemokines were incubated with media derived from 25-HC treated or control-RAW264.7 cells for 30 h, respectively. The following procedure was performed according to the manufacturer's instructions. The semi-quantification of the dots was analyzed by ImageJ software.

Human serum specimens

Archived samples from patients with cancer patients and health controls used in this study were collected in accordance with and approved by Clinical Research Ethical Committee of Renmin Hospital of Wuhan University. All participants provided written informed consent. Archived fasting serum samples were collected from female patients with BC and female control subjects without cancer. The clinical samples from female patients with BC and control volunteers were obtained from Renmin Hospital of Wuhan University. Serum EVs were isolated using size exclusion chromatography (SEC)⁶¹. Briefly, samples were thawed at 37 °C and centrifuged at 3000 $\times$ g and room temperature for 10 min. The supernatant (500 μ L) was applied onto a SEC column (qEV original 70 nm, Izon Science) and eluted by progressive addition of freshly filtered phosphate buffered saline (PBS), which allowed for separation and collection of 3 distinct 1.5 mL fractions where the intermediate tube contained EVs. In addition, serum EVs for small RNA sequencing are isolated using EXODUS⁶² (Shenzhen Huixin Life Technology). Trace plasma samples (20 μ L) were diluted in 5 mL with 1 $\times$ PBS and filtered through a 0.22 μ m syringe filter (Millipore). The filtered plasma solutions were then loaded into the EXODUS device. Protein fragments, lipids, nucleic acids, and other small impurities pass through nanopores under the alternating negative pressing with EVs (diameter > 20 nm) remaining inside the chamber. The EVs were then resuspended in 1 $\times$ PBS up to 200 μ L. All the clinical information was seen in Supplementary Table 7.

Software

Equipment build-in software were used for data collection, including the CFX Manager Software version 2.0 for real-time PCR data; Prism 9

was used for most data analysis and statistics; GSEA 4.3.2 was used for gene set enrichment analysis of RNA-seq data; <http://cistrome.org/db/#/> is used to predict transcription factors for M2 marker genes. CytoFLEX2.4 was used for flow cytometry analysis for cells and characterization for EVs.

Statistics and reproducibility

All quantitative data are presented as mean $\pm$ standard deviation unless otherwise stated. All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software). Two-tailed Student's *t* tests were used for comparison of means of data between two groups. For multiple independent groups, one-way or two-way ANOVA with post hoc Tukey tests were used. Values of $P < 0.05$ were considered significant. Sample size was generally chosen based on preliminary data indicating the variance within each group and the differences between groups. Pearson correlation analysis was employed for analyzing the strength and direction of the linear relationship between two variables and represented as R value. All samples that have received the proper procedures with confidence were included for analyses. Animals were randomized before treatments. Western blots are repeated independently three times with similar results, and representative images are shown. The tissue slice contained three independent samples and representative images are shown.

Data availability

The RNA-seq data generated in this study has been deposited in the NCBI Gene Expression Omnibus (GEO) under accession code [GSE222708](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE222708) which includes GSE222622 (mice liver tissue receiving EV injection model), GSE222707 (mice liver tissue from xenografted tumor model) and GSE222623 (25-HC and vehicle treated RAW264.7 cells). The flow cytometry data has been deposited in Figshare (https://figshare.com/articles/online_resource/FACS/27537747). LC-MS/MS-detected oxysterols are summarized in Supplementary Data 1. All data are included in the Supplementary Information or available from the authors, as are unique reagents used in this Article. The raw numbers for charts and graphs are available in the Source Data file whenever possible. Source data are provided with this paper.

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Author contributions

W.Y. is the primary contact for this paper. W.Y. conceived ideas, W.Y. and L.M.Z. contributed to project planning. W.Y., M.X.L. and S.H. designed and performed most of the experiments. H.H.L. and L.M.Z. assisted with mass spectrometry analysis. J.J.L. provided clinical samples. H.J.L. and X.J.H. assisted with SRS imaging of liver tissue. X.H.Z. assisted with electron microscopy. M.Y., W.K.H., X.L., W.Q.J. and X.T.W. assisted with cell treatment and mouse experiments. B.W.X. and T.X.Y. assisted with cell line construction. D.F. assisted with liver-specific knockout CH25H mice. W.Y., M.X.L. and S.H. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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