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The Role of Fibrin(ogen) in Transendothelial Cell Migration During Breast Cancer Metastasis

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1. Introduction

Despite all the modern advances in treatment for breast cancer, metastatic disease remains the hurdle to surmount in curing breast cancer or, at least, in significantly reducing morbidity and mortality to improve long-term survival and quality of life. For over a century, inflammation and thrombosis have been linked to metastatic cancer (Boccaccio & Medico, 2006). In addition to being known for describing the factors leading to venous thromboembolism (alterations in blood flow, vascular endothelial injury, and hypercoagulability) as Virchow’s triad, in 1863 Virchow noted a connection between chronic inflammation and cancer based on the recruitment of leukocytes to cancerous lesions (reviewed in (Balkwill & Mantovani, 2001)) (Fig. 1).

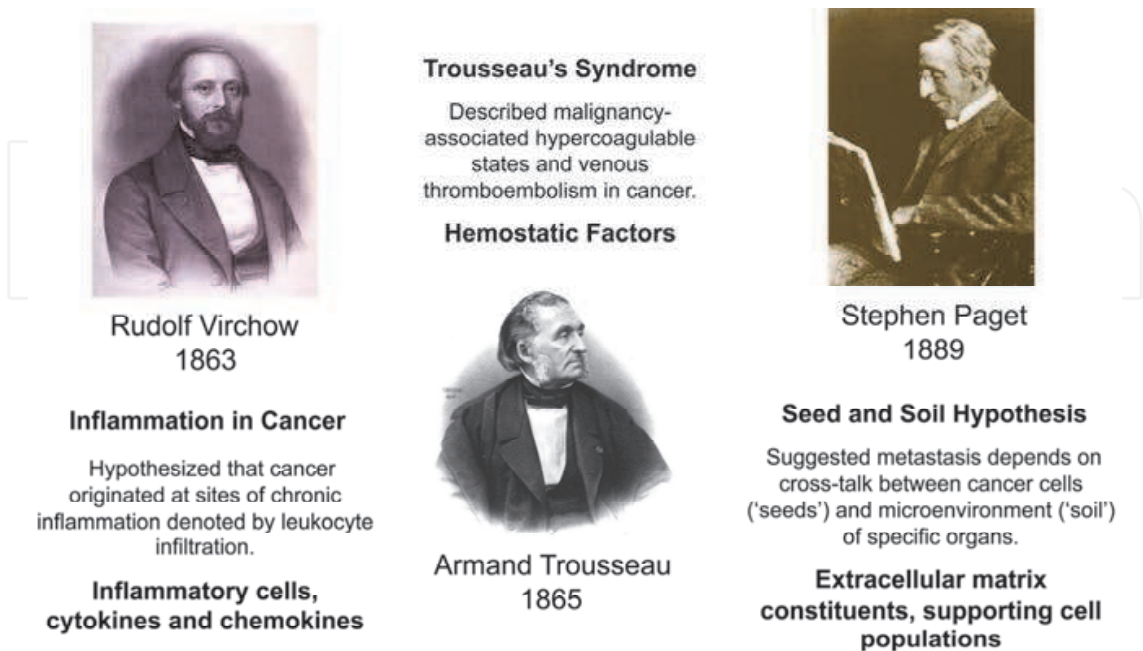


Fig. 1. The three faces of cancer metastasis. (Portraits obtained from public domain).

Rudolf Virchow, Armand Trousseau and Stephen Paget each provided valuable insight into the pathophysiology of invasive carcinomas—these theories still hold today to explain molecular mechanisms of cancer metastasis. Hypercoagulability is often diagnosed before identification of a coexisting malignancy, and is associated with increased thromboembolic risk (Sorensen et al., 2000). Armand Trousseau (Trousseau, 1865) (**Fig. 1**) identified and described the association between cancer and clot formation in 1865 and, shortly thereafter, self-identified these findings as a consequence of gastric cancer from which he later succumbed (Varki, 2007). Trousseau’s Syndrome is associated with hypercoagulability and thromboembolic events in adenocarcinomas (Starakis et al., 2010). Another important contribution that has lead to better understanding of the mechanisms of cancer metastasis was provided by Stephen Paget in 1889 (Paget, 1889) when he propose the seed and soil concept of cancer metastasis (**Fig. 1**). By examining countless autopsy specimen from breast cancer patients, Paget determined that cancer cells, the “seed”, had a preference to metastasize to distinct organs of the body based on favorable interactions with the stromal microenvironment, the “soil”. As reviewed by Langley and Fiddler (Langley & Fidler, 2011), it is clear that cancer therapy is targeted to either the “seed” through chemotherapy with cytotoxic drugs or the “soil” by manipulating stromal contributions favorable to metastatic growth such as inhibiting angiogenesis.

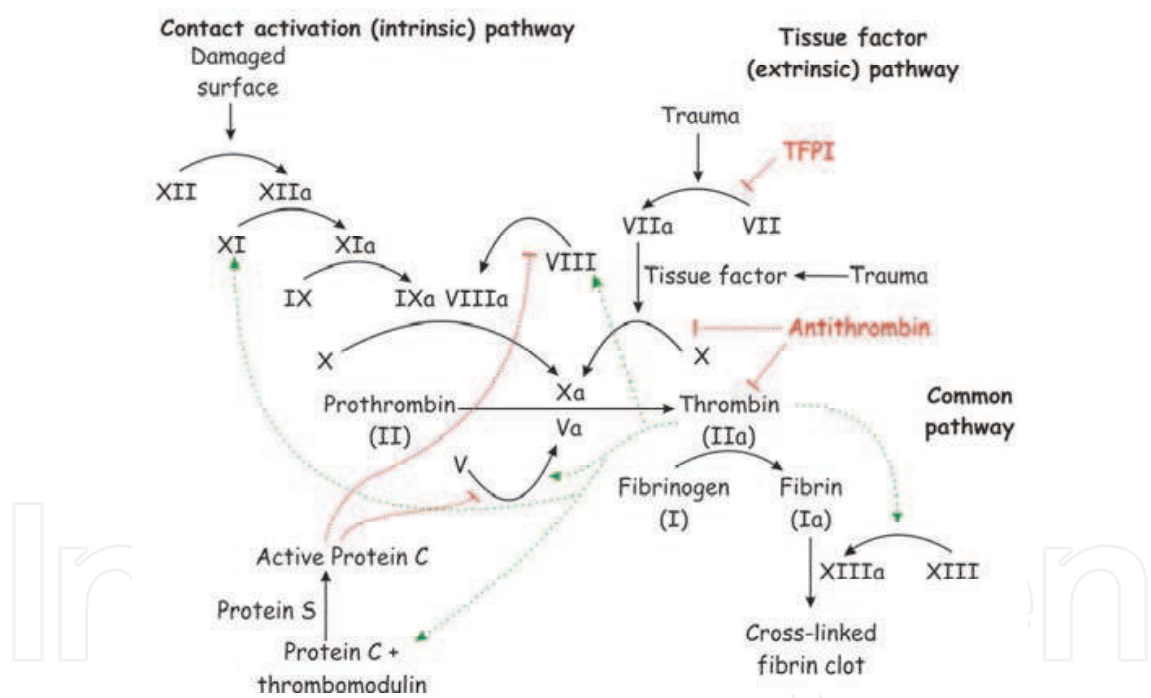


Fig. 2. Schematic view of intrinsic and extrinsic coagulation pathways.

Red lines denote pathway inhibitors of coagulation and green lines denote thrombin activation of hemostatic factors. (Reproduced from public domain image). Appropriate activation of the clotting cascade is fundamental to arrest bleeding in response to vascular injury. The immediate response, known as primary hemostasis, involves vasoconstriction of blood vessels and activation and aggregation of platelets to form a plug at the site of vascular injury. Activated platelets release a panoply of stored constituents including: chemokines (IL-8) and growth factors such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and

transforming growth factor (TGF)- β ; adhesive glycoproteins, including fibrinogen (Fg), fibronectin and von Willebrand factor; and lipid mediators such as lysophosphatidic acid, platelet-activating factor, leukotriene B₄, and thromboxanes. During secondary hemostasis, coagulation is activated either through the extrinsic pathway via tissue factor (TF)-Factor VII (FVII)/activated FVII (FVIIa) or the intrinsic pathway through Factor XII/FXIIa (**Fig. 2**). These pathways converge at the formation of the tenase complex that activates FX to FXa leading to thrombin activation. Thrombin cleaves soluble plasma Fg into fibrin monomers that form the insoluble fibrin clot after fibrin monomer polymerization and covalent crosslinking and stabilization by activated FXIII (FXIIIa). The fibrin clot provides a provisional matrix upon which injured endothelial cells adhere, proliferate and migrate to restore an intact endothelium lining blood vessels. Furthermore, fibrin and Fg provide a reservoir for sequestration of growth factors including FGF-2 (Sahni et al., 1998; Sahni et al., 1999), VEGF (Sahni & Francis, 2000), and TGF- β (Schachtrup et al., 2010), as well as an adhesive substrate for recruitment of leukocytes and stromal fibroblasts to aid in wound repair (Rybarczyk et al., 2003; Ugarova & Yakubenko, 2001). Normal wound repair is self-limiting as the provisional fibrin matrix is dissolved by various proteases, *e.g.*, plasmin, upon resolution of the vascular injury, reduction of inflammation and restoration of normal function (**Fig. 2**).

In the 1980s, however, Dvorak likened cancer progression to “wounds that never heal” in which Fg and fibrin also play prominent roles (Dvorak, 1986), and as reviewed by Coussens and Werb (Coussens & Werb, 2002). Several key steps in normal wound repair are also manifested during cancer progression (**Fig. 3**). As discussed above, a heightened state of coagulation occurs immediately after wound injury, and the release of chemokines and cytokines from activated platelets to recruit and activate proinflammatory cell types to the wound site amplify the inflammatory response system wide.

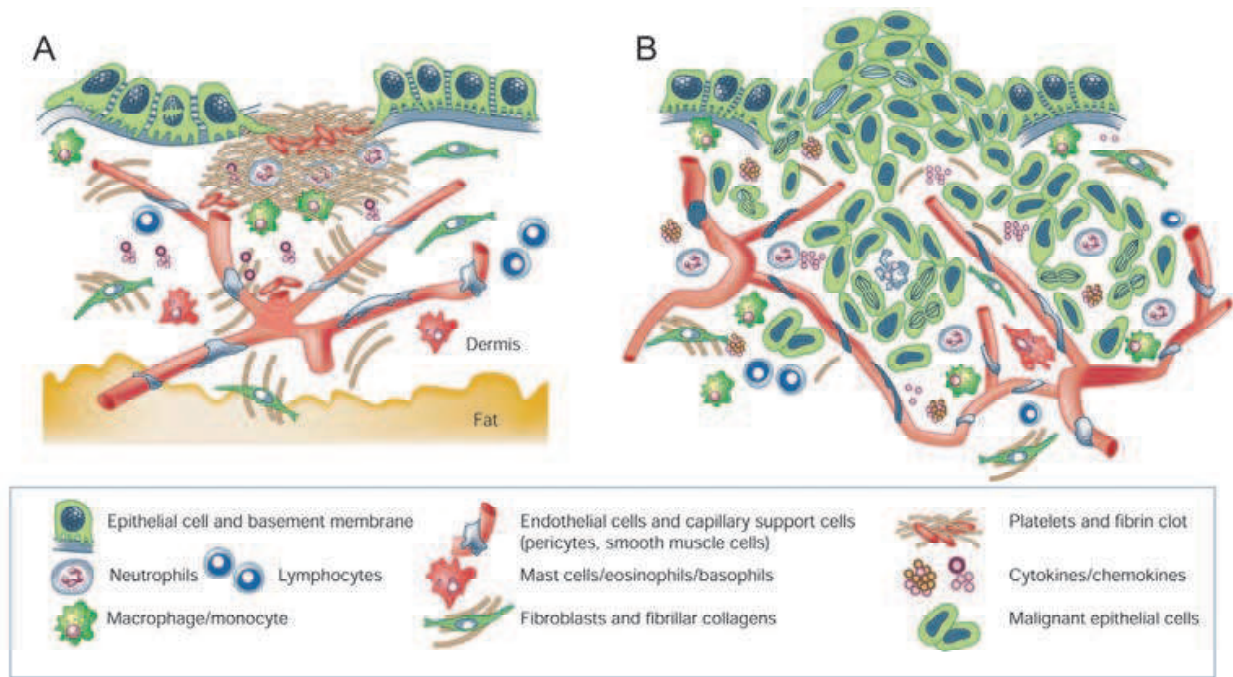


Fig. 3. Normal wound repair is depicted in panel A and mechanisms of wound repair left unchecked in cancer are depicted in Panel B. (Figure reprinted from (Coussens & Werb, 2002) with permission from Nature Publishing Group).

Systemic inflammation is best characterized by the innate acute phase response to injury or infection whereby the synthesis of a host of plasma proteins by the liver is altered to immediately respond to disruptions of homeostasis (Baumann & Gauldie, 1994). Of note, C-reactive protein and Fg are two positive (upregulated) acute phase proteins whose expression is also elevated in malignancies (Jones et al., 2006; Yamaguchi et al., 1998; Yigit et al., 2008). Coagulation and deposition of a provisional fibrin matrix occurs within minutes of vascular injury, and changes in expression of adhesion molecules on the surface of activated endothelium leads to the rolling and slowing of circulating leukocytes, firm attachment and the processes of diapedesis, *i.e.*, transmigration across the endothelial cell barrier into interstitial spaces. Neutrophils are the first proinflammatory cells to appear in the wound space where they release molecules to kill invading microorganisms and promote recruitment of stromal cells such as fibroblasts and endothelial cells to the wound space. Locally deposited growth factors promote cell proliferation and migration leading to the formation of granulation tissue over several days to a few weeks, which is the result of fibroblasts/myofibroblasts depositing extracellular matrix constituents (*e.g.*, collagens) and endothelial cells forming new blood vessels to facilitate wound closure. In the case of cutaneous wounds, re-epithelialization begins to close the wound, the provisional fibrin matrix is dissolved, and infiltrating monocytes/macrophages clean up wound debris in preparation for matrix remodelling, deposition of a complete basement membrane (*e.g.*, laminin) and, over weeks to months, gradual restoration of the tensile strength of the tissue (Coussens & Werb, 2002). In contrast, the orderly array of signaling components that turn on and off cell migration, cell proliferation, and angiogenesis during wound repair goes awry during cancer such that cell growth is unchecked, mechanisms of apoptosis are overridden and the stromal compartment is dramatically altered to perpetuate angiogenesis, tumor growth and cell migration to promote metastasis (**Fig. 3**).

Metastatic disease remains the prevailing reason for treatment failure and death from solid tumors including breast cancers. Only recently have three major areas of research outside the realm of the primary tumor cells themselves been considered viable for development of new therapeutic strategies to prevent the initiation, progression and metastasis of tumors. These include hemostatic factors, the tumor stromal microenvironment, and chronic inflammation. The blood coagulation protein Fg and its insoluble counterpart, fibrin, play central roles in inflammation, venous thromboembolism, and as components of the extracellular matrix. The goals of this chapter are three-fold: first, to review the current understanding of the roles of Fg and/or fibrin {commonly referred to as fibrin(ogen)} in cancer progression in general; second, to provide evidence that fibrin(ogen) likely plays a critical role in the metastatic spread of breast cancer; and third, to propose new therapies for treatment and future avenues of research to elucidate the molecular mechanisms that promote the phenotypic switch of breast epithelial cells to a metastatic cell phenotype.

2. Fibrin(ogen) in cancer progression

2.1 Hemostatic factors and vascular cells promote tumor metastasis

Molecules and cells linked to the prothrombotic state of Trousseau's syndrome that also facilitate cancer metastasis including thrombin, TF, selectins, platelets, endothelial cells and fibrin (Varki, 2007). It is well known that thrombin contributes to the severity of cancer progression by promoting tumor angiogenesis, cancer cell proliferation and metastasis by mechanisms other than just thrombin generation of fibrin (Nierodzik & Karparkin, 2006).

Cell-associated TF expression by cancer cells correlates with disease severity and poor prognosis {reviewed in (Palumbo & Degen, 2007)}. Although tumor cell-associated TF expression is not required for the growth of primary tumors, it is necessary for their metastatic spread (Palumbo et al., 2007). Similarly, FXIII and Fg are important for the metastatic spread of tumor cells through both the circulation and lymphatic systems but not primary tumor growth (Palumbo et al., 2008; Palumbo & Degen, 2001; Palumbo & Degen, 2007; Palumbo et al., 2000; Palumbo et al., 2002; Palumbo et al., 2007; Palumbo et al., 2005). Moreover, FXIII, Fg and platelets are important substrates or cell targets for thrombin action demonstrating the critical role played by the hemostatic system in promoting cancer metastasis. Degen and colleagues suggest that tumor cell-associated TF mediates thrombin generation to support the early survival of micrometastases by at least two mechanisms: 1) the formation of platelet-fibrin microthrombi to protect newly formed micrometastases from natural killer (NK) cell-mediated cytotoxicity, and 2) by promoting mechanical stability of tumor cell emboli within vascular beds at distant metastatic sites (Palumbo et al., 2008; Palumbo & Degen, 2001; Palumbo & Degen, 2007; Palumbo et al., 2000; Palumbo et al., 2002; Palumbo et al., 2007; Palumbo et al., 2005).

2.2 Chronic inflammation is associated with cancer initiation and progression

Systemic inflammation is clearly linked with adverse prognosis in patients with cancer, and is characterized by elevated expression of pro-inflammatory mediators including interleukin (IL)-6 (Gao et al., 2007; Knupfer & Preiss, 2007). IL-6 is the major cytokine responsible for upregulation of specific plasma proteins in the liver during an acute phase response (Baumann & Gauldie, 1994), and also in chronic inflammation (Barton, 2001; Lin & Karin, 2007; Neurath & Finotto, 2011). IL-6 induces expression of target genes, including Fg, by activation of Stat3 (Duan & Simpson-Haidaris, 2003); Stat3 is often constitutively active in breast cancer, and tumor growth can become dependent on Stat3 signaling (Pensa et al., 2009). Both IL-6 and Fg levels are elevated in patients with advanced lung cancer (Yamaguchi et al., 1998). In breast cancer patients, serum IL-6 correlates with increasing numbers of involved sites, liver metastasis, and disease progression (Knupfer & Preiss, 2007; Salgado et al., 2003). In 2002, Drix et al demonstrated that IL-6, VEGF and D-dimer levels are elevated in patients with progressive breast cancer; these markers correlate positively with disease severity, and serum IL-6 is an independent prognostic factor in patients with metastatic disease (Dirix et al., 2002). Elevated levels of Fg, D-dimers, IL-6, VEGF and soluble P-selectin, an indicator of platelet activation, were also found in the plasma of breast cancer patients by Caine et al, who furthered demonstrated that IL-6 induces dose-dependent release of VEGF from platelets in vitro (Caine et al., 2004). Steinbrecher et al demonstrated a direct link between fibrin(ogen), elevated IL-6 levels and the development of inflammation-driven cancer using a mouse model of colitis-associated cancer (Steinbrecher et al., 2010). IL-6 serves as a marker to predict which patients will respond poorly to anti-endocrine chemotherapy (Zhang & Adachi, 1999), as a marker of tumor staging and a predictor of micrometastases (Ravishankaran & Karunanithi, 2011). IL-6 also induces VEGF expression (Cohen et al., 1996) and invasion and migration of breast cancer cells (Walter et al., 2009). Furthermore, overexpression of Her2 in breast cancer cells upregulates IL-6 leading to Stat3 activation and altered gene expression resulting in an autocrine feedback loop promoting cell survival (Hartman et al., 2011). Together, these reports substantiate the importance of fibrin(ogen) and inflammation in cancer metastasis.

2.3 Fibrin(ogen) functions as a bridging molecule in cell-cell interactions during coagulation and inflammatory cell trafficking

Excessive fibrin deposition is accompanied by local expression of proinflammatory mediators, vascular leakage, and inflammatory cell recruitment and activation, leading to amplification of the inflammatory response (Clark, 1996; Simpson-Haidaris & Rybarczyk, 2001; van Hinsbergh et al., 2001). Specific structural features of fibrin(ogen) modulate the functions of a variety of different cell types including endothelial, epithelial, leukocytes, platelets and fibroblasts (**Fig. 4**). Cell receptors that bind to fibrin(ogen) include: $\beta 3$ integrins ($\alpha IIb\beta 3$ and $\alpha v\beta 3$) (Bennett et al., 2009); $\beta 2$ integrins (CD11a/CD18 and CD11b/CD18) (Altieri et al., 1993; Flick et al., 2004; Lishko et al., 2004; Loike et al., 1991; Ugarova et al., 2003; Yakovlev et al., 2005); and $\beta 1$ integrin, $\alpha 5\beta 1$ (Asakura et al., 1997; Suehiro et al., 1997). Nonintegrin adhesion molecules that bind to fibrin(ogen) include intercellular adhesion molecule (ICAM)-1 (Languino et al., 1993; Pluskota & D'Souza, 2000), vascular endothelial (VE)-cadherin (Bach et al., 1998b) and heparan sulfate proteoglycans (HSPG) (Odrliin et al., 1996a; Odrliin et al., 1996b). Fibrin(ogen) also modulates a number of signaling molecules important in innate immunity. Fg-bound FGF-2 induces expression of uPA, uPA receptor and PAI-1, and fibrin(ogen) induce IL-8, MCP-1 or IL-1 β expression in endothelial cells (Guo et al., 2004; Harley & Powell, 1999; Kuhns et al., 2001; Lee et al., 2001; Qi & Kreutzer, 1995; Ramsby & Kreutzer, 1994; Sahni et al., 2004). Fg and fibrin activate NF- κ B and AP-1 (Guo et al., 2004; Sitrin et al., 1998), transcription factors critical for propagation of inflammation.

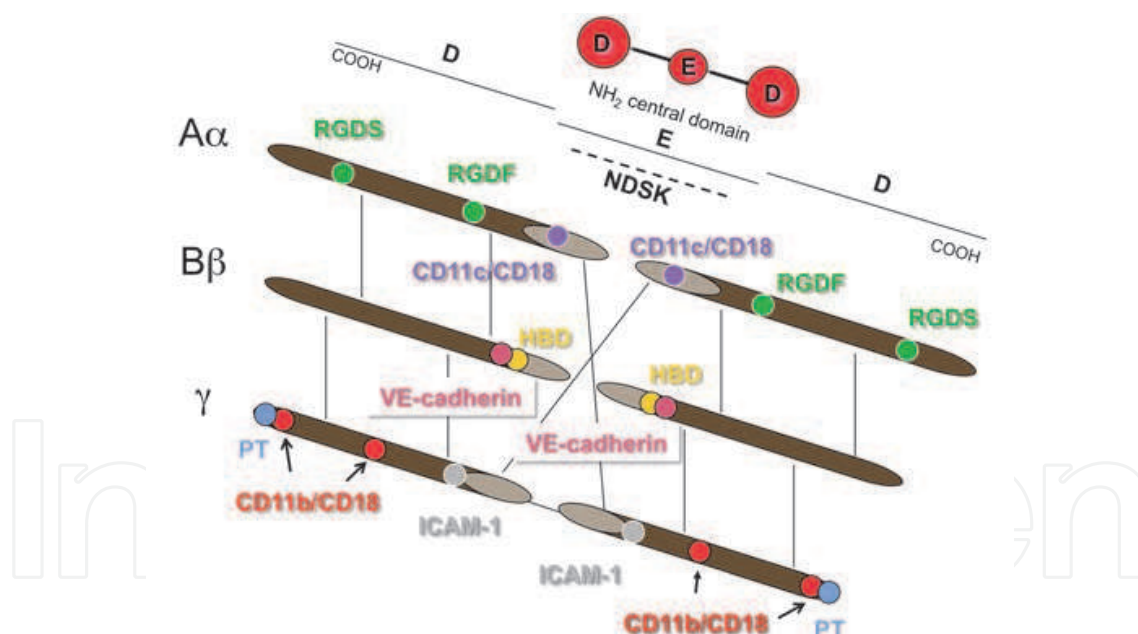


Fig. 4. Fibrin(ogen) enzyme and CNBr cleavage fragments and cell recognition domains.

Fg A α , B β and γ chains are held together by 29 pairs of disulfide bonds (approximated by the vertical lines) with the N-termini of all six chains held together in the central domain. Electron microscopy studies indicate that the dimeric Fg molecule appears as a trinodular structure as depicted by the red ball and stick cartoon. Thrombin release of fibrinopeptides, FPA and FPB, from A α and B β N-termini, respectively, produces soluble fibrin leading to fibrin polymerization into an insoluble gel stabilized by FXIIIa-mediated crosslinks between γ - γ and α - γ chains. Lines below the ball and stick cartoon denote N-terminal

plasmin cleavage fragment E and C-terminal fragments D. N-terminal disulfide knot (NDSK) (dashed line) is the minimal sequence of the central domain after CNBr cleavage and is structurally similar to plasmin E fragment. Residues on Fg for receptor-cell binding domains are: CD11c/CD18, A α ¹⁷⁻¹⁹; integrin RGDF, A α ⁹⁵⁻⁹⁸ and RGDS, A α ⁵⁷²⁻⁵⁷⁵; ICAM-1, γ ¹¹⁷⁻¹³³; CD11b/CD18, γ ¹⁹⁰⁻²⁰², γ ²²⁸⁻²⁵³ and γ ³⁹⁰⁻³⁹⁶; platelet (PT) binding, γ ⁴⁰⁰⁻⁴¹¹. The heparin binding domain (HBD) at β ¹⁵⁻⁴² overlaps the VE-cadherin binding site. The first fibrin degradation products (FDPs) released by plasmin cleavage are the β ¹⁵⁻⁴² domain and the C-terminal 2/3rd of the A α chain, termed α C, which contain several cell binding domains.

2.4 Fibrin(ogen) in the stromal microenvironment in breast cancer

The tumor microenvironment is a complex entity composed not only of extracellular matrix (ECM) constituents including: i) growth factors; ii) cytokines and chemokines; iii) proteases; and iv) matrix glycoproteins, glycosaminoglycans and proteoglycans—but also diverse cell populations that influence the behavior of cancer cells including: v) immune cells such as lymphocytes, NK cells, dendritic cells, macrophages and neutrophils; vi) stromal fibroblasts/myofibroblasts, adipocytes and stem cells; and vii) cells of the vasculature including endothelial cells, pericytes and smooth muscle cells (reviewed in (Andre et al., 2010; Anton & Glod, 2009; De Wever et al., 2008; Deryugina & Quigley, 2006; Tlsty & Coussens, 2006; Ulisse et al., 2009)). Although activated inflammatory cells in the tumor microenvironment play important roles in cancer initiation, progression, angiogenesis and metastasis, they are not the most numerous. Cancer-associated fibroblasts, similar to myofibroblasts of healing wounds, are the most abundant stromal cells in the tumor microenvironment (Tlsty & Coussens, 2006), and contribute significantly to chronic inflammation by production of chemokines, cytokines, and pro-angiogenic factors and deposition of matrix constituents that support new blood vessel formation required for tumor growth, cell migration and metastasis (De Wever et al., 2008). Solid tumors need to develop their own blood supply for nutrient delivery and removal of toxic waste. Angiogenesis, the formation of new blood vessels from existing vasculature, requires activation of proteases leading to degradation of the basement membrane, endothelial cell sprouting and pericyte attachment for vessel stabilization. Cancer-associated fibroblasts play an important role in synchronizing these events (De Wever et al., 2008). Furthermore, the topography of the ECM mediates vascular development and regulates the speed at which cells migrate during angiogenesis (Bauer et al., 2009). Vascular endothelial cells play a pivotal role in regulating leukocyte recruitment during inflammation (McGettrick et al., 2007). In most cases, cancers exploit pro-inflammatory mediators and recruited inflammatory cells to benefit their own survival (Lorusso & Ruegg, 2008) (also as reviewed in (Simpson-Haidaris et al., 2010)).

Fg and fibrin deposition is found within the stroma of most solid tumors (Simpson-Haidaris & Rybarczyk, 2001), and elevated levels of plasma Fg and fibrin degradation products (FDPs) correlate positively with lymph node involvement and metastatic spread of colorectal, ovarian, lung and breast cancers (Sahni et al., 2009; Varki, 2007). Fibrin deposition at the tumor-normal host cell interface as well as in the stroma of primary tumors is well documented, and is thought to protect tumors from infiltrating inflammatory cells by acting as a barrier thereby preventing inflammatory reactions directed towards the tumor cells (reviewed in (Simpson-Haidaris & Rybarczyk, 2001)). The presence of D-dimer, a fibrin degradation product indicative of pathological fibrin formation and dissolution, correlates

with poor prognosis in most solid tumors including colon, prostate, lung and breast (Batschauer et al., 2010; Kilic et al., 2008; Knowlson et al., 2010). However, in some malignancies, including breast, evidence demonstrating deposition of fibrin within the primary tumor is lacking (reviewed in (Simpson-Haidaris & Rybarczyk, 2001)). Instead, abundant Fg deposition occurs in breast tumor stroma in the absence of thrombin generation (Costantini et al., 1991).

2.5 Cancer cells, including breast, synthesize and secrete fibrinogen

The origin of tumor-associated fibrin(ogen) and fibrin(ogen) degradation products has historically been thought to be from exudation of plasma Fg due to the increased vascular permeability and subsequent procoagulant or fibrinolytic activity at the tumor site (Rybarczyk & Simpson-Haidaris, 2000). However, because Fg deposition in the stroma, but not fibrin formation, is considered a hallmark of breast cancer (Costantini et al., 1991), we hypothesized that breast cancer cells were capable of endogenous synthesis and secretion of Fg. We demonstrated that human MCF-7 cells are capable of synthesizing Fg chains, although assembly of intact Fg is defective due to degradation of the B β chain (Rybarczyk & Simpson-Haidaris, 2000). In addition, we have shown that lung, prostate and breast cancer epithelial cells synthesize and secrete Fg that enhances FGF-2-mediated cell proliferation, assembles into the ECM and binds to cancer cell surface receptors (Rybarczyk & Simpson-Haidaris, 2000; Sahni et al., 2008; Simpson-Haidaris, 1997; Simpson-Haidaris & Rybarczyk, 2001). Others have shown Fg production in cervical (Lee et al., 1996) and intestinal (Molmenti et al., 1993) cancer cell lines. Expression array profiling studies confirmed that Fg genes are expressed in breast (Pentecost et al., 2005) and lung carcinomas (Tan et al., 2005) from patients. Thus, Fg synthesized by cancer cells promotes growth of the primary tumor and supports tumor-associated angiogenesis characterized by localized VEGF production and leaky vessels (Dvorak, 2006). The importance of VEGF in promoting tumor vascular permeability, angiogenesis and leakage of plasma Fg into the perivascular space to induce tumor stroma desmoplasia is well known. However, whether *tumor-associated fibrin(ogen)* contributes to permeability of tumor vessels and breast cancer metastasis is unknown.

2.6 Fibrinogen is an extracellular matrix protein

Although Fg is known for its hemostatic role, we showed that Fg, not fibrin, is a component of the insoluble fibrillar ECM of fibroblasts, alveolar epithelial cells, endothelial cells and breast epithelial cells (Guadiz et al., 1997; Pereira et al., 2002; Sahni et al., 2009; Simpson-Haidaris et al., 2010; Simpson-Haidaris & Sahni, 2010). Upon assembly into matrix fibrils, Fg undergoes conformational changes exposing the cryptic β^{15-42} epitope in the absence of thrombin cleavage or covalent crosslinking (Guadiz et al., 1997; Simpson-Haidaris & Sahni, 2010). When Fg is pre-established in the ECM of adventitial fibroblasts prior to wounding, increased cell proliferation and migration enhance wound closure (Rybarczyk et al., 2003), which is dependent on *de novo* protein synthesis (Pereira & Simpson-Haidaris, 2001) but independent of added growth factors, PDGF and FGF-2 (Rybarczyk et al., 2003). However, assembly of Fg into mature matrix fibrils of breast epithelial cells appears to correlate negatively with the increasing invasive potential of the cell (Fig. 5). We also determined whether the cryptic HBD in soluble Fg (Odrliin et al., 1996b) was accessible in matrix Fg using a specific MoAb (T2G1) (Kudryk et al., 1984). Whereas the T2G1 epitope (β^{15-21}) within β^{15-42} is not accessible for antibody binding in soluble Fg or Fg immobilized to a surface, the

results indicated that β^{15-21} is exposed on Fg assembled into matrix fibrils (Guadiz et al., 1997; Rybarczyk et al., 2003). Together these data suggest that matrix Fg possesses “fibrin-like” properties in the absence of fibrin polymerization and that Fg deposition rapidly changes the topology of the ECM to provide a surface for cell migration and matrix remodeling during wound repair. However, the mechanisms by which β^{15-42} modulates cell-cell or cell-matrix adhesion are not well understood.

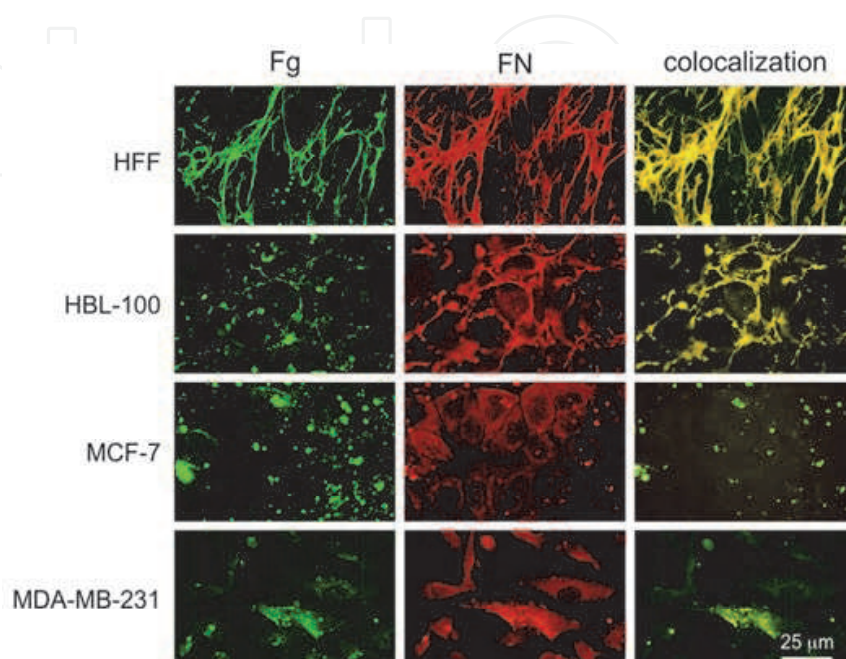


Fig. 5. Plasma fibrinogen assembles into mature matrix fibrils of nonmalignant cells (HFF and HBL-100) but poorly assembles in the matrix of malignant breast cancer cells (MCF-7 and MDA-MB-231). Primary human fibroblasts (HFF), a nonmalignant human breast cancer cell line (HBL-100) and two human breast cancer cell lines (MCF-7 and MDA-MB-231) were grown on gelatin-coated glass coverslips and treated with Fg conjugated to Oregon GreenTM (30 $\mu\text{g}/\text{ml}$) for 24 hr. The cells were washed, fixed, stained with anti-fibronectin (FN) polyclonal antibodies followed by rhodamine-goat anti-rabbit secondary antibodies, and visualized by epifluorescence microscopy. Green fluorescence is Fg-specific and red fluorescence denotes FN staining. Colocalization of Fg and FN results in yellow fluorescence. The loss of FN in the more invasive cell lines (MCF-7 and MDA-MB-231) is likely an explanation for purified plasma Fg binding to the surface of cells but failure to assemble into mature matrix fibrils, as we have shown that assembly of Fg into an elaborate fibrillar ECM depends on the assembly of FN fibrils as well (Pereira et al., 2002).

3. Role of Fibrin(ogen) in breast cancer metastasis

3.1 Importance of Fg peptide β^{15-42} in Fg-endothelial cell interactions

Fibrin(ogen) β^{15-42} sequences support a diverse array of biological functions mediated by fibrin(ogen). Although the primary structure of fibrinopeptide B (FPB) is poorly conserved across species, the fibrin β^{15-42} domain is highly conserved, implying evolutionary conservation of function (Courtney et al., 1994). The β^{15-42} region constitutes a cryptic domain in soluble Fg that is exposed in fibrin after thrombin cleavage (Odrlić et al., 1996b). Both the HBD and overlapping binding site for VE-cadherin are localized to β^{15-42} . VE-

cadherin mediates homophilic cell-cell adhesion critical for the maintenance of barrier integrity of the endothelium. Disruption of VE-cadherin-mediated endothelial barrier function leads to altered vascular permeability found in a number of diseases including ischemia-reperfusion (IR) injury, inflammation, angiogenesis, and cancer growth and metastasis (discussed in (Sahni et al., 2009)). Exposure of β^{15-42} and binding by VE-cadherin is also required for endothelial capillary tube formation in fibrin gels (Bach et al., 1998a; Chalupowicz et al., 1995); portions of the third extracellular domain (EC3) of VE-cadherin constitute a fibrin β^{15-42} receptor (Bach et al., 1998b; Yakovlev & Medved, 2009). Newly exposed β chain residues, β^{15} -GHRP-18, play a critical role in fibrin monomer aggregation during polymerization and clot formation during secondary hemostasis (Mosesson, 2005). Furthermore, exposure of the β^{15-42} domain mediates heparin-dependent fibrin binding to endothelial cell surfaces (Odrliin et al., 1996a); promotes endothelial cell adhesion and spreading (Bunce et al., 1992); promotes the release of endothelial cell-specific markers of endothelial activation (Ribes et al., 1989); and stimulates proliferation of endothelial cells, fibroblasts and cancer cells (Rybarczyk et al., 2003; Sahni et al., 2008; Sporn et al., 1995).

3.2 Fibrin β^{15-42} protects the myocardium from Ischemic-Reperfusion (IR) injury

A synthetic peptide of fibrin residues β^{15-42} has been implicated as a potential therapeutic agent to reduce tissue damage and scarring after a heart attack (Hirschfield & Pepys, 2003; Petzelbauer et al., 2005b; Roesner et al., 2007; Zacharowski et al., 2006; Zacharowski et al., 2007). Peptide β^{15-42} works by inhibiting leukocyte migration across the endothelium into heart tissue, which prevents excessive inflammation and tissue damage. Peptide β^{15-42} -mediated reduction of tissue injury depends on its ability to bind to VE-cadherin. Peptide β^{15-42} competes with FDP (e.g., the plasmin E domain of fibrin as depicted in **Fig. 4**) for binding to VE-cadherin to prevent transendothelial cell migration (TEM) of leukocytes during myocardial IR injury (Petzelbauer et al., 2005b; Roesner et al., 2007; Zacharowski et al., 2006; Zacharowski et al., 2007). These published reports demonstrate the physiologic efficacy of fibrin β^{15-42} for treating IR injury. *However, the molecular mechanisms induced by fibrin(ogen) β^{15-42} binding to VE-cadherin to mediate enhanced paracellular permeability and whether fibrinogen-induced cancer metastasis involves binding interactions with fibrin(ogen) β^{15-42} have not been previously studied.*

3.3 Fibrin(ogen) β^{15-42} induces endothelial barrier permeability via VE-cadherin binding interactions

In a recent report (Sahni et al., 2009), we sought to determine whether fibrin(ogen) β^{15-42} binding to VE-cadherin induced endothelial cell permeability, and whether fibrinogen-induced cancer metastasis involves binding interactions between VE-cadherin and fibrin(ogen) β^{15-42} . Using transwell insert culture systems, we showed that Fg β^{15-42} and VE-cadherin binding interactions promote endothelial cell barrier permeability (Sahni et al., 2009) (**Fig. 6**). Peptides containing or missing residues β^{15-17} critical for β^{15-42} binding to VE-cadherin (Gorlatov & Medved, 2002) and neutralizing antibodies that bind to Fg β^{15-21} (T2G1) and VE-cadherin (BV9) (**Fig. 7A**) were used to induce or inhibit permeability. Fg induced dose-dependent permeability of human umbilical vein endothelial cells (HUVEC) and microvascular endothelial cells (HMEC-1) (**Fig. 6**), but not epithelial cell barriers (as shown in Fig. 1 in ref (Sahni et al., 2009)), which could be inhibited by neutralizing antibodies against β^{15-21} (T2G1) and VE-cadherin (BV9) and synthetic peptides (not shown).

However, the neutralizing antibodies (T2G1 and BV9) did not completely inhibit Fg-induced permeability (**Fig. 7B**), suggesting that additional cell recognition domains on Fg participate in fibrin(ogen)-induced vascular permeability.

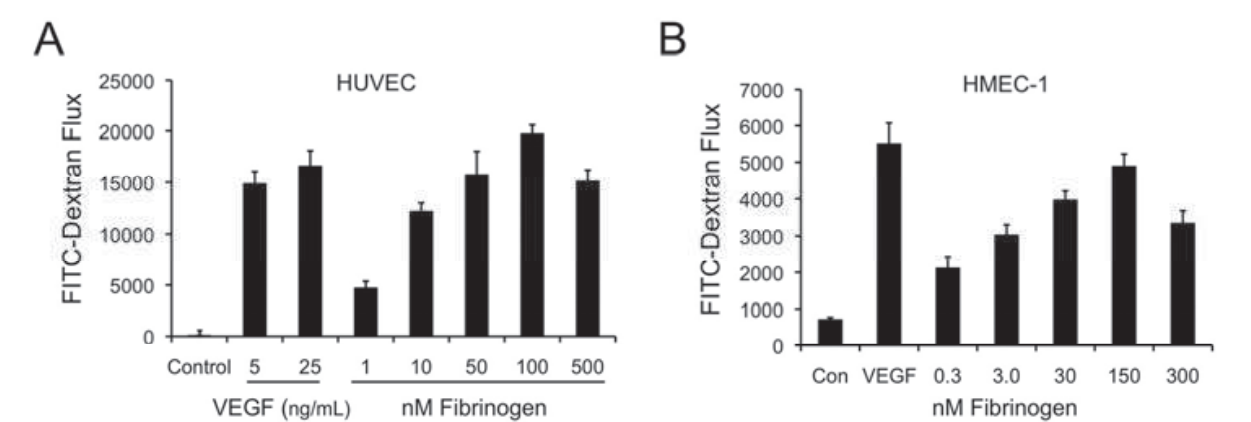


Fig. 6. Fg-induced EC permeability involves Fg β^{15-42} and VE-cadherin. Cells were grown to confluency on Millicell™ 24-well cell culture inserts. Panel 6A, HUVEC were left untreated (control) or treated for 15 min with increasing concentrations of Fg or VEGF as indicated. Panel 6B, HUVEC were treated with 30 nM of Fg plus 1 mg/ml FITC-Dextran for the times indicated. The FITC-Dextran flux to the bottom chamber was measured by fluorometry and the data presented as the mean relative FITC-Dextran Flux \pm SEM. Data points were derived from 3 or more independent experiments with the total number of replicates per condition ranging from 6-13. (Reprinted from (Sahni et al., 2009) with permission). P-values can be found in ref (Sahni et al., 2009).

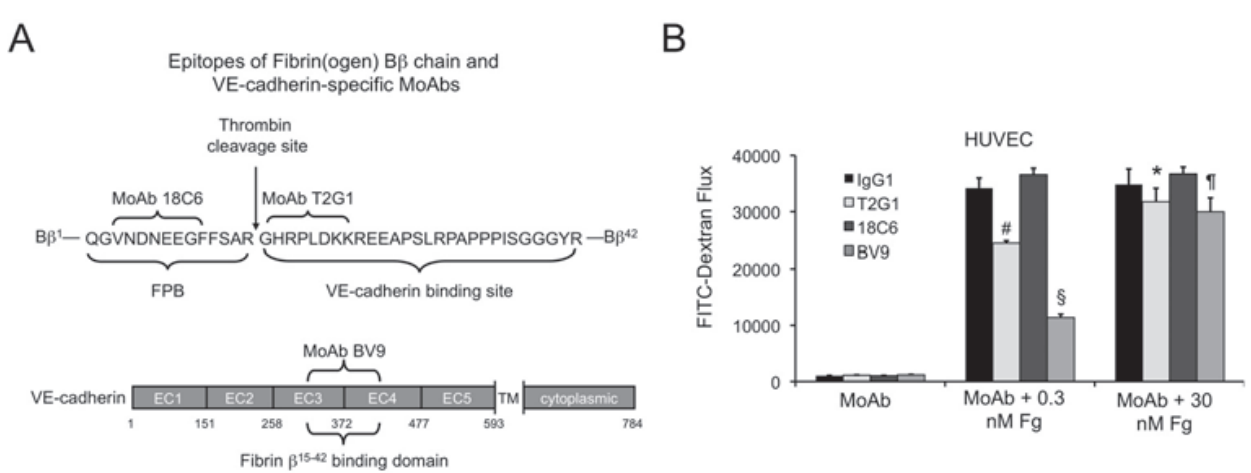


Fig. 7. Fg-induced EC permeability involves Fg β^{15-42} sequences and VE-cadherin. Panel 7A, schematics of the aminotermius of the fibrin(ogen) B β chain and the domain structure of VE-cadherin are depicted. The arrow denotes the thrombin cleavage site for release of FPB. The 18C6 epitope maps to FPB, the T2G1 epitope maps to β^{15-21} and the VE-cadherin binding site on fibrin maps to β^{15-42} . The epitope of the VE-cadherin-specific monoclonal antibody BV9 maps to the third and fourth extracellular domains (EC3-EC4). The fibrin β^{15-42} binding site on VE cadherin maps to EC3 near the EC3-EC4 junction. TM, transmembrane domain. Panel 7B, all monoclonal antibodies used are IgG₁ isotype murine antibodies and

nonimmune IgG₁ was used for the control. Monoclonal antibodies were used at 3 nM in the absence of Fg, or with 0.3 nM or 30 nM Fg for 45 min. The data were plotted as the mean \pm SEM of relative FITC-Dextran Flux and were obtained from three independent experiments with a total sample size of 6-9 per condition. (Reprinted from (Sahni et al., 2009) with permission). P-values can be found in ref (Sahni et al., 2009).

3.4 VE-cadherin binding domain of Fg (β^{15-42}) enhances transendothelial migration of malignant breast epithelial cells

Because plasma Fg promotes metastasis of some types of cancer and Fg β^{15-42} sequences promote endothelial cell permeability, we hypothesized Fg β^{15-42} sequences would play a role in promoting TEM of breast cancer cells. To test this hypothesis, breast cancer cells were labeled with a fluorescence cell-tracking dye (DiI) before they were mixed with increasing concentrations Fg. Breast cancer cells and Fg were allowed to pre-incubate for 15 minutes prior to addition to the upper chamber of a barrier monolayer of endothelial cells. After 45 minutes incubation, the relative number of breast cancer cells migrating to the underside of the transwell insert membrane were quantified by relative fluorescence and

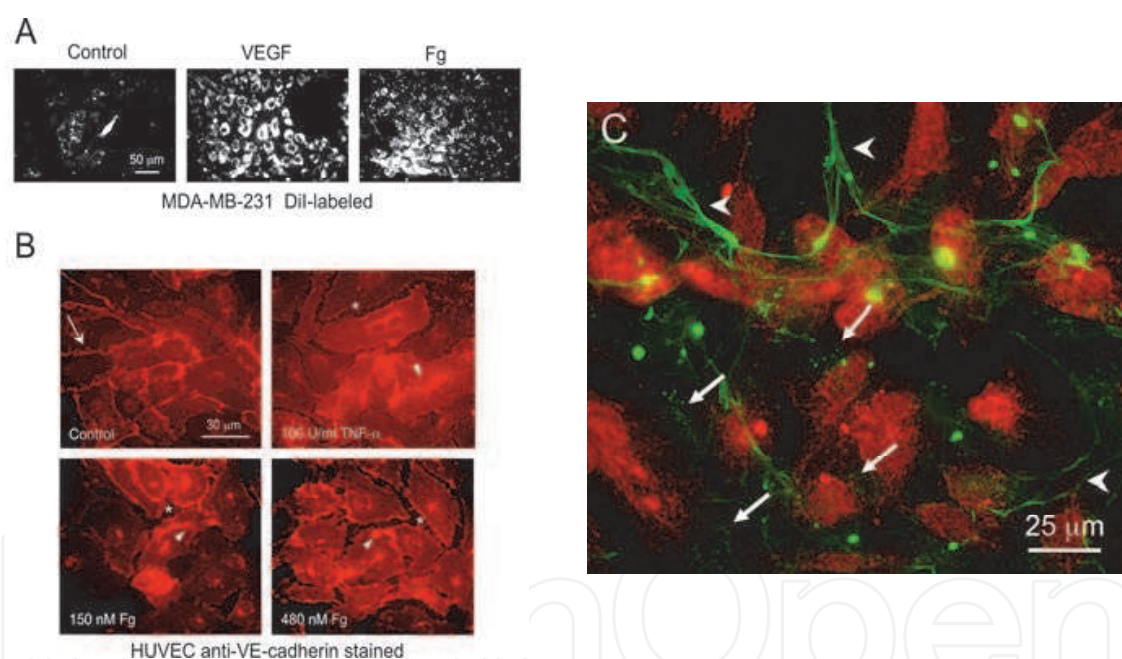


Fig. 8. Fg enhances TEM of malignant breast epithelial cells (Panel A), induces gap formation between adjacent endothelial cells (Panel B, asterisks), promotes intracellular relocalization (Panel B, arrowheads) of VE-cadherin at membrane cell-cell junctions (Panel B, Control, arrow), assembles into ECM (Panel C, arrowhead), and shows punctate, cell surface receptor-like binding between adjacent endothelial cells (Panel C, arrows). Cells in Panels A and B were treated as described in Section 3.3. In Panel C, endothelial cells were treated for 24 hours with purified plasma Fg conjugated to Oregon Green. Cells were fixed, permeabilized and stained with anti-FGF-2 (red fluorescence). After staining, the coverslip was mounted upside down on a microscope slide so that the basolateral aspect (bottom of cells) and the subendothelial ECM appear as the “top” of the cells. Matrix Fg and receptor bound Fg are shown in green fluorescence. Cover Figure ref (Sahni et al., 2009).

visualized by microscopy. VEGF was used as a positive control to induce endothelial cell permeability and TEM of breast cancer cells. The results indicated that TEM of both MCF-7 and MDA-MB-231 cells was increased in a Fg-concentration-dependent manner (see Fig. 3a of ref (Sahni et al., 2009)) and as visualized by immunofluorescence microscopy showing MDA-MB-231 cells adhered to the bottom side of the transwell filter (**Fig. 8A**).

To determine whether VE-cadherin and/or Fg β^{15-42} were involved in Fg-enhanced TEM of MDA-MB-231 cells, the assay was repeated in the presence of the neutralizing and control antibodies (as shown in Fig. 3c of ref (Sahni et al., 2009)). To determine whether Fg promoted gap formation between cells, confluent HUVEC were treated with 150 or 480 nM Fg or 100 Units/ml TNF- α , a known inducer of endothelial permeability and gap formation, for 30 minutes then cells were fixed, permeabilized and immunostained with an anti-VE-cadherin. Fg treatment induced gap formation between adjacent endothelial cells, and such treatment promoted the subcellular relocation of VE-cadherin from the cell periphery as in control cells into the cytoplasm in Fg- and TNF- α -treated cells (**Fig. 8B**). Indirect evidence for Fg binding at endothelial cell-cell junctions was obtained by fluorescence microscopy. The data reveal that Fg binds to endothelial cell-cell junctions in a punctate pattern, consistent with cell surface receptor binding to the cell-cell adhesion receptor, VE-cadherin (**Fig. 8C, arrows**). Fg also assembles as part of the fibrillar subendothelial ECM (**Fig. 8C, arrowhead**). Taken together, the data in **Fig. 6-8** demonstrate that the VE-cadherin binding domain defined by residues 15-42 on the β -chain of human Fg induces permeability of endothelial but not epithelial cell barriers and enhances TEM of malignant breast cancer cells by a VE-cadherin-dependent mechanism. In contrast, the basal level of TEM of nonmalignant breast epithelial cells was not enhanced by Fg treatment (Sahni et al., 2009).

3.5 Fibrinogen potentiates endothelial cell permeability at low doses of VEGF

Both FGF-2 and VEGF bind to fibrin(ogen) at distinct sites with high affinity (Sahni & Francis, 2000; Sahni et al., 1998). Fg bound-FGF-2 potentiates endothelial cell proliferation over FGF-2 alone (Sahni et al., 2003; Sahni & Francis, 2004; Sahni et al., 2006; Sahni et al., 1999). Although Fg-bound VEGF remains active, it does not potentiate endothelial cell proliferation over VEGF alone (Sahni & Francis, 2000). Because Fg induces endothelial cell permeability through VE-cadherin binding interactions (Sahni et al., 2009) and VEGF binds to Fg (Sahni & Francis, 2000), we tested the hypothesis that Fg would potentiate VEGF-induced EC permeability (**Fig. 9**).

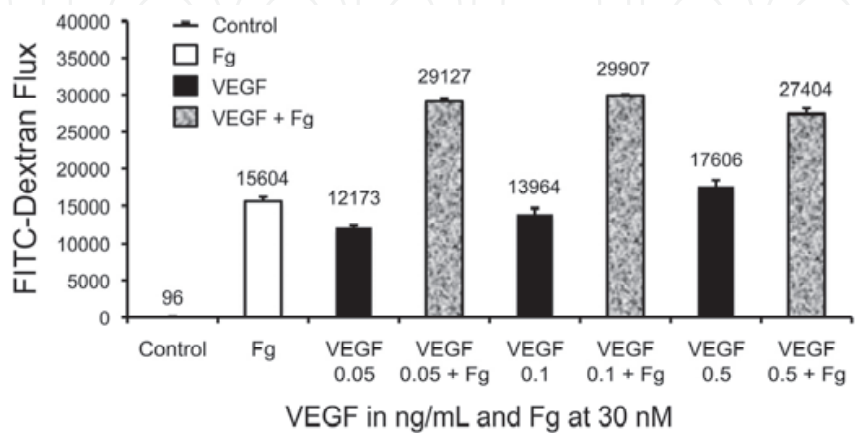


Fig. 9. Fg enhances permeability induced by low concentrations of VEGF.

The data indicate that 10 $\mu\text{g/ml}$ (30 nM) Fg enhanced the flux of FITC-dextran to the bottom chamber of the transwell plate at low doses of VEGF (0.05 and 0.1 ng/ml); however, the additive effect on induction of endothelial cell permeability was lost at 0.5 ng/ml and higher concentrations of VEGF (**Fig. 9**). Fg-enhancement of VEGF-induced permeability is rapid and saturated within 5 min, whereas 5 ng/ml of VEGF is required to induce a similar amount of FITC-dextran flux as 30 nM Fg + 0.05 ng/ml, *i.e.*, 100-fold less VEGF. Studies by others suggest that low-dose VEGF mediates inflammation to promote cell survival of vascular and nonvascular cells such as those of the CNS, prior to induction of angiogenesis (Abumiya et al., 2005; Croll et al., 2004). Furthermore, VEGF colocalizes with exuded Fg at sites of edema in renal cell carcinoma (Verheul et al., 2010). Together with the aforementioned published data, our results suggest that Fg may regulate vascular permeability induced by low doses of VEGF without inducing EC proliferation—such a response would be conducive to fibrinogen induction of breast cancer cell TEM.

4. Summary, therapeutic strategies and future research to elucidate fibrin(ogen)-mediated mechanisms of breast cancer metastasis

4.1 Summary and therapeutic strategy using free peptide β^{15-42} to inhibit breast cancer metastasis as depicted in Fig. 10, Steps 1-11

Regardless of the subtype of breast cancer, once the primary tumor becomes established (*Step 1*), it needs to develop its own blood supply for nutrient delivery and removal of toxic waste (*Step 2*). Breast cancer cells produce VEGF, which initiates permeability of nearby blood vessels allowing plasma Fg to leak into the tumor stroma promoting desmoplasia and deposition of a provisional fibrin(ogen) matrix in the tumor microenvironment (*Step 2*). Alternatively, endogenous synthesis of Fg by breast cancer cells could induce cancer progression. Thus, the innate immune response is activated to defend the host against this neoplastic insult. Release of IL-6 systemically leads to increased production of plasma Fg and fibrin formation resulting in exposure of β^{15-42} and binding to VE-cadherin, a step critical for angiogenesis (Bach et al., 1998b; Martinez et al., 2001). Furthermore, VEGF binds to Fg and fibrin with high affinity (Sahni & Francis, 2000), which may be necessary for Fg to enhance VEGF-mediated endothelial cell permeability without potentiating endothelial cell proliferation. In contrast, VE-cadherin and VEGF receptor-2 form a signaling complex to promote endothelial cell proliferation (Carmeliet et al., 1999; Dejana, 2004; Esser et al., 1998). Fibrin(ogen) potentiates FGF-2- but not VEGF-induced proliferation of endothelial cells, angiogenesis and cancer cell growth (Rybarczyk & Simpson-Haidaris, 2000; Sahni & Francis, 2000; Sahni et al., 2006; Sahni et al., 2008; Sahni et al., 1999; Simpson-Haidaris, 1997; Simpson-Haidaris & Rybarczyk, 2001). Furthermore, fibrin(ogen) enhances cell migration and cancer invasion through tumor stroma, and TEM, *i.e.*, intravasation of breast cancer cells into the blood stream (*Step 3*) (Roche et al., 2003; Rybarczyk et al., 2003; Sahni et al., 2009). Fg and fibrin can bridge between cells of the same or different kinds (Kloczewiak et al., 1983; Languino et al., 1995; Languino et al., 1993; Saito et al., 2002; Sriramaraio et al., 1996) and form aggregates or tumor emboli coated with fibrin(ogen) (*Step 4*). Because the host immune system does not recognize fibrin(ogen)-coated tumor emboli (Palumbo et al., 2005), immune-mediated destruction of tumor cells does not occur and these tumor emboli travel through the circulation to sites favorable for metastatic growth (*Steps 5 & 6*) such as lung. To establish metastatic growth, tumor emboli need to leave the circulation and enter lung tissue (*Steps 7 and 8*) where they find a receptive niche (*Step 9*) to begin the process again. Tumor

cell proliferation and angiogenesis (*Step 10*) in lung results in metastatic disease (*Step 11*). We *hypothesize* that free peptide β^{15-42} will bind to VE-cadherin between endothelial cells to block endothelial cell binding to β^{15-42} on intact fibrin(ogen) found in the tumor stroma or tumor vessels, thereby inhibiting tumor-associated angiogenesis (*Step 2*), intravasation (*Step 3*), extravasation (*Step 8*), and angiogenesis at metastatic tumor sites (*Step 10*) (as denoted by the lightning bolts at these steps in Fig. 10).

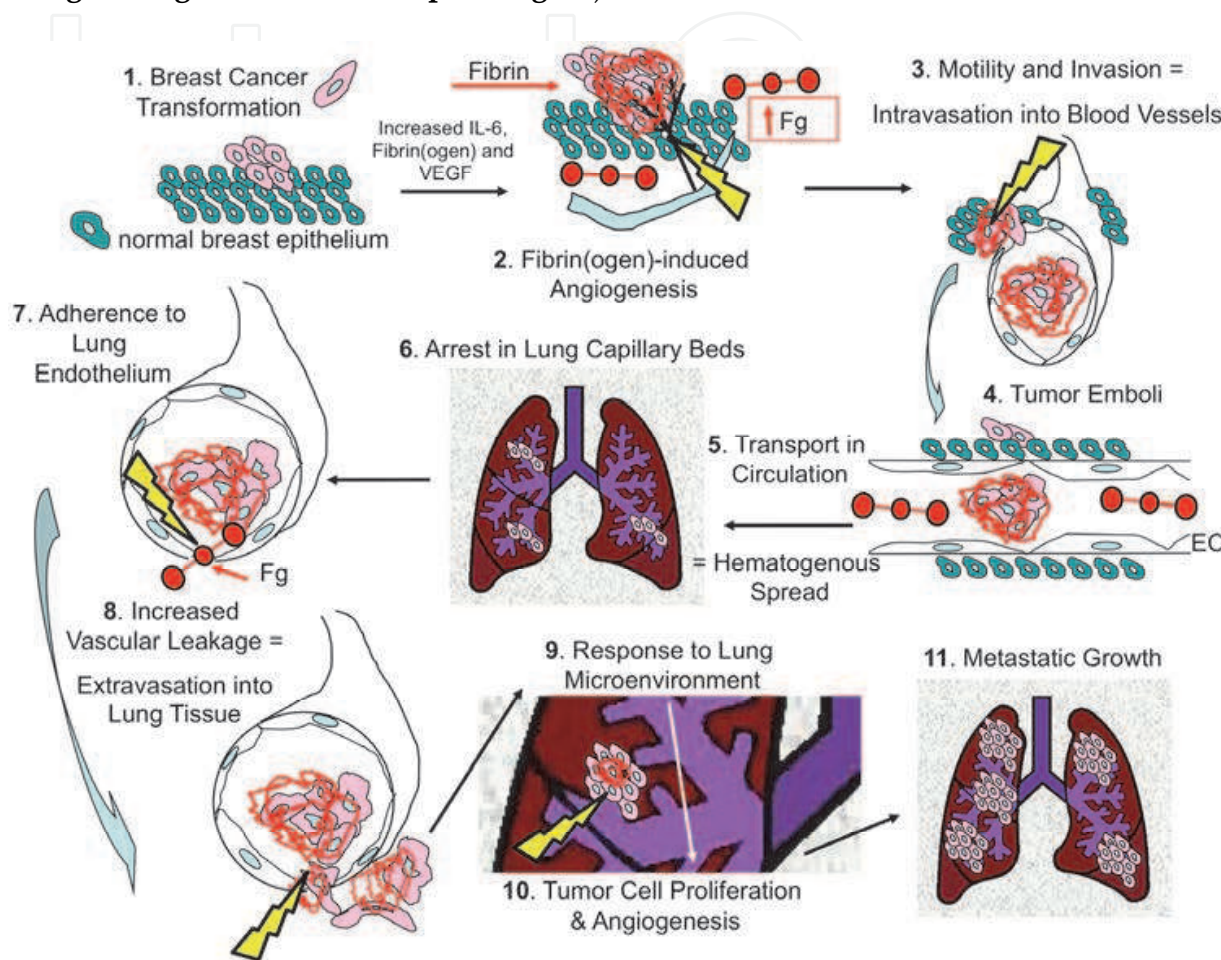


Fig. 10. Schematic summarizing role of fibrin(ogen) β^{15-42} in breast cancer metastasis and hypothesis development for employing free peptide β^{15-42} as a therapeutic strategy to treat metastatic breast cancers.

Successful demonstration of peptide β^{15-42} as an inhibitor of breast cancer metastasis and tumor-associated inflammation and angiogenesis *in vivo* would significantly impact breast cancer treatment in a timely manner. Peptide β^{15-42} , an endogenous fragment of fibrin, is already shown to be well tolerated in humans and effective in reducing damage to heart muscle after a heart attack in preclinical models of IR injury. However, until now, no one has proposed the use of peptide β^{15-42} as an inhibitor of breast cancer metastasis. A precedent and pipeline for production of viable therapeutics based on peptide β^{15-42} exists for treatment of damaged heart tissue, and Phase I and Phase II clinical trials are ongoing to test the safety and efficacy, respectively, of free β^{15-42} peptide for IR injury (Hallen et al., 2010; Petzelbauer et al., 2005a; Petzelbauer et al., 2005b; Roesner et al., 2007; Roesner et al., 2009; Wiedemann et al., 2010; Zacharowski et al., 2006). Therefore, the timeline for

successful translational to a therapeutic agent to treat metastatic disease in breast cancer patients with different subtypes of the disease would be significantly shortened. Moreover, even if the primary tumor develops its own blood supply before adjuvant therapy with peptide β^{15-42} is begun, we predict that free peptide β^{15-42} will prevent subsequent steps required for metastatic spread and growth of breast cancers. Another advantage to this therapeutic strategy is that peptide β^{15-42} functions outside the cell, precluding the need to deliver the peptide inside cells. Identifying molecular targets for therapeutic intervention of breast cancer metastasis, recruitment of inflammatory cells and angiogenesis will increase long-term disease-free survival and improve the quality of life for breast cancer patients.

4.2 Putative mechanisms whereby nonmalignant breast epithelial cells switch to a metastatic breast cancer cell phenotype responsive to fibrinogen induced TEM

A class of molecules found in the ECM, inside cells and attached to cell surfaces, called heparan sulfate proteoglycans (HSPG), contribute to breast cancer progression by promoting cancer cell proliferation, TEM, and tumor-associated angiogenesis (Koo et al., 2008). The ability to affect any one of these functions would help to reduce breast cancer metastasis; however, if all three of the functions could be targeted with one therapeutic approach, the morbidity and mortality due to metastatic breast cancer could be significantly reduced. Heparin is widely used as an anticoagulant, but it also inhibits HSPG-dependent mechanisms of cancer metastasis (Levy-Adam et al., 2005). However, anti-metastatic heparins that also inhibit blood coagulation are, therefore, not good candidates for widespread use to treat metastatic breast cancer due to bleeding complications. Thus, another molecular target to inhibit the prometastatic effects of HSPG but not inhibit coagulation is greatly needed. Spontaneous blood-borne and lymphatic metastasis of tumor emboli requires fibrin(ogen) (Palumbo et al., 2002). In addition to binding to VE-cadherin (Yakovlev et al., 2003), Fg β^{15-42} also binds to heparin and HSPG on endothelial cells with high affinity (Odrlić et al., 1996a; Odrlić et al., 1996b); however, a role for HSPG in Fg-mediated breast cancer metastasis has not been studied. Fg binding to heparin and HSPG involves residues β^{15-42} , and β^{15-42} -dependent fibrin binding to EC surfaces can be inhibited with heparin and heparan sulfate but not with chondroitin sulfate, indicating that Fg- β^{15-42} represents a HBD (Odrlić et al., 1996a; Odrlić et al., 1996b). The Fg HBD was later mapped to residues β^{15-57} , which includes the β^{15-42} VE-cadherin binding domain (Yakovlev et al., 2003; Yakovlev & Medved, 2009). In our recent publication (Sahni et al., 2009), we unexpectedly discovered that Fg enhanced TEM of only malignant breast cancer cells (MCF-7 and MDA-MB-231) but not nonmalignant breast epithelial cells (MCF-10A), suggesting inherent differences in the ability of cancer vs. normal breast epithelial cells to interact with fibrin(ogen). Because TEM of nonmalignant epithelial cells (MCF-10A) could not be enhanced in the presence of Fg (Sahni et al., 2009), we hypothesize that loss of HSPG from the surface of premalignant breast epithelial cells serves as a molecular switch to induce a highly aggressive, metastatic breast cancer phenotype (**Fig. 11A**). We plan to investigate this hypothesis in future studies.

Another mechanism to regulate Fg-enhanced TEM of malignant breast cancer cells is a gain in function of cancer-associated Mucin-1 (MUC1), which is a membrane-associated mucin expressed at low levels on the apical surface of normal polarized epithelial cells. MUC1 is a tumor-associated glycoprotein aberrantly expressed in >90% of breast cancers (Singh & Bandyopadhyay, 2007), promotes cancer cell proliferation and metastasis, and is associated

with poor survival (Hattrup & Gendler, 2006; Yuan et al., 2007). MUC1 is upregulated and hypoglycosylated in breast cancers. The polarized expression of MUC1 is lost on cancer cells such that it is expressed on the entire cell surface (Kondo et al., 1998; Moase et al., 2001; Wesseling et al., 1996; Yang et al., 2007). The MUC1 extracellular domain protrudes ~200 nm above the cell surface, whereas most cell surface receptors are ~35 nm long (Wesseling et al., 1996). When MUC1 is interspersed between adhesion molecules, it nonspecifically reduces cell-cell and cell-ECM interactions *in vitro* and *in vivo*, likely by steric hindrance caused by the extreme length and high density of the MUC1 at the cell surface (Wesseling et al., 1996) (**Fig. 11B**). MUC1 expression is found on MCF-7, MDA-MB-231, as well as other types of breast cancer cells, particularly on those isolated from patients with a highly aggressive subtype called inflammatory breast cancer (Alpaugh et al., 2002; Schroeder et al., 2003; Walsh et al., 1999); elevated expression of MUC1 contributes to lymphovascular tumor invasion of inflammatory breast cancer cells (Alpaugh et al., 2002).

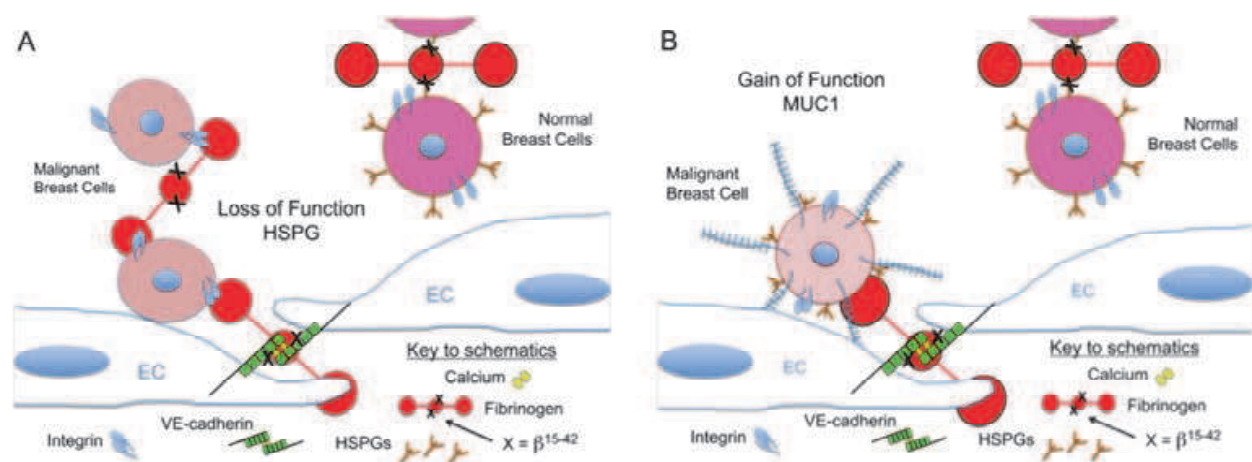


Fig. 11. Putative mechanisms whereby nonmalignant breast epithelial cells switch to a metastatic breast cancer cell phenotype responsive to fibrinogen-induced TEM. Panel A, schematic depicting loss of function due to release of cell-surface HSPG. Panel B, schematic depicting gain of function by overexpression of MUC1 leading to loss of polarity and cell-cell adhesion in breast epithelial cells.

We predict that Fg could bind to normal breast cell surface HSPG through Fg β^{15-42} , thus preventing Fg β^{15-42} binding to VE-cadherin extracellular domain 3 (EC3) and inhibition of TEM. Enhanced heparanase expression and enzymatic digestion of HSPG in human tumors correlates with metastatic potential, tumor vascularity, and reduced postoperative survival of cancer patients (Vlodavsky et al., 2008). Heparanase-induced loss of breast epithelial cell surface HSPG during conversion of non- or pre-malignant to malignant breast cancers would allow Fg β^{15-42} binding to VE-cadherin at cell-cell junctions to induce EC permeability. Fg would also bind to breast cancer cell integrins via binding sites on Fg C-terminal domains (see **Fig. 4**) then movement of VE-cadherin (induced by Fg binding to VE-cadherin as shown in **Fig. 8B**) in the endothelial cell membrane would induce paracellular transfer of Fg-bound breast cancer cells across the endothelial cell barrier to promote cancer metastasis. A precedent for this mechanism is already established; Fg binding to a counter adhesion molecule facilitates neutrophil TEM 20- to 30-fold (Languino et al., 1995). Overexpression of MUC1 could block accessibility of HSPG on breast cancer cells, which

would also prevent Fg β^{15-42} -HSPG binding interactions leaving Fg β^{15-42} available for binding to VE-cadherin. Alternatively, loss of cell surface HSPG and elevated expression of MUC1 may contribute to Fg-enhanced TEM of malignant compared to nonmalignant breast epithelial cells. These possibilities will be addressed by future experiments.

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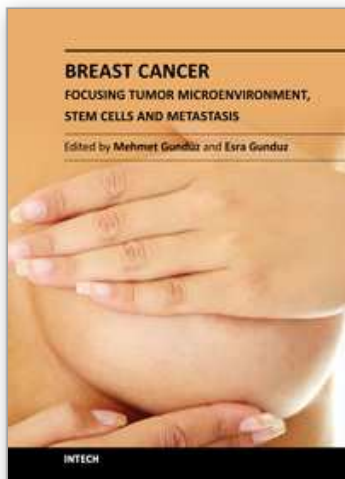
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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