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Cancerous Epithelial Cell Lines Shed Extracellular Vesicles With a Bimodal Size Distribution that is Sensitive to Glutamine Inhibition

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Abstract

Extracellular shed vesicles (ESVs) facilitate a unique mode of cell cell communication wherein vesicle uptake can induce a change in the recipient cell's state. Despite the intensity of ESV research, currently reported data represent bulk characterization of concentrated vesicle samples with little attention paid to heterogeneity. ESV populations likely represent diversity in mechanisms of formation, cargo, and size. To better understand ESV subpopulations and the signaling cascades implicated in their formation, we characterize ESV size distributions to identify subpopulations in normal and cancerous epithelial cells. We discovered that cancer cells exhibit bimodal ESV distributions, one small-diameter and another large-diameter population, suggesting that two mechanisms may govern ESV formation, an exosome population and a cancer-specific microvesicle population. Altered glutamine metabolism in cancer is thought to fuel cancer growth but may also support metastatic niche formation through microvesicle production. We describe the role of a glutaminase inhibitor, compound 968, in ESV production. We discovered that inhibiting glutamine metabolism significantly impairs large-diameter microvesicle production in cancer cells.

Keywords

Extracellular Shed Vesicle; Microvesicle; Exosome; Cellular Communication; Biomarker; Cancer; Pancreatic Cancer; KRAS; Microenvironment; Oncosome

1. Introduction

Cells shed heterogeneous vesicular structures into their local environment and throughout the body (1-5). These vesicles facilitate a unique mode of cell cell communication, akin to paracrine signaling, wherein cargo-laden packages are submitted from the originating, or parent, cell to the recipient cell. The uptake of extracellular shed vesicles (ESVs) can induce a change in the recipients' state and thus its behavior and function (6-9). The changes induced by ESV uptake are as diverse as the family of messengers. ESVs, first described in

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the literature as exosomes in the 1970s and 1980s (10-13), have been identified and interrogated throughout the years (1, 4, 5, 14-18). ESVs have been described with multiple names (2) including exosomes (19, 20), microvesicles (21, 22), microparticles (23, 24), and oncosomes (25, 26), among others; the nomenclature is not currently standardized (4). The given names, to some degree, indicate provenance, function, or properties. The term *exosome* typically refers to intraendosomal vesicles released by the cell (12), whereas the term *microvesicle* typically refers to structures that bud directly from cancer cell surfaces (1, 2, 22). Tumor-released exosomes have been implicated in cancer immunity (15), whereas tumor-derived microvesicles are implicated in the development of the metastatic niche (1, 9, 18, 22, 27-30). Importantly, microvesicles are different from apoptotic bodies (31), as their contents do not merely represent a random sampling of cell constituents, but rather specifically packaged cargo (1, 31). Many assays have been executed to characterize exosome and microvesicle content (32-34), mechanisms of formation (2, 18), and biological activity (7, 19). Although ESV interrogation (2, 35-39) and clinical and commercial application (ExoQuick™; Exo-Flow™; ExoELISA™) (40-42) represent areas of intense research and activity, little has been done to characterize ESV *subpopulations* emanating from a single cell source (22, 43). Limitations in processing techniques are presently responsible for the sparseness of subpopulation analysis to date.

Currently reported data represent bulk characterization of concentrated ESV samples with little or no attention paid to heterogeneity. ESV populations, even those that emanate from a single cell type, likely represent a diverse population with unique cargo and mechanisms of formation. To better describe ESV populations, an understanding of constituent subpopulations and the signaling cascades implicated in their formation and shedding is necessary (22). Of particular interest is the dissemination of cancer cell-derived microvesicles and their role in priming the metastatic niche. We are unaware of any thorough characterization of cancer cell-derived ESV size distributions. In this work, we characterize ESV size distributions of species sourced from model cancer cell lines to identify distinct subpopulations with a goal of informing subsequent interrogation. We compare these results, in the case of pancreatic cancer, to ESV signatures from a model normal epithelial pancreas cell line.

Among cancers, pancreatic cancer is the fourth-leading cause of cancer death in the United States (44) and the most lethal malignancy, with pancreatic ductal adenocarcinoma (PDAC) being the most common (45). The overall survival rate of pancreatic cancer is less than 5% (46). These abysmal outcomes result, in part, from a typically asymptomatic progression until late-stage cancer has developed. Clinically recommended means of early detection do not exist, even though early dissemination of tumor cells has been implicated in the low survival rates and rapid progression of pancreatic cancer (47, 48). In murine xenograft models, the interaction between cancer cells and normal pancreas cells promotes pancreatic cancer progression (49, 50); and microvesicles harvested from cancer cells interact with and change the state of stromal cells (1, 51, 52). These results indicate that, in pancreatic cancer, cancer cell-derived microvesicles can transform normal cells and prime the tumor microenvironment.

Pancreatic cancer often results from mutations in the *RAS* family of genes, typically *KRAS* (47, 53-55). In cells, the binding of guanosine triphosphate (GTP) to KRAS results in its activation (54, 56) and ability to initiate signaling cascades that promote cell proliferation, migration, and differentiation (54, 56). Mutations that cause KRAS to be in a persistently active, GTP-bound state send excessive signals that stimulate cell growth, thus contributing to tumor formation (54, 57, 58). Recently, modification of glutamine metabolism by oncogenic *KRAS* has been identified as a primary player in maintaining tumor growth and survival (59-64). Consequently, the ability to metabolize glutamine is markedly increased as the cell relies more heavily upon anabolic processes (60, 65, 66). These alterations in cellular metabolism are thought to provide the fuel necessary not only for cancer cell growth but also for microvesicle (MV) production. Given that altered glutamine metabolism results from the ubiquitous KRAS mutations, among other causes (67, 68), in pancreatic cancer, we investigated the effect that treating model pancreatic cancer cell lines with glutaminase inhibitor, compound 968, would have on ESV production. We discovered that inhibiting glutamine metabolism blocked the ability of pancreatic cancer cell lines to generate MVs. These findings underscore the functional connections between the altered metabolic state of cancer cells and their ability to generate ESVs.

2. Methods

2.1. Cells and Culture

U87 MG (U87, HTB-14™, glioblastoma), MDAMB231 (HTB-26™, metastatic mammary gland adenocarcinoma), PANC-1 (CRL-1469™, pancreatic ductal carcinoma), BxPC-3 (CRL-1687™, pancreatic adenocarcinoma), and hTERT-HPNE (CRL-4023™, hTERT-immortalized normal pancreatic duct) model cell lines were obtained from the American Type Culture Collection (ATCC®, Manassas, Virginia). U87 cells were transfected so as to stably express epidermal growth factor receptor variant III (EGFRvIII), see Section 2.2, which is associated with increased proliferation in glioma cells (1, 69, 70); this cell line will subsequently be referred to as U87+EGFRvIII.

2.1.1. Cell Culture—MDAMB231, U87, U87+EGFRvIII, and BxPC-3 cell lines were grown in Roswell Park Memorial Institute (RPMI-1640; Lonza, Walkersville, MD) cell medium containing 10% fetal bovine serum (FBS; Gemini BioProducts, West Sacramento, CA). The PANC-1 cell line was grown in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Manassas, VA) containing 10% FBS. The hTERT-HPNE cells were grown in a medium consisting of 75% DMEM (Sigma-Aldrich, St. Louis, MO) and 25% M3™Base (Incell, San Antonio, TX) containing 5% FBS. All lines were maintained at 37 °C in a humidified, 5% carbon dioxide environment. The medium in each flask was exchanged every 2-3 days and rinsed with phosphate buffered saline (PBS) according to standard sterile techniques. All cell cultures are maintained in 25 cm^2 rectangular cell culture flasks.

2.2. Generation of Stable Cell Lines

The pcDNA3 construct encoding human EGFRvIII was transfected into U87 cells using Lipofectamine (Invitrogen, Carlsbad, CA). Clones of U87 cells stably expressing EGFRvIII were selected by culturing the cells in RPMI-1640 containing 10% FBS and 1 μg mL^{-1}

puromycin. Once individual clones expressing EGFRvIII were obtained, the cells were then maintained in the same growth medium supplemented with 0.3 μg mL^{-1} puromycin.

2.3. ESV Harvesting Protocol

Prior to obtaining the ESVs, nearly confluent culture flasks were rinsed with PBS and then subjected to serum-free medium culture conditions for 12 hours. The resultant conditioned media, each from approximately 2.5×10^6 serum-starved cells, was collected for analysis. Conditioned media was centrifuged in two stages, 300×g for 10 minutes and 12000×g for 20 minutes, to pellet intact cells and cell debris, respectively. 500 μL aliquots of the supernatant were extracted for measurement.

2.3.1. Cell Treatment with a Glutaminase Inhibitor—A glutaminase inhibitor, compound 968 (968; EMD Millipore, Billerica, MA), was prepared by dissolving 968 in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) at 30 mM. 36 hours prior to ESV harvesting, cells were treated with 968 at 10 μM (71, 72); a total of 1.67 μL of 968 in DMSO was added to 5 mL of culture medium (0.03% by volume). Following the protocol described in Section 2.3, cells were treated with 10 μM 968 under serum-starved conditions for 12 hours. Subsequent harvesting and centrifugation steps are identical to those previously described.

2.4. Immunoblot Analysis

Cultures of cells treated as indicated were rinsed with PBS and then lysed with cell lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM β-glycerol phosphate, 1 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin). To generate ESV lysates, the partially clarified conditioned medium (medium cleared of intact cells and cell debris), see Section 2.3, was filtered using a Steri-Flip PVDF filter with a pore size of 0.2 μM (Millipore Corporation, Billerica, MA). The ESVs retained by the filter were washed thoroughly with PBS and then lysed with 300 μL cell lysis buffer. An equal number of cells from each cell line (5*e*3 cells) and a corresponding equal ratio of ESVs generated by the cell lines were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to PVDF membranes. The membranes were blocked in 5% dry milk diluted in TBST (20 mM Tris, 135 mM NaCl, and 0.02% Tween 20), and then were incubated with Flottilin-2 (Cell Signaling, Inc., Danvers, MA), RhoC (Cell Signaling, Inc.), or actin (Sigma-Aldrich) antibodies prepared in TBST. Horseradish-peroxidase conjugated secondary antibodies were used to detect the primary antibodies, followed by extensive washing with TBST and then exposure to enhanced chemiluminescence (ECL) reagent.

2.5. Dynamic Light Scattering

ESV preparations were characterized at 25 °C using dynamic light scattering (DLS; He-Ne laser, 633 nm; 173°backscattered light detection) on a Nano Series Zetasizer (zetasizer, Malvern Instruments, Southborough, MA). 500 μL samples were loaded into a microcuvette (ZEN0118, Malvern Instruments) for measurement. Each measurement represents 3 unique preparations with 3 runs per preparation with at least 12 unique measurements per run. All data reported represent 108 total measurements.

2.6. Data Analysis

All data were processed using an author-scripted MATLAB® routine. This routine employs a nonlinear least-squares regression on multiple 4-parameter skew-normal distributions to fit each data set. The *relative scattering intensity [a.u.]* of each size distribution figure represents the volume distribution, as reported by the zetasizer which converts intensity distribution data using Mie theory; the magnitude of the figures are scaled by an arbitrary factor to enable facile comparisons among data sets. The peak amplitudes correspond to the total volume represented by the associated particle size. In all figures, unless stated otherwise, error bars represent standard error of the mean for 9 samples.

3. Results

The purpose of these experiments is to identify and establish distinct ESV subpopulations shed from normal and cancerous epithelial cells (MDAMB231; U87, U87+EGFRvIII; PANC-1, BxPC-3, and hTERT-HPNE, Figure 1) by means of dynamic light scattering (DLS). DLS determines the size distribution of particles in solution by measuring their Brownian motion over time and measures relative sample concentrations through total recorded backscattered light. As particles move, light impinging on the particle is scattered; the time-associated scattered light readings are used to generate an autocorrelation curve. From this data, a diffusion coefficient is extracted, and thus sizes can be inferred from the Stokes-Einstein relation. A hallmark feature revealed by this investigation is the characteristic bimodal distribution of ESVs, derived from three distinct types of cancer (breast, brain, and pancreatic cancer), see Figure 1. Across all of the cancer cell lines, the small-diameter population exhibits a peak position of 88 ± 19 nm and an α, skewness, of 3.11 ± 1.17; the large-diameter population exhibits an average peak position of 462 ± 58 nm and an α of 2.85 ± 1.18. The skewness parameters indicate that both ESV subpopulations are dominated by the presence of the relatively larger diameter vesicles. Upon comparing the normal epithelial cell line, hTERT-HPNE, large-diameter peak position (417 ± 11 nm) and magnitude to those of the cancer cell lines, there is no statistically significant difference between the large-diameter peak position of hTERT-HPNE vesicles as compared to those of cancer cells, but approximately an order-of-magnitude difference between the peak amplitudes.

Western blots, as shown in Figure 2, were carried-out on the cells and the MVs that the cells shed into the medium to verify that the particles examined with DLS are ESVs. Flotillin-2 is a protein associated with membrane transport and fusion (8, 73) and should be present in ESVs (1, 17, 17, 70). Actin, a major component of the cytoskeleton, is abundantly expressed in both whole cell lysates and ESV lysates (17, 20, 74). In the case of large-diameter ESVs that bud directly from the cell surface, it is possible that actin is essential in the maturation of budding vesicles (17). RhoC, which is involved in extracellular matrix assembly, cytoskeletal reorganization, and cell migration (75, 76), is expressed in the cytosol but is not involved in ESV formation (18). As can be seen in Figure 1 and 2, the immortalized normal epithelial cells (hTERT-HPNE) make almost undetectable levels of MVs as determined by DLS and flotillin-2 and actin staining. Furthermore, ESV preparations are devoid of

cytosolic contamination as determined by the fact that RhoC is exclusively present in whole cell lysates (WCLs) and not in ESV lysates (ESVLs).

To explore the conjecture that MVs can be related to oncogenic processes, we asked whether glutamine metabolism, which is significantly upregulated in cancer, is important for the ability of cancer cells to generate MVs. Both cancerous epithelial cell lines, PANC-1 and BxPC-3, which rely heavily on glutamine for survival (67, 77), produced significantly more ESVs than the normal epithelial line, hTERT-HPNE. PANC-1 cells produced approximately twice as many ESVs as BxPC-3 cells. Compound 968, a glutaminase inhibitor, was added to the model pancreas lines and its impact upon ESV production was determined by DLS. We found that treating cells with compound 968 drastically altered ESV size distributions, as seen in Figure 3, and diminished microvesicle production, as seen in Figure 3 and Figure 4. The total calculated ESV volume for cancer cells was significantly reduced upon exposure to compound 968, but ESV volume in normal pancreas cells was not significantly affected. Relative to the non-treated cases, there was a 96.00 ± 30.97 % reduction in total PANC-1 vesicle volume, a 97.61 ± 28.49 % reduction in total BxPC-3 vesicle volume, and no statistically significant change in total hTERT-HPNE vesicle volume following treatment with compound 968, see Figure 4.

4. Discussion

We used dynamic light scattering to identify distinct cancer cell-derived ESV subpopulations. DLS has the benefit of extracting particle size distributions in a repeatable fashion and is an established, effective tool for characterizing particle sizes, including exosome populations in blood (36, 43, 78). Compared with other measurement approaches, DLS measures ESVs without requiring sample pelleting or dehydration, which could damage ESVs and alter their geometric parameters (73, 79), but does not directly report absolute particle concentration, number, or volume (38). A relative measure of total ESV volume can be calculated by integrating the product of volume percent backscattered light and ESV volume. This calculated total volume is proportional to the total vesicle volume (cargo) that could be delivered to recipient cells. Changes in total vesicle volume resulting from cell treatment or modification, such as exposure to compound 968, provides insight into changes in total ESV production resulting from treatment.

We have demonstrated the presence of two distinct subpopulations within cancer cell-derived vesicles. As suggested in other reports (1, 5, 34, 38, 80), vesicles shed by cancer cells exhibit a size range from approximately 20 nm to over 1 μm; our findings, for breast, brain, and pancreas cells, as shown in Figure 1, are consistent with this reported range. For each cancer cell line examined herein, the peak locations, skewness, and areas of the bimodal ESV distributions were quantified by fitting multiple 4-parameter skew-normal distributions by nonlinear least squares regression. Given the narrow range of peak positions and magnitudes for each cancer cell-derived ESV subpopulation, these data suggest that the processes governing ESV production for both small- and large-diameter vesicles may be tightly regulated and conserved across cancer types (22, 73). Despite similarity in ESV size distributions among cancer types, PANC-1 cells, which express oncogenic KRAS, produce substantially more ESVs than do BxPC-3 cells, which express wild-type KRAS (81). The

presence of small- and large-diameter populations in cancerous epithelial cell lines suggests that two unique mechanisms may govern the biogenesis of each unique subpopulation (2, 22). It is possible, although yet to be determined, that the small-diameter population is representative of a normal cell exosome signature and that the large-diameter vesicles represent an aberrant, cancer-related microvesicle signature. Evidence in a small cohort of stomach and liver cancer patients (82, 83) further bolsters this claim.

We determined that in the cell lines considered large-diameter ESVs (microvesicles) are a cancer-specific signature associated with dysregulated glutamine metabolism. Cancer activates, upregulates, modifies, or creates specific signaling cascades that are dysregulated versions of host pathways not expressed under normal conditions (18, 63, 84, 85). Given the role of glutamine metabolism in pancreatic cancer (54, 58-60, 67, 68), we explored the impact of a glutaminase inhibitor, compound 968, on microvesicle production. Compound 968 has been shown to inhibit cancer growth but has no impact on the proliferation of normal cells (71, 86, 87). We found that the results of treating cells with this glutaminase inhibitor included a preferential reduction in the large-diameter vesicle population, see Figure 3, and a drastic reduction in total vesicle production, as shown in Figure 3 and Figure 4. This outcome is consistent with the hypothesis that large-diameter ESVs (microvesicles) are cancer-specific, as the introduction of a glutaminase inhibitor significantly disrupted their production in cancer cells but only had a limited effect on normal epithelial cells, see Figure 3. These data also suggest that treating cancer cells with glutaminase inhibitors may serve as a means not only to preferentially starve, and perhaps eliminate, cancer cells (71, 88), but also as a way to restrict their MV production, which could reduce their ability to prepare the metastatic niche (7, 28, 78, 89). More work is necessary to appropriately interrogate and describe the role of glutaminase inhibitors in ESV production in cancer.

5. Conclusion

Understanding the mechanisms of ESV generation, as well as their cargo and properties, is essential. This is particularly crucial, as ESVs putatively induce changes of state in recipient cells and prime local environments for primary and metastatic tumor establishment and growth. As metastases are responsible for 90% of cancer-related death (7, 90), unlocking the processes by which ESVs are prepared provides insight into cancer treatment and inhibition of its progression. We discovered that inhibition of glutamine metabolism in model cancer cell lines significantly impairs large-diameter microvesicle production. This result suggests that vesicle formation in cancer cells can be reduced by applying a metabolic inhibitor and indicates that the large-diameter population may be representative of cancer cell-derived microvesicles. We have also characterized the size distributions and total relative volumes of ESVs from multiple model cell lines of primary tumor and metastatic origin, as well as a normal epithelial cell line. We discovered that each cancer cell population exhibits a bimodal distribution of vesicles, including one small population of particles with diameters less than approximately 200 nm and another large population bearing a diameter range from approximately 200 nm to 1.10 μm. The presence of a significant large-diameter ESV population appears only in cancer cells and not in normal epithelial cells. This feature of ESV subpopulations bolsters the argument that there may be two mechanisms governing ESV formation, a small-diameter, exosome, population and a large-diameter, cancer-

specific, microvesicle population. Further investigations are required to definitively establish the specific signaling cascades that govern ESV formation processes, as this could elucidate both mechanisms of vesicle formation and vesicle roles in priming the metastatic niche.

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Abbreviations list

ESVs	Extracellular shed vesicles
MVs	Microvesicles
GTP	Guanosine triphosphate
PDAC	pancreatic ductal adenocarcinoma
EGFRvIII	Epidermal Growth Factor Receptor variant III
DLS	Dynamic light scattering
ESVLs	Extracellular shed vesicles lysates
WCLs	Whole cell lysates

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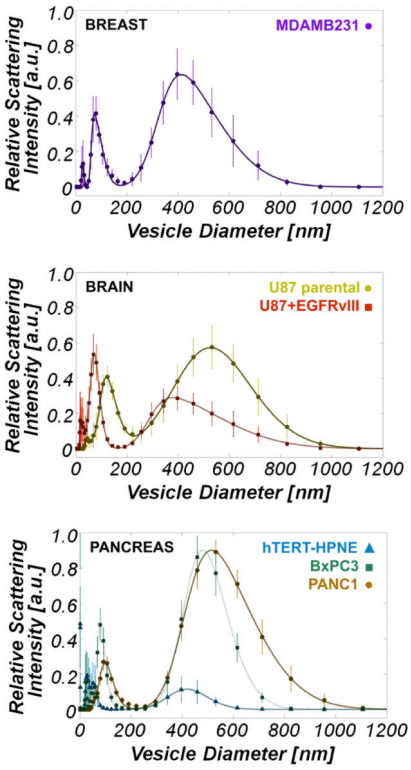

Figure 1.

Dynamic light scattering measurements reveal a bimodal vesicle population among cancer cell types examined. *(a)* ESV size distribution in MDAMB231 (□) cell lines. MDAMB231 peaks are located at 73 ± 1 nm and 413 ± 4 nm. *(b)* ESV size distribution in U87 (●) and U87+EGFRvIII (■) cell lines. U87 peaks are located at 120 ± 1 nm and 525 ± 5 nm. U87+EGFRvIII peaks are located at 70 ± 3 nm and 378 ± 2 nm. *(c)* ESV size distribution in PANC-1 (●) , BxPC-3 (▪), and hTERT-HPNE (▲) cell lines. The PANC-1 peaks are located at 98 ± 3 nm and 515 ± 3 nm. The BxPC-3 peaks are located at 80 ± 1 nm and 480 ± 2 nm. The hTERT-HPNE peaks are located at 31 ± 1 nm and 51 ± 1 nm, and 417 ± 11 nm. Of particular interest is the striking difference in ESV signatures between the normal pancreas cell line, hTERT-HPNE, and those of the two pancreatic cancer lines, BxPC-3 and PANC-1. Peaks at approximately 30 nm for the brain, breast, and BxPC-3 (pancreas) lines are an artifact of the culture medium (RPMI-1640). All deviations from the peak locations represent those values falling within the 95% confidence interval predicted by nonlinear least squares regression.

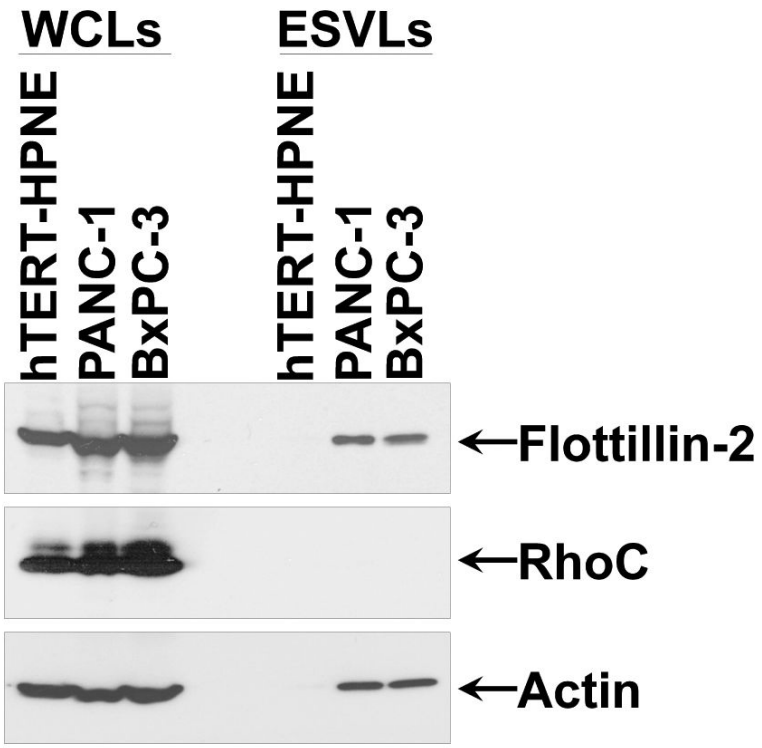

Figure 2.

Immunoblot Assay. Serum-starved hTERT-HPNE, PANC-1, and BxPC-3 cells were lysed, and the ESVs shed into the medium by the cells were isolated and lysed as well. The whole cell lysates (WCLs) and the ESV lysates (ESVLs) were subjected to western blot analysis with antibodies against the ESV marker flotillin-2, the cytosolic-specific marker RhoC, and the loading control actin. Two blank channels separate WCLs and ESVLs.

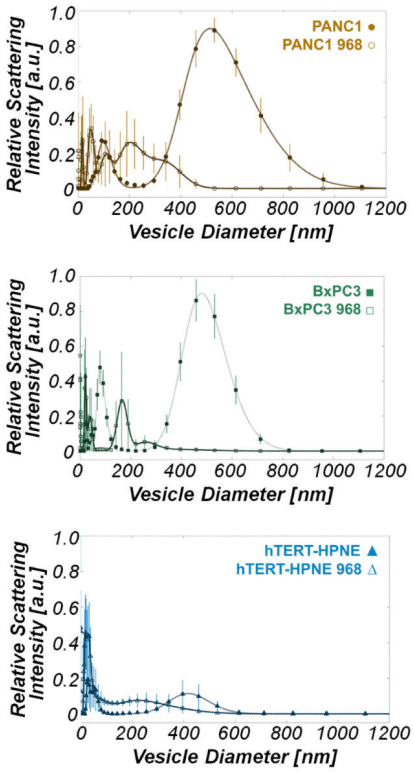

Figure 3.

Dynamic light scattering measurements demonstrate that treatment of cancer cells with compound 968 substantially reduces large-diameter microvesicle production. *(a)* ESV size distribution in untreated PANC-1 (●) and 968-treated PANC-1 (○) cells. *(b)* ESV size distribution in BxPC-3 (■) and 968-treated BxPC-3 (□) cells. *(c)* ESV size distribution in hTERT-HPNE (▲) and 968-treated hTERT-HPNE (△) cells.

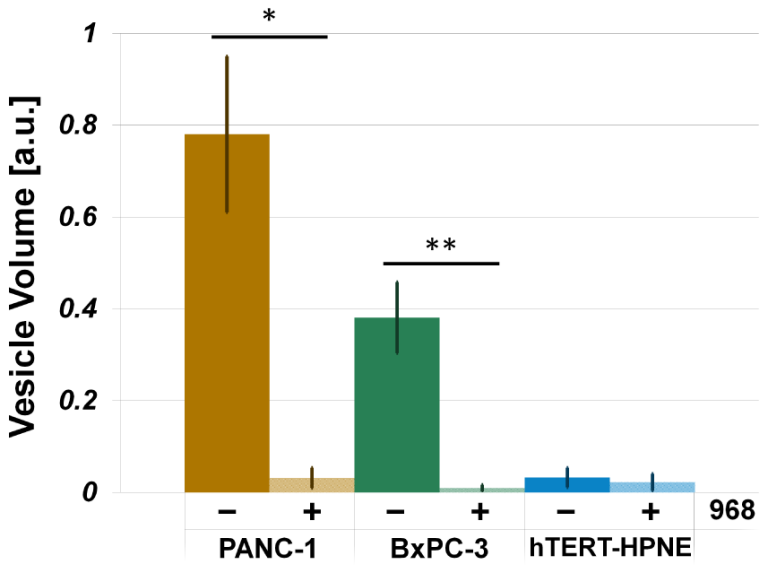

Figure 4.

Total vesicle volume analysis demonstrates that treatment of cancer cells with compound 968 dramatically reduces vesicle production in cancer cells (PANC-1, $^{*}p = 0.0006$; BxPC-3, $^{**}p = 0.0002$) and has no statistically significant effect on vesicle production in normal epithelial cells (hTERT-HPNE, $p = 0.7$).