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Supplemental Information

Glioblastoma-Derived Extracellular Vesicles Facilitate Transformation of Astrocytes via Reprogramming Oncogenic Metabolism

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TRANSPARENT METHODS

Cell cultures

Human low-passage GBM stem cells (GSCs) named GBM8 and GBM4 (established from female patients; gifts from Dr. Hiroaki Wakamoto, MGH) were cultured as neurospheres in serum-free Neurobasal medium (Gibco) supplemented with 3 mM GlutaMAX (Gibco), 1x B-27 supplement (Gibco), 0.5x N-2 (Gibco), 20 ng mL^{-1} EGF (R&D systems, MN), 20 ng mL^{-1} FGF (PEPROTECH, NJ) and 1% Antibiotic-Antimycotic Solution (Corning). The cells were passaged by dissociation using NeuroCult Chemical Dissociation Kit-Mouse (Stemcell Technologies, Canada) following the manual. PalmGFP+ GBM8 cells were produced by transduction with PalmGFP lentivirus (a gift from Dr. Xandra O. Breakefield, MGH) and sorted with FACS. Human glioma LN229 (female) and U251 (male) cell lines (from ATCC) were maintained in DMEM (Corning) supplemented with 10% FBS (Gibco) and 1% Antibiotic-Antimycotic Solution. For primary mouse astrocyte cultures, cortical tissues were dissected from P1 pups (of both sexes) of double-transgenic mice bearing EGFR mutation (EGFRvIII) and CDKN2A deletion ($CDKN2A^{-/-}$) (designated as EC astrocytes) and triple-transgenic mice bearing EGFRvIII, $CDKN2A^{-/-}$ and PTEN deletion ($PTEN^{-/-}$) (designated as ECP astrocytes) (Zhu et al., 2009). For primary human astrocyte cultures (pHA), fetal female cortical tissues were provided by Advanced Bioscience Resources, Inc. (Alameda CA). Mouse and human tissues were cut to small pieces and dissociated with 0.25% Trypsin (Gibco) and 0.1 mg/mL DNase I (Roche) for 15 min at 37 °C with swirling every 3min. The cells were then washed by centrifugation three times and seeded in poly-D-lysine coated T25 flask at ~80,000 cells per cm^2 in culture medium consisting of DMEM-F12 (Corning), 10% FBS (routine FBS from Gibco for pHA, or Tetracycline-free FBS from Clontech for EC and ECP cells), and 1% Antibiotic-Antimycotic Solution (Corning). Three days later, the media was replaced to fresh culture media, and then changed every 5-7 days. When the cells reach confluence, the flasks were shaken (200 rpm at 37 °C) overnight 3 times to remove microglia. pHA cells were transduced after two passages by the cocktail of SV40 large T antigen (SV40), RasG12V (Ras) and TERT lentiviruses for three consecutive days. Human transformed astrocytes (HTAs) were isolated from the resulting colonies. Two days before conditioned medium was harvest, FBS was replaced by vesicle-depleted FBS that was ultracentrifugated at 100,000 g, 4 °C for 24 h using an Optima L-90K ultracentrifuge (Beckman Coulter, CA) and a SW 28 rotor (28,000 rpm) (Wei et al., 2016). All cells were authenticated and negative for mycoplasma contamination. Human cells were used in accordance with the policies of institutional review boards at Brigham and Women's Hospital.

Virus production

293-T cells were cultured in DMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic Solution, and the medium was replaced to contain no antibiotics once the cells reached 70% confluence. The cells grown in 75 cm^2 flasks were transfected with a mixture of 3 plasmids (6 μg psPAX2, 3 μg pVSV-G and 10 μg lentiviral expression vector) and 60 μl Lipofectamine 2000 (Invitrogen). The

conditioned media was collected every 24 hr for 3 consecutive days, cells and cell debris removed by centrifugation at 300 g for 10 min and 2,000 g for 15 min, followed by 0.45 μm filtration (EMD Millipore), and collection of the pseudoviral particles.

Preparation of CM and transmission electron microscopy

Samples of CM, collected from GSCs cultured in serum-free conditions or glioma cells cultured with vesicle-depleted FBS, were spun at 300 g for 10 min and 2,000 g for 15 min at 4°C as described previously (Wei et al., 2017). The 40 ml supernatants were further filtered through 0.8 μm pores (filter diameter of 25mm; EMD Millipore, MA) and divided into two equal aliquots. The first aliquot was designated as the EV-containing conditioned medium (EV(+) CM). The second aliquot was further filtered through the 20 nm pores (filter diameter of 25mm; GE Healthcare) to remove EVs using a self-designed mechanical syringe pump (Wei et al., 2017) with up to 75 psi pressure applied, and was designated as EV(-) CM (Fig. 1A). In some experiments, CM was further concentrated on 3 kDa Amicon Ultra Centrifugal Filters (EMD Millipore) at 4000 g, 4 °C, for 60 min. For EV imaging, the filtrate was ultracentrifuged at 100,000×g for 80 min at 4 °C using an Optima L-90K ultracentrifuge (Beckman Coulter, CA) in a SW 28 rotor (28,000 rpm), and the pellet was resuspended with 100 uL DPBS (Witwer et al., 2013). Transmission electron microscopy of EVs was carried out as previously reported (Wei et al., 2017).

Transformation assay

Low passage pHAs were plated in 25 cm^2 flasks at ~10% density, and transduced by 6 mL cocktail of SV40, Ras and TERT lentiviruses (1:1:1) for three consecutive days. At 24 hr after the last transduction, culture media was replaced to either GBM EV(+) CM or EV(-) CM for a four-day period, followed by the additional three weeks of culturing in a regular medium. The cells were then fixed in 4% formaldehyde and stained with crystal violet to count the colonies.

Neurosphere formation

ECs, ECPs, and HTAs were dissociated to single-cell suspension with 0.25% trypsin (Corning), plated in low-attachment 96-well plates at 5, 12, 40 or 100 cells per well, and cultured in either EV(+) or EV(-) CM for 2 weeks. To monitor the neurosphere size and number, 96-well plates were scanned by GE IN Cell Analyzer 2200, and the images of each well stitched and analyzed by ImageJ software.

Soft agar and plate colony formation

For soft agar colony formation, 10^5 EC, ECP or HTA cells per well were seeded in 6-well plates, in a 0.25% soft agar solution supplemented with concentrated, U251 glioma-derived EV(+) or EV(-) CM, and on top of a 1% agarose basal layer. The corresponding CM was also added to soft agar and refreshed every two days. For plate colony formation, 500 cells per well were seeded in 6-well plates. And 24 hours later, the culture medium was replaced with either EV(+) or EV(-) CM and refreshed weekly

for 2 weeks. The cells were then fixed in 4% formaldehyde and stained with crystal violet for colony counting.

Immunofluorescence

For Ki-67 detection, disassociated EC, ECP, or HTA cells were resuspended in 10x EV(+) CM or 10x EV(-) CM and cultured in low-attachment culture flasks (Greiner Bio-one) for 2 days. The formed neurospheres were spun onto charged slides for 15 min at 2,000 rpm by StatSpin Cytofuge II, fixed in formaldehyde 4% for 30 min, and permeabilized with Triton 0.5% for 15 min at 4 °C. The samples were then blocked with 10% BSA in PBS for 1 hour at room temperature, incubated with primary Ki-67 antibody (Abcam, ab16667, 1:100) overnight at 4 °C, and secondary antibody (Alexa Fluro 594 goat anti-mouse IgG,1:1000) for 2 hours at 4°C. For the EVs uptake assay, ECs, ECPs, and HTAs were treated with either EV(+) or EV(-) CM collected from PalmGFP^{+} GBM8 cells. After 2 or 6 hours, the CM was removed, and the cells washed twice with DPBS for 5 minutes. Nuclei were stained with DAPI for 5 min at room temperature, and the samples scanned by the ZEISS LSM710 confocal microscope.

Metabolic assays

Seahorse XF Glycolytic rate assays have been performed based on the Agilent protocol. Approximately 15x10^{3} EC, ECP, or HTA cells per well were seeded in 96-well plates pre-coated with polylysine. On the next day, the cells were washed with PBS and then 100 µl of concentrated (10x) GBM8-derived EV(+) or EV(-) CM was added to the wells for 2-5 days. The cells were washed with PBS prior to the assays, and 180 µl Seahorse XF DMEM supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose was added per well. Proton efflux rate (PER) and mitochondrial respiration were measured by Seahorse XFe96 Analyzer after the injections of 20 µl Rotenone/antimycin A (5 µM) and 22 µl 2-DG (500 µM), respectively. The assays were performed with 5 replicates per group, and PER and Oxygen consumption rate (OCR)/mitochondrial respiration measurements normalized to the amount of cellular protein per well, as measured by micro BCA assay (Thermo Fisher Scientific).

RNA isolation and qRT-PCR

Cells and extracellular fractions were washed with cold DPBS (Corning) three times, and RNA was isolated from MVs, Exos, and RNPs as previously described (Wei et al., 2017). For isolation of extracellular RNA, cells and debris were removed by centrifugation, the supernatants supplemented with SUPERase In RNase Inhibitor (Ambion), and then filtered sequentially through 0.8 µm (EMD Millipore), 0.22 µm (EMD Millipore) and 0.02 µm (GE Healthcare). The last flow-through fraction was concentrated using 3 kDa Amicon Ultra Centrifugal Filters (EMD Millipore). RNA was then isolated using the Total RNA Purification Kit with on-column DNase treatment (Norgen Biotek, Canada). NanoDrop 2000 Spectrophotometer and Quant-iT RiboGreen RNA Assay Kit (Thermo Fisher Scientific) were employed to measure concentrations of cellular and extracellular RNA, respectively. For quantitative mRNA analysis, 10 ng of total RNA was used in 10 µl RT reaction with iScript Reverse

Transcription Supermix (Bio-Rad), followed by the PCR reactions with ExiLENT SYBR Green master mix and mRNA-specific primers. Sequences of specific PCR primers are listed in Supplementary Table 1.

Full-length RNA RT-PCR amplification

A 20 ng of total RNA was used as the template for the cDNA synthesis with Maxima Reverse Transcriptase (100U; Thermo Fisher Scientific), oligo(dT), and random hexamers in a 10 μl reaction. 1 ng cDNA was used in 10 μl PCR reaction mixture containing Phire Hot Start II PCR Master Mix (Thermo Fisher Scientific) and primers (0.5 μM each). Depending on the lengths of the amplicons, extension steps were employed for either 15 sec or 30 sec, and the PCR products were visualized on 1% or 1.5% agarose gels (Thermo Fisher Scientific). Primer sequences are listed in Supplementary Table 1.

RNA sequencing and data analysis

ECs, ECPs, and HTAs cells were resuspended in 10x EV(+) or EV(-) CM and cultured in suspension culture flasks (Greiner Bio-one) for 6 days, and the total RNA was isolated and quality controlled by Agilent 2100 Bioanalyzer. The libraries were prepared and sequenced on Illumina HiSeq X with PE150 mode to produce approximately 20 M reads per sample by Novogene Co., Ltd. The reads were quality controlled with FastQC, trimmed with Trimmomatic, aligned with HiSat2 to hg38 or mm10, and quantified with HTSeq-count using the Galaxy platform. Read counts were processed for differential expression analysis using the R package DEBrowser with DESeq2. Heatmap analysis was performed and visualized with the R package ComplexHeatmap. The comparative analysis of cellular RNA and extracellular RNA fractions was performed and visualized with R package corrplot. Gene set enrichment analysis was performed using the GSEA software (Broad Institute). The Gene Set Variation Analysis and the survival analysis on TCGA and CGGA datasets were performed and visualized with the R package GSVA and R package survival, respectively.

Tumor growth *in vivo*

EC or ECP cells (2×10^6) were resuspended in 100 μl 20x EV(+) or EV(-) CM mixed with 100 μl Matrigel and implanted subcutaneously to both flanks of 8 weeks old female athymic nude mice. Tumor growth was monitored with a digital caliper 2-3 times per week and its volume estimated using the formula (V= length × width). Four mice and eight tumors per group have been included. The animals have been sacrificed when the tumors reached diameter larger than 20 mm. All animal experiments were approved by the Animal Care Committee of Brigham and Women's Hospital.

Statistics

Data are expressed as mean ± SEM. Numbers of experimental replicates are given in the figure legends. A two-sided Student's t-test was employed to determine statistical significance between the two groups. Before performing t-test, normal distribution was

verified by 1-sample K-S test and equality of variances was verified by Levene's test using SPSS (IBM, NY). The Pearson correlation was used to evaluate the linear relationship between two continuous variables. The Kaplan–Meier analysis was used to estimate the survival differences between the two groups. A $P < 0.05$ was considered to be statistically significant.

SUPPLEMENTARY FIGURE LEGENDS

SF1. GBM EVs are taken up by pre-transformed astrocytes and facilitate their renewal and anchorage-independent growth, Related to Figure 1.

A. A number of secondary colonies formed by HTA cells increases when the cells are grown in EV(+) GBM CM.

B. There is no difference in a number of colonies produced by HTA cells grown in EV(+) and EV(-) fresh medium.

C. Transmission electron microscopy of EV(+) and EV(-) GBM CM. TEMs were replicated 3 times (upper panel). NTA-based quantification of EVs numbers in EV(+) CM was depicted as lower panel. TEMs and NTAs were replicated 3 times.

D. The uptake of GFP^+ EVs derived from $PalmGFP^+$ GBM8 cells by EC, ECP, and HTA recipient cell cultures. Confocal images of the recipient cultures treated with $PalmGFP^+$ EV(+) CM for 2 and 6 hours, or EV(-) CM from the same donor cells (scale bar=14 μm). Quantification of the GFP^+ recipient EC, ECP, and HTA cells demonstrates efficient EV uptake.

E. Treatment with GBM8 EV(+) CM increases HTA self-renew ability, as indicated by neurosphere formation assay, compared to treatment with GBM8 EV(-) CM, whereas no difference was observed between neurosphere formation in EV(+) and EV(-) fresh medium.

F. Microscopy of ECP colonies grown in soft agar with 10x EV(+) or EV(-) fresh medium for 2 weeks. No difference was observed between these conditions, as indicated by the count of colonies shown in the right panel.

* $P<0.05$; ** $P<0.01$; two-tailed t-test. Data are represented as mean ± SEM.

SF2. EC cells are not tumorigenic in nude mice, Related to Figure 2.

The EC cells injected alone, or resuspended in concentrated EV(+) or EV(-) GBM CM, have not formed tumors during 40 days post-implantation.

Data are represented as mean ± SEM.

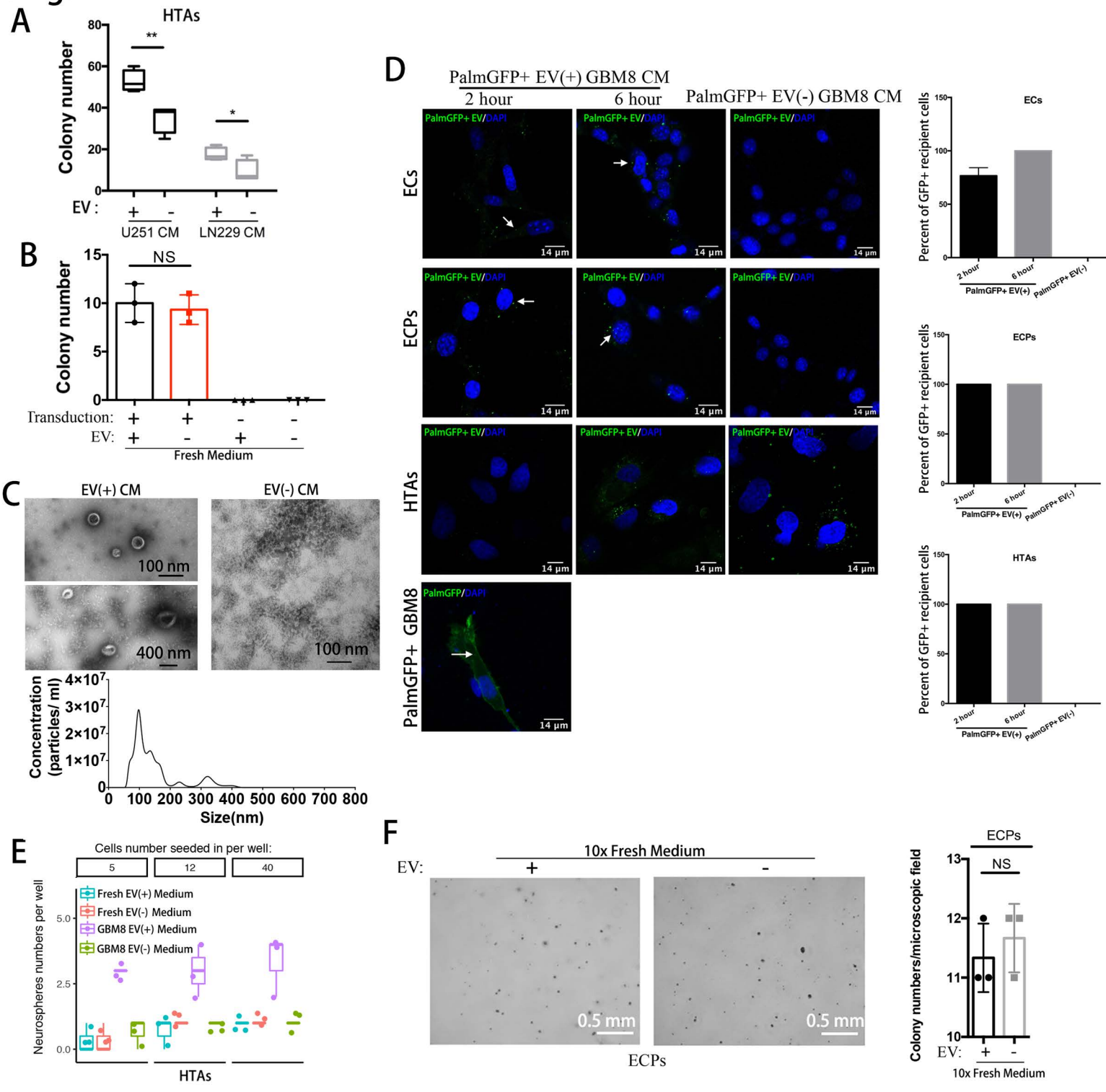
SF3. Analysis of the most abundant mRNAs in GBM EVs, Related to Figure 5.

A. Venn diagrams depict the number of common mRNA species among 200 top abundant mRNAs in four types of GBM MVs, resulting in a list of 92 mRNAs.

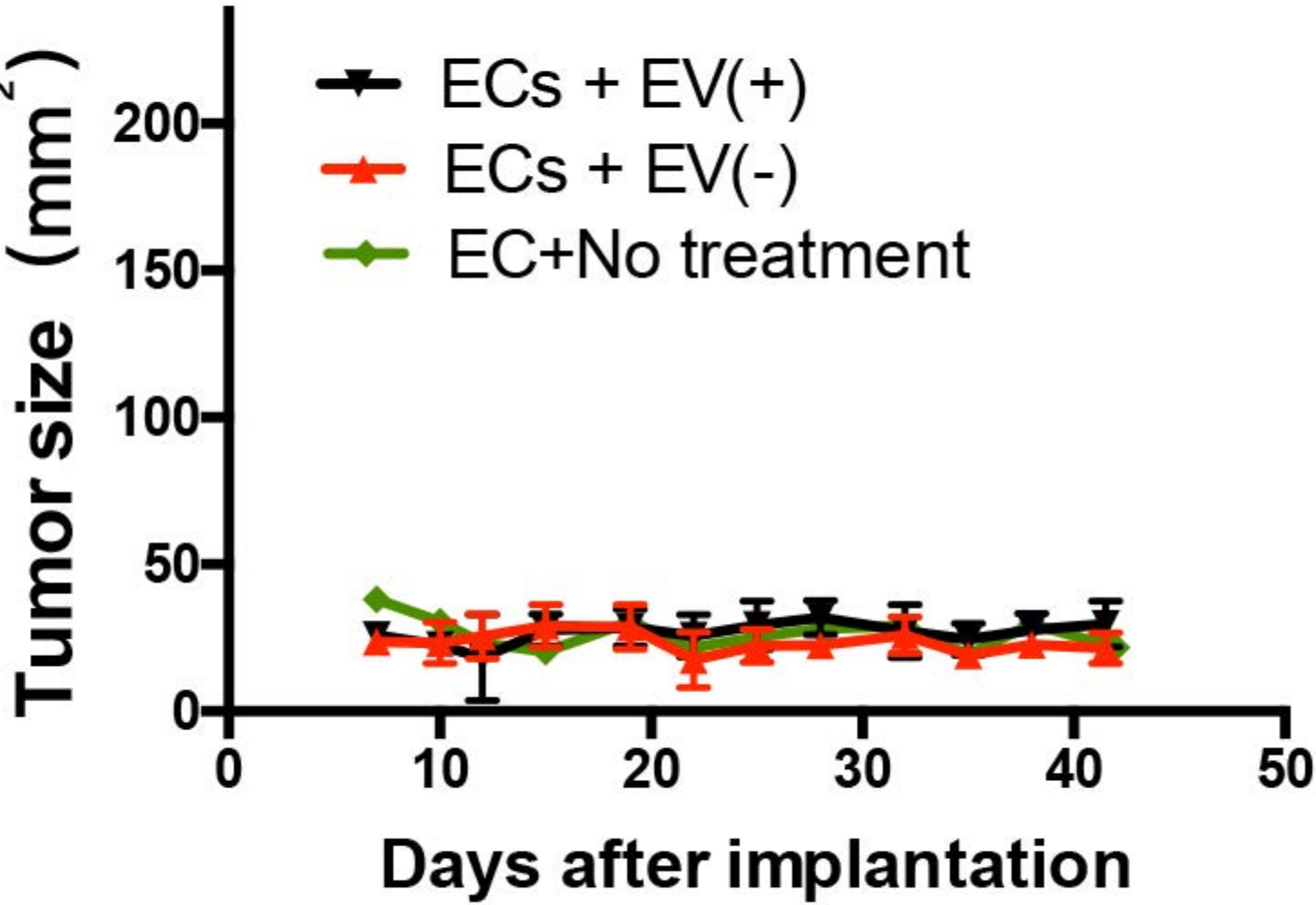
B. Venn diagrams depict the number of common mRNA species among 200 top abundant mRNAs in four types of GBM Exosomes, resulting in a list of 37 mRNAs.

C. Heatmap analysis of GBM samples from the TCGA and control brains from TARGET GTEx datasets depicts the global upregulation of RP, OXPHOS, and glycolysis genes in GBM relative to the normal brain tissues.

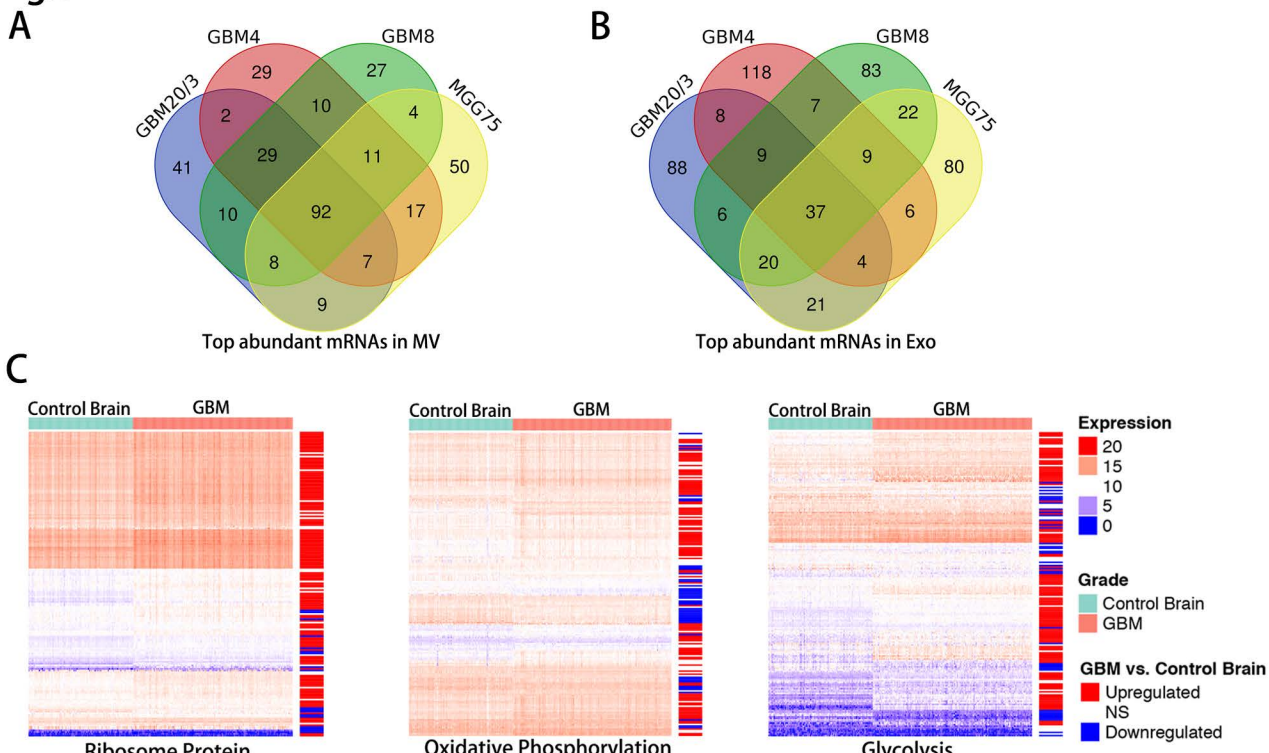
SFig.1


A
HTAs
Colony number
EV :
U251 CM
LN229 CM
B
NS
Colony number
Transduction:
EV:
Fresh Medium
C
EV(+) CM
EV(-) CM
100 nm
400 nm
Concentration (particles/ ml)
Size(nm)
D
PalmGFP+ EV(+) GBM8 CM
2 hour
6 hour
PalmGFP+ EV(-) GBM8 CM
ECs
ECPs
HTAs
PalmGFP+ GBM8
PalmGFP+ EV/DAPI
PalmGFP/DAPI
14 μm
Percent of GFP+ recipient cells
E
Cells number seeded in per well:
Fresh EV(+) Medium
Fresh EV(-) Medium
GBM8 EV(+) Medium
GBM8 EV(-) Medium
Neurospheres numbers per well
HTAs
F
10x Fresh Medium
EV:
0.5 mm
ECPs
Colony numbers/microscopic field
NS
10x Fresh Medium

SFig.2


ECs + EV(+)
ECs + EV(-)
EC+No treatment
Tumor size (mm2)
200
150
100
50
0
0
10
20
30
40
50
Days after implantation

SFig.3


A
GBM4
GBM8
GBM20/3
MGG75
29
27
2
10
4
41
29
11
50
10
92
17
8
7
9
Top abundant mRNAs in MV
B
GBM4
GBM8
GBM20/3
MGG75
118
83
8
7
22
88
9
9
80
6
37
6
20
4
21
Top abundant mRNAs in Exo
C
Control Brain
GBM
Ribosome Protein
Oxidative Phosphorylation
Glycolysis
Expression
20
15
10
5
0
Grade
Control Brain
GBM
GBM vs. Control Brain
Upregulated
NS
Downregulated