Review Article

Stem Cell INVESTIGATION

Exosomes, cancer's little army

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Abstract: In an attempt to conceptualize the process of cancer formation, Hanahan and Weinberg [2000] have outlined six universal characteristics of tumorigenesis, and labelled them as the "hallmarks of cancer". These hallmarks include; unlimited proliferation, evading growth suppressors, resisting cell death, replicative immortality, inducing angiogenesis, initiating invasion and metastasis. Cancer cell signalling is crucial for initiating and controlling cellular pathways that are involved in these hallmarks. The intricate network of communication between cancer cells and other cancer or non-cancer cells is still being investigated, and is yet to be fully understood. Initially it was proposed that the main form of communication between cells within the tumour microenvironment are soluble growth factors, and gap junctions. Then, researchers reported another form of cell-to-cell communication, through the release of spherical particles called exosomes. It is believed that these exosomes enable communication through the transfer of active components from the releasing cell, and off-loading it into the recipient cell. As researchers continue to examine the development of the cancer hallmarks and the pathways involved, it became evident that cancer cell-derived exosomes play a major role in almost all of them. This review will examine the role played by cancer cell-derived exosomes in development of cancer.

Keywords: Cancer; exosomes; proliferation; apoptosis; telomerase activation; angiogenesis; metastasis

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Introduction

According to the National Cancer Institute (Rockville, Maryland, USA), there are more than 150 different types of cancers found in humans. These Cancers are usually classified based on the organ of the body they appear in, and the type of cells undergoing tumorigenic transformation. The process of cancer formation is an intricate, multi-step process that could start anywhere in the body. The pathways where a healthy tissue is transformed into a cancerous tissue have been the focus of cancer research for many decades. In order to grasp a better understanding of the complex transformation, Hanahan and Weinberg (2000, further revised in 2011) outlined six universal characteristics of tumorigenesis, or six main biological traits that will develop in a given tissue during the process of tumour formation. These traits were called the "hallmarks of cancer" and they are; unlimited proliferation, evading growth suppressors, resisting cell death, replicative immortality, inducing angiogenesis, initiating invasion and metastasis (1,2). Cell-to-cell communication is an important participant in delivering messengers that control these hallmarks. The classic cell-to-cell communication is mainly orchestrated by soluble growth factors (3), and gap junctions (4). However, research over the past decades suggested the presence of another form of cell-to-cell communication; that is

exosomes.

Exosomes are a sub-type of extracellular vehicles (EVs), EVs are spherical shaped particles released by cells and enclosed by a phospholipid bilayer. Although there are no known specific exosomes markers, the distinction of exosomes from the other EVs subtypes realize on its cellular origin, protein content, density, in addition to size and morphology (5,6). Regarding cellular origin, exosomes originate from the endocytic pathway, when the multivesicular bodies fuse with the plasma membrane and release its content, i.e., the intraluminal vesicles (ILVs), into the extracellular space, the released ILVs will become the exosomes (7,8). As for protein content, exosomes contain characteristic proteins that can be found in any cell-released exosome, and can be used to distinguish them from other EVs subtypes (9). On the other hand, exosomes could also contain customized proteins that reflects its specific cellular origin (10). Identifying the exosomal protein content is still a work in progress. A web-based record of exosomal cargo called "ExoCarta" (www.exocarta.org), was established and is directed and updated by expert biologist. This database covers all exosomal cargo that includes; proteins, lipids, microRNA (miRNA) and messenger RNA (mRNA) (11,12). Whereas for density, size and morphology; exosomes are characterized by its 1.1–1.2 g/mL density in sucrose, cup-shaped appearance, and are 30 to 150 nm in diameter by electron microscopy (13).

Almost all mammalian cells, in normal physiological conditions, produce exosomes that carry out numerous of biological functions, the most crucial function is facilitating intercellular communication. It is believed that exosomes enable communication between cells by transferring its cargo from the releasing cell and off-loading it into the recipient cell (14). Cancer cells and cells of the tumour micro-environment, were also found to release exosomes. An increasing amount of recently published literature confirms that cancer and cancer-associated cells use exosomes to commute their message to malignant and non-malignant cells, again through the transfer of functional components, in order to initiate pathways that are necessary for tumor survival and propagation. As researchers investigate the development and propagation of the cancer hallmarks, it became evident that cancer cell-derived exosomes play a major role in almost all of them. For the scope of this review, we will examine the published literature on the role played by cancer cell-derived exosomes in enabling cancer cell proliferation and resistance to apoptosis, replicative immortality, induction of angiogenesis and metastasis, which collectively serve as the hallmarks of cancer.

Proliferation and resistance to apoptosis

Studies have reported that cancer cell-derived exosomes prompted cancer cell proliferation based on different *in vitro* assays. One study reported that exosomes derived from the gastric cancer cell line (SGC7901) significantly increased the proliferation of the two gastric cancer cell line cells; SGC7901 and BGC823, based on MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay. The study postulated that the increase was, in part, due to PI3K/Akt activation (expression up-regulation via Western blot) (15). In another study on gastric cancer cells, SGC-7901-derived exosomes significantly increased SGC-7901 proliferation, based on the same assay, in a dose-dependent manner. SGC-7901-derived exosomes were also found to increase SGC-7901 invasion based on a Matrigel coated Transwell assay. Utilizing a number of assays, the study concluded that the significant increase in both functions was a result of SGC-7901 exosomes activating the MAPK signalling pathway through the transfer of miRNAs [via microarray, reverse transcription polymerase chain reaction (RT-PCR) and Kyoto encyclopedia of genes and genomes pathway analysis, followed by Western blot expression measurement for confirmation] (16). Whereas a study on triple negative breast cancer (TNBC) reported that exosomes from TNBC cell lines [Hs578T and its invasive Hs578Ts(i)8 variant] significantly increased the proliferation (Neubauer chamber cell counting assay), migration (scratch assay) and invasion (coated Transwell assay) capacity of all three different recipient breast cancer cell lines (SKBR3, MDA-MB-231 and HCC1954). Moreover, Hs578Ts(i)$_8$-derived exosomes increased the invasiveness of its parent Hs578T cells. The same study also reported that exosomes derived from TNBC patients' sera had the same effect of increasing cell invasion compared with exosomes derived from control healthy sera (17). Another study on breast cancer cell lines reported that exosomes derived from the metastatic breast cancer cell line MDA-MB-231 significantly increased MDA-MB-231 and ZR-75-1, another breast cancer cell line, proliferations based on cell counting kit-8 (CCK-8) assay. When the two cell lines were treated with noncancerous mammary epithelial line MCF-10A-derived exosomes, there was no significant effect. The study suggested that the increase is due to non-coding RNA MALAT1 (detected

via RT-PCR) transfer by cancer cell-derived exosomes to recipient cells (18). Moreover, it was reported that attenuating the level of exosome production by MCF-7 breast cancer cell line via Shikonin treatment, reduced the rate of its cell proliferation based on CCK-8 assay (19). In addition to promoting proliferation, cancer cell-derived exosomes are thought to be involved in inhibiting cancer cell apoptosis. It was reported that exosomes derived from the bladder cancer cell line T24, promoted the proliferation of the two recipient bladder cancer cell lines T24 and 5637, based on cell CCK-8 assay. Also, T24-derived exosomes were able to significantly inhibit T24 and 5637 apoptosis, in a dose dependent manner, based on annexin V apoptosis assay. The study suggested that this effect is gained through exosomes activating the Akt and ERK pathways in cells (expression up-regulation detected via Western blot) (20).

Replicative immortality

Replicative immortality or telomerase activation, is a very distinctive characteristic of tumor cells. Telomeres are repeats of the TTAGGG sequence located at the chromosomes' ends to protect them from degradation (21). With each cell replication the telomere region gets shorter until the cell reaches replicative senescence, or the "non-dividing stage" (22). Telomere length is controlled by the enzyme telomerase, and the telomerase activity is measured by the catalytic subunit of the enzyme "TERT", encoded by the telomerase reverse transcriptase gene (23). In addition to TERT, telomerase is made up of two other subunits, telomerase RNA (TERC) and telomerase associated protein (TEP1). TERC and TEP1 are constitutively expressed, hence, telomerase activity is dependent on the third subunit, TERT (24). Telomerase is silenced after birth (25), hence mature somatic cells lack the amount of telomerase required to maintain the telomere region, this leads to telomere shortening and causes the aging process and aging-associated diseases (26). On the other hand, high levels of telomerase are considered as a hallmark of cancer (27). Elevated cell-free, serum human TERT (hTERT) mRNA levels is associated with many forms of cancer, and it correlates with its progression, hence it was suggested to be used for cancer detection and monitoring (28-31). More recently, Goldvaser *et al.* [2017] reported that for cancer patients' diagnosis, measuring the hTERT mRNA levels contained within sera-derived exosomes is more reliable technique than measuring free hTERT mRNA levels in serum (32). Since cancer cell-derived exosomes were reported to contain hTERT mRNA, and TERT, the catalytic sub unit of telomerase, can be regulated through epigenetic mechanisms including non-coding RNAs, this indicates that the transfer of hTERT mRNA from cancer to non-cancer cells could enable replicative immortality. The delivered mRNAs can bind to the 3'-UTR of TERT mRNA, of recipient cells and regulate its expression in a post-transcriptional manner (33). Indeed, Gutkin *et al.* [2016], have demonstrated that four cancer cell line-derived exosomes (Jurkat, K562, MCF7 and HCT116), plus sera-derived exosomes from pancreatic and lung cancer patients, all contained hTERT mRNA. The level of hTERT mRNA in these exosomes was related to the telomerase activity in the parent cell. When cancer cell line-derived exosomes were added to fibroblasts, the hTERT mRNA within the exosomes was up taken by the fibroblast. This activated the telomerase in the recipient cells, transforming them into telomerase positive fibroblasts (based on hTERT mRNA expression analysis via by RT-PCR, telomerase protein levels via Western blot and telomerase activity measurement via Q-TRAP assay). Activation of telomerase, increased fibroblasts proliferation (Trypan blue exclusion assay) and extended its life span, which in turn delayed cell senescence (β-galactosidase activity assay, nuclei morphology and γH2AX foci staining for DNA damage) (34).

Angiogenesis

Angiogenesis is a significant feature of cancer, which refers to aberrant formation of new blood vessels to support tumour growth, migration and metastasis. There is now an increasing number of studies reporting that cancer cell-derived exosomes are a key player in vascular cells activation during tumour formation. For example, it was reported that melanoma (B16-F10), TNBC (Hs578T) and hepatocellular carcinoma (HepG2) cell line-derived exosomes all stimulate human umbilical vein endothelial cells (HUVECs) tube formation (tube formation assay) (17,35,36). A study on ovarian cancer reported that exosomes from the ovarian cancer cell line (CAOV3) promoted HUVECs viability and migration based on CCK8 assay and Transwell migration assay. Also, these exosomes promote tube formation by HUVECs both *in vitro* (tube formation assay) and *in vivo* [chorioallantoic membranes (CAMs) assay]. Proteomic mass spectrometry analysis of CAOV3-derived exosomal proteins identified 10 proteins related to angiogenesis, including ATF2 and MTA1 (37). Whereas a study on hepatocellular carcinoma reported that exosomes derived

from four hepatocellular carcinoma cell lines (QGY-7703, HepG2, SK-Hep-1, and Huh-7), enhanced *in vitro* tube formation of HUVECs (tube formation assay). Exosomal miR-210 was indicated to cause this effect, indeed, when anti-miR-210 was introduced, the enhanced tube formation by exosome treated HUVECs was attenuated. A follow-up *in vivo* assay showed the same outcomes, as higher tubulogenesis was seen in a Matrigel plug and subcutaneous tumor xenografts model treated with exosomal miR-210. miR-210 TargetScan and miRanda databases predicted that miR-210 inhibits the expression of SMAD4 and STAT6, which leads to enhanced angiogenesis (38). Another mechanism of action study reported that analysis of miRNAs encapsulated in exosomes derived from the nasopharyngeal carcinoma (NPC) cell lines (CNE1, CNE2, 5-8F, and 6-10B), revealed overexpression of miR-23a (via RT-PCR). This have caused these exosomes to induce angiogenesis both *in vitro* (HUVECs tube formation assay) and *in vivo* (Matrigel plug assay and Zebrafish model). The study provided evidences (via RT-PCR) that miR-23a regulated angiogenesis by directly targeting testis-specific gene antigen (TSGA10) (39). It was also reported that NPC cell line (C666-1)-derived exosomes promoted HUVECs angiogenesis through enhancing migration (scratch assay), invasion (Matrigel invasion chambers assay) and tubulogenesis (tube formation assay) in a dose-dependent manner. Subsequently, an iTRAQ-based quantitative proteomics was used to identify the differentially expressed proteins in the C666-1 exosomes. As expected, pro-angiogenic proteins including intercellular adhesion molecule-1 (ICAM-1) and CD44 variant isoform 5 (CD44v5) are among the up-regulated proteins, whereas angio-suppressive protein, thrombospondin-1 (TSP-1) was down-regulated in C666-1 exosomes (40). A study on mesenchymal stem cells (MSCs) reported that the condition media of bone marrow MSCs (BMMSCs) treated with prostate cancer cell line (DU145)-derived exosomes had elevated levels of the pro-angiogenic factors; vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), based on enzyme-linked immunosorbent assay (ELISA) assay. Also, the exosomes induced BMMSC differentiation into myofibroblasts, as single stimulation with exosomes resulted in an increase the myofibroblastic marker alpha-smooth muscle actin (α-SMA) positive cells. This was thought to be through a transforming growth factor beta 1 (TGF-β1) dependent mechanism. The condition media of exosome-differentiated MSC, in turn, enhanced HUVECs cell survival (flow cytometry of viability markers), migration (scratch assay) and tubule-formation (tube formation assay) (41).

Furthermore, in the study where the TNBC line-derived exosomes were examined, it was reported that the invasive $Hs578Ts(i)_8$-derived exosomes had a significantly greater effect on endothelial tubules formation, compared to Hs578T-derived exosomes (17). It was also found out that leukaemia cell line (K562)-derived exosomes significantly increased HUVECs angiogenic capacity compared to the control, in a tube formation assay. Yet, hypoxic leukaemia cell line-derived exosomes had a greater effect on HUVECs in the same assay compared to non-hypoxic exosomes. It was proposed that this is due to the fact that hypoxic leukemia cell line-derived exosomes contain higher levels of miR-210 (TaqMan low-density miRNA array) (42). In another study, primary human glioma cells were subjected to hypoxia prior to exosome isolation, to reflect the tumour's hypoxic environment. These exosomes significantly increased tube formation by HUVECs and brain microvascular endothelia cells. In addition, glioma cell-derived exosomes induce microvascular sprouting in aortic ring sprouting assay (43). Same wise, it was reported that exosomes derived from a number of hypoxic lung cancer cell lines (CL1-5, NCI-H1437, -H1648, -H1792 and -H2087) significantly increased the migration (based on QCM 24-well Cell Migration System) and tube formation of HUVECs (tube formation assay), compared with exosomes derived from normal bronchial cells or normoxic lung cancer cells (44). The study suggested that this is due to increased miR-23a levels in these hypoxic exosomes. miR-23a will suppress its target, prolyl hydroxylase 1 and 2 (PHD1 and 2), which causes accumulation of hypoxia-inducible factor-1α (HIF-1α) in endothelial cells. In addition, exosomal miR-23a also inhibits tight junction protein ZO-1, causing an increase in vascular permeability and cancer transendothelial migration. This was confirmed when exosomes of hypoxic lung cancer cell lines increased endothelial permeability, based on *in vitro* vascular permeability assay, and trans-endothelial migration of cancer cells, leading to cancer cell intravasation/extravasation (44). Then, the outcomes were confirmed further when an *in vivo* Matrigel plug angiogenesis assay showed that exosomes derived from hypoxic CL1-5 cells further increased the formation of neovessels in the gel

plug compared to exosomes from normoxic CL1-5 (44).

Metastasis

Tumor metastasis is a very intricate process, it was projected that cancer cell-derived exosomes enable metastasis by initiating epithelial-mesenchymal transition (EMT) in the neoplastic epithelial cells, within the tumor microenvironment. It is also projected that cancer cell-derived exosomes will travel through the circulation and be selectively up-taken by the distal metastasis site, to initiate pre-metastatic niche formation (45). The first step in the metastasis cascade, is initiating the EMT process. During EMT the epithelial tumor cells acquire mesenchymal cell characteristics including; loss of cell-cell junctions and adhesion to the stroma, as well as increased migration and invasion capacity (46). As mentioned, exosomes mediate communication within the tumor microenvironment through the transfer of proteins and miRNAs. Promoters of the EMT cascade such as TGF-β1, matrix metalloproteinases and β-catenin were all identified within exosomes (47-50). Moreover, Vimentin which is also an EMT implicated protein (51), was found, in higher concentrations, in exosomes derived from PC14HM, a highly metastatic human lung cancer cell line, both at the gene (qRT-PCR) and protein (Western blot) levels. This was in comparison to exosomes derived from PC14, the original cell line. In addition, when treated with PC14HM-derived exosomes, human bronchial epithelial cells had higher migration (wound healing assay), invasion (Matrigel assays) and proliferation (EZ-Cytox cell viability assay) rates compared to cells treated with PC14-exosomes (52). In another study, it was found out that exosomes derived from hypoxic human oral squamous cell carcinoma (OSCC) cell lines (SCC-9 and CAL-27) increased the migration and invasion (via scratch assay and Matrigel invasion chambers assay) of OSCC cells. miRNA sequencing (Illumina HiSeq 2500 high-throughput sequencing) of normoxic *vs.* hypoxic OSCC-derived exosomes revealed a significant up-regulation of miR-21. Cancer-derived exosomal miR-21 was found to markedly increase the expression of vimentin and snail, also implicated in EMT (53), while at the same time, significantly decrease E-cadherin levels in OSCC cells (Western blot) (54).

In addition to EMT, cancer cell-derived exosomes will facilitate metastasis by establishing the pre-metastatic niche. Cancer exosomes promote the mobilization of cells that constitute the pre-metastatic niche. It is believed that exosomes will do that by up-regulating pro-inflammatory molecules and vascular leakiness at the site of metastasis. Then, when the pre-metastatic niche is formed, cancer cells will be deposited at the secondary organ in a selective manner, as cancer cells could be found in multiple organs vasculature, but only selective sites develop metastasis (55). This projected role of exosomes was confirmed by a number of studies, for example, it was reported that exosomes derived from primary cultures of murine pancreatic cancer cells, induced liver pre-metastatic niche formation in naive mice. This in turn increased liver metastatic burden. The study also reported that this was a result of selective exosome uptake by the liver Kupffer cells, which caused TGF-β1 release, which then led to hepatic stellate cells activation, creating a fibrotic microenvironment and caused increased immune cell infiltration (56). Another study reported that exosomes derived from highly malignant melanoma cell lines (B16-F10, SK-Mel-28 and SK-Mel-202) mobilize bone marrow-derived cells (BMDCs) by up regulating the oncoprotein tyrosine kinase-Met. This was confirmed when reduction of Met expression in these exosomes diminished the pro-metastatic behavior of BMDCs *in vivo* (lung metastasis mouse model). Also, in the same study, it was reported that injecting B16-F10 exosomes into mice, increased lung endothelial permeability, based on the presence of extravasated dextran, compared to exosomes from non-metastatic cell lines (57). Another reported mechanism of action, based on an *in vivo* study (lung metastatic mouse model), is that exosomes derived from primary tumor cells activate the toll-like receptor (TLR) 3 in lung epithelial cells, which causes these cells to increase chemokine production. This led to neutrophil recruitment and initiated pre-metastatic niche formation. This was confirmed when TLR3-deficient mice showed less lung metastasis events (58).

Following the pre-metastatic niche formation, metastatic cells will travel from the primary tumor site, through the blood circulation, to the site of metastasis. It is also believed that exosomes play a role in this step of metastasis by allowing cancer cells to escape immune surveillance in the blood circulation. Cancer cell-derived exosomes are also believed to play a role in the adaptation of cancer metastatic cells into the secondary organ, again through immune modulation. The most reported modulating action of cancer cell-derived exosomes, on cellular immunity, is through the up regulation of regulatory T cells and the inhabitation of effector T cells. One study reported that NPC cell lines (C15 and C17)-derived exosomes increased T reg cells

recruitment both *in vitro* (Boyden chamber assay) and *in vivo* (xenograft SCID mouse model) (59). Another study reported that incubating $CD4^+$ conventional T cells. with head and neck squamous cell carcinoma cell line (PCI-13)-derived exosomes caused an up-regulation of inhibitory genes (qRT-PCR). This have caused the cells to loss CD69 surface expression (activation marker measurement via flowcytometry) which causes the functional decline (60). Whereas another mechanism of action study revealed that a tumour maintains its stimulating inflammatory environment through exosomes, as it was demonstrated that breast carcinoma cell line (MCF-7)-derived exosomes promoted monocytes survival via the activation of the MAPK pathway (expression up-regulation of survival-associated molecules detected via Western blot) (61). Finally, at the metastatic site, cancer cells will either enter a dormant state or go through the mesenchymal to epithelial reverting transition (MErT) process. In which, tumor cells will re-acquire a phenotype which enable them to form macro-metastases at the secondary site (62). Exosome probably play a role in regulating this process, however, up tell now, there isn't much literature on this subject matter.

Closing remarks

It is well established by now that cancer cell-derived exosomes play a key role in almost all aspects of cancer development, propagation and maintenance. Numerous studies reported that cancer cells transmit and maintain their malignant characteristics through the release of exosomes. After binding, absorption, and internalization into the recipient cells, these exosomes could modulate pathways related to a number of biological actions including; cell cycle, apoptosis and invasiveness. However, the diverse mechanisms of exosomal-mediated-actions still require an extensive amount of investigation. Understanding the role of cancer cell-derived exosomes, in cancer, will greatly aid the diagnosis, management and hopefully the eradication of this disease. Almost a decade ago, researchers started reporting on the presence of "tumor-associated miRNAs" encapsulated within exosomes in patients' sera. These tumor-associated miRNAs are distinct from the miRNAs in nonmalignant-cell-derived exosomes and could be used as biomarkers for different forms of malignancies such as melanoma (63), ovarian cancer (64) and lung cancer (65). Nowadays, researchers are also reporting on exosome-based cancer management approaches that involve; inhibiting exosome release by cancer cells, or inhibiting cancer cell-derived exosomes uptake by recipient cells, through a number of compounds, drugs and antibodies (66). Yet again, for us to realize the full potential of cancer cell-derived exosomes in cancer diagnosis and treatment, more studies still need to be conducted to have a better grasp of the cancer exosome-cancer hallmark relation, and the implicated pathways.

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Footnote

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