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Explicating Exosomes: Reclassifying the Rising Stars of Intercellular Communication

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There is growing interest surrounding the diagnostic and therapeutic potential of exosomes, but a definitive description of these extracellular vesicles remains elusive. In this issue, Jeppesen et al. characterize exosomes following a strict isolation protocol and in so doing challenge several of the accepted properties of these agents of intercellular communication.

The secretion of membranous extracellular vesicles (EVs) by cells is a phenomenon well conserved across biological domains. Although the physiological function of these EVs has been recognized in a few specific contexts, such as neurotransmission or endocrine signaling, the broader process of vesicle secretion had been largely dismissed as a waste-disposal mechanism. Nevertheless, with mounting evidence of vesicles trafficking biologically active cargoes that vary in response to their microenvironment, EVs have come to be accepted as ubiquitous mediators of intercellular communication among all cell types. EVs transfer non-coding and coding RNAs (Valadi et al., 2007), mediate immune signaling through the transfer of cytokine-receptor complexes (Cossetti et al., 2014), and even function as fully independent metabolic units (Iraci et al., 2017). Several roles have been ascribed to EVs in influencing a diversity of physiological and pathological conditions, from the potentiation of chemotherapeutic resistance (Au Yeung et al., 2016) and induction of immunological responses (Théry et al., 2002) to the spread of prions (Fevrier et al., 2004). Accordingly, there is growing interest in the use of EVs as both biomarkers of disease (Ibsen et al., 2017) and as therapeutic drug-delivery vehicles (Alvarez-Erviti et al., 2011). To date, much of this attention has concerned the specific sub-type of EVs known as exosomes, yet a definitive characterization of what classifies as an exosome has proven elusive due to the heterogeneity of EV species and the assortment of non-specific isolation techniques. In this issue of *Cell*, Jeppesen et al. (2019) provide a much-needed reappraisal of what constitutes a bona fide exosome through a highly stringent and novel isolation methodology.

EVs more generally encompass a variety of vesicle types that differ in their route of biogenesis, size, and composition, with exosomes typically being defined as small vesicles, 40–150 nm in size, arising from a multivesicular endosomal (MVE) pathway. A number of different techniques are routinely used to isolate exosomes from biological fluids, including differential centrifugation, density-gradient centrifugation, and ultrafiltration. Each technique comes with its own advantages and disadvantages, and critically, they yield preparations of small EVs that differ in purity and heterogeneity. Thus, characterizations of the composition, cargo, and functionality of these populations are likely to reflect a diversity of vesicular and non-vesicular components rather than exosomes specifically. This heterogeneity is one key factor driving controversies surrounding the properties of exosomes, such as whether they are capable of cell-specific uptake and if their nucleic acid cargoes are functional upon delivery. A more specific understanding of the biological role and therapeutic potential of exosomes and other EVs necessitates a more discriminating isolation and classification, which is what Jeppesen et al. (2019) now report.

The authors first remove cell debris and large EVs by centrifugation before employing a two-step isolation procedure on the resultant crude small EV (sEV) preparation: high-resolution density-gradient fractionation to separate sEVs from non-vesicular material followed by separation of exosomes from other (non-exosomal) sEVs by direct immunoaffinity capture (DIC). The end result of this process is a population of vesicles of the appropriate density and bearing the tetraspanin exosomal markers CD9, CD63, and CD81. The composition of these classical exosomes differs from that of orthodox exosome preparations in a number of ways. First, classical exosomes are found to be absent luminal proteins commonly though ubiquitous to exosomes, including enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase and the chaperone heat shock protein 90 (HSP90). Cytoskeletal proteins are also not found by the authors inside classical exosomes, which, combined with the absence of metabolic enzymes, speaks against random encapsulation of the parent cell's cytosol during exosome biogenesis and instead implies controlled cargo packaging mechanisms. Additionally, membrane-bound annexins A1 and A2—often considered characteristic of exosomes—are instead to be markers of non-exosomal sEVs (Figure 1).

This work also addresses some of the contentious issues surrounding exosomal miRNA functionality, with classical exosomes found to be absent the Argonaute (Ago) proteins 1–4, components of the RNA-induced silencing complex, and all other miRNA-associated enzymes (e.g., Dicer, Drosha). Thus, in contrast to previous findings (Melo et al., 2014), classical exosomes lack the necessary componentry to facilitate cell-independent miRNA biogenesis. Indeed, with the exception of traces of Ago1–4 being detected in

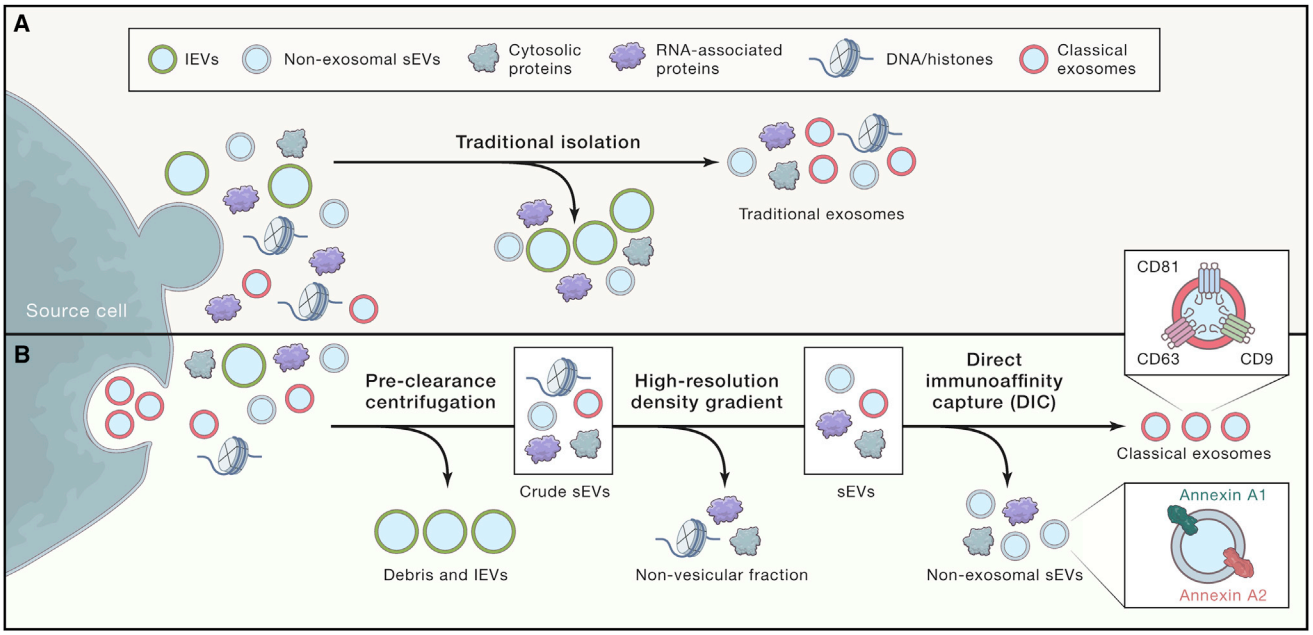

Figure 1. Traditional versus Revised Exosome Isolation Protocols

(A) Traditional exosome isolation methods yield a mixture of vesicular and non-vesicular products enriched in exosomes and free of large extracellular vesicles (lEVs).

(B) The method of Jeppesen et al. (2019) uses a two-step method to first separate non-vesicular fractions from small extracellular vesicles (sEVs) using a high-resolution density gradient before isolating bona fide classical exosomes from non-exosomal sEVs through direct immunoaffinity capture (DIC). Classical exosomes are marked by the expression of the tetraspanins CD9, CD63, and CD81, whereas non-exosomal sEVs are marked by annexins A1 and A2.

non-exosomal sEVs from a single cell line, components of the miRNA machinery are not observed in any sEVs. Patterns of miRNA abundance differ between cellular and extracellular sources, with the latter showing further disparity between non-vesicular and sEV fractions. Furthermore, none of the frequently cited extracellular RNA-binding proteins are found to be associated with EV fractions isolated by DIC, which suggests that classical exosomes may not be a significant source of extracellular RNA.

Perhaps most significantly, the authors demonstrate that extracellular double-stranded DNA is not associated with classical exosomes, or indeed any sEVs, but is instead co-purified with these EVs during standard isolation techniques. An EV-independent extracellular secretion mechanism is instead proposed, wherein DNA and histones are actively released alongside sEVs in an autophagy- and MVE-dependent pathway. Given the growing interest in extracellular DNA as a disease marker in liquid biopsies, a re-assessment of what is actually being measured may be due. Indeed, future studies should ideally demonstrate that components or functions of interest are indeed characteristic of a specific EV versus non-vesicular population. However, such precision may come at a cost—i.e., the methodology used in this study is more costly and less efficient than traditional methods with respect to time and yield. Ultimately, there is a need for greater standardization of isolation and purification techniques, or even a revision of the current classification and nomenclature. At a deeper level, this work highlights the need for a deeper understanding of the mechanisms underlying the biogenesis and loading of EVs, with issues such as the lipid composition of the exosomes and the mechanisms of miRNA packaging needing further study.

Jeppesen et al. (2019) show that bona fide exosomes have a substantially more restricted repertoire of biomolecular constituents than has been generally accepted. While the criterion for classifying exosomes is intrinsically arbitrary, precision in scientific nomenclature is vital for ensuring consistency among experimental observations.

Correctly attributing functionality to the appropriate extracellular entity will be imperative for their successful use as biomarkers or therapeutics.

DECLARATION OF INTERESTS

Stefano Pluchino owns >5% of CITC Ltd.; Jayden A Smith is an employee at CITC Ltd.

REFERENCES

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M.J. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat. Biotechnol. *29*, 341–345.

Au Yeung, C.L., Co, N.N., Tsuruga, T., Yeung, T.L., Kwan, S.Y., Leung, C.S., Li, Y., Lu, E.S., Kwan, K., Wong, K.K., et al. (2016). Exosomal transfer of stroma-derived miR21 confers paclitaxel resistance in ovarian cancer cells through targeting APAF1. Nat. Commun. *7*, 11150.

Cossetti, C., Iraci, N., Mercer, T.R., Leonardi, T., Alpi, E., Drago, D., Alfaro-Cervello, C., Saini, H.K., Davis, M.P., Schaeffer, J., et al. (2014). Extracellular vesicles from neural stem cells transfer IFN-γ via Ifngr1 to activate Stat1 signaling in target cells. Mol. Cell *56*, 193–204.

Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., Laude, H., and Raposo, G. (2004).

Cells release prions in association with exosomes. Proc. Natl. Acad. Sci. USA *101*, 9683–9688.

Ibsen, S.D., Wright, J., Lewis, J.M., Kim, S., Ko, S.Y., Ong, J., Manouchehri, S., Vyas, A., Akers, J., Chen, C.C., et al. (2017). Rapid Isolation and Detection of Exosomes and Associated Biomarkers from Plasma. ACS Nano *11*, 6641–6651.

Iraci, N., Gaude, E., Leonardi, T., Costa, A.S.H., Cossetti, C., Peruzzotti-Jametti, L., Bernstock, J.D., Saini, H.K., Gelati, M., Vescovi, A.L., et al. (2017). Extracellular vesicles are independent metabolic units with asparaginase activity. Nat. Chem. Biol. *13*, 951–955.

Jeppesen, D.K., Fenix, A.M., Franklin, J.L., Higginbotham, J.N., Zhang, Q., Zimmerman, L.J., Liebler, D.C., Ping, J., Liu, Q., Evans, R., et al. (2019). Reassessment of Exosome Composition. Cell *177*, this issue, 428–445.

Melo, S.A., Sugimoto, H., O'Connell, J.T., Kato, N., Villanueva, A., Vidal, A., Qiu, L., Vitkin, E., Perelman, L.T., Melo, C.A., et al. (2014). Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. Cancer Cell *26*, 707–721.

Théry, C., Duban, L., Segura, E., Véron, P., Lantz, O., and Amigorena, S. (2002). Indirect activation of naïve CD4+ T cells by dendritic cell-derived exosomes. Nat. Immunol. *3*, 1156–1162.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., and Lötvall, J.O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. *9*, 654–659.