Development and characterization of cancer cell line exosomes as reference standards in cancer research

Siddhartha Paul, PhD,[1] Nanda Mahashetty, MS,[1] Heather Branscome, MS,[2] Dezhong Yin, PhD,[1] Fang Tian, PhD[1]
[1]ATCC Cell Systems, Gaithersburg, MD 20877; [2]ATCC, Manassas, VA 20110

Credible leads to Incredible

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Abstract

Exosomes are subcellular nanoparticles (50–200 nm in size) that are released from cells through a fusion of multicellular bodies with plasma membrane. Exosomes are currently being evaluated as potential diagnostic tools in a number of diseases including cancer. Exosomes are stable carriers of cell-free cargo in the form of DNA, RNA, and protein, thereby making them an attractive candidate for early detection of cancer via liquid biopsy. Tumor exosomes have also been linked to stimulation of tumor cell growth, angiogenesis, metastasis, and suppression of the immune system. However, isolating a consistent population of exosomes can be challenging and the need exists for highly characterized exosomes for use as reference standards for research and diagnostic applications. We have developed a novel method employing tangential flow filtration for isolation of large quantities of pure and sterile exosomes from cell culture media. Exosomes from cancer cell lines representing the most prevalent cancer types including PC3 (ATCC® CRL-1435™) and LNCaP (ATCC® CRL-1740™) prostate cells, HCT116 (ATCC® CCL-247™) colorectal cells, MDA-MB-231 (ATCC® HTB-26™) breast cells, A549 (ATCC® CCL-185™) lung cells, and U87-MG (ATCC® HTB-14™) glioblastoma cells were isolated and characterized in this study. We employed a stringent quality control approach in order to define and characterize these exosomes. Our method gave us high yield of >1×10^{10} exosomes/mL and average protein equivalent of 2 mg/mL. Our data demonstrated expression of a number of different exosome proteins including tetraspanin confirmed through Western blotting analysis. Isolated exosomes had a median size of around 102 nm through nanoparticle tracking analysis (NTA). Our western blot data showed differential protein marker expression of CD63, CD81, CD9, flotillin-1, and TSG101 protein and this expression profile is unique in differing tumor cell lines. We show the functionality of these exosomes through cellular uptake, anchorage-independent growth assay, and angiogenesis assay in this study. We also evaluated the quality and integrity of RNA from these purified exosomes and the RNA information demonstrates the presence of small RNAs (20-200nt) as expected; these RNA could be useful in biomarker studies. We show that exosomes isolated by our method not only had high exosome yield and quality but also were functional in nature and thereby make ideal reference standards/controls in the field of cancer research and diagnostics development.

Process map for Exosome Isolation

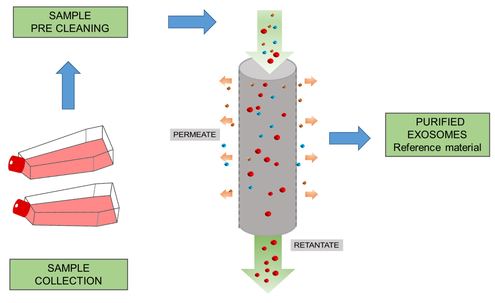
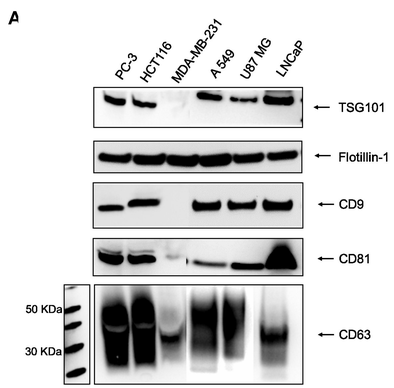


Figure 1. Process map of exosome isolation. Large-scale conditioned medium is collected from cell culture for exosome isolation. The medium is then centrifuged to remove dead cells/debris (pre-cleaning). The clarified medium is filtered, washed, and concentrated by using a tangential flow filtration system (TFF). The retentate fraction contains purified exosomes, which are used for downstream exosome characterization and analysis.

Results

Characterization of Exosomes



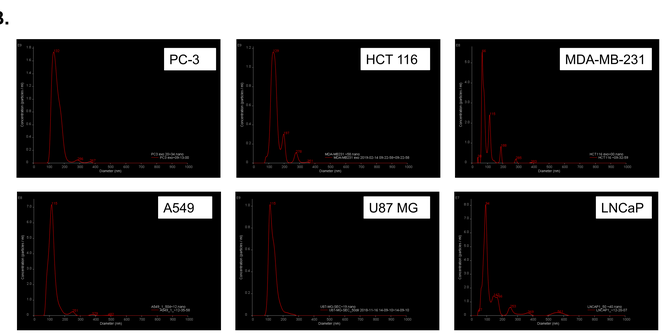


Figure 2. Protein maker expression and Size distribution analysis. A) Common exosome protein markers were analyzed on the purified exosomes from different cancer cell lines. Same amount of proteins were used (10ug) for western blotting for each of these markers. As seen from the blots, exosomes isolated from different cell lines have different expression profile for the protein markers. . **B)** Analysis was performed using NTA. Representative histograms of purified exosomes from different cancer lines are shown.

Table 1: Concentration of purified exosomes. Exosome concentration was analyzed by NTA

Sample	Exosomes (particles/mL)	Exosome size diameter (median)
PC-3	1.4 x 10^{11}	140 nm
HCT 116	1.3 x 10^{10}	71 nm
MDA-MB-231	5.9 x 10^{10}	122 nm
A549	4 x 10^{10}	140 nm
U87-MG	5 x 10^{10}	122 nm
LNCaP	4 x 10^{10}	95 nm

Exosome RNA analysis

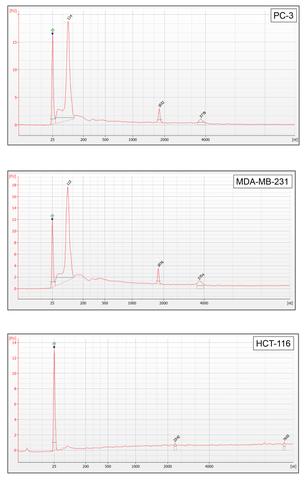


Figure 4. Size distribution of total RNA from cancer cell lines exosomes. Exosomal RNA sizing was performed using Agilent Bioanalyzer RNA 6000 Pico Kit™. Most of the exosomes contain small RNAs (20–200 nt). The major peaks are at 113 nt. The samples also contain low levels of ribosomal RNA (peaks at 1386 and 3754)

Functional assay – Anchorage-independent growth assay

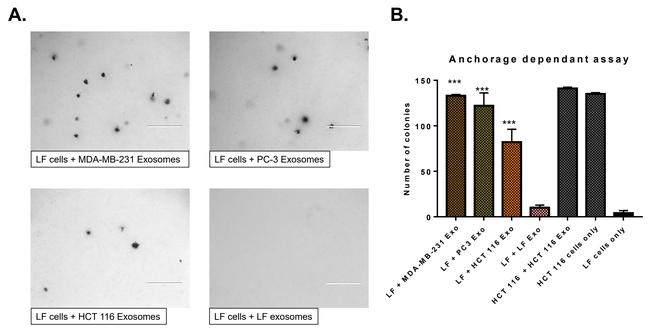


Figure 5. Exosome-mediated induction of cell growth soft-agar. Human primary lung fibroblasts (ATCC® PCS-201-013™) were treated for 7 days with 100 µg/mL protein equivalent concentration of exosomes from MDA-MB-231, PC-3, HCT116, and lung fibroblasts. After 7 days of treatment, cells were harvested and utilized in soft-agar assays. Cells in soft-agar plates were incubated for 21 hours and were stained with crystal violet stain overnight. Cell colonies were counted with 4 fields/well of a 6-well culture plate by using phase contrast microscope. (A) Crystal violet-stained colonies. Scale bars = 400 µm. (B) The total number of colonies for each treatment. An unpaired t-test was performed to analyze the soft agar colony formation capabilities of exosomes from cancer lines compared to exosomes from lung fibroblasts. MDA-MB-231 and PC-3 showed ***p <0.0006 and HCT116 showed *** p <0.0003 compared to LF exosome. p <0.010 indicates significant cell transformation capabilities of cancer exosomes.

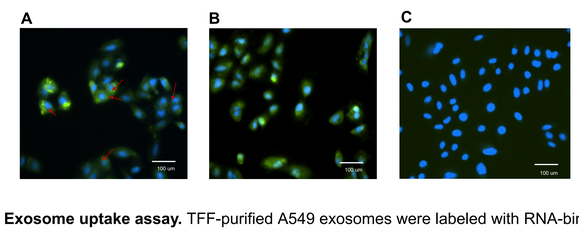


Figure 3. Exosome uptake assay. TFF-purified A549 exosomes were labeled with RNA-binding dye (green) and added to the recipient cell line (A549). Cells were then fixed and stained with DAPI (blue). Images were captured with a fluorescent microscope. Images depict (A) dye-labeled exosomes added to the cells, (B) only dye added to the cells, and (C) cells without any dye or labeled exosomes. Scale bars = 100 µm.

Functional Testing – Angiogenesis assay

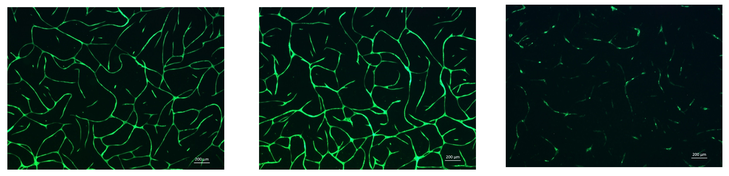


Figure 6. Effect of TFF-purified exosomes on tubular formation in an *in vitro* angiogenesis assay. Representative photos imaged under a fluorescent microscope show tubular formation after 7 days in culture for A549 exosome-treated GFP-labeled endothelial cells (ATCC® CRL-4054™). Cells were treated with 100 µg/mL protein equivalent concentration of exosomes for all the different types of exosomes. Untreated cells received no exosomes and for positive control, cells were treated with MSC exosomes. The ATCC Angio-***Ready***™ Angiogenesis Assay System (ATCC® ACS-2001-2™) was used for this assay.

Summary

- This study demonstrates isolation and characterization of exosomes from well-characterized ATCC cancer cell lines by using a novel TFF isolation technique.
- Purified exosomes exhibited the expected size distribution and expressed characteristic protein markers with a unique profile depending on the cell type.
- The RNA profile of the exosomes displayed the expected size distribution of exosomal RNA.
- TFF-purified exosomes exhibited functionality when evaluated via anchorage-independent and angiogenesis assays, thereby demonstrating their use in a variety of downstream applications for cancer research.

ATCC 10801 University Boulevard, Manassas, Virginia 20110-2209 **Phone:** 800.638.6597 **Email:** SalesRep@atcc.org **Web:** www.atcc.org