

Exosomes: an overview of biogenesis, composition and role in ovarian cancer

Beach *et al.*



REVIEW

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Exosomes: an overview of biogenesis, composition and role in ovarian cancer

Allison Beach¹, Huang-Ge Zhang², Mariusz Z Ratajczak³ and Sham S Kakar^{3*}

Abstract

Exosomes are tiny membrane-bound vesicles that are over produced by most proliferating cell types during normal and pathological states. Their levels are up-regulated during pregnancy and disease states such as cancer. Exosomes contain a wide variety of proteins, lipids, RNAs, non-transcribed RNAs, microRNAs and small RNAs that are representative to their cellular origin and shuttle from a donor cell to a recipient cell. From intercellular communication to tumor proliferation, exosomes carry out a diverse range of functions, both helpful and harmful. Useful as biomarkers, exosomes may be applicable in diagnostic assessments as well as cell-free anti-tumor vaccines. Exosomes of ovarian cancer contain different set of proteins and miRNAs compared to exosomes of normal, cancer-free individuals. These molecules may be used as multiple “barcode” for the development of a diagnostic tool for early detection of ovarian cancer.

Keywords: Exosome, Ovarian cancer, Cancer, Microvesicle, Cancer diagnosis

Introduction

Exosomes, like the intraluminal vesicles range approximately from 30-100 nm in diameter secreted by live cells and were first observed in the early 1980s. Secretion of exosomes from cells was proposed to be a mechanism through which cells discard their garbage [1-4]. However, in recent years, exosomes have emerged as important molecules for inter-cellular communication that are involved both in normal and in pathophysiological conditions, such as lactation [5], immune response [6] and neuronal function [5], and also in the development and progression of diseases such as liver disease [7], neurodegenerative diseases [8] and cancer [9,10]. They exhibit a round, saucer-like morphology and are encapsulated by a lipid bilayer [10,11]. Exosomes are found in higher concentrations in the peripheral circulation during pregnancy and cancer [12]. A large number of biological functions of exosomes have been demonstrated however, in this review we will focus discussing on the functions of exosomes in ovarian cancer and immune tolerance related to cancer.

Exosome biogenesis and secretion

Exosomes in eukaryotic cells, throughout their life cycle periodically engulf small amounts of intracellular fluid, forming a small intracellular body called an endosome [13,14]. As the early endosome matures and develops into the late endosome, it becomes characterized by the formation of intraluminal vesicles (ILV) inside the lumen of the endosome. Ranging from 30-100 nm in diameter, the ILVs are formed by inward budding of the endosomal membrane, randomly engulfing portions of the cytosolic contents and incorporating transmembrane and peripheral proteins into the invaginating membrane. The transformation into the late endosome can also be detected by observing a change in its shape and location: the early endosome is tube-like in shape and is usually located in the outer portion of the cytoplasm, whereas, the late endosome is spherical and found closer to the nucleus [14]. The late endosome containing ILVs is also called as multivesicular body (MVB) [15,16].

The fate of the MVB may vary. Typically, it fuses with the lysosome and its contents are degraded by the hydrolysis inside the lysosome. It has been proposed that the contents of the MVB are sorted; proteins destined for degradation by the lysosome and are found in the ILVs, whereas proteins that may have another function or role are found outside the ILVs of the MVB. However, the mechanism by

* Correspondence: ssakaka01@louisville.edu

³Department of Medicine, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA

Full list of author information is available at the end of the article

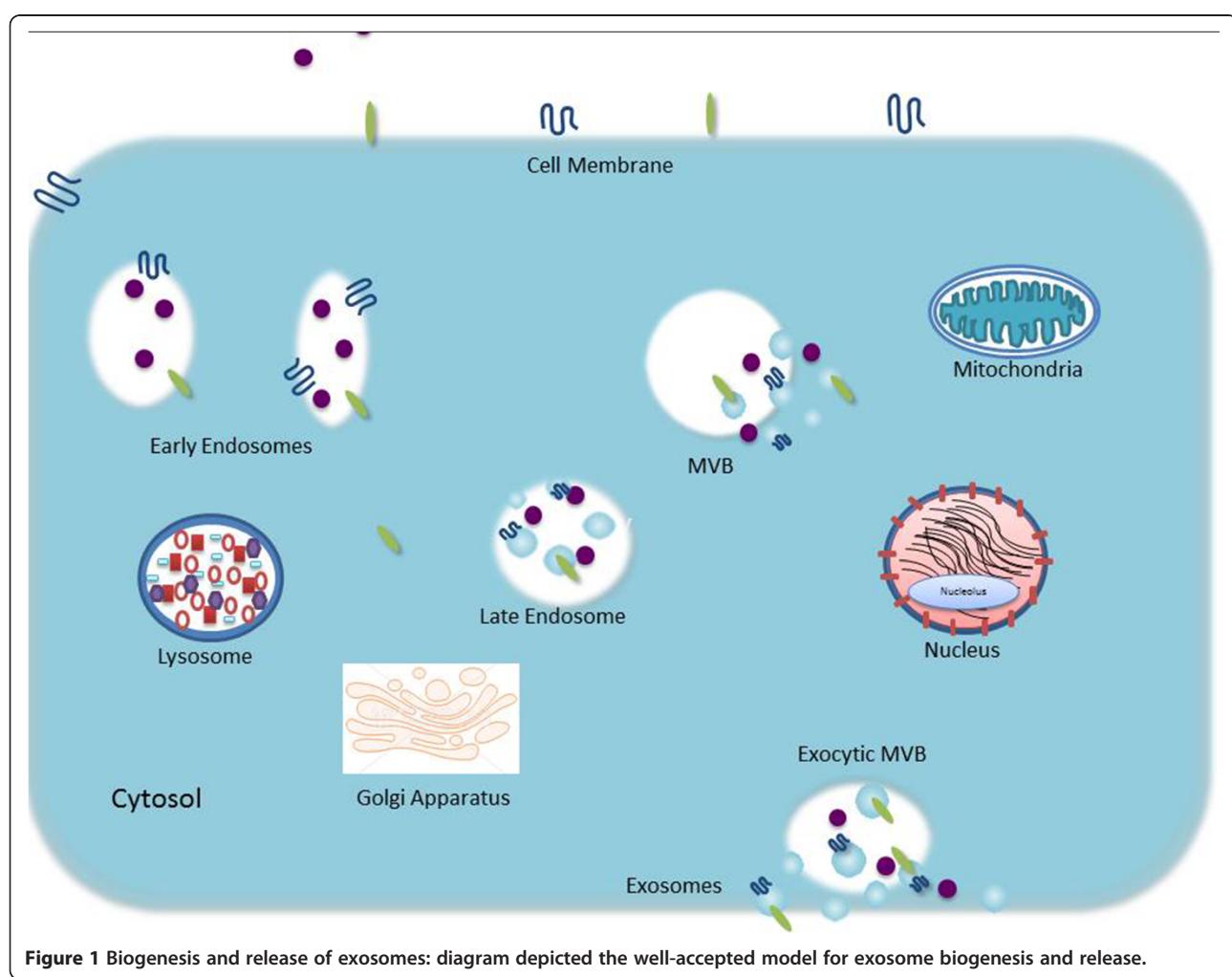
which this process occurs is not yet completely understood. Possible theories involving the sorting of proteins have been proposed [17,18].

As an alternative to fusion with the lysosome, the MVB may fuse with the plasma membrane of the cell in a proposed ion-dependent manner instead, releasing its ILVs in an exocytotic fashion to the extracellular environment (Figure 1). These vesicles are then referred as exosomes [15]. Demonstrated both *in vivo* and *in vitro*, exosomes are secreted during both normal and pathological conditions and by a wide array of cell types [19]. First discovered in maturing mammalian reticulocyte (immature red blood cells) exosomes are secreted by wide range of mammalian cell types, including B and T cells [20], dendritic cells [16], mesenchymal stem cells [21], epithelial cells [22,23], astrocytes [24], endothelial cells [25] and cancer cells [12,26-37]. Exosomes have also been identified in most bodily fluids including urine and amniotic fluid [38], blood [39], serum [12,40], saliva [41,42], ascites [28,43], breast milk [6], cerebrospinal fluid [44,45] and nasal secretion [46]. Importantly,

cancer cells have been shown to secrete exosomes in greater amounts than normal cells [12,47].

Exosomes are mainly secreted by two different mechanisms, constitutive release via the Trans-Golgi network and inducible release [48,49]. Studies from Thery and colleagues demonstrated that a number of Rab family proteins, including Rab27a and Rab27b, act as key regulators of exosomes secretion [50]. Apart from Rab 27a and 27b, another Rab family member, Rab 35 and Rab 11 have also been shown to regulate the secretion of exosomes by interacting with GTPase-activating protein TBC1 domain family member 10A-C (TBC1D10A-C) [51,52]. The Rab family of proteins are often mutated (constitutively active) or over-expressed in cancer cells.

It has also been shown that activation of the tumor suppressor protein, p53, stimulates and increases the rate of exosome secretion by regulating transcription of the various genes such as TSAP6 and CHMP4C, which activate exosome production [53]. If the cell experiences stresses such as hypoxia or toxic stress, DNA may become damaged. The p53 protein responds to this



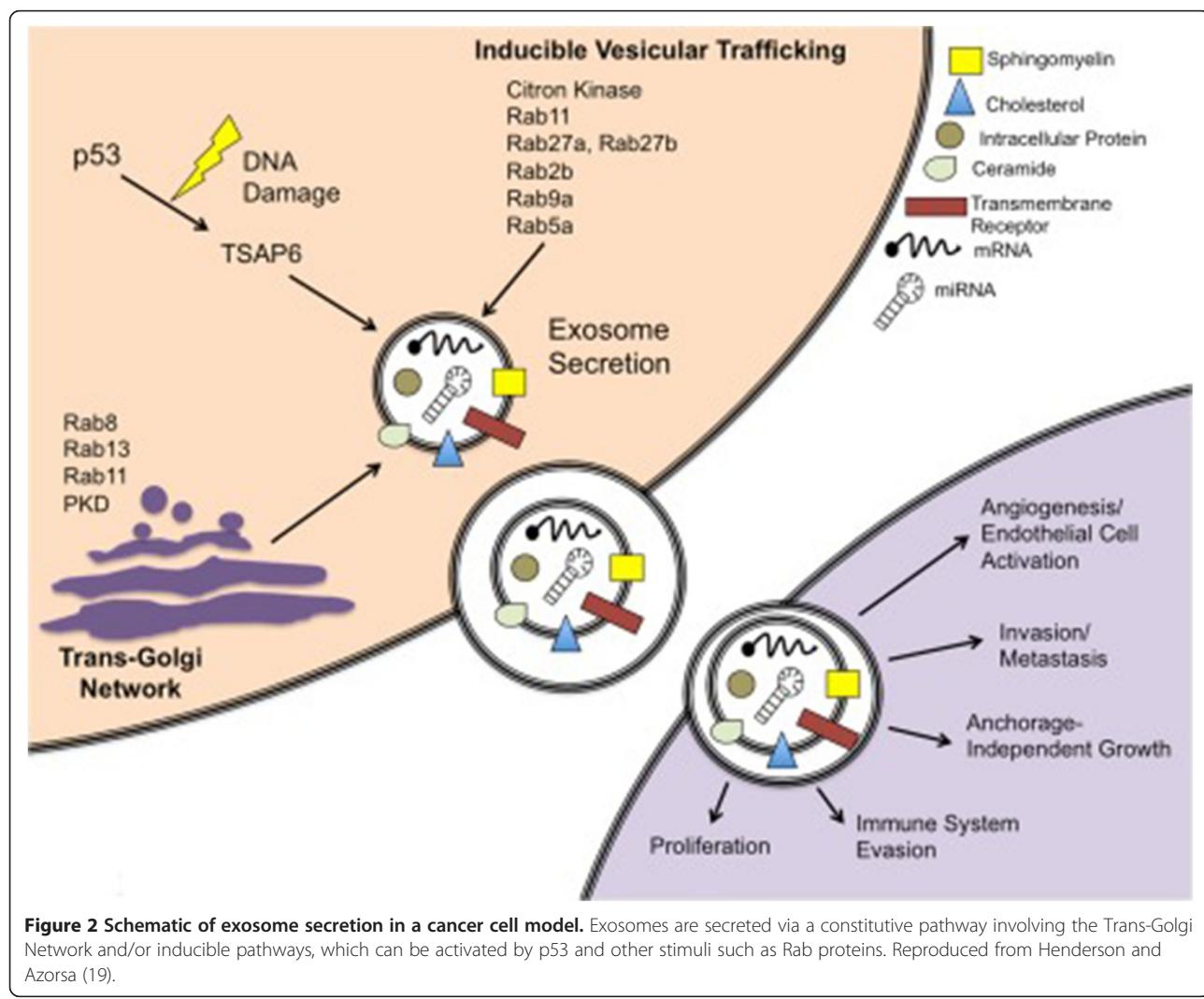
process by regulating the transcription of number of genes (Figure 2). This results in what is known as “by-stander effect”, during which the cell secretes certain proteins that communicate with nearby cells to help in recruiting them for a response to compensate the stress [54]. Irradiation of human prostate cancer cells strongly indicated DNA damage that could indeed induce a p53-dependent increase in exosome secretion by activating the cells [54]. In addition, secretion of exosomes appears to be increased by K⁺-induced depolarization in neuronal cells, and the crosslinking of CD3 with T cells, and intracellular Ca²⁺ levels [14,15]. Various other stimuli and change in membrane pH have also been shown to trigger the secretion of exosomes from various cell types [55,56].

Once exosomes are released from the parent cells, they carry out a variety of functions and may eventually be taken up by another cell [57] (Figure 2). It has been reported that certain exosomes, such as those that have been secreted by tumor cells, carry phosphatidylserine

(PS) on their membranes. PS acts as a signal and has a large role in exosome uptake by certain cells [58]. When an exosome is taken up by another cell, the entire exosome may be endocytosed and re-located to clathrin-coated pits, or may simply release its inner contents into the cell and remain united with the cell membrane [57].

Composition of exosomes

A large amount of exosomal proteins have been identified, and the consistency among exosomes stemming from like-cell types suggests that a sorting mechanism is acting during exosomal development. TSG101 and Alix have been identified in exosomal analysis and are known components of the *endosomal sorting complex required for transport* (ESCRT) machinery. ESCRT functions to sort the cargo proteins of ILVs. It works at the endosomal/MVB membrane and recognizes ubiquitinylated proteins that are associated with tetraspanins and possibly those which are GPI-anchored, arranges them in



such a way on the membrane that they are fated to become contents of exosomes [15,17].

The function of the original cell from which an exosome originate can be deduced by the makeup of proteins, lipids, miRNAs, mRNA, non-coding RNAs and other molecules found in a particular exosome. Exosomes contain many different cell surface molecules and are able to engage many different cell receptors simultaneously. This allows them to participate in the exchange of materials between cells, for example proteins, lipids, carbohydrates, and pathogens [13]. Recent information from different cell type reveals that exosomes contains 4,563 proteins, 194 lipids, 1639 mRNA and 764 microRNA [59,60] demonstrating their complexity. Exosomes are rich in molecules that are involved in antigen presentation, such as CD1 and the major histocompatibility molecules (MHC) class I and II, which play a key role in immune-regulation by possessing antigenic peptides [61]. MHC I also may be involved in the presentation of antigen fragments to T cells [62,63]. Also exosomes contain tetraspanins CD9, CD63, CD81, and CD82, as well as co-stimulatory molecules (CD86) and adhesion molecules CD11b and CD54 [33,61,62]. Other common exosomal proteins include heat shock proteins HSP70 and HSP90, which expedite peptide loading onto MHC I and II, as well as play a role in the cellular response to environmental stresses. Inside the cell, HSPs assist in protein folding and trafficking, acting as chaperones [62,63]. Exosomes in general also contain cytoplasmic proteins such as annexins and rab proteins, both of which may promote the fusion of MVB with the cell membrane and expulsion of exosomes. In the last few years, exosomes have been reported to contain nucleic acid such as DNA, RNA, miRNA and none coding RNA [12,58,59,64-69] in addition to proteins. RNA containing exosomes may represent alternate pathway of cellular communication with significant implications in the modification of cell phenotypes. The mRNAs found in exosomes are found to be functional and could be transferred to target cells and translated into proteins [70,71]. Specific miRNA such as let-7, miR-1, miR-15, miR-16, miR-151 and miR 375 which play role in angiogenesis, hematopoiesis, exocytosis and tumorigenesis have been reported in exosomes [65]. Along with DNA, RNA, miRNA, cytoplasmic tubulin, actin, and actin-binding proteins are also commonly found in exosomes [12,58,59]. Membrane proteins CD55 (decay accelerating factor) and CD59 have been reported in exosomes which provide protection against the complement system and thus are an instrumental part in the stability of the exosome outside the cell [15]. Exosomes also commonly contain signal transduction proteins such as G-proteins and protein kinases [58,59]. In addition, exosomes tend to have a rich content of proteolysis enzymes, suggesting

that exosomes may increase cell migration [10,13]. It is to note that exosomes do not contain mitochondrial, nuclear, and endoplasmic proteins [13].

Exosomes derived from B cells, T cells, dendritic cells, intestinal epithelial cells and many other cell types contain specific-proteins pertaining to their particular functions in addition to commonly found proteins in exosomes. Exosomes secreted by intestinal epithelial cells display syntaxin 3, C26, and the A33 antigen, depending upon which side of the epithelial membrane it is released such as apical or basolateral [15]. Exosomes from T lymphocytes contain many T cell receptors, indicating exosomes role in intercellular communication. Exosomes secreted by dendritic cells contain CD80 and CD86 proteins, which are involved in the activation of naïve CD4⁺ T cells [10]. Microglial cells appear to release exosomes, which display CD13, the active aminopeptidase that cleaves neuropeptides [15]. Some of the major proteins reported in the exosomes are shown in Table 1.

Functions of exosomes

Exosomes are involved in many physiological processes, both beneficial and pathological. Initially, exosomes were characterized as a means for the maturing reticulocyte to get rid of superfluous proteins. Studies have now shown that exosomes are involved in elimination of unnecessary proteins or unwanted molecules from the cell, exchange of materials between cells, intercellular communication, propagation of pathogens, functions of the immune system (both stimulatory and inhibitory), antigen presentation, and many more. By observing the varying protein contents, it is quite easy to understand how exosomes that come from different cell types can have differential functions [13,62] and prove to be highly useful molecules in various biological functions.

Exosomes in ovarian tumor

There is a paradox about exosomes that some may have beneficial effects, such as those released from B cells and dendritic cells. These have powerful anti-tumor and immune-stimulatory effects and the latter also provides co-stimulatory proteins that are used to activate T cells when presented with tumor antigens. On the other hand, exosomes secreted from tumor cells have been observed to enhance tumor invasiveness and angiogenesis, while suppressing the immune response [72]. Breast cancer tumor-derived exosomes and prostasomes (exosome-like vesicles released by the prostate) can also promote angiogenesis in individuals with cancer, whereas prostasomes in a healthy individual inhibit angiogenesis, enhance sperm motility, and delay and stabilize the acrosome reaction, as well as act immune suppressive in its local environment [35,57]. It is not only cancer cell-

Table 1 Some of the common proteins in exosomes

Antigen presentation	MVB formation	Signal transduction	Heat shock	Tetraspanin	Adhesion molecules
MHC I	Alix	Gi2a, Gi3a, Gsa, FRL, Erk2, Fyn	Hsp70	CD9	CD146, CD166
MHC II	TSG101		Hsp84	CD37	ICAM-1, ALCAM
CD86	Gag	Sh2 phosphatase	Hsp90	CD53	MAC-1, Integrins a3, a4, aM, b1, b2
		RhoA, C, GDI		CD63	
		Syntenin, CBL		CD81	LFA3, CD53,
		Catenin, LCK		CD82	CD326
					CD11a, b, c
Membrane	Cytoskeletal	Miscellaneous		Lipid Raft Associated	Enzymes
Transport & Fusion	Proteins				
AP-1, Arp2/3, SNAP	Radixin	Thioredoxine	P-Selectin	LBPA	ATP Citrate lyase
Syntaxin	Advillin	peroxidase	CD18, CD147	Cholesterol	ATPase
			Clathrin		
Dynamin	Vimentin	Histone 1, 2, 3	Complement factor 3	Flotillin-1	G6P Isomerase
Rab5,7, Rap1B	Talin, CAP1	MVP, CD26, CD13	CD55	Stomatin	Peroxiredoxin 1
RabGDI	Ezrin, Actin		CD59		Asp amino- transferase
Annexins (I, II, IV, V, VI)	Tubulin	Ferritin light chain 1, 2			a-enolase
	Moesin				Aldehyde reductase
	Cofilin				FA Synthase
References:	15, 6, 72,73				Pyruvate kinase

derived exosomes that have pervasive qualities; exosomes secreted by platelets have demonstrated to promote tumor growth and metastasis of lung cancer cells [55].

Tumor cell-secreted exosomes include many of the common exosomal proteins; they also contain tumor antigens that are reflective of the tumors they are derived from [13]. The heat shock proteins (HSP) found in tumor exosomes are taken up by dendritic cells and macrophages, which process and present the HSP to the lymph nodes. The HSP then release the tumor-specific antigens they hold, which are presented to cytotoxic T cells leading to their activation. Studies have demonstrated the elimination of various cancers using HSP-stimulated immune cells [62].

Exosomes released by tumor cells are quite unique and have recently come into the spotlight, with many groundbreaking discoveries of their unique proteins and their potential to be diagnostic biomarkers. Tumor exosomes are known to have a significant role in the communication and interaction with tumor cells, immune cells and its surrounding environment. In cancer cells, exosomes entail the transfer of cancer-promoting cellular contents to surrounding cells within the tumor microenvironment or to the circulation to act at distant sites, thereby enabling cancer progression. Several studies have demonstrated the presence of exosomes in

ovarian cancer cell cultures and ovarian cancer patients' plasma/serum or ascites [12,38,39,43,58,62,63,73-76] in addition to many other cancers. Three to 4-fold higher amount of exosomes has been reported in circulation of patients with ovarian cancer compared to normal individuals [77]. Ovarian cancer is one of the most lethal forms of cancer in women, and until recently, lacked consistent biomarkers useful for its detection. Approximately 70% of cases of ovarian cancer are diagnosed in advanced stage, which has only a 20% survival rate within 5 years of diagnosis. However, if diagnosed at early stage (at Stage I), survival rate is over 90% [12]. Therefore, early diagnosis using exosomal contents may be highly important and may save large number of patients dying from ovarian cancer due to late diagnosis. Exosomes provide stable, disease-specific markers for detection, disease characterization, and disease prognosis [73]. Even though change in exosome content in circulation can be used as a diagnostic tool to detect cancer, but measuring the content of circulating exosomes is not very specific and authentic technique. However, proteomic and genetic profiling of circulating exosomes can provide better diagnosis and monitoring of therapeutic response and diagnostic tool [73].

Recent discoveries of the presence of microRNAs, epithelial cell adhesion molecule (EpCAM) and CD24 in ovarian tumor-derived exosomes have been highly promising

alternatives for early detection of ovarian cancer. EpCAM is a glycoprotein that facilitates homo-typical adhesion of cells. It has been found in pseudo-stratified, transitional and simple epithelia on the basolateral surfaces and has been identified as a cargo protein in exosomes. EpCAM is expressed to a certain degree in the normal epithelia of many organs and is highly overexpressed in multiple types of carcinomas. The overexpression is correlated with an escalation of epithelial cell proliferation, as occurs in tumor development [66]. Studies by some investigators have used the magnetic activated cell sorting procedure (MACS) using magnetic micro-beads coated with EpCAM antibodies to isolate exosomes that specifically express EpCAM [12,77]. Results from these studies showed very low levels of EpCAM (0.039 ± 0.030 mg/ml) in exosomes of normal individuals and an increase in levels related to stage and severity of ovarian carcinoma, reaching a significantly higher level of 1.42 ± 0.228 mg/ml for Stage IV ovarian carcinomas. This indicates that EpCAM can be useful as a biomarker for the diagnosis of ovarian cancer as well as the detection of tumor-derived exosomes [12,66]. High EpCAM concentration is usually associated with a high concentration of CD24 [43]. CD24 is recognized as a tumor marker and is associated with poor prognosis of ovarian carcinomas. It is also expressed by many other types of malignancies as well as by some normal cell types. CD24 is a glycoprotein linked to the cell membrane via a GPI-anchor. CD24 can also be found in the cytoplasm inside MVBs and released into the extracellular environment via exosomes, in which case it is correlated with more aggressive forms of ovarian carcinoma, worsening the prognoses and therefore, shortening patients' survival times [43].

Presently, ~2,000 microRNAs have been described in human [78] microRNAs have been reported to play important role in cellular and developmental processes, including developmental timing, organ development, differentiation, proliferation, apoptosis and immune regulation [67]. Recently, Taylor and his colleagues reported the presence of diagnostic miRNAs from EpCAM-positive exosomes from ovarian cancer patients' serum [12]. The discoveries for the presence of microRNAs, EpCAM, and CD24 in ovarian tumor-derived exosomes have provided promising alternatives to early detection. MicroRNAs are small non-coding RNAs ([22-25] nucleotides) that are important in the regulation of cellular processes such as differentiation, proliferation, cell death, and maturation. MicroRNAs from normal cells and tumor cells of the same tissue type have remarkably different makeup. These findings, along with the previous knowledge of the secretion of exosomes by tumor cells, have recently led to research that microRNAs may be useful as biomarkers. Ideal microRNAs used for diagnosis purpose would be absent or present at non-detectable level in the circulating exosomes of cancer-free individuals, but are aberrantly expressed in ovarian cancer and it is postulated

that they may be useful in the diagnosis and prognosis of ovarian cancer. There are 8 microRNAs (miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205 and miR-214) that are confirmed to be diagnostic [12]. The levels of these microRNAs in serum differ slightly between the early and late stages of ovarian cancer, but differ significantly between exosomes from individuals with benign disease and those with advance stages of malignancy. Specific microRNAs have been profiled from a direct tumor tissue samples and compared to microRNAs from exosomes from the peripheral circulation. Results showed that the microRNA from tumor-derived exosomes are a correct reflection of the microRNA from the tumor, indicating that profiling the exosomal microRNA may serve as a useful tool for early diagnosis of this deadly disease instead of sampling of ovarian cancer tissues [12].

Exosomes in immune tolerance

While some exosomes have demonstrated immune-stimulatory effects, whereas, others, such as exosomes released by intestinal epithelial cells have demonstrated the ability to induce antigen-specific tolerance. In a key study by Karlsson, these so-called "tolerosomes" were shown to carry MHC II molecules and thus assist the body in building a tolerance to food antigens [36]. Exosomes have also been reported to stimulate an immune tolerance during pregnancy [79-82].

Interestingly, in contrast to intestinal epithelial cell-derived exosomes helping to create antigen-specific tolerance, exosomes released from dendritic cells that are mounted with tumor peptides produce a specific anti-tumor response *in vitro* in human [10]. Findings by Andre and colleagues used exosomes from dendritic cells that had been pulsed with tumor peptides showed a tumor-rejection effect in patients with melanoma, prostate cancer, lymphoma, and renal cancer [33], indicating that exosomes secreted by tumor cells have the ability to stimulate an anti-tumor response by transferring tumor-specific antigens to dendritic or other antigen-presenting cells, initiating a specific immune response and thus have much potential in cancer immunotherapy [10,11]. In order for a T cell-mediated immune response to a tumor to occur, the antigen-presenting dendritic cells must be presented and then process a tumor antigen, which then may be cross-presented on MHC-I molecules to activate a T cell response, which has been demonstrated in an *in vitro* model [37].

In addition to exosomes acting as immune-stimulants, they have also demonstrated the ability to encourage tolerance in the immune system. Exosomes introduced to a patient from a donor prior to transplant surgery allow the patient longer transplant acceptance time by the immune system [71]. Studies using a rat model have shown that rat treated with bone marrow-derived exosomes prior to a

heart allograft procedure showed an increase in alloantibody production, but also experienced a decreased anti-donor T cell response, and longer graft survival [83].

Another means of tumor promotion by exosomes is their capability to suppress the immune response. Experiments performed by Liu et al. [84] indicated an exosome-mediated reduction of natural killer (NK) T cells, unrelated to actions of T-regulatory cells. The same group found tumor exosomes derived from myeloid-derived suppressor cells (MDSCs) [85] and Clayton and colleagues found tumor exosomes derived from mesothelioma [85] to express (membrane-associated) Transforming Growth Factor- β (TGF β ₁). Instead of acting as a cell cycle regulator in normal cells to induce apoptosis or cease cell proliferation, TGF β ₁ from tumor exosomes assists in immune response evasion by tumor cells and also induces an antiproliferative effect on tumor growth and lymphocytes. The exosomal, membrane-associated form of TGF β ₁ was more potent in its inhibitory effect than the soluble form at significantly lower doses [86].

Another proposed method of immune evasion by tumor exosomes is induction of T cell apoptosis. It is known that tumor exosomes are able to inhibit T cell proliferation. Many cancers coincide with notable amounts of Fas Ligand (FasL), which is correlated with poor prognosis [87]. FasL is a transmembrane protein in the tumor necrosis factor family. FasL is found in the membrane of exosomes that are released from tumors and it has been concluded that FasL does induce apoptosis of T cells, suppressing the immune response [16,85,88,89].

Another mechanism of immune defense involves antigen-presenting cells. Dendritic cell-derived exosomes express MHC I and II and T cell co-stimulatory molecules and deliver these complexes to dendritic cells, which are capable of activating CD4 $^{+}$ and CD8 $^{+}$ T cells and suppressing tumor growth [16,90,91] (Table 1). However, if antigen-presenting cells such as dendritic cells are not involved, tumor-derived exosomes may possess a self-promoting function. Studies have recently stressed the importance of microvesicles (exosomes) secreted by tumor cells an important means of communication between tumor cells and their environment.

Exosomes and microvesicles

In this review we focused on exosomes that are secreted from the inner cell membrane compartment. We did not discuss involvement of larger vesicles (100 nm-1 μ m) that are secreted from the cell surface membranes. Augmenting evidence indicates that both types of vesicles - smaller (exosomes) and larger (microvesicles) are secreted from the activated cells. Since ovarian cancer cells express highly tissue factor (TF) [92], they can easily activate blood platelets that are rich source of both exosomes and microvesicles. It has

been demonstrated that TF-directed activation of platelets enhances in exosome/microvesicle-dependent manner metastasis of several types of cancer cells [86,93,94]. Moreover, a concept has been presented that tumor growth and expansion is directed by a network of extracellular vesicles secreted by various types of normal and malignant cells present in tumor microenvironment [95]. Its involvement in progression of ovarian cancer needs further studies.

Exosomes and therapies

Research and clinical studies have already proven exosomes to be an accessible and reliable source of biomarkers and early diagnosis of disease, with many more avenues yet to be explored. Exosomes bode well in their role as a means of intercellular communication; they contain many different sets of molecules that have roles in various cellular processes and possess the ability to carry antigenic information. They have the advantage of being nonliving and they are easily recovered from biological fluids [15]. Perhaps the most advantageous of exosomal possibilities is their potential to serve as a key factor in finding a cure for cancer. Tumor-derived exosomes have been used to carry tumor antigens and present them to T cells, priming them to induce the anti-tumor response, resulting in tumor cell death [10,15,62]. Their ability to do this suggests that tumor-derived exosomes may be useful in developing a cell-free anti-cancer vaccine [96,97].

With the growing knowledge of exosomes functions and the inner workings of the immune response, immunotherapeutic treatments are continuously being investigated and developed. Vaccines are being created on the basis of the knowledge of antigen presenting dendritic cells that have the ability to activate CD4 $^{+}$ and CD8 $^{+}$ T cells, as well as natural killer cells. In a phase I clinical trial, dendritic cell exosomes demonstrated the ability to activate NK cell proliferation and activation, shown by an increased quantity of circulating NK cells in patients. By observing such role of exosomes, it is plausible that in near future, a cell-free anticancer vaccine will be available.

Conclusions

Because of multiple biological and therapeutic functions of exosomes, studies on application of exosomes are moving forward with new applications of exosomes. Identification of new proteins and biomolecules in exosomes and novel mechanisms by which they communicate and deliver their functions are of great interest for diagnosis, drug delivery and therapeutic purposes.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AB prepared the first draft of the manuscript; SSK and MZR participated in its design and coordination, and wrote and revised the manuscript; HGZ provided in depth input and edited the manuscript. All authors read and approved the final manuscript.

Author details

¹Department of Physiology and Biophysics, James Graham Brown Cancer Center, University of Louisville, 505 South Hancock Street, Louisville, KY 40202, USA. ²Department of Microbiology and Immunology, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA.
³Department of Medicine, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA.

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