7

Platelets in Hematogenous Breast Cancer Metastasis: Partners in Crime

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Cite this chapter as: Free SR, Carraway KL III. Platelets in Hematogenous Breast Cancer Metastasis: Partners in Crime. In: Mayrovitz HN. editor. *Breast Cancer*. Brisbane (AU): Exon Publications. Online first 10 Jun 2022.

Doi: https://doi.org/10.36255/exon-publications-breast-cancer-platelets

Abstract: Distant metastasis is the primary driver of breast cancer-associated mortality, and research into the mechanisms underlying hematogenous tumor cell dissemination could give rise to the development of novel and more effective therapeutic agents and strategies. Platelets are activated directly by tumor cell interaction and indirectly by tumor-secreted factors to trigger platelet aggregation, degranulation, and the subsequent release of pro-tumorigenic factors. Platelet presence within the primary tumor, bloodstream, and metastatic sites allows for continuous exposure of breast cancer cells to these factors, making platelets a powerful partner in tumor cell dissemination. Platelet-tumor cell crosstalk contributes to hematogenous breast cancer metastasis by providing physical and biochemical support to metastasizing cells via mechanisms including protection from shear forces, anoikis, and immune attack, and enhancement of angiogenesis, migration, and pro-tumorigenic inflammation. Here, we review platelets and their many benefits to metastatic breast cancer, their role in facilitating paraneoplastic thrombosis, and current research regarding their potential as a breast cancer therapeutic target.

Keywords: antiplatelet therapy; breast cancer; hematogenous metastasis; paraneoplastic thrombosis; platelets in breast cancer

In: Mayrovitz HN, editor. *Breast Cancer*. Brisbane (AU): Exon Publications.
ISBN: 978-0-6453320-3-2. Doi: https://doi.org/10.36255/exon-publications-breast-cancer

INTRODUCTION

One hundred and fifty years ago, Armand Trousseau first drew the insightful connection between platelets and cancer when he noted an increased incidence of thrombosis in patients with visceral cancer (1). Ninety years later, Gabriel and Tatiana Gasic observed that neuraminidase, a potent inducer of thrombocytopenia, reduced pulmonary metastasis in mouse models of cancer (2–4). Since then, the contribution of platelets to the progression and dissemination of cancer has been established across cancer types and tissues of origin. In breast cancer (BC) specifically, where patients with advanced, distantly metastatic disease face a 5-year survival rate of only 30% (5), the ability of platelets to support tumor cell survival and aggressiveness throughout the metastatic cascade is an important area of research and therapeutic consideration. Furthermore, high co-incidence of coagulopathies and cancer—acknowledged by Trousseau—poses an additional risk to patients and may be mitigated by platelet-directed therapies (6, 7). Herein, we review the role of platelets in promoting growth and invasion in primary breast tumors, enhancing circulating tumor cell (CTC) survival and extravasation from the bloodstream, enabling the seeding and growth of distant metastatic lesions, and fostering BC-associated coagulopathy.

PLATELETS

With their tremendous abundance (1.5–3.5 × 10^8/mL of human blood) and unique ability to rapidly aggregate and release hundreds of factors in response to activation, platelets are the first responders to arrive on the scene of vascular injury and begin the process of clot formation and healing (8). Though small (~2 μm in diameter) and anucleate, platelets contain ribosomes, mitochondria, a cytoskeletal network, in addition to distinct α-granules, lysosomal granules, and dense-granules, which store biologically active factors essential to injury response and hemostatic maintenance (9). These factors can be fully synthesized within platelet precursor megakaryocytes, acquired by receptor-mediated endocytosis or pinocytosis, or even synthesized within the platelets themselves using pre-mRNA generated in megakaryocytes; this allows for flexibility and diversity in platelet storage contents (9, 10). Release of granular contents is mediated by platelet activation and degranulation, which can be triggered by a variety of agonists (e.g., adhesive proteins such as Von Willebrand Factor (VWF) in the vascular wall, soluble factors such as thrombin, adenosine diphosphate (ADP), or thromboxane A_2 (TXA_2)) (8).

Unfortunately, platelets are unable to discern between vascular injury and cancer, which has been described as the "wound that does not heal" (11). Cancer cells secrete several platelet agonists, including ADP and TXA_2, which induce constitutive reciprocal signaling with platelets that not only supports cancer growth and metastasis, but also induces a hypercoagulative state throughout the body (12). Cancer-specific signals stimulate changes in nearby platelet storage contents, creating "tumor-educated platelets" that may be useful as surrogate biomarkers for identifying tumor type, location, and even mutational profile (13, 14).

PLATELETS AND THE PRIMARY TUMOR

Primary breast tumor cells are exposed to platelets and platelet signals, as demonstrated by robust platelet marker CD42b staining of patient BC samples at the tumor's leading edge (15, Figure 1A). As a result, platelets secrete factors within the primary tumor compartment which contribute to tumor cell survival, proliferation, and aggressiveness (discussed below). Accordingly, retrospective analysis of tumor biopsies from BC patients who subsequently received treatment and neo-adjuvant chemotherapy revealed that patients with CD42b-rich tumors were significantly less likely to achieve pathological complete response to chemotherapy (15).

Tumor growth and dissemination are dependent on the acquisition of intratumor vasculature to provide oxygen and nutrients to rapidly dividing cancer cells. Angiogenesis, the process by which tumors create new or co-opt existing endothelial vasculature, is mediated by a complex balance of numerous pro- and anti-angiogenic factors (16). Platelets are an important source of these factors, which they store in α-granules until agonists trigger their release. When cancer cells secrete agonists such as ADP and thrombin, nearby platelets are activated and release pro-angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietin-1, and tumor necrosis factor-alpha (TNF-α) (17). Platelet-depleted tumors show hyperpermeable vasculature and poor tumor perfusion due to increased angiopoeitin-2 levels and diminished pericyte recruitment to

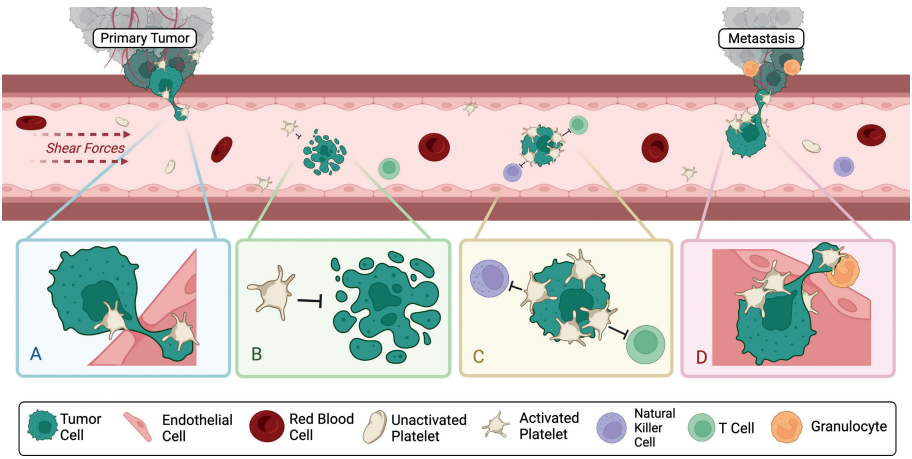

Figure 1. Platelets aid BC cells throughout the metastatic cascade. A, Platelets help primary tumor cells migrate and intravasate by secreting factors that promote epithelial-to-mesenchymal transition and remodeling of the extracellular matrix (ECM; (20, 23)). **B,** Platelets protect BC cells from shear-induced apoptosis and encourage resistance to anoikis caused by loss of ECM attachment (55, 58, 59). **C,** Platelets contribute to BC immune evasion by directly inhibiting local immune cell activation, masking immune ligands on tumor cells, and conferring "pseudo-expression" of immune-inactivating molecules to tumor cells (26, 31, 34, 60–64). **D,** Platelets support arrest and trans-endothelial migration of BC cells and help establish the pre-metastatic niche by recruiting granulocytes to the area (72–75). Figure created with biorender.com.

hemorrhagic blood vessels (18). On the other hand, tumor cell-activated platelets enhance endothelial tube formation *in vitro*, and increase tumor vascularization and metastatic burden *in vivo* (19, 20). Patients with BC display significantly increased levels of systemic platelet-sequestered angiogenic factors VEGF, platelet-derived growth factor (PDGF), and transforming growth factor-beta (TGF-β; Figure 2) compared to healthy controls; this upregulation is correlated with higher incidence of lymph node metastasis and advanced disease stage (21).

To disseminate, tumor cells must invade the surrounding tissue and extracellular matrix (ECM) and penetrate the vasculature (Figure 1A). In one form of metastatic progression, tumor cells undergo epithelial-to-mesenchymal transition (EMT), wherein single epithelial tumor cells acquire invasive mesenchymal morphology and behavior (22). Breast tumors showing greater platelet presence tend to show more EMT-like characteristics (e.g., loss of apical-basal polarity, loss of E-cadherin expression, gain of vimentin expression) than those with less platelet activity (15). This is mediated largely through increased concentrations of intratumor TGF-β, which is abundantly secreted via platelet α-granules and activates transcription of EMT-related genes across cancer types (20, 23, 24). Platelets also engage EMT through direct interaction with cancer cell α2β1 surface integrins, which activates the Wnt-β-catenin pathway and upregulates autocrine TGF-β production by cancer cells (25). Furthermore, platelets constitutively express the cell surface TGF-β-docking Glycoprotein A Repetitions Predominant (GARP) receptor, which is essential for the conversion of TGF-β from its latent to active form and increases the pool of active TGF-β within the primary tumor (Figure 2) (26).

Whether mediated by EMT or collective cell migration (CCM), in which cells retain epithelial characteristics and invade as a group (27), remodeling of the ECM in the primary tumor compartment is an essential step in metastasis and is stimulated by bidirectional signaling between platelets and tumor cells (20). Platelet-stimulated paracrine and autocrine TGF-β signaling upregulates BC cell expression of matrix metalloproteinases-2 and -9 (MMP-2 and -9) (18, 20, 25), plasminogen activator inhibitor-1 (PAI-1) (18), and various proteases (18, 20, 25), which encourage the degradation of the ECM to enable cellular migration and invasion. Conversely, sustained TGF-β activity enhances tumor cell production of pro-tumorigenic ECM molecules like fibrin, fibronectin, and collagen, which enable cellular communication and migration (20, 25, 28).

To survive and spread, BC cells must evade immune detection and destruction (29). Many cancers avoid immunosurveillance by acquiring expression of programmed death-ligand 1 (PD-L1), which negatively regulates $CD8^+$ T cell function; as a result, PD-L1 checkpoint blockade treatments have become an attractive therapeutic approach in recent years (30). Zaslavsky et al. observed that platelets express PD-L1—particularly after activation by tumor cells—and can confer PD-L1 "pseudo-expression" to PD-$L1^{-/-}$ mouse tumors, resulting in decreased T cell-driven cytotoxicity and increased tumor burden (31). Likewise, patients with PD-L1-negative lung cancer who responded to PD-L1 checkpoint inhibition tended to have greater platelet infiltration in biopsied tumors, suggesting that platelet presence sensitizes PD-L1-deficient cancers to PD-L1-directed therapies, though these results need to be confirmed on a larger cohort of patient samples (31). Other groups have observed that platelets also suppress cytotoxic T cell function in the tumor microenvironment through platelet-GARP driven activation

of TGF-β (Figure 2B) (26), which inhibits T cell proliferation and activity through a diverse array of mechanisms reviewed by Gorelik and Flavell (32) and Thomas and Massagué (33). Furthermore, platelets provide well-documented protection of tumor cells from natural killer (NK) cells (34). Though this phenomenon likely exists within the primary tumor, it has been most thoroughly documented in circulating tumor cells (CTCs) and will be described in more detail below.

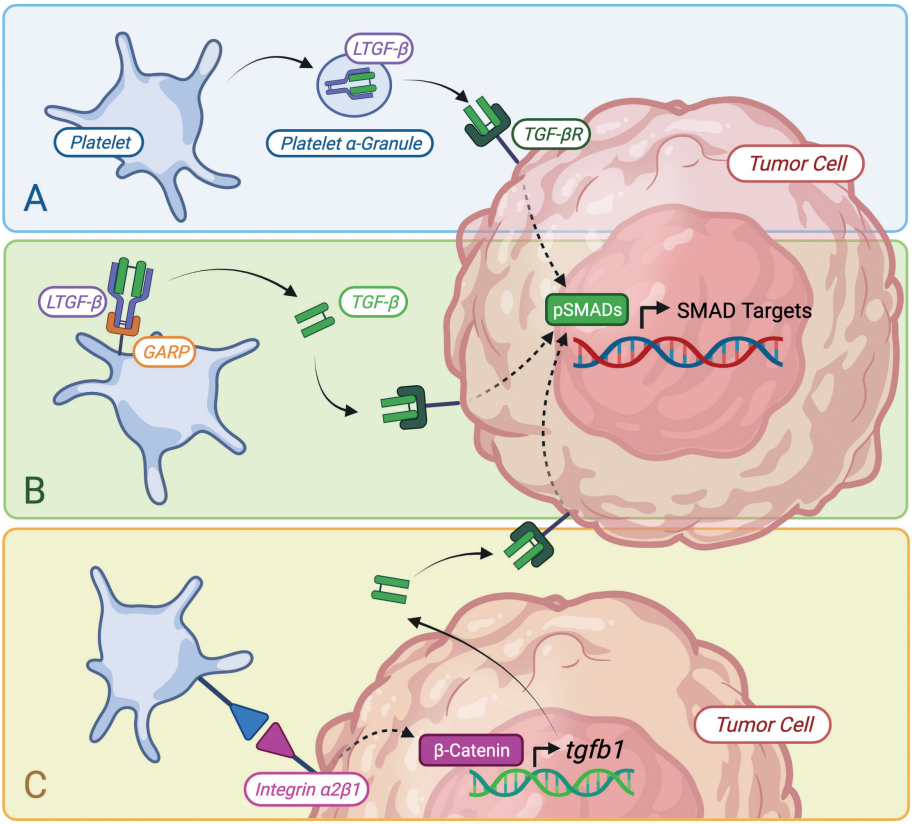

Figure 2. Platelets increase the local pool of active TGF-β. A, Platelets are the single greatest source of TGF-β in blood plasma, secreting huge amounts of latent TGF-β (LTGF-β) complexes in α-granules upon activation, which are then processed and activated by factors found on platelets and other cell types as shown in **B** (24). **B,** Platelets constitutively express the "Glycoprotein A Repetitions Predominant" (GARP) receptor, which docks LTGF-β (derived from platelets or other cell types) for activation. This substantially increases the local reservoir of active TGF-β (26). **C,** Direct contact between platelets and tumor cells via the cancer cell surface integrin α2β1 stimulates the Wnt-β-Catenin signaling pathway. This upregulates downstream transcription of *tgfb1*, resulting in increased TGF-β secretion by tumor cells (25). Active TGF-β secreted by platelets and tumor cells is bound by the TGF-β receptor on tumor cells, driving SMAD phosphorylation and downstream signaling that increases transcription of genes related to EMT, ECM remodeling, and more. TGF-βR, TGF-β receptor; pSMADs, phosphorylated "Suppressor of Mothers against Decapentaplegic" factors. Figure created with biorender.com

PLATELETS AND CIRCULATING TUMOR CELLS

BC cells metastasize to distant organs by directly intravasating into blood vessels or indirectly entering into the lymphatic system which drains into the bloodstream (35). Upon hematogenous entry, an array of threats create a survival bottleneck for metastatic cells, and very few CTCs survive and successfully seed metastases (36). To survive these dangers, CTCs rapidly recruit platelets to provide physical protection and biochemical enhancements (37). In fact, platelets are the first cells in circulation to adhere to CTCs, forming microthrombi in seconds which then recruit fibrin, neutrophils, monocytes, and macrophages for additional aid (38).

Under normal physiologic conditions, platelets are recruited to sites of injury upon disruption of the basement membrane, which exposes subendothelial procoagulation factors (e.g., tissue factor (TF), VWF, collagen) typically unavailable in circulation (8). CTCs co-opt platelet activity by expressing these same factors. For example, widespread TF overexpression by cancer cells is driven by signaling programs associated with oncogenic transformation and EMT (39, 40). As a receptor for key components of the coagulation cascade, TF expression by cancer cells encourages localized thrombus formation and is associated with increased metastatic success and worse patient outcomes (41, 42). Likewise, aberrant expression of the adhesive glycoprotein VWF has been observed in BC (43) and other cancer types (44, 45). Tumor cell VWF secretion increases overall plasma VWF concentrations, contributing to paraneoplastic hypercoagulopathy; furthermore, tumor cells can directly bind VWF via $\alpha_v\beta_3$ integrins (46) or glycoprotein Ib/V/IX complexes (47), allowing them to self-aggregate, adhere to platelets, and arrest under flow conditions (45, 46).

In addition to the indirect modes of platelet-CTC aggregation described above, where cells need not interact directly but aggregate through mutual binding of coagulation factors, direct platelet-CTC binding can occur through a variety of receptor-ligand interactions. Platelet P-selectin interaction with CTC mucins (48–50), platelet glycoprotein VI (GPVI) interaction with CTC galectin-3 (51), and platelet receptor FcγRIIa interaction with CTC immunoglobulin G (IgG) (52) have all been described (53). While these unique receptor-ligand dynamics present enticing therapeutic targets for inhibition of platelet-CTC interactions, the sheer number of molecules which can redundantly promote CTC binding and platelet activation may thwart these attempts.

BC cells in circulation are susceptible to anoikis, a type of programmed cell death triggered by loss of attachment to the ECM (54). Secreted platelet proteins promote anoikis resistance by upregulating CTC expression of the GTPase RhoA, thereby activating the transcription co-activator YAP1 and inducing expression of downstream genes involved in proliferation and apoptotic resistance (55). It is also widely believed that platelets physically guard CTCs from the shear forces of the bloodstream, which can induce cell cycle arrest (56), apoptosis, and necrosis (57). Although limited experimental evidence exists to support platelet-mediated shear force protection (58), *in silico* modeling of CTC-platelet interactions under shear indicates that platelets may reduce the total shear force magnitude applied to any single region of a tumor cell, decreasing deformation and damage of the membrane and preventing apoptosis (59, Figure 1B).

Perhaps the most widely studied benefit conferred by platelets to tumor cells is NK cell evasion, which is achieved by manipulating the behavior of both tumor cells and the NK cells themselves. Kopp and colleagues observed that platelet-secreted TGF-β downregulates expression of the activating immunoreceptor NKG2D by NK cells, diminishing their cytotoxic effects (60). Platelets also impair NK cell production of interferon gamma (IFNγ), which is a key effector molecule through which NK cells induce an adaptive immune response (61, 62). In addition, platelets release sheddases ADAM10 and ADAM17 which cleave NK ligands from the surface of tumor cells and prevent them from being targeted (63). Platelets can also confer "pseudo-expression" of the major histocompatibility complex (MHC) class I to tumor cells, which allows tumor cells to avoid NK targeting without triggering T-cell surveillance (64, Figure 1C).

Soon after entry into the bloodstream, CTCs travel to distant sites where they arrest and begin to extravasate. Vascular arrest occurs through both passive means (i.e., size-restricted arrest, where CTCs are physically trapped within small capillaries) (65) and active mechanisms (i.e., adhesion to the endothelium or subendothelial matrix) (66, 67); platelets have been implicated in both processes, though their roles are controversial (68). While platelets have been observed in CTC clusters mechanically arrested by size-restriction, it is unclear whether platelets are necessary for this entrapment, or if they simply pile up around CTCs that are blocking their passage (69). Likewise, direct CTC binding to both the endothelium (70) and the subendothelial matrix (67) has been noted even in the absence of preliminary platelet thrombus formation, suggesting that platelets are not strictly necessary for initial arrest. However, platelets have been implicated in tumor cell tethering, rolling, and adhesion to the endothelium via P-selectin interactions (71), suggesting that they may provide stability to CTC attachment.

PLATELETS AND DISTANT METASTASIS

Regardless of their role in vascular arrest, platelets significantly bolster the process of extravasation. In addition to the migratory advantage platelet-induced TGF-β signaling confers to tumor cells (23), platelets potentiate tumor cell-induced endothelial retraction (72) and induce the loss of endothelial tight junctions (73). These changes support trans-endothelial migration by tumor cells, which is further evidenced by observations that platelet inhibition significantly reduces tumor cell extravasation (73, Figure 1D).

Because they are rare and transient *in vivo* and difficult to model *in vitro*, relatively little is known about the initial stages of BC metastatic colonization, and specifically how these events are impacted by platelets. It is likely that many of the same platelet-tumor cell signaling programs that occur during primary tumor growth and intravasation are useful to disseminated tumor cells (e.g., platelet-induced tumor cell MMP secretion for ECM degradation, platelet expression of pro-angiogenic factors, etc.), though few of these dynamics have been demonstrated specifically during early metastatic colonization. On the other hand, platelets have been specifically implicated as early founders of the "pre-metastatic niche". Labelle et al. observed that granulocytes are recruited to the vicinity of CTC-platelet aggregates arrested in the vasculature, where they

help prepare a microenvironment amenable to tumor cell seeding (74, Figure 1D). Granulocyte recruitment is dependent on chemokine signaling by activated platelets, and metastatic success is significantly attenuated by depletion of either cell type. Another study found that by inhibiting platelet activation, deposition of fibronectin in metastatic sites in the lung was significantly decreased, resulting in a less hospitable metastatic environment and fewer lesions (75). These results support the notion that while platelets may be dispensable for initial vascular arrest, they are necessary for successful vascular retention and metastatic outgrowth (68).

PLATELETS AND PARANEOPLASTIC COAGULOPATHY

BC cells express high levels of pro-coagulant factors like TF and phosphatidylserine on their surfaces, or secrete them in extracellular vesicles (76). BC is also associated with increased platelet counts overall, likely stimulated by increased cytokine-driven thrombopoiesis, creating a positive feedback loop of platelet hyperactivation and systemic hypercoagulation (77). Consequently, patients with BC are at significantly higher risk than healthy patients of venous and arterial thromboembolism, myocardial infarction, and ischemic stroke, particularly following chemotherapeutic treatment (7). In fact, thromboembolic complications are one of the leading causes of death in patients undergoing chemotherapy (78). To mitigate the thrombotic risk associated with cancer and cancer treatment, clinicians regularly prescribe prophylactic anticoagulants like heparin as a supplement to cancer treatment regimens (79).

PLATELETS AS A THERAPEUTIC TARGET IN BREAST CANCER

Given their well-established localization to and support of BC metastases, platelets are an attractive anti-cancer target. In one approach, platelets are directly inhibited through antagonism of platelet receptors (e.g., P-selectin, GPVI, P2Y12) via antibodies or small molecules (80). Numerous such drugs exist and several have been approved for the treatment of cardiovascular disease (81), though their use in BC treatment is largely limited to pre-clinical studies. However, administration of anti-platelet agents alone or in combination with standard-of-care therapies has shown promise in metastatic prevention across studies and cancer types, as reviewed by Xu et al. (80).

One anti-platelet drug that has been thoroughly studied in the context of cancer is aspirin. Aspirin is a cyclooxygenase inhibitor that acts by inhibiting synthesis of platelet prostanoids, which promote activation of other nearby platelets. Aspirin treatment prevents BC cell-induced platelet activation and subsequent release of pro-tumorigenic factors *in vitro* (82), and leads to reduced metastasis in an *in vivo* mouse model of metastatic BC by suppressing platelet-enhanced anoikis resistance (83). In the clinic, observational studies of BC patients prescribed aspirin after cancer diagnosis show mixed effects on patient

outcomes, though meta-analyses indicate that aspirin use is associated with reduced risk of BC death (84). Strikingly, in randomized controlled trials of aspirin for the prevention of cardiovascular disease, allocation to the aspirin group reduced the incidence of distantly metastatic adenocarcinoma by nearly half during trial follow-ups (85). This translated to significantly higher patient survival, regardless of whether the trials tested high or low-dose aspirin. These results suggest that low-dose aspirin may be useful in the prevention of distant metastasis, though therapeutic success may depend on preventative rather than responsive administration.

In another therapeutic approach, platelet localization to the tumor is exploited to allow enhanced delivery of therapeutic molecules into the intratumoral space. Bahmani et al. found that by encapsulating the toll-like receptor agonist resiquimod in platelet membrane-coated nanoparticles (PNPs), they enhanced uptake and retention of the drug in the tumors of a 4T1 mouse BC model, increasing intratumor immunity and decreasing tumor growth better than the drug alone (86). PNPs have also been used *in vitro* to effectively deliver chemotherapeutic agents, siRNAs, and photosensitizers to primary tumors and CTCs (87). Though PNPs are not currently being used in the clinic, they present a promising new method to increase the efficacy of existing therapeutics.

CONCLUSION

Platelets are essential partners to BC cells throughout the course of hematogenous metastasis. Through a multitude of reciprocal signaling events, tumor cells activate platelets, encouraging their aggregation and degranulation. In return, platelets secrete factors which enhance tumor cell survival and aggressiveness. This crosstalk threatens patients not only by its ability to foster metastatic disease, but also in its induction of a systemwide hypercoagulative state. Therapies which target the platelet-tumor cell interaction have the potential to mitigate the danger of BC metastasis, while also preventing cancer-associated thromboembolism. Therapeutic options for the prevention and treatment of distantly metastatic BC will be increased as researchers work to better understand and target the unique mechanisms of cancer-platelet cooperation.

Acknowledgement: We thank Michelle Hu for her comments and insights on this chapter. This work was supported by NIH grants CA230742 and CA250211 (K.L.C.).

Conflict of Interest: The authors declare no potential conflict of interest with respect to research, authorship and/or publication of this chapter.

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