Exosomes: biogenesis, function and therapeutic potential in cancer

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Abstract

Exosomes are extracellular vesicles that are secreted by eukaryotic cells to mediate cell communication, immune responses and cell signaling for regeneration and cell differentiation. Exosomes are between 30-150nm in size and are transported via body fluids to their recipient cells. Currently, there are two known pathways for exosome production, namely the ESCRT pathway and a ceramide-dependent pathway. Exosomes can be internalized by ligand-receptor interaction, fusion with the outer membrane via lipid raft endocytosis, clathrin-dependent endocytosis and phagocytosis. Tumor-derived exosomes can drive cancer progression by facilitating metastasis, angiogenesis and have immunosuppressive effects. Additionally, tumor-derived exosomes consist of tumor antigens that can be utilized to trigger an anti-tumor immune response. This review provides an overview of the biogenesis, function and therapeutic potential of exosomes to treat human cancer cells.

Abbreviations: **CAFs**: Cancer-associated fibroblasts; **CTL**: Cytotoxic T-lymphocyte; **DCs**: Dendritic cells; **DC_{ex}**: Dendritic cell-derived exosomes; **EMT**: Epithelial to mesenchymal transition; **ESCRT**: Endosomal sorting complex required for transport; **Gag**: Group-specific antigen; **HNSCC**: head and neck squamous cell carcinoma; **iDC_{ex}**: Immature dendritic cell-derived exosomes; **ILVs**: intraluminal vesicles; **mDC_{ex}**: Mature dendritic cell-derived exosomes; **MHC**: major histocompatibility complex; **MSC**: mesenchymal stem cells; **MVB**: multivesicular bodies; **Nef**: Negative regulatory factor; **NK**: natural killer; **OVA**: ovalbumin; **PDAC**: pancreatic ductal adenocarcinoma; **TAA**: Tumor-associated antigen; **TEX**: tumor-derived exosomes; **TGF-β**: transforming growth factor-β; **TSA**: Tumor-specific antigen

Exosome biogenesis

Exosomes are a type of extracellular vesicles (EVs) that are secreted by almost every eukaryotic cell type. Exosomes transport nucleic acids, proteins, lipids and metabolites to distant parts of the body to mediate cell-cell communication, differentiation, regeneration and immune responses [1]. Exosomes derived from endosomal vesicles have a diameter between 30-150nm and consist of a lipid bilayer covered with a variety of surface molecules, such as cholesterol, ceramide and proteins. These surface proteins protect the exosome from being degraded by digestive enzymes, enzymes in the bodily fluids and immune cells. The composition of these surface molecules differs between exosomes originated from different cell types [2].

Before being secreted into the extracellular matrix, exosomes are localized in multivesicular bodies (MVB) as intraluminal vesicles (ILVs). ILVs are formed when the membrane of an early endosome invaginates and buds inwards into the MVB lumen. The invagination can be facilitated by the endosomal sorting complex required for transport (ESCRT)-dependent pathway or by the ESCRT-independent pathway. The ESCRT-dependent pathway consists of multiple proteins that form complexes with each other, including, ESCRT-0, 1, 2, 3 and AAA ATPase Vsp4 that each play a different role in ILV formation (**Fig. 1**). When ILVs are formed by the ESCRT-dependent pathway, clathrin is recruited by ESCRT-0. Together they bind and cluster ubiquitinated proteins. Subsequently, ESCRT-1 interacts with ESCRT-2, which is important for the downstream functions of ESCRT-2. ESCRT-2 is probably responsible for connecting MVBs to microtubules[3]. Next, ESCRT-3 polymerizes which facilitates membrane invagination and vesicle formation. Finally, Vsp4 depolymerizes ESCRT-3, which causes vesicle abscission [3][4].

Invagination can also be facilitated by the sphingolipid ceramide. This process is independent of the ESCRT-complex but rather on microdomains that are present in the endosomal membrane. These microdomains are enriched in sphingolipids such as ceramides. Ceramide triggers the formation of larger domains that induce membrane budding and vesicle abscission. ILVs formed via this

mechanism can't be degraded by lysosomes but only secreted into the extracellular matrix as exosomes [5][6].

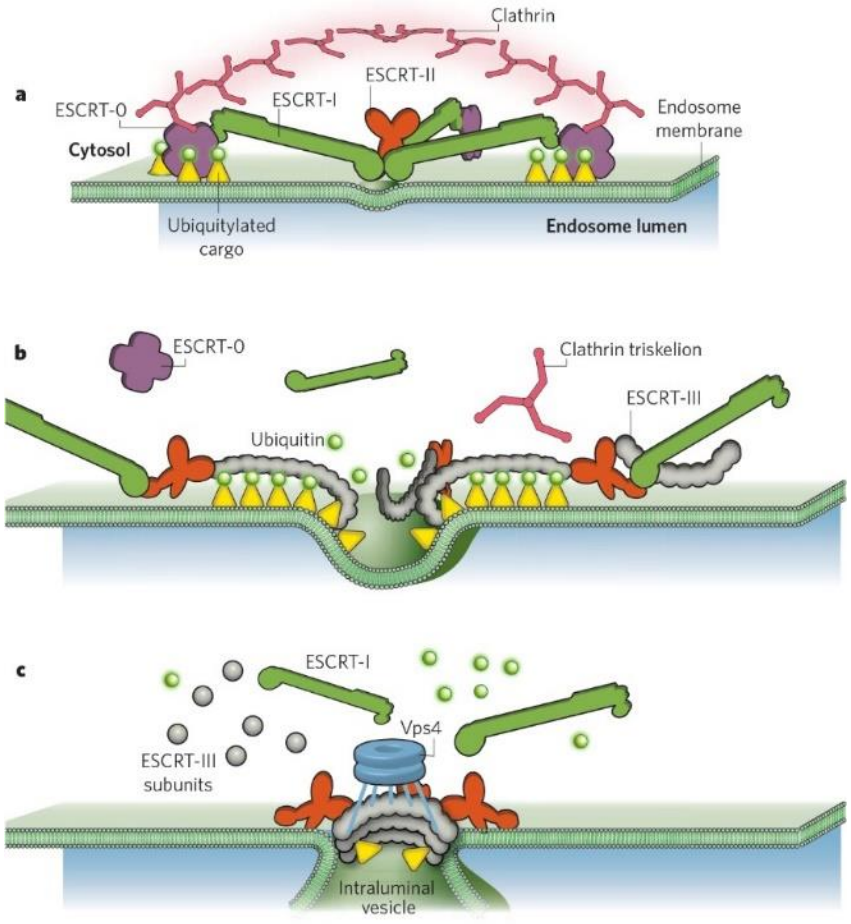

Figure 1 The sorting of ubiquitylated membrane proteins by the ESCRT complex. ***a*** *ESCRT-0 binds and clusters ubiquitylated proteins and recruits clathrin. ESCRT-0 recruits ESCRT-1 which recruits ESCRT- 2.* ***b*** *Membrane deformation occurs by the binding of ESCRT-3 to ESCRT-2 and the polymerization of ESCRT-3. During this process, cargo proteins are deubiquitylated.* ***c*** *Membrane abscission occurs and Vps4 depolymerizes ESCRT-3, ensuring that its subunits can be recycled* [7].

After endosome invagination and maturation, an MVB is formed. An MVB can either fuse with a lysosome to degrade its contents or fuse with the outer cell membrane and thereby release the ILVs as exosomes. In the latter process, the Rab-GTP and SNARE family proteins are involved [8]. When exosomes are secreted into the extracellular matrix, they can be transported via body fluids to distant parts of the body or target neighboring cells [9]. Once exosomes arrive at the target cell/organ, they can be taken up by ligand-receptor interaction or by fusion with the outer membrane via lipid raft endocytosis, clathrin-dependent endocytosis and phagocytosis.

Role of exosomes in cancer

Exosomes play an important role in cancer development. It is known that tumor-derived exosomes (TEX) contribute to angiogenesis, epithelial-to-mesenchymal transition and metastasis (**Fig. 2**) [12]. Sufficient blood supply is essential for tumor growth. In the absence of vascular support tumors only grow up to 1-2mm^3 in diameter and can even become necrotic or apoptotic. Therefore, angiogenesis is an important process for tumor cell proliferation and cancer progression [11]. Tumor cells stimulate angiogenesis by releasing exosomes containing microRNAs, in particular miR-141. Shuangshuang *et al.* observed that exosomes from small cell lung cancer patients expressed high levels of miR-141 [12]. These miR-141-exosomes were able to promote proliferation, migration, invasion and tube formation and induced micro-vessel sprouting in human umbilical vein endothelial cells by preventing the translation of Krüppel-like factor 12 [12]. TEX not only regulate angiogenesis with exosomal miRNAs but can also regulate angiogenesis by transporting proangiogenic proteins, such as

angiogenin, vascular endothelial growth factor, fibroblast growth factor, interleukin-6, interleukin-8 and metalloproteinases 1 and 2 to neighboring epithelial cells [13].

Next to angiogenesis, epithelial to mesenchymal transition (EMT) is a key process for cancer progression and metastasis. EMT alters the phenotype of cancer cells and improves their ability to migrate, invade and seed to distant organs. Transforming growth factor-beta is considered as a master inducer of EMT, invasion and metastasis by regulating the expression of genes related to cytoskeleton assembly, cell-cell attachment and extracellular matrix remodeling [14]. It has been known that the transforming growth factor-β (TGF-β) receptor contains sequences used by cell machinery as signals for internalization[15]. Clement *et al.* showed that TGF-β receptors can be directed to endosomes and activate the SMAD-dependent pathway before being recycled to the plasma membrane [15]. Since endosomes are the precursor of exosomes it is reasonable to assume that exosomes contain TGB-β. Indeed, cancer-associated fibroblasts (CAFs)-derived exosomes show high levels of TGF-β. After treatment, CAF-derived exosomes were able to increase the expression of TGFβR1, TGFβR2 and SMAD2 in SW620 human colon adenocarcinoma cells. Increased TGF-β signaling results in the upregulation of stemness-associated genes such as Nanog, SOX2, Oct4 and CD133, promoting tumor stemness [16]. Tumor stemness is considered unfavorable because makes the tumor cells more resistant to proton/photon radiation therapy. Exosomes from tumor-educated mesenchymal stem cells (MSC) contained high levels of TGF-β, semaphorins and complement factors, enhancing the immunosuppressive activity and M2-polarization in myeloid cells [17]. In addition, TGF-β de-repressed PD-1 in tumor infiltration lymphocytes by downregulating SATB1, which resulted in higher PD-1 expression in murine T cells [17]. Therefore, cellular re-programming with EXO^{MSC} is considered as an important step toward the development of an immunosuppressive tumor microenvironment.

In addition to promoting proliferation, migration, invasion and metastasis, TEX mediate immune-suppressive responses which promote tumor cell immune evasion. Various studies reported that TEX express different immunosuppressive proteins, such as CD73, CD39, PD-1, PD-L1, TRAIL and FasL. Exosomes derived from head and neck squamous cell carcinoma(HNSCC) were able to hydrolyze extracellular ATP into immunosuppressive adenosine and AMP [18]. Shuler *et al.* showed that HNSCC-derived exosomes were able to exchange exosomal CD73 onto CD4+ CD39+ regulatory t-cells. Which led to increased extracellular adenosine levels and the development of an immunosuppressive tumor microenvironment. The PD-L1/PD-1 immune checkpoint regulates the proliferation of antigen-specific T-cells by transmitting inhibitory signals to new cytotoxic T-cells in the lymph nodes, preventing recruitment to the tumor. HNSCC derived exosomes express both PD-1 and PD-L1. EXO^{HNSCC} were able to downregulate CD69 surface expression on activated T-cells after co-incubation with $PD\text{-}L1^{High}$ exosomes, preventing T-cell activation. Exosomal PD-L1 levels are highly correlated with the evidence of advanced disease and tumor stage[19]. TRAIL and FasL are two immunosuppressive proteins that are expressed on cytotoxic T cells and natural killer (NK) cells, eliminating unwanted cells and autoreactive lymphocytes. Binding to their respective cognate DD-containing receptors, initiating the caspase-dependent apoptotic route [20]. Exosomes isolated from ovarian cancer patients exhibit elevated expression of FasL and were able to induce T-cell apoptosis[21]. The same goes for melanoma-derived exosomes that also induced T-/NK cell killing via FasL and TRAIL signaling [22].

In contrast, TEX are also capable of initiating an anti-tumor immune response. TEX are reported to efficiently deliver a variety of tumor antigens to dendritic cells (DCs) [23][24]. Heat shock protein 70(HSP70) and carbohydrate antigen 125 are highly expressed tumor-associated antigens (TAAs) in lung cancer, both being closely related to tumor growth and metastasis. Wang *et al.* determined that lung cancer-derived exosomes transport both these TAAs to DCs, resulting in a 3-fold increased tumor-specific INF-γ production. Increased INF-γ production led to an enhanced Th1 response and cytotoxic T lymphocyte(CTL) response, suppressing tumor growth and increased overall survival in mice[23]. The ability of DCs to produce major histocompatibility complex (MHC) class 1 and 2 expressing exosomes play an important role in CD8+ T cell activation. DC-derived exosomes (DC_{ex}) express peptide-loaded MHC class 1, MHC class 2 and co-stimulatory molecules, such as CD86 and intracellular adhesion molecule-1 [25]. Admyre *et al.* indicated that mature-DC_{ex} (mDC_{ex}) stimulated $CD8^{+}$ T cell activation to a greater extent than immature-DC-derived exosomes (iDC_{ex}), because of lower MHC-1, MHC-2 and co-stimulatory molecule levels [26]. However, further research

demonstrated that DC_{ex} are not very efficient in the initial priming of naive T cells, but rather operated as a restimulation mechanism of activated T cells and memory T cells [27].

FasL and TRAIL not only induce apoptosis in tumor-infiltrating lymphocytes but also show anti-tumor effects. DC_{ex} express a variety of TNF superfamily ligands, such as FasL and TRAIL. FasL and TRAIL levels were higher in mDC_{ex} than in iDC_{ex}, resulting in increased cancer cell killing in mDC_{ex} treated B16 melanoma. Interestingly, mDC_{ex} were able to induce cancer cell killing in other tumor cell types, including squamous lung carcinoma KLN205 cells and colon carcinoma MC38 cells, while exosomes derived from immature DCs were not able to kill these tumor cells.

The examples mentioned above about the role of TEX in cancer demonstrate that exosomes have a dual role where they can promote and inhibit cancer progression. The mode of action of exosomes is determined by their molecular composition, the cell they originate from and the stage of tumor progression [8].

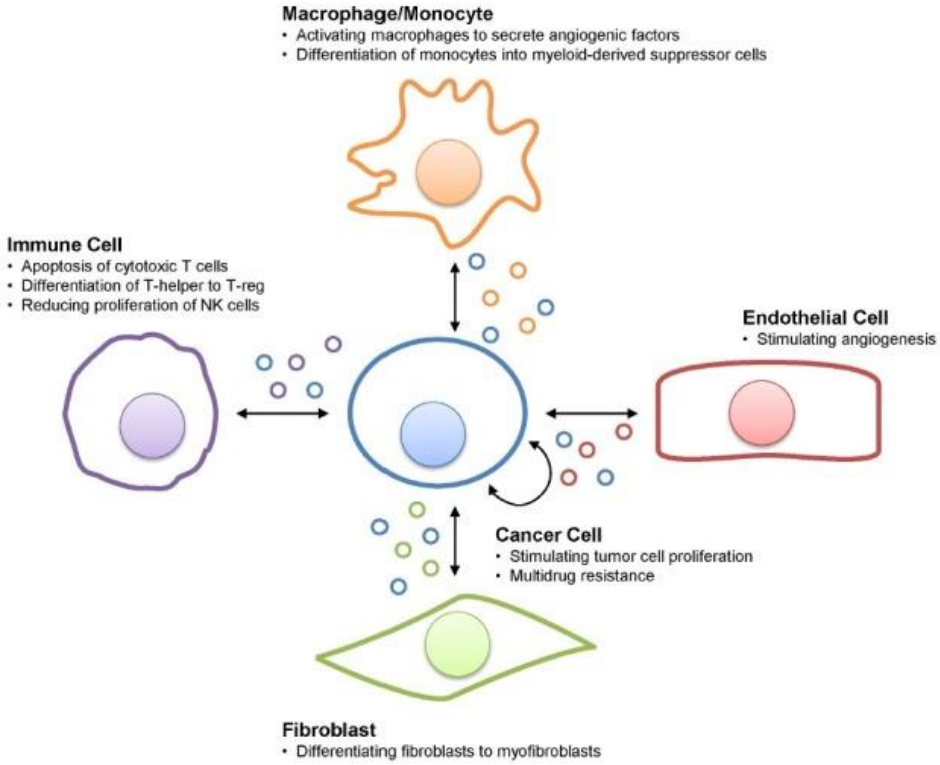

Figure. 2. The role of tumor-derived exosomes in the progression of cancer. *Tumor-derived exosomes target multiple cell types to promote cancer growth and survival. Tumor-derived exosomes stimulate angiogenesis, modulate the proliferation, migration, invasion of endothelial cells and activate macrophages to secrete angiogenic factors or differentiate into myeloid-derived suppressor cells. They reduce the natural killer cell population, induce apoptosis in cytotoxic T-cells and promote the differentiation of T-helper cells into T-reg cells* [28].

Exosomes in cancer diagnostics

Exosomes show great therapeutic potential. They can be used as cargo transporters, immune modulators and for cancer diagnostics. As mentioned before, the composition of exosomes is cell-type-specific and therefore reflects the cell they originate from. By analyzing the composition of exosomes including, mRNA, miRNA, lipids and proteins their origin can be determined [29]. When translating this to cancer diagnostics, exosomes can be used to determine the tumor type and its progression stage. Early detection of cancer will improve clinical outcome and increase the life expectancy of these patients. Researchers are finding more and more cancer-specific exosome markers. For instance, serum exosomes enriched in miR-21 has been associated with glioblastomas, pancreatic, colorectal, colon, liver, breast, ovarian and esophageal cancers [30]. In addition, elevated urine-derived exosomal miR-21 has been associated with bladder and prostate cancer [30]. Noboru *et. al.* demonstrated the diagnostic potential of exomes in the detection of pancreatic cancer [31].

Exosomes isolated from pancreatic fluid in patients with pancreatic ductal adenocarcinoma (PDAC) showed higher exosomal miR-21 levels than patients with chronic pancreatitis. Indicating that exosomal miR-21 in pancreatic fluid can be used as a pancreatic cancer marker. In addition, Shiyu *et al.* demonstrated by protein analysis that CLDN4, EPCAM, CD151, LGALS3BP, HIST2H2BE and HIST2H2BF were highly expressed in exosomes from patients with PDAC [32]. Demonstrating that miRNAs and proteins are excellent cancer markers. These were two examples of detecting PDAC in an early stage but there are many more known exosomal markers for the diagnosis of lung cancer, breast cancer, ovarian cancer, rectal cancer, gastric cancer, hepatocellular carcinoma, multiple myeloma and melanoma [32]. With many more exosomal cancer markers yet to be discovered.

Different steps are needed for TEX analysis including, isolation, purification and analysis of exosomal contents. Exosomes can be isolated from different biological fluids, such as blood, saliva and urine. The most common method for exosome purification from the biological fluids is with the use of ultrahigh-speed centrifugation techniques. Ultrahigh-speed centrifugation separated exosomes based on their density and size from other components in biological fluids by subjecting the fluids to different centrifugal forces [33]. After purification, several techniques including, western blotting, trypsin digestion, flow cytometry, mass spectrometry, ELISA, RT-qPCR can be used to analyze exosomal composition. Based on the composition cancer type and stage can be determined

Exosomes as cargo transporter

Exosomes are non-immunogenic and can therefore act as efficient and save cargo transporters. Cancers are predominately treated with Taxol, a chemotherapeutic agent, that prevents cell division [34]. Treatment of cancers with Taxol often causes side-effects such as neutropenia, diarrhea and hair loss [35]. By loading Taxol in exosomes a 1000-fold reduced concentration can be used to achieve the same tumor shrinkage and fewer side effects as conventional Taxol treatment [36]. Similar results were obtained by Y. Si *et al.* who conjugated an anti-SSTR2 antibody on the surface of romidepsin-loaded exosomes to specifically target neuroendocrine cancer [37]. This method is even more specific and increases exosome buildup and uptake at the tumor site.

In addition to delivering therapeutic agents, exosomes can also be used for the transport of siRNA. Naked siRNAs are quickly degraded in human plasma with a half-life of minutes. By loading siRNA in exosomes this problem can be overcome. Z. Yang *et al.* showed that downregulation of cyclin-dependent kinase 4 using siRNA-loaded exosomes significantly inhibited tumor growth in MCF-7 mice [38].

Exosomes as immune modulators

Next to loading exosomes with a siRNA or drugs, exosomes can also function as a vehicle to block receptors and modulate immunological pathways. Many cancers, including ovarian cancer, thyroid cancer and prostate cancer, show elevated expression of the transmembrane protein CD47 [39]. CD47 binds to SIRPα on macrophages, preventing the tumor cells from being phagocytosed by macrophages [40]. M2 macrophages are the most abundant type of macrophages at the tumor site, contributing to an immunosuppressive tumor microenvironment. In contrast to M2 macrophages, M1 macrophages have anti-tumor effects and thus the preferred type of macrophages at the tumor site [41]. Exosomes from M1 macrophages contain M2 reprogramming cytokines such as TNF-α and interferon-γ. K. Pu and H.-Y. Xie developed an exosome-nano bioconjugate by conjugating antibodies against CD47 and SIRPα to M1-derived exosomes, linked by a pH-sensitive benzoic-imine bond. In the acidic tumor microenvironment this bond breaks, releasing the anti-CD47 and anti-SIRPα antibodies into the extracellular matrix. Antibody-CD47 and SIRPα binding prevent CD47-SIRPα interactions, resulting in increased phagocytosis of tumor cells. Moreover, the remaining M1 exosomes will be taken up by M2 macrophages, promoting reprogramming into M1 macrophages [42].

One of the mechanisms cancer cells make use of to escape the immune system is the development of $CD8^+$ T-cell tolerance against TAAs. However, repeated stimulation with TAAs can cause a $CD8^+$ T-cell immune response. This is due to the presence of low affinity self-reactive $CD8^+$ T-cells that

undergo avidity maturation after iterative stimulation [43]. As mentioned before, exosomes can be used to transport TAAs to DCs and prime T-cells but the concentration of TAAs inside the exosomes is quite low. Which resulted in a weak anti-cancer immune response. Exosomal TAA levels can be increased by transfecting exosome-producing cells with a molecular construct consisting of an exosome surface protein (lactadherin, LAMP2b, mutant Nef) fused to a TAA or tumor-specific antigen (TSA). Negative regulatory factor (Nef) is a virulence factor, which originates from the human immunodeficiency virus (HIV). Mutant Nef is a functionally defective protein lacking the patholofical effects of Nef that gets incorporated at high levels in the outer leaflet of exosomes [44][45]. Anticoli *et al.* fused Nef to HER2, a TAA overexpressed in breast cancer, and transfected this construct in murine muscle cells. After transfection, the murine muscle cells produced high amounts of exosomes with HER2-nef fusion protein, targeting DCs and thereby inducing a $CD8^+$ T-cells immune response against HER2-overexpressing tumor cells [46]. It is also possible to fuse TAAs to the inner leaflet of exosomes by fusing an antigen of interest to virus-derived group-specific antigen (Gag). Gag binds to phosphatidylinositol-4,5-bisphosphate present in the inner leaflet of exosomes. Arima *et al.* engineered a Gag-ovalbumin (OVA) fusion protein that gets incorporated at high levels in the inner leaflet of exosomes. Results showed that OVA-Gag exosomes were less prone to lysosomal degradation compared to exosomes loaded with OVA-lactadherin fusion proteins binding to phosphatidylserine on the outer membrane of exosomes. DCs treated with Gag-OVA exosomes showed higher OVA antigen presentation and more IL-2 production when incubated with CD8-OVA T-cells. Leading to an increased anti-cancer immune response [47].

In contrast to the TAA/TSA fusion protein-based strategy, TEX can also directly activate dendritic cells. *In vitro* stimulation of immature DCs with TEX promotes DC maturation. TEX are phagocytized, broken down into small peptides and subsequently, presented on the MHC complex in DCs. Finally, the now mature DCs can be subcutaneous or intra-organ injected in the patient to induce a $CD8^+$ T-cell anti-tumor immune response. This approach significantly regressed tumor growth in dogs with canine transmissible venereal cancer [48]. *In vitro* loading of DCs with TEX inhibited tumor growth significantly more in comparison to direct administration with TEX [49]. This can be explained by the fact that TEX/exosomes can be taken up by a large variety of cells next to DCs which results in a less effective antitumor immune response [50].

Conclusion/discussion

Exosomes are complex nanovesicles that regulates cell-cell communication, differentiation, regeneration and immune responses. Their function is determined by several factors such as their composition, origin and target cell. TEX show both pro-tumor and anti-tumor effects. On one hand, exosomes can promote angiogenesis, epithelial-to-mesenchymal transition and metastasis in cancer cells, but on the other hand, exosomes can induce anti-cancer immune responses by presenting tumor antigens to DCs. It is known that the composition of exosomes can be affected by various stress conditions. TEX produced under external stress factors such as chemotherapeutic drugs, heat shock and irradiation can induce various anti-cancer immune responses [51]. While exosomes released from hypoxic cancer cells promote migration, invasion and proliferation of cancer cells. These two opposite effects of TEX demonstrate that external factors play a huge role in the function of TEX. In addition, these external factors such as stress responses have a different effect on the function of TEX derived from different cell types. This is one of the main reasons why exosomes are such complex molecules. The effects of external factors on the function of TEX in different tissues should be determined. With that knowledge, you could manipulate the biogenesis of exosomes in such a way to elicit an anti-cancer immune response.

In this article I have mentioned several therapeutic approaches of exosomes with some having greater potential than the other. 1) Exosomes loaded with siRNA can prevent oncogene-related proteins from being translated. Yet, some mRNA molecules are still being translated. Besides, it only temporarily inhibits tumor cell growth. 2) Administrating exosomes loaded with drugs, such as Taxol, reduce side-

effects because a lower Taxol concentration can be used. However, just like conventional chemotherapy, not every tumor cell gets killed, which makes a recurrence more likely. 3) Increasing TAA/TSA levels in exosomes by fusing them to exosome surface proteins results in an increased $CD8^+$ T-cell response. The limitation of this approach is the requirement of a known TSA/TAA, which is not always the case. 4) Vaccination with DC-pulsed TEX circumvents this problem and induces an immune response against multiple different TAAs. Personally, I think that the latter two approaches show the most promise as they both elicit an anti-tumor immune response, leading to cancer remission. However, as previously mentioned TEX can both stimulate and inhibit cancer developments based on its contents. Therefore, incorrect utilization of exosomes may potentially be dangerous and can for instance stimulate cancer progression. A better understanding of the factors that contribute to the function of exosomes is needed for the development of a potent exosome-based anti-cancer vaccine. Based on these factors the biogenesis/composition of TEX can be manipulated in a way to increase the efficacy of an exosome-based anti-cancer vaccine.

Exosomes show great potential in cancer diagnostics and can be immediately used for this purpose. There are already a few known exosomal cancer markers and more yet to be discovered. Tumor markers in the blood such as prostate-specific antigen for the detection of prostate cancer and cancer antigen 125 for the detection of ovarian cancer are already being used for cancer diagnosis. Recent studies have demonstrated that both PSA and cancer antigen 125 are highly expressed in exosomes from patients with prostate cancer and ovarian cancer [52][53]. Both studies indicated that exosomes were superior in distinguishing cancer patients from healthy individuals because of its high specificity and sensitivity, outperforming conventional PSA and cancer antigen 125 blood tests. However, there are still some challenges to overcome for the use of exosomes in cancer diagnostics, concerning the isolation and identification of exosomes. As previously mentioned, ultracentrifugation is the most common method for the isolation of exosomes. But this technique is time-consuming, cumbersome and may damage the exosomes, which makes this technique not suitable for diagnostic purposes. There are other techniques such as microfluidics-derived chip isolation which separates exosomes based on the difference between biochemical properties such as density, size and immunoaffinity. But the downside of this technique is that it has a low sample yield. Currently, there is no isolation/identification technique suitable for the use cancer diagnostics. A standardized exosome isolation/identification technique must be developed that is cost-effective, fast and accurate. With still some small hurdles to overcome exosomes show a bright future in cancer diagnostics and will undoubtedly become an important tool in the diagnostic field.

References

[1] I.-M. Chung, G. Rajakumar, B. Venkidasamy, U. Subramanian, and M. Thiruvengadam, "Exosomes: Current Use and Future Applications," *Clin. Chim. Acta*, 2019.

[2] P. Akuma, O. D. Okagu, and C. C. Udenigwe, "Naturally Occurring Exosome Vesicles as Potential Delivery Vehicle for Bioactive Compounds," *Front. Sustain. Food Syst.*, vol. 3, p. 23, Apr. 2019.

[3] M. Babst, "MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between," *Current Opinion in Cell Biology*, vol. 23, no. 4. pp. 452–457, Aug-2011.

[4] J. H. Hurley, "The ESCRT complexes," *Critical Reviews in Biochemistry and Molecular Biology*, vol. 45, no. 6. pp. 463–487, Dec-2010.

[5] K. Trajkovic *et al.*, "Ceramide triggers budding of exosome vesicles into multivesicular endosomes," *Science (80-.).*, vol. 319, no. 5867, pp. 1244–1247, Feb. 2008.

[6] N. P. Hessvik and A. Llorente, "Current knowledge on exosome biogenesis and release," *Cellular and Molecular Life Sciences*, vol. 75, no. 2. Birkhauser Verlag AG, pp. 193–208, 01-Jan-2018.

[7] C. Raiborg and H. Stenmark, "The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins," *Nature*, vol. 458, no. 7237. pp. 445–452, 26-Mar-2009.

[8] D. Kołat, R. Hammouz, A. K. Bednarek, and E. Płuciennik, "Exosomes as carriers transporting long non-coding RNAs: Molecular characteristics and their function in cancer (Review)," *Mol. Med. Rep.*, vol. 20, no. 2, pp. 851–862, 2019.

[9] I.-M. Chung, G. Rajakumar, B. Venkidasamy, U. Subramanian, and M. Thiruvengadam, "Exosomes: Current Use and Future Applications," *Clin. Chim. Acta*, 2019.

[10] A. Bobrie and C. Théry, "Unraveling the physiological functions of exosome secretion by tumors," *Oncoimmunology*, vol. 2, no. 1, Jan. 2013.

[11] N. Nishida, H. Yano, T. Nishida, T. Kamura, and M. Kojiro, "Angiogenesis in cancer," *Vascular Health and Risk Management*, vol. 2, no. 3. Dove Press, pp. 213–219, 2006.

[12] S. Mao *et al.*, "Exosomal miR-141 promotes tumor angiogenesis via KLF12 in small cell lung cancer," *J. Exp. Clin. Cancer Res.*, vol. 39, no. 1, p. 193, Dec. 2020.

[13] W. Olejarz, G. Kubiak-Tomaszewska, A. Chrzanowska, and T. Lorenc, "Exosomes in angiogenesis and anti-angiogenic therapy in cancers," *Int. J. Mol. Sci.*, vol. 21, no. 16, pp. 1–25, Aug. 2020.

[14] A. F. Teixeira, P. ten Dijke, and H. J. Zhu, "On-Target Anti-TGF-β Therapies Are Not Succeeding in Clinical Cancer Treatments: What Are Remaining Challenges?," *Frontiers in Cell and Developmental Biology*, vol. 8. Frontiers Media S.A., 08-Jul-2020.

[15] C. A. Clement *et al.*, "TGF-β Signaling Is Associated with Endocytosis at the Pocket Region of the Primary Cilium," *Cell Rep.*, vol. 3, no. 6, pp. 1806–1814, Mar. 2013.

[16] L. Liu *et al.*, "Cancer associated fibroblasts-derived exosomes contribute to radioresistance through promoting colorectal cancer stem cells phenotype," 2020.

[17] S. Biswas *et al.*, "Exosomes Produced by Mesenchymal Stem Cells Drive Differentiation of Myeloid Cells into Immunosuppressive M2-Polarized Macrophages in Breast Cancer," *J. Immunol.*, vol. 203, no. 12, pp. 3447–3460, Dec. 2019.

[18] P. J. Schuler *et al.*, "Human CD4+CD39+ regulatory T cells produce adenosine upon co-expression of surface CD73 or contact with CD73+ exosomes or CD73+ cells," *Clin. Exp. Immunol.*, vol. 177, no. 2, pp. 531–543, 2014.

[19] M. N. Theodoraki, S. S. Yerneni, T. K. Hoffmann, W. E. Gooding, and T. L. Whiteside, "Clinical significance of PD-L1 þ exosomes in plasma of head and neck cancer patients," *Clin. Cancer Res.*, vol. 24, no. 4, pp. 896–905, Feb. 2018.

[20] A. Rossin, G. Miloro, and A. O. Hueber, "TRAIL and FasL functions in cancer and autoimmune diseases: Towards an increasing complexity," *Cancers (Basel).*, vol. 11, no. 5, May 2019.

[21] P. Peng, Y. Yan, and S. Keng, "Exosomes in the ascites of ovarian cancer patients: Origin and effects on anti-tumor immunity," *Oncol. Rep.*, vol. 25, no. 3, pp. 749–762, Mar. 2011.

[22] M. J. Martínez-Lorenzo *et al.*, "The human melanoma cell line MelJuSo secretes bioactive

FasL and APO2L/TRAIL on the surface of microvesicles. Possible contribution to tumor counterattack," *Exp. Cell Res.*, vol. 295, no. 2, pp. 315–329, May 2004.
[23] C. Wang, X. Huang, Y. Wu, J. Wang, F. Li, and G. Guo, "Tumor cell-associated exosomes robustly elicit anti-tumor immune responses through modulating dendritic cell vaccines in lung tumor," *Int. J. Biol. Sci.*, vol. 16, no. 4, pp. 633–643, 2020.
[24] N. M. Mahaweni, M. E. H. Kaijen-Lambers, J. Dekkers, J. G. J. V. Aerts, and J. P. J. J. Hegmans, "Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma," *J. Extracell. Vesicles*, vol. 2, no. 1, 2013.
[25] P. D. Robbins and A. E. Morelli, "Regulation of immune responses by extracellular vesicles," *Nature Reviews Immunology*, vol. 14, no. 3. Nature Publishing Group, pp. 195–208, 25-Mar-2014.
[26] C. Admyre, S. M. Johansson, S. Paulie, and S. Gabrielsson, "Direct exosome stimulation of peripheral human T cells detected by ELISPOT," *Eur. J. Immunol.*, vol. 36, no. 7, pp. 1772–1781, Jul. 2006.
[27] J. M. Pitt *et al.*, "Dendritic cell-derived exosomes for cancer therapy," *Journal of Clinical Investigation*, vol. 126, no. 4. American Society for Clinical Investigation, pp. 1224–1232, 01-Apr-2016.
[28] Y. J. Yoon, O. Y. Kim, and Y. S. Gho, "Extracellular vesicles as emerging intercellular communicasomes," *BMB Reports*, vol. 47, no. 10. The Biochemical Society of the Republic of Korea, pp. 531–539, 2014.
[29] G. Ailuno, S. Baldassari, F. Lai, T. Florio, and G. Caviglioli, "Exosomes and Extracellular Vesicles as Emerging Theranostic Platforms in Cancer Research," *Cells*, vol. 9, no. 12, p. 2569, Dec. 2020.
[30] R. Kalluri and V. S. LeBleu, "The biology, function, and biomedical applications of exosomes," *Science*, vol. 367, no. 6478. American Association for the Advancement of Science, 07-Feb-2020.
[31] N. Ideno, Y. Mori, M. Nakamura, and T. Ohtsuka, "Early Detection of Pancreatic Cancer: Role of Biomarkers in Pancreatic Fluid Samples," *Diagnostics*, vol. 10, no. 12, p. 1056, Dec. 2020.
[32] S. Li, M. Yi, B. Dong, X. Tan, S. Luo, and K. Wu, "The role of exosomes in liquid biopsy for cancer diagnosis and prognosis prediction," *International Journal of Cancer*. Wiley-Liss Inc., 2020.
[33] X. X. Yang, C. Sun, L. Wang, and X. L. Guo, "New insight into isolation, identification techniques and medical applications of exosomes," *Journal of Controlled Release*, vol. 308. Elsevier B.V., pp. 119–129, 28-Aug-2019.
[34] M. A. Jordan, "Mechanism of action of antitumor drugs that interact with microtubules and tubulin," *Current Medicinal Chemistry - Anti-Cancer Agents*, vol. 2, no. 1. pp. 1–17, 2002.
[35] D. Mavroudis *et al.*, "Phase I study of paclitaxel (Taxol) and pegylated liposomal doxorubicin (Caelyx) administered every 2 weeks in patients with advanced solid tumors," *Oncology*, vol. 62, no. 3, pp. 216–222, 2002.
[36] C. Melzer, V. Rehn, Y. Yang, H. Bähre, J. von der Ohe, and R. Hass, "Taxol-loaded MSC-Derived exosomes provide a therapeutic vehicle to target metastatic breast cancer and other carcinoma cells," *Cancers (Basel).*, vol. 11, no. 6, Jun. 2019.
[37] Y. Si *et al.*, "Targeted Exosomes for Drug Delivery: Biomanufacturing, Surface Tagging, and Validation," *Biotechnol. J.*, Jan. 2019.
[38] Z. Yang *et al.*, "Functional exosome-mimic for delivery of siRNA to cancer: in vitro and in vivo evaluation," *J. Control. Release*, vol. 243, pp. 160–171, Dec. 2016.
[39] "Expression of CD47 in cancer - Summary - The Human Protein Atlas." [Online]. Available: https://www.proteinatlas.org/ENSG00000196776-CD47/pathology. [Accessed: 03-Sep-2020].
[40] E. Tzatzarakis, B. Hissa, C. Reissfelder, and S. Schölch, "The overall potential of CD47 in cancer immunotherapy: with a focus on gastrointestinal tumors," *Expert Rev. Anticancer Ther.*
[41] K. Mehla and P. K. Singh, "Metabolic Regulation of Macrophage Polarization in Cancer," *Trends in Cancer*, Nov. 2019.
[42] K. Pu and H.-Y. Xie, "Responsive exosome nano-bioconjugates for synergistic cancer therapy," *Angew. Chemie - Int. Ed.*

[43] C. E. Nelson *et al.*, “Robust Iterative Stimulation with Self-Antigens Overcomes CD8+ T Cell Tolerance to Self- and Tumor Antigens,” *Cell Rep.*, 2019.
[44] P. Di Bonito *et al.*, “Antitumor HPV E7-specific CTL activity elicited by in vivo engineered exosomes produced through DNA inoculation,” *Int. J. Nanomedicine*, vol. 12, pp. 4579–4591, Jun. 2017.
[45] P. Di Bonito, L. Accardi, L. Galati, F. Ferrantelli, and M. Federico, “Anti-cancer vaccine for HPV-associated neoplasms: Focus on a therapeutic HPV vaccine based on a novel tumor antigen delivery method using endogenously engineered exosomes,” *Cancers (Basel).*, vol. 11, no. 2, Jan. 2019.
[46] S. Anticoli *et al.*, “Engineered exosomes emerging from muscle cells break immune tolerance to HER2 in transgenic mice and induce antigen-specific CTLs upon challenge by human dendritic cells,” *J. Mol. Med.*, vol. 96, no. 2, pp. 211–221, Feb. 2018.
[47] Y. Arima, W. Liu, Y. Takahashi, M. Nishikawa, and Y. Takakura, “Effects of Localization of Antigen Proteins in Antigen-Loaded Exosomes on Efficiency of Antigen Presentation,” *Mol. Pharm.*, vol. 16, no. 6, pp. 2309–2314, Jun. 2019.
[48] Y. Ramos-Zayas *et al.*, “Immunotherapy for the treatment of canine transmissible venereal tumor based in dendritic cells pulsed with tumoral exosomes,” *Immunopharmacol. Immunotoxicol.*, vol. 41, no. 1, pp. 48–54, Jan. 2019.
[49] H. Zhang *et al.*, “Cell-free tumor microparticle vaccines stimulate dendritic cells via cGAS/STING signaling,” *Cancer Immunol. Res.*, vol. 3, no. 2, pp. 196–205, Feb. 2015.
[50] O. Markov, A. Oshchepkova, and N. Mironova, “Immunotherapy based on dendritic cell-targeted/-derived extracellular vesicles — A novel strategy for enhancement of the anti-tumor immune response,” *Front. Pharmacol.*, vol. 10, 2019.
[51] W. S. Kim *et al.*, “Comparison of exosomes derived from non-and gamma-irradiated melanoma cancer cells as a potential antigenic and immunogenic source for dendritic cell-based immunotherapeutic vaccine,” *Vaccines*, vol. 8, no. 4, pp. 1–14, Dec. 2020.
[52] Z. Chen *et al.*, “Exosomal CA125 as A promising biomarker for ovarian cancer diagnosis,” *J. Cancer*, vol. 11, no. 21, pp. 6445–6453, 2020.
[53] M. Logozzi *et al.*, “Increased plasmatic levels of psa-expressing exosomes distinguish prostate cancer patients from benign prostatic hyperplasia: A prospective study,” *Cancers (Basel).*, vol. 11, no. 10, Oct. 2019.