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THEME | *Extracellular Vesicles in Cell Physiology*

Stem cells and extracellular vesicles: biological regulators of physiology and disease

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Borgovan T, Crawford L, Nwizu C, Quesenberry P. Stem cells and extracellular vesicles: biological regulators of physiology and disease. *Am J Physiol Cell Physiol* 317: C155–C166, 2019. First published March 27, 2019; doi:10.1152/ajpcell.00017.2019.—Many different subpopulations of subcellular extracellular vesicles (EVs) have been described. EVs are released from all cell types and have been shown to regulate normal physiological homeostasis, as well as pathological states by influencing cell proliferation, differentiation, organ homing, injury and recovery, as well as disease progression. In this review, we focus on the bidirectional actions of vesicles from normal and diseased cells on normal or leukemic target cells; and on the leukemic microenvironment as a whole. EVs from human bone marrow mesenchymal stem cells (MSC) can have a healing effect, reversing the malignant phenotype in prostate and colorectal cancer, as well as mitigating radiation damage to marrow. The role of EVs in leukemia and their bimodal cross talk with the encompassing microenvironment remains to be fully characterized. This may provide insight for clinical advances via the application of EVs as potential therapy and the employment of statistical and machine learning models to capture the pleiotropic effects EVs endow to a dynamic microenvironment, possibly allowing for precise therapeutic intervention.

biomarkers; extracellular vesicles; leukemia; machine learning; stem cells

GENERAL VESICLE BIOLOGY

Classification, Biogenesis, and Release

Extracellular vesicles (EVs) are heterogeneous, naturally occurring, membrane enclosed spheres of varying size that are secreted by most cell types. They may be broadly classified into exosomes, microvesicles, and apoptotic bodies according to their biogenesis, cellular origin, and size (Table 1). Larger populations of microvesicles specific to certain cancer are termed oncosomes and contain oncogenic cargo and unique signatures of the tumor cells from which they emerge (11).

The biogenesis of microvesicles distinctly differs from that of exosomes, and at times DNA is detectable in extracellular vesicles. The former is generated by membrane lipid microdomains that bleb out of the cell membrane, allowing retention of the membrane proteins of the parent cell (which has implication in biomarker utility). The latter is a more homogenous population, derived by GTPase-dependent fusion and inward budding of endosomes/multivesicular bodies (MVB) with the plasma membrane via the endolysosomal pathway (Fig. 1) (11).

Cargo and Effects on Target Cells

EVs contain numerous proteins, bioactive lipids, DNA and RNA species that are capable of entering target cells and altering the transcription and expression of genes and proteins related to numerous cellular functions. EVs are released from almost all tissues and have been shown to regulate normal physiological homeostasis, as well as pathological states, by facilitating cell-to-cell communication in a paracrine fashion. As we will discuss, EVs may also act in an endocrine signaling fashion, having effects on distant cell niches. Vesicles have been noted to have positive and negative effects on cell proliferation, differentiation, viability, organ homing, and injury recovery, in addition to disease progression (2, 3, 7, 35). Their ability to manifest varying, sometimes polar, effects is due to their vast heterogeneity and specificity. EV cargo composition is selectively packaged via a complex endosomal sorting complex required for transport (ESCRT), such that the same cell will release varying cargos depending on its physiological state (24, 26). Furthermore, EV quantity and size distribution, as well as effector function, are all downstream effects of the specific cell type and disease type they manifested from.

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Table 1. *Biogenesis pathways of extracellular vesicles are distinct*

Vesicle Type	Size, nm	Origin	Content	Marker
Exosomes	40–120	Endocytic pathway	Proteins, lipids, and nucleic acids (mRNA, miRNA, and other noncoding RNAs)	Alix, Tsg101, tetraspanins (CD81, CD63, CD9), flotillin
Microvesicles	50–1,000	Plasma membrane blebbing	Proteins, lipids, and nucleic acids (mRNA, miRNA, and other noncoding RNAs)	Integrins, selectins, CD40
Apoptotic bodies	500–2,000	Plasma membrane blebbing	Nuclear fractions, cell organelles	Annexin V, phosphatidylserine

Once at the effector cell, EVs impart cellular effects by several purported mechanisms including *1*) direct binding and activation of cell surface receptors by proteins and lipid ligands, or *2*) fusion and uptake (phagocytosis/endocytosis) of vesicle contents into the recipient cells. Effector molecules (e.g., mRNA), non-coding regulatory RNAs (e.g., microRNAs or miRNAs), proteins, and transcription factors can all be delivered, each having short- and long-term implications on effector cell phenotype and function (33). Our own studies exploring the effect of EVs from lung and bone marrow sources showed that there was an initial transfer of lung-derived mRNA for surfactants to the target marrow cells, as well as the transfer of a factor that induced donor cell, lung-specific mRNA characteristics on the target cell (1). However, with time in cytokine-supported culture, the lung-specific mRNA was derived from the target marrow cells—presumptively representing a stable epigenetic change. We produced identical data measuring albumin mRNA specific to rats and mice. In this setting, rat and mouse albumin-specific primers were utilized, liver vesicles were incubated with marrow cells, and the resulting target cell albumin mRNA was analyzed for whether it was mRNA transferred from donor cell or mRNA produced in the target cell. As with the lung experiments, initially there was evidence for transfer of donor cell mRNA as well as a transcriptional inducer, but with time in culture, only target cell albumin mRNA was found (1). Thus, the mechanism here was also a stable epigenetic change. Various studies have also highlighted the ability of EVs to directly transfer relatively larger molecules such as cellular receptors, major histocompatibility complex (MHC) molecules, antigens, as well as entire organelles. Johnson et al. (23) demonstrated that B cell precursor acute lymphoblastic leukemia (ALL) cells release large EVs (<6 am) that contain fully intact mitochondria, lysosomes, Golgi, and intermediate filaments (Fig. 2).

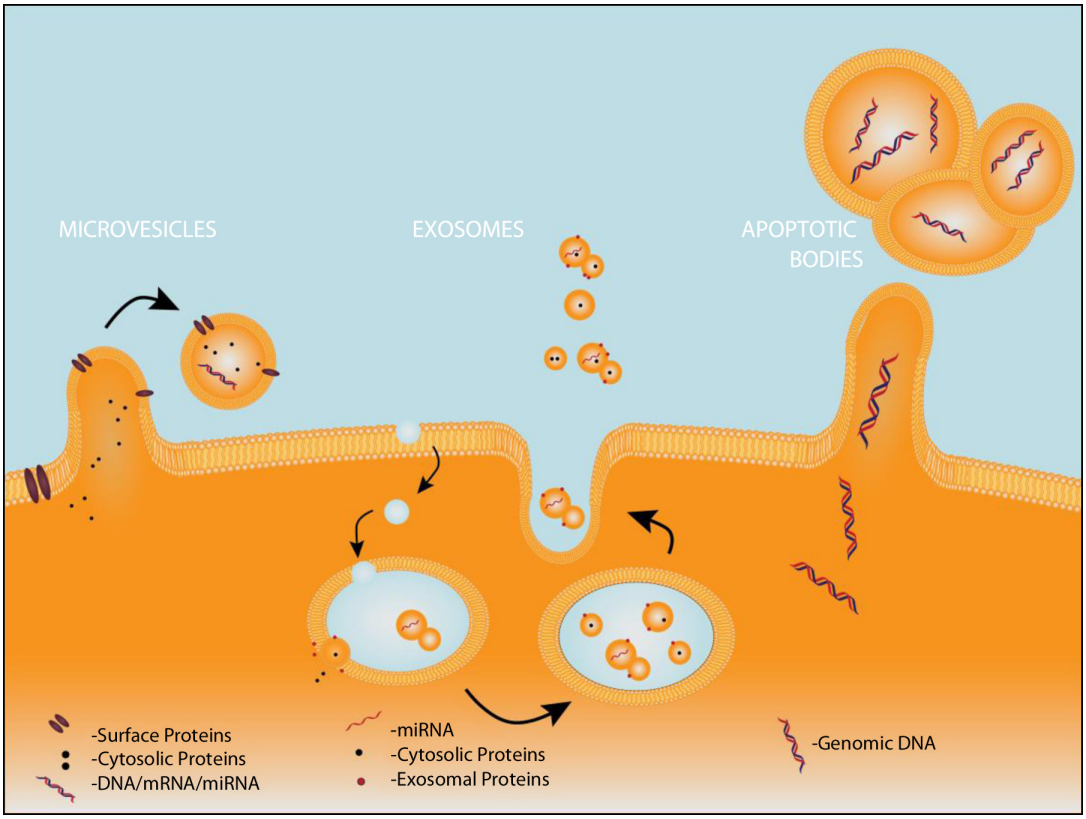

Fig. 1. A schematic of microvesicle blebbing and exosome multivesicular bodies fusing with the plasma membrane.

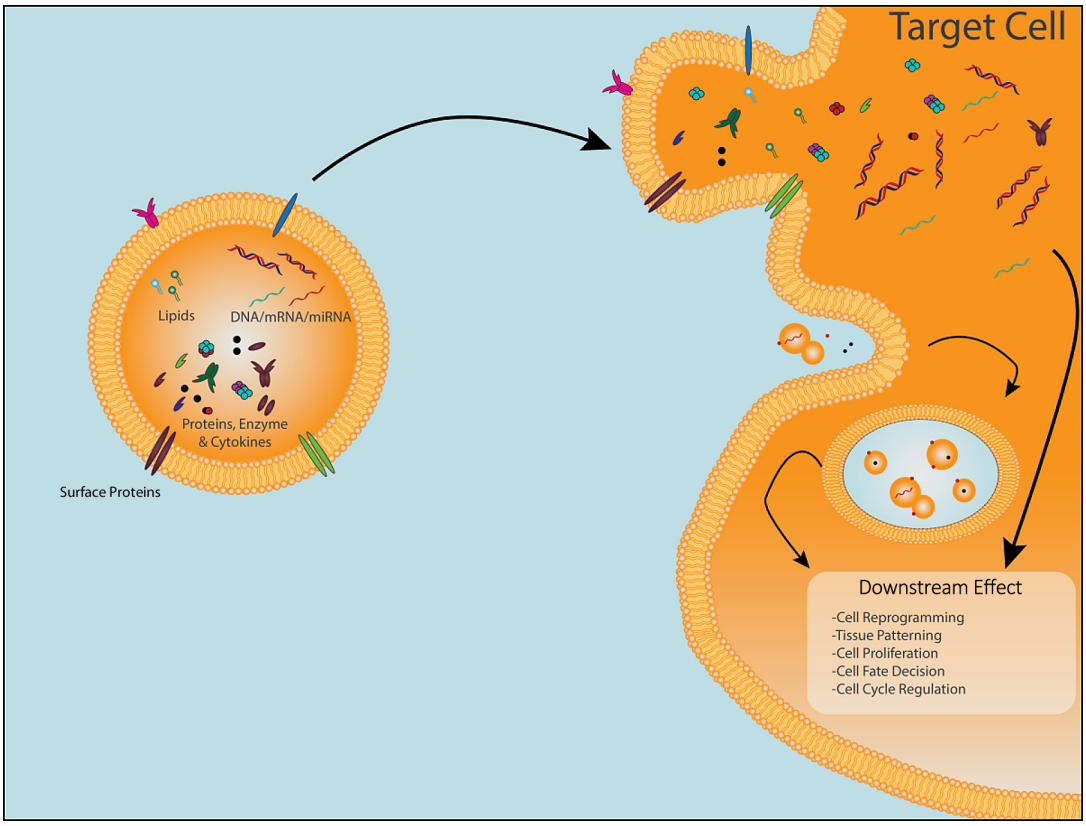

Fig. 2. Extracellular vesicle selective packaging, effects at target cell membrane, and release of internal contents.

THE EV PARADOX: GOOD AND BAD EVs

As EVs provide a vast range of biological potential, their functional cargo must therefore provide comparable variability. The originator cells' phenotype and biological state dictates EV number, cargo, and distribution. Hence, depending on these variables, not all EVs are created equal, suggesting an evolving paradigm that indicates the existence of "good" and "bad" vesicles across a number of different disease models. When the vesicles evolve from the diseased or abnormal tissue, they tend to make the disease worse; while when they come from normal tissue, they tend to exert a healing effect. By utilizing their innate variety of effector mechanisms and functions, vesicles can have drastic and far reaching implications on the basic normal function and homeostatic maintenance of an organism. This includes (but is not limited to) immune detection, cancer inhibition, inflammation regulation, metabolic feedback, and management of the cellular microenvironment in both physiological and pathological conditions. As such, EVs are being recognized as important mediators of cellular phenotypes and potential experimental therapeutic strategies for a number of disorders. Our own studies have provided evidence that salivary vesicles serve as a noninvasive, early, and reliable source of biomarker for traumatic brain injury (TBI) (13). The patterns of candidate biomarkers in this study have great potential for risk stratifying TBI patients prone to develop post-concussion syndrome (or post-traumatic encephalopathy), and their expression might allow for risk stratification and serve as a maker of symptomatic and neurophysiologic recovery following TBI.

Mesenchymal Stem Cells EVs and Disease

Hundreds of clinical trials involving the use of human bone marrow mesenchymal stem cells (MSCs) for the treatment of a variety of different human diseases have been registered (55). MSC-derived EVs can function in both tissue regeneration and immunosuppression, and exert their therapeutic functions in a paracrine fashion, rather than one based on direct cellular contact. Depending on cancer type, stage, and aggressiveness, EVs have been implicated in the inhibition of breast cancer cell migration and invasion, as well as in halting tumor growth in models of Kaposi's sarcoma and of hepatoma (30). A myriad of studies, including many of our own on radiated bone marrow stem cell populations, have shown that that both murine or human-derived vesicles have similar effects within the tested murine disease model, suggesting that EVs lack species specificity (42, 57).

Our past work has also illustrated that human bone marrow EVs can have a healing effect across multiple disease paradigms: reversing the malignant phenotype in prostate and colorectal cancer, recovering function in a murine model of acute kidney injury (AKI), as well as mitigating radiation damage to marrow (48, 57). A salient study performed by Camussie et al. (39) has generated data to indicate a potent healing effect for single doses of MSC-derived EVs obtained from different sources in multiple models of AKI recovery.

The mechanisms appear multiple. At the cellular level we have seen MSC-EVs promote proliferation and survival of healthy resident niche cells, limit inflammation, oxidation and vessel destruction (39). We have extended the therapeutic role of MSC-derived EVs into other disease states as well. In studies of monocrotaline induced murine pulmonary hypertension, studies have shown that exosomes from monocrotaline-treated mice could induce pulmonary hypertension in normal mice (2). This appeared to be secondary to genomic changes induced by different species of miRNA. However, the pulmonary hypertension induced by EVs isolated from sick mice could be prevented or reversed by exposure of the mice to healthy MSC-derived EVs, constituting a Yin and Yang plurality of vesicle effects. When exosomes from the mice with pulmonary hypertension were infused into normal mice, they induced pulmonary hypertension as measured by right cardiac ventricle free wall-to-left ventricle and septum ratio. Here again, the mechanisms of pathogenesis or prevention are likely multiple. These include the possibility that, in addition to genetic and phenotypic alteration caused by EVs, there are cellular mediators that are also influenced by them. The cellular mediators thought to be responsible for these phenomena were vascular progenitors. These cells are quite radiosensitive at levels that are well tolerated by both mice and humans (100 cGy). Exposure of mice with monocrotaline-induced pulmonary hypertension to 100 cGy whole body irradiation both prevented and reversed the disease, a finding thought to be due to the loss of these cellular mediators (3).

Our group is developing similar models using cocultured Kasumi-1 cells, a human leukemic cell line harboring an 8;21 chromosome translocation, with various concentrations of human mesenchymal stem cell-derived EVs. Here, we have demonstrated that these EVs alter the leukemic phenotype, stunt neoplastic proliferation, and induce apoptotic cellular death. The apoptotic-inducing effects of MSC-EVs that we have shown have been observed across multiple cancer models. Liu and colleagues (60) clearly demonstrated that human Wharton's jelly MSC-derived EVs (hWJMSC-EVs) inhibited the growth of bladder cancer in vitro and in an animal transplantation model for tumorigenesis. This work was extended to echo findings similar to those of Zhang et al. (61) showing that hWJMSC-MVs exhibit proapoptotic properties against explant tumor cells. Palatine tonsil-derived MSCs (TD-MSCs) when cocultured with head and neck squamous cell carcinoma cell lines similarly resulted in apoptosis induction and G_1 phase arrest of the cancer cell lines.

Explaining the Mixed Results in MSC Studies

There has been abounding criticism aimed at the mixed results seen across studies utilizing the therapeutic potential of MSCs. Perhaps the most capricious variable leading to discrepant results is that the tumor models used across publications naturally vary in multiple ways (50, 51, 54). This variability is only heightened when considering the varied, nonuniform, procedural, and technical methods across various laboratories (50, 51, 54). Tumor immunity, hypoxia, angiogenesis, and cytokine secretion are all highly variable in the models described in these studies. Even greater is the patient-to-patient variability in studies exploring MSC isolates.

There are also increasingly heterogeneous populations of the MSC cell types, all from variable sources. This heterogeneity is likely a prime contributor to why the role of MSCs in cancer settings has given varied and inconsistent results. Numerous publications isolated MSCs on the basis of plastic adherence without using appropriate cell surface marker expression as well as multipotency (51, 55). More selective and better characterized cell surface markers that permit isolation of a more homogenous population are needed. In addition, not all studies looking at MSC vesicles adhered to (now) accepted EV isolation protocols.

The large distribution of cell culture variability has been reported to alter the MSC phenotype. These include seeding MSC in high FBS media or supplementing with multiple growth factors, as well as allowing cells to clump or reach too high of a confluence. MSC cells that have been passed for multiple generations have also been reported to alter their phenotype, and ultimately differentiate.

Publications have purported that MSCs harbor the ability to spontaneously transform into a malignant phenotype. Not only has this never been directly observed or characterized, but Klopp et al. (26a), as well as other authors, addressed the issue with a very plausible explanation, citing that a high proportion of "MSC cell lines are contaminated" with tumor cells (e.g., reported contamination with osteosarcoma and glioma cells used in the same laboratories).

The experimental techniques by which MSC cells are introduced into the tumor microenvironment also portend variability. The bulk of the in vivo studies reporting tumor promotion mixed MSCs with tumor cells and coinjected the cells together. The effect is thought to be orchestrated in part by the hypothesis that cancer cells are able to more readily incorporate the MSC cells into tumor stroma, as both are competing to establish a niche (50, 51). This would be synonymous to late-stage acute myeloid leukemia (AML), during which AML blasts are able to outcompete and alter the MSC phenotype and EV population towards a pro-leukemic state. To some degree, this is likely due to sheer numbers: the cancer cell, with its inherent survival advantage, increased replication rate and higher quantity of EVs, has an immediate influence over a smaller MSC population at a time when the structure of the microenvironment is being capitulated. The end result means cancer cells can outcompete and easily incorporate MSCs, and likely other cell types of the cancer niche, into stromatogenesis.

This is in stark contrast to the delivery of MSCs alone into an established cancer microenvironment. The majority of the studies that introduced MSCs into established tumors reported tumor growth inhibition. These are identical findings to our preliminary work incorporating human bone marrow-derived MSC-EVs in early nascent AML inhibition.

Lastly, there have been over 1,000 patients involved in numerous clinical trials exploring the therapeutic use of MSCs for multiple indications. There has been no reported major adverse event or any indication of tumor formation in any of these patients.

Cancer-Derived EVs and Normal Tissue

EVs secreted by tumor cells have multiple implications in cancer pathology and progression.

Systemically, EVs shed by cancer cells are able to promote thrombus formation via expression of tissue factor (TF), which is a potent trigger of the coagulation cascade and thrombotic events. In addition, various cancers, as well as oncology treatments, can lead to the release and activation of platelet-derived EVs, further stimulating vascular remodeling and clot formation (41).

Tumor-derived EVs have been shown to have modulating effects impacting a variety of cancer hallmarks, all of which have been implicated in establishing a pro-tumor locale. The tumor microenvironment requires robust access to nutrition and oxygen. EVs promote endothelial remodeling and the formation of new blood vessels (angiogenesis) via the delivery of cytokines, growth factors, and miRNA cargo that potentiates long-term expression of angiogenic growth factors and cytokines (36). Cell signaling involved in modulation of cellular differentiation and proliferation can also be augmented by EVs. Panagopoulos et al. (64) showed that vesicles from both in vivo prostate cancer cell and explant cultured prostate cancer cells can induce cellular changes that produce a neoplastic phenotype in normal prostate cell lines. Similar studies in colorectal cancer have demonstrated the ability of EVs derived from a malignant colon cancer cell line and malignant patient tissue to induce the malignant phenotype in nonmalignant colon cells. These results were reproduced using vesicles from patients with other malignancies, namely lung, and prostate (55). Furthermore, these vesicles induced a tissue-specific genetic change in normal human marrow cells, which ultimately altered cellular phenotype.

Hematological Malignancies and EVs

EVs have also been shown to contribute to various hematological malignancies. Apart from their impact in acute leukemia (discussed in detail below), the role of EVs in chronic leukemia types has highlighted the broad functional potential EVs endow to their surroundings.

Chronic lymphocytic leukemia (CLL)-derived EVs can promote the survival of for CLL B cells by activating the Akt target of rapamycin/p70S6K/hypoxia-inducible factor-1α axis in CLL bone marrow stromal cells, with subsequent production of vascular endothelial growth factor and new blood vessel formation. CLL EVs can have many other biological and phenotypic changes on stromal cells, creating a niche promoting CLL cell adhesion, survival, and growth (15, 47).

In a similar fashion, chronic myeloid leukemia (CML) EVs injected into rat models can induce CML-like characteristics via the transfer of their fusion gene BCR/ABL DNA cargo, stimulating bone marrow stromal cells to produce interleukin (IL)-8 (mRNA and protein), a potent proangiogenic factor that modulates both in vitro and in vivo the leukemia cell malignant phenotype (15, 48).

EVs and Chemoresistance

EVs present in various models of cancer, including hematological malignancies, have direct associations with transferring chemoresistance.

We have shown that EVs from explant prostate cancer induce a neoplastic phenotype in normal prostate cell lines. In other publications, the coculture of EVs isolated from patient biopsied prostate tumor samples significantly induced changes towards a neoplastic phenotype and increased tumorigenic soft agar colony formation of non-malignant prostate cells, concluding that EVs derived from solid tumor cells can drive cancer progression and enhance resistance to certain chemotherapies (56).

Apart from altering phenotype, the potential of EVs to transfer multidrug resistance-associated proteins have also been highlighted in some of our own studies. In addition, we have also shown the contrary: the reversal of taxane resistance and tumorigenic phenotype in a human prostate carcinoma cell line (as well as human explants) can be accomplished by treatment with healthy MSC-derived EVs (56). In our leukemia model, we have expanded the use of proliferation and apoptosis assaying techniques to explore the potential of human bone marrow MSC-derived EVs as a direct adjunct therapy for AML. Our primary work indicates similar effects to what was observed in our solid cancer paradigms—that the killing potential of cytarabine, at even relatively low doses, is potentiated by the addition of healthy MSC-derived EVs. This trend is seen as early as day 1 of coculture and is increasingly apparent by day 6. In solid tissue cancers, the rescuing ability of EVs derived from healthy cells was observed: EVs isolated from normal prostate cells acquired via patient biopsy reverse the resistance of malignant prostate cells to various drugs. Out of all sources, EVs have a direct effect on phenotypic and genotypic changes, highlighting the central role of EVs in disease progressions and reversal.

Multiple Cellular Pathways and Proteins Are Directly Modulated by EVs

The mechanisms by which EVs abate or intensify sensitivity to chemotherapeutic drugs in various cancer models are vast. In many hematological malignancies, EVs impart chemoresistant properties on the microenvironment by EV-guided horizontal information transfer, thereby modulating the surrounding stroma to supporting oncogenic growth and drug resistance of leukemia cells (19, 53). In various models, including those exploring multiple myeloma (MM), bone marrow stromal cell-derived exosomes mediate cellular communication by transferring mRNAs, miRNAs, and proteins important in proliferation, survival, and chemoresistance (58).

In another murine MM model, the coculturing of 5T33 bone marrow MSC-derived exosomes with MM cells upregulated multiple antiapoptotic pathways that promoted MM cell viability. These exosomes, derived from stromal cells within a microenvironment amidst developing active cancer, were also able to induce drug resistance to the proteasome inhibitor bortezomib via activation of several survival relevant pathways, including c-Jun N-terminal kinase, p38, p53, and Akt. Pathways involved in angiogenesis have been shown to modulate cancer progression and chemotherapeutic evasion in the various cancer models we have discussed as well as multiple other (54). In vivo mouse matrigel plug models have shown that angiogenesis can be induced by immortalized myelogenous leukemia cell line K562 exosomes. These K562 exosomes induced Src phosphorylation and activation of downstream Src pathway proteins in human umbilical endothelial cells. Src is a kinase family predominantly inactive in cells. When it is activated, as with diseased EVs, multiple cellular signaling cascades involved in cell adhesion/integrin signaling

and growth proliferation are activated. Interestingly, the effect seen in this study was abrogated by treatment with oral dasatinib, a dual Src/Abl kinase inhibitor.

In vitro work with apoptosis-resistant primary AML blasts showed other modalities by which cells evade death. These apoptosis-resistant primary AML blasts were able to upregulate BCL-2 expression when compared with sensitive AML cells (53). There was also varied expression of other apoptosis-regulating proteins carried within the EVs of these cell lines. Proteins such as MCL-1, BCL-X, BAX, as well as BCL-2 have roles in regulating apoptosis and are plausible ways by which the cell can escape death and become resistant (4). Direct transfer of EVs from resistant to sensitive cells was observed via confocal-microscopy-based colocalization studies.

P-glycoprotein (P-gp), a plasma membrane multidrug efflux transporter, is another EV-bound protein that has been implicated in the transfer of drug resistance. When EVs isolated from drug-resistant leukemia cells (VLB100) were cocultured with drug-sensitive cells (CCRF-CEM), EV-contained P-gp was confirmed by using flow cytometry and Western blotting. The transfer of EVs with P-gp was then assayed using whole cell drug accumulation assays of rhodamine 123 and daunorubicin (37). Cells that had successfully incorporated EV-delivered P-gp exhibited increased drug resistance. The shaping of the leukemic niche is also dependent on other proteins that support leukemic growth. Galectin-3, a multifunctional galactose-binding soluble lectin protein, is expressed on the surface of bone marrow stromal cells. Other groups have also discovered that stromal cells can also secrete Galectin-3, as well as package this soluble protein into exosomes. Overexpression of this protein has been linked to enhanced tumor cell adhesion, angiogenesis, and immune evasion. When challenged with cytotoxic chemotherapy, stromal cells in ALL models begin to transcribe and deliver Galacetin-3-loaded EVs to dividing ALL cells, leading to increased drug resistance via tonic NF-κB pathway activation, which itself regulates Galectin-3 signaling (37, 40).

In other leukemic studies, cultured CLL B cells exposed to EVs derived from a stroma encompassing a developing leukemic niche showed heightened chemoresistance to several drugs. The cargo of these stromal EVs was related to various cell signaling pathways involved in inflammation, oxidative stress, NF-κB, and phosphatidylinositol 3-kinase/Akt pathway activation. Additionally, there were numerous preponderant proteins in these EVs strongly implicated in the transfer of chemoresistance, including S100-A9. Multiple works have discovered that the NF-κB pathway can be activated by S100-A9 expression and mediate CLL promotion (39).

Apart From Proteins, Exosomes Impart Chemoresistant Properties Via Multiple miRNAs

Other leukemia models have demonstrated the importance of B cell receptor (BCR) signaling in disease progression and therapeutic resistance. Much of this is postulated to be EV dependent. Clinical trials exploring the potential of the tyrosine kinase inhibitor ibrutinib highlighted the drug's ability to inhibit IgM-stimulated exosome release and subsequent BCR inactivation in B cells. Analysis of plasma samples collected from untreated CLL patients showed BCR activation, which seemed to induce exosomes bearing unique microRNA prolife including miR-29 family, miR-150, miR-155, and miR-223. This unique miRNA profile was vastly different from exosome profiles of patients undergoing ibrutinib treatment that have BCR suppression (37, 62). This implicates EVs not only in the pathogenesis of disease progression but also as possible surrogate biomarkers.

Exosomes can carry multiple miRNA profiles. EVs packed with miR-221/222, from tamoxifen-resistant MCF-7 breast cancer cells, can shuttle their cargo to sensitive cells of the same type, thereby transferring resistance. Utilizing PKH67 fluorescent labeling, other investigators were able to track the miRNA-loaded EVs as they entered their target cells. The elevated miR-221/222 effectively reduced the gene expression of p27 and estrogen receptor-α in target cells increasing drug resistance (26a).

Bouvy and colleagues (5) demonstrated similar EV-miRNA-mediated chemoresistance across other cancer resistance models, including salient work with promyelocytic leukemia HL60 cells. EVs shed by multiresistant strains (HL60/AR) that overexpressed the multidrug resistance protein 1 (MRP-1) were able to successfully transfer their chemoresistance to resistance cells via the exchange of MRP-1, multiple nucleic acids, and multiple miRNA species including miR-19b and miR-20a.

EV-Mediated Alteration in Cellular Pathways and Transfer of Proteins and miRNA Ultimately Leads to Phenotypic Change

As discussed, the bone marrow stroma can be recapitulated by active cancer via multiple EV-dependent mechanisms. Multiple cellular pathways implicated in carcinogenesis are directed by the direct transfer of exosomal protein and microRNA. In ex vivo and in vivo leukemic models the net effect of EV-modulation translated to a phenotypic change of the bone marrow stromal cells towards a more inflammatory signature that resembles the phenotype of cancer-associated fibroblasts (CAFs). As a result, stromal cells exposed to a leukemic microenvironment show enhanced proliferation, migration, and secretion of inflammatory cytokines, all contributing to a tumor-supportive niche (39, 43). As we will further discuss below, the CAF phenotype in CLL models also has immune-mediated consequences.

THE ROLE OF EVs IN THE PROGRESSION OF AML

Human MSC-Derived EVs: Effects on Stroma

The tumor microenvironment has a paramount role in tumor defense and progression. The cell-to-cell interaction of the tumor microenvironment represent only one mode of cellular communication. EVs harness immense biological activity and present a novel means by which to induce target cell activity and phenotypic manipulation that is independent of cell-to-cell contact. The microenvironment harbors multiple cell types and EV populations, each with distinct origin, cargo, and homing—ultimately, implying multiple simultaneous and competing signaling cascades. EVs present a "noncontact" means by which cells horizontally exchange genetic information, and infer metabolic and phenotypic effects on one another—a "moveable niche" of sort.

Across both liquid and solid cancer types, there is a mutualistic cross talk between the primary cancer and its proximate

surrounding stroma (the microenvironment). Multiple groups across an array of cancer types have shown that the cell-to-cell communication stromal cells maintain with the primary tumors is necessary to support their survival, localization, proliferation, and spread. Stromal and tumor-derived EVs represent a new component of this supportive microenvironment and are implicated in modulating the tumor microenvironment within the local niche, and at distant sites to prepare for cancer metastasis.

Other works have postulated the tumor-suppressive role that a healthy microenvironment may have on nascent cancer, one in which healthy stromal EVs are involved in the reprogramming of tumor cells toward a more benign phenotype. This phenomenon has been observed across multiple models in our previous discussion of EV-mediated chemosensitivity and resistance (34, 37, 44). The contrary has also been studied: as cancer grows, it subsequently imparts changes to its surroundings, reshaping its own stroma to favor its own survival. Leukemia-derived EVs have been implicated in the physiological transition of hematopoietic stem cells to cancer-associated fibroblasts to help shape the pro-leukemic niche (5, 37, 56, 60).

Early On the Bone Marrow Protects the Niche

MSC-EVs are multifaceted bioactive vehicles that have proven to harbor regenerative and therapeutic roles in numerous in vitro and in vivo models. Experimental models including Kaposi's sarcoma, glioma, breast cancer, and non-Hodgkin's lymphoma have all taken advantage of the innate tumor-suppressive effects harbored by bone marrow MSCs (6, 36). Our own studies have validated these properties in the setting of colorectal and prostate cancer, as well as the mitigation of radiation damage to bone marrow (1, 42).

In our developing leukemic models, similar results were observed: we successfully isolated human bone marrow MSC EVs and have shown that these impart an antiproliferative and proapoptotic effect on leukemic cells in vitro. These findings illustrate the potential therapeutic uses of EVs as an adjunct to conventional AML therapies, which our early preliminary data suggest is a promising application. As they can be sequestered and isolated from patients' tissue, they are likely to be well tolerated as a therapeutic platform.

In vitro and in vivo MM studies looking at the molecular cargo of exosomes transferred from bone marrow-derived MSCs to clonal plasma cells demonstrate that healthy bone marrow-derived MSC exosomes (not under the influence of a developing cancer microenvironment) inhibited the growth of MM cells (37). In contrast, MSCs exposed to a bone marrow influenced by progressive MM secreted exosomes harboring a very different physiology, leading to the survival and growth of MM cells (54). This dichotomy indicates an early protective role of the bone marrow stromal microenvironment that is altered and abrogated by developing cancer—a common theme translatable across multiple cancer platforms, including our own studies exploring the early development of the leukemic niche. Other works have validated this dynamic variation in exosomal cargo, including proteins and microRNA, between normal MSCs and those exposed to progressive cancer, showing that MM-exposed bone marrow MSCs hold higher levels of oncogenic proteins, cytokines, and adhesion molecules than those not exposed.

Human MSC-Derived EVs and Early Nascent Leukemia

There is a paucity of data to describe the likely suppressive effects that EVs derived from a healthy bone marrow microenvironment impart on early nascent AML modes. More recently, we have established a reproducible model to explore the function of such healthy vesicles on the growth of various AML cell lines. In these functional studies, we cocultured Kasumi-1 cells with various concentrations of human (h)MSC-derived EVs. Vesicles were isolated using an established differential centrifugation technique and were cocultured with Kasumi-1 cells for varying amounts of time. To study cellular proliferation, we employed a fluorescence-based method for quantifying viable, proliferating cells. Our preliminary data suggest that the addition of human mesenchymal stem cell-derived EVs inhibits the proliferation of the Kasumi-1 AML cell line in vitro. This effect is seen as early as one day of coculture and persists out to three and six days. In addition, we have employed a tri-fluorescent assay that allows the quantification of multiple modes of death and have established that apoptosis is the primary mechanism by which hMSC-EVs impart their antiproliferative effects on leukemic cells. hMSC EVs were not shown to illicit significant amounts of necrosis. To add, we have also shown that these "good" MSC-derived EVs enhance the in vitro killing properties of the antimetabolite chemotherapeutic drug cytarabine.

AML-Derived EVs and The Bone Marrow Niche

As cancer progresses it nullifies this MSC-directed protection by changing the microenvironment in an EV-mediated manner. As there are "good" players in this cancer paradigm, there are numerous nefarious players competing in the same niche. CLL-EVs are perhaps the best studied source of "bad" EVs; they serve as a prime example of the oncogenic properties leukemic-EV can impart on surrounding stroma. As CLL cells begin to thrive and outcompete their surrounding populations, CLL-derived EVs rapidly deliver their biologic cargo to the surrounding stromal cells in response to which bone marrow-MSCs have been shown to acquire a CAF phenotype with enhanced proliferative and migratory properties (3, 39). CAF-derived factors have an immunogenic effect on the T and myeloid cells, altering their phenotypes toward immunosuppressive and tumor-promoting Th2/M2-like cells, respectively. Other work has supported this, implicating CAF in contributing to the defective T cell and myeloid cell immune responses and an inflammatory milieu characteristic of CLL promotion (16, 18). We believe as AML begins to thrive it shapes its own microenvironment in a similar fashion.

AML is characterized by rapid growth of abnormal blast cells that accumulate in the bone marrow and interfere with normal hematopoiesis. The pathogenesis of AML is associated with a tumor-supportive microenvironment and an aberrant immune response. Disease recurrence occurs in most patients with AML within 3 years after diagnosis, and the outcome in older patients who are unable to receive intensive chemotherapy without significant side effects remains dismal, with a median survival of only 5 to 10 months.

Leukemic EVs have far reaching implications on all components of the leukemic microenvironment, and harness therapeutic potential to ameliorate clinical outcomes in AML.

Effects on Stem Cells

AML, and other leukemias, shed EVs that have been shown to target healthy stem cells. Surprisingly they do not promote cell death of the surrounding stroma, but rather, enhance the survival of MSCs while altering their plasticity, development, and ability to undergo normal hematopoiesis (60). The mutualistic cross talk between human stem cells (HSCs) and the numerous cells of the bone marrow microenvironment is necessary for the maintenance of downstream progenitors and normal hematopoiesis. Schepers et al. (44) and Shin et al. (45) have shown that AML cells (likely via an EV-directed mechanism) cause numerous chromosomal anomalies and genetic mutations within the surrounding stroma, thereby altering the biology of the stem cell continuum away from normal hematopoiesis, and towards transforming bone marrow stem cells towards immature progenitors that will subsequently develop into leukemic blasts or altered cancer-stem cells capable of supporting a pro-leukemic environment.

Experiments conducted by Viola and colleagues (52) have demonstrated that bone marrow MSCs exposed to AML contribute a pro-leukemic niche via an EV-directed mechanism. RT-PCR quantitative analysis of EVs isolated from the stromal cells of AML marrow aspirates revealed altered expression in bone marrow MSC EVs for CXCL12, KITLG, and CXCL1, as well as for genes previously reported in modified stroma in myelodysplastic syndrome (*IGFBP4*, *ANGPTL4*) (53). All of these genes have implications in leukemic progression.

Hong and colleagues (19a) found similar variations in EVs derived from MSCs exposed to leukemia. Specifically, transforming growth factor-β1 (TGF-β1) levels reflected clinical responses to chemotherapy, decreased after induction of chemotherapy and increased during consolidation treatment (48). When EVs derived from MSCs within a thriving CLL microenvironment are cultured with CLL B cells, the latter show an increase in their chemoresistance to several drugs, including fludarabine, ibrutinib, idelalisib and venetoclax after 24 hours (15, 49). EVs from MSCs of leukemic patients can also rescue leukemic cells from spontaneous or drug-induced apoptosis. Additionally, these EVs induce a higher migration and also a stronger gene modification of relevant survival genes (as compared with the EVs of healthy MSCs) via stroma cell-derived factor 1-α (39).

Recent publications have investigated the effect of leukemia microvesicles on healthy umbilical cord blood HSCs. The total number of total EVs in the plasma of newly diagnosed AML patients was found to be higher than that of healthy controls across most of these studies. In a study using patient-derived cancerous EVs that were cocultured with healthy umbilical cord blood hematopoietic stem cells, the experimental results noted increased plasticity/stemness when performing colony-forming unit assays and looking at the microRNA gene expression of EV-treated stem cells versus non-EV treated controls. The group investigated hematopoietic stem cell-specific cluster of differentiation (CD) markers, noting that $CD34^+$, $CD34^+CD38^-$, $CD90^+$, and $CD117^+$ phenotypes were higher in treated cells, again indicating an upregulated "stemness" sign (43). Rather than simply destroying stem cells, it seems that leukemic EVs cause stem cell deregulation to promote oncogenes, specifically by increasing levels of microRNA-21 and microRNA-29a; increased stem cell plasticity and their ability to adapt; while also increasing the total number of stem cells all together (43). The end-result is an increased number of oncogenic stem cells better adapted to handle multiple survival stressors such as chemotherapy and immune detection.

In vitro work with various AML cell lines and the plasma from mice bearing AML xenografts has shown that diseased animals release leukemic EVs laden with multiple stem cell-related microRNAs (miR-155, miR-150, and miR-375) that target the downregulation of C-MYB, a transcription factor involved in stem and progenitor growth and differentiation (21, 53). Further works have looked closer at other AML-derived EV microRNA targets. Via coimmunoprecipitation and subsequent high-throughput sequencing, the identified targets of miR-155 have revealed mechanisms of AML directed inhibition of specific stem cell transcription factors; a loss of CXCR4 and c-kit expression; and the manipulation of several proteins with established roles in malignancy (TP53, BRCA1, and MYC) and hematopoiesis (CTNNB1 and RUNX1; SOCS1). FOXP1 and Gab1 are also targets of miR-150, which is necessary for the proper development of B cell precursors, and is dysregulated in leukemia (21, 53). This mode of action is preserved across other leukemias as well. MiR-150 has also been shown to play a role in CLL progression via impairment of multiple signaling pathways that influence B cell receptors, having implications in uncontrolled cell growth and resistance against monoclonal antibody therapies that specifically target the BCR.

The Importance of CXCR4

Exosome-mediated RNA transfer from leukemia to the microenvironment has a direct effect on stem cell localization. The stromal cell-derived factor 1 (SDF1), also called CXCL12, is a chemokine protein on stromal cells and possesses angiogenic properties and is involved in the outgrowth and metastasis of CXCR4-expressing tumor cells. CXCR4's ligand, SDF-1, is known to be important in hematopoietic stem cell homing to the bone marrow and in hematopoietic stem cell quiescence (21). The CXCR4-CXCL12 axis keeps HSCs localized in the niche. Studies suggest that leukemia-derived exosomes may promote leukemic progeny growth by interfering with canonical CXCR4-CXCl12 signaling. Leukemic EVs carry and deliver various microRNAs, such as the aforementioned miR-150, which directly targets CXCR3, downregulating its expression and allowing for cell mobilization and chemotaxis (10, 21, 22). Models of ALL show similar findings. Studies utilizing in vivo confocal microscopy show that ALL cells target vasculature within the endothelial niche that expresses the adhesion molecule E-selectin and the chemoattractant, CXCL12. Moreover, when these vascular targets were changed or damaged, ALL cells lost their homing capability to engraft into these endothelial compartments. There has been impetus to create drugs that block the CXCL12-CXCR4 interaction, as various in vitro studies across multiple cancer settings, including AML, have shown that CXCR4 inhibition reduces chemoresistance of AML and CLL cells both in vitro and in xenograft models (10, 21, 22).

Cell-to-cell contact is clearly important in biologic regulation, and manipulating this mode of cellular cross talk has many of the same downstream implications that have been discussed in EV-mediated communication. Decreased expression of CXCL12 and increased G-CSF, IL-1α, IL-1β, IL-6, TNF-α, LIF, and chemokines CCL3 and CCL4 have been implicated in enhancing leukemic survival at the expense of normal HSC survival (20, 34). This is akin to the survival advantage imparted by cancerous leukemic EVs onto marrow stem cells. In human MDS samples we can appreciate similar mechanisms. Isolated MSCs were found to overexpress multiple disease propagating factors such as CDH2 (N-cadherin), insulin-like growth factor binding protein 2 (IGFBP2), VEGFA, and LIF (20, 34). The aberrant MDS stem cells (which likely have an innate survival advantage and quickly outnumber the normal native bone marrow stem cells) were cocultured with healthy MSCs, causing deregulated signaling in the healthy cell population likely via an EV-regulated mechanism. This resulted in phenotypic alteration in which healthy MSCs were transformed towards a phenotype more representative of aberrant MDS stem cells (20, 34).

Effects on the Endosteal Bone Marrow Niche

The endosteal bone marrow (BM) niche (osteocytes/osteoblasts/osteoclasts) is also influenced as the leukemic microenvironment develops. The multipotency of MSCs means that they are naturally involved in directing osteoblastic lineages within the bone marrow microenvironment. AML and other myeloproliferative neoplasms can reprogram their endosteal BM niche to support leukemic growth and support BM fibrosis. Under the influence of AML-derived EVs, MSCs and an array of osteoblastic lineages show a depressed expression of HSC retention factors (e.g., CXCL12, SCF, Lepr, Angpt1, Cdh1, Slit1, and Tgf-β2). These cells also harbor an altered expression of TGF-β, Notch signaling, and an expressive and phenotypic change toward inflammatory myelofibrotic cells, culminating in an overall a loss of endosteal stem cell support and normally hematopoiesis (14, 15, 28). These transformed osteoblastic lineage cells in turn increase levels of factors such as thrombopoietin (TPO), Tgf-β2, and IL-1β that stimulate myeloid differentiation and proinflammatory cytokines such as CCL3, IL-6 and IL-1β. Normal hematopoiesis is deterred. The altered cells effectively support small populations of leukemia stem cells that sustain AML. Krause et al. (27) found similar alterations of the BM specifically by osteoblastic cell-specific activation of the parathyroid hormone receptor, enhancing MLL-AF9 oncogene-induction of AML in mouse transplantation models.

Effects on the Endothelium

Leukemic EVs affect the sinusoidal endothelium. Leukemia-derived EVs are able to promote metastasis and disturb the architecture of multiple tight junction proteins. Zhou et al. (62) reported that breast cancer-secreted exosomes are enriched in miR-105, which destroys vascular endothelial barrier, allowing cancer to enter the circulation. Leukemia can also bolster angiogenesis (62). In vitro studies, first reported by Umezu et al. (51), clearly showed leukemic cell to endothelial cell communication via exosomal miRNAs. The human immortalized myelogenous leukemia cell line K562 was transfected with fluorophore (Cy3)-labeled pre-miR-92a and cocultured with human umbilical vein endothelial cells (HUVECs). Twenty-four hours later there was clear transfer of the labeled pre-miR-92a into the HUVEC cytoplasm and the fluorophore signal clearly colocalized with the exosomal marker CD63 (52).

Various cancer models also take advantage of the naturally occurring hypoxia present in the deepest compartments of the bone marrow niche. Cancer-derived EVs, in various leukemic cancers and MM, are enriched in in miR-135b, a microRNA that targets and inhibits hypoxia-inducible factor 1 (HIF-1) gene in endothelial cells, having effects on multiple signaling cascades including the factor inhibiting HIF (FIH) signaling pathway that allows cancer cells to adapt to hypoxic conditions. HIF activation is, in part, EV-dependent and occurs in almost all types of cancer. In vitro and in vivo nude mouse models have confirmed much of this work (46).

Effects on the Stromal Cells

In addition to altering MSCs, the bone marrow niche, and endothelial cells, leukemic EVs also directly communicate with the different stroma cells of the microenvironment. Stromal cells have been shown to directly take up cargo from and communicate with leukemic EVs. In a construct that entailed green fluorescent protein (GFP)-expressing murine OP9 BM stromal cells, Huan et al. (22) reported the direct localization and uptake of dye-stained AML EVs by the GFP-expressing murine OP9 BM stromal cells. When the cargo of the oncogenic EVs was assayed it was found to carry RNA transcripts such as NPM1, FLT3, CXCR4, MMP9, and IGF-1R many of which have relevance in leukemic tumorigenicity and development (10, 22). This cross talk is reciprocal, such that when stromal cells are influenced by oncogenic EV signaling the stromal cells themselves in turn activate STAT1 and NOTCH3 signaling in breast cancer cells and expand cancer-initiating cell populations responsible for drug resistance and nascent tumor formation (30, 58).

The implications of targeting exosomes toward phenotypic modulation extend beyond the endothelial matrix of the bone marrow microenvironment. EVs from CLL, and other leukemic cells lines and patient sera, are incorporated into endothelial and mesenchymal stem cells ex vivo and in vivo. The miRNA and proteins in these EVs do not potentiate a killing effect on the marrow stroma but rather upregulate an inflammatory response in the target cells. Chronic inflammasome activation itself is tied to leukemic dysregulation and the phenotypic mutagenesis of normal stem cells into CAFs. CAFs proliferate faster than normal stroma and themselves perpetuate the upregulation, expression, and release of inflammatory cytokines (48, 52). They also have secondary effects on endothelial cells, increasing angiogenesis. In turn, the leukemia-modified stroma favors leukemic blast proliferation while stymieing normal hematopoiesis (48, 52).

Effects on the Immune Cells

Leukemic blasts also have numerous suppressive effects across the population of immune cells that reside in the microenvironment. When studying the peripheral blood from newly diagnosed patients with AML, multiple studies have observed an increase in the absolute number of peripheral

blood T cells (43, 47, 59). However, when compared with healthy controls these immune cells exhibited aberrant T cell activation patterns on gene expression profiling. Genes involved in actin cytoskeletal formation were identified, and therefore the ability of T cells from AML patients to form immunologic synapses with autologous blasts was significantly impaired. In effect, AML-exposed aberrant T cells were not able to communicate effectively with normal antigen-presenting cells due to their inability to recruit necessary phosphotyrosine signaling molecules to the synapse (43, 47, 59). CLL models have shown similar immune-suppressing effects. However, when similar studies were performed on CLL patients, gene expression changes differed from those observed in AML immunosuppression, suggesting multiple mechanism and pleiotropic effects at play.

In work by Szczepanski and colleagues (46, 58), AML blast-derived exosomes from the sera of cancer patients decreased natural killer cell cytotoxicity and downregulated expression of NKG2D in normal natural killer cells Sera from patients with acute myeloid leukemia also contained elevated levels of transforming growth factor-β, a potent immunosuppressive molecule for NK cell cytotoxicity.

PROSPECTS

Translational Biomarkers and Machine Learning Models

AML remains a difficult and frequently lethal disease despite standard chemotherapy and stem cell transplantation. Results in older populations of patients are particularly dismal. As we have seen, MSC EVs have been demonstrated to reverse disease manifestations in a number of disease settings and especially in various cancers. Vesicle treatment of AML could represent a significant clinical step forward, as could studies focusing on disease-promoting vesicles to identify cellular mediators, or disease-promoting cells that could then be targeted therapeutically as a mechanism for disease modulation—a theme more widely applicable in various neoplastic conditions mediated by EV biology.

Along this same regard, plasma exosomes can possibly serve as markers of therapeutic response in patients with AML. Similar works in the area of lymphoma have explored the utility of vesicles towards this end point (36). Studies of solid cancer have provided some headway. In a paper fraught with controversial results, Melo et al. (34a) reported that the pancreatic cancer exosomes express a proteoglycan (glypican-1; GPC1) on their surface, and that $GPC1^+$-circulating exosome levels correlated with tumor burden and the survival of afflicted patients before and after surgery (12, 31). Moreover, plasma exosome concentration (measured by the protein concentration present within the sample) was significantly increased compared with control plasma in a disease burden dependent manner and was decreased after conventional consolidation chemotherapy (12, 31).

The emergence of technology, allowing clinicians to identify and isolate circulating cancer DNA, introduces the prospect of "liquid biopsy." Genetic profiling studies on circulating DNA demonstrated that the DNA in circulation is mainly contained in EVs. In addition to blood and cerebral spinal fluid, the ubiquity of EVs means that they may be detected by relatively noninvasive means, such as the collection of saliva or urine. The material packaged inside is also protected from degradation, serving as yet another advantage over conventional biopsy or serum markers.

As discussed, EV cargo contains unique molecules specifically derived from the parent cell. The exosome subset also originates from the endocytic compartment and reflects the membrane markers of the cell of origin. Numerous publications have shown that inflammation and disease greatly increase the total EV quantity, thus making detection and isolation of pathological EVs feasible (44). The detection of tumor-specific EVs and their cargo has been accomplished in numerous cancers including glioblastoma, prostate, and leukemia (36, 37, 56). However, the methods in these studies are not without their limitations. EV collection from bodily fluid is a heterogeneous mixture of exosomes derived from numerous healthy and diseased cells. Sorting through this "contaminated" milieu of healthy EVs for isolation has been a major challenge. The ability of GPC1 as a surrogate pancreatic cancer marker is marred by the fact that GPC1 is a surface proteoglycan that interacts with many proteins; thus, such analyses remain a challenge due to the lack of specificity that allows researchers to differentiate cancer exosomes from exosomes produced by other cell types. Additionally, there is lack of capture reagents with sufficiently high avidity and specificity for the unique markers expressed by diseased cells.

Our preliminary data suggest that the separation and absolute purification of diseased EVs from those excreted by healthy cells may not be necessary. Total plasma EVs from human serum, although a very heterogeneous amalgamation, is (perhaps) the most biologically relevant and accurate representation of the state of organisms. There exists finite, but characterizable, differences within the total plasma EV pool of a healthy organism versus that of a diseased one if appropriately captured by effective machine learning algorithms. Employing mathematical and statistical models to quantify and predict the magnitude of these changes allows for precise therapeutic intervention.

Indeed, multinomial logistic regression can be used to classify various EV populations, while conventional *k*-means clustering algorithms can allow us to visualize potentially unique subpopulations of EVs. Here, we propose that the same goals may be better accomplished with nonlinear machine learning-based algorithms [e.g., support vector machines (SVMs), Gaussian processes, deep neural networks, decision tree frameworks] (31). Previous studies have used similar types of tools to assess the quality of induced pluripotent stem cells, as well as to differentiate malignant colon cancer cells from nonneoplastic immune cells within a given patient by simply analyzing the patient's biophysical cellular data (12). Much optimization remains, but there is promising progress and unique, clinically relevant potential in merging the vast biologically rich information EVs harbor with sophisticated technology such as neural networks and machine learning.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.B., C.N., and P.Q. prepared figures; T.B., L.C., C.N., and P.Q. drafted manuscript; T.B., L.C., C.N., and P.Q. edited and revised manuscript; T.B., L.C., C.N., and P.Q. approved final version of manuscript.

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