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## Anchorage Independent Growth of Breast Carcinoma Cells is Mediated by Serum Exosomes

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### Abstract

We hereby report studies that suggest a role for serum exosomes in the anchorage independent growth (AIG) of tumor cells. In AIG assays, fetal bovine serum is one of the critical ingredients. We therefore purified exosomes from fetal bovine serum and examined their potential to promote growth of breast carcinoma cells in soft agar and Matrigel after reconstituting them into growth medium (EEM). In all the assays, viable colonies were formed only in the presence of exosomes. Some of the exosomal proteins we identified, have been documented by others and could be considered exosomal markers. Labeled purified exosomes were up-taken by the tumor cells, a process that could be competed out with excess unlabeled vesicles. Our data also suggested that once endocytosed by a cell, the exosomes could be recycled back to the conditioned medium from where they can be up-taken by other cells. We also demonstrated that low concentrations of exosomes activate MAP kinases, suggesting a mechanism by which they maintain the growth of the tumor cells in soft agar. Taken together, our data demonstrate that serum exosomes form a growth promoting platform for AIG of tumor cells and may open a new vista into cancer cell growth *in vivo*.

### Keywords

Breast; Carcinoma; Exosomes; Anchorage; Growth; MAP Kinase; Soft Agar; Matrigel

### Introduction

The *in vitro* two dimensional growth of cultured cells including tumor cells is well characterized, particularly the growth signals emanating from the integrin/extracellular matrix interaction [1]. For a number of years, ideas have bounced back and forth as to whether the growth signaling mechanisms in two-dimensional tissue culture can be extrapolated to the growth cues *in vivo* [2]. A number of *in vitro* culture systems have been developed including organotypic culture system, that takes into account the three-dimensional *in vivo* growth of tumor cells [3]. Suffice to say, the *in vivo* growth of tumor cells is complex because not only is it three-dimensional in character, there are numerous variables including stromal factors that affect growth [4]. Apart from the limitations imposed on it, the anchorage independent growth

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of tumor cells in soft agar bears the closest resemblance to the *in vivo* growth when compared to the two-dimensional growth on plastic [5].

Serum, particularly fetal bovine serum is routinely used to supplement cell growth media. Serum has a number of adhesion or attachment factors which allow the cells to adhere and spread on the bottom of the culture flask to initiate growth signals. Apart from the well characterized adhesion proteins such as fibronectin, laminin, and collagen [6], serum also contains a myriad of factors whose role in cell growth is either defined or yet to be determined [7]. There are over 1,000 proteins in serum, where some are at nanomolar or lower concentrations and others at micro molar or higher concentrations. In studies designed to measure the contribution of a particular growth factor, it is customary to use serum free medium supplemented with graded doses of the protein or factor. Such studies have been done to determine the growth factor most suited to support anchorage independent growth [8]. Interestingly, in majority of such studies, in addition to the growth factor of interest, the medium was also supplemented with fetal bovine serum [9]. Serum proteins appear to be particularly important requirement for anchorage independent growth. We have made an effort in the present study to identify the protein or group of proteins “platform” associated with anchorage independent growth in serum.

We hypothesized that fetuin-A or a group of proteins which form a complex with or co-purify with it is/are responsible for anchorage independent growth in serum. We had demonstrated that fetuin-A is one of the serum proteins that could support anchorage independent growth in breast carcinoma cells [10]. Unfortunately fetuin-A easily associates with other serum proteins making it difficult to single it out as the principal driver of growth. Recent studies indicated that exosomes (nanovesicles) purified from HIV infected monocyte-derived macrophages and urine, contain fetuin-A among other serum proteins [11,12]. We hereby present data that demonstrate that serum proteins or factors (including fetuin-A) that confer anchorage independent growth are associated with exosomes.

## Materials and Methods

Cells: Breast carcinoma cell lines MDA-MB-435, and a sub-clone of BT-549 stably transfected with galectin-3 and re-named BT-549Gal-3, were kindly donated by Dr. Avraham Raz, Karmanos Cancer Research Institute. MCF-10A and MDA-MB-231, were purchased from ATCC. The cells were routinely cultured in DMEM/F12 supplemented with essential and non-essential amino acids, 100 µg/ml penicillin-streptomycin, 2.5 µg/ml Fungizone, 20 ng/ml epidermal growth factor, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 98 ng/ml cholera toxin, and 10% heat inactivated fetal bovine serum at 37°C, 5% CO<sub>2</sub>, in a humidified incubator. GFP-annexin-II expression plasmid was kindly donated to us by Dr Carl E. Creutz (University of Virginia). All the other reagents unless otherwise stated were purchased from Sigma (St Louis, MO).

## Exosome Purification Protocol

To purify exosomes from fetal bovine serum, we used one batch of FBS from Gemini Bioproducts (West Sacramento, CA), one from PAA Laboratories, Inc. (New Bedford, MA), and two batches from Atlanta biologicals, Lawrenceville, GA. The serum (100 ml) was mixed with RPMI-1640 medium at a ratio of 50:50. This was centrifuged at 20,000 × g to remove micro vesicles and other cellular debris and the resultant supernatant was centrifuged at 100,000 × g for 10 h. The supernatant (exosome free fraction) was carefully decanted and named exosome free medium (EFM) while the resulting precipitate was dissolved in PBS and centrifuged at 200,000 × g for 1 hr. The resulting precipitate was dissolved in RPM-1640 and constituted the exosome enriched medium (EEM). Both EEM and EFM were adjusted to a concentration of 6 mg/ml using serum free DMEM/F12 medium.

### Anchorage Independent growth Assays

The anchorage independent growth assay (AIG) was modified for the 96-well culture plates. Aliquots of agar Noble (Difco) (0.8% w/v in water; 100 ml) were sterilized and stored at room temperature in conical flasks. The agar was melted, and kept in water bath at 50°C. To make the bottom layer, one part of the melted 0.8% agar (700 µl) was mixed with 560 µl of 2× DMEM/F12 serum free medium containing either 140 µl of exosome enriched medium (EEM) or exosome free medium (EFM). This final mixture (0.4% agar) was then added to wells at 100 µl/well and allowed to gel at room temperature. To prepare the top layer (0.3% soft agar) 700 µl of 0.6% agar was mixed with 560 µl of 2× SFM containing the cells (500 cells/well) and either 140 µl of EEM or EFM. This mixture was then added to the bottom layer (100µl/well) and allowed to gel at room temperature. After three weeks of growth the colonies were photographed. Alamar Blue (Biosource, Camarillo, CA) was also added to the wells to measure the growth potential of the colonies by monitoring the release of reducing metabolites as a read-out. In some experiments we used agarose (Invitrogen) instead of soft agar. We also assayed for 3-dimensional growth of tumor cells in Matrigel. Growth factor reduced Matrigel (BD Biosciences, Bedford, MA) from frozen stock was kept overnight at 4°C to liquefy. This was then added to 96-well culture plate (100 µl/well) and allowed to solidify by incubating at 37°C for at least 20 min. Serum free medium (SFM), EFM, and EEM were added (200 µl/well) on top of Matrigel followed by BT-549Gal3 cells (500 cells/well). The plates were then incubated in a humidified CO<sub>2</sub> incubator at 37°C for two weeks.

### Proteomic Analysis of the proteins in Exosomes

**In-Gel Digestion**—Equal amounts of proteins from EEM and EFM were loaded into SDS-PAGE (4-12% gradient gel), resolved and stained with coomassie brilliant blue. Each lane was cut into 4 regions and each subjected to in-gel tryptic digestion [13]. Briefly, the gel regions were excised and washed with 100 mM ammonium bicarbonate for 15 minutes. The liquid was discarded and replaced with fresh 100 mM ammonium bicarbonate and the proteins reduced with 5 mM DTT for 20 minutes at 55° C. After cooling to room temperature, iodoacetamide was added to 10 mM final concentration and placed in the dark for 20 minutes at room temperature. The solution was discarded and the gel pieces washed with 50% acetonitrile/50 mM ammonium bicarbonate for 20 minutes, followed by dehydration with 100 % acetonitrile. The liquid was removed and the gel pieces were completely dried, re-swelled with 0.3 µg of modified trypsin (Promega) in 100 mM NH<sub>4</sub>HCO<sub>3</sub>, and digested overnight at 37°C. Peptides were extracted by three changes of 60% acetonitrile/0.1% TFA, and extracts from each region were combined separately, generating 4 samples per lane, and dried *in vacuo*. Samples were reconstituted in 30 µL 0.1 % formic acid for LC-MS-MS analysis.

### LC-MS-MS Analysis and Protein Identification

Resulting peptides were analyzed using a Thermo Finnigan LTQ ion trap instrument equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 2.0 SR2 instrument control. Peptides were separated on a packed capillary tip (Polymicro Technologies, 100 µm × 11 cm) with Jupiter C18 resin (5 µm, 300 Å, Phenomenex) using an in-line solid-phase extraction column (100 µm × 6 cm) packed with the same C18 resin [using a frit generated with liquid silicate] similar to that previously described [14]. The flow from the HPLC pump was split prior to the injection valve to achieve flow-rates of 700 nL-1000 µL min<sup>-1</sup> at the column tip. Mobile phase A consisted of 0.1 % formic acid and Mobile phase B consisted of 0.1% formic acid in acetonitrile. A 95 min gradient was performed with a 15 min washing period (100 % A for the first 10 min followed by a gradient to 98% A at 15 minutes) to allow for solid-phase extraction and removal of any residual salts. Following the washing period, the gradient was increased to 25% B by 50 min, followed by an increase to 90 % B by 65 min and held for 9 min before returning to the initial conditions. Tandem spectra

were acquired using a data dependent scanning mode in which one full MS scan ( $m/z$  400-2000) was followed by 9 MS-MS scans. Tandem spectra were searched against the bovine subset of the UniRef100 database using the SEQUEST algorithm. The database was concatenated with the reverse sequences of all proteins in the database to allow for the determination of false positive rates. Protein matches were preliminarily filtered using the following criteria: cross-correlation ( $X_{corr}$ ) value of  $\geq 1.0$  for singly charged ions,  $\geq 1.8$  for doubly charged ions, and  $\geq 2.5$  for triply charged ions. A ranking of primary score (RSp) of  $\leq 5$  and a preliminary score (Sp) of  $\geq 350$  were also required for positive peptide identifications. Once filtered based on these scores, all proteins identified by less than two peptides were eliminated, resulting in false positive rates of  $<1\%$ . The Sequest output was also filtered using IDPicker using a false positive ID threshold (default is 0.05 or 5% false positives) based on reverse sequence hits in the database.

The use of sampling statistics has been shown to be a useful measure of relative protein abundance in label-free-LC-MS/MS shotgun proteomics. Recent studies have revealed a direct relationship between protein abundance and sampling statistics, such as percent sequence coverage, peptide count and spectral count. Most notably, the spectral count is the total number of tandem mass spectra (MS/MS) that are matched to the peptides of a protein. Liu et al. [15], demonstrated that spectral counts could be a reliable indicator of protein abundance with a linear range over 2 orders of magnitude by using a spectral count based measure; spectral sampling. In contrast to spectral counts, other sampling measures such as percent sequence coverage and number of peptides identified per protein did not have nearly as good of a linear correlation to actual protein abundance [15]. Moreover, spectral count has the highest technical reproducibility, followed by the less-reproducible peptide count and relatively non-reproducible sequence coverage [16]. Protein reassembly from identified peptide sequences is done with the aid of a parsimony method recently described by Zhang et al. [17] which identifies indiscernible proteins (protein groups) that can account for the identified peptides. Relative protein abundance (fold ratio changes) between exosome free medium (EFM) and exosome enriched medium (EEM), were compared based on spectral counts from two independent LC-MS/MS runs corresponding to two aliquots from the same preparation run in separate lanes of SDS-PAGE gels (Table 2).

### Transmission Electron Microscopy of the Exosomes

The exosomal sample was fixed in 2.5% paraformaldehyde for at least 1 hr, followed by 2 buffer washes (TBS) for 5 min each. The samples were then incubated with 50% ethanol for 15 min followed by 70% ethanol for 30 min. They were then mixed 1:1 with LR white resin in 100% ethanol for 30 min followed by 2:1 mix with LR white resin in 100% ethanol for 30 min. They were then immersed in pure LR white resin for 60 min and then another fresh LR white resin for 60 min followed by LR white resin overnight in a rotator. They were then placed in gelatin capsules with fresh LR white resin and cured at 50 degrees for 48 hr. The sections were cut to 100 nm thickness and within 30 min, staining process was started. The sections were washed in TBS buffer 3 times for 30 seconds each then the grids blocked using 2% BSA for 30 min. The sections were then fixed in 2.5 % glutaraldehyde for 30 seconds, washed in buffer and then 3 times in water for 30 seconds to remove salts and then stained for 30 seconds with 1% Uranyl acetate. The moisture was wicked away and then examined using a Phillips CM 12 TEM at 80 KV.

### Up-take of Purified Serum Exosomes by Tumor Cells

Exosomes purified as described above were labeled with rhodamine isothiocyanate (Sigma) as described [18] and incubated with adherent BT-549Gal3 cells. Briefly, the total exosomal proteins were determined by the Bradford assay and 100  $\mu\text{g}/\text{assay}$  were equilibrated in 100 mM carbonate buffer pH 9.0 by centrifugation at  $10,000 \times g$  for 15 min in an ultra-filtration

unit (MCO-100,000). The equilibrated exosomes were re-suspended in 100  $\mu$ l of the same buffer and mixed with 100  $\mu$ g/assay of rhodamine isothiocyanate in DMSO. This was then incubated 2 h at room temperature and in the dark. After labeling, the exosomes were washed abundantly by centrifugation in the ultra-filtration unit to remove un-reacted rhodamine. Adherent breast tumor cells were grown on microscope cover slips, starved for 48 h by growth in DMEM supplemented with 0.5 % FBS, washed twice with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free Dulbecco PBS (Invitrogen) and once with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free HBSS (Invitrogen). Cells were then incubated for 30 min in HBSS containing 10  $\mu$ g/ml rhodamine-labeled exosomes, 1 mM of divalent ions  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , and the indicated concentrations of unlabeled exosomes. The cells were rinsed twice with cold PBS before and after fixation with  $-20^{\circ}\text{C}$  cold methanol for 10 min and the cover slips mounted on slides using Prolong with DAPI (Invitrogen). Up-take was visualized by fluorescent microscopy using a Nikon Eclipse TE-2000-E inverted microscope equipped with NIS-Elements AR software (Nikon, Melville, NY, USA)

### Recycling of Serum Exosomes by Tumor Cells

BT-549Gal-3 cells were transfected with GFP-annexin A2 or mock transfected using Fugene6 according to the manufacturer (Roche) and serum-starved for 48 h. The mock transfected cells were incubated with rhodamine-labeled exosomes for 30 min at  $37^{\circ}\text{C}$ , then washed twice with PBS at RT. The GFP-annexinA2 transfected and the rhodamine labeled cells were respectively harvested by treatment with 2 mM EDTA and washed in cold PBS and once in HBSS supplemented with 1 mM  $\text{Mg}^{2+}$  and 1 mM  $\text{Ca}^{2+}$ . These were then mixed at a ratio of 1:3 (GFP-transfected cells to rhodamine-labeled cells), plated in six-well plates with cover slips and incubated for another 2 hr at  $37^{\circ}\text{C}$ . The cells were rinsed twice with cold PBS before and after fixation in  $-20^{\circ}\text{C}$  cold methanol for 10 min and the cover slips mounted on slides using Prolong with DAPI (Invitrogen). Up-take/recycling were visualized by fluorescent microscopy using a Nikon Eclipse TE-2000-E inverted microscope equipped with NIS-Elements AR software (Nikon, Melville, NY, USA)

### MAP kinase Signaling Mediated by Exosomes

Cells were plated in 10 cm dishes and serum-starved as above for 48h. The adherent cells were then washed twice with PBS and once with 10 mM HEPES pH 7.2, 140 mM NaCl, 4.7 mM KCl (HBS) and incubated in HBS containing 1 mM  $\text{Mg}^{2+}$  and 1 mM  $\text{Ca}^{2+}$  without or with 0.1 mg/ml of exosomes for the indicated time points at  $37^{\circ}\text{C}$ . The medium was rapidly discarded, replaced with ice-cold PBS and the dishes maintained on ice until the cells were harvested by scraping. These were washed in the ice-cold PBS and the cell pellets stored at  $-80^{\circ}\text{C}$  until needed. In parallel experiments, cell culture dishes were pre-treated with poly(HEMA) as previously described [19]. The poly(HEMA)-treated dishes were sterilized by UV then washed twice with PBS. About  $2 \times 10^6$  cells/dish that have been serum-starved as above were plated on the poly(HEMA)-treated dishes in HBS containing 1 mM  $\text{Mg}^{2+}$ , 1 mM  $\text{Ca}^{2+}$  without and with the indicated concentrations of exosomes (mg/ml) for 10 min or following a time course at  $37^{\circ}\text{C}$ . MAP kinase activation was visualized by the detection of phosphorylated ERK1/2 by western blotting using antibodies to the dually phosphorylated ERK1 and ERK2. Total ERK as the loading control was detected using antibodies to either ERK2 or ERK1/2.

## Results

### Role of Serum Exosomes on Anchorage dependent and independent growth of Breast Carcinoma Cells

Our objective here was to determine the serum fraction (exosome free or enriched) that supports anchorage independent growth of breast carcinoma cells. The breast carcinoma cells were suspended in soft agar and allowed to grow either in the absence or presence of serum exosomes for at least 3 weeks. The data clearly show that AIG is robust only in those wells containing



exosomes (Fig. 1). This serum fraction which we have named exosome enriched medium (EEM) in addition to common serum proteins such as albumin, also contains a unique set of proteins that are absent in exosome free medium (EFM) (Table 1). The AIG assay was repeated at least 4 times with similar results. All the batches of fetal bovine serum we employed yielded exosomes that promoted AIG and the colonies grew in both soft agar and agarose at a similar rate. Not only did the cells growing in the presence of exosomes form large colonies with time, they were also healthy and actively released reducing metabolites into the conditioned medium, a hall mark of metabolic activity in growing cells (Fig. 1B). It can be argued that MDA-MB-435 is not an authentic breast cancer cell line [20]. However, its growth patterns in soft agar being similar to the other breast carcinoma cell lines, demonstrate that the ability of exosomes to influence anchorage independent growth can be extrapolated to other tumor cells such as melanoma.

Tumor cells in serum free medium failed to grow in growth factor reduced Matrigel after two weeks of incubation (Fig. 2A). However, in EFM, the cells grew mainly as a monolayer on top of the Matrigel with extensive spreading. In EEM on the other hand, there were distinctive three dimensional stellate colonies (Fig. 2C). These stellate colonies are typically observed when tumorigenic cells are grown in Matrigel [21]. Apart from anchorage independent growth in soft agar and Matrigel, we also examined the influence of exosomes on the growth of breast epithelial cells on plastic. In the presence of the vesicles, the tumor cells adhered and spread rapidly on plastic and with extended period of growth (seven days), the cells grew in tight aggregates on plastic (Fig 3B). This type of growth is not uncommon in tumor cells [22], further re-enforcing the potential role of these vesicles on AIG. Due to the inability of normal breast epithelial cells (MCF-10A) to grow in soft agar, we speculated that they would also fail to grow on plastic in the presence of exosomes. On the contrary, the cells proliferated in the presence of the vesicles and as expected in vesicle free medium (EFM). However, in serum free medium, growth was minimal (Fig 3A).

### **Analysis of Proteins identified by mass-spectroscopy in both EEM and EFM**

Transmission electron microscopy (TEM) revealed vesicles in EEM whose average diameter was approximately 150 nm (Fig. 4A). This average diameter was larger than that of bonafide cell derived exosomes with an average in diameter of between 60-100 nm [23]. Serum vesicles (micro-vesicles) precipitated at approximately  $20,000 \times g$  lacked any biological activity such as support of anchorage independent growth (data not shown). An examination of the distribution of two of the more abundant proteins in the EEM by sucrose gradient centrifugation revealed that while alpha 2-microglobulin was associated with the dense portion of the sucrose gradient, fetuin-A is associated with the less dense portion of the gradient (data not shown).

When the proteins in EEM and EFM were resolved on SDS-PAGE, it was evident that whereas most of the serum proteins appeared in both fractions, some were more concentrated in EEM (Fig. 4B, arrows). Among the proteins recovered in the EEM lane, a number of them were found only in exosomes by mass spectroscopy (LC-MS/MS) (Table 1). The relative abundance of these proteins in either EFM or EEM was determined by use of spectral counts. The presence of at least two spectral counts was used as the threshold for peptide/protein identification. Given that the stringency during the LC-MS/MS was high and resulted in a measurable false positive rate of less than 1%, we conclude that a small spectral count is indicative of low abundance of the peptide, yet a high degree of confidence in the presence of that peptide in the sample. Interestingly, some of these proteins have also been reported in exosomes purified from other sources and are considered exosomal marker proteins. These include heat shock proteins HSP-90 [24], alpha tubulin [11], peptidyl prolyl trans isomerase [25,26], elongation factor 1 alpha [11], galectin-3 binding proteins [27], glyceraldehyde-3-phosphate dehydrogenase [11], and chloride intracellular channel protein 1 [11]. By far the most abundant protein

associated with serum exosomes is alpha 2-macroglobulin (Fig. 4C and Table 2). Interestingly other acute phase proteins synthesized in the liver such as fetuin-A and heat labile complement factors were also associated with the exosomes (Table 2). The concentration of fetuin-A in EFM was more or less comparable to its concentration in EEM (Table 2 and Fig. 4C). It is likely that the high enrichment of serum proteins in bovine serum exosomes may account for their rather large size compared to exosomes purified from cells.

### Up-take of Purified Exosomes by Tumor Cells

Exosomes can be up-taken or endocytosed by cells via a mechanism that is not well characterized in the literature [28]. We show here that the purified labeled bovine exosomes can be endocytosed by breast carcinoma cells. After about 2 hr of up-take, the exosomes appear to concentrate in the late endosomal compartment (Fig. 5A). This pattern of up-take is similar to that which has been observed by others [28]. Interestingly, when we incubated the cells with the labeled exosomes and excess unlabeled exosomes (Fig. 5B and 5C), the up-take of the former was blocked, suggesting that exosomes are endocytosed via a cell surface receptor/s.

### Re-cycling of Serum Exosomes by Tumor Cells

It was interesting to observe that by giving the tumor cells just enough exosomes ~0.1 mg/ml, the cells were able to grow in soft agar for up to three weeks without the addition of more exosomes. This prompted us to question whether endocytosed exosomes can be re-cycled to maintain growth. To test this working hypothesis, we transfected BT-549-Gal3 cells with GFP-annexinA2 and incubated the cells with rhodamine-isothiocyanate labeled exosomes. For the first 30 min. we could see only red and green cells (Fig. 6A). However, after 3 hr of incubation, we mostly observed red and orange cells (Fig. 6B and 6C). This means that the cells that had endocytosed exosomes, released some back to the conditioned medium where they were up-taken by GFP-annexinA2-transfected cells (Fig. 6B and 6C). In Fig. 6C, it is evident that the two GFP-annexinA2 transfected cells had endocytosed the released exosomes that were now concentrated in the late endosomal compartment, where they co-localized with annexinA2 which is also secreted from the cells via the re-cycling pathway of exosomes. We further demonstrated this by isolating and purifying exosomes from GFP-annexinA2 transfected cells and showing that GFP-annexinA2 is indeed recovered in the exosomal fraction (Fig. 6D). Members of annexin family of membrane proteins are routinely observed in cellular exosomes and can be considered markers of cellular exosomes [29]. In addition, galectin-3, another marker of cellular exosomes [29], was shown to be present in the exosomes purified from the tumor cells (Fig. 6E).

### Map Kinase Signaling Mediated by Exosomes

We next investigated the role of exosomes in transmitting growth signals to cells on plastic or non-adhered cells growing on poly-(HEMA) coated dishes. It was interesting to note that on plastic and in the presence of  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ions, ERK was activated (phospho-ERK) within 10 min of exosome addition and the activity diminished after 90 min. (Fig. 7A). This is the pattern of activation that is usually observed when one plots a time course of ERK activation [30]. However in the presence of a low concentration of exosomes (0.1 mg/ml), ERK was again activated within 5 min. but the signal remained strong for at least 240 minutes (Fig. 7A). This mode of activation is in line with adhesion and spreading of the cells on plastic in the presence of exosomes. In non-adherent cells, ERK activation was observed in the presence of low concentrations of exosomes (0.05 mg/ml) but at elevated concentrations approaching 0.5 mg/ml, activation of ERK was inhibited (Fig. 7B). This has been a consistent observation in at least 3 different experiments. Again as observed in adhered cells, in the presence of low concentrations of exosomes (0.1 mg/ml), the phosphorylated signal in ERK1/2 remained strong for at least 2 h (Fig 7C). Surprisingly the ERK1/2 activation signal in anchorage independent

cells in the presence of exosomes (0.1 mg/ml) was evident even after 18 hours of growth (data not shown). A similar sustained activation of ERK in non-adhered cells was recently reported in ovarian cancer cells [31].

## Discussion

In the present studies, we have re-visited a long standing question regarding the identity of factor(s) in fetal bovine serum that mediates anchorage independent growth of tumor cells. A number of growth factors such as IGF, EGF, TGF- $\beta$ ,  $\beta$ FGF, PDGF have individually and as a group, been shown to play a role in anchorage independent growth of tumor cells [8,32,33]. However, even after all these growth factors were included as a cocktail in serum free medium and mixed with soft agar, colony formation by tumor cells was still less compared to growth in the medium containing serum [34]. It is in this background of looking for serum proteins that may influence anchorage independent growth that we questioned whether fetuin-A (a serum glycoprotein) plays a role in anchorage independent growth of tumor cells. In our earlier studies we determined that antibodies to this glycoprotein reduced anchorage independent growth potential in soft agar (data not shown). However, fetuin-A in addition to being secreted into the blood as a free protein, is also bound to other proteins [35] and has been shown to be associated with exosomes in the serum [11,12].

In the present analyses, our goal was to identify the serum fraction (exosome free or exosome enriched) that supports anchorage independent growth (AIG) of breast carcinoma cells. The data clearly show that the membranous fraction of fetal bovine serum (contains exosomes) is the one that mediates AIG of the tumor cells. The *in vitro* growth of tumor cells in soft agar is regarded as an excellent predictor of tumor growth *in vivo* [5]. The ability of exosomes to mediate the growth of stellate colonies [21] in Matrigel further underscores the potential role of exosomes in the *in vivo* tumor growth. Whereas Matrigel also supported the growth of tumor cells in EFM, the cells mainly grew in 2-dimensions and not inwards or upwards in this medium.

Therefore in order for us to dissect *in vivo* tumor growth mechanisms, the interaction between these exosomes and carcinoma cells deserves a rigorous analysis. The exosomes are able to promote the growth of both normal (MCF-10A) and breast carcinoma cells. Growth signals for anchorage independent growth of tumor cells are well documented in the literature [2]. In normal cells (such as MCF-10A) that are attached or adhered to the substratum, the engagement of integrins by extracellular matrix proteins transmits growth signals that can also crosstalk with those emanating from growth factor/receptor interaction again resulting in cell growth [2]. However, in normal cells, lack of adhesion to the substratum results in anoikis or programmed cell death in case of epithelial and endothelial cells [2]. Transformed cells on the other hand are capable of transmitting growth signals whether or not they are attached to the substratum. However, the precise mechanism by which transformed cells coordinate the cross talks among the various growth signals while simultaneously inhibiting the apoptotic signals is poorly understood.

Despite the anchorage independence of transformed cells, they still require a number of growth factors, some of which the cells synthesize (autocrine) and some obtained from the serum. Our data suggest that in addition to providing the breast carcinoma cells with the growth factors such as TGF, the membranous portion of serum (contains exosomes) provide unique growth platforms. The vesicles could in theory concentrate growth factors on the surfaces of tumor cells. They could also promote cell to cell aggregation resulting in the enhancement of growth signals. The bovine vesicles just like the human exosomes, could also be derived from a variety of cell types including epithelial cells [36], macrophages [37], and platelets [38]. Some of the proteins could be imbedded in the vesicles while others such as fetuin-A could be on the surface. A number of serum proteins associated with exosomes have been implicated in anchorage



independent growth of tumor cells in soft agar. Studies by Calhoun et al [39], have demonstrated that knockdown of cyclophilin A expression using RNAi in U2OS cells resulted in disruption of the F-actin structure and decreased anchorage independent growth, proliferation and migration. Interestingly, Emmprin (CD 147), a cell surface glycoprotein that plays a critical role in cellular transformation and anchorage independent growth, is a receptor for cyclophilin A [40]. The other protein that is abundantly expressed in the exosomes, HSP-90 alpha also plays a role in anchorage independent growth together with the other members of the family. The majority of known substrates for the 90 kDa heat shock protein (HSP-90) are signal intermediates of clinical importance such as kinases [41,42]. For example, activated Cd42-associated kinase (AcK1) expression in LNCaP cells enhanced anchorage-independent growth *in vitro* and accelerated tumorigenicity in nude mice. However, its activity required the binding of HSP-90 beta [43]. Furthermore, the expression of the small heat shock protein (HSP-27) is associated with increased anchorage independent growth, increased invasiveness and resistance to chemotherapeutic drugs, poor prognosis and reduced disease free survival [44]. Studies by Anand et al. [45] have demonstrated that the gene encoding protein elongation factor (eEF-1 alpha) is amplified in 25% of primary ovarian tumors and is highly expressed in approximately 30% of ovarian tumors. They further showed that eEF-1alpha enhances focus formation, enhances anchorage independent growth and decreases doubling time of rodent fibroblasts.

Apart from the above mentioned serum proteins that are exclusively associated with exosomes, others such as alpha2-macroglobulin are concentrated in them but also occur in the exosome free fraction of serum. Another group of proteins which include fetuin-A, is more or less equally distributed in both EEM and EFM. Fetuin-A may after all, act as an opsonin to facilitate the up-take of the exosomes by the tumor cells [46]. Some of the exosomal associated serum proteins, particularly those that are loosely attached to their surfaces, may have specific receptors on the tumor cells through which they transmit their growth signals. However, the transit time of the proteins on the cell surface may be limited because by 10 min of adding exosomes to the cells, they are endocytosed. Once inside the cells, the exosomal proteins could directly promote anchorage independent growth and then channeled to the degradative pathway [47]. They could also be diverted into the re-cycling pathway of exosomes [48]. There are bovine serum proteins including fetuin-A that are routinely observed in exosomes purified from cells [11]. The assumption therefore is that these are bovine serum exosomal proteins that are part of the re-cycling exosomal pathway.

We have directly demonstrated that labeled bovine exosomes taken up by one breast tumor cell can eventually exit this cell and be up-taken by another. As the serum exosomes including their assortment of proteins are up-taken by cells on hand, the released exosomes on the other hand bring out with them a unique set of cellular proteins. For example, we have demonstrated that galectin-3, annexin-2GFP (in transfected cells) and endogenous annexin-2 are part of the repertoire of proteins secreted in exosomes from tumor cells. Both of these have been observed in cellular exosomes [29]. Galectin-3 is one of the cellular proteins that have been implicated in anchorage independent growth of tumor cells in soft agar [49]. It is thus tempting to speculate that exosomal galectin-3 is one of the key proteins with anchorage independent growth promoting properties. The up-take of serum exosomes by tumor cells could in theory speed up the release of the cellular exosomes (bearing these unique proteins) which in turn are up-taken by neighboring cells to influence anchorage independent growth.

In order to begin to understand mechanistic pathways by which exosomes influence anchorage independent growth in tumor cells, we analyzed MAP kinase signaling in the interaction between tumor cells and purified exosomes. This is one of the key signaling pathway in tumor cells and in particular breast carcinomas that is responsible for maintenance of AIG [50,51]. Obviously there are cross-talks between MAP kinase pathways and other pathways that have

also been heavily implicated in anchorage independent growth such as PI3 kinase/Akt [52]. The strong activation of ERK1/2 in the tumor cells by the bovine exosomes in the absence of growth factors such as EGF, suggest that these vesicles act in a synergistic manner with other growth factors to promote AIG. The ability of low concentrations of exosomes to maintain anchorage independent growth in the tumor cells argues for re-cycling of the exosomal derived growth factors and is consistent with sustained activation of ERK1/2.

In summary, we have demonstrated that serum factors necessary for anchorage independent growth of tumor cells in soft agar are concentrated in the exosomal compartment of serum and that these exosomes are most likely recycled to maintain growth. It is highly likely that human serum exosomes will also display a similar protein profile as the bovine vesicles, and with the potential to drive the *in vivo* growth of tumors particularly of the breast. Work is on going in our laboratory to identify cell surface receptor/s responsible for the endocytic up-take of the bovine exosomes. The identity of the key proteins that drive anchorage independent growth, whether originating from the exosomal compartment of tumor cells or bovine serum, is another focus area.

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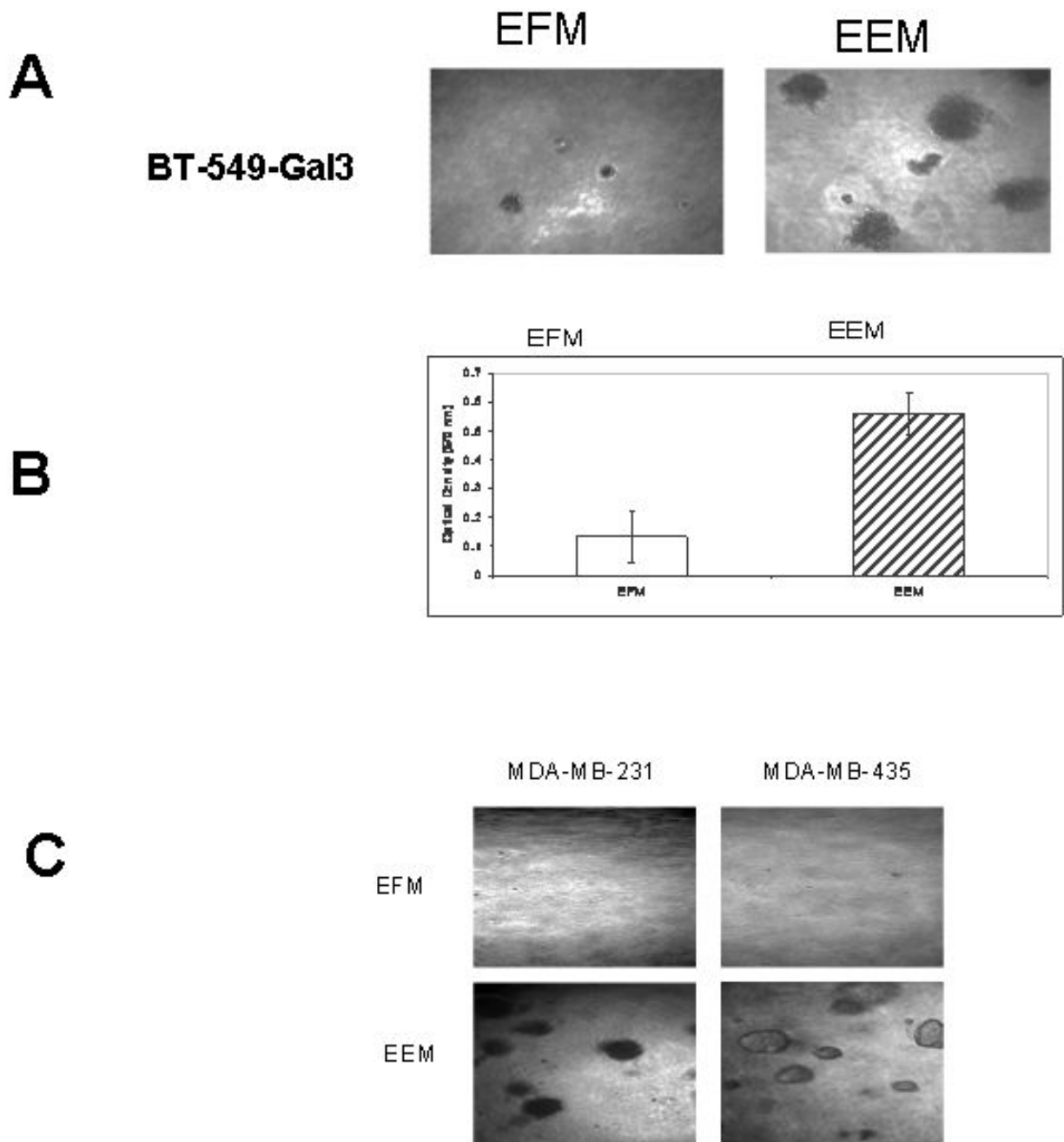
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## Abbreviations

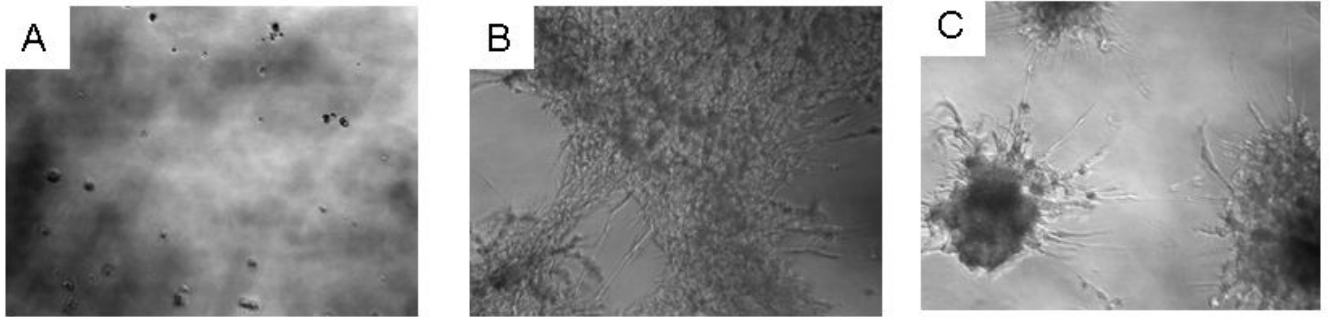
<b>EEM</b>	exosome enriched medium
<b>EFM</b>	exosome free medium
<b>HEMA</b>	2-hydroxyethylmethacrylate
<b>AIG</b>	anchorage independent growth





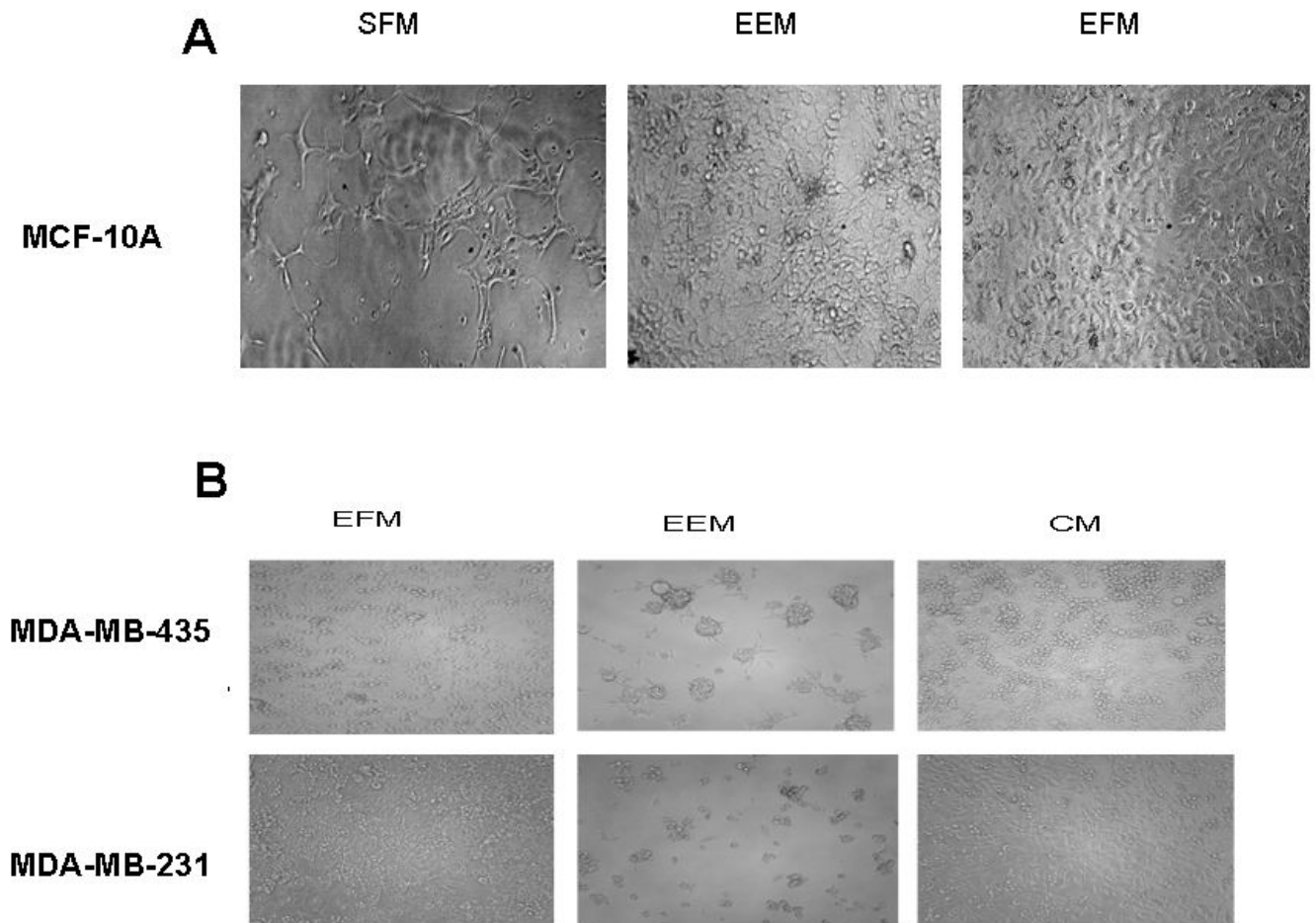
**Figure 1. Bovine serum exosome mediated anchorage independent growth of breast cancer cells in soft agar**

Soft agar growth assays were set up in 96-well micro titer plates as described in Materials and Methods. The cells were allowed to grow in soft agar either in the presence (EEM) or absence (EFM) of purified bovine exosomes. After 3 weeks the colonies were either photographed (panel A) or Alamar blue was added to the control wells and to the wells containing exosomes. After 4 hours of color change, the plates were read at 570 nm (panel B). The growth promoting properties of the exosomes were also determined using other breast cancer cell lines (panel C).

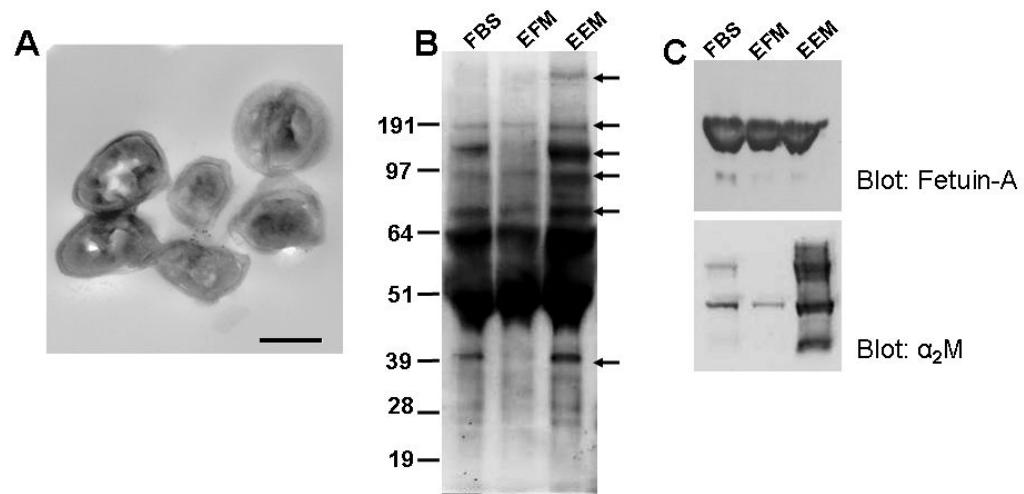


**Figure 2. Bovine serum exosome mediated growth of stellate colonies of breast cancer cells in Matrigel**

Liquid Matrigel (100 $\mu$ l/well) was allowed to solidify at 37°C in 96-well micro titer plate. The carcinoma cells were then added on the Matrigel (500 cells/well) in the presence of serum free medium (panel A), exosome free medium (panel B) and exosome enriched medium (panel C). The cells were then incubated in a humidified CO<sub>2</sub> incubator for 14 days and then photographed.

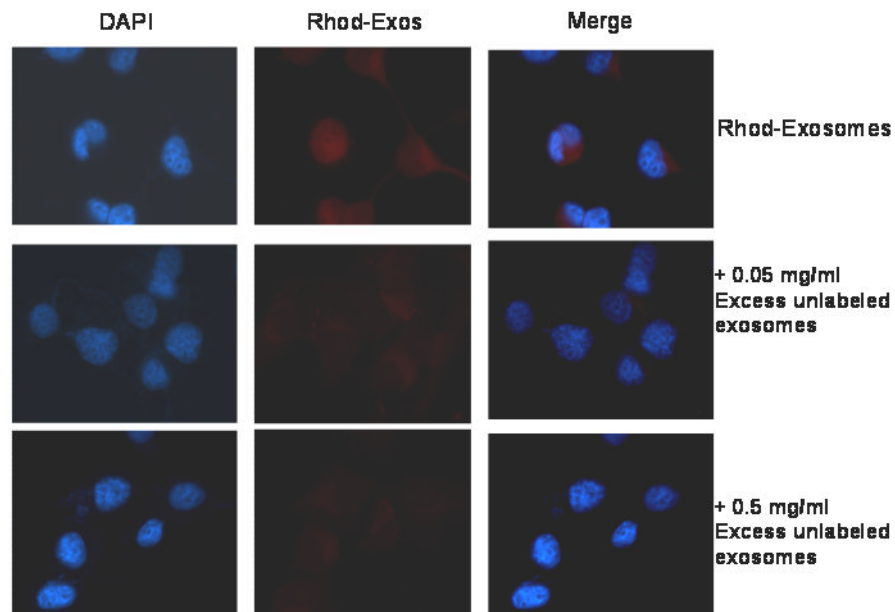


**Figure 3. Bovine serum exosome mediated growth of normal and breast cancer cells on plastic**  
In panel A, MCF-10A were at  $2 \times 10^4$  cells/well in 96-well micro titer plates in serum free medium, exosomal enriched medium (EEM) and exosomal free medium (EFM). The cells were allowed to grow for 5 days and then photographed. In panel B, one breast carcinoma cell line, MDA-MB-231 and a melanoma cell line, MDA-MB-435 were likewise plated at  $2 \times 10^4$  cells/well in 96-well micro titer plates in EEM and EFM and in complete medium (CM) containing 10% fetal bovine serum.



**Figure 4. Proteins Associated with bovine serum exosomes**

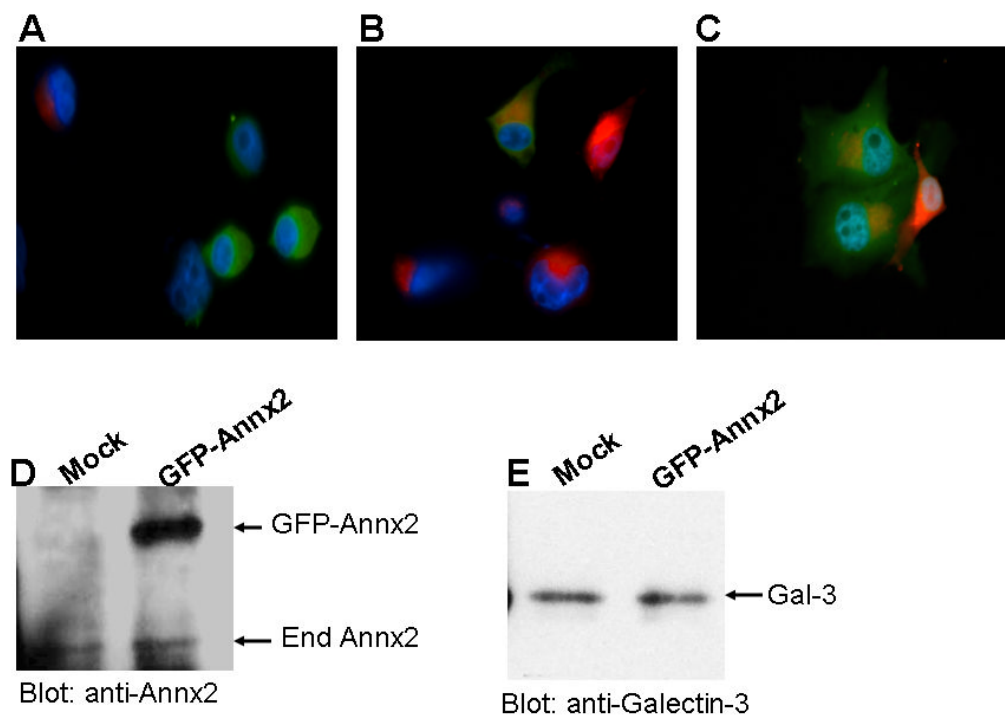
Panel A, transmission electron micrograph of exosomes purified from fetal bovine serum. The black line represents 100 nm. Panel B, aliquots of bovine serum, exosome free serum, and exosomes were resolved in gradient SDS-PAGE and stained with coomassie brilliant blue. The protein bands were photographed and then all the proteins in each lane cut and analyzed by mass spec as described in Materials and Methods. Panel C, proteins from bovine serum, exosome-free serum and exosomes were resolved in SDS-PAGE and then transferred to immobilon paper and probed for fetuin-A and alpha 2 macroglobulin.



**Figure 5. Up-take of purified bovine serum exosomes by tumor cells**

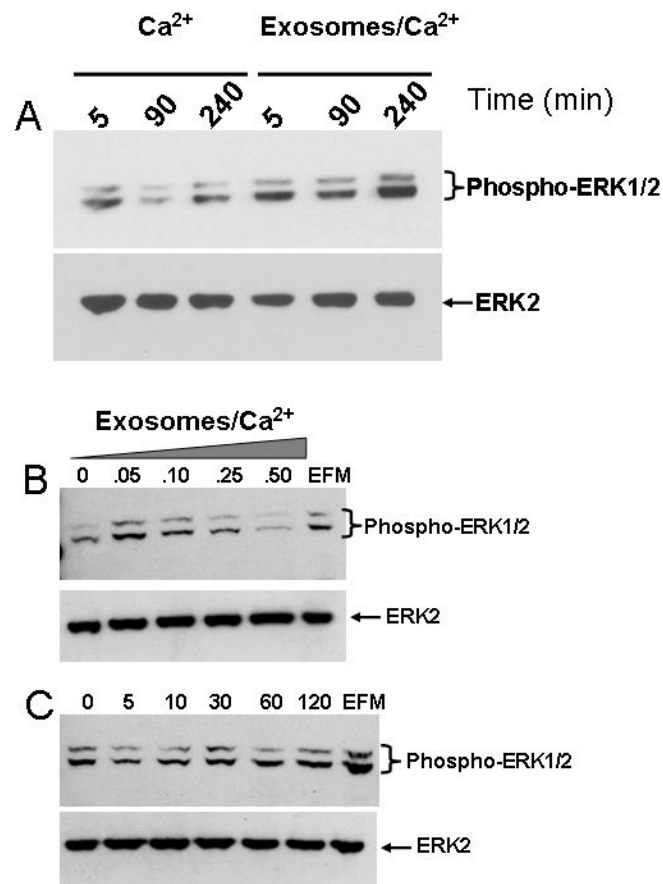
Purified bovine exosomes were labeled with rhodamine isothiocyanate, purified again by ultra-filtration to remove excess unreacted rhodamine and then added to BT-549Gal-3 cells on cover-slips which had been serum starved for 48 h. The cells were incubated with labeled exosomes for 30 min in the absence and presence of excess unlabeled exosomes (0.05 mg/ml) and (0.5 mg/ml) respectively in HBSS containing divalent ions. The cells were rinsed twice with cold PBS and then fixed with  $-20^{\circ}\text{C}$  cold methanol for 10 min, cover-slipped and visualized by fluorescent microscopy. The experiment was repeated with MDA-MB-231 and MCF-10A cells with similar results.





**Figure 6. Recycling of bovine serum exosomes by tumor cells**

BT-549Gal-3 cells were transfected with GFP-annexin-A2 or mock transfected using Fugene6 and serum starved for 48 h. The mock transfected cells were then incubated with rhodamine-labeled exosomes for 30 min at 37°C followed by washing with PBS at room temperature. The GFP-annexin-A2 transfected cells (green cells) and rhodamine labeled (red) cells were harvested with 2 mM EDTA, washed in cold PBS and mixed at a ratio of 1:3 (green cells to red cells), plated in six-well plates on coverslips and incubated for 10 min (Panel A) and then for 2hr at 37°C (Panels B and C). The GFP-annexin-A2 and mock transfected cells were also detached with EDTA and incubated separately in HBSS in siliconized tubes for 2 h at 37°C. Exosomes were isolated and purified from the conditioned medium as described in Materials and Methods. The cellular exosomes were resolved in SDS-PAGE, transferred to immobilon membranes and probed with annexin-2 (Panel D) and galectin-3 (Panel E) antibodies.



**Figure 7. MAP Kinase signaling mediated by bovine serum exosomes**

BT-549Gal3 cells were plated in 10 cm. dishes and serum starved for 48 h. The adherent cells were incubated in HBS containing 1 mM of the divalent ions ( $\text{Mg}^{2+}/\text{Ca}^{2+}$ ) for the indicated time points (Panel A). The cells were scrapped, washed in ice-cold PBS and stored at  $-80^{\circ}\text{C}$  until needed. In parallel experiments, cells ( $2 \times 10^6$  cells/dish) were plated on poly(HEMA)-treated dishes in HBS containing the divalent ions ( $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) without or with indicated concentrations of exosomes (mg/ml) for 10 min (Panel B). EFM (0.5 mg/ml) was also included as a positive control. In Panel C, the cells were incubated with 0.1 mg/ml of exosomes on poly(HEMA) coated wells for the indicated time points. The EFM (0.1 mg/ml) was also incubated with the cells for 120 min as a control. MAP kinase activation was visualized by detection of phosphorylated ERK1/2 by western blotting using antibodies to the dually phosphorylated ERK1 and ERK2. Total ERK (loading control) was detected using antibodies to ERK2.

**Table 1**  
**Proteins Found only in Exosome Enriched Medium (EEM)**

Accession Number	spectral counts		Description
	EEM	EEM	
UniRef100_UPI00005C0899	0	17	similar to Filamin A
UniRef100_UPI00005C2264	0	8	similar to myosin, heavy polypeptide 9, non-muscle, partial
UniRef100_P62935	0	6	Peptidyl-prolyl cis-trans isomerase A
UniRef100_Q2KJF4	0	6	Similar to Galectin-3 binding protein
UniRef100_Q76LV2	0	5	Heat shock protein HSP 90-alpha
UniRef100_Q27967	0	5	Secreted phosphoprotein 24 precursor
UniRef100_Q5E9F5	0	4	Transgelin-2
UniRef100_Q1RMK2	0	4	IGHM protein
UniRef100_P01267	0	4	Thyroglobulin precursor
UniRef100_UPI000051DE08	0	4	Tubulin alpha chain
UniRef100_O02717	0	4	Non-muscle myosin heavy chain
UniRef100_UPI00005BC732	0	4	similar to fibulin 1
UniRef100_O18787	0	4	Elongation factor 1 alpha
UniRef100_UPI00005BFC16	0	3	similar to Complement C4 precursor, partial
UniRef100_Q28107	0	3	Coagulation factor V precursor
UniRef100_P10096	0	3	Glyceraldehyde-3-phosphate dehydrogenase
UniRef100_UPI00005BC368	0	3	similar to alpha 3 type VI collagen isoform 1 precursor
UniRef100_O46415	0	3	Ferritin light chain
UniRef100_UPI00005BC752	0	3	similar to Plexin B2 precursor (MM1)
UniRef100_Q3SZZ9	0	3	FGG protein
UniRef100_UPI00005BEE04	0	3	similar to Stem cell growth factor precursor
UniRef100_UPI00005BC072	0	3	similar to extracellular matrix protein 1
UniRef100_Q76LV1	0	2	Heat shock protein HSP 90-beta
UniRef100_Q32LP0	0	2	Unc-112-related protein 2
UniRef100_Q05443	0	2	Lumican precursor
UniRef100_UPI00005BDFA6	0	2	similar to beta tubulin 1, class VI
UniRef100_UPI00005BE617	0	2	similar to mannan-binding lectin serine protease 2
UniRef100_UPI00005BD7A2	0	2	similar to talin 2
UniRef100_P28801	0	2	Glutathione S-transferase P
UniRef100_UPI00005C03A6	0	2	similar to ubiquitin-activating enzyme E1 isoform 3
UniRef100_Q17QH6	0	2	Similar to collectin sub-family member 11 isoform a
UniRef100_UPI00005C030B	0	2	similar to deleted in malignant brain tumors 1
UniRef100_P33672	0	2	Proteasome subunit beta type 3
UniRef100_Q3MHN2	0	2	Complement component C9 precursor
UniRef100_Q29451	0	2	Lysosomal alpha-mannosidase A peptide
UniRef100_UPI00005A1C47	0	2	similar to karyopherin beta 1
UniRef100_P02676	0	1	Fibrinogen beta chain precursor
UniRef100_Q28106	0	1	Contactin-1 precursor
UniRef100_UPI00005C1679	0	1	similar to Neogenin precursor, partial

Accession Number	spectral counts		Description
	EFM	EEM	
UniRef100_UPI00005C18F6	0	1	Similar:C1q and tumor necrosis factor related protein 3
UniRef100_UPI00005C18F7	0	1	similar to collagenous repeat-containing
UniRef100_Q3ZCJ2	0	1	Aldo-keto reductase family 1, member A1
UniRef100_Q2KJ63	0	1	Kallikrein B, plasma (Fletcher factor) 1
UniRef100_Q5E9B7	0	1	Chloride intracellular channel protein 1
UniRef100_Q3MHN0	0	1	Proteasome subunit beta type 6 precursor
UniRef100_O02808	0	1	Von Willebrand factor
UniRef100_UPI00005C164A	0	1	similar to ADAM metallopeptidase
UniRef100_UPI00005C052B	0	1	similar to Afamin precursor
UniRef100_UPI00005BB7FD	0	1	similar to Carboxypeptidase N subunit 2 precursor
UniRef100_Q3T149	0	1	Heat-shock protein beta-1
UniRef100_UPI0000562815	0	1	heat shock 27kDa protein 1

Spectral counts are representative of two independent LC-MS/MS runs corresponding to two aliquots from the same preparation run in separate lanes of SDS-PAGE gels.

**Table 2**  
**Relative Fold Change Ratios of proteins in Exosome Enriched Medium (EEM) versus Exosome Free Medium (EFM)**

Accession Number	Spectral Counts		Ratio	Description
	EFM	EEM		
UniRef100_UPI00005BC629	5	95	19	similar to Alpha-2-macroglobulin precursor
UniRef100_UPI00005BC5E5	1	15	15	similar to pregnancy-zone protein
UniRef100_P07589	4	33	8.3	Fibronectin
UniRef100_UPI00005BB909	1	8	8	similar to Ceruloplasmin precursor (Ferroxidase)
UniRef100_UPI00006830EC	5	35	7	ANTITHROMBIN III
UniRef100_Q3SZV7	1	7	7	Similar to hemopexin
UniRef100_P00735	5	32	6.4	Prothrombin precursor (Coagulation factor II)
UniRef100_UPI00005B628A	6	37	6.2	hypothetical protein LOC540261
UniRef100_P97280	2	12	6	Inter-alpha-trypsin inhib. heavy chain H3 prec
UniRef100_Q2PQT6	1	6	6	Vinculin
UniRef100_UPI0000110A3C	5	27	5.4	BETA-ACTIN
UniRef100_Q28194	3	16	5.3	Thrombospondin-1
UniRef100_Q3ZBS7	3	15	5	Vitronectin
UniRef100_UPI00005BC623	185	861	4.7	Similar:Alpha-2-macroglobulin precur. (A2-M)
UniRef100_UPI0000693DE0	66	298	4.5	complement component 3
UniRef100_Q2UVX4	74	325	4.4	Complement C3 precursor
UniRef100_UPI00005C2942	2	8	4	Similar:Ig lambda chain V-I region BL2 precur.
UniRef100_Q32T06	1	4	4	Endopin 2C
UniRef100_Q0VCX1	1	4	4	Complement component 1, s subcomponent
UniRef100_UPI00005C201D	1	4	4	Similar:C4b-binding prot alpha chain precur
UniRef100_UPI00005C27D5	1	4	4	Similar:gamma-2a immunoglobulin heavy chain
UniRef100_Q06805	1	4	4	Tyrosine-protein kinase receptor Tie-1 precursor
UniRef100_Q95121	4	14	3.5	Pigment epithelium-derived factor precursor
UniRef100_Q03247	14	47	3.4	Apolipoprotein E precursor
UniRef100_UPI00005BC731	3	10	3.3	similar to Fibulin-1 precursor isoform 1
UniRef100_P02584	3	10	3.3	Profilin-1
UniRef100_P17697	7	23	3.3	Clusterin precursor (Glycoprotein III)
UniRef100_P01966	22	72	3.3	Hemoglobin subunit alpha
UniRef100_Q28178	9	27	3	Thrombospondin-1 precursor
UniRef100_UPI00005BE9E4	2	6	3	similar to Heparin cofactor II precursor (HC-II)
UniRef100_Q9N2I2	2	6	3	Plasma serine protease inhibitor precursor
UniRef100_P17690	1	3	3	Beta-2-glycoprotein 1 precursor
UniRef100_P02081	50	138	2.8	Hemoglobin fetal subunit beta
UniRef100_Q5E9B1	3	8	2.7	L-lactate dehydrogenase B chain
UniRef100_P02662	3	8	2.7	Alpha-S1-casein cursor
UniRef100_Q29RQ1	8	20	2.5	Complement component C7 precursor
UniRef100_UPI00005C18A3	2	5	2.5	similar to Complement factor H precursor
UniRef100_UPI00005C1B6C	2	5	2.5	Perlecan
UniRef100_Q05B55	5	12	2.4	Hypothetical protein



Accession Number	Spectral Counts			Description
	EFM	EEM	Ratio	
UniRef100_UPI00005BFBFC	15	34	2.3	similar to Complement C4 precursor
UniRef100_UPI00005C211E	13	28	2.2	similar to Ig gamma-1 chain C region
UniRef100_Q0VCM5	15	31	2.1	Similar:Inter-alpha-trypsin inhib. heavy chain H1
UniRef100_P02070	31	62	2	Hemoglobin subunit beta
UniRef100_P22226	5	10	2	Cyclic dodecapeptide precursor
UniRef100_Q3SWW8	3	6	2	Similar to thrombospondin 4
UniRef100_Q2HJF0	3	6	2	Similar to Serotransferrin
rUniRef100_UPI00005C24B2	2	4	2	similar to Laminin alpha-3 chain precursor
rUniRef100_UPI00005C16C9	1	2	2	similar to CG9164-PA, isoform A
UniRef100_UPI00005BDE5A	1	2	2	similar to Neuropilin-1 precursor
UniRef100_UPI00005BD502	1	2	2	Similar:Cofactor for Sp1 trans activation sub 3
UniRef100_Q5E956	1	2	2	Triosephosphate isomerase
UniRef100_UPI00005BFBFF	30	54	1.8	similar to Complement C4 precursor, partial
UniRef100_Q3SZH5	10	18	1.8	Similar to Angiotensinogen
UniRef100_P06868	32	57	1.8	Plasminogen precursor (EC 3.4.21.7)
UniRef100_Q2KIF5	7	12	1.7	V11a protein
UniRef100_P02672	15	25	1.7	Fibrinogen alpha chain precursor
UniRef100_P01030	29	44	1.5	Complement C4 precursor
UniRef100_Q9BG13	2	3	1.5	Peroxiredoxin-2
UniRef100_Q5EA67	57	84	1.5	Inter-alpha (Globulin) inhibitor H4
UniRef100_Q28085	22	32	1.5	Complement factor H precursor
UniRef100_Q3T052	58	84	1.5	Inter-alpha (Globulin) inhibitor H4
UniRef100_Q3SX14	24	33	1.4	Similar to Gelsolin
UniRef100_Q0IHK2	289	390	1.4	Transferrin
UniRef100_Q9GLY6	18	24	1.3	Inter-alpha-trypsin inhibitor heavy chain2
UniRef100_Q3SZ57	25	33	1.3	Alpha-fetoprotein precursor
UniRef100_Q2KIU6	44	57	1.3	Similar to Complement factor B
UniRef100_P28800	14	18	1.3	Alpha-2-antiplasmin precursor
UniRef100_Q1RMN8	7	9	1.3	Similar to Ig lambda-like polypep. 1
UniRef100_P00978	27	34	1.3	AMBp protein precursor
UniRef100_Q3SZZ4	26	32	1.2	AMBp protein
UniRef100_UPI00005BDE33	82	99	1.2	similar to inter-alpha globulin inhib. H2 polypep
UniRef100_Q2KIS7	5	6	1.2	Tetranectin precursor
UniRef100_P02453	5	6	1.2	Collagen alpha-1(I) chain precursor
UniRef100_Q3UZM0	23	27	1.2	8 days embryo whole body cDNA, RIKEN
UniRef100_P15497	30	35	1.2	Apolipoprotein A-I precursor
UniRef100_UPI00001116D7	13	15	1.2	TRYPSIN
UniRef100_Q0V8M9	62	69	1.1	Inter-alpha (Globulin) inhibitor H3
UniRef100_Q46375	30	32	1.1	Transthyretin precursor
UniRef100_UPI00000718F2	39	41	1.1	keratin 10
UniRef100_UPI00006830E5	17	17	1	TRYPSIN

Accession Number	Spectral Counts			Description
	EFM	EEM	Ratio	
UniRef100_Q58CQ9	4	4	1	Pantetheinase precursor
UniRef100_Q6LBN7	3	3	1	Lactoferrin
r-UniRef100_Q2KI19	2	2	1	Similar to CG10053-PA
UniRef100_Q2KIW5	1	1	1	Centromere protein U
UniRef100_UPI00005C2368	1	1	1	similar to HGF activator preproprotein
UniRef100_UPI00005C11DB	1	1	1	similar to Spectrin beta chain
UniRef100_UPI000016C268	1404	1389	1	Albumin
UniRef100_UPI00001AE6F7	55	51	0.9	keratin 9
UniRef100_Q3MHN5	53	45	0.9	Vitamin D-binding protein precursor
UniRef100_Q2KJF1	18	15	0.8	Alpha-1-B glycoprotein
UniRef100_UPI0000E2470B	65	54	0.8	Cytokeratin-14
UniRef100_Q58D62	47	39	0.8	Fetuin-B precursor
UniRef100_P34955	158	130	0.8	Alpha-1-antiproteinase precursor
UniRef100_P18902	13	10	0.8	Plasma retinol-binding protein
UniRef100_UPI00001A389F	13	10	0.8	Plasma retinol-binding protein
UniRef100_Q2KIT0	4	3	0.8	Similar to collagen, type X, alpha 1
UniRef100_UPI0000D9CC8C	48	35	0.7	similar to keratin 1 isoform 7
UniRef100_UPI0000167B80	62	45	0.7	keratin 1
UniRef100_UPI0000110894	7	5	0.7	BETA-LACTOGLOBULIN
UniRef100_Q3ZEJ6	3	2	0.7	Serpina 3-3
UniRef100_Q4VAQ2	43	27	0.6	Keratin 2
UniRef100_UPI00005A1BEF	8	5	0.6	similar to keratin 25D
UniRef100_P12763	400	235	0.6	Alpha-2-HS-glycoprotein precursor
UniRef100_Q3SZR3	12	7	0.6	Alpha-1 acid glycoprotein
UniRef100_UPI0000D9CC7B	15	8	0.5	keratin 6A
UniRef100_P01044-2	22	11	0.5	Isoform LMW of P01044
UniRef100_UPI0000167F3F	22	11	0.5	kininogen 1
UniRef100_Q9TTJ5	4	2	0.5	Regucalcin
UniRef100_P02465	4	2	0.5	Collagen alpha-2(I) chain precursor
UniRef100_UPI00006A12E2	4	2	0.5	keratin 24
rUniRef100_UPI00005C1B5E	2	1	0.5	similar to dynein, cytoplasmic, heavy polypep1
UniRef100_Q08E14	2	1	0.5	Similar to Collagen alpha 1(III) chain
UniRef100_P01035	2	1	0.5	Cystatin C precursor
UniRef100_UPI00006D113E	7	3	0.4	keratin 5 isoform 20
UniRef100_Q2KJ62	23	9	0.4	KNG protein
UniRef100_UPI00005C202E	13	5	0.4	similar to Apolipoprotein B-100 precursor
UniRef100_P08779	9	3	0.3	Keratin, type I cytoskeletal 16
UniRef100_UPI00005BDB2A	6	2	0.3	similar to Apolipoprotein B-100 precursor
UniRef100_Q2KIQ9	6	2	0.3	SERPINA3 protein
UniRef100_Q5DPW9	3	0	0	Cystatin E/M
UniRef100_P35445	2	0	0	Cartilage oligomeric matrix protein

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Accession Number	Spectral Counts		Ratio	Description
	EFM	EEM		
UniRef100_P42916	2	0	0	Collectin-43 precursor
UniRef100_UPI00005C2037	2	0	0	similar to Collagen alpha 1(V) chain precursor

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Fold change ratio is calculated by the total number of spectral counts of a peptide/protein in the EEM divided by spectral count of EFM. Spectral counts are representative of two independent LC-MS/MS runs corresponding to two aliquots from the same preparation run in separate lanes of SDS-PAGE gels.