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Examination of the specificity of tumor cell derived exosomes with tumor cells in vitro

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ABSTRACT

Small endogenous vesicles called exosomes are beginning to be explored as drug delivery vehicles. The *in vivo* targets of exosomes are poorly understood; however, they are believed to be important in cell-to-cell communication and may play a prominent role in cancer metastasis. We aimed to elucidate whether cancer derived exosomes can be used as drug delivery vehicles that innately target tumors over normal tissue. Our *in vitro* results suggest that while there is some specificity towards cancer cells over "immortalized" cells, it is unclear if the difference is sufficient to achieve precise *in vivo* targeting. Additionally, we found that exosomes associate with their cellular targets to a significantly greater extent (>10-fold) than liposomes of a similar size. Studies on the association of liposomes mimicking the unique lipid content of exosomes revealed that the lipid composition contributes significantly to cellular adherence/internalization. Cleavage of exosome surface proteins yielded exosomes exhibiting reduced association with their cellular targets, demonstrating the importance of proteins in binding/internalization. Furthermore, although acidic conditions are known to augment the metastatic potential of tumors, we found that cells cultured at low pH released exosomes with significantly less potential for cellular association than cells cultured at physiological pH.

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1. Introduction

Exosomes, once thought to be nothing more than cell debris, are now believed to be an integral component of extracellular communication [1,2]. Exosomes are characterized as 50–100 nm vehicles synthesized through the reverse budding of the late endosomal membrane, forming a multivesicular body (MVB). Fusion of MVBs with the plasma membrane releases exosomes into the extracellular environment [3], where they can interact and associate with target cells. Proteomic analysis of exosomes reveals elevated levels of adhesion proteins that promote adsorption to the cell surface [4,5]. Exosomes have also recently been shown to attach to recipient cells via phosphatidylserine receptors [6,7]. After exosomes associate with their target cells, they are internalized through both endocytotic [8–10] and phagocytotic [11] pathways allowing for effective delivery of functional cargo to recipient cells [12–14]. Work by Al-Nedawi et al. demonstrated the ability of exosomes derived from highly aggressive brain gliomas to transfer the mutated epidermal growth factor receptor (EGFRvIII) to cancerous cells lacking EGFRvIII, thereby endowing cells with oncogenic activity [12]. Similarly, functional mRNA found in exosomes derived from a mouse mast cell line can be transferred to human mast cells. After transfer, new mouse proteins were translated in the recipient human cells [13]. These latter findings, combined with the observation that serum from cancer patients contains elevated levels of exosomes, are consistent with a role for exosomes in tumor progression [15].

Exosomes' innate ability to deliver their cargo to recipient cells makes them an intriguing candidate for use as a drug delivery vehicle. In fact, exosomes have recently been used to deliver siRNA to the brains of mice. Targeting to the brain was achieved through fusion of the neuron-specific RVF peptide to the lamp2b protein found on dendritic cell derived exosomes. Successful siRNA delivery resulted in a 62% knockdown in the therapeutic target [16]. In another recent study, mouse lymphoma cell-derived exosomes loaded with the anti-inflammatory drug curcumin were able to protect mice from a lipopolysaccharide septic shock challenge by delivering curcumin to activated myeloid cells. In contrast, curcumin incorporated into liposomes was only slightly more efficient than free curcumin in preventing mortality [17].

Previous studies have suggested that exosomes are more readily associated with cancer cells as compared to normal cells [18]. Considering the purported role of exosomes in intercellular communication within tumors, it follows that exosomes might be particularly effective for delivery to cancer cells, especially to the parent cell line that produced the exosomes. Tumor derived exosomes have a distinct protein and lipid composition resembling that of the cells from which they are derived, suggesting that exosomes may be uniquely suited to interact

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with their parent cells line in comparison to cells of various origins. In this study we quantify the association of exosomes with both cancer and immortalized cell lines, and compare this to the adherence/internalization of similarly-sized liposomes. Furthermore, we perform experiments to determine the extent to which the lipid and protein components contribute to exosome association with recipient cells.

In addition, it has been suggested by Parolini et al. [18] that low extracellular pH in which cells are grown increases the propensity for exosomes to fuse with target cells. Because the tumor microenvironment is known to be acidified [19], it is possible that the physiological properties of exosomes produced under these conditions may also be affected. We rigorously test this hypothesis by comparing the association of exosomes with recipient cells harvested from cells cultured at different pHs. To further elucidate any potential role of an acidic microenvironment on exosome association, the media of recipient cells was also acidified to assess its effect on adherence/internalization.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modification of Eagle's Medium (DMEM, with 4.5 g/L glucose, L-glutamine, and sodium pyruvate), Dulbecco's phosphate-buffered saline (DPBS), Fetal Bovine Serum (FBS), Trypsin, and Penicillin-Streptomycin were all purchased from Mediatech, Inc. (Manassas, VA). Plasmocin™ was obtained from InvivoGen (San Diego, CA). FBS was ultra-centrifuged for 10 h at 120,000 ×g to remove contaminating exosomes. All media was filtered with 0.22 μm low protein binding cellulose acetate filter from Thermo Fisher Scientific, Inc. (Rockford, IL) before use. MCF-7 cells and PC3 cells were purchased from ATCC. H460 and ARPE-19 cell lines were a gift from Dr. Uday Kompella. 16HBE cells were a generous gift from Dr. Brian Day. SK-Mel-5 and MDA-MB-231 cells were a gift from Dr. David Ross. WPMY cells were a gift from Dr. Isabel Schlaepfer. Uryanl Acetate was ordered from Electron Microscopy Sciences (Hatfield, PA). Formvar/carbon-coated EM grids were made at the University of Colorado, Denver Electron Microscopy Core. Sodium phosphate monobasic monohydrate, sodium phosphate dibasic and bis-tris (Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane) were purchased from Sigma-Aldrich (St. Louis, MO). Complete Mini EDTA-Free Protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Anti-calnexin was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-cytochrome C was purchased from Bio Legend (San Diego, CA). Anti-HSP70 was purchased from Cell Signalling Technology (Danvers, MA). Anti-HSP90 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD63 and Anti-CD9 were purchased from Abcam (Cambridge, MA). The chemiluminescent kit, ECL™ Plus Western Blot Detection System and both secondary antibodies, ECL™ Anti-mouse IgG horseradish peroxidase linked $F(ab')_2$, and ECL™ Anti-rabbit IgG horseradish peroxidase linked $F(ab')_2$ were obtained from GE Healthcare (UK Little Chalfont Buckinghamshire). Carnation powdered milk was purchased from Nestle (Solon, OH). Millipore Immobilon Polyvinylidene Fluoride (PVDF) Transfer Membranes were purchased from Thermo Fisher Scientific, Inc. (Rockford, IL). D(+)-Sucrose, 99.7%, for biochemistry, was purchased from Acros Organics (Fairlawn, New Jersey). L-α-phosphatidylcholine (Chicken Egg) (PC), L-α-phosphatidylserine (Porcine Brain) (PS), Sphingomyelin (Porcine Brain) (SM), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Proteinase K was obtained from Qiagen (Valencia, CA) and NEB2 buffer was purchased from New England BioLabs (Ipswich, MA). BCA Protein Assay Reagent, HPLC grade methanol and chloroform were purchased from Thermo Fisher Scientific, Inc. (Rockford, IL). DID (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and rhodamine DHPE (Rhodamine B 1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine, Triethylammonium Salt) were obtained from Invitrogen (Carlsbad, CA). Fluorescein was purchased from J.T Baker (Center Valley, PA). Hydrochloric acid was obtained from RICCA Chemical Company (Arlington, TX). Sequencing grade trypsin was purchased from Promega Corporation (Madison, WI).

2.2. Cell culture

All cancer (PC3, MCF-7, MDA-MB-231, SK-Mel-5, H460) and non-cancer immortalized (16HBE, WPMY-1, ARPE-19) cell lines were grown in DMEM, 10% FBS, supplemented with Pen-Strep and Plasmocin™ at 5 μg/ml. Media conditions were identical across all cell lines to prevent any media related effects on the extent of exosome association with the various cell lines. All cells appeared healthy and maintained consistent doubling times under these conditions. Identical media conditions were used for experiments in which the pH of the media was shifted from pH 7.4 to pH 6.8 and 6.3, with the addition of 30 mM Bis-Tris. After the addition of Bis-Tris to the media, HCl was used to lower the pH to 6.8 and 6.3, while no HCl was added to the media of cells cultured at pH 7.4.

2.3. Exosome isolation

Exosomes were isolated from the supernatant of PC3, MCF-7 and MDA, MB-231 cells lines. All cell lines were grown to 50% confluency in 225 cm^2 BD cell culture flasks, before the media was replaced by exosome-free DMEM 10% FBS media. After 48 h, the supernatant was collected and purified by a series of centrifugation steps: 10 min 300 ×g, 20 min 15,000 ×g, and 10 h 100,000 ×g. Concentrated exosomes were then washed in PBS and centrifuged at 200,000 ×*g* for 10 h on a sucrose density cushion. The sucrose cushion consisted of three distinct layers, 12 % sucrose, 30 % sucrose and 50% sucrose. Exosomes have been previously reported to have a density between 1.1 and 1.2 g/cm^3 [13,20,21]. Subsequently, the 30% sucrose fraction and the top of the 50% sucrose fraction were collected, washed with PBS, and centrifuged at 100,000 ×g for 10 h. The pelleted exosomes were re-suspended in 500 μl PBS. Exosome protein content was quantified using the BCA protein assay. It is well known that proteins account for 25–75% of the weight of various biological membranes, with most membranes possessing approximately 50% protein [22]. However, the exact protein-to-lipid weight ratio of exosome has not been quantified. For our experiments we assumed exosomes to be equal parts protein and lipid by weight, thus the total weight of exosomes was estimated by doubling BCA protein assay results. While assuming a 50/50 weight ratio of proteins to lipid in exosomes may result in slightly erroneous values when comparing to the equivalent amount of liposomes, the > 10-fold differences reported in Fig. 3 are significantly greater than could be accounted for by more extreme protein-to-lipid ratios in exosomes. Exosomes were then labeled with specific concentrations of DID based on the weight of each sample. Unincorporated DID was removed through density gradient centrifugation as described above.

2.4. Electron microscopy

Electron microscopy was performed on a Technai G2 Bio-twin made by FEI. Isolated exosomes, re-suspended in phosphate buffer, were deposited on formvar/carbon-coated EM grids and left to dry in ambient air. Phosphate buffer containing 2.0% uranyl acetate was added to the exosome-coated grids and left for 10 min before washing with phosphate buffer containing 0.4% uranyl acetate.

2.5. Confocal microscopy

Confocal microscopy was performed on a Nikon Eclipse TE2000-E microscope. Images were analyzed using Nikon EZ-C1 FreeViewer Version 390 software. Exosomes were labeled with the fluorescent molecule DID. Long-chain dialkylcarbocyanines, such as DID, are used

extensively to label biological membranes [23,24]. Five microliters of DID labeled exosomes, in PBS, were placed on a cover slip and imaged.

2.6. Fluorescent imaging of exosome uptake

Fluorescent images were taken using an OperettaTM High Content Imaging System instrument (Perkin Elmer; Waltham, MA). MCF-7 cells were grown on a 96-well black walled plate made by Greiner Bio-One (Monroe, NC). Five micrograms MCF-7 exosomes in 100 μl DMEM supplemented with 10% FBS, labeled with DID, were incubated with cells for 4 h. Cell media was subsequently removed and cells were rinsed with PBS twice. Cells were stained with Hoechst 33342 nucleic acid stain at a concentration of 10 μg/ml in PBS for 15 min. Cells were rinsed with PBS to remove free Hoeschst 33342 stain. Cells were then fixed for 10 min with 3.7% formalin. Formalin was removed by washing with PBS. Images were analyzed using Harmony 3.5.1 software manufactured by Perkin Elmer.

2.7. Western blotting

Cells and exosomes were lysed with lysis buffer containing protease inhibitors and subsequently centrifuged for 10 min at 500 × g to remove debris. Twenty micrograms of protein from cells or exosomes were separated by SDS-PAGE. Gels were blotted onto polyvinylidene fluoride (PVDF) membranes and incubated with Tris-buffered saline (TBS) containing 5% powdered milk for 1 h, followed by overnight incubation with 5% powdered milk and primary antibody. The PDVF membrane was then washed twice with TBS containing 0.05% Tween 20 before the addition of TBS with 5% powdered milk and the corresponding secondary antibody for 1 h. Chemiluminescence was used to identify the presence of the protein of interest. Chemiluminescence was imaged using a Molecular Dynamics Storm 860.

2.8. Mass spectrometry analysis

One hundred microgram of isolated exosomes from MCF-7 and PC3 cells were subjected to in solution digestions using performic acid. Performic acid solution was prepared in a 1:19 ratio of 30% hydrogen peroxide:formic acid. Performic acid solution was warmed to 55 °C for 3 min immediately prior to use. Three volumes of performic acid were added to the exosome sample and incubated on ice for 3 h. The reaction was quenched using 5 volumes of ice cold double distilled water. Samples were dried to completion using a speed vacuum and resuspended in 50 μl of 50 mM ammonium bicarbonate. Sequencing grade trypsin was added to the sample in a 1:50 trypsin:protein ratio and incubated overnight at 37 °C. The next morning the samples were dried to completion using a speed vacuum and the pellet resuspended in 30 μl of 0.1% formic acid in water. Tryptic digests were separated using 5–50% ACN gradient over 120 min on a C18 column (Michrocom, Agilent). MS/MS spectra were collected using the Amazon Speed ion ETD trap equipped with CaptiveSpray nanoBooster ionization source (Bruker Daltonics) at the University of Colorado School of Pharmacy Mass Spectrometry Core. Acetonitrile enriched nitrogen gas was used as a sheath gas to increase the charge state of peptide ions and enhance identifications. Data processing was performed using ProteinScape 3.1. Database searches were performed against all of the human entries in the Swiss Prot database using the Mascot Server using 0.6 Da peptide mass tolerance and 0.5 Da MS/MS tolerance allowing for 1 missed cleavage and modifications for dioxidation of methionine and trioxidation of cysteine. Protein identification was considered significant if at least 2 unique peptides were used for identification.

2.9. Sucrose density gradients

To identify the density of isolated exosomes, exosomes were placed on top of a discontinuous sucrose density gradient and centrifuged at 200 k × g for 10 h. The sucrose gradient was composed of 6 distinct sucrose density layers ranging from 1.05 g/cm^3 to 1.23 g/cm^3.

2.10. Lipid extraction

Lipids from PC3 cells and PC3-derived exosomes were extracted using the Bligh and Dyer method [25]. Briefly, approximately 300 μg exosomes or cells (based on BCA protein content) in 200 μl PBS were added to 1.9 ml $CHCl_3$:MeOH 1:2 and vortexed. Subsequently, 0.625 ml $CHCl_3$ was added and vortexed, followed by addition of 0.625 ml dH_2O. Samples were then centrifuged to separate the organic and aqueous phases. The organic phase was recovered and dried in pre-weighed HPLC vials. The HPLC vials were re-weighed on a Mettler Toledo MX5 Micro Balance. The difference in weight allowed for an accurate weight of total lipids extracted from PC3 cells and PC3 derived exosomes.

2.11. Liposome formulations

All liposome formulations were prepared by mixing lipids at specific ratios with DID in chloroform in pre-weighed 300 ul glass HPLC vials. Lipid mixtures in HPLC vials were dried under nitrogen gas and placed under vacuum to remove residual chloroform. As with exosome and cell lipid extracts, each liposome formulation in HPLC vials was re-weighed on a Mettler Toledo MX5 Micro Balance to obtain an accurate weight of total lipids. To the HPLC vials containing the dried lipids, 500 μl PBS was added, and subsequently sonicated to remove lipids from the HPLC vial walls. The lipid/PBS mixture was removed and extruded through 100 nm pore size polycarbonate membranes (Avestin, Ottawa, ON). All liposome formulations had a mean diameter of 105 ± 10 nm (data not shown).

2.12. Protein cleavage on exosomes

To cleave membrane bound proteins from the surface of PC3-derived exosomes labeled with DID, 200 μg exosomes (based on BCA protein content) were incubated with proteinase K at 50 μg/ml and 1x NEB2 buffer at 37 °C for 1 h, via a protocol modified from Escrevente et al. [9]. One microgram of control exosomes and proteinase K-treated exosomes were then immediately incubated with PC3 and MCF-7 cell lines for 4 h; control experiments indicated that the low level of proteinase K had no detectable effect on cell detachment/viability. Cells were then analyzed with flow cytometry to determine the extent of exosome association.

2.13. Sizing

Exosomes and liposomes were sized using a NanoSight LM20. Exosomes were diluted 1/1000 from stock before analysis. Measurements were done in triplicate, using the same stock of exosomes. The size and standard deviation from each of the three individual measurements was then averaged.

2.14. Flow cytometry

All flow cytometry experiments were performed on a Becton Dikinson FACScan. Briefly, between 1 and 10 ug of DID or rhodamine DHPE-labeled exosomes or liposomes were added to cells cultured in Costar® 96 well cell culture flat bottom tissue culture flasks. After 4 h incubation with either exosomes or liposomes, cells were rinsed with PBS, removed by trypsinization, suspended in PBS, and put on ice. A minimum of ≥5,000 events were acquired per sample. The small amount of exosomes harvested in any individual preparation require that experiments be replicated a minimum of three times with exosomes from different preparations. While the absolute numbers for association did vary among different exosome preparations, consistent

trends were observed. Results from an individual experiment are reported, and the differences were statistically analyzed with FlowJo software as detailed below. The y-axis of flow cytometry data is presented as a count of cells, where each data set is fit to have equivalent maximum peak heights in order to facilitate visual comparison between samples. Using this type of analysis, the area under the curve does not indicate the absolute number of cells analyzed. The extent of exosomes association with recipient cells was quantified by monitoring the percentage of cells that had increased fluorescence when compared to control cells ("positive"). Probability binning, built into Flowjo software, was use to statistically analyze samples of the same cell line, while t-tests were used to statistically analyze the differences among samples across multiple cell lines.

3. Results

3.1. Characterization of exosomes isolated from cell culture

Exosomes isolated from the supernatant of cultured MCF-7 cells were evaluated using electron microscopy. Fig. 1A depicts vesicular structures, uniform in size (50 -100 nm), and comparable to previously described exosomes [21,26–28]. Further, the presence of HSP90, HSP70, calnexin, cytochrome c, CD9, and CD63 in MCF-7-derived exosomes was assessed by western blot analysis. Exosomes are known to be selectively enriched in the cytosolic proteins HSP90 and HSP70 and the tetraspan glycoproteins CD9 and CD63, while proteins associated with the endoplasmic reticulum and mitochondria are not actively incorporated into exosomes [26,29–33]. MCF-7 cell lysates were positive for all proteins analyzed, while MCF-7-derived exosome samples were devoid of cytochrome c and had minimal calnexin expression (Fig. 1B). The lack of ER and mitochondrial proteins in exosome preparations demonstrates that the vesicles observed with electron microscopy are isolated exosomes that are relatively free of cell debris. Post isolation, exosomes were labeled with the lipophilic fluorescent probe DID (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate). To visualize the uptake of MCF-7 exosomes labeled with DID, MCF-7 exosomes were incubated with MCF-7 cells for 4 h. Post incubation, cells were washed with PBS and the nucleus of cells were fluorescently stained. Uptake of 4 T1 exosomes can be seen in Fig. 1C.

Table 1
Exosome mean sizes and standard deviations are calculated from three separate measurements on the preparations used in our experiments. PC:Cholesterol (mole ratio 2:1) liposomes were extruded through 100 nm pore size polycarbonate membranes.

	Size (nm)	S.D.
MCF-7	138 ± 5	55 ± 9
MDA-MB-231	142 ± 10	50 ± 1
PC3	146 ± 13	62 ± 7
Liposome	105 ± 10	35 ± 2

To further characterize the presence of isolated exosomes, LC/MS was performed on both MCF-7 and PC3 exosomes. We identified 59 unique proteins in MCF-7 exosome samples (Supplemental Table 1) and 80 unique proteins from PC3 (Supplemental Table 2). To each table we have added the subcellular localization and function of each protein identified in MCF-7 and PC3 exosomes. Analysis of the data revealed the presence of the canonical markers of exosomes in both MCF-7 and PC3 exosome samples including: Heat Shock Proteins 70, 90, and 27; CD9, Ras-related protein 13 (RAB-13 and others);

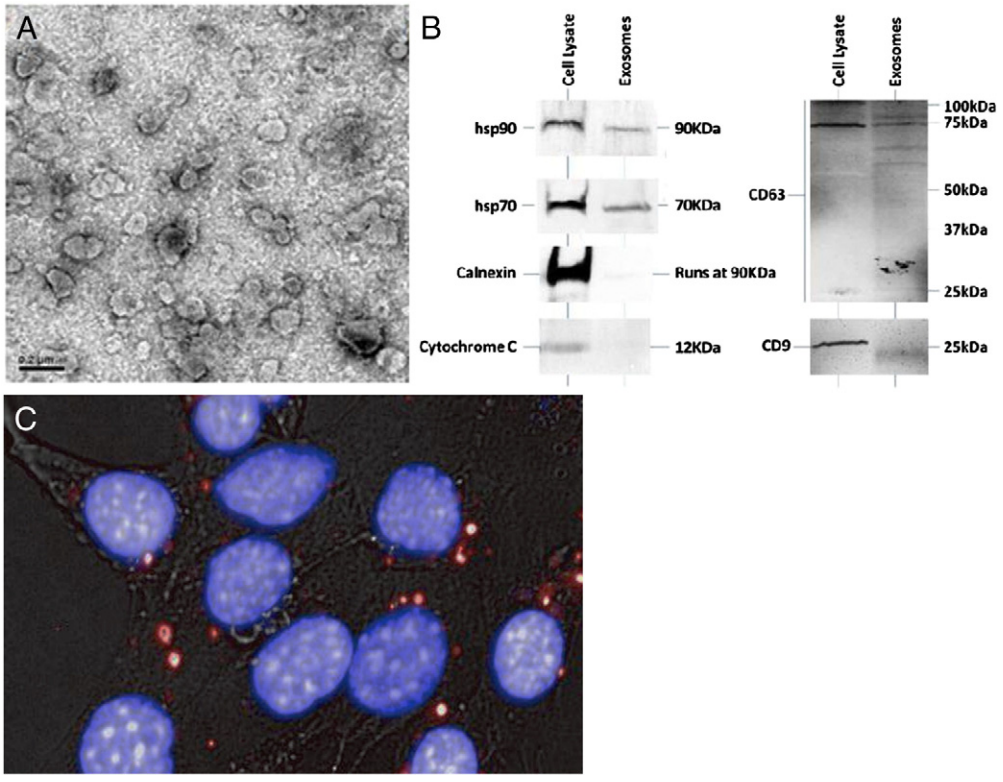

Fig. 1. Characterization of exosomes. A) Electron microscope image of exosomes derived from MCF-7 cells. Scale bar = 0.2 μm. B) Biochemical characterization of exosomes by western blot. MCF-7 exosomes and MCF-7 cells were lysed with lysis buffer, and debris removed. The protein content of MCF-7 exosomes and MCF-7 cell samples were quantified using the BCA protein assay. Twenty micrograms of exosome protein or cell proteins were separated using SDS-PAGE and subsequently subjected to western blotting with antibodies for HSP70, HSP90, calnexin, cytochrome c, CD9 and CD63. Isolated exosomes were positive for HSP90, HSP70, CD9 and CD63, but were void of cytochrome c and had negligible calnexin compared to cell lysate, which was positive for all proteins. CD63 proteins in both exosomes and cell lysate samples self-associated to make higher molecular weight species. The western blot demonstrates successful separation of exosomes from cells and cell debris. C) MCF-7 cells were incubated for 4 h with DID-labeled exosomes (red). MCF-7 nuclei were stained with Hoeschst 33342 (blue). Bright field images are overlaid to visualize cells.

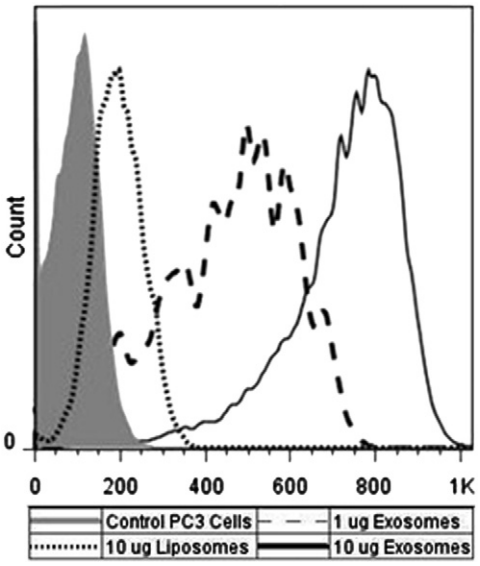

Fig. 2. Association of exosomes and liposomes with PC3 cells. PC:Cholesterol (2:1 mole ratio) liposomes or PC3-derived exosomes labeled with 0.01% DID were incubated with PC3 cells for 4 h at 37 °C. Extent of association was measured using flow cytometry. Mean autofluorescence for PC3 control cells was 102, mean fluorescent association by PC3 cells incubated with 10 μg of liposomes was 194; PC3 cells incubated with 10 μg exosomes had a mean fluorescence of 718, and cells incubated with 1 μg exosome had a mean fluorescence of 425.

Annexins 1, 2, 5, and 7; Pyruvate kinase (PKM); Alpha-enolase (ENO1); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Glucose-6-phosphate 1-dehydrogenase (G6PD); and actin [33]. Furthermore, a search of Vesiclepedia (http://microvesicles.org/) in June 2014, revealed that every protein identified, using LC/MS, in both MCF-7 and PC3 exosomes had been previously found associated with exosomes. In addition, the vast majority of proteins identified also are classified in the UniProtKB Gene Ontology Cellular Component sections as members of "extracellular vesicles exosomes", further supporting the notion that our protein cohorts are derived from exosomes (http://www.uniprot.org/).

3.2. Comparison of exosome and liposome cellular association

The biological role of exosomes is unclear; however, one potential function is cell-to-cell communication. Numerous groups have published on the ability of exosomes to be endocytosed by both the "parent" cell line [9,10,34] and by cells of various origin [11,13,35–38]. Here we examine the extent of exosome adherence/internalization by the "parent" cell line in comparison to 105 nm liposomes (Table 1) composed of PC:Cholesterol, mole ratio 2:1. This lipid composition was chosen because it has been studied extensively and has a similar composition to Daunoxomes, a commercial liposome product used to treat cancer. Ten micrograms of liposomes and either 1 ug or 10 ug PC3-derived exosomes labeled with 0.1% of the fluorescent dye DID were incubated with PC3 cells for 4 h at 37 °C. The degree of association was analyzed using flow cytometry (Fig. 2). The higher mean fluorescence intensity of the cells incubated with 1 μg of exosomes, in comparison to cells incubated with 10 μg liposomes, suggests that considerably greater than ten times more exosomes were associated with PC3 cell than PC:Cholesterol liposomes. This would indicate the physical/chemical properties of exosomes promote association to cells, and reaffirms the notion of exosomes' importance in cell-to-cell communication.

3.3. Extent of exosome adherence/internalization by various cells lines

Cell-to-cell communication via exosomes may not only be important for normal cell function, but also may play a role in cancer progression and metastasis. This is consistent with the elevated exosome concentrations in the blood of cancer patients [15,39]. To assess whether exosomes are associated with their "parent" cell line to a greater extent than by cells of various origins, and to determine if exosomes adhere/internalize with cancer cells (independent of origin) more than with "normal" immortalized cells, exosomes derived from three cancer cell lines were added back to the media of five cancer cell lines and three

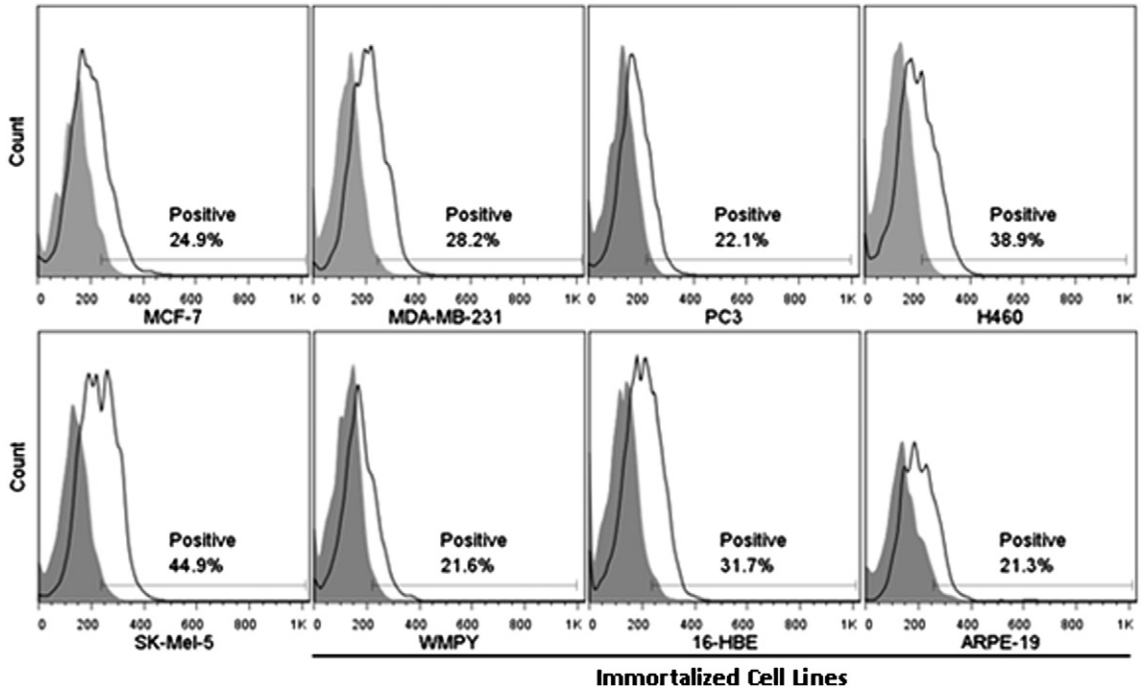

Fig. 3. Extent of PC:Cholesterol (2:1 mole ratio) liposomes association with five cancer cell lines (MCF-7, MDA-MB-231, PC3, H460 and SK-Mel-5) and three immortalized cell lines (WMPY, 16-HBE and ARPE-19). Liposomes labeled with 0.01% DID were incubated with all cell lines for 4 h at 37 °C. Extent of association was measured using flow cytometry. Association was quantified by monitoring the percentage of cells that had increased fluorescence when compared to control cells ("Positive").

Table 2
Extent of exosome association with recipient cells, as seen in Figs. 3 and 4, are compiled for comparison. Adherence/internalization experiments of liposomes and exosomes were performed simultaneously to prevent day-to-day variations in the extent of association. Bolded numbers indicate association of "daughter" exosomes by the "parent" cell line. Both liposome association and exosome association values for both cancer and immortalized cell lines are averaged. Data from Fig. 4 is further normalized as described in the text to account for differences in non-specific association when comparing exosome association between cancer and immortalized cell lines.

	Percent Positive Uptake				Normalized Percent Positive			
Cell line	Liposome	**MCF-7 Exo**	MDA Exo	PC3 Exo	Relative Liposome Uptake	**MCF-7 Exo**	MDA Exo	PC3 Exo
MCF-7	24.9	**34.1**	32.4	16.4	0.9	**38.6**	36.7	18.6
MDA-MB-23**1**	28.2	40.6	**18.0**	**17.2**	1.0	40.6	**18.0**	17.2
PC3	22.1	32.8	24.3	**18.0**	0.8	41.9	31.0	**23.0**
H460	38.9	45.1	32.2	20.3	1.4	32.7	23.3	14.7
SK-Mel-5	44.9	39.8	22.1	16.9	**1.6**	25.0	13.9	10.6
WPMY	21.6	29.8	14.7	9.5	0.8	38.9	19.2	12.4
16-HBE	31.7	15.5	7.7	6.1	1.1	13.8	6.9	5.4
ARPE-19	21.3	45.7	16.2	14.0	0.8	60.5	21.4	18.5
Average	29.2	35.4	21.0	14.8	_	36.5	21.3	15.1
Average Liposome Cancer % Positive				31.8	Average Normalized Cancer % Positive			25.7
Average Liposome Immortalized % Positive				24.9	Average Normalized Immortalized % Positive			21.9
Average Exosome Cancer % Positive				27.3	Average Normalized WPMY and ARPE-19 % Positive			28.5
Average Exosome Immortalized % Positive				17.7				
Average Exosome WPMY and ARPE-19 % Positive				21.7				
Average Liposome WPMY and ARPE-19 % Positive				21.5				

immortalized cell lines. Comparison of particle association with numerous cell lines is complicated by differences in cell doubling times, size, and morphology, and by the rate of endocytosis. Cells chosen for analysis were derived from a range of tissues. Because cells were derived from many different tissues, we expect their peripheral lipid and protein content to be distinct from each other. Additionally, cells were chosen for study based on their growth rate and physical characteristics. All eight of the cell lines used for our experiments doubled approximately every two days (data not shown). Further, each cell line's size and morphology was scrutinized using microscopy. The cell lines selected for our experiments all grew as a monolayer, while cell lines with exceptionally large or small cell morphology were excluded from our experiments due to concerns that cells with significantly greater surface area might exhibit artificially enhanced association *per cell* as determined by flow cytometry.

Additionally, to ensure that any differences in the extent of exosome adherence/internalization by the various cells was not a result of varying levels of non-specific endocytosis, 10 μg of neutral (PC:Cholesterol 2:1 mole ratio) liposomes were incubated with all eight cell lines. The extent of liposome association was monitored by flow cytometry and used to assess non-specific association in each cell line (Fig. 3). The eight cell lines monitored had a baseline auto-fluorescence of 129 ± 8.8. Increase in cell fluorescence, as a result of liposome association, was quantified by monitoring the percentage of cells that had increased fluorescence when compared to control cells, and was reported as percent positive. The five cancer cell lines, from a wide range of tissues, (MCF-7 — mammary gland/breast, MDA-MB-231 — mammary gland/breast, PC3 — prostate, H460 — lung, and SK-Mel-5 — skin) on average took up liposomes to a slightly greater extent than the three immortalized cell lines (WPMY — prostate/stroma, 16-HBE- bronchial epithelial cells, and ARPE-19 — retinal pigmented epithelium cells) (Table 2). We assume that these rates of liposome association reflect differences in non-specific association, and relative values are used to account for differences in binding/endocytotic activity among cell lines (see below).

Exosomes collected from two metastatic cancer cell lines, MDA-MB-231 and PC3, and one non-metastatic cell line MCF-7, were labeled with DID. Fluorescently-labeled exosomes were added back to the media of both cancer and immortalized cells. After a 4-h incubation, cells were removed and analyzed for changes in fluorescence by flow cytometry. As described above for liposomes, association was quantified by monitoring the percentage of cells that had increased fluorescence when compared to control cells.

Analysis of the data in Fig. 4 suggests that exosomes derived from MCF-7, MDA-MB-231, and PC3 cancer cell lines are not preferentially associated with their "parent" cell lines. When taken as an average, the three immortalized cell lines have decreased exosome association as compared to the five cancer cell lines, as seen in Fig. 4. However, non-specific adherence/internalization by immortalized cells is also lower than that of the five cancer cell lines (Fig. 3). In order to compare the extent of exosome association with the various cell lines, and account for the differences in non-specific adherence/internalization, the data were normalized by calculating the ratio of percent positive liposome adherence/internalization for all cell lines relative to MDA-MB-231 cells, and that value was used to normalize all exosome association data (Table 2). Normalization revealed that on average, the three immortalized cell lines accumulated exosomes to a lesser extent than the cancer cell lines. But, further examination shows that 16-HBE cells had significantly decreased exosome association in comparison to cancer cells, while WPMY and ARPE-19 cells had relatively similar extents of exosome association as the five cancer cell lines.

Although there does not appear to be increased exosome association with their "parent" cell lines or a distinct difference in exosome association between cancer and immortalized cells, adherence/internalization of MCF-7 exosomes was significantly greater than exosomes derived from MDA-MB-231 and PC3 cells. To ensure that the increased adherence/internalization of MCF-7 exosomes was not due to increased size or density resulting in enhanced settling on monolayer cells causing more cell contact, exosomes from MCF-7, MDA-MB-231 and PC3 cells were sized using a NanoSight LM20. As seen in Table 2, exosomes derived from each cell line have similar sizes and standard deviations. Additionally, MCF-7, MDA-MB-231, and PC3 exosomes all partitioned into the sucrose gradient layer corresponding to 1.18 g/cm^3 (data not shown), indicating that the isolated exosomes from the three cell lines have similar densities. These data indicated that increased association of MCF-7-derived exosomes was not likely a result of differences in their physical properties. Instead, these data suggest that the constituents of MCF-7 exosomes are responsible for their greater adherence/internalization.

Fig. 4. Exosome association with cancer and immortalized cell lines. Exosomes isolated from the supernatant of MCF-7, MDA-MB-231, and PC3 cell lines were added back to the media of five cancer cell lines (MCF-7, MDA-MB-231, PC3, H460 and SK-Mel-5) and three immortalized cell lines (WMPY, 16-HBE and ARPE-19). Exosomes were labeled with 0.003% DID by weight; the greater association of exosomes as compared to liposomes required lower levels of DID to avoid detector saturation. Extent of association was measured using flow cytometry and quantified by monitoring the percentage of cells that had increased fluorescence when compared to control cells ("Positive"). Results from a single experiment are presented; repeat experiments yielded virtually identical results.

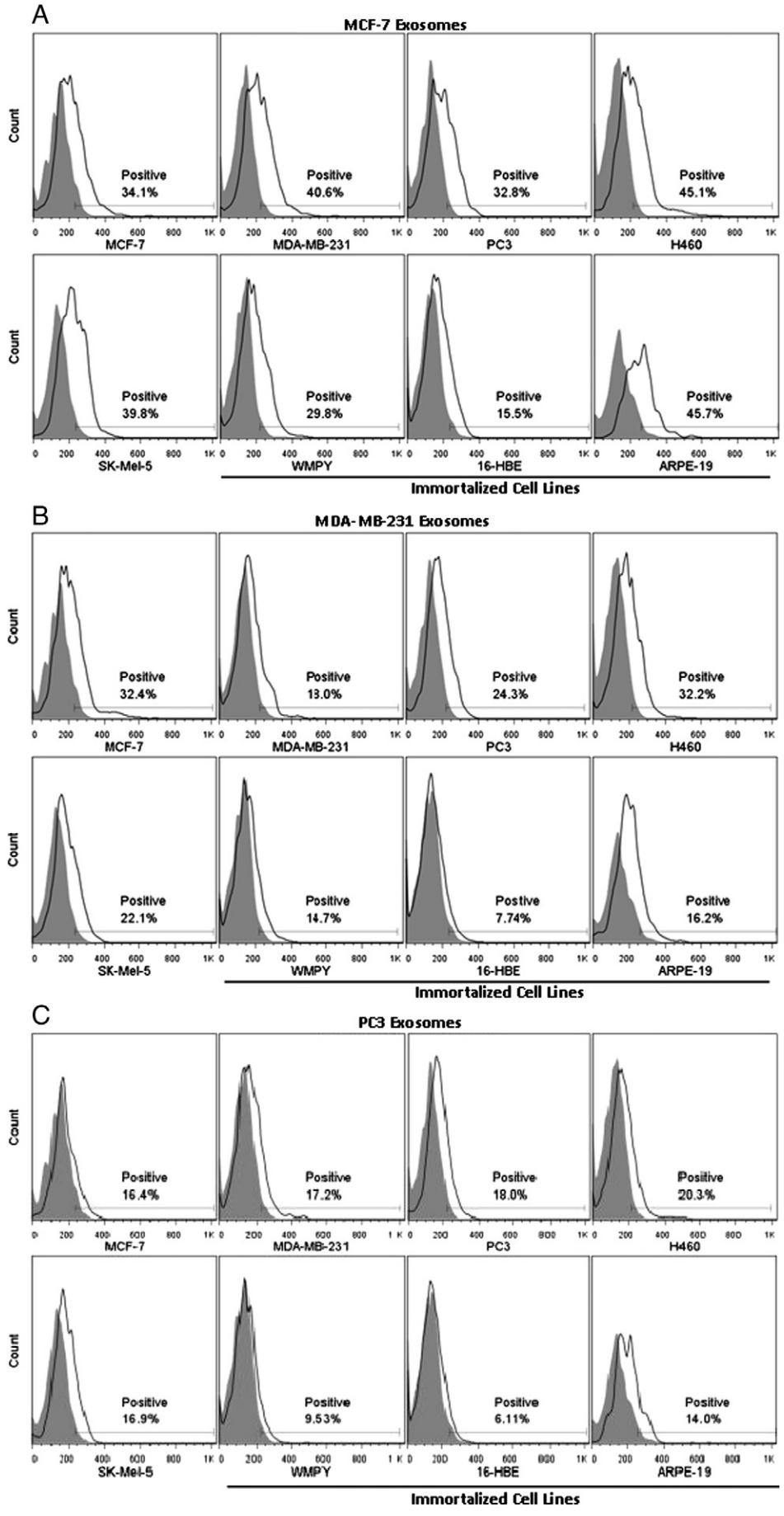
A
MCF-7 Exosomes
Count
Positive 34.1%
Positive 40.6%
Positive 32.8%
Positive 45.1%
MCF-7
MDA-MB-231
PC3
H460
Positive 39.8%
Positive 29.8%
Positive 15.5%
Positive 45.7%
SK-Mel-5
WMPY
16-HBE
ARPE-19
Immortalized Cell Lines
B
MDA-MB-231 Exosomes
Positive 32.4%
Positive 13.0%
Positive 24.3%
Positive 32.2%
Positive 22.1%
Positive 14.7%
Postive 7.74%
Positive 16.2%
C
PC3 Exosomes
Positive 16.4%
Positive 17.2%
Positive 18.0%
Positive 20.3%
Positive 16.9%
Positive 9.53%
Positive 6.11%
Positive 14.0%

Table 3
Representative cholesterol and phospholipid composition of exosomes and cells. Mole percentages were compiled from the works of Wubbolts et al. 2003(37), Laulagnier et al. 2004(38) and 2005(39), Subra, Laulagnier et al. 2007(40), Trajkovic et al. 2008(41), and Parolini et al. 2009(18).

	Cholesterol		Phospholipids	
Mole % Cell	15		85	
Mole % Exosome	30		70	
Change From Cell Membrane	2×		.82×	
Phospholipids	Sphingomyelin	PSPI	PC	PE
Mole % Cell	10	10	55	25
Mole % Exosome	25	20	30	25
Change From Cell Membrane	2.5×	2×	.54×	1×

An additional goal of the study was to compare the association of prostate cancer-derived exosomes with cancer and immortalized prostate cells. The data indicate that there is a significantly greater association of PC3-derived exosomes by the prostate cancer (PC3) cells as compared to the immortalized prostate (WPMY) cells ($p < .05$). This finding suggests that in the tumor microenvironment, PC3 exosomes may preferentially associate with cancer cells over normal cells, and thus PC3-derived exosomes may serve as selective delivery vehicles for prostate cancer therapeutics. Unfortunately, we were unable to do a similar comparison with breast cancer cell lines because immortalized breast cells (MCF-12 A and MCF-10A) did not fit the growth criteria to be included in the experiment. As a result, we cannot draw definitive conclusions regarding differential exosome adherence/internalization in breast cancer cells versus immortalized breast cells.

3.4. Influence of lipids and proteins on exosome cellular association

To elucidate why exosomes adhere/internalize to a significantly greater extent (>10-fold) than PC:Cholesterol liposomes, we investigated how the lipid and protein components of exosomes affect adherence/internalization. A number of studies have concluded that exosomes have a unique lipid composition, distinct from the plasma membrane and organelles of the "parent" cell line [18,24,40–43]. Currently, no consensus on the lipid composition of exosomes derived from various cells has been reached; however, there does appear to be a number of trends conserved across the various cell types. Table 3 is a compilation of the lipid composition of exosomes reported in the literature. Evaluation of the trends reported for exosome lipid composition revealed a two-fold increase in the mole percentage of cholesterol in exosomes as compared to "parent" cells. Additionally, the mole percentage of phospholipids is altered considerably in exosomes. Exosomes also contain 2.5-fold more sphingomyelin (SM) and two-fold more anionic lipids (PS/PI) compared to their "parent" cells. Phosphatidylcholine (PC) decreases from 55% to 30% of the phospholipid content, and phosphatidylethanolamine (PE) remains relatively unchanged in exosomes with respect to the "parent" cell line.

To assess if the lipid composition of exosomes affects their propensity for cellular association, fluorescently-labeled liposomes with specific cholesterol/phospholipid compositions mimicking either the "parent" cell lipid content or the exosome lipid composition (Table 3) were incubated with PC3 cells for 4 h at 37 °C. Flow cytometry analysis indicated that liposomes formulated to mimic exosomes (30/70 cholesterol/phospholipid mole ratio) adhere/internalize significantly more than liposomes formulated to mimic the "parent" cell lipid composition (15/85 cholesterol/phospholipid mole ratio; Fig. 5) to their cellular targets. In order to determine whether the increase in cholesterol content or the change in phospholipid ratios is responsible for the increased association, a third formulation having the phospholipid content of the "parent" cell but increased cholesterol content (30%) was evaluated. Results with the high cholesterol "parent" cell liposomes (30/70 cholesterol/phospholipid mole ratio) suggest that although the increased cholesterol content improved adherence/internalization, the phospholipids also contribute to the enhanced adherence/internalization (Fig. 5A). PS receptors on phagocytic cells have been shown to bind PS on liposomes and exosomes [6,7,44,45], suggesting a similar receptor-mediated interaction with PS maybe augmenting the association of liposomes and exosomes with recipient cells.

To further characterize the importance of the exosome lipid composition with respect to association with recipient cells, lipids from PC3 cells and PC3-derived exosomes were extracted and used to prepare fluorescently-labeled liposomes. After incubation with PC3 cells, data from flow cytometry showed that liposomes prepared from exosome lipid extracts associate to a significantly greater extent with PC3 cells compared to liposomes prepared from total cell lipid extracts (Fig. 5B). Similarly, liposomes prepared from the lipid extracts of exosomes adhered/internalized significantly more than PC:Cholesterol liposomes (Fig. 5C). These data further indicate that the lipid composition of exosomes contributes to their enhanced cell association.

In addition to the lipid composition, the protein content of exosomes is known to contain elevated levels of adhesion/targeting, transmembrane, and transport/fusion proteins relative to the "parent" cell [46,47]. To investigate the importance of proteins with respect to exosome endocytosis, PC3-derived exosomes were incubated with proteinase K to cleave transmembrane proteins protruding from the surface of exosomes or proteins otherwise adhering to exosome surfaces. Incubation of PC3-derived exosomes subjected to proteinase K digestion revealed a significant decrease in their association with both PC3 and MCF-7 cells as compared to untreated PC3-derived exosomes (Fig. 6). Results are similar to those seen by Escrevente et al. [9]. No significant change in the size of exosomes, pre- and post-proteinase K

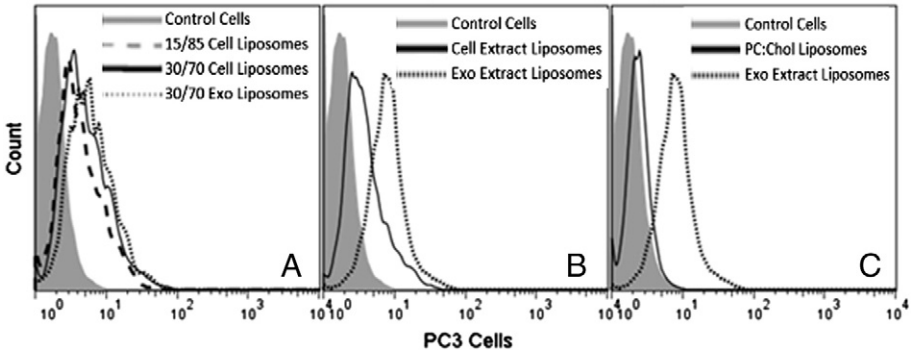

Fig. 5. Effect of lipid content of liposome association. A) Liposomes with specific cholesterol/phospholipid content simulating the lipid content of either exosomes or cells were incubated with PC3 cells. Association of 15/85 Cell Liposomes is significantly lower than 30/70 Exosome Liposomes ($p < .05$). B) Liposomes prepared from the lipid extracts of PC3-derived exosomes and PC3 cells were incubated with PC3 cells. Association of the liposomes composed of exosome lipids are significantly greater ($p < .05$). C) Association of PC:Cholesterol liposomes (2:1 mole ratio) was compared to liposomes prepared from exosome lipid extracts. Association of liposomes prepared from exosome lipids was significantly higher ($p < .05$).

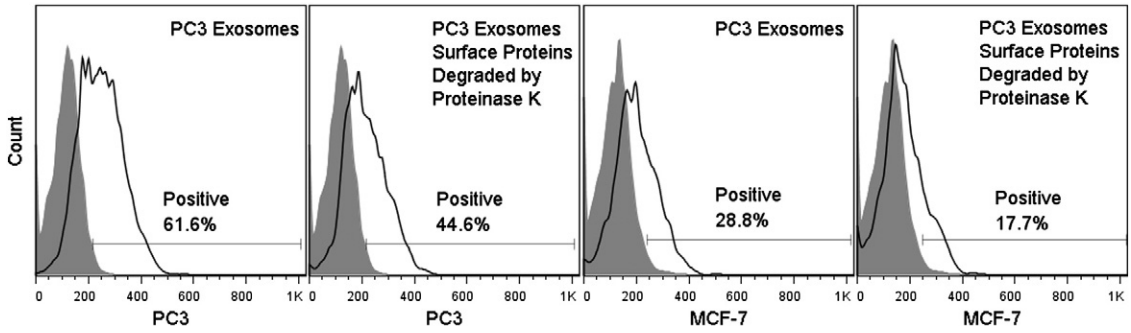

Fig. 6. Effect of proteins on exosome association. PC3-derived exosomes were treated with proteinase K to remove surface protein moieties. PC3-derived exosomes (untreated or subjected to proteinase K) were incubated with PC3 and MCF-7 cells for 4 h at 37 °C. Association was quantified by monitoring the percentage of cells that had increased fluorescence when compared to control cells ("Positive"). For both PC3 and MCF-7 cells, untreated PC3 exosomes were associated with their cellular targets to a significantly greater extent than PC3 exosomes subjected to proteinase K ($p < .05$).

digestion was observed (data not shown). At this time, it is unclear which proteins are most involved in exosome–cell interactions. Furthermore, it is possible that not all proteins are cleaved from surface of exosomes using this technique. Thus our results may be underestimating the extent to which proteins facilitate exosome association with recipient cells.

3.5. pH cell conditions influence exosome cellular association

Tumor microenvironments are known to be acidified reaching a pH of as low as 6.6 [19]; conditions known to affect numerous biological properties of cells [48–50]. This suggests that exosomes released from cells cultured below physiological pH may also have distinct properties, including their propensity for association with recipient cells. To elucidate how the pH of the microenvironment affects the properties of the "daughter" exosomes, MDA-MB-231 and PC3 cells were cultured at pH 7.4, 6.8, and 6.3 for 48 h. Subsequently, exosomes were isolated from the media of both cell lines cultured at the three different pHs. All exosomes were characterized based on size and density to determine if differences in physical properties resulted from culture in acidic conditions. Size measurements of exosomes released from both MDA-MB-231 cells and PC3 cells cultured at all three pHs revealed no significant differences (Table 4). Additionally, exosomes derived from PC3 cells cultured at pH 7.4, 6.8, and 6.3 were placed on a sucrose gradient and centrifuged at 200 k × g for 10 h. The majority of exosomes derived from each pH accumulated into the sucrose layer corresponding to a density of 1.18 g/cm^3 (Fig. 7). The density of exosomes has been previously characterized to be between 1.12 and 1.20 g/cm^3 [21,51]. The similar sizes and densities suggest that any differences in adherence/internalization do not result from altered physical properties.

Exosomes derived from MDA-MB-231 and PC3 cells cultured at pH 7.4, 6.8, and 6.3 were labeled with the fluorescent probe DID at 0.01% by weight. One microgram of labeled exosomes was added back to the media of their "parent" cell line growing at pH 7.4, 6.8, and 6.3 for 4 h at 37 °C (Fig. 8) to assess any differences in exosome adherence/internalization. Exosomes isolated from MDA-MB-231 and PC3 cells cultured at pH 7.4 were associated with their cellular targets to a slightly greater extent than exosomes isolated from cells cultured at pH 6.8. The most drastic difference was seen when comparing both pH 7.4- and pH 6.8-derived exosomes to exosomes isolated from cells cultured at pH 6.3. The pH 6.3-derived exosomes were associated significantly less than pH 7.4- and pH 6.8-derived exosomes by MDA-MB-231 cells cultured at all pHs. Taken collectively, the results identify major differences between the exosomes derived from cells cultured at pH 7.4 and pH 6.3 with respect to association with their parent cell line. In contrast, the pH in which cells are growing does not dramatically alter their association profile regardless of the conditions under which exosomes were harvested.

Table 4
Exosome mean sizes and standard deviations were calculated from three separate measurements on preparations harvested from cells grown at the indicated pH.

	pH 7.4		pH 6.8		pH 6.3	
	Size (nm)	S.D.	Size (nm)	S.D.	Size (nm)	S.D.
MDA-MB-231	152 ± 8	78 ± 11	130 ± 8	69 ± 11	152 ± 8	78 ± 11
PC3	156 ± 18	64 ± 10	151 ± 10	77 ± 19	138 ± 10	62 ± 20

4. Discussion

The scope of exosomes' biological functions is still being unraveled, and cell-to-cell communication is frequently implicated. Previous studies have shown that exosomes are capable of delivering functional cargo *in vitro* [12–14] and *in vivo* [16,17], leading others [47,52,53] to propose the use of exosomes as efficient drug delivery vehicles. Considering reports of exosome uptake by the parent cell line [9,10,34], and the potential to develop exosomes derived from cancer cells as tumor-homing delivery vehicles, the main goal of this study was to determine if exosomes are endowed with properties that allow for preferential association with their parent cell line or cancer cells in general. To this end, our *in vitro* studies have identified differences in the adherence/internalization of exosomes by cancer and immortalized cell lines. Differences in the non-specific association with the various cell lines may also affect the extent of exosome adherence/internalization, a factor that has not been considered in prior studies. Normalization of the data to account for non-specific (liposome) association suggests that exosomes are not selectively associated with a specific cell line(s). However, because exosomes associate with their cellular targets to a significantly greater extent than liposomes (at least 10-fold) in all cell lines, the adherence/internalization mechanisms may be completely different for these vehicles. While the greater association of exosomes could potentially be exploited for drug delivery, the differences in adherence/internalization between cancer and immortalized cell lines in culture may not be sufficient for cancer-derived exosomes to specifically target their "parent" cell line, suggesting that targeting moieties may need to be incorporated into exosomes to more efficiently target tumors[16].

The unique composition of exosomes [18,24,40–43] likely contributes to the enhanced association as compared to liposomes. Our results are consistent with this suggestion, and indicate that liposomes composed of exosomal lipids are more readily associated with recipient cells (Fig. 5). It is important to recognize that exosomes are enriched in cholesterol, SM, and anionic phospholipids, most notably PS, with a corresponding decrease in PC. Work with model membranes suggests that high cholesterol and SM concentrations endow exosomes with significant rigidity and stability [54–57]. Elevated cholesterol concentrations increase the cohesive properties of the membrane resulting in decreased permeability [54]. In addition, cholesterol and SM are known to form intermolecular hydrogen bonds [55,56] in a 1/1 stoichiometric

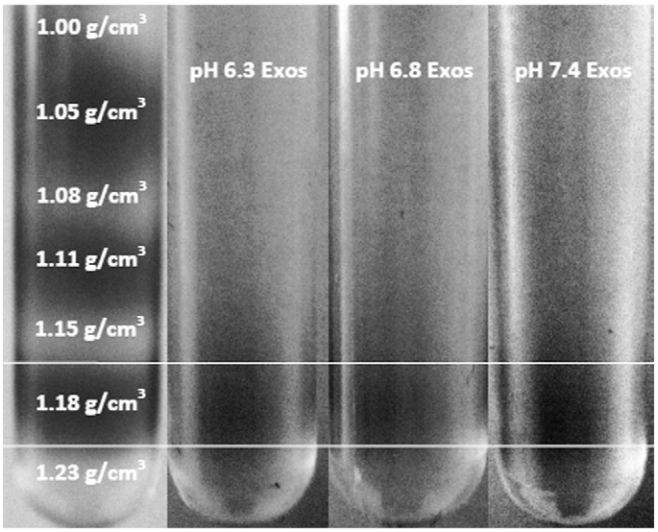

Fig. 7. Density of isolated exosomes. Exosomes labeled with 0.01% DID in PBS loaded onto a continuous sucrose gradient and centrifuged at 200 k ×g for 10 h. The fluorescent dye, DID, allowed for visual identification of exosomes. In order to distinguish between each sucrose layer, a control sucrose gradient was run (far left) where every other sucrose layer was stained with fluorescein. Exosomes isolated from the supernatant of PC3 cells cultured at pH 6.3, 6.8 and 7.4 all partitioned into the sucrose layer corresponding to a density of 1.18 g/cm3.

complex [54], forming lipid raft domains [57]. Recent studies from our laboratory have suggested that cholesterol domains increase gene delivery both *in vitro* [58,59] and *in vivo* [60], implicating lipid domains as important modulators of intracellular trafficking. The stability and rigidity imparted by cholesterol and SM also likely enable exosomes to circulate in the blood for extended periods as cholesterol and SM together incorporated into model liposomes decrease the rate of clearance by the RES (reticuloendothelial system) [56,61,62]. The tight packing of the membrane also potentially inhibits the transfer of exosome lipids to high-density lipoproteins (HDL) [56]. While cholesterol and SM promote long *in vivo* circulation times of lipid vesicles, PS is present on the exterior of apoptotic bodies and foreign material which signals macrophages to engulf these particles [62]. Recently, PS receptors on phagocytic cells have been seen to bind PS in exosomes, which would likely compromise circulation times [6,7]. It is interesting that work by Haque et al. reported that a neutral lipid molar ratio of 35/30/15/20 for PC/PE/SM/cholesterol, respectively, was optimal for liposome fusion and stability [63]. It is intriguing that this lipid composition closely resembles the neutral lipid molar ratios of exosomes, suggesting that nature has optimized the lipid components of exosomes for maximum *in vivo* stability and delivery.

We have demonstrated the importance of exosome lipids in facilitating adherence/internalization with recipient cells. However, the protein components of exosomes may also augment exosome adherence/internalization. Exosomes are enriched in transmembrane proteins, including adhesion proteins, tetraspanins, and the ICAM family of proteins. Adhesion proteins, most notably integrins, are abundant in exosomes and are important cell adhesion receptors [64,65]. Integrins on the outer surface of B cell-derived exosomes have been shown to promote binding to infant foreskin fibroblasts [66]. Another important class of transmembrane proteins are tetraspanins, which are believed to be responsible for target cell selection and interactions [67]. Furthermore, ICAMs found on exosomes may facilitate capture at the cell surface [4,5]. Our results demonstrating that cleavage of exosomal proteins severely diminished the extent of exosome association, suggesting that proteins also play a role in adherence/internalization of exosomes by the recipient cell (Fig. 6).

Low extracellular pH is a distinguishing phenotype of solid tumors [68]. Poor vascularization results in hypoxic regions and acidification of the tumor microenvironment [69,70]. Hypoxic conditions lead to the release of proteins (predominately in exosomes) involved in angiogenesis, adhesion, and immune recruitment resulting in enhanced metastatic potential [69]. This is consistent with the notion that low pH selects for cancer cells with metastatic phenotypes [70,71]. Furthermore, exosomes have been implicated to play a role in cancer metastasis

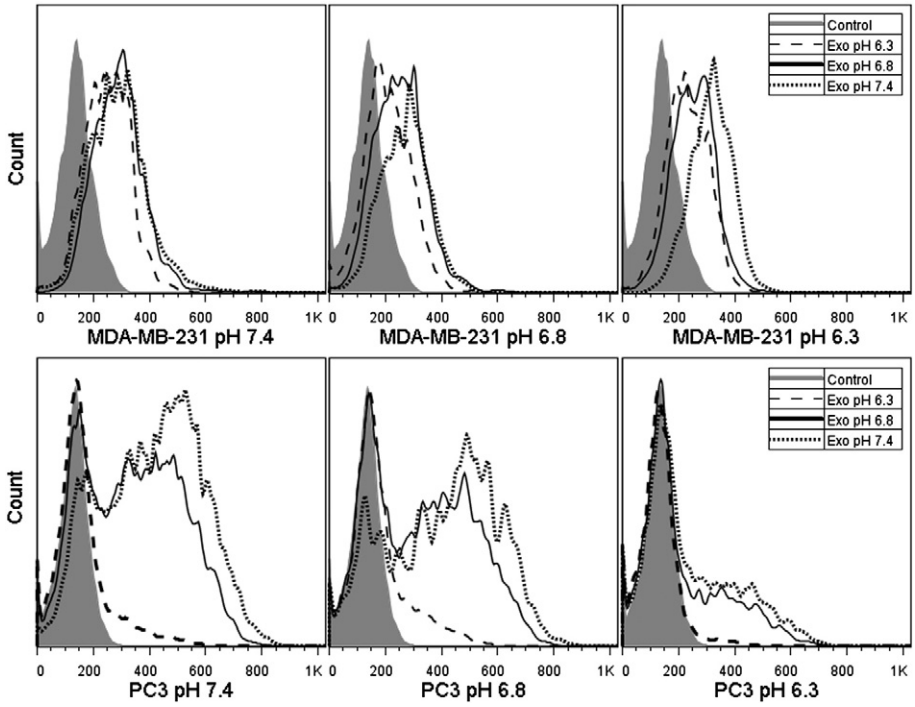

Fig. 8. Effect of pH on exosome uptake. Top: Exosomes derived from MDA-MB-231 cells cultured at pH 7.4, 6.8 and 6.3 were incubated with MDA-MB-231 cells growing at pH 7.4, 6.8, and 6.3. Bottom: Exosomes derived from PC3 cells cultured at pH 7.4, 6.8 and 6.3 were incubated with PC3 cells growing at pH 7.4, 6.8, and 6.3. All exosomes were labeled with 0.01% DID. For each panel, association with pH 6.3 exosomes was significantly less than with pH 7.4 exosomes ($p < .05$).

[72,73]. Thus, it is reasonable to suggest that cells cultured in acidic conditions might release exosomes with increased metastatic potential. Because we aimed to investigate the potential of exosomes as a drug delivery vehicle, our goal was to determine whether low pH-derived exosomes are associated to a greater extent than exosomes derived from cells cultured at physiological pH. Parolini et al. [18] concluded that exosomes derived from cells cultured at pH 6.0 had a greater fusion efficiency than exosomes harvested from cells cultured at physiological pH. In contrast, our results demonstrated that exosomes harvested from cells cultured at pH 7.4 associated more readily than those from cells cultured at pH 6.3. These data suggest that harvesting exosomes from cells grown under acidic conditions does not enhance their potential for drug delivery, at least *in vitro*. A possible explanation for the observed differences between our results and those of Parolini et al. [18] is the condition of the cells at low pH. Parolini et al. [18] reports that growth of melanoma cells is unaffected when cultured at low pH. In contrast MDA-MB-231 and PC3 cells, used in our experiments exhibit a reduced rate of doubling (data not shown) suggesting cells were not thriving in acidified conditions. These observations indicate that the behavior of our cell lines under acidified conditions differs from that used by Parolini et al., in that stressed cells may not release exosomes with a composition that enhances exosome association with recipient cells.

5. Conclusions

This work has highlighted the enhanced association of cancer cell-derived exosomes as compared to liposomes of a similar size by both cancer and immortalized cell lines. While we do not observe preferential association of exosomes by their parent cell line, cancer cells take up exosomes to a greater extent than immortalized cell lines. However, the enhanced adherence/internalization of exosomes by the cancer cell lines is not significant when differences in non-specific association are accounted for (presumably due to higher baseline levels of endocytosis/phagocytosis by cancer cells), but PC3 exosomes did exhibit significant preferential association to prostate cancer cells. In addition, we found that the unique lipid and protein composition of exosomes both contribute to facilitate adherence/internalization. While our results suggest that exosomes may still need to be targeted to enhance delivery specifically to cancer cells, the fact that exosomes are associated to a significantly greater extent by all cells suggests that their physical/chemical properties might be exploited to improve drug delivery. Lastly, we demonstrated that acidification of the microenvironment to which cells are exposed during exosome harvest and/or during adherence/internalization did not significantly increase association.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.07.026.

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