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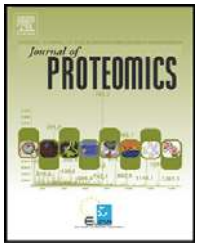

Review

Exosomes: Extracellular organelles important in intercellular communication

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ABSTRACT

In addition to intracellular organelles, eukaryotic cells also contain extracellular organelles that are released, or shed, into the microenvironment. These membranous extracellular organelles include exosomes, shedding microvesicles (SMVs) and apoptotic blebs (ABs), many of which exhibit pleiotropic biological functions. Because extracellular organelle terminology is often confounding, with many preparations reported in the literature being mixtures of extracellular vesicles, there is a growing need to clarify nomenclature and to improve purification strategies in order to discriminate the biochemical and functional activities of these moieties. Exosomes are formed by the inward budding of multivesicular bodies (MVBs) and are released from the cell into the microenvironment following the fusion of MVBs with the plasma membrane (PM). In this review we focus on various strategies for purifying exosomes and discuss their biophysical and biochemical properties. An update on proteomic analysis of exosomes from various cell types and body fluids is provided and host-cell specific proteomic signatures are also discussed. Because the ectodomain of ~42% of exosomal integral membrane proteins are also found in the secretome, these vesicles provide a potential source of serum-based membrane protein biomarkers that are reflective of the host cell. ExoCarta, an exosomal protein and RNA database (http://exocarta.ludwig.edu.au), is described.

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Contents

Abbreviations: MVBs, multivesicular bodies; ILVs, intraluminal vesicles; ESCRT, Endosomal Sorting Complexes Required for Transport; PM, plasma membrane; LBPA, lyosbisphosphatidic acid; SMVs, shedding microvesicles; ABs, apoptotic blebs.

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1. Introduction

Molecules that perform specific cellular functions are segregated and compartmentalized into dynamic and distinctly structured organelles, which are composed of both resident and transient molecules that carry out specific functions. The molecular components of organelles are exchanged constantly with the rest of the cell and fluctuate with physiological perturbations [1]. While the majority of organelles reside within the cell, some, such as exosomes, shedding microvesicles (SMVs) and apoptotic blebs (ABs) are released into the extracellular space.

2. Exosomes

Exosomes are 40–100 nm diameter membranous vesicles of endocytic origin that are released by a variety of cell types into the extracellular space [2]. Exosomes were first reported in 1983 by Johnstone and colleagues while culturing reticulocytes [3]. Inward budding of endosomal membranes results in the progressive accumulation of intraluminal vesicles (ILVs) within large multivesicular bodies (MVBs). Transmembrane proteins are incorporated into the invaginating membrane while the cytosolic components are engulfed within the ILVs [4]. Based on their biochemical properties, intracellular MVBs can either traffic to lysosomes where they are subjected to proteosomal degradation (i.e., 'degradative MVBs') or, alternatively, to the plasma membrane (PM) where upon fusion with the PM they release their contents (ILVs) into the extracellular space (the so-called 'exocytic MVBs' — Fig. 1A); ILVs released into the extracellular space are referred to as 'exosomes' [5]. While this 'degradative'/'exocytic' MVB phenomenon has been reported to occur in oligodendrocytes and involves changes in the ceramide chemistry of MVB membranes [6], it is not clear whether this phenomenon is generally applicable and occurs in all cell types. Furthermore, it is not clear whether there are two classes of MVBs (i.e., exocytic and degradative) or whether MVBs contain exocytic and degradative ILVs. To date, exosomes are the only type of membranous vesicles originating from intracellular compartments such as the MVBs. Endosomal Sorting Complexes Required for Transport (ESCRTs), multiprotein complexes, are involved in the mechanism governing the biogenesis/degradation of MVBs [7,8] in a ubiquitinylation-dependent [9] manner. With the aid of three-dimensional structural studies, key links between the components of the ESCRT multiprotein complex, phospholipids and ubiquitin are beginning to shed new insights into MVB biogenesis and trafficking [10].

2.1. *Shedding microvesicles (SMVs)*

SMVs are large membranous vesicles (>100 nm diameter) that are shed from the PM of a wide variety of cell types [11–13]. Following blebbing (outward protrusion) of the PM, fission of the PM stalk detaches the cytoplasmic protrusions, resulting in the formation of SMVs [14]. Regulation of this process involves several enzymes such as calpain, flippase, floppase, scramblase and gelsolin [15]. Platelet-derived SMVs are reported to contain components of membrane lipid rafts (e.g., flotilin-1 (FLOT1)), lineage markers (e.g., platelet/endothelial cell adhesion molecule (PECAM-1) [15]), while oncogenic growth factor receptors (e.g., EGFRvIII) and tissue factor (CD142) are present in SMVs from gliomas [12] and plasma [16,17], respectively.

2.2. *Apoptotic blebs (ABs)*

Apoptotic or dying cells release membrane vesicles into the extracellular environment via blebbing of the PM. These membrane vesicles, which are condensed remnants of the shrinking apoptotic cell [18], are referred to as apoptotic blebs (ABs). While SMVs are also released during the early stages of apoptosis, ABs are released during the late stages of cell death [19]. SMVs and ABs are 100–1000 nm and 50–500 nm in diameter, respectively, and are heterogeneous in shape; by contrast, exosomes are much smaller in size (40–100 nm diameter) and homogeneous with respect to shape. Exosomes are cup-shaped and float at a density of 1.10–1.21 g/mL in sucrose gradient, which uniquely differentiates them from non-exosomal vesicles that are of irregular shape and float at higher densities (>1.23 g/mL) [20].

3. Microvesicles: the case for more stringent nomenclature

The extracellular microenvironment including body fluids such as ascites and blood contains a mixed population of exosomes, SMVs and ABs [14]. These microvesicles have been studied over the years using a variety of isolation strategies and have been categorized by their distinct structural and biochemical properties [2]. However, studies aimed at elucidating the mechanism of their biogenesis are under represented; moreover, the heterogeneous materials from which

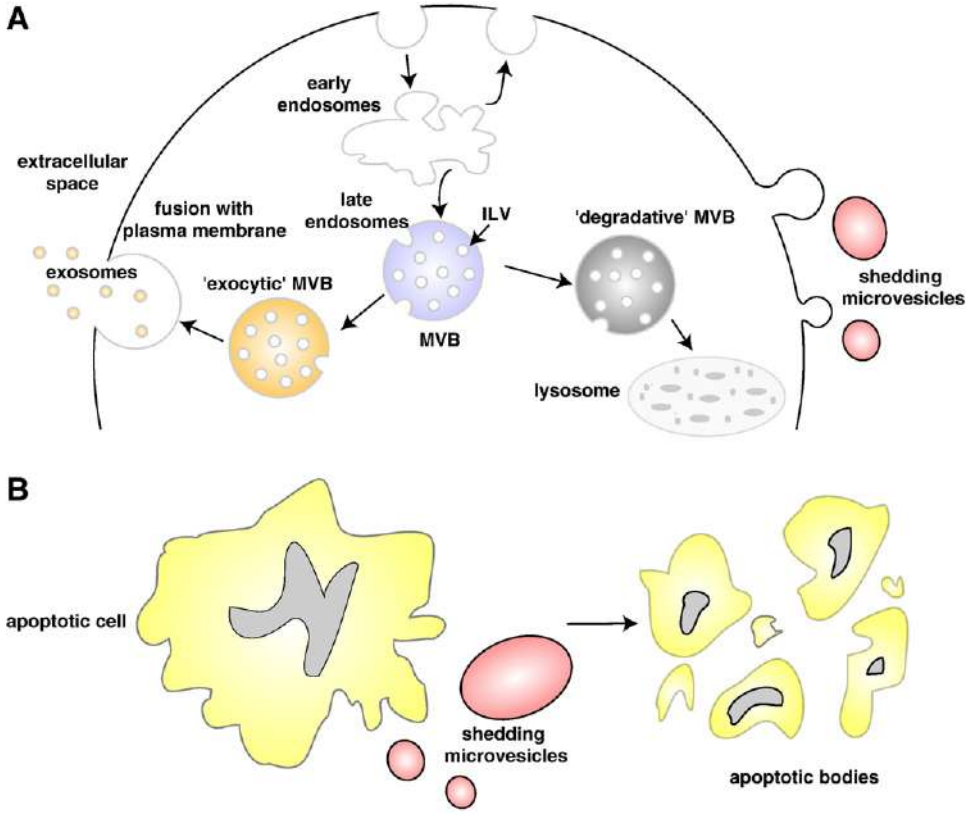

Fig. 1 – Schematic representation of the release of extracellular membranous microvesicles into the extracellular space. A, Release of exosomes and SMVs is shown. In early endosomes, proteins are either recycled to the PM or sequested in ILVs of the larger MVBs. ILVs of MVBs are generated by budding from the limiting membrane into the lumen of endosomes [96]. Owing to the biophysical properties, MVBs either can be degradative (evolving into lysosomes) regulated by ESCRT or ubiquitination [9,97,98] or can be exocytic (i.e., fuse with PM with sub sequel release of their contents — exosomes). SMVs are released by the process of blebbing or shedding from the PM. B, Apoptotic or dying cells with cell shrinkage, a hallmark of apoptosis, leads in generation of ABs. These vesicles are remnants of the degrading apoptotic cell with nuclear and cytoplasmic content.

samples were derived have led to confusing terminologies. For example, different nomenclatures have been used to name secreted vesicles, resulting in diverse terminologies such as exosomes, microparticles, nanoparticles, microvesicles, shedding microvesicles, ectosomes, exosome-like vesicles, apoptotic blebs, promininosomes, prostasomes, dexosomes, texosomes, dex, tex, epididimosomes, argosomes, archeosomes and oncosomes [21]. Confusion in terminology has led to typical exosome preparations sometimes being referred as microvesicles and *vice versa*. However, these terminologies need to be refined and general consensus in the nomenclature agreed upon. As a first step towards standardizing the nomenclature, it is worthwhile to take into account the three known mechanisms by which membrane vesicles are released into the extracellular microenvironment: exocytic fusion of MVBs resulting in exosomes, budding of vesicles directly from the PM resulting in SMVs (Fig. 1A) and cell death leading to ABs and SMVs (Fig. 1B). Perhaps, the term 'microvesicles' should be used to indicate a mixed population of vesicles that contains exosomes, SMVs and ABs. When isolating microvesicles it is of paramount importance to clearly distinguish exosomes from SMVs and ABs to avoid cross contamination, which will undoubtedly confound interpretation of biochemical data. Table 1 summarizes various attributes of microvesicles. The biochemical and functional aspects of ABs and SMVs, which are documented in previous reviews [14,21,22], will not be discussed in detail in this review.

4. Current status of exosome protein composition

Exosomes contain a distinct set of proteins such as the Alix, TSG101, HSP70 and the tetraspanins CD63, CD81 and CD9. The protein content of exosomes has been extensively analyzed from various cell types and body fluids by MS, Western blotting, fluorescence-activated cell sorting and immunoelectron microscopy. A detailed analysis of 19 proteomic studies (each qualified study identified at least 30 proteins) revealed a more generic outlook of exosomal proteins (Fig. 2). Proteins identified in at least 26% (5/19) of the studies are depicted in Fig. 2. The 19 exosomal studies used for this analysis were derived from dendritic cells [20], melanoma cells [23], urine [24,25], microglia [26], mast cells [27], colorectal cancer cells [28,29], mesothelioma cells [30], brain tumor [31], oligodendrocytes [32], tracheobronchial cells [33], hepatocytes

Table 1 – Attributes of extracellular membranous microvesicles.

	Exosomes	SMVs	ABs
Size (diameter)	30–100 nm	100–1000 nm	50–500 nm
Flotation density (rate zonal centrifugation)	1.10–1.21 g/mL	NA	1.16–1.28 g/mL [20]
Morphology	Cup-shaped	Various shapes	Heterogeneous
Lipid composition	LBPA, low phosphatidylserine exposure, cholesterol, ceramide, contains lipid rafts, sphingomyelin	High phosphatidylserine exposure, cholesterol	High phosphatidylserine exposure
Protein markers	Alix, TSG101, HSC70, CD63, CD81, CD9	Selectins, integrins, CD40, metalloproteinases	Histones
Site of origin	MVBs	Plasma membrane	–
Mode of extracellular release	Constitutive and regulated	Regulated	Regulated
Mechanism of discharge	Exocytosis of MVBs	Budding from plasma membrane	Cell shrinkage and death
Composition	Proteins, miRNA, mRNA	Proteins, miRNA, mRNA	Proteins, DNA, miRNA, mRNA

SMVs, shedding microvesicles; ABs, apoptotic blebs; LBPA, lyosbisphosphatidic acid; MVBs, multivesicular bodies; NA, not known.

[34], neuroglial cell [35], plasma [36], breast milk [37], breast cancer cells [38], saliva [39] and embryonic fibroblast cells [40]. ExoCarta [41], a database of exosomal proteins and RNA, was used to download the protein data pertaining to these 19 exosomal proteomic studies. Exosome protein composition varies depending on the cell type of origin and a unique tissue/cell type signature for exosomes was revealed [29]. In addition, a conserved set of proteins were identified in exosomes in

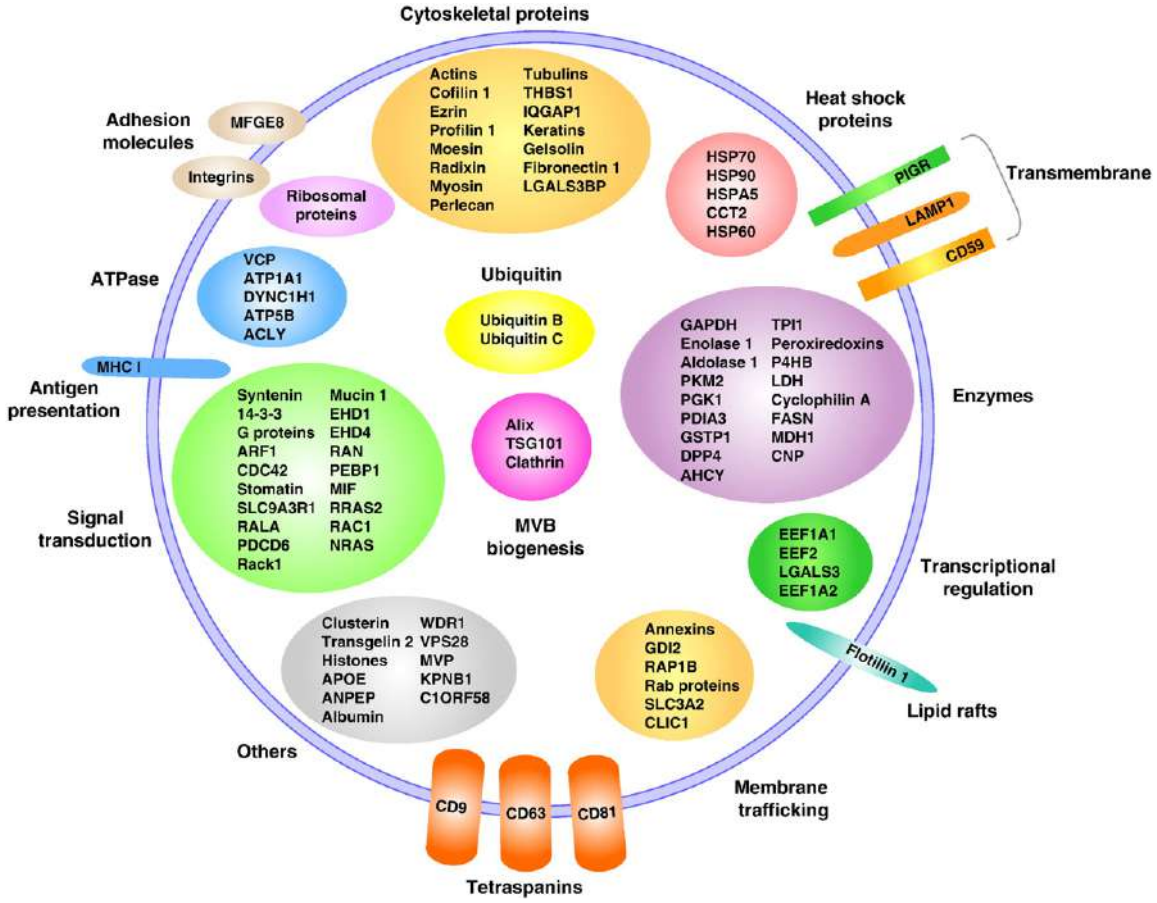

Fig. 2 – A graphical representation of the protein composition of exosomes categorized as per the function performed. ExoCarta [41] was used to download 19 exosomal proteomic studies that had identified at least 30 proteins. Protein molecules that are identified in more than 26% of the proteomic studies are depicted in the figure as gene symbols or protein names.

spite of their cellular origin. As shown in Fig. 2, MVB biogenesis molecules such as Alix (identified in 68% of the studies), TSG101 (37%) and clathrin (37%) are highly associated with exosomes. Similarly, HSP70 is identified in 89% of the proteomic studies. Table 2 provides the occurrences of each molecule in the 19 exosomal proteomic studies depicted in Fig. 2.

One class of cytosolic proteins commonly seen in exosomes includes the Rabs, the largest family of small GTPases, which regulate exosome docking and membrane fusion [23]. Active Rabs interact with proteins involved in vesicular transport and protein complexes that regulate vesicle fusion with acceptor membranes [42]. RAB5 localized in early endosomes mediates endocytosis and endosome fusion of clathrin-coated vesicles [43]. Interestingly, studies using green fluorescent protein-tagged endosomal GTPases showed the existence of mechanisms for segregating Rab GTPases into membrane domains with distinct functions [43]. Such studies revealed the presence of multiple combinations of RAB4, RAB5 and RAB11 membrane domains in early and recycling endosomes in contrast to RAB7 and RAB9 membrane domains presence in late endosomes. Browsing for Rab proteins in ExoCarta revealed as many as 40 Rab proteins that are identified in various exosome studies.

In addition to Rabs, exosomes are rich in annexins (annexins I, II, IV, V, VI, VII and X1) which aid in membrane trafficking and fusion events [44]. Exosomes are also enriched with tetraspanins (CD63, CD81 and CD9) [29] and heat-shock proteins (HSP60, HSP70, HSPA5, CCT2 and HSP90), and contain cell-type-specific proteins such as A33 (colon epithelial-derived) [29], MHC II (antigen presenting cells-derived) [45,46], CD86 (antigen-presenting cells) [47,48] and MFG-E8/lactadherin (immature dendritic cells) [49]. Interestingly, colorectal cancer-derived exosomes showed a significant enrichment of coiled-coil, RAS and MIRO domain containing proteins [29]. Coiled coil motifs play a vital role in localization of proteins to early endosomes [50] and vesicular transport [51] while RAS and MIRO domains are found in small GTPases (Rabs) and Rho GTPases.

Other exosomal proteins include the metabolic enzymes (GAPDH, enolase 1, aldolase 1, PKM2, PGK1, PDIA3, GSTP1, DPP4, AHCY, TPL1, peroxiredoxins, P4HB, LDH, cyclophilin A, FASN, MDH1 and CNP), ribosomal proteins (RPS3), transmembrane (PIGR, LAMP1 and CD59), signal transduction (syntenin, 14-3-3, G proteins, ARF1, CDC42, stomatin, SLC9A3R1, RALA, PDCD6, rack1, mucin 1, EHD1, RAN, PEBP1, MIF, RRAS2, RAC1, NRAS and EHD4), adhesion (MFGE8 and integrins), ATPases (VCP, ATP1A1, DYNC1H1, ATP5B and ACLY), cytoskeletal (actins, tubulins, cofilin 1, ezrin, profilin 1, moesin, radixin, myosin, perlecan, THBS1, IQGAP1, keratins, gelsolin, fibronectin 1 and LGALS3BP) and ubiquitin molecules (ubiquitins B and C) [29]. Additionally, lipid compositions of exosomes are characteristic of the cell origin and play a vital role in exosome biogenesis [52]. Lipid composition analysis has been performed with exosomes derived from dendritic cells [53], mast cells [53], reticulocytes [54] and B cells [55]. Interestingly, internal membranes of MVBs are shown to be enriched with lipids such as lyosbisphosphatidic acid (LBPA) [56]. LBPA plays an important role in exosome biogenesis, especially ILV formation [52].

Table 2 – Proteins identified at least 26% (5) of the 19 proteomic studies from ExoCarta.

	Gene symbol	Protein name	Number of identifications in 19 proteomic studies
1	HSPA8	heat-shock 70 kDa protein 8	17
2	ACTB	actin, beta	16
3	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	16
4	ENO1	enolase 1, (alpha)	15
5	ANXA2	annexin A2	14
6	CFL1	cofilin 1 (non-muscle)	14
7	MSN	moesin	14
8	PDCD6IP	programmed cell death 6 interacting protein	13
9	SDCBP	syndecan binding protein (syntenin)	13
10	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	13
11	CD63	CD63 molecule	12
12	EEF1A1	eukaryotic translation elongation factor 1 alpha 1	12
13	YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	12
14	ANXA5	annexin A5	11
15	CD81	CD81 molecule	11
16	PGK1	phosphoglycerate kinase 1	11
17	PKM2	pyruvate kinase, muscle	11
18	PPIA	peptidylprolyl isomerase A (cyclophilin A)	11
19	YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	11
20	ALDOA	aldolase A, fructose-bisphosphate	10
21	ANXA6	annexin A6	10
22	EZR	ezrin	10
23	GDI2	GDP dissociation inhibitor 2	10
24	HSP90AA1	heat-shock protein 90 kDa alpha (cytosolic), class A member 1	10
25	HSP90AB1	heat-shock protein 90 kDa alpha (cytosolic), class B member 1	10
26	HSPA1A	heat-shock 70 kDa protein 1A	10
27	HSPA1B	heat-shock 70 kDa protein 1B	10
28	MFGE8	milk fat globule-EGF factor 8 protein	10
29	ACTG1	actin, gamma 1	9
30	ALB	albumin	9
31	ANXA4	annexin A4	9
32	CD9	CD9 molecule	9
33	EEF2	eukaryotic translation elongation factor 2	9
34	GNAI2	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	9

(continued on next page)

Table 2 (continued)

	Gene symbol	Protein name	Number of identifications in 19 proteomic studies
35	GNB2	guanine nucleotide binding protein (G protein), beta polypeptide 2	9
36	GSTP1	glutathione S-transferase pi 1	9
37	MYH9	myosin, heavy chain 9, non-muscle	9
38	PFN1	profilin 1	9
39	RDX	radixin	9
40	YWHAB	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, beta polypeptide	9
41	ANXA1	annexin A1	8
42	ARF1	ADP-ribosylation factor 1	8
43	GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1	8
44	HSPA5	heat-shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	8
45	IQGAP1	IQ motif containing GTPase activating protein 1	8
46	PRDX1	peroxiredoxin 1	8
47	RAB5C	RAB5C, member RAS oncogene family	8
48	RAP1B	RAP1B, member of RAS oncogene family	8
49	THBS1	thrombospondin 1	8
50	TPI1	triosephosphate isomerase 1	8
51	TUBA1A	tubulin, alpha 1a	8
52	VCP	valosin-containing protein	8
53	YWHAH	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, eta polypeptide	8
54	CCT2	chaperonin containing TCP1, subunit 2 (beta)	7
55	CLIC1	chloride intracellular channel 1	7
56	CLTC	clathrin, heavy chain (Hc)	7
57	CLU	clusterin	7
58	EHD1	EH-domain containing 1	7
59	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	7
60	LDHB	lactate dehydrogenase B	7
61	MUC1	mucin 1, cell surface associated	7
62	P4HB	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	7
63	PIGR	polymeric immunoglobulin receptor	7
64	RAB11B	RAB11B, member RAS oncogene family	7
65	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	7

Table 2 (continued)

	Gene symbol	Protein name	Number of identifications in 19 proteomic studies
66	TSG101	tumor susceptibility gene 101	7
67	TUBA1C	tubulin, alpha 1c	7
68	TUBB5	tubulin, beta 5	7
69	YWHAQ	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, theta polypeptide	7
70	ACTN4	actinin, alpha 4	6
71	ANPEP	alanyl (membrane) aminopeptidase	6
72	ANXA11	annexin A11	6
73	APOE	apolipoprotein E	6
74	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	6
75	CDC42	cell division cycle 42 (GTP binding protein, 25 kDa)	6
76	EHD4	EH-domain containing 4	6
77	FASN	fatty acid synthase	6
78	FN1	fibronectin 1	6
79	GNAI3	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	6
80	GNAQ	guanine nucleotide binding protein (G protein), q polypeptide	6
81	GNAS	GNAS complex locus	6
82	GSN	gelsolin (amyloidosis, Finnish type)	6
83	HIST4H4	histone cluster 4, H4	6
84	KRT10	keratin 10	6
85	LDHA	lactate dehydrogenase A	6
86	MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	6
87	PEBP1	phosphatidylethanolamine binding protein 1	6
88	PRDX2	peroxiredoxin 2	6
89	RAB11A	RAB11A, member RAS oncogene family	6
90	RAN	RAN, member RAS oncogene family	6
91	TAGLN2	transgelin 2	6
92	UBB	ubiquitin B	6
93	ACLY	ATP citrate lyase	5
94	AHCY	S-adenosylhomocysteine hydrolase	5
95	ANXA7	annexin A7	5
96	ARF3	ADP-ribosylation factor 3	5
97	ATP5B	ATP synthase, H+transporting, mitochondrial F1 complex, beta polypeptide	5
98	C1ORF58	chromosome 1 open reading frame 58	5
99	CD59	CD59 molecule, complement regulatory protein	5
100	CNP	2′,3′-cyclic nucleotide 3′ phosphodiesterase	5

Table 2 (continued)

	Gene symbol	Protein name	Number of identifications in 19 proteomic studies
101	DPP4	dipeptidyl-peptidase 4	5
102	DYNC1H1	dynein, cytoplasmic 1, heavy chain 1	5
103	EEF1A2	eukaryotic translation elongation factor 1 alpha 2	5
104	FLOT1	flotillin 1	5
105	GNA11	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	5
106	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	5
107	HIST1H4A	histone cluster 1, H4a	5
108	HIST1H4B	histone cluster 1, H4b	5
109	HLA-A	major histocompatibility complex, class I, A	5
110	HSPD1	heat-shock 60 kDa protein 1 (chaperonin)	5
111	HSPG2	heparan sulfate proteoglycan 2	5
112	ITGA6	integrin, alpha 6	5
113	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	5
114	KPNB1	karyopherin (importin) beta 1	5
115	KRT15	keratin 15	5
116	KRT5	keratin 5	5
117	LAMP1	lysosomal-associated membrane protein 1	5
118	LGALS3	lectin, galactoside-binding, soluble, 3	5
119	LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	5
120	MDH1	malate dehydrogenase 1, NAD (soluble)	5
121	MVP	major vault protein	5
122	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	5
123	PDCD6	programmed cell death 6	5
124	PDIA3	protein disulfide isomerase family A, member 3	5
125	RAB10	RAB10, member RAS oncogene family	5
126	RAB13	RAB13, member RAS oncogene family	5
127	RAB14	RAB14, member RAS oncogene family	5
128	RAB35	RAB35, member RAS oncogene family	5
129	RAB5A	RAB5A, member RAS oncogene family	5
130	RAB5B	RAB5B, member RAS oncogene family	5
131	RAB7	RAB7, member RAS oncogene family	5
132	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	5
133	RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	5
134	RPS3	ribosomal protein S3	5
135	RRAS2	related RAS viral (r-ras) oncogene homolog 2	5
136	SFN	stratifin	5
137	SLC9A3R1	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	5
138	STOM	stomatin	5
139	TUBA1B	tubulin, alpha 1b	5
140	TUBB4	tubulin, beta 4	5
141	UBC	ubiquitin C	5
142	VPS28	vacuolar protein sorting 28 homolog (S. cerevisiae)	5
143	WDR1	WD repeat domain 1	5

5. Exosomes mediate cell-to-cell communication

Cellular interactions are pivotal for the progression, angiogenesis and invasiveness of tumors [21]. Such interactions are presumed to be regulated by membrane surface molecules (e.g., EGFR) and soluble secreted proteins (e.g., IL-12) that activate the target cells by interacting with the target cell surface receptors. Recently, another mode of intercellular communication that had gained immense scientific interest is mediated by exosomes. Possible mechanisms by which exosomes communicate with the target cell are shown in Fig. 3. As shown in Fig. 3A, exosomal membrane proteins can interact with the target cell in a juxtacrine fashion, thereby activating the target cell. Likewise, exosomal membrane proteins can be cleaved by proteases and the resulting fragment can act as ligands for cell surface receptor in the target cell (Fig. 3B). Interestingly, some of the exosomal membrane proteins are not identified in the cell surface of the originating cell (e.g., LAMP-2) [20]. Perhaps, this observation holds immense promise in terms of juxtacrine and ectodomain cleavage mediated cell-to-cell signaling events. In addition to juxtacrine and ectodomain cleavage-based signaling, exosomes can fuse with the target cell resulting in the non-selective transfer of exosomal proteins and RNA (Fig. 3C) to the target cell. Additionally, such fusion might change some of the membrane features of the target cell (e.g., arachidonic acid transfer from platelets-derived SMVs to leukocytes and endothelial cells [57]) including varied lipid concentrations and the transfer of exosomal membrane proteins on the target cell surface (e.g., CD41 antigen from platelets-derived SMVs to tumor and endothelia cell surface [58,59]).

6. Exosome purification protocols

Detailed biochemical and functional analyses of exosomes are confounded by the technical difficulty in isolating and

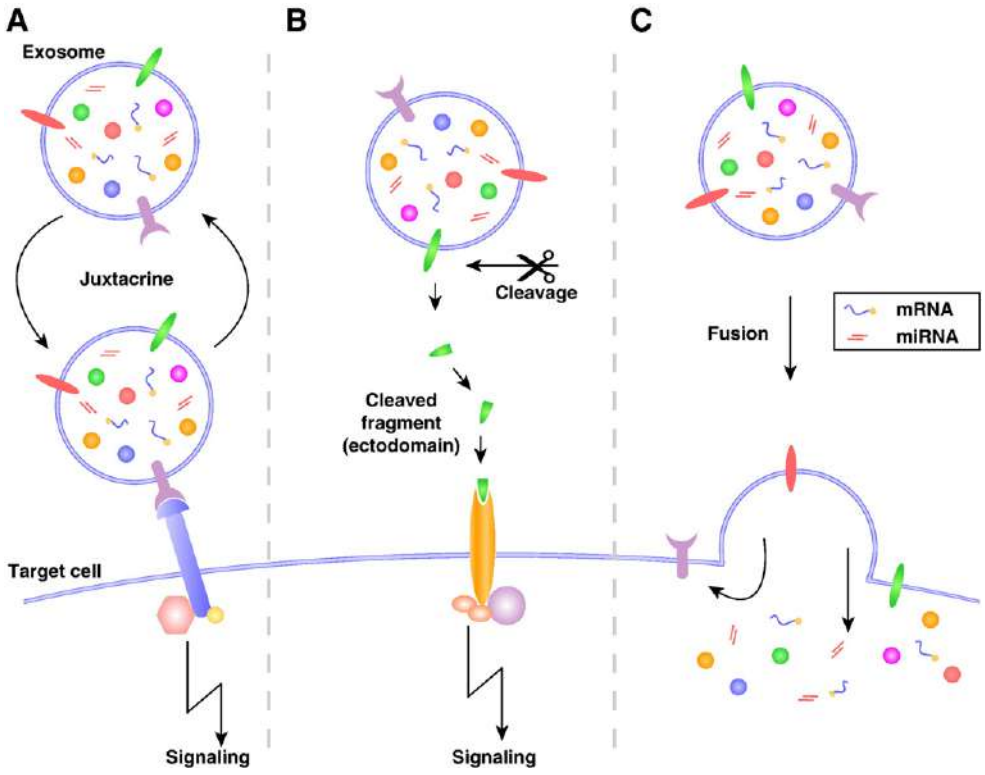

Fig. 3 – Possible mechanisms of intercellular communication by exosomes. A, Exosomal membrane proteins can interact with receptors in a target cell and activate intracellular signaling (juxtacrine fashion). B, Exosomal membrane proteins can be cleaved by proteases in the extracellular space. Cleaved fragments can then act as a soluble ligand which binds to the target cell surface receptor. This mechanism in turn activates the signaling cascade within the target cell. C, Exosomes can fuse with the target cell membrane and release their contents inside the recipient target cell in a non-selective manner. The surface membrane of the target cells in turn can be modified by the addition of new membrane receptors (from exosome membranes) and different lipid compositions. Exosomal molecules (protein, mRNA and miRNA) can activate a multitude of signaling events in the recipient target cell.

purifying them to homogeneity. Without stringent purification, exosomes are typically contaminated by other membranous vesicles such as SMVs and ABs. In contrast to intracellular organelles, which are purified from complex tissue homogenates, extracellular organelle 'exosomes' are relatively easy to purify. For example, by combining differential centrifugation, membrane filtration, concentration, rate zonal centrifugation and immunocapture, exosomes can be isolated from a multitude of cell line and body fluids [2]. Characterization of isolated exosomes is typically performed using electron microscopy, FACS, LC-MS/MS and Western blotting [2,4]. A major issue with these studies that limits comparative analyses is that diverse purification strategies are employed. For example, many exosome preparations are heavily contaminated with SMVs and ABs, and *vice versa*. Even though the biophysical properties of exosomes and other vesicles are distinct (Table 1), relatively a few studies have exploited biophysical properties such as flotation density to isolate and characterize exosome preparations (sucrose or OptiPrep density gradient). The majority of studies employ a simpler approach of differential centrifugation wherein the preparations can be significantly contaminated by SMVs, ABs and cellular debris. The far simpler approach of using membrane filters (0.1–0.2 μm) in combination with differential centrifugation could eliminate the large vesicles and result in a more

Table 3 – Recommended procedures for isolation/ purification of exosomes.

Method	Comments
Differential centrifugation	Differential centrifugation coupled with membrane filtration (0.1–0.2 μm) can eliminate large contaminating extracellular vesicles (SMVs). Small contaminating vesicles (ABs) can still be present in the preparation.
Rate zonal centrifugation	Pure exosomal preparation can be obtained (usually 1.10–1.21 g/mL). Exosomal marker proteins can be used to identify the flotation density of the exosomal population.
Immunoaffinity capture	Based on the specificity and availability of the antibodies, pure exosomal preparation can be obtained. The yield will be low as exosome subpopulations with the localized antigen of choice will be isolated.

reliable exosome preparation [40]. Table 3 lists the recommended exosome isolation and purification procedures.

Immunoaffinity capture using magnetic beads has been employed recently to isolate highly-purified exosomes [60,61]. For example, immunoaffinity capture based on the HER2 antibody was used to isolate tumor exosomes from breast adenocarcinoma cell line culture supernatant and ascites from ovarian cancer patients [61]. Similarly, A33 [62–64]-expressing tumor exosomes were collected from the culture supernatant of the colon carcinoma cell lines, LIM1215 [29] and LIM1863 (Simpson, unpublished data) using A33-antibody coated Dynabeads. Immunoaffinity capture-based exosome isolation can be performed in cell culture media containing fetal calf serum and high-M_r protein oligomers (e.g., haptoglobin), which may otherwise contaminate conventional preparations. By these means, exosomes can be purified free of contaminating large-M_r proteins and oligomers (e.g., proteosomal complexes) that co-sediment with exosomes at high centrifugal force [29]. Interestingly, the immunoaffinity capture approach resulted in depletion of histones (markers of ABs) as compared to crude exosomes (prepared by differential centrifugation and membrane filtration) which, presumably, resulted from contaminating ABs that are in the same size range as exosomes (50–100 nm diameter).

7. Clinical studies involving exosomes

It is well known that a variety of solid human tumors are spontaneously infiltrated by T cells and that memory effector T cells associate with favorable clinical outcomes while overwhelming regulatory T cells compromise long-term survival [65]. This has led to the identification of many tumor-associated antigens capable of eliciting cytotoxic T cell responses and vaccine immunotherapy as an approach to cancer treatment. Recently, there has been much interest in the application of exosomes as a potential viable vaccine for clinical immunotherapy; dendritic cell-derived exosomes are enriched in the components necessary to function as an antigen presenting entities [66]. Exosome-based clinical studies have been carried out on lung cancer and melanoma patients [67–70]. These clinical studies employed a robust macroscale-method, developed by Lamparski and colleagues for isolating clinical-grade dendritic cell-derived exosomes [68]. The method relies on a combination of ultrafiltration (500 K membrane) and ultracentrifugation into a 30% sucrose/deuterium oxide (98%) cushion (density, 1.21 g/mL) to harvest exosomes from dendritic cell culture supernatant in high yield (40–50%). Two Phase 1 clinical trials for melanoma and lung cancer, using peptide antigens [67,68], were completed using this purification strategy. Phase I clinical studies in melanoma patients showed that the injection of dendritic cells-derived exosomes is safe and associated with some tumor regression and long-term stabilization [69]. Importantly, the study revealed that the injected dendritic cells-derived exosomes significantly increased circulating natural killer cells and natural killer group 2 member D-dependent functions in the majority of melanoma patients [71]. Similarly, Phase I clinical trial that evaluated the tolerance of dendritic cell-derived exosomes in patients with stage III/IV lung cancer showed that the exosome injection is safe and allowed long-term stabilization in 4 of the 12 patients [70] (for a further commentary on clinical trials and application of exosomes, discussed at the 'Workshop on the Biological Significance of Exosomes in Montreal, Canada, May 20–21, 2005', refer to the review by Johnstone [72]).

A potential confounding factor in exosome isolation, particularly for clinical applications, is possible retroviral contamination. For example, exosomes and HIV-1 particles have similar biophysical properties such as size (30–100 nm and ~100 nm, respectively) and buoyant density (1.13–1.21 g/L [20,55] and 1.13–1.21 g/L [73], respectively), as well as their ability to activate immune cells. While earlier studies reported that exosomes carried virion cargo [35,74,75], recent exosome purification strategies deploying immunoaffinity capture [76] or a combination of immunoaffinity capture and density gradient centrifugation using iodixanol (OptiPrep™) [77] demonstrate that exosomes from haematopoietic cells can be purified free of virions like HIV-1.

Exosomes have been reported in diverse physiological fluids such as blood and ascites fluid. One of the first studies to provide evidence for exosomes in blood of healthy donors was performed by Caby and colleagues [78] using differential centrifugation or immunocapture using anti-CD63 mAb latex beads. These exosomes were characterized by electron microscopy (50–90 nm diameter), flotation density (1.15–1.27 g/mL) and proteome analysis (e.g., tetraspanins CD63, CD9, CD81, and LAMP-2 and class I and II MHC molecules). More recently, circulating EpCAM-positive exosomes were isolated from sera of patients with early stage ovarian cancer by a modified activated cell sorting (MACS) procedure using antibody beads [79]. Subsequent miRNA profiling of these exosomes indicate the potential use of circulating tumor exosomes as surrogate diagnostic markers for biopsy profiling and possible utility to screen asymptomatic populations [79]. In 2008, Levine and coworkers [80] reported the presence of 48 K TNFR1-containing exosome-like vesicles (density of 1.09 to 1.11 g/mL on sucrose gradient) in human plasma and also sera where they co-segregate with LDL particles. In more recent studies using a multidimensional purification scheme incorporating gel-exclusion chromatography, rate zonal centrifugation through continuous sucrose gradients and high-speed centrifugation, the same group reports a distinct population of PPARγ-containing exosomes in circulating human plasma [36]. Using anti-HLA class II mAb coated Dynabeads™, Klibi and colleagues report the immunomagnetic capture of galectin-9-containing exosomes in the plasma of nasopharyngeal (NPC)-patients or mice xenografted with NPC cell lines [81].

8. Exosomes as a rich source for discovering potential blood-based biomarkers

The release of exosomes into the extracellular space affords an opportunity to examine exosomes in body fluids such as blood, urine and malignant ascites. Accessing these bioactive vesicles in a non-invasive manner may lead to potential

diagnostic biomarkers of disease conditions. Exosomes were observed *in vivo* in blood from healthy donors [78] with similar biophysical properties as that of previously described exosomes released from various cell types *in vitro*. A magnetic bead immune capture strategy was employed to isolate circulating epithelial cell adhesion molecule-positive exosomes from the plasma of ovarian cancer [79] and lung cancer [82] patients. Interestingly, these studies revealed that plasma-exosome levels were increased in patients with advanced disease (e.g., mean 2.85 mg/mL exosomes for lung cancer adenocarcinoma patients compared with 0.77 mg/mL exosomes in the blood of normal volunteers [82]). These studies suggest that circulating exosomes in the body may play a role in pathogenesis and cell–cell or organ–organ communications by transporting molecules that need to reach distant cell targets [78]. The increased levels of tumor-derived exosomes in plasma and malignant effusions of patients with cancer [83] suggest that exosomes can be a rich source for the discovery of blood-based diagnostic biomarkers of disease (Fig. 4). Exosomes have also been isolated from physiological fluids such as normal urine [24,25,84,85], malignant and pleural effusions [83,86], bronchial lavage fluid [87], ocular fluids [88], human semen [89,90], amniotic fluid [91], human saliva [39,92], breast milk [37], pregnancy-associated sera [93] and synovial fluid [94]. Urine contains exosomes that are derived from various types of kidney cells that come into contact with the urinary space, including glomerular podocytes and renal tubule cells. In a recent study, prostate cancer biomarkers, PCA-3 and TMPRSS2: ERG, were detected in exosomes isolated from the urine of prostate cancer patients [95].

A possible obstacle in such body fluid based exosome analysis is the presence of contaminating exosomes secreted by normal cells (e.g., normal colon cells as compared to colon cancer cells) and other cell types (e.g., non colon cell types). To better understand the molecular properties of disease cell-derived exosomes, such exosomes need to be isolated free of normal cell-derived exosomes from the complex mixture in blood. Purification strategies that discriminate between complex exosome mixtures in blood could be aided by the knowledge of exosome tissue-specific signatures, especially disease cell signature, which can be obtained from the ExoCarta [41] compendium of exosomal proteins and RNA.

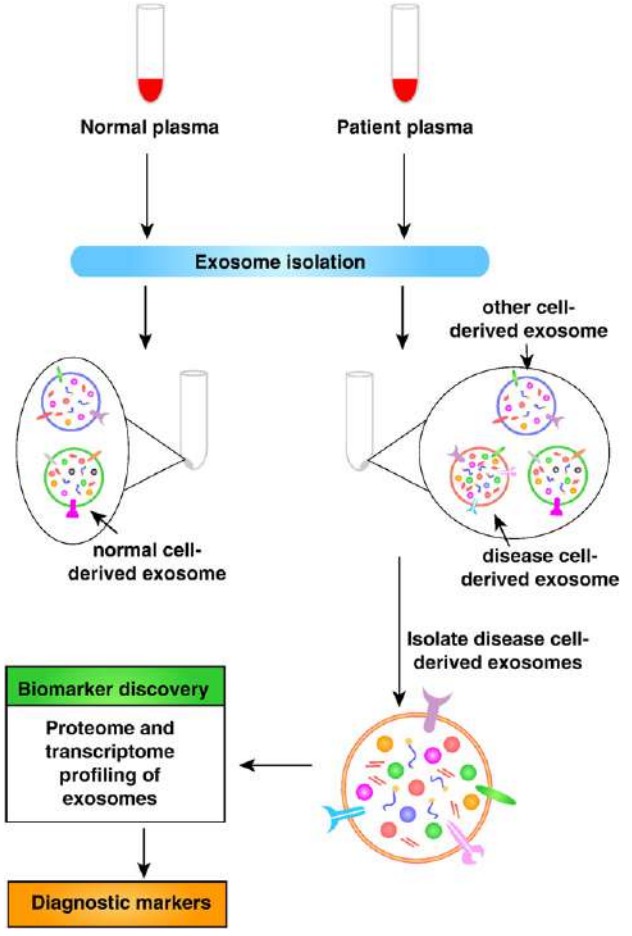

Fig. 4 – Circulating exosomes can be a rich source for identifying potential biomarkers. Patient plasma contains exosomes that are released by disease cells (e.g., colorectal cancer cells), normal counterpart (e.g., normal colon cells) and other normal cells (e.g., liver). Exosomal tissue signatures can be used to isolate disease cell-derived exosomes for proteomic and transcriptomic profiling.

9. Exocarta: a manually curated database of exosomal proteins and RNA

ExoCarta is a database of previous exosomal proteomic and transcriptomic studies. It is a manually curated repository containing proteins and RNA that were cataloged from 75 published exosome studies. A total of 2624 proteins, 901 mRNA and 274 miRNA from 4 different organisms are present in this compendium. Additionally, the compendium lists proteins that are more often identified in exosomal studies based on the number of occurrences of these molecules in the 75 published proteomic studies. Some of these molecules can be used as reliable exosomal markers, which is currently one of the important steps in characterizing the presence of exosomes in the preparation. Interestingly, tissue-specific proteins that are identified in exosomes are also listed in ExoCarta. For example, the A33-antigen is specific to colorectal cancer-derived exosomes [29] and future studies based on immunoaffinity capture of colon-specific exosomes can be performed with this knowledge. ExoCarta is freely available for the scientific community and the entire data encompassing 75 studies can be freely downloaded (http://exocarta.ludwig.edu.au).

10. Summary

Exosomes are secreted by various cell types and play important roles in cellular communication. Heterogeneous microvesicle studies over the years had led to confounding terminologies due to the cross-contamination of SMVs and, possibly, ABs. However, the known underlying mechanisms by which microvesicles are released into the extracellular space are limited to three: exocytic exosomes from the intracellular MVBs, SMVs from the PM and ABs from cells

undergoing apoptosis. Consensus in the nomenclature of naming these microvesicles is needed at this juncture and the onus is on the investigators to name the vesicle population correctly in order to avoid any further confusion. In contrast to SMVs and ABs, exosomes are relatively smaller in size and float at a different density (1.10 g/mL–1.21 g/mL). In addition to a common set of proteins, in spite of their cellular origin, exosomes also have tissue- or cell-type-specific protein/RNA molecules. Such attributes coupled with the extracellular location of exosomes, make them ideal starting materials for the identification of candidate biomarkers relevant to the pathophysiology of a specific disease. For approximately 42% of integral PM proteins found in exosomes, their corresponding soluble ectodomain can be found in the secretome (Mathivanan and Simpson, unpublished observations). A simple non-invasive approach can be employed to isolate exosomes for the purpose of identifying several candidate protein diagnostic biomarkers.

Keynotes

- Exosomes are defined as 40–100-nm diameter membrane vesicles of endocytic origin that are released from most cell types upon fusion of multivesicular bodies (MVBs) with the plasma membrane (PM). Within the MVBs, exosomes are referred to as intraluminal vesicles (ILVs); it is only upon release of MVB contents into the microenvironment that ILVs are referred to as exosomes. The nomenclature of exosomes can be confusing since typical exosomes are often referred to in the literature as 'microvesicles'.
- Exosomes have a cup-shaped appearance by electron microscopy, sediment at 100,000 *g*, and have a buoyant density in sucrose of 1.10–1.21 g/mL. While exosomes have a lipid bilayer with the same topography as plasma membranes, they are also reported to expose phosphatidylserine on their surface.
- It has been recently reported in oligodendrocytes that formation of MVBs destined for passage to the plasma membrane and release of their exosomes cargo into the microenvironment or those tagged for degradation by the proteosome machinery in lysosomes involve different ceramide-based molecular machineries. It is not clear whether this observation extrapolates to other cell types and whether there are two types of MVBs (exocytic and degradative MVBs) or different types of ILVs within MVBs.
- The protein composition of typical exosomes is often confounded by the exactness of the strategy employed in their purification. Because many high-M_r oligomeric proteins and viruses co-sediment with exosomes at 100,000 *g*, more robust purification methods such as immune capture and/or rate zonal centrifugation are essential for obtaining high-purity exosomes.
- Proteomic cataloging of highly-purified exosomes from urinary, mast cell and colorectal cancer cell lines has identified 31 membrane and cytosolic proteins common to these cell types. Additionally, exosomes exhibit protein signatures that reflect the originating cell type. A searchable compendium of exosomal proteins and RNA is now accessible at ExoCarta (http://exocarta.ludwig.edu.au). Typical protein markers for exosomes include Alix, TSG101, CD63, CD9, CD81 and HSP70.
- Exosomes contain inactive forms of both mRNA and microRNAs that can be transferred to a neighboring cell, conferring new functional properties to the recipient cell after the acquisition of the exosomal genetic material. MicroRNA profiling studies of disease cell-derived exosomes and exosomes circulating in blood offer the potential of exosomal microRNA profiles for use as diagnostic biomarkers of disease through non-invasive blood tests.
- Exosomes have pleiotropic effects that influence the physiology of neighboring cells. Of these, the best studied (*in vitro*) are the roles of exosomes in various stages of the immune response (interactions with immune cells). These range from exosomes being a vehicle for antigen presentation to antigen-independent roles that can inhibit (immunosuppressive properties) or promote immune responses (immune-activating properties). Additionally, exosomes play role in intercellular communication, being conveyors of proteins and lipids that affect downstream signaling events in recipient cells. They can also deliver genetic material that affects the physiology of recipient cells.

Box 1

Extracellular membranous vesicles: Membranous vesicles (exosomes, shedding microvesicles (SMVs) and apoptotic blebs (ABs)) that are secreted/shed by cells into the extracellular space.

Exosomes: Upon fusion of the MVBs with the plasma membrane (PM), the intraluminal vesicles (ILVs) are released into the microenvironment and are referred to as exosomes.

Shedding microvesicles (SMVs): Vesicles that are shed directly from the PM into the extracellular space.

Apoptotic blebs (ABs): Vesicles that are released by dying/apoptotic cells.

Microvesicles: Mixed population of exosomes, SMVs and ABs.

Box 2

ExoCarta: a database for exosomal proteins and RNAs

- ExoCarta is a web-based freely accessible compendium the published protein, mRNA and miRNA content of purified exosomes from a multitude of cell line and body fluids.
- While the onus for the *quality* of data contained in the database resides with the researchers who published the data, the database catalogs data from manual curation of scientific literature.
- Every molecule entry in ExoCarta is accompanied by information pertaining to the gene information, database cross references, gene ontology annotations, protein–protein interactions and experiment description.

- ExoCarta provides biomedical researchers with information pertaining to isolation/purification methods employed in the published exosomal studies (this information allows researchers to evaluate the purity of any exosome preparation).
- Data present in ExoCarta can be freely downloaded from the ExoCarta website.

What ExoCarta is not?

- ExoCarta does not address data quality — the onus of data quality resides with the researcher(s) responsible for acquiring and publishing the data.
- ExoCarta has not cataloged proteins/RNA from non-exosome extracellular membranous vesicle studies.

ExoCarta URL: http://exocarta.ludwig.edu.au

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