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TITLE: “Role of Extracellular miR-122 in Breast Cancer Metastasis”

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Report Title: Role of Extracellular miR-122 in Breast Cancer Metastasis

Abstract:
Reprogrammed glucose metabolism due to increased glycolysis and glucose uptake is a hallmark of cancer. The purpose of this study is to study the role of cancer-secreted miR-122 in adapting the pre-metastatic niche through the down-regulation of the glycolytic enzyme pyruvate kinase M2 (PKM2). We show that cancer cells can suppress glucose metabolism in lung fibroblasts and brain astrocyte niche cells through secreting extracellular miR-122, a miRNA whose level in the circulation predicts metastasis of breast cancer. Our results demonstrate that cancer cells are capable of reprogramming how niche cells metabolize glucose through exosome secretion of miR-122 and the consequent down-regulation of glucose metabolic enzymes in niche cells leading to reduced glucose utilization. In vivo treatment with anti-miR-122 oligos restores glucose uptake in distant organs, including brain and lungs, while decreasing the incidence of metastasis. Our results thereby demonstrate an important function of cancer-derived extracellular miR-122 in adapting glucose utilization of recipient niche cells, and thus reprograms systemic energy metabolism to facilitate disease progression.

Subject Terms: breast cancer, exosome, miR-122, glucose metabolism, PKM2, GLUT1, niche adaption

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1. **INTRODUCTION:**

Our data indicates that 1) miR-122 is highly secreted by BC cells and its level in the circulation is a marker for predicting metastatic progression in early-stage BC patients; 2) miR-122 down-regulates the glycolytic gene through targeting a conserved site in the 3’UTR; 3) suppression of PKM2 expression by miR-122 reduced the level of the GLUT1 causing reduced glucose uptake; and 4) anti-miR-122 therapy suppressed metastasis in a xenograft mouse model of human BC. Therefore, we hypothesized that BC-derived exosomal miR-122, in part by targeting PKM, reprograms systemic glucose utilization in niche cells for the creation of a favorable pre-metastatic environment to facilitate BC progression and metastasis. In depth functional analysis of cancer-secreted miR-122, which contributes to the adaptations of cancer-hosting niche, will provide novel information in the dynamic environmental crosstalk between cancer and host during disease progression. Furthermore, this study demonstrated the feasibility of targeting miRNAs to block the intercellular communication at an early stage and prevent cancer-directed reprogramming of pre-metastatic niches, specifically the brain and lung. Our future objectives are to validate the miR-122 pathway in glucose metabolism in primary BC, to identify patients that may benefit from anti-miR-122 treatment strategies, to understand the system-wide effects of BC-secreted miR-122 on energy metabolism reprogramming, and to identify how metastatic niche adaption occurs by circulating tumor cells.

2. **KEYWORDS:** breast cancer, exosome, miRNA, glucose metabolism, PKM2, GLUT1, niche adaption

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
  - Major Task 1: Exosome treatment of lung fibroblasts, brain astrocytes, and endothelial cells (niche cells)
    - Milestone Achieved: identification of specific gene targets of miR-122 and mechanism of glucose allocation in various niche cells. 100% completed in 2014.
  - Major Task 2: Exosome injection in NSG mice
- **Milestone Achievement:** Identification of miR-122 induced metastatic niche selection by circulating tumor cells. 100% completed.

- **Major Task 3:** Orthotopic xenograft tumors expressing high miR-122 and effectiveness of anti-miR-122 intervention
  - **Milestone Achievement:** Characterization of effects of breast cancer-secreted miR-122 on systemic glucose allocation reprogramming; 1-2 peer-reviewed articles. 100% completed.

- **What was accomplished under these goals?**
  1) Major activities include characterizing the extracellular vesicles isolated by ultracentrifugation from MCF10A/vec (control cell line), MCF10A/miR-122 (miR-122 specific over-expressing cell line), and breast cancer cell line MDA-MB-231 (which has high miR-122) by use of AF4 and sucrose gradient buoyant velocity (Fig. 1). MiR-122 over-expression was demonstrated to regulate glucose metabolism through the down-regulation of PKM2 (Fig. 2). Restoration of PKM2 reversed this suppression (Fig. 2). The 3 producer cell lines mentioned above were used to determine the effect of extracellular miR-122 on lung fibroblasts and brain astrocytes. Extracellular vesicles (EV) were labeled with DiI to measure EV uptake in niche cells (Fig. 3-4). EV were also isolated from the aforementioned cell lines and used to treat NSG mice i.v (Fig. 5). Xenograft tumors were established in NSG mice for over-expression of miR-122 in MCFDCIS cells (Fig. 6) and miR-122 intervention (Fig. 7).

  2) The specific objective was to examine the effect of extracellular miR-122 on PKM2, CS, GLUT1 expression (Fig. 2-5). Glucose metabolism was examined in EV-fed niche cells by 2-NBDG uptake and media metabolite measurement (Fig. 3-4). EV-treated mice were injected with 2-NBDG to measure glucose uptake (Fig. 5a-c). Cell type specific markers were used to identify astrocytes (GFAP) and fibroblasts (FSP-1). Human specific CD63 was used as an exosome marker. MiR-122, PKM, and GLUT1 expression was also measured (Fig 4d-e). Circulating tumor cells were injected intracardiac to measure metastatic colonization of the pre-metastatic niche. Functional studies to characterize miR-122 over-expression were performed in a poorly metastatic model with
endogenously low miR-122 expression (MCFDCIS, Fig. 6). The effectiveness of miR-122 intervention was performed using highly metastatic MDA-MB-231-HM xenograft tumors with treatment of anti-miR-122 oligos (Fig. 7).

3) Results indicate that extracellular miR-122 reduces PKM RNA and protein (Fig. 3-5) which can be alleviated by treating fibroblasts and astrocyte in vitro or mice with anti-miR-122 oligos. The reduction of GLUT1 as a by-production of reduced PKM resulted in reduced glucose uptake measured by 2-NBDG uptake and media metabolite analysis (Fig. 2-4). Restoration of PKM or GLUT1 restored glucose uptake (Fig. 2I-L). To determine if the reduction of PKM or GLUT1 alone was sufficient to reduce glucose uptake, siRNA against PKM2 or GLUT1 was used in lung fibroblasts and cells were treated with 2-NBDG. Knock-down of either PKM2 or GLUT1 was sufficient to reduce glucose uptake (Fig. 3k). Additionally when conditioned media was collected from fibroblasts transfected with siRNA and fed to cancer cells, the additional glucose in the media resulted in an increase of cancer cell proliferation measured by BrdU incorporation (Fig. 3I). Metastatic colonization of the lung and brain was enhanced in mice pre-treated with extracellular vesicles high in miR-122 (Fig. 5f-g). The reduction of PKM and GLUT1 was sufficient to reduce glucose uptake in brain and lung tissues to promote metastasis (Fig. 5). Over-expression of miR-122 reduced primary tumor growth through the down-regulation of PKM2 and GLUT1 resulting in lower ATP levels (Fig. 6a-h). We also observed enhanced metastasis to the brain and lungs of mice bearing miR-122 expressing tumors (Fig. 6i-j). The effect of miR-122 to re-program systemic glucose allocation and promote metastasis was alleviated by treating mice with anti-miR-122 oligos (Fig. 7).

- What opportunities for training and professional development has the project provided?
  - I have had training activities including one-on-one work with my mentor.
  - I presented at the bi-monthly department seminar series and an international poster session at Keystone Symposium: Integrating Metabolism and Tumor Biology in January 2015.
- Presentation Training class, February – August 2015.
- Trained a summer undergraduate student and continuing to train pre-doctoral graduate students.

- **How were the results disseminated to communities of interest?**
  - Poster session at Keystone Symposium.
  - Publication of manuscript in *Nature Cell Biology* 17: 183-194.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - The regulation of miR-122 will be examined as proposed.
  - Attend “Cancer Biology” and “Scientific grant writing for the basic scientist” courses at City of Hope.
  - Continue training pre-doctoral students.

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?**
     - Nothing to report.
   - **What was the impact on other disciplines?**
     - Nothing to report.
   - **What was the impact on technology transfer?**
     - Nothing to report.
   - **What was the impact on society beyond science and technology?**
     - Nothing to report

5. **CHANGES/PROBLEMS:**
   - Changes in approach and reasons for change
     - Nothing to report.
   - **Actual or anticipated problems or delays and actions or plans to resolve them**
     - Nothing to report.
- Changes that had a significant impact on expenditures
  - Nothing to report.
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
  - Nothing to report.

6. PRODUCTS:
- Publications, conference papers, and presentations
  - Journal publications.
  - Books or other non-periodical, one-time publications.
    - Nothing to report
  - Other publications, conference papers, and presentations.
    - Keystone Symposium: Integrating Metabolism and Tumor Biology, Poster J1 1047 “The role of miR-122 in breast cancer metastasis.” Vancouver, BC, Canada.
    - Department of Cancer Biology Seminar Series, 2015 (Local)
- Website(s) or other Internet site(s)
  - Nothing to report
- Technologies or techniques
  - Nothing to report
- Inventions, patent applications, and/or licenses
  - Nothing to report.
- Other Products
  - Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
- What individuals have worked on the project?

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<tr>
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<th>Miranda Fong</th>
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- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report

- **What other organizations were involved as partners?**
  - Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS:** Nothing to report

9. **APPENDICES:**

Keystone Symposium Poster Abstract:

In our previous study, higher levels of miR-122 were associated with metastatic progression in early-stage BC patients, suggesting a pro-malignant role of miR-122. Sequence analysis predicted a single, species-conserved miR-122 binding site in the 3’UTRs of PKM and CS. Therefore, we cloned the miR-122 binding sites in these two genes as well as their seed-sequence-mutated version into a reporter luciferase
plasmid. The wild-type but not mutated site responded to exogenously expressed miR-122 by directing ~50% reduction of reporter gene expression. The critical role of PKM1/2 and CS in glycolysis and TCA cycle urged us to explore the function of miR-122 in regulating glucose metabolism. MCF10A/miR-122 but not MCF10A/vec cells exhibited significantly reduced PKM2, CS, and GLUT1. Metabolome analysis of the cells by NMR spectroscopy revealed significantly decreased intracellular glucose and pyruvate in MCF10A/miR-122 cells. We next analyzed changes of metabolites in the media of MCF10A cells with or without miR-122 over-expression after 72 h of cell culture. Cells over-expressing miR-122 displayed ~50% decreased glucose uptake from the media and ~40% reduced lactate production after normalization to cell number. Modestly diminished glutamine metabolism was also observed in these cells. We established an xenograft BC model using MDA-MB-231-HM cells in the mammary fat pad of NSG mice. Mice were treated with PBS, mismatch control oligos, or anti-miR-122 oligos. Bioluminescence imaging (BLI) at week 5 started to reveal suppressed metastasis in mice receiving anti-miR-122 treatment, particularly to the lung and brain. The mechanism of miR-122 to promote metastasis by downregulating these targets is currently under investigation.

Published manuscript below.
Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis

Miranda Y. Fong, Weiyiing Zhou, Liang Liu, Aileen Y. Alontaga, Manasa Chandra, Jonathan Ashby, Amy Chow, Sean Timothy Francis O’Connor, Shasha Li, Andrew R. Chin, George Somlo, Melanie Palomares, Zhuo Li, Jacob R. Tremblay, Akihiro Tsuyada, Guoqiang Sun, Michael A. Reid, Xiwei Wu, Piotr Swiderski, Xiubao Ren, Yanhong Shi, Mei Kong, Wenwan Zhong, Yuan Chen and Shizhen Emily Wang

Reprogrammed glucose metabolism as a result of increased glycolysis and glucose uptake is a hallmark of cancer. Here we show that cancer cells can suppress glucose uptake by non-tumour cells in the premetastatic niche, by secreting vesicles that carry high levels of the miR-122 microRNA. High miR-122 levels in the circulation have been associated with metastasis in breast cancer patients, and we show that cancer-cell-secreted miR-122 facilitates metastasis by increasing nutrient availability in the premetastatic niche. Mechanistically, cancer-cell-derived miR-122 suppresses glucose uptake by niche cells in vitro and in vivo by downregulating the glycolytic enzyme pyruvate kinase. In vivo inhibition of miR-122 restores glucose uptake in distant organs, including brain and lungs, and decreases the incidence of metastasis. These results demonstrate that, by modifying glucose utilization by recipient premetastatic niche cells, cancer-derived extracellular miR-122 is able to reprogram systemic energy metabolism to facilitate disease progression.

Reprogrammed energy metabolism to fuel rapid cell growth and proliferation is an emerging hallmark of cancer. Most cancer cells use aerobic glycolysis with reduced mitochondrial oxidative phosphorylation for glucose metabolism even when oxygen is sufficient. This phenomenon, known as the ‘Warburg effect’, favours the uptake and incorporation of nutrients needed to produce a new cell. To compensate for the consequent reduction in ATP production, cancer cells often adopt mechanisms to increase glucose uptake and utilization. One mechanism involves the regulation of glucose transporters, among which GLUT1 (also known as SLC2A1) is responsible for basal levels of glucose uptake in all cells. GLUT1 can be regulated by the PI3K/AKT/mTOR pathway, which is frequently activated in cancer. Furthermore, hypoxia can stimulate glucose uptake and metabolism through HIF-1 by inducing GLUT3 and glycolytic genes ALDA, PGK1 and PKM (pyruvate kinase; refs 6,7). It was recently reported that phosphorylation or sumoylation of PKM2 leads to translocation to the nucleus, where it acts as a transcriptional co-activator to induce GLUT1, PDK1 and HK1 (refs 8–13). Here we focus on a mechanism mediated by a cancer-secreted microRNA (miRNA) that reallocates glucose to favour uptake by cancer cells.

MiRNA negatively regulates gene expression by binding to the 3’ untranslated region (3’ UTR) of messenger RNA, leading to degradation or translation blockade. Deregulation of miRNA is tightly linked to cancer, and circulating miRNA has emerged as a potential biomarker for cancer diagnosis and prognosis. MiRNA can be secreted into the extracellular environment through membrane-enclosed vesicles (such as exosomes) or in

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miR-122 is highly secreted by cancer cells. (a,b) RNA was extracted from the 110,000g medium pellet (a) and PBS-washed cells (b) and analysed for miR-122 by quantitative PCR with reverse transcription (RT-qPCR). Data were normalized to levels of total proteins (secreted; a) or U6 (cellular; b), and compared with the non-tumour line MCF10A (n=6 extracts). (c) Representative electron microscopy images of vesicles in the 110,000g medium pellet. Scale bar: 100 nm. (d) Size distribution of vesicles identified in the 110,000g medium pellets (n=25 vesicles for MCF10A/vec; n=38 for MCF10A/miR-122; n=94 for MDA-MB-231). (e) Fractogram (UV absorption at 280 nm) for the AF4 eluates characterizing the MDA-MB-231 110,000g medium pellet. (f) A representative electron microscopy image of MDA-MB-231-derived vesicles in the fraction eluted at 18–25 min. The measured diameter of vesicles is shown as mean ± s.d. (n=41). Scale bar: 100 nm. (g) RT-qPCR-determined levels of miR-122 and miR-16 in MDA-MB-231-derived protein and vesicle fractions separated by AF4 (n=6 extracts). Absolute miRNA levels are calculated on the basis of standard curves. ND: not detected. (h) After sucrose gradient centrifugation of MDA-MB-231-derived 110,000g medium pellet, the absolute miRNA level in each gradient fraction was determined by RT-qPCR and calculated on the basis of standard curves (n=6 extracts). A representative electron microscopy image of MDA-MB-231-derived vesicles in sucrose fraction 5 (F5) is shown. Scale bar: 100 nm. *P<0.05 for all panels, derived from Kruskal–Wallis test. Data are represented as mean ± s.d. in all panels except c–e.

complexes with protein or lipid-based carriers. Accumulating evidence demonstrates that miRNA as well as proteins can be transferred to neighbouring or distant cells in these secretory forms to modulate cell function. Extracellular miRNA is therefore emerging as a new group of messengers and effectors in intercellular communication.

Several miRNAs have been implicated in metabolism and metabolic disorders. Among them, miR-122 regulates cholesterol efflux, liver triglyceride content and the rate of β-oxidation. Potential miR-122 targets have been analysed by luciferase-reporter-based 3’UTR screening, identifying PKM as one of the targets, which suggests that miR-122 may play a role in glucose metabolism. Our recent study in breast cancer (BC) patients identified higher levels of circulating miR-122 as a marker for predicting metastatic progression in early-stage BC (ref. 18). This urged us to investigate the function of extracellular miR-122 in cancer progression and metastasis. Here we demonstrate that cancer-secreted miR-122 can be transferred to normal cells in the premetastatic niche, thereby suppressing glucose utilization in these cells to accommodate the massive energy needs of cancer cells during metastatic growth.
Figure 2 miR-122 suppresses glucose metabolism by downregulating PKM. (a) BrdU uptake in indicated cells was analysed by flow cytometry (n=6 biological replicates). (b) Quantification of intracellular metabolites by NMR spectroscopy (n=3 biological replicates). (c) Glycogen staining (red) in MCF10A/vec and MCF10A/miR-122 cells. Scale bar: 100 μm. (d) Change of metabolites in the CM after 72 h culture (n=6 biological replicates). (e) The predicted miR-122 binding site in the 3′UTR of the human PKM and CS genes. The corresponding sequence in the mutated (mt) version is also shown. (f) The psiCHECK reporters containing 3′UTR of human PKM and CS genes with wild-type (wt) or mutated (mt) miR-122 binding sites were used to transfect MCF10A cells stably expressing miR-122 or the empty vector (as control). Luciferase activity was analysed at 48 h post-transfection (n=6 extracts) and the ratio between Renilla luciferase and firefly luciferase activities (Rluc/Fluc) is shown. (g) Determination of PKM isoforms expressed in MCF10A and MDA-MB-231. RNA was subjected to RT-PCR followed by digestion with Ncol (N), PstI (P) or both enzymes (NP), plus an uncut control (U). Products were separated on an agarose gel with SYBR Safe. The presence of a PstI digestion site indicates the splicing isoform M2, whereas the Ncol site indicates isoform M1. Sizes of markers (in base pairs) are indicated. (h) RT-qPCR analysis showing the relative expression of indicated genes in MCF10A/miR-122 and MCF10A/vec cells (n=6 extracts). (i) Western blot analysis in MCF10A/miR-122 and MCF10A/vec cells with restored expression of PKM2 and CS. Sizes of markers (in kilodaltons, kDa) are indicated. (j) RT-qPCR analysis in selected colonies with restored expression of PKM2 and CS (n=6 extracts). (k) PKM activity (units) in 5 μg protein in indicated cells (n=6 extracts). (l) Change of glucose in the medium after 72 h culture of selected clones normalized to cell number (n=3 biological replicates). P < 0.05, **P < 0.01 for all panels, derived from Kruskal–Wallis test. Data are represented as mean ± s.d. in all panels except c,e,g and i. Uncropped images of blots and gels are shown in Supplementary Fig. 5.
Figure 3 Cancer-secreted miR-122 downregulates glucose uptake in lung fibroblasts. (a) Uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethyldisocarbocyanine perchlorate (DiI)-labelled exosome-containing EVs (prepared from the indicated producer cells) at 48 h. Scale bar: 60 µm. (b) Levels of miRNAs in fibroblasts pretreated with 10 nM RNA polymerase II inhibitor 5,6-dichloro-1-β-D-ribofuranoside (DRB) for 2 h followed by treatment with EVs for 16 h. miR-122 levels were normalized to U6 (n=6 extracts). (c) Determination of PKM isoforms in fibroblasts and mouse tissues by RT-PCR as indicated in Fig. 2g. Sizes of markers (in base pairs) are indicated. (d) Fibroblasts were treated with two doses of EVs from the indicated producer cells given 48 h apart and transfected with anti-miR-122 oligonucleotides or mismatch control oligonucleotides, before analysis at 96 h by (e) western blot analysis (with marker size indicated in kDa), (f) PKM activity assay using 5 µg of proteins (n=5 biological replicates), (g) 2-NBDG uptake (n=6 biological replicates) and (h) change of glucose in the CM (n=9 biological replicates). (i) Change of glucose in the CM (n=6 biological replicates). Fluorescent intensity was compared with the first column, which served as a control. (j) CM was collected from siRNA-transfected fibroblasts cultured for 72 h and the glucose concentration measured. The CM was then fed to MDA-MB-231-HM cells before proliferation was assessed by BrdU incorporation at 72 h (n=6 biological replicates). (m) Circulating EVs were extracted from pooled healthy donor sera and from BC patients’ sera with low or high vesicular miR-122 and used to treat fibroblasts, and 2-NBDG uptake was measured (n=8 biological replicates). Fluorescent intensity was compared with the first column, which served as a control. *P < 0.05, **P < 0.01 for all panels, derived from Kruskal–Wallis test. Data are represented as mean ± s.d. in all panels except a,c,e and j. Uncropped images of blots and gels are shown in Supplementary Fig. 5.
RESULTS

miR-122 is highly secreted by cancer cells

We first examined the conditioned medium (CM) of various breast cell lines for miR-122 secretion. We focused on the 110,000g medium pellet that is known to contain extracellular vesicles (EVs) including exosomes, and that carried the majority of extracellular miR-122, compared with the supernatant fraction (Supplementary Fig. 1a). All BC lines secreted significantly elevated miR-122 when compared with non-cancerous MCF10A (Fig. 1a). This was not accompanied by an elevated intracellular level, as most cancer lines exhibited reduced intracellular miR-122 (Fig. 1b). Whereas MCF10A-derived vesicles all exhibited a diameter of 30–100 nm, representing exosomes, vesicles from the BC line MDA-MB-231 were more heterogeneous and contained more than 50% exosomes, with the rest being microvesicles larger than 100 nm (Fig. 1c,d), consistent with a previous study.11 Further characterization of the medium pellet by asymmetrical flow field flow fractionation (AF4; ref. 32) revealed two peaks representing proteins (eluted at 8–11 min) and vesicles (eluted at 18–25 min) measuring 30–60 nm (averaged ≈ 35 nm for MDA-MB-231), but a lack of high-density lipoproteins (eluted at 11–16 min; Fig. 1e,f and Supplementary Fig. 1b, ref. 32). For MDA-MB-231, miR-122 was exclusively detected in the vesicle but not the protein fraction, whereas forced overexpression of miR-122 in MCF10A increased miR-122 secretion predominantly in vesicles, with a slight induction also detected in protein-associated form (Fig. 1g and Supplementary Fig. 1c). Secretion of miR-122 by MCF10A cells engineered to stably overexpress the control vector (MCF10A/vec) was below the detection limit in fractionated samples. By gradient centrifugation of the medium pellet we further determined that, for both MDA-MB-231 and MCF10A-derived lines, miR-122 and miR-16 peaked in fractions 5 and 6, which contained vesicles measuring 30–100 nm (Fig. 1h and Supplementary Fig. 1d,e). Overall, our results indicate that cancer cells specifically secrete high levels of miR-122 into EVs including exosomes, and suggest that the potential effect of cancer-derived miR-122 may be ectopically observed in the recipient cells on EV-mediated transfer rather than in the cancer cells producing it.

miR-122 suppresses glucose metabolism by downregulating PKM

To study the function of miR-122, we first used MCF10A cells engineered to stably overexpress miR-122 (MCF10A/miR-122) or the control vector (MCF10A/vec). MCF10A/miR-122 had significantly reduced proliferation measured by 5-bromodeoxyuridine (BrdU) incorporation (Fig. 2a). Metabolome analysis of the cells revealed significantly decreased intracellular glucose and pyruvate in MCF10A/miR-122 (Fig. 2b and Supplementary Fig. 2), along with increased UDP-glucose (Fig. 2b) and glycogen staining (Fig. 2c) that is probably due to the excessive glucose spared from glycolysis going towards storage. Furthermore, the ATP level in MCF10A/miR-122 was not significantly decreased intracellular glucose and pyruvate in MCF10A/miR-122 (Fig. 1b). Whereas MCF10A-derived vesicles all exhibited a diameter of 30–100 nm, representing exosomes, vesicles from the BC line MDA-MB-231 were more heterogeneous and contained more than 50% exosomes, with the rest being microvesicles larger than 100 nm (Fig. 1c,d), consistent with a previous study.11 Further characterization of the medium pellet by asymmetrical flow field flow fractionation (AF4; ref. 32) revealed two peaks representing proteins (eluted at 8–11 min) and vesicles (eluted at 18–25 min) measuring 30–60 nm (averaged ≈ 35 nm for MDA-MB-231), but a lack of high-density lipoproteins (eluted at 11–16 min; Fig. 1e,f and Supplementary Fig. 1b, ref. 32). For MDA-MB-231, miR-122 was exclusively detected in the vesicle but not the protein fraction, whereas forced overexpression of miR-122 in MCF10A increased miR-122 secretion predominantly in vesicles, with a slight induction also detected in protein-associated form (Fig. 1g and Supplementary Fig. 1c). Secretion of miR-122 by MCF10A cells engineered to stably overexpress the control vector (MCF10A/vec) was below the detection limit in fractionated samples. By gradient centrifugation of the medium pellet we further determined that, for both MDA-MB-231 and MCF10A-derived lines, miR-122 and miR-16 peaked in fractions 5 and 6, which contained vesicles measuring 30–100 nm (Fig. 1h and Supplementary Fig. 1d,e). Overall, our results indicate that cancer cells specifically secrete high levels of miR-122 into EVs including exosomes, and suggest that the potential effect of cancer-derived miR-122 may be ectopically observed in the recipient cells on EV-mediated transfer rather than in the cancer cells producing it.

The metabolomic changes observed in MCF10A/miR-122 suggested a role for miR-122 in glucose metabolism. TargetScan and microRNA.org algorithms predicted a single, species-conserved miR-122 binding site in the 3′ UTRs of pyruvate kinase (PKM) and citrate synthase (CS) genes (Fig. 2e). Therefore, we PCR-cloned the 3′ UTRs and their seed-sequence-mutated versions downstream of the open reading frame (ORF) of a Renilla luciferase reporter gene and assessed the ability of miR-122 to downregulate luciferase expression. For both PKM and CS, the wild-type but not the mutated 3′UTR responded to miR-122 by directing about 50% reduction of reporter gene expression (Fig. 2f). Among a panel of genes controlling glucose metabolism, MCF10A/miR-122 exhibited significantly reduced PKM2 (isoform determined in Fig. 2g), CS and GLUT1 (Fig. 2h–j). Consistent with PKM2 downregulation, miR-122 also caused a significant reduction of PKM enzymatic activity (Fig. 2k).

To further determine if the miR-122-induced decrease in glucose consumption was mediated by PKM2 and/or CS downregulation, we restored the expression of these genes in MCF10A/miR-122 by overexpressing the PKM2 or CS complementary DNA that lacked the 3′ UTR. Both clones with fully or partially restored PKM activity (Fig. 2k) showed restored GLUT1 expression and glucose uptake from the medium that were comparable to those with MCF10A/vec (Fig. 2i,j). This is consistent with the previously reported ability of PKM2 to induce c-Myc and GLUT1 expression as a nuclear co-activator of β-catenin.9 We noticed that restoration of CS by exogenous expression was always accompanied by elevated expression of endogenous PKM2 (Fig. 2i,j), possibly reflecting a natural feedback mechanism to accommodate the increased need for pyruvate by enhanced CS activity. Although this hindered us from dissecting the role of CS downregulation in mediating miR-122’s function without the concomitant regulation of PKM2, our results indicate that restoration of PKM2 alone is sufficient to abolish the effect of miR-122 on glucose uptake; therefore, we chose to focus on this miR-122/PKM-mediated effect for the rest of this study.

Cancer-secreted miR-122 downregulates glucose consumption in niche cells

To study the ectopic effect of cancer-secreted miR-122, we focused on lung fibroblasts, brain astrocytes and neurons that are abundantly present in the premetastatic sites of BC. Primary lung fibroblasts exhibited efficient uptake of exosome-containing EVs regardless of the producer cells, as indicated by the internalization of Dil-labelled EVs (Fig. 3a). In these cells, EVs that are high in miR-122 caused significantly increased intracellular miR-122, which was not affected by an RNA polymerase II inhibitor (Fig. 3b), indicating that this increase of miR-122 reflects the EV-mediated miRNA transfer but not an induction of miR-122’s endogenous expression. Also observed in high-miR-122 EV-treated fibroblasts were decreased expression of PKM2 and GLUT1 (Fig. 3c–e), along with decreased PKM activity (Fig. 3f). In addition, high-miR-122 EVs significantly reduced recipient cells’ uptake of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), a fluorescent analogue of glucose, which has been used to assess glucose transport in various cell types15–16 (Fig. 3g), as well as their glucose consumption from the medium (Fig. 3h). These effects were significantly suppressed by treating recipient cells with anti-miR-122 (Fig. 3d–h).
Restored expression of PKM2 or GLUT1 by transfecting fibroblasts with the corresponding cDNA construct lacking 3′UTR abolished the effect of high-miR-122 EVs on fibroblast glucose consumption (Fig. 3i,j). In contrast, knockdown of either PKM2 or GLUT1 significantly reduced 2-NBDG uptake (Fig. 3k). We next assessed the effect of fibroblast glucose metabolism on proliferation of cancer cells sharing the same medium. Medium from fibroblasts treated with high-miR-122 EVs had significantly increased 2-NBDG uptake (Fig. 3k). We further examined the effect of circulating EVs isolated from the sera of healthy donors or BC patients. High-miR-122 EVs derived from a BC patient significantly reduced 2-NBDG uptake (Fig. 3l). We next assessed the effect of fibroblast glucose metabolism on proliferation of cancer cells sharing the same medium. Medium from fibroblasts treated with high-miR-122 EVs had significantly increased 2-NBDG uptake (Fig. 3k). We further examined the effect of circulating EVs isolated from the sera of healthy donors or BC patients. High-miR-122 EVs derived from a BC patient significantly reduced 2-NBDG uptake (Fig. 3l). We next assessed the effect of fibroblast glucose metabolism on proliferation of cancer cells sharing the same medium. Medium from fibroblasts treated with high-miR-122 EVs had significantly increased 2-NBDG uptake (Fig. 3k). We further examined the effect of circulating EVs isolated from the sera of healthy donors or BC patients. High-miR-122 EVs derived from a BC patient significantly reduced 2-NBDG uptake (Fig. 3l).

Cancer-secreted miR-122 reprograms glucose consumption in niche tissues and promotes metastasis

To verify that cancer-secreted miR-122 modulates glucose metabolism in the premetastatic niches in vivo, we intravenously injected exosome-containing EVs with low or high levels of miR-122 into mice and measured glucose uptake in brain and lungs. Co-immunofluorescence
Figure 5 Vesicular transfer of miR-122 alters glucose uptake in niche tissues. Indicated EVs were intravenously injected into the tail veins of NOD/SCID/IL2Rγ-null (NSG) mice biweekly for 3.5 weeks. (a) Co-immunofluorescence of exosome marker CD63 (detected by a human-specific antibody; red) with astrocyte marker GFAP (white) or fibroblast marker FSP-1 (white) in brain and lung tissues of mice injected with 2-NBDG (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). White bar: 20 µm. (b) Quantification of 2-NBDG uptake in brain (n=20 fields from four mice). (c) Quantification of 2-NBDG uptake in lung (n=20 fields from four mice). (d,e) RT-qPCR in brain (d) and lungs (e) (n=9 extracts from three mice). (f,g) Luciferase qPCR for the detection of metastases in the brain (f) and lungs (g) of mice pretreated with EVs followed by an intracardiac injection with luciferase-labelled MDA-MB-231-HM tumour cells (n=15 extracts from five mice). (h) miR-122 levels determined by RT-qPCR in the serum of non-tumour-bearing mice (group 1), mice bearing MCFDCIS or MDA-MB-231-HM tumours (groups 2 and 3) and non-tumour-bearing mice treated with EVs from MCF10A/vec, MCF10A/miR-122 or MDA-MB-231 cells (groups 4–6) (n=15 extracts from five mice). Data were normalized to the levels of miR-16. Data are represented as mean ± s.d. for all panels except a. ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001 for all panels, derived from Kruskal–Wallis test.
Figure 6  In vivo effect of miR-122 on primary tumour growth and metastasis.

(a) Tumour growth curve in mice carrying MCFDCIS/vec and MCFDCIS/miR-122 orthotopic xenografts (n = 7 mice). (b) Bioluminescence imaging (BLI) at week 3. (c) Luciferase quantification of b (n=7 mice), p/s: photons per second. (d) Immunohistochemistry (IHC) for Ki67, PKM1/2 and GLUT1 in tumour, brain and lung sections. Scale bar: 100 µm. (e) Quantification of Ki67+ tumour cells from three fields per tumour (n = 6 mice per group). (f) Intratumoral levels of ATP assessed in tumour lysates by ENLIGHTEN assay (normalized to U6). (g) miR-122 expression (normalized to U6). (h) 2-NBDG uptake quantification in the tumour, brain and lungs (n = 12 fields from four mice per group). (i,j) Luciferase qPCR in the brain (i, n = 24 extracts from eight mice per group) and lungs (j, n = 18 extracts from six mice per group) of MCFDCIS tumour-bearing mice. (k) Tumour growth curve in mice carrying orthotopic xenografts of MDA-MB-231 with stable knockdown of miR-122 (MDA-MB-231/122KD) and also receiving EV treatments as indicated (n = 7 mice per group). No significant difference (P > 0.05) between groups, on the basis of the Kruskal–Wallis test. (I,m) Luciferase qPCR for detection of metastases in the brain (l) and lungs (m) of mice bearing MDA-MB-231/122KD tumours and treated with the indicated EVs (n=12 extracts from four mice per group). *P < 0.05, **P < 0.01, ***P < 0.001 for all panels, derived from Kruskal–Wallis test. Data are represented as mean ± s.d. in all panels except b and d.
**Figure 7** miR-122 intervention alleviates cancer-induced glucose reallocation *in vivo* and reduces metastasis. Luciferase-labelled MDA-MB-231-HM cells were injected into the no 4 mammary fat pad of NSG mice. Mice were divided into three groups (*n*=8 mice per group) for treatment with PBS, anti-miR-122 or mismatch control oligonucleotides. (a) Tumour growth curve (*n*=8 mice). No significant difference (*P* > 0.05) between groups, on the basis of the Kruskal–Wallis test. (b) 2-NBDG uptake in the tumour and tumour-adjacent stroma (*n*=20 fields from four mice per group). (c) Primary tumour sections were analysed by IHC (for PKM2 and GLUT1) and *in situ* hybridization (ISH) (for miR-122). For 2-NBDG (green) uptake, sections were counterstained with DAPI (blue) to show nuclei. The dotted lines delineate tumour (T) from stroma (S). White bar: 100 μm. (d) BLI at week 5 indicating extensive brain and lung metastases in PBS and mismatch groups and reduced incidence of metastasis in anti-miR-122 group. (e) Quantification of BLI at week 5 (*n*=8 mice per group). p/s: photons per second. (f) Representative images of 2-NBDG uptake in brain and lungs. White bar: 60 μm. (g) Quantification of 2-NBDG uptake in the brain and lungs of tumour-free (normal) NSG mice and tumour-bearing mice that were untreated when killed at week 3 after tumour cell implantation or that were untreated when killed at week 3 after tumour cell implantation or treated as indicated and killed at week 6 (*n*=20 fields from four mice per group). (h,i) RT-qPCR in brain (h) and lungs (i) of tumour-free and tumour-bearing mice (*n*=12 extracts from four mice per group). (j) GLUT1 IHC in brain and lungs. Scale bar: 60 μm. Data are represented as mean ± s.d. in all panels except c,d,f and j. *P* < 0.05, **P** < 0.01, ***P*** < 0.001 for all panels, derived from Kruskal–Wallis test.
of cell-type-specific markers for astrocytes and fibroblasts and a
human-specific exosomal marker, CD63, demonstrated that human-
derived EVs can be received by these niche cell types in vivo
(Fig. 5a). Both brain and lungs showed reduced 2-NBDG uptake as a
consequence of receiving vesicular miR-122, which resulted in reduced expression of PKM and GLUT1 (Fig. 5b–e).

In another experiment, mice were pretreated with EVs before an
intracardiac injection of luciferase-labelled MDA-MB-231-HM cells.
Three weeks later, metastases in the brain and lungs were quantified
by luciferase qPCR and confirmed by histology. Among mice receiving
high-miR-122 EVs, all exhibited significant metastatic colonization
in lungs and brain, whereas no metastases were observed in mice
treated with MCF10A/vec EVs or PBS (Fig. 5f,g). Serum miR-122
levels were comparable between mice receiving high-miR-122 EVs and
those bearing MDA-MB-231-HM tumours that naturally secrete miR-
122 (Fig. 5h). Therefore, vesicular miR-122 results in reprogramming
of niche tissue glucose utilization as a possible mechanism to promote
circulating tumour cell colonization.

miR-122 overexpression reduces primary tumour growth while
enhancing metastasis
To determine if the miR-122 level in primary tumours regulates
tumour growth and if the primary-tumour-secreted miR-122
adapts the premetastatic niches to promote metastasis, we stably
overexpressed miR-122 in an MCF10A-derived tumorigenic line,
MCFCDCIS, which forms comedo ductal carcinoma in situ-like
lesions that spontaneously progress to invasive tumours39. miR-122
overexpression reduced cell proliferation, glucose uptake and the
expression of PKM2, CS and GLUT1 in vitro (Supplementary
Fig. 3a–d). Orthotopic xenograft tumours of MCFCDCIS/miR-122
were significantly smaller than MCFCDCIS/vec tumours (Fig. 6a–c),
containing decreased numbers of Ki67+ tumour cells, reduced
expression of PKM2 and GLUT1 and decreased ATP level (Fig. 6d–f).

Mice bearing MCFCDCIS/miR-122 tumours had increased miR-122
and decreased PKM1/2 and GLUT1 in the brain and lungs as well
as significantly reduced 2-NBDG uptake in the brain, which was not
observed in the lungs at the time of tissue collection (Fig. 6d,g,h).
These mice also had significantly enhanced metastases to the brain
and lungs (Fig. 6i), suggesting that miR-122 reduces primary tumour
cell proliferation by restricting glucose uptake while simultaneously
reprogramming the premetastatic niches to promote tumour cell
colonization and metastatic formation.

To further focus on the niche-adapting effect of extracellular
miR-122 by blocking its function inside cancer cells, we generated
Luciferase-labelled MDA-MB-231 cells stably expressing an anti-miR-
122, which exhibited significantly reduced intracellular and secreted
miR-122 and increased PKM2 (Supplementary Fig. 3e). Orthotopic
xenograft tumours of these cells were established in mice that also
received EV treatments. Although no difference in primary tumour
growth was observed among all groups, mice receiving high-miR-122
EVs developed more metastases in brain and lungs (Fig. 6k–m).

Systemic miR-122 intervention alleviates cancer-induced
glucose reallocation in vivo and reduces metastasis
To study the in vivo effect of systemic miR-122 intervention, xenograft
tumours of MDA-MB-231-HM that naturally secreted high-miR-122
EVs were established, and mice were randomized into three treatment
groups, which received PBS, mismatch control oligonucleotides or
anti-miR-122 oligonucleotides. Although there was no difference
in primary tumour size among the three groups (Fig. 7a and
Supplementary Fig. 4), increased 2-NBDG uptake and enhanced
staining of PKM2 and GLUT1 were observed in tumour-adjacent
stromal cells in anti-miR-122-treated mice (Fig. 7b,c). At week 5 we started to observe a lower incidence of metastasis
to the brain and lungs in mice receiving anti-miR-122 treatment
(Fig. 7d,e). At the premetastatic stage, when there were no detectable
metastases by luciferase PCR, both organs showed reduced 2-NBDG
uptake when compared with non-tumour-bearing mice (Fig. 7f,g),
suggesting that factors secreted by the primary tumour can regulate
glucose utilization in a distant organ in preparation for metastasis.
This effect became more pronounced as cancer progressed, as a further
reduction in 2-NBDG uptake was observed in the metastasis-free
areas of brain and lungs at week 6 when metastases had developed
in these organs (Fig. 7f,g; PBS group). Notably, treatment with anti-
miR-122 but not the mismatch control oligonucleotides significantly
alleviated tumour-derived suppression of glucose uptake in the brain,
although the restoration was not significant in the lungs (Fig. 7f,g). In
the brain, miR-122 levels increased with tumour progression, with a
significant reduction in mice receiving anti-miR-122 oligonucleotides.
Supporting our in vitro data, we noticed a concomitant decrease in the
levels of PKM1 (isoform determined in Fig. 4c) and GLUT1 during
tumour progression, which was alleviated by anti-miR-122 treatment
(Fig. 7h,i). In the lung, anti-miR-122 oligonucleotides alleviated
primary tumour-derived suppression of PKM2 and GLUT1 (Fig. 7j,i).
Taken together, our in vitro and in vivo data indicate that cancer cells
can induce glucose reallocation in the premetastatic microenvironment
by suppressing glucose utilization in niche cells and enabling more
glucose to be available to cancer cells, thereby facilitating metastatic
cancer growth. This effect is at least partially mediated by cancer-
secreted miR-122.

DISCUSSION
Our study demonstrates that miR-122 is highly secreted by BC cells
and can promote metastasis by adapting the metabolic environment
in a premetastatic niche, providing an understanding of our previous
observation that miR-122 levels in the circulation are associated
with metastatic progression in BC patients40. In addition to EVs,
other protein and lipoprotein carriers of circulating miRNAs have been identified45.46. Although we cannot exclude the potential role
for these other forms of extracellular miR-122 from our mechanism
identified herein, characterization of the 110,000g medium pellet
used in our study by AF4 and gradient centrifugation indicates
that EVs of 30–100 nm are a major component of this material
and capable of transferring miR-122 from cancer to normal niche
cells to promote metastasis. An interesting phenomenon of stromal–
epithelial metabolic coupling, termed the reverse Warburg effect
for stromal glycolysis and cancer cell oxidative phosphorylation,
have been recently recognized43. In BC, cancer-associated stromal
cells rely on glycolysis to provide energy metabolites to cancer cells
through monocarboxylate transporters during disease progression43.
Endothelial cells also rely on glycolytic metabolism to support vessel
sprouting for angiogenesis43. Although extracellular miR-122 does not

seem to contribute to increased glycolysis in cells in the primary tumour microenvironment, on the basis of the concomitant decrease in lactate production on miR-122-mediated reduction of glucose uptake (Fig. 2d and Supplementary Fig. 3b) and lack of change in most tricarboxylic acid cycle metabolites (Fig. 2b), other miRNAs in cancer-secreted EVs may contribute to this effect, which would be an interesting future direction. In exploring what caused the increase of isocitrate in MCF10A/miR-122 (Fig. 2b), we found that isocitrate dehydrogenase (IDH1) 1 and 2 were both downregulated by miR-122 (Fig. 2i). Although a search of the 3′UTR of IDH1/2 did not reveal any miR-122 binding site, restoration of PKM2 was able to restore IDH1/2 levels in MCF10A/miR-122, suggesting that, similarly to GLUT1, IDH1/2 might be directly or indirectly regulated by PKM2. We also examined the NMR spectrum for α-ketoglutarate, which could not be reliably quantified owing to the low concentrations. However, the spectrum did suggest a reduction of α-ketoglutarate in MCF10A/miR-122 (Supplementary Fig. 2e), which is consistent with decreased IDH1/2 and increased isocitrate in these cells.

Adaptation of a premetastatic niche, initially defined by Kaplan and Lyden et al.44,45, before the arrival of tumour cells, has been recognized as an important means for cancers to facilitate their sustained growth and metastasis46,47. Exosomes from highly metastatic melanomas ‘educate’ bone marrow progenitor cells towards a premetastatic phenotype and induce vascular leakiness at premetastatic sites to facilitate metastasis46. Melanoma-derived exosomes also prepare sentinel lymph nodes for metastasis by inducing cell recruitment, extracellular matrix remodelling and vascular growth factors48. In renal cell carcinoma, microvesicles derived from CD105+ tumour-initiating cells trigger angiogenesis, which serves to enhance lung metastasis49. Further studies have highlighted the importance of other tumour-secreted factors resulting in establishment of a premetastatic niche49-54.

Here we added a unique aspect of nutrient utilization to this paradigm of cancer–host crosstalk. Enhanced glucose uptake is common in cancer as a result of the high energy demand in cancer cells and the low ATP-generating efficiency due to the Warburg effect. GLUT1 and glycolytic enzymes have been shown to be upregulated in BC (refs 55,56) as potential mechanisms for increasing glucose uptake. Cancer cells also develop strategies to increase their availability to glucose, such as angiogenesis to gain nutrients from the blood. Here we provide evidence that cancer cells also systemically suppress the nutrient utilization by other cell types to favour themselves. This miR-122-mediated mechanism may be more important at an early stage before cancer-induced angiogenesis, when the availability of nutrients in the tumour microenvironment becomes limited to sustain tumour growth, and when disseminated tumour cells arrive at a distant tissue to prepare for rapid expansion around the surrounding normal niche cells, which are native competitors for nutrients. Indeed, we observed that BC cells at the primary site were able to affect glucose uptake by brain and lungs at a premetastatic stage (Fig. 7f-j). Importantly, miR-122 intervention using antisense oligonucleotides significantly reduced BC metastasis to brain and lungs (Fig. 7d,e). Thus, our previous18 and current studies indicate that cancer-derived circulating miR-122 has the potential to be both a predictive marker and a therapeutic target for metastatic BC. As miR-122 antagonists are in clinical trials for patients with hepatitis C infection and exhibit good tolerance with a low propensity for drug interactions57,58, miR-122-targeted therapy in cancer patients seems highly feasible, and the non-invasive blood test for circulating miR-122 would enable accurate selection of patients who may benefit from this treatment.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

ACKNOWLEDGEMENTS
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AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS

Cells, plasmids and viruses. Human cancer cell lines and the non-cancerous cell line MCF10A were obtained from the American Type Culture Collection (ATCC). Cells, plasmids and viruses.

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Cells, plasmids and viruses. Human cancer cell lines and the non-cancerous cell line MCF10A were obtained from the American Type Culture Collection (ATCC). Cells, plasmids and viruses.
Medium metabolite analysis. MCF10A-derived cells seeded at equal number were cultured in growth medium containing 3 g l\(^{-1}\) glucose but no pyruvate for 72 h before CM was collected, cleared by centrifugation, and subjected to metabolite measurement using a BioProfile 100 Plus (Nova Biomedical). Medium collected from cell-free plates after 72 h incubation was used as the baseline control to calculate the consumption or production of each metabolite, which was further normalized to the cell number in each plate determined at the time of CM collection. MCFDCIS-derived cells were cultured for 48 h before CM was collected for analysis.

Cell metabolome analysis by NMR spectroscopy. Sample preparation, NMR spectroscopy and data analyses were carried out as described\(^ \text{66} \). Hydrophilic metabolites dried from the methanol–water fractions were resuspended in 500 µl 100% D\(_2\)O containing 3.2 µM 4,4-dimethyl-4-silapentane-1-sulfonic acid, which serves as an internal chemical shift reference and a concentration standard. One-dimensional NMR spectra were acquired at 25 °C on a Bruker Avance spectrometer equipped with a cryoprobe operating at 600.19 MHz. 1H frequency. Presaturation was used to suppress water signal, and the spectra were collected with spectral width of 10 kHz, 32 k data points, 3 s relaxation delay and 1,024 transients. 1H NMR spectra of the samples were processed using the Chenomx NMR Suite Processor (version 7.5, Chenomx), and the metabolites were identified and quantified using the Chenomx NMR Suite Profiler. Standard deviation was calculated from triplicate samples.

Animal models: EV conditioning and xenografts. All animal procedures were approved by the Institutional Animal Care and Use Committee at City of Hope and in compliance with ethical regulations. Female NSG mice six to eight weeks old were used here. Exosome-containing EVs were isolated from MCF10A/vec, MCF10A/miR-122 and MDA-MB-231 by the above procedure (for preparation of old were used here. Exosome-containing EVs were isolated from MCF10A/vec, MCF10A/miR-122 and MDA-MB-231 by the above procedure (for preparation of MCF10A/miR-122 and MDA-MB-231 by the above procedure (for preparation of EVs) and 110,000 g MCF10A/miR-122 and MDA-MB-231 by the above procedure (for preparation of EVs) and 110,000 g MCF10A/miR-122 and MDA-MB-231 by the above procedure (for preparation of EVs) were resuspended in growth medium containing 3 g l\(^{-1}\) glucose but no pyruvate for 72 h before incubation with rabbit anti-mouse GFAP diluted 1:647 IgG diluted 1:300 (catalogue no A-21244) and goat anti-mouse Alexa 594 IgG diluted 1:300 (catalogue no A-11032, Life Technologies). Images were obtained by fluorescence microscopy then pseudo-coloured and merged using Image-Pro Premier software.

Immunohistochemistry and in situ hybridization. IHC was carried out as previously described\(^ \text{67} \) using a 1:400 antibody dilution for PKM2 (C-11), 1:600 dilution for PKM1 (catalogue no NBP2-14833, Novus Biologicals) and 1:250 dilution for GLUT1 (Abcam; catalogue no ab652). ISH was carried out as described\(^ \text{67} \) using an LNA microRNA ISH miR-122 optimization kit (catalogue no 90003, Exiqon) followed by incubation of sheep anti-digoxigenin-AP (catalogue no 11993274910, Roche Diagnostics), and developed with NBT:BCIP (catalogue no SK-5400, Vector Laboratories) at 30 °C overnight. Nuclear Fast Red was used to counterstain nuclei (catalogue no H-3403, Vector Laboratories).

Glycogen staining. Glycogen staining was carried out using a periodic acid–Schiff kit (Sigma-Aldrich) following the manufacturer’s protocol.

Statistical analyses. All quantitative data are presented as mean ± s.d. For all quantitative data, statistical analyses were carried out using Kruskal–Wallis tests. Values of P < 0.05 were considered significant. Sample size was generally chosen on the basis of preliminary data indicating the variance within each group and the differences between groups. For animal studies, sample size was predetermined to enable an 80% power to detect a difference of 50%. All samples/animals that have received the proper procedures with confidence were included for the analyses. Animals were randomized before treatments in Figs 5–7. For animal studies, the investigators were blinded to allocation during outcome assessment. For every figure, statistical tests are justified as appropriate, and the data meet the assumptions of the tests.

Repeatability of experiments. For the experiments in which no quantification is shown, images representative of at least three independent experiments are shown

Supplementary Figure 1 Characterization of the 110,000 xg medium pellet. (a) For each cell line, RNA were extracted from the 110,000 xg medium pellet and concentrated supernatant obtained from equivalent volume of CM and analysed for miR-122 and miR-16 by RT-qPCR. Absolute miRNA levels are calculated based on standard curves (n = 6 extracts). (b) Representative EM images of vesicles in the AF4 fraction eluted at 18–25 min. The measured diameter of vesicles was shown as mean ± SD (n = 126 for MCF10A/vec-derived vesicles; n = 222 for MCF10A/miR-122-derived vesicles; n = 41 for MDA-MB-231-derived vesicles). Bars equal 100 nm. (c) RT-qPCR-determined levels of miRNAs in MCF10A/vec- and MCF10A/miR-122-derived protein and vesicle fractions separated by AF4. Absolute miRNA levels are shown (n = 6 extracts). ND: not detected. (d) Absolute miRNA levels in each gradient fraction after sucrose gradient centrifugation of MCF10A/miR-122-derived 110,000 xg medium pellet were determined by RT-qPCR (n = 6 extracts). (e) A representative EM image of MCF10A/miR-122-derived vesicles in sucrose fraction 6 (F6) (n = 362 vesicles). Bar equals 100 nm. * p < 0.05 for all panels derived from Kruskal-Wallis test. Data are represented as mean ± SD in all panels except (b & e).
Supplementary Figure 2. Examples of peak assignment based on NMR spectra. For all spectrum snapshots, black represents MCF10A/vec, red represents MCF10A/miR-122, and blue represents library entry. In general, each metabolite produces multiple resonances in different regions of the spectrum. We only analyzed those with some of the resonances that can be resolved unambiguously. An example is given by glucose as shown in (a). One of the glucose resonances overlaps with that of O-acetylcholine, lactose, glutathione, homoserine and ethanolamine at around 3.8 ppm, but another glucose resonance is well resolved at around 3.4 ppm. Another example is given by lactate that has peaks in two regions of the spectrum that do not overlap with other resonances (b). The unique patterns of NMR resonances, due to J-coupling, are used for de-convolution of overlapping resonances. An example is given in (c), which shows that one set of peaks of isocitrate overlaps with that of glutathione, but the patterns of the peaks from the two metabolites are different, and at least one peak of the isocitrate resonance can be well resolved for de-convolution to extract the concentrations. Similarly, the pyruvate peak can be well resolved from those of glutamate (d). Examples of assignments of some other resolved peaks are given in (e).
Supplementary Figure 3 Characterization of cell lines with modified miR-122 levels used for in vivo studies. (a) Cell number counts of MCFDCIS/miR-122 and MCFDCIS/vec cells at indicated time points (n = 6 biological replicates). (b) Medium metabolite analysis after 48 h of culture (n = 6 biological replicates). (c) RT-qPCR analysis showing the relative expression of indicated genes in MCFDCIS/miR-122 and MCFDCIS/vec cells (n = 6 extracts). (d) Western blot analysis in MCFDCIS/miR-122 and MCFDCIS/vec cells. Size of markers (in kDa) are indicated. (e) RT-qPCR-determined levels of intracellular and secreted miR-122 as well as PKM expression in MDA-MB-231 cells with stable knockdown of miR-122 (MDA-MB-231/122KD) compared to the control cells (n = 6 extracts). *p < 0.05 for all panels derived from Kruskal-Wallis test. Data are represented as mean ± SD in all panels except (d). Uncropped, unprocessed images of blots are shown in Supplementary Fig. 5.
Supplementary Figure 4 BLI of the primary tumours established with MDA-MB-231-HM and treated with anti-miR-122 oligos. (a) BLI images at week 3. (b) Quantification of (a) using Living Image Software (n = 8 mice per group). No significant difference (p > 0.05) between groups based on Kruskal-Wallis test.
Supplementary Figure 5  Uncropped, unprocessed images of blots and gels.
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<td>Mouse Cs</td>
<td>AGGCTAGACTGCTGCACACAAT</td>
<td>AGGACAGCTAGGGTGTGAAGAAG</td>
<td></td>
</tr>
<tr>
<td>Mouse Glut1 (Slc2a1)</td>
<td>GGCCCTAAGGTCACATGAG</td>
<td>CCAATTTAAGCCGAACTG</td>
<td></td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>CGAGGCCCAGAGCAAGAG</td>
<td>CGGTTGGCCTTAAGGTCAG</td>
<td></td>
</tr>
</tbody>
</table>

**Supplementary Table 1** RT-qPCR primer sequences for human and mouse genes.