Membrane Lipids Define Small Extracellular Vesicle Subtypes Secreted By Mesenchymal Stromal Cell

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Abstract

Therapeutic efficacy of the mesenchymal stromal cell (MSC), a multipotent progenitor cell, is attributed to small (50 to 200 nm) extracellular vesicles (EVs). The presence of a lipid membrane differentiates exosomes and EVs from other macromolecules. Analysis of this lipid membrane revealed three distinct small MSC EV subtypes each with a differential affinity for cholera toxin B chain (CTB), annexin V (AV), and shiga toxin B chain (ST) that binds GM1 ganglioside, phosphatidylserine, and globotriaosylceramide, respectively. Similar EV subtypes are also found in biologic fluids and are independent sources of disease biomarkers. Here, we compared and contrasted these three EV subtypes. All subtypes carry β-actin, but only CTB-binding EVs (CTB-EVs) are true exosomes, enriched with exosome proteins and derived from endosomes. No unique protein has been identified yet in AV-binding EVs (AV-EVs); ST-binding EVs (ST-EVs) carry RNA and a high level of extra domain A (EDA)–containing fibronectin. Based on the CTB, AV, and ST subcellular binding sites, the origins of CTB-, AV- and ST-EV biogenesis are the plasma membrane, cytoplasm, and nucleus, respectively. The differentiation of EV subtypes through membrane lipids underlies the importance of membrane lipids in defining EVs and implies an influence on EV biology and functions.

Keywords: cholesterol/trafficking, endocytosis, lipids, membranes, secretion, exosome, cholera toxin B chain, annexin V, shiga toxin B chain

Introduction

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells that are the most widely used candidate cell in regenerative stem cell with nearly 500 MSC clinical trials (http://www.clinicaltrials.gov/; accessed May 2015). The therapeutic efficacy of MSCs has been increasingly attributed to a paracrine secretion that ameliorates cellular injury and enhances repair (1). Among the active agents proposed to date were two EV types, namely 50-1000 nm microvesicles (2) by Giovanni Camussi 's group and 50-100 nm exosomes (3) by our group.

MSC exosomes are small lipid membrane vesicles

MSC exosomes were first described in 2010 (3). They are presently classified as a member of the family of small EVs that comprises secreted lipid membrane vesicles of 50-200 nm. The size range of exosomes and small EVs has not been precisely defined, and there are slight variations in the ranges used by different groups. In the early days, EV sizes are often estimated by electron microscopy and the sizes tended to be underestimated especially if the samples are dehydrated during processing for electron microscopy. On the hand, dynamic light scattering analysis tends to overestimate the size due to the presence of a hydration shell around the vesicles. In a recent review by Clothide Thery, a pioneer in exosome research, she observed that a 2000*g* centrifugation which is commonly used to remove debris prior to exosome/small EV purification removed the majority of EVs of diameter larger than 200 nm (4). For the purpose of this review, the small EVs will be defined as those with a size range of 50-200 nm.

The presence of a lipid membrane differentiate exosomes and EVs from other biological macromolecules and contributes significantly to biological potency to exosomes and EVs. Disruption of the membrane structure abolished the therapeutic activity of the exosomes(5). In the first description of MSC exosomes, the presence of a lipid membrane was demonstrated through several lines of evidence. First, the proteins in the preparation had a flotation density of 1.10 to 1.18 g ml^{-1} that lies between the liposome density of 1.04-1.05 g ml^{-1} (6) and the estimated protein density of 1.35 to 1.42 g ml^{-1} (7). Second, tetraspanin

proteins CD 9 and CD81 were enriched in the exosome preparation and were shown partially resistant to trypsin digest. Limiting tryptic digest of the exosome preparation generates partial CD9 tryptic peptides (3) and these peptides mapped to intra-membrane and intra-luminal regions (unpublished data). This partial resistance was abolished by pre-incubation with detergent. RNA in the exosome preparation also have a much lighter density of ~1.11 g ml^{-1} than the expected >1.8 g ml^{-1}(8). The RNA was also resistant to RNase activity and this resistance was abolished by pre-treatment with phospholipase. Together, these observations demonstrate that the proteins and RNAs are encapsulated with a lipid membrane structure. Third, major plasma membrane phospholipids such as cholesterol, sphingomyelin and phosphatidylcholine were enriched in the preparation. The molar ratio of sphingomyelin to phosphatidylcholine in the secretion i.e. conditioned medium was 3.3:1 versus 1.6:1 in the cells (3). This corresponds well with the previous reported observation that the proportion of sphingomyelin to phosphatidylcholine was twice as high in exosomes as in the corresponding cells(9). Moreover, the molar concentration of cholesterol, sphingomyelin and phosphatidylcholine per unit weight of proteins in the secretion was 4-13 times higher than that in the cells (3) and this corresponds very well with 8.4-fold enrichment of lipids per mg of protein in exosomes(10).

In contrast to MSC exosomes, the MSC microvesicles as first described encompasses EVs across a wide size range (2) and are likely to include both large and small EVs. Consistent with the presence of small EVs, one of the two sources of the microvesicles appeared to originate to be the mulitvesicular bodies where exosomes are made. The other is from blebbling at the plasma membrane. Apart from having a much wider size range, this microvesicle preparation have several other features that contrasted significantly with those in the exosome preparation. For example, this preparation contained mRNA and mammalian RNA have an estimated median length of 1.4 kb(11). This contrasted significantly with the <300 nt RNA in MSC exosomes (3). Furthermore, unlike the RNA in MSC exosomes, the RNA in the MSC microvesicles could be degraded by RNase without compromising integrity or structural morphology of MVs (2, 12). The miRNA composition was also significantly different between those

present in MSC exosomes and MSC microvesicles. The former is enriched in pre-miRNA (8) while the latter is enriched in mature miRNA(13). A possible reason for the differences in the MSC microvesicle preparation and MSC exosome preparation could be the inclusion of the >200nm EVs whose larger cargo volume may dominate the features of the preparation. Since this review is focussed on the small MSC EVs, the MSC microvesicles will be not included in this review.

MSC exosomes carry endocytosed CTB and transferrin

Exosomes are the only EV type known to have an endosomal biogenesis. The biogenesis of exosomes begins when the membrane of endosomes invaginates to form a multivesicular body (MVB) with numerous intraluminal vesicles (ILVs). When MVBs fuse with the plasma membrane, the ILVs in the MVB are released as exosomes. As a result, exosomes are typified by a conserved set of proteins that are associated with endocytosis and endosomal trafficking such as caveolins, clathrin, transferrin receptors, tetraspanins (CD81, CD63, CD9), Alix, Tsg101 (reviewed(14)).

The MSC exosome preparation as first described in 2010 was uniformly sized with a hydrodynamic radius of 55-65 nm. It should be noted that the estimated size of EVs is influenced by the method of measurement or instrumentation. For instance, size, as estimated by electron microscopy, tends to be smaller due to dehydration during sample preparation. Consistent with the initial characterization of the preparation as an exosome preparation, this preparation was subsequently confirmed to contain bona fide exosomes i.e. derived from endosomes by demonstrating through pulse-chase experiments that MSCs secrete a fraction of endocytosed extracellular ligands such as biotin-conjugated transferrin or biotin-conjugated cholera toxin B chain (CTB) in small EVs(15). Transferrin is the ligand for transferrin receptor, the first exosome marker to be identified (16, 17) and transferrin receptor 2 has been reported to be localized in lipid rafts and released into exosomes with CD81(18). On the other hand, CTB binds GM1 ganglioside, a lipid that is highly enriched in lipid rafts (reviewed (19)).

The pulse-chase of transferrin and CTB into MSC exosomes is consistent with the well documented "lipid raft" characteristic of exosomes. Exosomes are long known to be enriched in lipid raft proteins e.g. flotillin-1, raft-associated proteins e.g. ezrin/villin-2, raft-associated lipids such as GM1 gangliosides (20, 21). The endocytosed transferrin and CTB on MSC exosomes were found to co-localize with each other and with other exosome markers. Proteome analysis also revealed when the proteins in proteome of CTB-EVs were clustered into pathways where they are known to function. Four of the top 20 pathways were endocytic or exocytic processes namely Caveolar-mediated Endocytosis Signaling, Virus Entry via Endocytic Pathways, Mechanisms of Viral Exit from Host Cells, and Clathrin-mediated Endocytosis Signalling (22). Together, the presence of a proteome enriched in proteins associated with endocytosis, endosome-associated activities or exocytosis, and the co-localization of endocytosed transferrin and CTB with exosome markers confirmed that CTB-binding EVs from MSCs are bona fide exosomes. This association between CTB and exosomes was further confirmed recently when cholera toxin was shown to be propagated from cell to cell through exosomes (23).

Small MSC EVs include exosomes and other small EV subtypes

The presence of non-exosomal EV subtypes in the first described MSC exosome preparation was suspected when CTB was found to extract only a fraction of the total protein (22). Subsequent testing using known membrane lipid-binding ligands led to the identification of two other EV subtypes, Annexin V-binding EVs (AV-EVs) and Shiga toxin B chain-binding EVs (ST-EVs). AV binds phosphatidylserine while ST binds globotriaosylceramide. The use of membrane lipid-binding ligands to identify other EVs was rationalized on the importance of the lipid membrane as the defining and physically delimiting feature of EVs. This will also ensure that lipid membrane-bound entities and not similarly sized macromolecules were isolated.

Like CTB-EVs, AV- and ST-EVs are ~100-200 nm vesicles that could be visualized by electron microscopy (22). Based on the distribution of proteins in MSC small EVs across a sucrose gradient of

1.09 to 1.15 g/ml, the density of CTB, AV- and ST-EVs is likely to be within this range. A retrospective analysis of published data revealed that CD9 in the MSC exosome prepration had a modal flotation density of 1.13g/ml (3) while the RNA in the MSC exosome had a modal flotation density of 1.11 g/ml(8). Since CTB-EVs carry almost all the CD9 and only the ST-EVs but not the CTB-or AV-EVs carry RNA (22), the CTB-EVs and ST-EVs may have different densities. The density of AV-EVs is presently not known. All the three EV types carry β-actin. The size distribution of proteins from each of the three EVs on a 1-D protein gel displayed discernible differences. Western blotting revealed that exosome proteins CD81, CD9, ALIX, and TSG101 were enriched relative to β-actin in the CTB-EVs, present at very low levels in AV-EVs and not detectable in ST-EVs. On the other hand, ST-EVs carry a high level of EDA-containing fibronectin and RNA, both of which are not detectable in CTB- and AV-EVs.

The unique fibronectin- and RNA-enriched cargo of ST-EVs demonstrate that ST-EVs are distinct from the CTB- and AV-EVs. However, the ST-EVs may not be the only RNA bearing EVs in the MSC exosome preparation as RNA sequencing revealed that the RNA in ST-EVs represent only a fraction of the total RNA in the MSC exosome preparation.

At the present, there is no unique protein that characterized the AV-EVs. The low level of exosome markers in AV-EVs was specific to AV-EVs and not contamination by CTB-EV as the level of the markers in AV-EVs were similar with or without a prior extraction with CTB (22).

Biogenesis of MSC EV subtypes

Aside from the CTB-EVs which were shown to be bona fide exosomes with an endosomal biogenesis, the biogenesis of AV-EVs and ST-EVs have not been elucidated. Since AV-EVs have exposed phosphatidylserines, a reasonable implication is that they were derived from apoptotic cells. However, it was observed that during staurosporine-induced apoptosis, MSCs secrete AV-EVs that have a much-

elevated level of CD9 relative to β-actin than AV-EVs produced by healthy MSCs, suggesting that AV-EVs was different from that by apoptotic MSCs.

As the key differentiating feature of CTB-, AV- and ST-EVs is the presence of specific membrane lipids, it is likely that their biogenesis occurs at sites in the plasma membrane or membrane organelles where the specific membrane lipids are present and also highly enriched to facilitate polyvalent binding. The latter is important as all three lipid binding proteins have multi-valent binding activities. Each AV molecule binds 4-8 phosphatidylserine molecule (24, 25). As both CTB and ST are pentamers, they each can potentially bind five or more lipid molecules. Each CTB monomer is known to bind one GM1 ganglioside (26) while each ST monomer binds one or more globotriaosylceramide (27). When permeabilized MSCs were stained with fluorescence-labeled CTB, AV or ST, it was observed that CTB staining co-localized with CD81 in the plasma membrane or in punctate cytoplasmic distribution, AV staining is concentrated in the cytoplasm at the perinuclear area with little co-localization with CD81 and ST staining was limited to the nucleus. The cellular distribution of CTB-binding activity is consistent with its association with bona fide exosomes that originate from endosomes.

The subcellular binding activities of AV did not coincide with the distribution of phosphatidylserine in the plasma membrane, endosomes and secretory vesicles(review (28)). A possible reason for this discrepancy is that AV binding of phosphatidylserine is context dependent i.e. the presence of other phospholipids such as phosphatidylethanolamine could affect the binding of phosphatidylserine by AV (29). Another possible reason is that AV is polyvalent and the phosphatidylserines other locations may be not enriched enough for polyvalent binding by AV. Although the subcellular distribution of AV binding sites did not coincide with the endosomal pathway, the presence of low level of exosomal markers in AV-EVs suggests that biogenesis of AV-EVs may involve endosomes and that AV-EVs may be a sub-population of exosomes.

In contrast to the CTB and AV binding, ST binding sites were localized to the nucleus. While this nuclear localization is consistent with its cargo of RNA and an enrichment of pre-miRNA (8), the mechanism for the biogenesis of ST-EVs with its cargo of RNA s and the enrichment of the ECM protein is at present not known. There is evidence of vesiculation in the nucleus particularly in the transport of riboprotein complexes and viruses out of the nucleus(30). It is conceivable that the viruses are then subsequently released out of the cell. While it is possible that the biogenesis of ST-EVs follows a similar pathway as nuclear vesiculation of viruses, there are no evidence to support this yet.

The differences in cargo load, membrane lipids and the subcellular distribution of EV membrane lipids strongly suggest that the three EV types, CTB-, AV- and ST-EVs are unique EV types. However, this should be confirmed directly through methods such as single EV analysis of double or triple labelled EVs.

Physiological relevance of CTB-, AV- and ST-EVs

The physiological relevance of the three EV subtypes to MSC biology has not been elucidated yet. Part of the challenge is the isolation of the EV subtypes away from the lipid-binding ligand for further characterisation. The strong affinity between the ligand and lipid generally precludes the disruption of this bond without breaking the EV. While there is preliminary evidence that other cell types also secrete EVs exhibiting similar affinity for the three lipid binding ligands and have similar subcellular binding sites for the three lipid binding ligands(22), the relatedness of each EV subtype from different cell types has not been investigated yet.

The discovery of three EV subtypes in MSC secretion that could be differentiated from each other by their binding affinity for specific lipid binding proteins led subsequently to the discovery that EVs with similar binding affinities were also present in biological fluids such as plasma and ascites (31-33). The protein cargo is substantially different among the EV subtypes (31). It also observed that the level of some proteins within each EV subtypes differed between those from normal and diseased patients. Thus each EV subtype from the plasma represents a unique source of disease biomarkers. Recently, it was

reported that TIMP metallopeptidase inhibitor 1 (TIMP-1) in plasma CTB-EVs and Plasminogen activator inhibitor-1 (PAI-1) in plasma AV-EVs complement plasma Placental growth factor (PlGF) in predicting pre-eclampsia in an 843-patient cohort of low-risk obstetric population with a strong predictive value as measured by a combined Area under the ROC curve (AUC) score of 0.96(32). Matrix metallopeptidase 9 (MMP-9) in the plasma of serous ovarian cancer was reported to preferentially localized in ascites AV-EVs (33). Together, these reports demonstrate that EV subtypes in biological fluids have different cargos, and as such, different EV subtypes may have a different biological function

Conclusion

The lipid membrane of an EV not only define and limit the physical structure of the EV, it is also integral to EV biogenesis and cargo loading. As reviewed here, differences in the membrane lipid composition of similarly sized EVs can denote a difference in biogenesis, cargo content and functions. As seen in EV subtypes from biological fluids of healthy and sick patients, EV cargo content is also dependent on the physiological state of the producing cells. Therefore, small EVs including those described as exosomes are essentially heterogenous mix of EV types. This has significant implications fro exosome/EV research. It is commonly assumed in the field that the components in an exosome/EV preparation is evenly distributed in each of the EVs in the preparation. Given that most EV preparations are inevitably a heterogenous mix of different EV types, (4), the components in an an exosome/EV preparation are differentially distributed among the different EV types present in the preparations. Therefore it is imperative that thesecomponents must first be properly segregated to the appropriate EV types as this is most fundamental to any attempts to elucidate the biogenesis or mechanism of action of any exosome/EV preparation. For example, the absence of RNA in MSC CTB-EVs which are shown to be bona fide exosomes demonstrate that a RNA cargo may not be a property of exosomes. Therefore, the loading of RNAs into EVs may not involve lipid rafts. Instead, the presence of RNA in ST-EVs suggest that RNA loading into EVs may involve nuclear vesiculation.

However, the elucidation of the biological function of each of these EV types has been technically very challenging as the binding affinity of CTB, AV and ST for the EVs are very high such that isolation of each EV type away from the binding ligand for biological investigations or even protein quantitation is impossible. Presently, efforts to analyse these EVs using single EV analytical techniques are underway

Conflict of interest and funding

SKL is the founder of Paracrine Therapeutics. The authors did not receive any funding or benefits from industry or any for-profit organization for this work.

References

1. Caplan, A. I. 2017. Mesenchymal Stem Cells: Time to Change the Name! *Stem Cells Translational Medicine* **6**: 1445-1451.

2. Bruno, S., C. Grange, M. C. Deregibus, R. A. Calogero, S. Saviozzi, F. Collino, L. Morando, A. Busca, M. Falda, B. Bussolati, C. Tetta, and G. Camussi. 2009. Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* **20**: 1053-1067.

3. Lai, R. C., F. Arslan, M. M. Lee, N. S. Sze, A. Choo, T. S. Chen, M. Salto-Tellez, L. Timmers, C. N. Lee, R. M. El Oakley, G. Pasterkamp, D. P. de Kleijn, and S. K. Lim. 2010. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* **4**: 214-222.

4. Tkach, M., J. Kowal, and C. Théry. 2018. Why the need and how to approach the functional diversity of extracellular vesicles. *Philosophical Transactions of the Royal Society B: Biological Sciences* **373**.

5. Arslan, F., R. C. Lai, M. B. Smeets, L. Akeroyd, A. Choo, E. N. Aguor, L. Timmers, H. V. van Rijen, P. A. Doevendans, and G. Pasterkamp. 2013. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem cell research* **10**: 301-312.

6. Minelli, C., A. Sikora, R. Garcia-Diez, K. Sparnacci, C. Gollwitzer, M. Krumrey, and A. G. Shard. 2018. Measuring the size and density of nanoparticles by centrifugal sedimentation and flotation. *Analytical Methods* **10**: 1725-1732.

7. Fischer, H., I. Polikarpov, and A. F. Craievich. 2004. Average protein density is a molecular-weight-dependent function. *Protein Science : A Publication of the Protein Society* **13**: 2825-2828.

8. Chen, T. S., R. C. Lai, M. M. Lee, A. B. Choo, C. N. Lee, and S. K. Lim. 2010. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res* **38**: 215-224.

9. Laulagnier, K., C. Motta, S. Hamdi, S. Roy, F. Fauvelle, J.-F. Pageaux, T. Kobayashi, J.-P. Salles, B. Perret, C. Bonnerot, and M. Record. 2004. Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. *Biochemical Journal* **380**: 161-171.

10. Llorente, A., T. Skotland, T. Sylvänne, D. Kauhanen, T. Róg, A. Orłowski, I. Vattulainen, K. Ekroos, and K. Sandvig. 2013. Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1831**: 1302-1309.

11. Sommer, S. S., and J. E. Cohen. 1980. The size distributions of proteins, mRNA, and nuclear RNA. *Journal of Molecular Evolution* **15**: 37-57.

12. Bruno, S., C. Grange, F. Collino, M. C. Deregibus, V. Cantaluppi, L. Biancone, C. Tetta, and G. Camussi. 2012. Microvesicles Derived from Mesenchymal Stem Cells Enhance Survival in a Lethal Model of Acute Kidney Injury. *PLOS ONE* **7**: e33115.

13. Collino, F., M. C. Deregibus, S. Bruno, L. Sterpone, G. Aghemo, L. Viltono, C. Tetta, and G. Camussi. 2010. Microvesicles Derived from Adult Human Bone Marrow and Tissue Specific Mesenchymal Stem Cells Shuttle Selected Pattern of miRNAs. *PLOS ONE* **5**: e11803.

14. van Niel, G., G. D'Angelo, and G. Raposo. 2018. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* **19**: 213-228.

15. Tan, S. S., Y. Yin, T. Lee, R. C. Lai, R. W. Yeo, B. Zhang, A. Choo, and S. K. Lim. 2013. Therapeutic MSC exosomes are derived from lipid raft microdomains in the plasma membrane. *J Extracell Vesicles* **2**.

16. Harding, C., J. Heuser, and P. Stahl. 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *The Journal of Cell Biology* **97**: 329-339.

17. Pan, B. T., and R. M. Johnstone. 1983. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* **33**: 967-978.

18. Calzolari, A., C. Raggi, S. Deaglio, N. M. Sposi, M. Stafsnes, K. Fecchi, I. Parolini, F. Malavasi, C. Peschle, M. Sargiacomo, and U. Testa. 2006. TfR2 localizes in lipid raft domains and is released in exosomes to activate signal transduction along the MAPK pathway. *J Cell Sci* **119**: 4486-4498.

19. and, D. A. B., and E. London. 1998. FUNCTIONS OF LIPID RAFTS IN BIOLOGICAL MEMBRANES. *Annual Review of Cell and Developmental Biology* **14**: 111-136.

20. de Gassart, A., C. Geminard, B. Fevrier, G. Raposo, and M. Vidal. 2003. Lipid raft-associated protein sorting in exosomes. *Blood* **102**: 4336-4344.

21. Simon, S., R. Hanieh, and H. Franz‐Georg. 2009. Proteomics of MUC1‐containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF‐7. *PROTEOMICS* **9**: 2820-2835.

22. Lai, R. C., S. S. Tan, R. W. Yeo, A. B. Choo, A. T. Reiner, Y. Su, Y. Shen, Z. Fu, L. Alexander, S. K. Sze, and S. K. Lim. 2016. MSC secretes at least 3 EV types each with a unique permutation of membrane lipid, protein and RNA. *J Extracell Vesicles* **5**: 29828.

23. Zanetti, C., A. Gallina, A. Fabbri, S. Parisi, A. Palermo, K. Fecchi, Z. Boussadia, M. Carollo, M. Falchi, L. Pasquini, M. L. Fiani, and M. Sargiacomo. 2018. Cell Propagation of Cholera Toxin CTA ADP-Ribosylating Factor by Exosome Mediated Transfer. *Int J Mol Sci* **19**.

24. Meers, P., and T. Mealy. 1993. Calcium-dependent annexin V binding to phospholipids: stoichiometry, specificity, and the role of negative charge. *Biochemistry* **32**: 11711-11721.

25. Sopkova, J., M. Renouard, and A. Lewit-Bentley. 1993. The crystal structure of a new high-calcium form of annexin V. *J Mol Biol* **234**: 816-825.

26. Merritt, E. A., S. Sarfaty, F. van den Akker, C. L'Hoir, J. A. Martial, and W. G. Hol. 1994. Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci* **3**: 166-175.

27. Ling, H., A. Boodhoo, B. Hazes, M. D. Cummings, G. D. Armstrong, J. L. Brunton, and R. J. Read. 1998. Structure of the Shiga-like Toxin I B-Pentamer Complexed with an Analogue of Its Receptor Gb3. *Biochemistry* **37**: 1777-1788.

28. Leventis, P. A., and S. Grinstein. 2010. The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys* **39**: 407-427.

29. Lizarbe, M., J. Barrasa, N. Olmo, F. Gavilanes, and J. Turnay. 2013. Annexin-Phospholipid Interactions. Functional Implications. *International Journal of Molecular Sciences* **14**: 2652.

30. Hagen, C., Kyle C. Dent, T. Zeev-Ben-Mordehai, M. Grange, Jens B. Bosse, C. Whittle, Barbara G. Klupp, C. A. Siebert, D. Vasishtan, Felix J. B. Bäuerlein, J. Cheleski, S. Werner, P. Guttmann, S. Rehbein, K. Henzler, J. Demmerle, B. Adler, U. Koszinowski, L. Schermelleh, G. Schneider, Lynn W. Enquist, Jürgen M. Plitzko, Thomas C. Mettenleiter, and K. Grünewald. 2015. Structural Basis of Vesicle Formation at the Inner Nuclear Membrane. *Cell* **163**: 1692-1701.

31. Tan, K. H., S. S. Tan, S. K. Sze, W. K. Lee, M. J. Ng, and S. K. Lim. 2014. Plasma biomarker discovery in preeclampsia using a novel differential isolation technology for circulating extracellular vesicles. *Am J Obstet Gynecol* **211**: 380 e381-313.

32. Tan, K. H., S. S. Tan, M. J. Ng, W. S. Tey, W. K. Sim, J. C. Allen, and S. K. Lim. 2017. Extracellular vesicles yield predictive pre-eclampsia biomarkers. *J Extracell Vesicles* **6**: 1408390.

33. Reiner, A. T., S. Tan, C. Agreiter, K. Auer, A. Bachmayr-Heyda, S. Aust, N. Pecha, M. Mandorfer, D. Pils, and A. R. Brisson. 2017. EV-Associated MMP9 in High-Grade Serous Ovarian Cancer Is Preferentially Localized to Annexin V-Binding EVs. *Disease Markers* **2017**.