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Abstract: Once thought of as inert remnants of cellular processes, the significance of membrane vesicles is now expanding as their capacity to package and transfer bioactive molecules during intercellular communication is established. This ability to serve as vectors in the trafficking of cellular cargo is of mounting interest in the context of cancer, particularly in the dissemination of deleterious cancer traits from parent cells to recipient cells. Although microparticles (MPs) contribute to the pathogenesis of cancer, their unique characteristics can also be also exploited in the context of cancer treatment. The detection of MPs in body fluids has the potential to provide an effective means for the diagnosis, prognosis and surveillance of cancer patients. The use of such easily accessible systemic biomarkers that reflect the characteristics of the parent cells circumvents the need for invasive biopsy procedures. In addition, the autologous nature of MPs may allow them to be used as novel therapeutic drug carriers. Thus the modulation of MP vesiculation, the detection of MPs in disease monitoring, and the application of MPs as therapeutic delivery vehicles present prospective clinical interventions in the treatment of cancer.

Revision Notes

1. The authors overviewed the contribution of microparticles (MPs) to the pathogenesis of cancer and the potential for clinical application of MPs. In contrast to the title of the manuscript "Microparticles in Cancer: A Review of Recent Developments and the Potential for Clinical Application.", a large part of the content of this manuscript occupied the refer and discussed on the publications of the authors' group. The authors should discuss more generally on state-of-the-art for clinical application of MPs or some other extracellular vesicles (EVs).

The authors have now included an extra section on Page 10 discussing the clinical applications of extracellular vesicles in cancer.

2. The authors should refer to sufficient publications in general, especially in cancer-derived extracellular vesicles.

Other references relevant to this study have now been added.

3. The term "microparticles" mainly used in this manuscript is not well documented. Is there any specific differences between MPs and EVs? If so please describe those appropriately in the text.

An explanation has now been added on Page 3 to describe the different membrane vesicles.

4. The authors described "exosomes" in the text in the section 4. However, there is no mentioned the differ from MPs. This is a very confusing.

An explanation of the different membrane vesicles has now been included on Page 3.

5. The content of the figure is a very limited information. Modify the figure more generously including more other findings on tumor metastasis and drug resistance.

Additional figures have now been included.

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Microparticles in Cancer: A Review of Recent Developments and the Potential for Clinical Application

Running Title: Clinical application of microparticles in cancer

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Abbreviations: Matrix metalloproteinases, MMPs; mesenchymal stem cells, MSC; MPs, microparticles; miRNA, microRNA; microvesicles, MVs; MDR, multidrug resistance; non-small cell lung carcinoma, NSCLC; P-gp, P-glycoprotein; PYK2, proline-rich tyrosine kinase 2.

Abstract

Once thought of as inert remnants of cellular processes, the significance of membrane vesicles is now expanding as their capacity to package and transfer bioactive molecules during intercellular communication is established. This ability to serve as vectors in the trafficking of cellular cargo is of mounting interest in the context of cancer, particularly in the dissemination of deleterious cancer traits from donor cells to recipient cells. Although microparticles (MPs) contribute to the pathogenesis of cancer, their unique characteristics can also be exploited in the context of cancer management. The detection of MPs in body fluids has the potential to provide an effective means for the diagnosis, prognosis and surveillance of cancer patients. The use of these readily accessible systemic biomarkers has the potential to circumvent the need for invasive biopsy procedures. In addition, the autologous nature of MPs may allow them to be used as novel drug delivery carriers. Consequently, the modulation of MP vesiculation to treat disease, the detection of MPs in disease monitoring, and the application of MPs as therapeutic delivery vehicles present prospective clinical interventions in the treatment of cancer.

Keywords: Biomarker; cancer; drug delivery; metastasis; microparticle inhibitors; microparticles; multidrug resistance; P-glycoprotein.

1. Introduction

Microparticles (MPs) are part of a general classification of extracellular vesicles termed microvesicles (MVs), which includes a population of membrane vesicles that are heterogeneous in shape, ranging in size from 0.1-1 μm and isolated from biological fluids or conditioned culture media [1]. Other extracellular vesicles include apoptotic bodies and exosomes, which differ on the basis of their size and origin. The irregularly shaped apoptotic bodies are released from cells undergoing apoptosis and fragmentation and range from 1-5 μm in size, whereas, exosomes (30-100 nm) are released by the fusion of multivesicular bodies with the cell membrane [1]. MPs, which are the focus of this review, are released from the surface of cells by the process of outward membrane budding through a loss of calcium-dependent membrane phospholipid asymmetry and cytoskeletal rearrangement [2]. MPs are therefore composed of fragments of the parent cell, which comprise the plasma membrane proteins and cytoplasmic and nucleic constituents of the parent cell. Once MPs bud from the parent cell, they are released into the systemic circulation, where they can effectively deliver their cargo long-range to recipient cells. In this way, MPs serve as systemic vehicles in mediating intercellular communication. MPs have been found to carry various bioactive molecules, proteins and nucleic acids including mRNA and microRNA (miRNA) [3-6]. Thus they are involved in multiple aspects of cancer progression including the development of drug resistance [5, 7-10] and metastases [11-13], tumor angiogenesis (by the dissemination of components such as sphingomyelin and VEGF) [14, 15] cellular survival (by the removal of cytosolic caspase 3) [16, 17] and evasion of immune surveillance via the expression of components such as latent membrane protein (LMP-1) [18] and Fas ligand [19, 20] (Figure 1). In this review, we will be focusing on recent developments in the role of MPs in cancer and how they can be utilized clinically in cancer management.

2. Microparticles provide a link between drug resistance and metastasis

MPs have been shown to confer and transfer multidrug resistance (MDR) in cancer cells [5, 7, 10, 21]. This we showed was mediated through the intercellular transfer of functional resistance proteins, such as P-glycoprotein (P-gp) and Multidrug resistance protein 1 (MRP-1) Figure 2 is a confocal image which shows the transfer of P-gp-EGFP fusion protein transferred via MPs to recipient drug sensitive cancer cells. We observe significant co-localization with the membrane intercalating dye PKH-26 following a 4 hour co-culture period. This is consistent with our previous reports showing functionality of transferred resistance proteins contributing to the acquisition of MDR in recipient cells [5, 7, 10]. The MP-mediated acquisition of MDR was also shown to be associated with the promotion of an enhanced metastatic capacity in recipient breast cancer cells [11]. The elucidation of this relationship is significant, as these two deleterious traits were previously considered independent. Although an association between the emergence of the two phenotypes had been alluded to previously [22-24], a definitive link and the mechanism behind this remained unknown. Our laboratory was the first to show that MPs serve as a conduit in mediating this relationship [11].

Recipient breast cancer cells, which were both lowly metastatic and responsive to drug treatment, acquired an enhanced metastatic capacity with the ability to resist drug treatment upon co-culture with MPs derived from highly metastatic, drug-resistant donor cells [11]. MPs derived from breast cancer MDR cells were shown to mediate migration, invasion and drug resistance in recipient breast cancer cells to yield a population that was metastatic and drug resistant (Figure 3).

The clinical relevance of MPs as the link between metastasis and drug resistance is that progression of either metastatic capacity or resistance may be indicative of progression of the other trait also. From a therapeutic perspective this provides opportunities for the prevention of these deleterious cancer phenotypes.

3. Microparticles mediate the enhancement of metastatic traits

Metastasis is an especially unfavorable aspect of cancer progression, whereby a localized population of cancer cells spreads through the lymphatic system or bloodstream to other parts of the body. MPs have been shown to play a role in the induction of an enhanced metastatic capacity in cancer in a variety of ways. MPs have been found to transfer matrix metalloproteinase's (MMPs), which are capable of degrading the extracellular matrix, allowing metastasis and invasion by cancer cells. MMP-2 and MMP-9 were found in MPs released from ovarian cancer cells and breast cancer cells and enhanced the metastatic capacity of recipient cells [12, 25, 26]. As mentioned above, we also showed that MPs formed are a conduit between drug resistance and metastasis. This appears to be mediated via the regulation of *miR-503* and proline-rich tyrosine kinase 2 (PYK2) to promote the migration and invasive capacity of recipient breast cancer cells [11].

Specifically, we confirmed that *miR-503* is required for the inhibition of migration and invasion in breast cancer as demonstrated by wound healing migration assays and Matrigel®-coated transwell invasion assays [11]. This finding supported previous observations of reduced migration and invasion following transfection of *miR-503* in hepatocellular carcinoma cells [27], acute myeloid leukemia cells [28], chronic myelogenous leukemia cells [29], osteosarcoma cells, colon cancer cells [30], head and neck carcinoma cells [31] and in endometrioid endometrial cancer cells [32]. Moreover, we showed for the first time that MPs were involved in mediating the effects of *miR-503* in breast cancer cells. One such mechanism for the down regulation of *miR-503* by MPs was proposed to be via the activation of the NF-κB signaling pathway, as NF-κB has been shown to suppress the expression of *miR-503* in epithelial cells [33]. Moreover, NF-κB has been associated with the promotion of migration and invasion in breast cancer [34].

Furthermore, PYK2 protein and mRNA was found to be upregulated in recipient cells following co-culture with MPs [11]. PYK2, a member of the focal adhesion kinase subfamily of cytoplasmic tyrosine kinases, was correlated with an increased metastatic capacity in a breast cancer cell line [35], a squamous cell carcinoma of the head and neck [36], hepatocellular carcinoma [37, 38] and prostate cancer [39]. Both PYK2 and *miR-503* may promote metastasis in recipient cells via regulation of the PI3K/AKT signaling pathway. The overexpression of PYK2 was associated with activation of the PI3K/AKT pathway and poor survival and metastasis in hepatocellular carcinoma [37]. Additionally, as *miR-503* targets and inhibits PI3K/AKT activation [40, 41], the suppression of *miR-503* in recipient cells may result in the up regulation of PI3K/AKT signaling and the subsequent promotion of metastasis.

Although up regulated in recipient cells, PYK2 was not found in the cargo of the MPs themselves [11]. This was the first demonstration that it was the dissemination of intermediary mediators that led to the MP-mediated regulation of PYK2 in recipient cells rather than the direct transfer of components packaged within the MP cargo. Therefore, the scope of MP involvement in the promotion of migration and invasion is continuously broadening.

4. Clinical applications of microparticles and other extracellular vesicles in cancer

Modulation of MP release in the management of cancer

The subject of MP inhibitors is an emerging focus in the field. Calcium channel blockers [42], ROCK inhibitors [43] and pantethine [44] have been shown to prevent the production and release of MPs in various cell types. This new class of compounds has potential to provide a novel strategy in circumventing MP-mediated dissemination of deleterious traits and preventing cancer progression [45, 46]. In a recent study, we elucidated the effects of Calpain inhibitor II, vitamin B5 derivatives and the calcium channel blocker Verapamil hydrochloride on modulating MP-

biosynthetic pathways. Interestingly, Calpain inhibitor II (ALLM) showed a significant inhibition of MP production in both resting as well as cells activated with a calcium ionophore, A23187, while a ROCK inhibitor (Y-27632) inhibited MP synthesis in activated cells only. Thereby, these novel molecules may serve as potential candidates in strategies employed for the prevention of MP-mediated disease progression in cancer [46].

MPs as novel drug delivery systems

In addition to their role as indicators of disease, the capacity of MPs to carry a multitude of components as part of their cargo can also be exploited in drug delivery. Since MPs naturally function as vehicles for the delivery of bioactive molecules, the refinement and modification of these processes may allow MPs to be used as novel therapeutic vehicles in the treatment of cancer.

MPs have been found to sequester chemotherapeutic drugs [9]. In doing so, they provided another means by which MPs facilitated cancer MDR. MPs do this by both passive and active mechanisms. Passive sequestration occurs via diffusion of clinically relevant chemotherapeutic drugs such as the anthracyclines daunorubicin and doxorubicin across the MP membrane followed by incorporation within the intravesicular cargo of nucleic acids and phospholipids [9]. Once trapped, the drugs are no longer freely available to the target site and thus cancer cells evaded therapy. Active sequestration occurs in MPs derived from drug-resistant cancer cells that carry the drug efflux transporter P-gp on their surface. A number of this P-gp is inside-out in orientation such that rather than its traditional function of effluxing drugs, this results in the actively influx of drugs into MPs where they become trapped.

Such a mechanism for drug trapping may be harnessed for therapeutic use in a similar manner to that employed by synthetic liposomes [47-50]. Indeed, a study conducted by Tang and colleagues in 2012 [51] showed that malignant cells incubated with chemotherapeutics drugs were able to package these drugs in the

MPs released from them. These drug-loaded MPs in turn were shown to have an anti-tumor effect in murine tumor models without the typical side effects [51].

The development of MPs as a viable mechanism for the delivery of therapeutic molecules would be advantageous over artificial vesicles as they can be isolated from the patient, loaded with the desired drug(s) and reintroduced into the patient during treatment. Therefore, complications associated with rejection and immunogenicity would be avoided through the use of these autologous and biocompatible vehicles. The potential for using such vehicles for therapeutic delivery has been described for exosomes [52]. The clinical viability of dendritic cell-derived exosomes was assessed in Phase I trials with melanoma patients [53] and in patients with non-small cell lung carcinoma [54], with results showing that the therapy was well tolerated and could produce the required therapeutic effects. In particular, there is mounting interest in the delivery of nucleic acids for cancer therapy using this same approach.

Loaded exosomes were used to deliver exogenous short interfering RNA to the brain tissue of mice, resulting in specific gene knockdown of *BACE1*, a therapeutic target in Alzheimer's disease, and reduction in β-amyloid 1-42 levels, a component of the amyloid plaques associated with Alzheimer's disease [55]. Furthermore, microvesicles harboring suicide gene mRNA and protein from donor cells reduced the growth of schwannoma tumors in an orthotopic mouse model [56]. MPs have also been shown to selectively package miRNAs and deliver them to recipient cells to regulate target gene expression and cellular function [6, 57, 58]. Therefore, there is extensive potential to use endogenously or exogenously loaded MPs in gene therapy as part of cancer therapy.

Role of MPs as biomarkers of cancer

In addition to the applications described above, there is emerging evidence for the role of extracellular vesicles in disease monitoring and diagnosis by serving as

biomarkers in cancer. MPs have been shown to have an incredible capacity to incorporate constituents from the parent cell and deliver them to recipient cells, contributing to cancer progression and resistance [5-7, 21]. It is this very capacity that makes MPs a promising biomarker for the diagnosis, prognosis and surveillance of cancer. Circulating MPs have been detected in the blood, urine and ascites of cancer patients [26, 59-62]. Furthermore, elevated levels of circulating MPs have been detected in patients with non-small cell lung carcinoma [63] and newly diagnosed glioblastoma patients [64] compared to healthy controls. Additionally, breast cancer patients have higher levels of MPs than healthy controls or patients with benign breast tumors [65-67]. This supports their potential as relevant diagnostic markers of malignancy.

The detection of MPs has been associated with the prognosis and clinical status of cancer patients. An example is provided in pancreatic cancer. Tissue factor is an initiator of the blood coagulation cascade and has been detected in MPs extracted from the plasma of patients. Increased levels of tissue factor expressing MPs were found to be indicative of the presence of an aggressive, metastatic and poorly differentiated malignant pancreatic state that could easily infiltrate peripancreatic vessels in patients [68]. Consistent with this was the strong association with thrombosis and increased mortality in patients with pancreaticobiliary cancers [69]. This study amongst many others lends support to the diagnostic and prognostic potential MPs have in cancer management.

Due to their presence in easily extracted body fluids and their capacity to reflect the characteristics of the parent cell, the proteomic and nucleic profile of MPs also has potential to be employed in diagnosis and prognosis. This may potential circumvent the need in the future for invasive biopsy procedures in staging and grading of cancers. The determination of the molecular status of tumors allows for detection of specific disease markers, surveillance of cancer progression and supports

approaches used in individualized and targeted therapies. This has potential for fast and efficient implementation of tailored interventions, resulting in improved clinical outcomes for the patient.

Clinical applications of other membrane vesicles in cancer

The immunotherapeutic effect of ascites derived exosomes in combination with GM-CSF has been assessed in Phase 1 trials for the treatment of colorectal cancer. In this study, exosomes could induce an antigen-specific anticancer cytotoxic T lymphocyte response in patients, with minimal toxicity and tolerated administration [70]. Furthermore, an ongoing Phase II trial which assesses the efficacy of dendritic cell derived exosomes as autologous therapeutic vaccines in advanced non-small cell lung carcinoma (NSCLC) patients to stimulate their natural defences to prevent tumor progression [71]. The preclinical [72] and clinical data [72] are promising showing that dendritic cell derived exosomes serve as maintenance immunotherapy in patients bearing inoperable NSCLS by T-cell priming and restoring T- and NK-cell functions in end stage patients. Likewise, mesenchymal stem cells (MSC) derived MPs were shown to induce cell cycle arrest and apoptosis of different tumor cells as well as inhibit *in vivo* tumor growth [73]. This approach is potentially beneficial as MP inhibition of disease progression occurs in the absence of MSC differentiation into stromal fibroblasts which would otherwise be conducive to tumor growth [74, 75]. Given their small size, lack of toxicity, target specificity and tolerance in host cells, membrane vesicles may serve as therapeutic agents for the treatment of cancer as well as clinical biomarkers for disease diagnosis and monitoring based on their cancer specific protein, nucleic acid and lipid cargo. Clinical applications of membrane vesicles are still in the developmental stage and their full potential waits to be explored.

5. Conclusion

Elucidation of the pathological role of MPs in cancer is ongoing. Understanding this role is critical in the development of interventions to prevent the progression of cancer, as well as in harnessing this natural mechanism and using it in clinical practice. In this way, there are three avenues by which MPs are being studied for improved clinical outcomes. Firstly, through the formulation of MP inhibitors as a novel therapeutic class in the treatment of numerous conditions arising from MP release and the MP-mediated intercellular communication. Secondly, harnessing MPs as natural vehicles in drug delivery. Utilizing circulating MPs as cancer biomarkers providing an effective and non-invasive form of cancer diagnosis, prognosis and surveillance to tailor and personalize therapy. These strategies highlight MPs as attractive therapeutic candidates in disease state management.

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8. Figure Legends

Figure 1: The role of MPs in cancer progression. MPs facilitate **(A)** the development of drug resistance through the transfer of functional drug resistance proteins such as P-gp and MRP-1, **(B)** the enhancement of metastatic potential enabled by the acquisition of proteases, miRNAs and protein tyrosine kinases **(C)** promotion of angiogenesis by the dissemination of components such as sphingomyelin and VEGF, and **(D)** cellular survival and evasion of immune surveillance via the expression of components such as caspase 3, latent membrane protein (LMP-1) and Fas ligand.

Figure 2: MPs transfer P-gp to drug sensitive cells. 100 µg of MPs derived from human breast adenocarcinoma cells (MCF-7) transfected with EGFP tagged P-gp, transfer P-gp to drug sensitive MCF-7 cells following a 4 hour co-culture period. Cells were fixed, labeled with PKH-26 membrane dye and the cell nuclei with DAPI as per the manufacturer's protocol (Sigma Aldrich, Australia). Panel A shows the merged channels captured, Panel B shows P-gp-EGFP in the 488 nm channel, Panel C shows PKH-26 in the 561 nm channel. Images were acquired on a Nikon A1 laser scanning confocal microscope at 100x magnification. Imaris 8 software (Bitplane AG) was used for 3D reconstruction of the images. Scale bar as indicated. Data are representative of a typical experiment.

Figure 3: MPs link the development of drug resistance to an enhanced metastatic capacity in cancer. (A) MPs shed from highly metastatic, drug-resistant donor cells transfer components such as P-gp protein, mRNA and associated miRNAs, PYK2 and *miR-503* to up regulate P-gp expression and metastatic capacity in lowly metastatic, drug-sensitive recipient cells. **(B)** Recipient cells acquire both drug resistant and metastatic traits to promote the evasion of chemotherapy and metastatic spread of cancer.

Figure 1

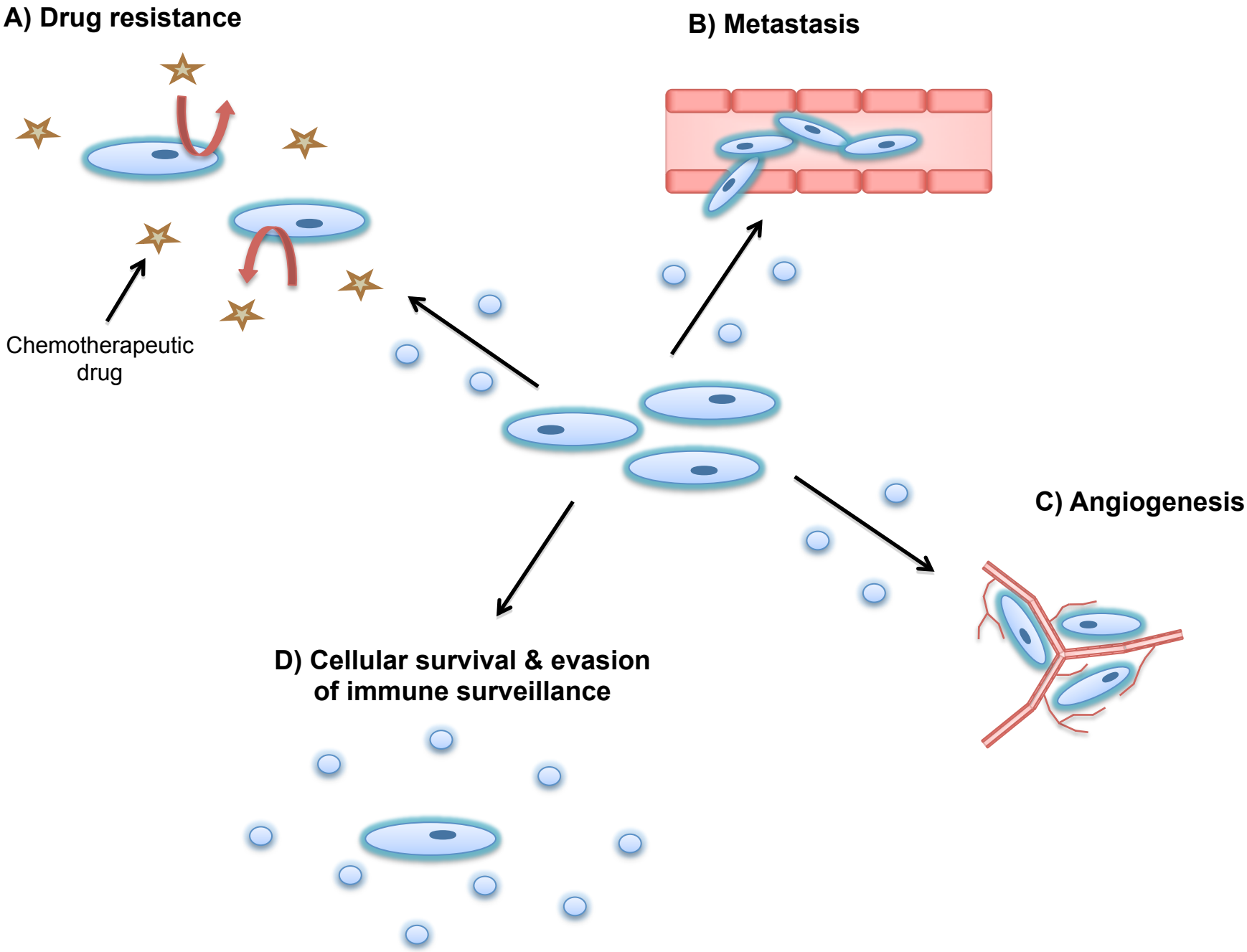

A) Drug resistance
B) Metastasis
Chemotherapeutic drug
C) Angiogenesis
D) Cellular survival & evasion of immune surveillance

Figure 3

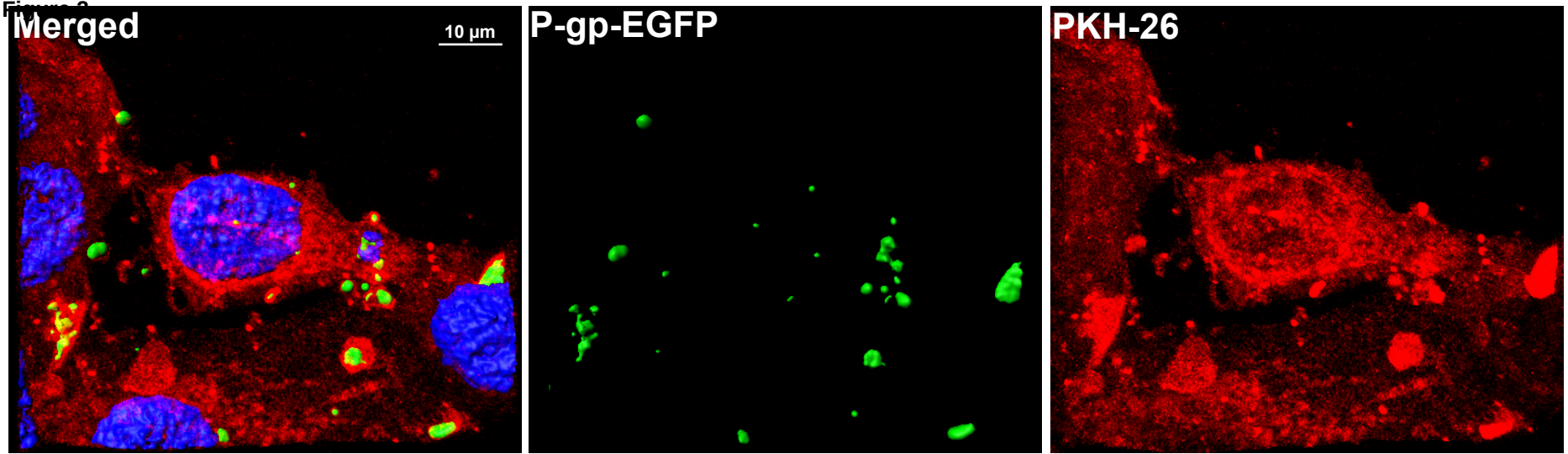
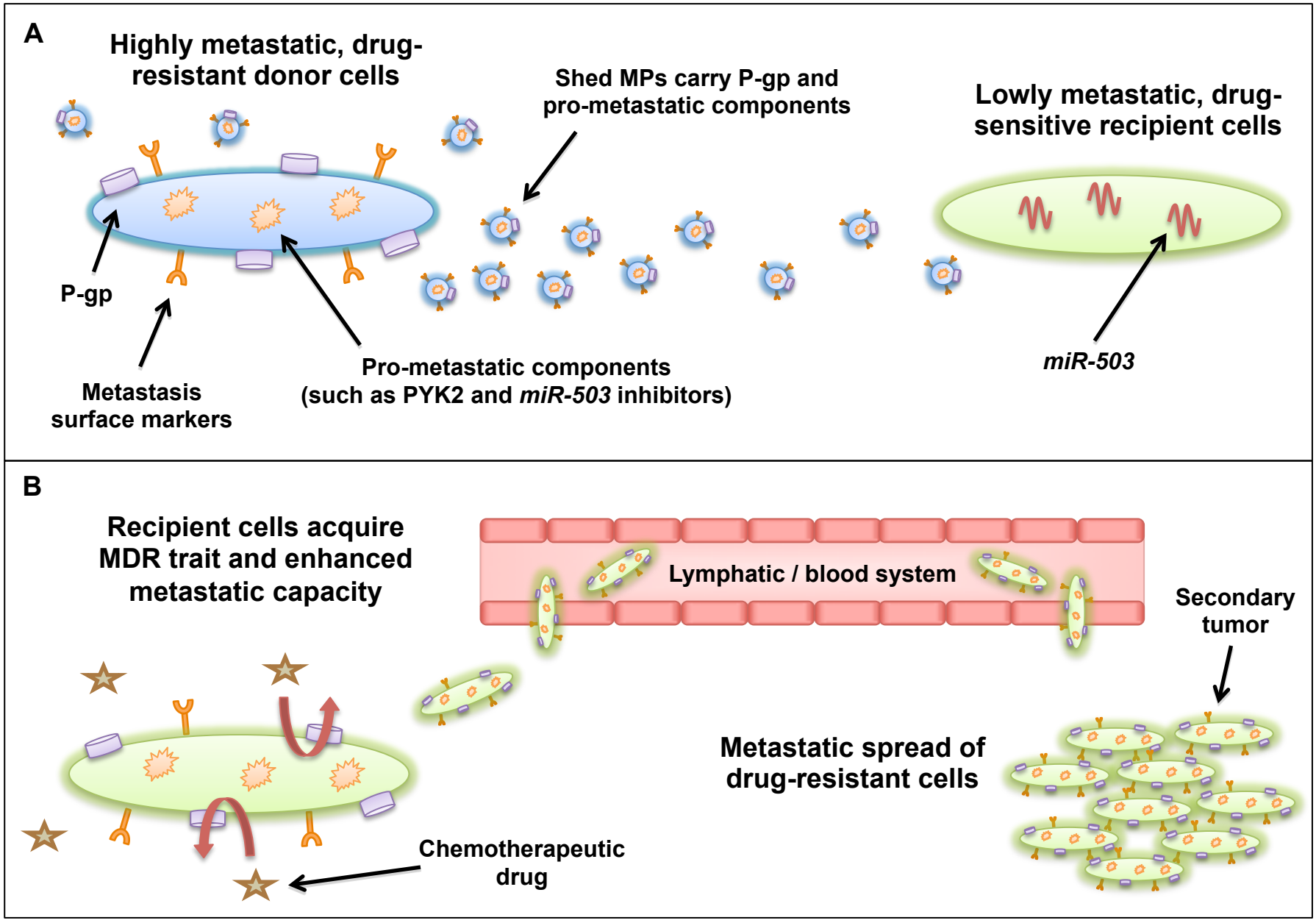


Figure 3


A
Highly metastatic, drug-resistant donor cells
Shed MPs carry P-gp and pro-metastatic components
Lowly metastatic, drug-sensitive recipient cells
P-gp
Metastasis surface markers
Pro-metastatic components (such as PYK2 and miR-503 inhibitors)
miR-503
B
Recipient cells acquire MDR trait and enhanced metastatic capacity
Lymphatic / blood system
Secondary tumor
Metastatic spread of drug-resistant cells
Chemotherapeutic drug