Author/s:
Gangoda, L;Liem, M;Ang, CS;Keerthikumar, S;Adda, CG;Parker, BS;Mathivanan, S

Title:
Proteomic Profiling of Exosomes Secreted by Breast Cancer Cells with Varying Metastatic Potential

Date:
2017-12-01

Citation:
Gangoda, L., Liem, M., Ang, C. S., Keerthikumar, S., Adda, C. G., Parker, B. S. & Mathivanan, S. (2017). Proteomic Profiling of Exosomes Secreted by Breast Cancer Cells with Varying Metastatic Potential. Proteomics, 17 (23-24), https://doi.org/10.1002/pmic.201600370.

Persistent Link:
https://hdl.handle.net/11343/293971

**Dataset Brief**

# Proteomic profiling of exosomes secreted by breast cancer cells with varying metastatic potential

Lahiru Gangoda[1], Michael Liem[1], Ching-Seng Ang[2], Shivakumar Keerthikumar[1], Christopher G. Adda[1], Belinda S. Parker[1] and Suresh Mathivanan[1,*]

[1]Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Victoria 3086, Australia

[2]Bio21 Institute, University of Melbourne, Victoria 3010, Australia

**To whom correspondence should be addressed:**

Dr. Suresh Mathivanan

Department of Biochemistry and Genetics,

La Trobe Institute for Molecular Science,

La Trobe University,

Bundoora, Victoria 3086, Australia

Tel: +61 03 9479 2565

Fax: +61 03 9479 1226

Email: S.Mathivanan@latrobe.edu.au

**Abbreviations:**

EVs : Extracellular vesicles

Rsc : Relative spectral count

TEM : Transmission electron microscopy

NTA : Nanoparticle tracking analysis

ECM : Extra cellular matrix

LC-MS/MS : Liquid chromatography–mass spectrometry

FCS : Fetal calf serum

SDS : Sodium dodecyl sulphate

PAGE : Polyacrylamide gel electrophoresis



**Keyword:** extracellular vesicles, breast cancer, metastasis, exosomes, proteomic cargo

**Total number of words:** 2,793

**Abstract**

Cancer cells actively release extracellular vesicles, including exosomes, into the surrounding microenvironment. Exosomes play pleiotropic roles in cancer progression and metastasis, including invasion, angiogenesis, and immune modulation. However, the proteome profile of exosomes isolated from cells with different metastatic potential and the role of these exosomes in driving metastasis remains unclear. Here, we conducted a comparative proteomic analysis of exosomes isolated from several genetically related mouse breast tumour lines with different metastatic propensity. The amount of exosomes produced and the extent of cancer-associated protein cargo varied significantly between non-metastatic and metastatic cell-derived exosomes. Metastatic cell-derived exosomes contain proteins that promote migration, proliferation, invasion and angiogenesis while the non-metastatic cell-derived exosomes contain proteins involved in cell-cell/cell-matrix adhesion and polarity maintenance. The metastatic exosomes contain a distinct set of membrane proteins including Ceruloplasmin and Metadherin which could presumably aid in targeting the primary cancer cells to specific metastatic sites. Hence, it can be concluded that the exosomes contain different protein cargo based on the host cells metastatic properties and can facilitate in the dissemination of the primary tumours to distant sites.

Breast cancer is the most common cancer in women worldwide with nearly 1.7 million new cases diagnosed in 2012 [1]. Breast cancer alone accounts to about 15% of all cancer deaths in women [1]. While some therapies are highly effective in removing primary tumours and preventing disease recurrence; advanced metastatic disease in distant organs is often untreatable and is the primary cause of mortality [2]. Breast cancer has a propensity to

metastasize to various organs including the lymph nodes, bone, lung, liver and brain [3]. However, factors that influence metastasis to certain organs are largely unknown. Recently, integrin subtypes in exosomes have been shown to regulate the distant site of metastasis [4, 5]. Exosomes are extracellular vesicles that are secreted by multiple cell types into the extracellular space [6-9]. The importance of exosomes in tumour progression and metastasis has been made quite evident in recent times [4, 10, 11]. Knockdown or overexpression of the integrin subtypes in the cells and thereby the secreted exosomes either attenuated metastasis or increased organ-specific metastasis, respectively [4]. However, how exosomes regulate these processes is largely unclear. Here, exosomes were isolated from a panel of Balb/c mammary tumour lines with different metastatic potential [12]. A follow up quantitative proteomic analysis was performed on the exosomes isolated from the tumour cells. The generated proteomic data was analysed to identify potential regulators of breast cancer metastasis.

Exosomes were isolated from the supernatants of breast cancer cells cultured in exosome depleted medium. The tumor lines used in the study were non-metastatic (67NR), weakly metastatic to lung (66cl4), or highly metastatic to lymph node, lung, and rarely bone (4T1). 4T1.2 a rare variant of 4T1 with preferential metastasis to the bone was also used in the study [12, 13]. To confirm the isolation of exosomes, Western blot analysis of exosomal enriched proteins TSG101 and Alix [14] was performed. As shown in Figure 1A, Alix and TSG101 were enriched in exosomes isolated from 4T1 and 4T1.2 compared to exosomes isolated from 67NR and 66cl4. However, the exosome marker CD9 was enriched in all exosomes compared to breast cancer cell lysates. In order to confirm the presence of exosomes (small EVs) by biophysical methods, the pellets obtained from differential centrifugation followed by ultracentrifugation were subjected to transmission electron microscopy (TEM). A homogenous population of membranous vesicles within the range of 30–150 nm in diameter, characteristic of exosomes [15], was present in the pellets obtained from all four breast cancer cells. TEM analysis revealed no significant differences in morphology/size of exosomes secreted by metastatic and non-metastatic cells (Figure 1B). Furthermore, nanoparticle tracking analysis (NTA) showed that the average peak for all the vesicles were in the range of 101-116 nm (Figure 1C). However, the number of particles secreted by the metastatic cells (4T1 and 4T1.2) were significantly more than that of non-metastatic (67NR) and weakly metastatic (66cl4) cells. The observation with the Western blotting (Figure 1A)

and the NTA (Figure 1C) is consistent with previous articles suggesting that metastatic cells secrete more exosomes compared to non-metastatic cells [11, 16, 17]. The amount of proteins per particles were significantly less in 66cl4 cell-derived exosomes compared to the other three cell line-derived exosomes (Figure 1D).

Next, the isolated exosomes were subjected to in-gel quantitative proteomics and analysed using FunRich tool [18, 19]. A total of 1950, 1399, 1496 and 1706 proteins were identified in exosomes isolated from 67NR, 66cl4, 4T1 and 4T1.2, respectively (Figure 2A) (Supplementary Table 1). Among these, 991 proteins were commonly detected in all exosomal proteomes. Spectral counting based label-free quantitation was performed to identify the relative abundance of proteins in exosomes. Exosomal protein abundance profile of metastatic cells 4T1 and 4T1.2 were similar to the non-metastatic cells based on hierarchical clustering (Figure 2B). Next, we focussed on the proteomic difference between the non-metastatic 67NR and highly metastatic (4T1 and 4T1.2) exosomes. Based on comparison between the non-metastatic and highly metastatic cell line-derived exosomes, 363 proteins were enriched in exosomes isolated from 4T1 compared to 67NR exosomes (Figure 2C). Out of these 363, 168 proteins are unique to 4T1 cell-derived exosomes while the remaining 195 is shared between the exosomes but more abundant in 4T1. Similarly, 358 proteins were enriched in exosomes isolated from 4T1.2 compared to 67NR exosomes (Figure 2D). Out of these 358, 211 proteins are unique to 4T1.2 cell-derived exosomes while the remaining 147 is shared between both types of exosomes but more abundant in 4T1.2.

Recently, integrins on exosomes are shown to regulate organ-specific metastasis [4]. Consistent with this finding, the exosomes from metastatic cells contained multiple integrins (Figure 2E). Next, to identify proteins that are differentially abundant in non-metastatic and metastatic exosomes, proteome profiles of 67NR and 4T1 were compared. 4T1 cell-derived exosomes were enriched with proteins implicated in cell migration (Cdc42, Krt14 and Rhoa) while 67NR cell-derived exosomes were enriched with proteins implicated in cell adhesion (Figure 2F). Proteins implicated in cell adhesion including Ctnna1 (47-fold), Lama5 (12-fold), Ctnna2 (10-fold), Fat1 (7.5-fold), Pcdh1 (6.5-fold), Lamc1 (5-fold), Cdh1 (4.4-fold), Ctnnd1 (3.7-fold) and Dsp (2.3-fold) were enriched in 67NR non-metastatic cell-derived exosomes. Western blotting analysis confirmed an increase in Ctnnd1 and Laminin, alpha 5 (Lama5) in 67NR cell-derived exosomes compared to those derived from 4T1 cells (Figure

2G). On the contrary, proteins implicated in migration such as Cdc42, Keratin 14 and Itgb1 were enriched in metastatic (4T1) cell-derived exosome (Figure 2G). While Ctnnd1, Keratin 14 and Lama5 were of similar abundance in cells and exosomes, Itgb1 and Cdc42 were specifically enriched in exosomes compared to the host cells.

Metabolic changes in primary tumours have a significant impact on tumour progression and on the development of the metastatic phenotype. Most cancer cells are programmed to increase glucose uptake, but to reduce the proportion of glucose oxidized in the Krebs cycle (Warburg effect). When comparing exosomal proteomic profiles of highly metastatic 4T1 and non-metastatic 67NR, it was evident that there were changes in enzymes involved in metabolic pathways (Supplementary Table 1). Lactate dehydrogenase A (Ldha) levels were 3-fold higher in 4T1 exosomes compared to 67NR exosomes. Many enzymes implicated in acetyl-Coenzyme A metabolism were also differentially abundant in metastatic and non-metastatic exosomes. Compared to 4T1, the enzymes Acacb (5.1-fold), Acat2 (3.7-fold), Aacs (3.7-fold), Acat3 (3-fold), Acat1 (2.3-fold) and Acaca (17.8-fold) were enriched in 67NR exosomes.

It has been previously established that exosomes shed by highly metastatic cancer cells can be taken up by distant cells to promote pre-metastatic niche formation [11]. To facilitate this process, exosomes may carry cancer-associated protein cargo [15]. When comparing the proteomic profiles of highly metastatic 4T1 and non-metastatic 67NR exosomes, it was evident that this was the case and indeed the 4T1 exosomes were more enriched with cancer-associated protein cargo (Supplementary Table 1). Both the fibroblast growth factor receptors, Fgfr2 and 3 were 14.7-fold enriched in 4T1 exosomes as compared to 67NR exosomes. 4T1 exosomes were also enriched in other growth factor receptors and accessory proteins such as Tgfbr1 (4.7-fold), Igf1r (2.2-fold), Fibp (2.9-fold), Frs2 (3.8-fold) and Il1rap (9.2-fold). In addition, angiogenic factor metadherin was also 8.4-fold enriched in 4T1 exosomes. Metadherin plays a very important role in anchorage independent growth of cancer cells and is known to promote progression and development of various cancers including breast cancer [20]. Ddah1, known to promote angiogenesis in lung cancer [21], was 9.2 fold enriched in 4T1 breast cancer exosomes. The 4T1 exosomes were enriched 9.2-fold for urokinase plasminogen activator (Plau) which could promote ECM degradation and cancer invasiveness [22]. These results indicate that tumour promoting mitogenic and

angiogenic factors were enriched in exosomes secreted by the metastatic 4T1 cells compared to non-metastatic 67NR cell-derived exosomes.

To understand whether metastatic cell-derived exosomes can stimulate migration in cancer cells, a wound healing assay was performed on 67NR and 4T1 cells. The cells were treated with exosomes and wound recovery area was measured and plotted (Figure 3A). Incubation of 4T1 cells with 4T1 exosomes induced significant wound closure compared to that of the untreated cells (Figure 3A). These data suggest that exosomes secreted by metastatic cells are enriched with cancer-associated protein cargo and can influence the migration of the cancer cells.

As exosomes have been implicated in organotropism [4], the membrane proteins present in metastatic exosomes might provide clues on critical regulators of organ-specific metastasis. Hence, proteins with transmembrane (TM) domains were predicted by TMHMM tool. Proteins with a TM domain that are at least 2-fold enriched in 4T1 exosomes compared to 67NR exosomes are plotted in Figure 3B. For this analysis, the membrane proteins of non-metastatic 67NR cell-derived exosomes were compared against the highly metastatic (4T1 and 4T1.2) cell-derived exosomes to identify critical regulators of metastasis. Ceruloplasmin (Cp), considered as a biomarker of breast cancer [23], was 31-fold and 19-fold enriched in exosomes isolated from 4T1 and 4T1.2 cells. Similarly, multiple membrane proteins including Cd38, Slco2a1, Acsl4, Mtdh, Fgfr and Tgfbr were enriched in exosomes secreted by metastatic cells. These data suggest that exosomes secreted by metastatic cells are enriched with membrane proteins that may aid in targeting the primary tumor cells to metastatic sites.

In summary, we used 67NR, 66cl4, 4T1 and 4T1.2 mammary epithelial carcinoma cells to characterize their exosome proteomic profile. Label-free quantitative proteomic analysis of 67NR and 4T1 cell-derived exosomes revealed that 4T1 exosomes are enriched in proteins with functions related to migration and invasion whereas 67NR exosomes were enriched in proteins involved in maintaining cell polarity and adhesion. Furthermore, exosomes secreted by metastatic cells were enriched with many membrane proteins. It is unclear whether any of these proteins may have a role in organ-specific metastasis. Follow up studies are needed to

understand the role of these membrane proteins in organotropism. These membrane proteins can be exploited as potential biomarkers for breast cancer and as targets to block metastasis.

**Acknowledgement**

Suresh Mathivanan is supported by Australian Research Council DECRA (DE150101777). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Conflict of interest**

The authors have declared no conflict of interest

**References**

[1] Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., Jemal, A., Global cancer statistics, 2012. *CA: a cancer journal for clinicians* 2015, *65*, 87-108.

[2] Sharma, G. N., Dave, R., Sanadya, J., Sharma, P., Sharma, K. K., Various types and management of breast cancer: an overview. *Journal of advanced pharmaceutical technology & research* 2010, *1*, 109-126.

[3] Lu, X., Kang, Y., Organotropism of breast cancer metastasis. *Journal of mammary gland biology and neoplasia* 2007, *12*, 153-162.

[4] Hoshino, A., Costa-Silva, B., Shen, T.-L., Rodrigues, G., Hashimoto, A., Mark, M. T., Molina, H., Kohsaka, S., Di Giannatale, A., Ceder, S., Tumour exosome integrins determine organotropic metastasis. *Nature* 2015, *527*, 329-335.

[5] Rak, J., CANCER Organ-seeking vesicles. *Nature* 2015, *527*, 312-314.

[6] Gangoda, L., Mathivanan, S., Cortactin enhances exosome secretion without altering cargo. *The Journal of cell biology* 2016, *214*, 129-131.

[7] Théry, C., Exosomes: secreted vesicles and intercellular communications. *F1000 Biol Rep* 2011, *3*, 130.

[8] Kalra, H., Drummen, G. P., Mathivanan, S., Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. *International journal of molecular sciences* 2016, *17*, 170.

[9] van Niel, G., Porto-Carreiro, I., Simoes, S., Raposo, G., Exosomes: a common pathway for a specialized function. *J. Biochem. (Tokyo)* 2006, *140*, 13-21.

[10] Gangoda, L., Boukouris, S., Liem, M., Kalra, H., Mathivanan, S., Extracellular vesicles including exosomes are mediators of signal transduction: are they protective or pathogenic? *Proteomics* 2015, *15*, 260-271.

[11] Peinado, H., Aleckovic, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., Garcia-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B. A., Callahan, M. K., Yuan, J., Martins, V. R., Skog, J., Kaplan, R. N., Brady, M. S., Wolchok, J. D., Chapman, P. B., Kang, Y., Bromberg, J., Lyden, D., Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* 2012, *18*, 883-891.

[12] Eckhardt, B. L., Parker, B. S., van Laar, R. K., Restall, C. M., Natoli, A. L., Tavaria, M. D., Stanley, K. L., Sloan, E. K., Moseley, J. M., Anderson, R. L., Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix. *Molecular cancer research : MCR* 2005, *3*, 1-13.

[13] Lelekakis, M., Moseley, J. M., Martin, T. J., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F. R., Slavin, J., Anderson, R. L., A novel orthotopic model of breast cancer metastasis to bone. *Clinical & experimental metastasis* 1999, *17*, 163-170.

[14] Lotvall, J., Hill, A. F., Hochberg, F., Buzas, E. I., Di Vizio, D., Gardiner, C., Gho, Y. S., Kurochkin, I. V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M. H., Witwer, K. W., Thery, C., Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles* 2014, *3*, 26913.

[15] Keerthikumar, S., Gangoda, L., Liem, M., Fonseka, P., Atukorala, I., Ozcitti, C., Mechler, A., Adda, C. G., Ang, C. S., Mathivanan, S., Proteogenomic analysis reveals exosomes are more oncogenic than ectosomes. *Oncotarget* 2015, *6*, 15375-15396.

[16] Ginestra, A., La Placa, M. D., Saladino, F., Cassara, D., Nagase, H., Vittorelli, M. L., The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer research* 1998, *18*, 3433-3437.

[17] Vardaki, I., Ceder, S., Rutishauser, D., Baltatzis, G., Foukakis, T., Panaretakis, T., Periostin is identified as a putative metastatic marker in breast cancer-derived exosomes. *Oncotarget* 2016, *7*, 74966-74978.

[18] Pathan, M., Keerthikumar, S., Ang, C. S., Gangoda, L., Quek, C. Y., Williamson, N. A., Mouradov, D., Sieber, O. M., Simpson, R. J., Salim, A., FunRich: An open access standalone

functional enrichment and interaction network analysis tool. *Proteomics* 2015, *15*, 2597-2601.

[19] Pathan, M., Keerthikumar, S., Chisanga, D., Alessandro, R., Ang, C. S., Askenase, P., Batagov, A. O., Benito-Martin, A., Camussi, G., Clayton, A., Collino, F., Di Vizio, D., Falcon-Perez, J. M., Fonseca, P., Fonseka, P., Fontana, S., Gho, Y. S., Hendrix, A., Nolte-'t Hoen, E., Iraci, N., Kastaniegaard, K., Kislinger, T., Kowal, J., Kurochkin, I. V., Leonardi, T., Liang, Y., Llorente, A., Lunavat, T. A., Maji, S., Monteleone, F., Øverbye, A., Panaretakis, T., Patel, T., Peinado, H., Pluchino, S., Principe, S., Ronquist, G., Royo, F., Sahoo, S., Spinelli, C., Stensballe, A., Théry, C., van Herwijnen, M. J. C., Wauben, M., Welton, J. W., Zhao, K., Mathivanan, S., A novel community driven software for functional enrichment analysis of extracellular vesicles data. *J Extracell Vesicles* 2017, *6*, 1321455.

[20] Liang, Y. J., Hu, J., Li, J. T., Liu, Y. J., Yu, J. Y., Zhuang, X. Q., Mu, L. L., Kong, X. Y., Hong, D. L., Yang, Q. F., Hu, G. H., Epigenetic Activation of TWIST1 by MTDH Promotes Cancer Stem-like Cell Traits in Breast Cancer. *Cancer Res.* 2015, *75*, 3672-3680.

[21] Shiozawa, T., Iyama, S., Toshima, S., Sakata, A., Usui, S., Minami, Y., Sato, Y., Hizawa, N., Noguchi, M., Dimethylarginine dimethylaminohydrolase 2 promotes tumor angiogenesis in lung adenocarcinoma. *Virchows Archiv : an international journal of pathology* 2016, *468*, 179-190.

[22] Dolo, V., D'Ascenzo, S., Violini, S., Pompucci, L., Festuccia, C., Ginestra, A., Vittorelli, M. L., Canevari, S., Pavan, A., Matrix-degrading proteinases are shed in membrane vesicles by ovarian cancer cells in vivo and in vitro. *Clinical & experimental metastasis* 1999, *17*, 131-140.

[23] Vaidya, S. M., Kamalakar, P. L., Copper and ceruloplasmin levels in serum of women with breast cancer. *Indian J Med Sci* 1998, *52*, 184-187.

**Figure legends**

**Figure 1**

**Isolation and characterization of exosomes**

(**A**) Western blot analysis (20 μg) of whole cell lysates and pellets obtained by differential centrifugation followed by ultracentrifugation from 67NR, 66cl4, 4T1 and 4T1.2 cells showed the presence of exosomal enriched proteins Alix, Tsg101 and CD9. (**B**) Transmission

electron microscope images of exosomes isolated from breast cancer cells showed vesicles in the range of 30-150 nm diameter consistent with exosomes. (**C**) Nanoparticle tracking analysis of vesicles obtained by differential and ultracentrifugation shows the presence of vesicles in the range of 30-150 nm. Equal number of cells were used in the analysis to quantify the number of particles present in the exosomal pellet isolated from metastatic and non-metastatic cells. (D) Bar graph showing protein amounts per $10^9$ particles. Error bars represent standard error of the mean, n=3, ** denotes significance ($p<0.05$).

# Figure 1

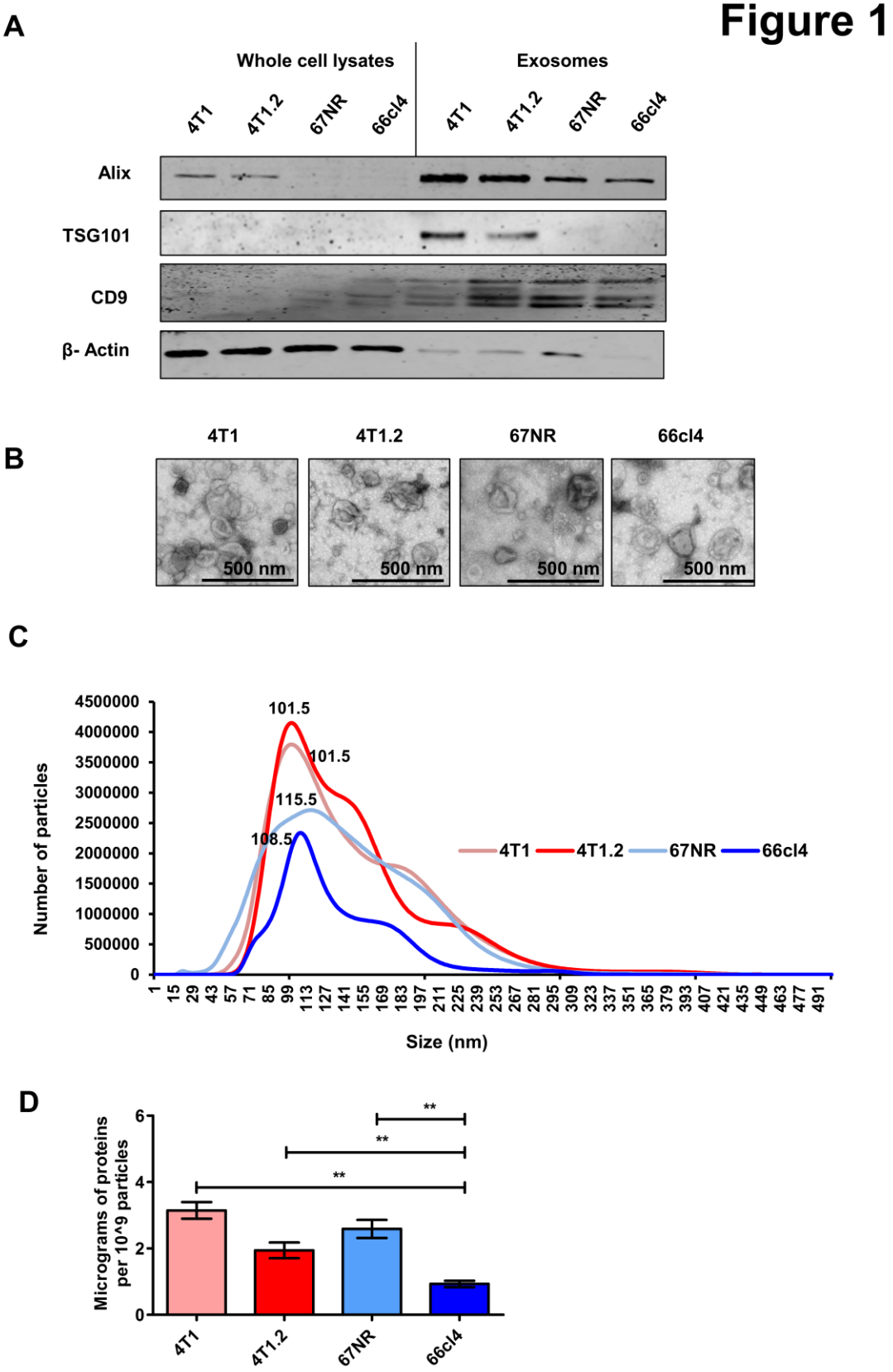


**Figure 2**

**Proteomic profile of exosomes isolated from breast cancer cells with different metastatic potential**

**(A)** Four-way Venn diagram of proteins identified in exosomes isolated from breast cancer cells. A total of 991 proteins were identified in common between all the exosomes irrespective of the metastatic potential. **(B)** Heatmap of proteins that are differentially abundant in exosomes secreted by the breast cancer cells. Exosome proteome profiles of the most highly metastatic 4T1 and 4T1.2 cells clustered together. (**C**) Venn diagram of differentially abundant proteins identified in 67NR and 4T1 exosomes showed 363 proteins to be enriched in 4T1 exosomes. (**D**) Venn diagram of differentially abundant proteins identified in 67NR and 4T1.2 exosomes showed 358 proteins to be enriched in 4T1.2 exosomes. (**E**) Heatmap depicting the integrin profile of the exosomes. (**F**) Heatmaps showing 67NR exosome are enriched for proteins involved in cell adhesion and 4T1 exosomes are enriched for proteins associated with migration. (**G**) Western blot validation of proteins found to be differentially enriched in 4T1 and 67NR exosomes.

# Figure 2

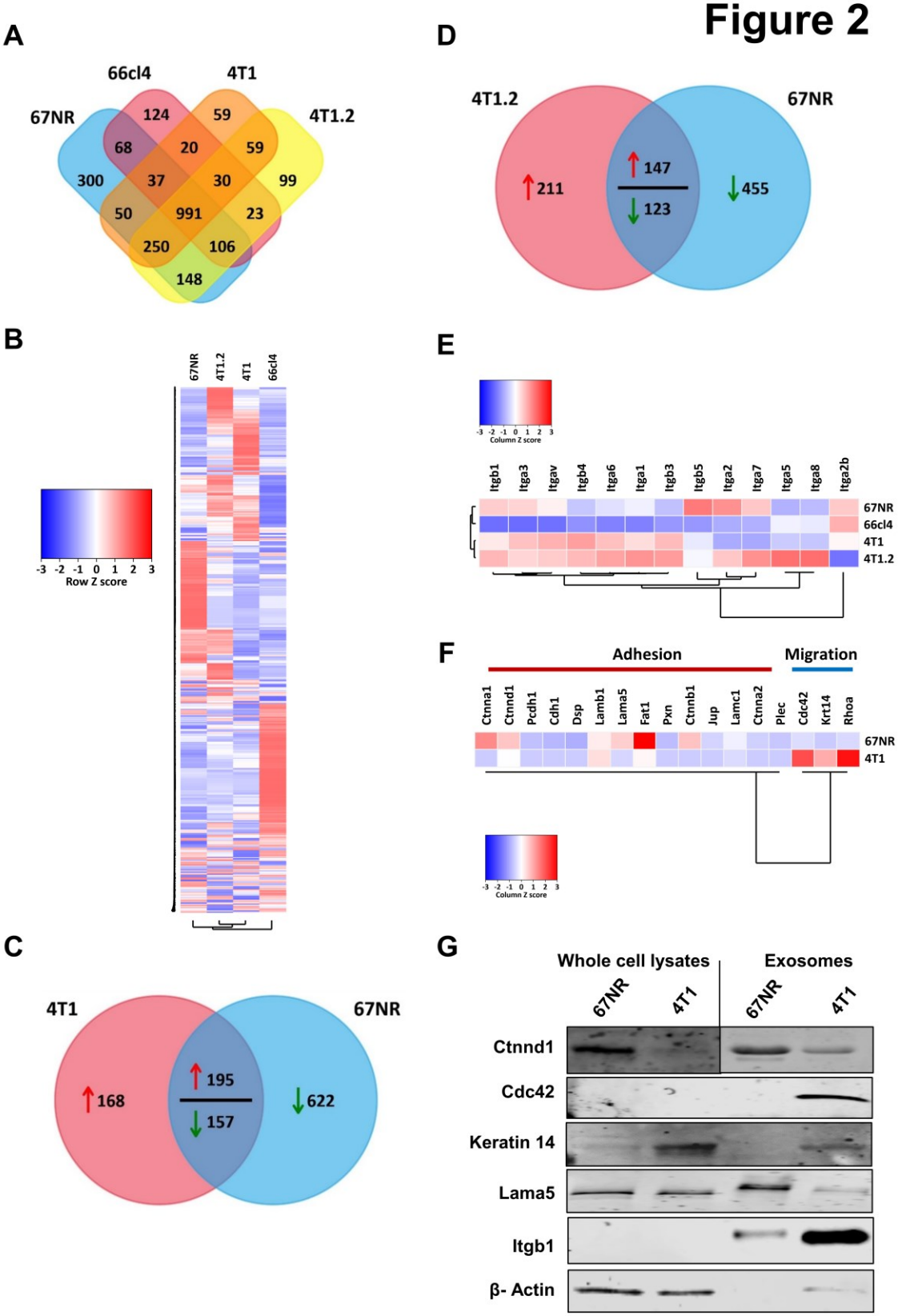

**Figure 3**

**Migratory potential and membrane proteome of exosomes**

(**A**) Wound healing assay of breast cancer cell lines are displayed. Wounds were created post reaching 100% confluence and cells were treated with either 67NR or 4T1 exosomes for 24 h. Migration was assessed at 24 h after wounding. Images were taken under the 10x objective of the light microscope. Quantification of wound closure showed that 4T1 exosomes induced more migration compared to 67NR exosomes. Error bars represent standard error of the mean, n=3, ** denotes significance ($p<0.05$). Student's t-test was used to evaluate statistically significant differences between the values. (**B**) Fold abundance of membrane proteins enriched in exosomes isolated from metastatic cells.

# Figure 3