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Small extracellular vesicles and particles (sEVPs) derived from tumor-free pre-metastatic organs promote breast cancer metastasis and support organotropism

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Abstract

Metastatic breast cancer remains largely incurable, partly due to our incomplete understanding of its intricate underlying mechanisms. Notably, intercellular communication mediated by small extracellular vesicles and particles (sEVPs) has emerged as a key feature of metastasis. While tumor-derived sEVPs have been extensively studied and are known to be pro-metastatic, the role of sEVPs from metastasis-prone normal tissue sites remains primarily undefined. Here, we characterized and studied the function of sEVPs secreted from tumor-free pre-metastatic organs (TuFMO-sEVPs) such as the brain and lungs in both immunocompetent and patient-derived xenograft models. TuFMO-sEVPs from the brain of mammary tumor-bearing mice were found to have a distinct protein content as compared to brain-sEVPs from tumor-free mice, suggesting that the primary tumor can systemically influence the cargo of TuFMO-sEVPs. Importantly, mice orthotopically injected with breast cancer cells which had been educated with either brain or lung TuFMO-sEVPs prior to transplantation showed significantly increased metastasis to the respective organ. We further demonstrated that TuFMO-sEVPs induced the expression of the enzyme dihydrofolate reductase (DHFR) upon uptake by breast cancer cells, leading to their enhanced metastatic capacity. Organ-specific signatures generated from TuFMO-sEVP educated tumor cells were found to be increased in metastatic samples from breast cancer patients as compared to the primary tumor or normal tissue samples and these signatures also significantly correlated with poorer patient outcome. Collectively, our data reveals a novel facet of the metastatic cascade, implicating a role for TuFMO-sEVPs in directing metastasis and providing a potential therapeutic strategy for targeting this process.

Keywords Small extracellular vesicles and particles (sEVPs), Breast cancer, Brain metastasis, Lung metastasis, Global proteomics analysis, Dihydrofolate reductase-mediated metastasis

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Background

Metastasis is a dauntingly complex process which remains a major obstacle in the treatment of solid cancers including breast cancer. Metastatic breast cancer (MBC) arises when the primary tumor disseminates pioneer cells which travel through the blood or lymphatic system to distant organs such as the brain, lungs, bones and liver to form new colonies [1]. Notably, cell-cell communication is a key feature of this multi-stage process [2]. Besides local communication within the primary tumor microenvironment, cancer cells can also signal over long distances to sites of future metastases inducing the formation of a hospitable microenvironment termed as the pre-metastatic niche (PMN) which fosters the survival and outgrowth of disseminated tumor cells upon their arrival to these secondary sites [3, 4].

Importantly, extracellular vesicles and particles (EVPs) have emerged as key mediators of intercellular communication, both locally and systemically [2]. EVPs encompass a heterogeneous group of membrane-bound vesicles including small EVPs (sEVPs) which are usually less than 200 nm in diameter [5]. sEVPs are secreted by most, if not all, normal and cancer cells and enclose a multitude of bioactive molecules ranging from nucleic acids to proteins, metabolites and lipids. Once released from donor cells, sEVPs can transfer their functional cargoes to proximal and distant recipient cells, influencing their phenotype and function [6].

In cancer, sEVP-mediated bidirectional communication occurs between both malignant and non-malignant cells in the tumor microenvironment [2]. Specifically, tumor-derived sEVPs have been shown to play pleiotropic roles in reprogramming the host tissue and promoting cancer progression [6, 7]. They are also known to drive the formation of the pre-metastatic niche through enhanced vasculogenesis, extracellular matrix remodeling and immune regulation [3, 4, 8]. Besides tumor-derived sEVPs, the cellular components of the immediate tumor microenvironment namely epithelial cells, cancer-associated fibroblasts (CAFs), adipocytes, endothelial cells and immune cells also release sEVPs and support cancer progression by modulating tumor cell proliferation and metastasis [2, 6]. Of note, both tumor- and non-tumor-derived sEVPs may serve as novel biomarkers given that they are widely distributed in body fluids and contain specific contents which can be used to identify their cell of origin [9].

Importantly though, the function of sEVPs derived from normal tissues including tumor-free pre-metastatic sites such as the brain and lungs in breast cancer remains poorly characterized. While brain and lung-derived sEVPs are known to play key roles in both normal tissue homeostasis and brain- and lung-related disorders respectively [10], little is currently known about the effects of these sEVPs on tumor cells, especially in the context of tumor cell dissemination and outgrowth during breast cancer metastasis. This highlights the need to uncover the potential functions of tumor-free pre-metastatic organ-derived sEVPs (TuFMO-sEVPs) to better understand and treat metastatic disease.

In this study, we provide functional evidence for the role of brain and lung TuFMO-sEVPs in promoting breast cancer metastasis, with potential future clinical implications.

Results

Isolation and characterization of TuFMO-sEVPs from in vivo breast cancer models

In order to study sEVPs released from tumor-free breast cancer metastatic-prone organs namely the brain and lungs, early-stage in vivo breast cancer models were employed as preclinical tools to model the pre-metastatic stage. Specifically, the patient-derived xenograft model PDX223 [11] and age-matched non-tumor-bearing controls were used to harvest pre-metastatic organs once the primary tumor was palpable in the PDX (Fig. 1A). The organs showed no detectable metastasis from bioluminescence imaging at the time of sEVP isolation (Supplementary Fig. 1A). This was further confirmed by flow cytometric analysis of the dissociated organs and digital droplet polymerase chain reaction (ddPCR) of both the dissociated and intact organs and the peripheral blood mononuclear cell (PBMC) fraction, whereby no human DNA from potential contaminating tumor cells was detected (Fig. 1B and Supplementary Fig. 1B-C). The isolated organs were cultured as tissue fragments and the released sEVPs were collected from the tissue explant conditioned medium via ultracentrifugation. The secreted brain and lung TuFMO-sEVPs displayed a classical cup-shaped morphology under the transmission electron microscope (Fig. 1C). Further characterization with nanoparticle tracking analysis confirmed an average size of 150 nm (Supplementary Fig. 1D & E). Brain-derived sEVPs isolated from mammary tumor-bearing mice had a slightly increased diameter as compared to sEVPs isolated from tumor-free control mice. The amount of sEVPs secreted from the brain and lungs of tumor-bearing mice was similar to controls (Supplementary Fig. 1F).

Besides characterizing the physical parameters, we also examined the content of the TuFMO-sEVPs. In general, sEVPs isolated from the brain and lungs of PDX223 had similar amount of RNA and protein as sEVPs isolated from the control mice (Supplementary Fig. 1G & H). In order to further determine the type of proteins present in the sEVPs, we performed global mass spectrometric-based proteomics analysis of brain-derived sEVPs. Interestingly, TuFMO-sEVPs secreted from the brain of tumor-bearing mice were found to contain different

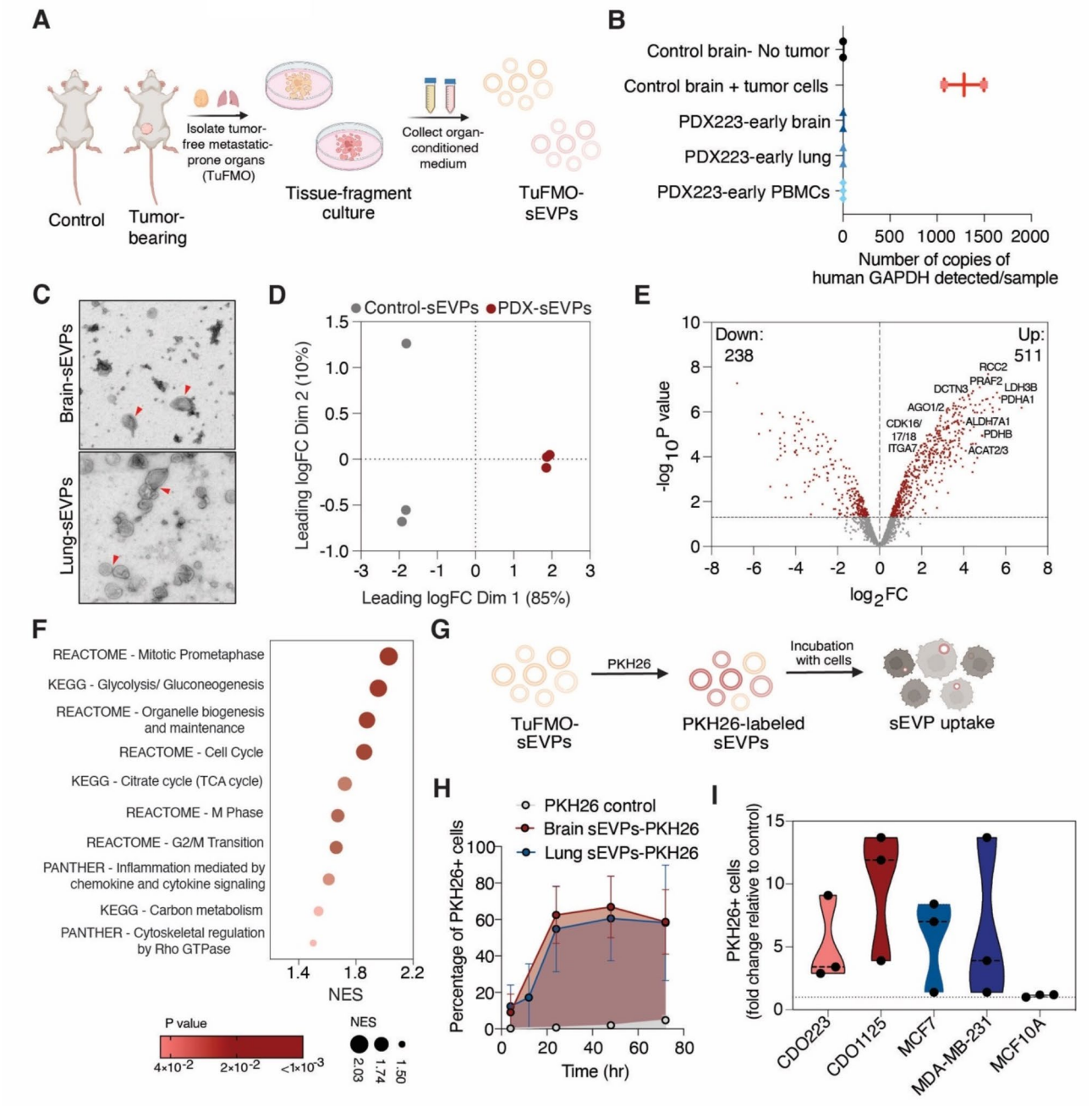

Fig. 1 Tumor-free breast cancer pre-metastatic organs secrete sEVPs (TuFMO-sEVPs) which can be taken up by tumor cells. (**A**) Experimental scheme for the isolation of TuFMO-sEVPs from breast cancer pre-metastatic organs of patient-derived xenograft (PDX) and age-matched non-tumor-bearing control. (**B**) Digital droplet PCR analysis to detect human GAPDH in the intact brain, lungs or peripheral blood mononuclear cell (PBMC) fraction, $n = 2–3$ per condition. (**C**) Representative transmission electron microscopy (TEM) images of brain and lung TuFMO-sEVPs, indicated by the red arrow. (**D**) Principal component analysis (PCA) of the protein content of brain sEVPs from control mice vs. PDX based on global mass spectrometric analysis, $n = 3$ mice per group. (**E**) Volcano plot of differentially enriched proteins in brain TuFMO-sEVPs from PDX as compared to control mice ($p < 0.05$ labeled in red). (**F**) Gene set enrichment analysis (GSEA) of proteins significantly enriched in brain TuFMO-sEVPs from PDX as compared to control mice, ranked by normalized enrichment score (NES). (**G**) Experimental scheme to measure TuFMO-sEVP uptake by tumor cells through PKH26 labeling. (**H**) Uptake of 10 µg brain- and lung-derived TuFMO-sEVPs by MCF7 cells as measured by the percentage of PKH26 + cells over time, $n = 3$. (**I**) Uptake of 10 µg brain TuFMO-sEVPs by different breast cancer and breast epithelial cell lines and CDOs after 3 h, $n = 3$. Data information: Scale-bars: 250 nm (TEM). Data is represented as mean ± SD, all replicates shown are biological replicates

types of proteins as compared to the brain-derived sEVPs coming from tumor-free controls (Fig. 1D). There was an enrichment of proteins known to play a role in metastasis such as proteins encoded by *Praf2*, *Dctn3*, *Ago1/2* and *Itga7* and proteins involved in metabolism including *Pdha1*, *Pdhb*, *Ldh3b*, *Aldh7a* and *Acat2/3* in the brain-derived TuFMO-sEVPs from PDX (Fig. 1E, Supplementary Fig. 1I and Supplementary Table 1). Concordantly, gene set enrichment analysis confirmed that the most significantly enriched biological processes included metabolism (glycolysis/gluconeogenesis and TCA cycle), mitosis and cell cycle, suggesting that brain-derived TuFMO-sEVPs from PDX contain proteins which could potentially promote metabolism and proliferation upon uptake by recipient cells (Fig. 1F). Overall, our findings illustrate that tumor-free pre-metastatic organs in tumor-bearing mice secrete sEVPs distinct from control sEVPs released from non-tumor bearing mice, indicating the potential systemic influence of the primary tumor on the cargo of TuFMO-sEVPs.

Uptake of TuFMO-sEVPs by breast cancer cells

Next, we investigated whether breast cancer cells were able to uptake TuFMO-sEVPs. sEVPs isolated from the metastasis-free brain and lungs of mammary tumor-bearing mice were labeled with the lipophilic dye PKH26 and co-incubated with breast cancer cell lines (Fig. 1G). Flow cytometric analysis confirmed uptake of the TuFMO-sEVPs by the luminal breast cancer cell line MCF7 over time (Fig. 1H and Supplementary Fig. 2A). Besides MCF7, the triple-negative breast cancer cell line MDA-MB-231 and CTC (circulating tumor cell)-derived organoids (CDOs) from metastatic breast cancer patients namely CDO223 (estrogen receptor-positive luminal subtype) and CDO1125 (triple-negative subtype) [12] also showed uptake of brain and lung TuFMO-sEVPs (Fig. 1I and Supplementary Fig. 2B). Interestingly, the non-malignant mammary epithelial cell line MCF10A showed limited sEVP uptake as compared to the breast cancer cell lines, indicating that TuFMO-sEVP uptake is likely enhanced in breast cancer cells. Besides measuring sEVP uptake via PKH26 labeling, we also labeled the protein cargo of TuFMO-sEVPs to confirm their uptake by tumor cells via microscopic analysis (Supplementary Fig. 2C). Altogether, these findings suggest that breast cancer cells are generally able to take in TuFMO-sEVPs.

TuFMO-sEVPs have metastasis-promoting function

Remarkably, MCF7 cells changed their epithelial morphology upon brain TuFMO-sEVP uptake, adopting a spindle-shaped structure associated with reduced polarity (Supplementary Fig. 3A). This phenotypic switch resembled cells undergoing epithelial-to-mesenchymal transition (EMT), a process that cancer cells usually hijack to become more pro-metastatic. Accordingly, tumor cells educated with brain TuFMO-sEVPs showed increased expression of the EMT marker vimentin and reduced expression of the epithelial marker E-cadherin (Supplementary Fig. 3B-C).

In order to evaluate the effect of TuFMO-sEVPs on tumor growth and metastasis, the luminal type CDO223 breast cancer cells were educated with TuFMO-sEVPs prior to mammary fat pad injection (Fig. 2A). Primary tumor growth over time was not impacted by pre-education with TuFMO-sEVPs (Supplementary Fig. 3D). Importantly though, prior education of CDO223 cells with brain TuFMO-sEVPs led to a pronounced increase in the brain metastatic burden, with no effect on the primary tumor and lung metastatic burden (Fig. 2B, Supplementary Fig. 3E-F). The sEVP-depleted secretome of the ex vivo brain fragment culture did not impact on brain metastasis suggesting that the observed effect is sEVP-specific and soluble factors do not necessarily play a similar role in this context (Fig. 2B). Furthermore, inhibition of the cellular uptake of TuFMO-sEVPs by prior treatment with heparin hindered brain TuFMO-sEVP mediated metastasis, confirming that the uptake of TuFMO-sEVPs by tumor cells is required for its metastasis-promoting effect. This finding was recapitulated in a different breast cancer PDX model of the HER2-positive subtype (PDX1058), whereby prior education with brain-derived TuFMO-sEVPs led to a rise in brain metastatic incidence without affecting lung metastasis and the primary tumor burden (Supplementary Fig. 3G-H).

In analogy to the brain TuFMO-sEVPs, pre-education of CDO223 cells with lung-derived TuFMO-sEVPs significantly promoted lung metastasis, without affecting the brain metastatic or primary tumor burden (Fig. 2C, Supplementary Fig. 3I-J). To corroborate our findings with a different approach, we orthotopically injected mVenus+ MDA-MB-231 cells, which had been educated with lung TuFMO-sEVPs, in a 1:1 ratio with uneducated tdTomato+ MDA-MB-231 control cells (Fig. 2D). The percentage of lung sEVP-educated mVenus + cells which metastasized to the lung was significantly higher as compared to the control tdTomato + cells at the primary tumor endpoint (Fig. 2E). Finally, we also recapitulated the pro-metastatic and organotropic effect of the TuFMO-sEVPs in the immunocompetent 4T1-BALB/c model. Prior education of 4T1 cells with brain or lung-derived TuFMO-sEVPs promoted metastasis to the organ from which the TuFMO-sEVPs were derived from without affecting the primary tumor burden (Fig. 2F-G, Supplementary Fig. 3K). Taken together, our data show that breast cancer cells are able to uptake brain and lung TuFMO-sEVPs, which in turn promote metastasis and support organotropism of the tumor cells to the site from which the TuFMO-sEVPs originated.

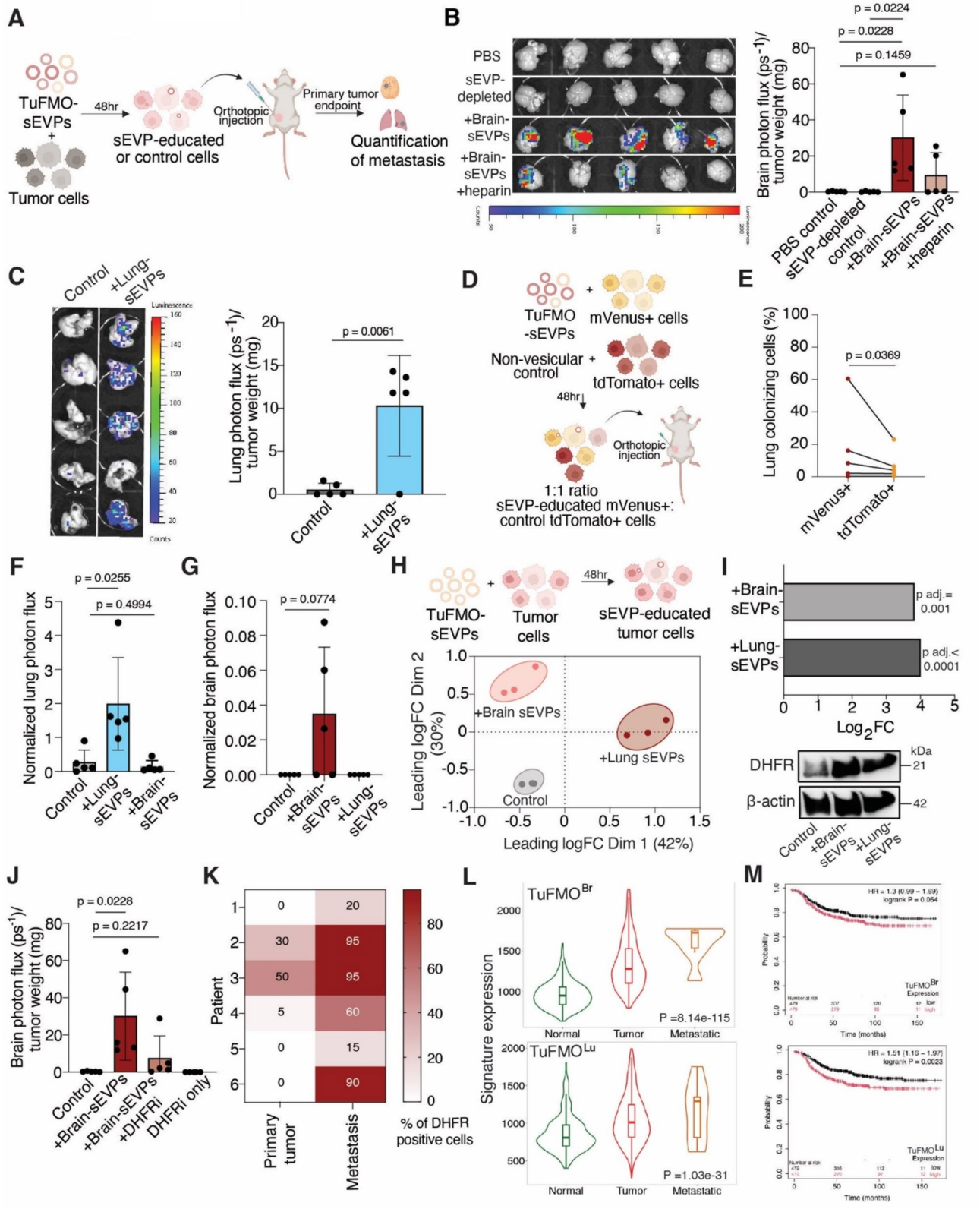

Fig. 2 (See legend on next page.)

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Fig. 2 Brain and lung TuFMO-sEVPs promote brain and lung metastasis respectively. (**A**) Experimental scheme to determine the effect of TuFMO-sEVPs on metastasis. (**B**) Representative brain bioluminescence images (left) and brain metastatic burden (right) in mice orthotopically injected with CDO223 cells pre-educated with brain TuFMO-sEVPs with/without 10 µg/ml heparin, PBS or sEVP-depleted secretome, $n = 5$ mice per group. (**C**) Representative lung bioluminescence images (left) and lung metastatic burden (right) in mice orthotopically injected with CDO223 cells pre-educated with lung TuFMO-sEVPs or PBS (control), $n = 5$ mice per group. (**D**) Experimental scheme and (**E**) Percentage (%) of lung colonizing cells in mice orthotopically injected with a 1:1 ratio of lung sEVP-educated mVenus+ MDA-MB-231 cells: control tdTomato+ MDA-MB-231 cells, $n = 5$. (**F**) Lung and (**G**) Brain metastatic burden in BALB/c mice orthotopically injected with 4T1 cells pre-educated with TuFMO-sEVPs or PBS (control), $n = 5$ mice per group. (**H**) Experimental scheme (top) and PCA (bottom) of the proteomic profile of patient-derived CDO223 cells educated with TuFMO-sEVPs based on global mass spectrometric analysis, $n = 3$ per condition. (**I**) Log fold change showing the expression of DHFR from mass spectrometric-based proteomics analysis of CDO223 cells (top) or immunoblots of 4T1 cells (bottom) after education with brain or lung TuFMO-sEVPs. β-actin is the loading control. (**J**) Brain metastatic burden in mice orthotopically injected with CDO223 cells pre-educated with brain TuFMO-sEVPs with/without 10 µM pyrimethamine (DHFR inhibitor), treated with 10 µM pyrimethamine only or PBS (control), $n = 5$ mice per group. (**K**) Percentage of DHFR-positive tumor cells in paired breast cancer patient primary tumor and metastatic samples, $n = 6$. (**L**) Expression of the signature derived from CDO223 cells educated with brain (top) or lung (bottom) TuFMO-sEVPs in TNMplot breast invasive carcinoma dataset comparing metastatic vs. primary tumor vs. normal samples, *p*-value from Kruskal-Wallis test. (**M**) Kaplan Meier plots of the distant metastasis-free survival of breast cancer patients with low (black line) or high (red line) brain ($TuFMO^{Br}$) (top) or lung ($TuFMO^{Lu}$) (bottom) signature using data from the Kaplan-Meier Plotter, n is indicated in each plot. Data information: 10 µg/ml sEVPs was used. Data is represented as mean ± SD, all replicates shown are biological replicates. Statistical significance was evaluated using two-tailed t-test (B, C, F, G, J) and two-tailed ratio paired t-test (E)

DHFR mediates TuFMO-sEVP directed breast cancer metastasis

In order to elucidate the molecular effects of TuFMO-sEVPs on metastasis, we performed unbiased global mass spectrometric-based proteomics analysis of CDO223 cells educated with pre-metastatic brain- or lung-derived TuFMO-sEVPs (Fig. 2H, top). Notably, prior education with TuFMO-sEVPs changed the proteomic profile of the breast cancer cells (Fig. 2H, bottom). Gene Ontology (GO) analysis revealed significant enrichment of pathways associated with brain morphogenesis, synapse organization, cell movement, signaling and metabolic modules important for survival in the brain specifically in tumor cells educated with brain-derived TuFMO-sEVPs (Supplementary Fig. 4A, left). Conversely, pathways related to JAK-STAT as well as interleukin signaling, taurine and alkanesulfonate metabolism were significantly enriched in tumor cells educated with lung TuFMO-sEVPs, indicating that specific TuFMO-sEVPs can potentially endow tumor cells with properties which promote their survival in the organs from which the sEVPs originate (Supplementary Fig. 4A, right).

To gain molecular insights into the general mechanism underlying TuFMO-sEVP mediated metastasis, we focused on candidate proteins which were elevated in the tumor cells upon uptake of brain and lung TuFMO-sEVPs. This revealed an enrichment of signaling proteins and proteins linked to metastasis such as *S100p*, *Dhfr* and *Mtfr2* while transport-related proteins like the solute carrier (SLC) family were found to be less abundant in tumor cells educated with TuFMO-sEVPs as compared to controls (Supplementary Fig. 4B, Supplementary Tables 2 & 3). Specifically, we confirmed an increase in the enzyme dihydrofolate reductase (DHFR) upon uptake of brain- and lung-derived TuFMO sEVPs by different breast cancer cell lines (Fig. 2I). Treatment of CDO223 cells educated with brain TuFMO-sEVPs with the FDA-approved DHFR inhibitor and antiparasitic drug pyrimethamine [13] abolished the metastatic promoting effect of the brain TuFMO-sEVPs without affecting the primary tumor burden (Fig. 2J, Supplementary Fig. 4C). This suggests a key role for DHFR in mediating the pro-metastatic effect of TuFMO-sEVPs in breast cancer. Accordingly, immunohistochemical staining of DHFR in paired longitudinal breast cancer patient samples of primary tumor vs. metastasis (CATCH cohort) [14] revealed a higher percentage of DHFR expressing tumor cells at the metastatic sites as compared to the primary tumor site (Fig. 2K, Supplementary Fig. 4D and Supplementary Table 4). This finding was further validated in a publicly available dataset [15] whereby a robustly higher expression of DHFR was found in the metastases of breast cancer patients as compared to the primary tumor or healthy samples (Supplementary Fig. 4E). Elevated DHFR expression was also associated with reduced distant metastasis-free survival in breast cancer patients (Supplementary Fig. 4F). Collectively, these findings demonstrate that TuFMO-sEVPs can induce specific molecular modules important for the survival of tumor cells in the organs from which they originate while also promoting DHFR expression in general, which then contributes to the enhanced metastatic capacity of the tumor cells.

TuFMO-sEVP driven organ-specific signature is enhanced in breast cancer metastasis

In order to determine the clinical relevance of our findings in breast cancer patients, we generated an organ-specific expression signature from the significantly enriched proteins identified in breast cancer cells educated with brain or lung TuFMO-sEVPs (Supplementary Table 5). Both brain and lung-related TuFMO-sEVP signatures ($TuFMO^{Br}$; $TuFMO^{Lu}$) were found to be more pronounced in breast cancer patients' metastatic samples as compared to the primary tumor or normal samples [15] (Fig. 2L). Furthermore, breast cancer patients whose tumors exhibited the $TuFMO^{Br}$ or $TuFMO^{Lu}$ signature

had shorter distant metastasis-free survival as well as overall survival (Fig. 2M, Supplementary Fig. 4G), indicating the contribution of TuFMO-sEVPs to breast cancer metastasis in the clinical setting as well.

Discussion

Metastasis is an intricate process relying heavily on intercellular communication, which has been proposed to be largely mediated by sEVPs shuttling between the tumor cells and the metastatic tissue [2]. While tumor-derived sEVPs have been widely shown to systemically modulate recipient cells [6, 7], the role of sEVPs secreted from pre-metastatic tumor-free organs have so far been overlooked and it remained unclear whether they promote or suppress metastasis. In this study, we provide evidence that sEVPs secreted from tumor-free metastatic-prone organs such as the brain and lung support metastasis via the reprogramming of breast cancer cells upon their uptake.

Herein, we used breast cancer PDX models to mimic the pre-metastatic stage, focusing on sEVPs secreted by tumor-free organs which will eventually be colonized such as the brain and lungs. Using our preparation method, we isolated vesicles showing a cup-shaped morphology with an average size of 150 nm. Small extracellular vesicles are generally heterogeneous in their physical and chemical properties and most standard techniques fail to accurately differentiate between the different types [5, 9]. Therefore, the term sEVPs was used as a shorthand to encompass all communicating small extracellular vesicles and particles which are less than 200 nm in size.

Intriguingly, our findings suggest that the primary tumor can remotely modulate the protein cargo of vesicles secreted by distant pre-metastatic organs such as the brain. Indeed, cancer is increasingly recognized as a systemic disease and tumor-derived secreted factors and sEVPs have been found to subvert non-cancerous cells at future metastatic sites at both the molecular and phenotypic level [2, 8]. Here, we demonstrated that sEVPs released from the pre-metastatic brain contain proteins which are typically involved in glycolysis, TCA cycle as well as cell cycle induction. This is in line with previous findings on the effect of tumor sEVP-derived miR-122 in reprogramming metabolism at the pre-metastatic niche in the brain [16]. Given the high energetic and biosynthetic demands of tumor cells, the metabolite-rich brain sEVPs could be especially beneficial to breast cancer cells disseminating to the brain.

Importantly, we confirmed the uptake of brain and lung-derived TuFMO-sEVPs by different types of breast cancer cells and demonstrated the metastatic organotropic effect of TuFMO-sEVPs on tumor cells. This pro-metastatic effect was observed in both immunocompromised PDX as well as immunocompetent models, suggesting that it is not necessarily restricted by the adaptive immune system. Upon uptake of TuFMO-sEVPs, the educated tumor cells adopted a distinct proteomic profile allowing for their survival and outgrowth at the respective metastatic site. Specifically, brain TuFMO-sEVP educated tumor cells showed an enrichment of proteins involved in pathways like synapse organization and fatty acid oxidation, which are processes known to be crucial for brain metastasis [17]. This also correlated with the presence of cargo proteins which are involved in these distinct processes such as ACAT2/3, PRAF2 and AGO1/2 in the brain TuFMO-sEVPs. Conversely, lung TuFMO-sEVP educated breast cancer cells showed a higher abundance of proteins involved in interleukin and STAT signaling in line with previous reports on the role of these signaling pathways in fostering lung metastasis [18]. We also found that in general, uptake of TuFMO-sEVPs led to increased DHFR level which was revealed to play a key role in mediating the pro-metastatic effect of TuFMO-sEVPs. DHFR is a folate cycle enzyme critical for the synthesis of DNA precursors such as purines and thymidylate which are in demand in highly proliferative cells and in line with previous studies [13], its inhibition by the FDA-approved antiparasitic drug pyrimethamine impeded metastasis, providing a potential therapeutic strategy for targeting this process.

Given that our model incorporates the use of sEVPs isolated from mouse organs with patient-derived breast cancer cells, we also developed an organ-specific signature from the enriched proteins in the TuFMO-sEVP educated tumor cells and validated the expression in a human metastatic breast cancer dataset showing an association with poor patient outcome [15]. It would be ethically challenging to isolate TuFMO-sEVPs from human pre-metastatic organs such as the brain and furthermore, using in vitro approaches would fail to capture intricate physiological processes which can only be modeled in vivo. Of note, bi-directional sEVP-driven communication has also been demonstrated between cancer cells and the hepatic niche using an all-human liver microphysiological system [19], supporting our findings that normal tissues secrete sEVPs which can be taken up by cancer cells. Interestingly though, in the reported study, hepatic sEVPs were found to suppress tumor cell proliferation, suggesting that the role of normal tissue-derived sEVPs can be context dependent. It is also noteworthy that both metastasis and metastatic organotropism are highly complex processes encompassing a multitude of governing factors and pathways. While we show here that brain and lung TuFMO-sEVPs have common pro-metastatic but distinct organotropic roles, the exact mechanisms underlying this organotropism remain a topic for future exploration given that this effect will likely be influenced by numerous other cellular and non-cellular factors within the metastatic cascade.

Conclusion

Our data collectively propounds that metastatic organs are not only passive recipients of tumor cells but that they can also secrete sEVPs which likely play a key role in the metastatic cascade. Given that tumor-derived sEVPs can travel to distant metastatic organs, it is plausible that TuFMO-sEVPs can conversely migrate to the primary tumor or interact with circulating tumor cells to promote the early stages of the metastatic cascade and metastatic organotropism. Future studies will be required to shed light on the physiological interactions between cancer cells and TuFMO-sEVPs from metastatic organs besides the brain and lungs and further explore the use of TuFMO-sEVPs as biomarkers in metastatic breast cancer patients. In conclusion, our study posits that the crosstalk of tumor cells with TuFMO-sEVPs helps in determining the cancer cell fate, providing a novel facet to our current understanding and management of metastatic organotropism and breast cancer metastasis.

Abbreviations

CDO	Circulating tumor cell-derived organoid
CTCs	Circulating tumor cells
EMT	Epithelial-to-mesenchymal transition
GO	Gene ontology
GSEA	Gene set enrichment analysis
HER2	Human epidermal growth factor receptor 2
MBC	Metastatic breast cancer
NTA	Nanoparticle tracking analysis
PBS	Phosphate buffered saline
PDX	Patient-derived xenograft
PMN	Pre-metastatic niche
sEVPs	Small extracellular vesicles and particles
TEM	Transmission electron microscopy
TuFMO	Tumor-free pre-metastatic organ

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

T.C. and A.T. conceived the study and designed the overall concept. T.C. performed all the molecular biology and mouse experiments and analyzed the data. R.Würth, P.S.K., T.V., R.Weber and S.L. assisted in experiments and data analysis. E.W. performed the immunohistochemical staining and analysis. D.H. and M.S. carried out and analyzed the results of the mass spectrometric analysis. K.N. assisted in the nanoparticle tracking analysis and M.N. and K.R. performed the electron microscopy. V.T. and L.L.M. provided clinical support and P.L. and A.S. helped in data interpretation. M.S. and A.T. supervised the project and discussed data and strategy. A.T. also acquired the funding for this study. The manuscript was written by T.C. and edited by M.S., R.Würth, P.S.K., M.N, K.R., D.H. and A.T. All authors read and approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Animal experiments were performed in compliance with the German legal regulations and were approved by the governmental review board of the state of Baden-Württemberg in Germany, operated by the local Animal Welfare Office (Regierungspräsidium Karlsruhe) under the license number G-169/22.

The use of patient-derived material for the establishment of PDX and CDOs used in this study was previously reported by our group (12) and the study was approved by the ethical committee of the University of Heidelberg (case number S-408/2013).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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