Supporting Information

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SI Materials and Methods

Human Specimens. ***Blood specimens.*** The specimens used for these experiments were distinct from our previous studies (1, 2) but are a subset of a collection more recently described (3), with more detail presented here. Blood samples from individuals with metastatic prostate cancer and prostate cancer-negative controls were collected after written informed consent was obtained. All participants signed a Human Subjects Committee approved Informed Consent Form for a peripheral blood draw. The research was approved and supervised by the University of Washington and Fred Hutchinson Cancer Research Center Institutional Review Boards (IRBs). Prostate cancer-negative donors ($n = 9$) were recruited among individuals undergoing screening for prostate cancer at the University of Washington and found to be negative by digital rectal examination and serum prostate-specific antigen (PSA) analysis. Individuals with metastatic prostate cancer were recruited among previously diagnosed patients undergoing treatment at the University of Washington (Figs. 1–4 and Fig. S3) ($n = 9$) or Seattle Cancer Care Alliance (Fig. S2) ($n = 6$) with PSA > 20 ng/mL. Blood was processed within 2 h of collection. To avoid skin cell contamination, samples used to prepare plasma were collected after at least 10 mL blood was collected into a separate draw tube (Figs. 1–4 and Fig. S3) or after blood was drawn into separate tubes for clinical patient care (Fig. S2).
Plasma. Venous blood was aseptically collected through a 21-gauge needle into 10 mL EDTA collection tubes for plasma (366643; BD). Tubes were then inverted five times (not shaken) and stored at 4 °C until processed. Specimens were then centrifuged for 15 min at $1,300 \times g$ (at tube bottom) and 4 °C in a Jouan CR412 centrifuge. Plasma was collected through a 3.0-mL pipet (357524; Falcon) while carefully avoiding the pelleted material. Aliquots were then prepared in 1.5-mL screw cap tubes with O rings (C-3354-1; ISC Bio Express) and stored at −80 °C.
Serum. Blood for serum was collected into 10 mL Vacutainer SST Plus Blood Collection Tubes (367985; BD), inverted as above, allowed to clot for a minimum of 30 min, and stored at 4 °C until processed. Tubes were then centrifuged for 15 min at $1,300 \times g$ in the above centrifuge at 4 °C, after which aliquots were prepared and stored as above.
Human cord blood collection and dendritic (Langerhans) cell derivation. All protocols using human cord blood samples were approved by the Seattle Biomedical Research Institute IRB. Written informed consent was obtained from each donor. Cord blood specimens were obtained without linkage to maternal records and deemed not to represent Human Subject Research (because materials would otherwise be discarded). Cord blood was obtained after normal caesarean section deliveries ($n = 3$) and kept at room temperature before processing. Leukocytes from cord blood were purified by histopaque centrifugation according to the manufacturer's instructions (Sigma-Aldrich). CD34+ cells were purified using a CD34+ Microbead Kit (Miltenyi Biotech). Isolated CD34+ cells were differentiated as independently published (4, 5). Specifically, cells were cultured at 1×10^4 cells/mL in Langerhans cell (LC) media consisting of X-Vivo 15 (Lonza) plus 100 ng/mL GM-CSF, 20 ng/mL stem cell factor, 2.5 ng/mL TNF-α, 0.5 ng/mL TGF-β1, and 100 ng/mL Flt3 ligand. All cytokines were purchased from PeproTech. Cells were cultured for 7 d, and LCs were further purified by gravity (5). Briefly, up to 8 mL cells were gently pipetted onto 6 mL 7.5% (wt/vol) BSA in PBS in 15-mL tubes. After 30 min on ice, tubes were aspirated to 3.5 mL to remove single cells. Purified clusters were concentrated by centrifugation at $300 \times g$, resuspended, and cultured at 5×10^5 cells/mL for 3 additional d in LC media that had been depleted of particles by ultracentrifugation at $100,000 \times g$ for 90 min. At the end of 3 d, culture media were used for exosome isolation.
Human semen collection and preparation. Approval for the study was given by the University of Washington IRB. Written informed consent was obtained from each donor. Ejaculates from healthy HIV-negative men ($n = 3$) were obtained by masturbation into a sterile container after a sexual abstinence period of >48 h, mixed with 3 mL RPMI media, and kept on ice until exosome purification was performed.

Isolation of Exosomes. ***From plasma and serum.*** Frozen samples were thawed on ice and gently homogenized by two cycles of rotation at 4 °C for 2 min and collection at $500 \times g$ for 30 s; 1 mL plasma or serum was added to 1 mL ice-cold PBS in a 2-mL Eppendorf tube, mixed by gentle inversion five times, and centrifuged at $12,000 \times g$ for 45 min at 4 °C in an Eppendorf 5424R centrifuge with an F-45–24-11 rotor. The cleared dilute plasma was then aspirated away from the pellet by pipetting carefully to avoid pelleted material, added to 3 mL ice-cold PBS in a 5-mL ultracentrifuge tube (326819; Beckman Coulter), and centrifuged at $120,000 \times g$ for 70 min at 4 °C in an SW-55 Ti swinging bucket rotor and L-90 ultracentrifuge (Beckman Coulter). Supernatants were then gently decanted, and exosome pellets were resuspended in 100 μL PBS; 1 mL supernatant and 90 μL exosome fraction were used for RNA extraction (see below).
From dendritic cell (LC) -conditioned medium and semen. Cells were removed by centrifugation at $1,000 \times g$ for 10 min. Cell debris was removed by centrifugation at $2,400 \times g$ for 30 min followed by sequential 0.45 and 0.22 μm syringe filtration (Millex HA). Vesicles were then purified by ultracentrifugation over a sucrose cushion using a method adapted from ref. 6. Up to 2.5 mL sample was underlayed with 300 μL 20 mM Tris/30% (wt/vol) sucrose/deuterium oxide cushion (pH 7.4). Samples were ultracentrifuged at $100,000 \times g$ for 90 min at 4 °C in an SW-50 swinging bucket rotor (Beckman). The 30% (wt/vol) sucrose cushion was then brought to 15 mL with PBS. The exosomes were washed and concentrated by centrifuging at $2,400 \times g$ through an Amicon ultracel 100-kDa cellulose centrifugal filter, replacing 10 mL PBS to further wash exosomes, to a final volume of 425 μL to 3.2 mL. Aliquots of exosomes were stored at −80 °C.
From human ovarian carcinoma cells. The 2008 ovarian cancer cells (7) were grown to ∼80% confluence in DMEM with 10% (vol/vol) FBS. Cultures were then washed in triplicate with serum-free medium and cultured in serum-free medium for 48 h. The conditioned media were then collected, centrifuged at $500 \times g$ for 10 min, and filtered through a 0.22-μm syringe filter (Nalgene). This precleared conditioned medium was then ultracentrifuged as above for preparation of exosomes from plasma. Resulting pellets were resuspended in PBS containing 0.1% BSA to prevent aggregation.
From mast cells. Exosomes were prepared from the HMC-1 cell line essentially as previously described in ref. 8 with the following modifications: 0.22-μm filtration was omitted, and the $120,000 \times g$ exosome pellet was washed in PBS with 0.1% BSA, recentrifuged, and resuspended in fresh PBS with 0.1% BSA.

EM. Samples of vesicle pellets prepared by ultracentrifugation were processed for visualization by transmission EM essentially as previously described (9) with the following modifications: vesicle pellets were resuspended in PBS, and a 10-μL aliquot was diluted to 25 μL with PBS and then fixed with 25 μL 4% (wt/vol) paraformaldehyde. The fixed material was centrifugally deposited

onto Formvar carbon-coated EM grids using an Airfuge ultracentrifuge and EM-90 rotor (Beckman Coulter) at 26 psi for 10 min. Karnovsky's fixative was substituted for 1% glutaraldehyde to subsequently fix the sample-coated grids after washing. Samples were visualized on a JEOL 1010 Transmission Electron Microscope.

RNA Isolation. Total RNA was isolated from all samples using the miRNeasy Kit (Qiagen) according to the manufacturer's protocol with the following modifications: liquid samples were combined with 5× sample volumes of Qiazol reagent and mixed with three cycles of vortexing for 10 s followed by inversion three times. Samples in Qiazol were then incubated at room temperature for 5 min to denature the sample and inactivate RNases. We then added synthetic *Caenorhabditis elegans* microRNA (miRNA) oligoribonucleotides (1, 10) prepared in Qiazol to each sample, mixed as above, froze on dry ice, and stored samples at −80 °C. Synthetic *C. elegans* miRNAs were added after sample denaturation was complete to avoid potential degradation of these unprotected normalizing controls by endogenous RNases present in the sample. After thawing samples on ice and homogenizing at room temperature as above, 0.2 volumes chloroform was added to each sample. At that point, the manufacturer's protocol was followed, with the entire aqueous phase from each sample loaded onto a single affinity column after the addition of ethanol. RNA was eluted into low-adhesion 1.7-mL tubes (C-3302-1; Genemate) to prevent losses caused by adsorption.

Individual Real-Time PCR Assays. Individual miRNAs were detected by real-time PCR as previously described (1, 10). Data from all miRNA real-time PCR arrays were analyzed, and cycle-threshold (C_T) values were calculated by the ViiA 7 signal-processing algorithm (v1.0; Applied Biosystems) to automatically call baseline and threshold with carboxy-x-rhodamine (ROX) passive reference dye normalization. TaqMan assays for human miRNAs *hsa*-miR-16, -miR-126, -miR-141, -miR-210, -miR-223, -miR-375, and -miR-720 in addition to *C. elegans* miRNA *cel*-miR-39 were obtained from Applied Biosystems. Oligoribonucleotides corresponding to the mature sequence of each miRNA were synthesized (Integrated DNA Technologies) and serially diluted for standard curves. For comparison of ultracentrifugation fractions, the copy number was then corrected to account for the volumetric differences of the fractions in the relative representation of the input plasma to allow for results to be directly comparable (i.e., total RNA was extracted from 1 mL supernatant, corresponding to 20% of input raw plasma lot, but total RNA was extracted from 180 μL exosomes, corresponding to 90% of the input raw plasma lot; thus, supernatant values were divided by 0.2, and exosome values were divided by 0.9). For miRNA/exosome stoichiometry (Figs. 3 and 4, Fig. S4, and Table S5), final values were corrected for miRNA yield from purification using copy numbers for the *cel*-miR-39 spike-in oligonucleotide relative to its known input quantity. For other measurements, *cel*-miR-39 was used only to normalize between samples in the analysis (no absolute recovery yield correction) as per our standard protocol (1, 10).

Fluorescence Microscopy Analysis. Exosomes from ovarian cancer cell-conditioned media were prepared as described above and subsequently washed with 15 mL PBS in a 100-kDa molecular mass cutoff centrifugal filter unit (UFC910024, Amicon Ultracel; EMD Millipore). The washed exosomes were then labeled with 10 μM Vybrant DiI lipophilic dye (V-22885; Life Technologies) for 30 min at room temperature and washed again as above. Yellow-green 100-nm beads (F8803, FluoSpheres; Life Technologies) were mixed with the exosomes, and 15 μL of the mixture was dried on a glass microscope slide. The spots were washed two times with 100 μL PBS and fixed in 100 μL 1% paraformaldehyde for 10 min. The paraformaldehyde was removed, and the slides were coverslipped with Prolong Gold Antifade Reagent (P10144; Life Technologies). After curing, the slides were imaged on a DeltaVision image restoration system fluorescence microscope (Applied Precision). Image stacks were deconvoluted with softWoRx (Applied Precision). After deconvolution, beads and exosomes were separately counted by hand (ImageJ), and the ratio of beads to exosomes was determined. At least 1,000 of each were counted for each technical replicate. Given the known concentration of beads used and relevant dilution factors, the concentration of exosomes was then calculated.

Droplet Digital PCR. Samples were analyzed using a recently described method (3) with minor modifications. Samples were reverse-transcribed using the TaqMan miRNA Reverse Transcription Kit and target-specific reverse transcription primers (Applied Biosystems, Inc.) as follows: 10× reverse transcription buffer (1 μL), RNase inhibitor (0.12 μL), 100 mM each dNTP (0.1 μL), Multiscribe reverse transcriptase (0.66 μL), 5× reverse transcription primer (2 μL), RNase/DNase-free H_2O (4.12 μL), and RNA (2 μL; total volume = 10 μL). Reverse transcription thermal conditions were 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min (Tetrad2 Peltier Thermal Cycler; Bio-Rad); 1 μL reverse transcription product for each concentration was combined with RNase/DNase-free H_2O (8 μL), the target-specific TaqMan Real Time PCR Primer Probe Set (1 μL 20× solution; Applied Biosystems), and droplet digital PCR (ddPCR) 2× Supermix for Probes (10 μL; Bio-Rad; total volume = 20 μL) and thoroughly mixed by repeated pipetting of at least one-half the total volume. It is important that the reverse transcription product be added to the ddPCR master mix (through liquid contact), because this order of addition minimizes transfer losses of the template. Each 20 μL PCR (see above) was loaded into an eight-channel, single-use consumable droplet generation cartridge; 70 μL ddPCR droplet generation oil (Bio-Rad) containing emulsion-stabilizing biocompatible surfactant was loaded into adjacent oil wells, and the microfluidic chip was loaded into a β-series prototype droplet generator. After vacuum application, the resulting water-in-oil emulsions were pipette-transferred from the outlet well to a 96-well skirted polypropylene plate (Eppendorf), heat-sealed with foil, and then amplified to the end point using a Tetrad2 Peltier Thermal Cycler (Bio-Rad) and the cycling protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (2.5 °C/s ramp rate) with a final 10 min hold at 98 °C. Plates containing amplified droplets were loaded into an early-access version of the commercially available QX100 Droplet Reader (Bio-Rad). For all of the miRNA assays, the global fluorescence threshold was set at 4,000 relative fluorescence units, regardless of the assay efficiency. Concentration estimates were based on the fraction of droplets where amplification has occurred (p) modeled as a Poisson distribution, and data analysis methods used the Quantasoft (1.1.1.0) data analysis package installed with the droplet reader. Detection efficiency was determined by calculating the ratio of copies detected in the standard curves to the theoretical inputs from the known concentration of stock miRNA oligoribonucleotides (provided by the vendor) used to prepare them, and this value was used as a correction factor for the values reported for experimental samples.

miRNA Profiling. Samples were profiled for the relative abundance of 375 miRNAs using miRNA Ready-to-Use PCR, Human Panel I, V2.M (Exiqon).

For plasma samples. To empirically determine the limit of quantification and the PCR efficiency for each miRNA assay in this system, we used dilution analysis of plasma miRNA essentially as described in ref. 2. Total RNA extracted from healthy human donor plasma as described above was pooled from three donors and serially twofold diluted to prepare a 13-point standard curve.

Total RNA extracted from exosome preparation fractions derived from the plasma of $n = 9$ prostate cancer patients was pooled ($n = 3$ individuals per pool; $n = 3$ pools). This replicate pool approach was chosen to minimize the skewing effects of individual outliers while still providing an estimate of variation; 13 μL each dilution series sample, pooled fraction sample, or water (as a nontemplate control) was used for reverse transcription with the miRCURY LNA Universal RT MicroRNA PCR Kit (Exiqon) as directed. The optional UniSp6 RNA spike-in oligoribonucleotide was added to each reaction as directed. Reverse transcription reaction products were combined with SYBR Green Master Mix (Exiqon) and loaded into the 384-well real-time PCR arrays. Real-time PCR was performed on a ViiA 7 instrument with v1.0 software (Applied Biosystems). In addition, each fraction pool RNA sample was independently assayed for the spike-in *C. elegans* oligoribonucleotide *cel*-miR-39 by TaqMan Real-Time PCR Assay as described above.

Other exosome samples. Individual total RNAs extracted from exosomes (semen exosomes, $n = 3$; dendritic cell exosomes, $n = 3$; mast cell exosomes, $n = 2$; ovarian carcinoma cell exosomes, $n = 2$) were combined into one pool per sample type and profiled as above.

miRNA Profiling Data Analysis. ***Analysis of plasma fractions.*** Data from miRNA real-time PCR arrays were imported into ExpressionSuite software (v1.0; Applied Biosystems), and C_T values were calculated in one study. For each dataset, miRNAs that were undetected in all fraction pools were filtered out. The remaining miRNA assays were normalized across plates based on the UniSp6 control assays and the values of the *cel*-miR-39 spike-in oligoribonucleotides as determined by TaqMan Real-Time PCR. For each assay on the array, the standard curve C_T values were plotted against the log of the dilution. Nonlinear assays were identified by visual inspection and removed. From the dilution series plots and no template control, the C_T value corresponding to the assay-specific limit of detection was determined by visual inspection. Within the assay linear range, the PCR efficiency was calculated as $e^{(-1/\text{slope})} - 1$. This process provided limit of detection and efficiency data for 182 miRNA assays. Experimental samples with C_T values beyond the limit of detection for the relevant assay were considered undetected, and assays where all samples were undetected were filtered out. Relative quantification (RQ) was performed on the remaining data using the method by Pfaffl (11) and the assay-specific PCR efficiency. The RQ was then corrected to account for the volumetric differences of the fractions in the relative representation of the input plasma to allow for results to be directly comparable (i.e., total RNA was extracted from 1 mL supernatant, corresponding to 20% of the input raw plasma, but total RNA was extracted from 180 μL exosomes, corresponding to 90% of the input raw plasma; thus, supernatant values were divided by 0.2, and exosome were values divided by 0.9). The final analysis yielded normalized RQ values for 146 (patient pool 1), 149 (patient pool 2), and 116 (patient pool 3) miRNA assays. Heat maps were generated using R.

Analysis of other exosome samples. Data from all miRNA real-time PCR arrays was analyzed, and C_T values were calculated by the ViiA 7 signal processing algorithm (v1.0; Applied Biosystems) to automatically call baseline and threshold with ROX normalization. C_T values were then ranked, and undetermined values were removed. It is worth noting that one of the miRNAs on the real-time PCR array, *hsa*-miR-720, has been removed from miRbase, because it is believed to be a tRNA-derived fragment. However, tRNA-derived fragments have been shown to be functional miRNAs, because they bind to Argonaute proteins (12) and inhibit protein translation in human cells (13); hence, we did not exclude *hsa*-miR-720 from our analyses.

Nanoparticle Tracking Analysis. Specimens were thawed on ice and gently resuspended immediately before sampling to prevent the settling of particles. Serial dilutions of each specimen were prepared in 0.02-μm nanofiltered (6809–2102; Whatman Anotop 25) molecular-grade water (SH30538.03; Thermo Scientific) using low-adhesion 1.7-mL tubes (C-3302-1; Genemate) to prevent exosome adsorption onto the walls of the tube. Diluted samples were loaded into the assembled sample chamber of a NanoSight LM10 through a 1-mL syringe, applying the sample slowly to expel air without bubble formation. The chamber was then connected to the instrument and laser-engaged, and microparticles were brought into focus using the thumbprint region as a reference; 60-s video images were acquired by a Hamamatsu C11440 ORCA-Flash 2.8 digital camera and analyzed by NanoSight NTA 2.3 software. Individual members of the prepared serial dilutions were analyzed until the raw concentration detected was within the recommended range for the instrument (1×10^8–1×10^9 particles/mL).

Statistical Analysis. Tests and parameters used are indicated in the figures. All statistical analyses were performed using Prism 5.0c software.

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13. Sobala A, Hutvagner G (2013) Small RNAs derived from the 5′ end of tRNA can inhibit protein translation in human cells. *RNA Biol* 10(4):553–563.

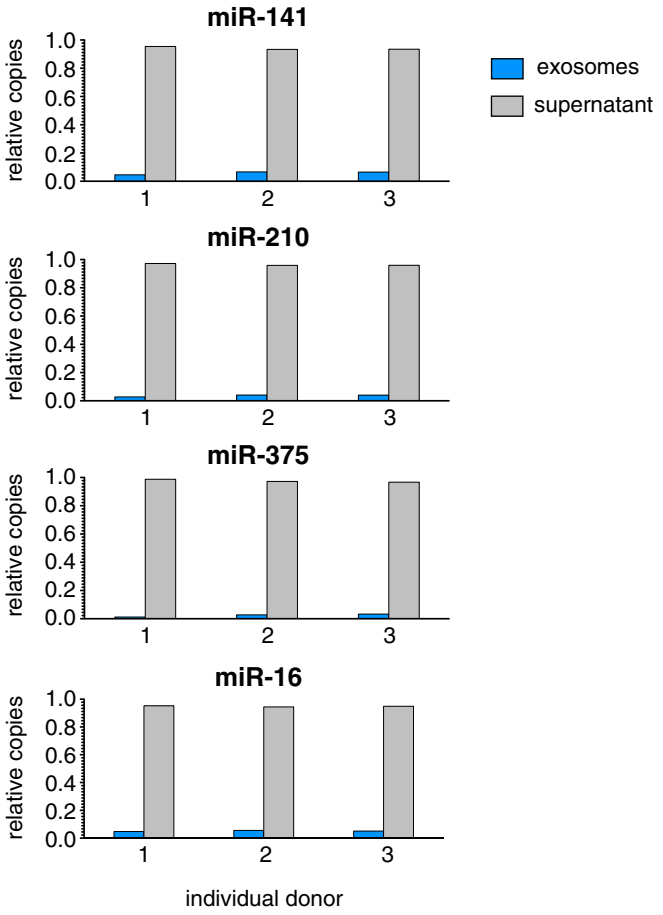

Fig. S1. Exosome preparations from cancer patient serum contain relatively low miRNA abundance. Exosomal fractions from prostate cancer patient sera ($N = 3$) were prepared as described in Fig. 1*A*. Total RNA was extracted from each fraction, and the relative abundance of the miRNAs indicated was quantified by real-time PCR.

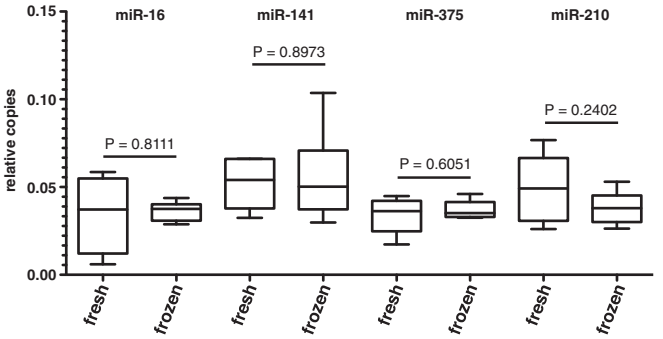

Fig. S2. Freezing does not significantly alter the abundance of miRNA in plasma exosomes. Plasma was freshly collected from individuals with prostate cancer ($n = 6$) and identical aliquots made for immediate lysis in a denaturing solution or storage at −80 °C. Frozen aliquots were subsequently thawed and lysed, and total RNA was extracted in parallel with freshly denatured samples. miRNA abundance was quantified by real-time PCR. Lines shows medians, boxes show 25th and 75th percentiles, and whiskers show 10th and 90th percentiles. *P* values were determined by paired *t* test (two tails).

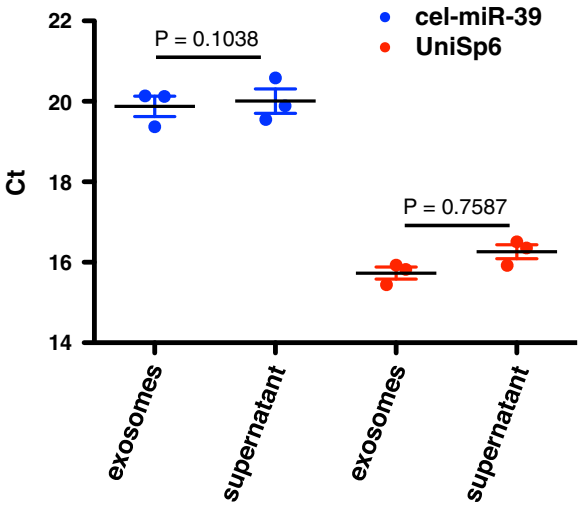

Fig. S3. miRNA purification, reverse transcription, and amplification efficiencies are not significantly different between exosome and supernatant samples. Synthetic *cel*-miR-39 oligoribonucleotides spiked into denatured samples before purification and UniSp6 oligoribonucleotide controls spiked into the reverse transcription reaction are not detected differently by real-time PCR analysis of pooled samples from Fig. 1*D*. *P* values were determined by unpaired *t* test with Welch's correction (two tails). Ct, cycle threshold.

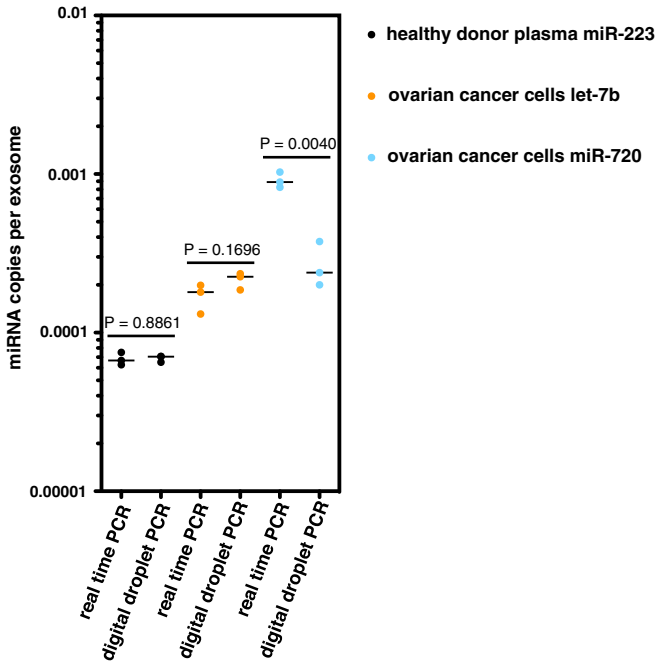

Fig. S4. Analysis of exosomal miRNA by ddPCR yields comparable results with analysis by real-time PCR. *P* values were determined by unpaired *t* test with Welch's correction (two tails).

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)
Table S5 (DOCX)