

## Breast Tumor Microenvironment: Proteomics Highlights the Treatments Targeting Secretome

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Tumor secreted substances (secretome), including extracellular matrix (ECM) components, act as mediators of tumor–host communication in the breast tumor microenvironment. Proteomic analysis has emphasized the value of the secretome as a source of prospective markers and drug targets for the treatment of breast cancers. Utilizing bioinformatics, our recent studies revealed global changes in protein expression after the activation of ECM-mediated signaling in breast cancer cells. A newly designed technique integrating a capillary ultrafiltration (CUF) probe with mass spectrometry was demonstrated to dynamically sample and identify *in vivo* and pure secretome from the tumor microenvironment. Such *in vivo* profiling of breast cancer secretomes may facilitate the development of novel drugs specifically targeting secretome.

**Keywords:** tumor microenvironment • breast cancer • secretome • extracellular matrix

### 1. Introduction

Breast cancer is the most common cancer and one of the leading causes of cancer-related death in women around the world. Because mortality from breast cancer is often due to distant metastasis, there is an urgent need to identify prospective biomarkers for early detection. Breast cancer cell proliferation and invasion involve the breaching of tissue barriers and the subsequent infiltration of cells into the surrounding tissue.<sup>1</sup> The growth and progression of breast tumor cells depend not only on their malignant potential, but also on the multidirectional interactions of secreted substances (secretome), including extracellular matrix (ECM), produced by all the cell types including tumor, stroma, endothelial cells, and immune system cells within the local microenvironment. Previous evidence demonstrated that a permissive tumor microenvironment is required for successful progression and

metastasis of tumor cells.<sup>2</sup> Secretomes are a rich source of new therapeutics and drug targets, and are becoming a major focus of drug discovery programs throughout the industry.<sup>3</sup> Although many anticancer drugs targeting the secretome have been developed,<sup>4,5</sup> a comprehensive secretome for breast cancer is still needed for further target identification and development of more potent and specific anticancer drugs. The identification of proteins or peptides released into the medium of tumor cells cultured *in vitro* is the most common method for determining a tumor secretome. However, the secretory pattern of cells *in vitro* is not always the same as the *in vivo* secretome. A novel technology using capillary ultrafiltration (CUF) probes is capable of sampling the secretome *in vivo* from various tissues at different time points.<sup>6–9</sup> More importantly, this CUF probe sampling technique provides a unique modality for localized secretome sampling in the tumor microenvironment, thus, providing insight into the localized concentrations of secretome proteins that cannot be achieved via sampling from circulating blood plasma. Tumor secretomes are normally a mixture of multiple proteins and peptides released either from tumor or host cells.<sup>6,10</sup> Mass spectrometry has been employed to identify several proteins in the CUF probe-collected secretome.<sup>6–9,11</sup> Secretome ECM is a crucial component for the communication between the tumor cells and their microenvironment. In our recent research, a proteomics analysis of a peptide-derived drug mediating the integrin signaling cascade in breast cancer cells has emphasized the significance of treatments targeting the tumor microenvironment.<sup>12,13</sup>

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## 2. Breast Tumor Microenvironment and Secretome

Studies of tumor microenvironment have emphasized evaluation of the tumor as an organ-like structure with complex, dynamic cross-talk.<sup>14</sup> It is now known that tumor cells and their stroma co-evolve during tumorigenesis and progression. Stroma consists of cells, extracellular substances (secretome) including secreted proteins/petides, other molecules, and ECM. Tumor–stroma interactions in breast cancer are dynamic networks between epithelial cells and a microenvironment consisting of stromal cells that include fibroblasts, inflammatory cells, innate and adaptive immune cells, adipocytes, vasculature, glial cells, and specialized mesenchymal cells.<sup>14,15</sup> Secretome in tumor microenvironment contains the ECM, constituted by proteins, receptors, proteoglycans, and adhesive molecules as well as a milieu of secreted proteins including cytokines, chemokines, growth factors, angiogenesis factors, and proteases at its surroundings.<sup>15,16</sup> The ECM, abundantly secreted by fibroblasts, provides supportive and structural architecture to cells and tissues. Fibroblasts are the most abundant cellular component in tumor microenvironment, and the population is even greater than tumor cells in some specific cancers.<sup>17,18</sup> The tumor-associated fibroblasts (TAFs) are phenotypically and functionally mimic to fibroblasts in wound healing but different from normal epithelial fibroblasts in the same tissue but not in the microenvironment.<sup>17,19</sup> TAFs can produce high  $\alpha$ -smooth muscle actin, which allows cells to recruit into inflammation region and contract for tissue reparation.<sup>20</sup> On the other hand, fibroblast activation protein (FAP) is a type II membrane protein most prominent expressed on tumor stroma fibroblasts.<sup>17</sup> FAP may represent an early detection biomarker, and a potential therapeutic target for breast cancer treatment.<sup>18</sup> From large-scale gene expression profiles of normal breast tissue and in situ and invasive breast carcinomas, the unique CXCL12 overexpressed in tumor activated myofibroblasts and augmented their proliferation, invasion, and migration. It implied that chemokines play a key role in breast tumor progression by acting as paracrine factors.<sup>21</sup>

The inflammatory cell consists of monocytes/macrophages, neutrophils, eosinophils, mast cells, and lymphocytes which are recruited to breast tumors preferentially in necrosis and hypoxia areas.<sup>22</sup> Tumor-infiltrating immune cells are originally regarded as cytotoxic to the tumor cells; however, current findings support that such tumor-associated leukocytes can contribute to cancer initiation, proliferation, metastasis due to the immune tolerance, or suppression associated with malignant disease.<sup>23,24</sup> Of these immune cells, tumor-associated macrophages (TAMs) represent the largest population and the most multifunctional bioactivities.<sup>22</sup> Circulating monocytes are attracted to tumor microenvironment by chemokines or chemoattractants, and once they have arrived at the tumor site, they differentiate into macrophages. TAMs belong to polarized M2 (F4/80<sup>+</sup>/CD206<sup>+</sup>) macrophage population and possess little cytotoxicity to tumor cells due to their restricted NO and proinflammatory cytokine productions. TAMs produce high interleukin (IL)-4, IL-13, and glucocorticoids which are capable of tuning inflammatory stimuli and Th2 type immunity.<sup>23</sup> TAMs can also promote tumor cell proliferation, matrix remodeling, intravasation, and spread by releasing epidermal growth factor (EGF), vascular endothelial growth factor (VEGF)-C, VEGF-D, VEGF receptor 3 (VEGFR-3), IL-8, matrix metalloproteinase (MMP)2, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), vascular cell adhesion molecule (VCAM)-1, and transforming growth factor (TGF)- $\beta$ .<sup>22,24</sup> The chemokine,

CCL5, was found to play a key role in recruitment of TAMs, and CCL5 blockage inhibited breast tumor growth.<sup>25</sup>

A vast amount of secretome including cytokines and growth factors is released both by the cancer and stromal cells. The secretome may reflect a variety of pathological conditions, thus, representing a rich source of biomarkers, which play critical roles in cell adhesion, intercellular interactions, and invasion at the level of cell and the whole organism. Thus, great interest is currently focused on the characterization of secretome from isolated cells and neoplastic tissues with the intention of identifying novel biomarkers. In 2007, Mbeunkui et al. have identified the secretome from a series of isogenic MCF10 breast cancer cell lines representing different aggressiveness by liquid chromatography–mass spectrometry (MS) MS (LC-MS/MS).<sup>26</sup> The most significant changes were observed for alpha-1-antichymotrypsin and galectin-3-binding protein that were highly secreted from more malignant cell lines, yet undetected in the premalignant cell lines. Other proteins showing increasing abundance in the more aggressive cell lines included alpha-1-antitrypsin, cathepsin D, and lysyl oxidase. The results suggest the potential usefulness of the secretome for identifying prospective markers for the early detection and aggressiveness/progression of cancer.<sup>26</sup> Celis et al. have demonstrated that the tumor interstitial fluid (TIF) perfusing the tumor microenvironment in invasive ductal carcinomas of the breast is a novel and potentially highly promising source of biomarkers.<sup>27</sup> In addition to providing the global view of the TIF proteome, their findings offer the systematic search of diagnostic biomarkers and targets for therapeutic intervention as well.<sup>27</sup> Shed proteins that perfuse the tumor microenvironment play a critical role in cell–cell and cell–matrix communication and may serve as a source of low molecular mass biomarkers for breast tumor. Proteins shed by vesicles contain matrix-degrading proteinases, beta 1 integrins, cathepsins B and D, IL-1 beta, BRCA1, and fibroblast growth factor (FGF)-2. Hanash et al. have indicated that heat shock proteins occur in the plasma via a nonclassical pathway.<sup>28</sup> The member of S100 superfamily, S100A4, was shown to be involved in the metastasis process in human breast cancer. The protein is externalized by the stroma cells to the fluid that perfuses the tumor microenvironment. High expression of S100A4 seems strongly associated with poor prognosis.<sup>29</sup> For the above studies, it should be stressed that the local microenvironment may be different in various stages of the tumor reflecting intratumor and intrastroma heterogeneity, and that new and more sensitive detection technologies in combination with tissue microdissection may be necessary to gain a better understanding of the biological events taking place in the local surroundings.

The complex network of cell types associated with tumor microenvironment produces either secreted or externalized proteins. The fluids generated in tumor lysates representing the cellular events and dynamic secretory processes affected in breast cancer may allow advanced cancer therapy, as well as further our understanding of the molecular mechanisms underlying breast cancer development and progression.<sup>30,31</sup> Even though many secreted proteins still remain to be determined, the biological activities of the proteins identified so far have provided us with a glance of the biological processes taking place in the tumor microenvironment.

## 3. *In Vivo* Sampling of Secretome via Ultrafiltration

Many challenges make it difficult to obtain a comprehensive profile of tumor secretome. Identification of tumor secretome

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according to traditional methods is to obtain the released proteins/peptides from the medium of *in vitro* tumor cell culture and then analyze their properties *in vivo*. However, the data from the *in vivo* animal systems rarely matches well with those that are *in vitro*. One alternative is to homogenize and spin down the tumor masses, and then harvest the tumor secretome from supernatants. Unfortunately, tumor secretomes collected from supernatants generally are contaminated by other proteins/peptides, which leak from the homogenization-damaged tumor or host cells. Additionally, it generally requires sacrificing many animals to obtain a dynamic secretion pattern of secretome. Microdialysis and ultrafiltration are two major methods that have been applied for dynamically sampling of *in vivo* secretome.<sup>32</sup>

Microdialysis has been used for sampling growth factors<sup>33</sup> and measuring interstitial tumor 5-fluorouracil (5-FU) pharmacokinetics in breast cancer.<sup>34</sup> Microdialysis sampling depends on the passive diffusion of substances across a semipermeable hollow membrane driven by a concentration gradient.<sup>35</sup> It is a commonly used means to obtain macromolecule-free samples from the extracellular fluids, although there is a challenge for microdialysis sampling to detect peptides and proteins. Peptide/proteins have relatively larger size and smaller aqueous diffusion coefficients, resulting in a lower recovery due to mass transport limitations through the semipermeable hollow membranes.<sup>36</sup> Recently, it has been shown that the addition of affinity-based trapping agents into the microdialysis perfusion fluid considerably enhanced the relative recovery via the binding reaction of larger substances such as peptides/proteins.<sup>35</sup> Furthermore, microdialysis method has been currently used for *in vivo* sampling of cytokines and growth factors. Because of the high biological activity of most cytokines, their concentration in extracellular fluids is usually in low-picomolar concentrations. Under normal circumstances, cytokines are typically undetectable in extracellular fluids or tissues. Cytokines are highly localized secreted proteins (approximately 8–80 kDa) produced by various cell types as part of an immunological response. Elevated expression of cytokines is a sign of activation of cytokine pathways associated with inflammation or disease progression. Enzyme linked immunoabsorbent assays (ELISAs) using a 'two-site' sandwich detection assay, that is, one antibody is used to capture cytokine antigen(s) and another detection antibody, are the most broadly used methods to quantify cytokines in biological matrices because of their acceptable specificity, sensitivity, rapid turnaround time, convenience, the ease of performance, and a relatively low cost. However, a typical ELISA can only measure one cytokine at a time and requires at least 100  $\mu$ L sample volumes. It is important to note that microdialysis sampling endows with localized-sampling and thus insight into localized concentrations of cytokines that cannot be accomplished via sampling from blood plasma.<sup>37</sup> This has been recently illustrated with the IL-6 where its interstitial fluid concentration was 100-fold higher than that found in the plasma.<sup>38</sup> Previous studies demonstrated that intraperitoneal implantation of a microdialysis probe into a lipopolysaccharide-induced inflammation mouse is capable of sampling cytokines *in vivo*.<sup>36</sup> The microdialysis probe was perfused with mouse inflammation cytokine antibody coated microspheres. The use of cytokine antibody coated microspheres as a means for microdialysis sampling significantly improves the detection of cytokines including tumor necrosis factor (TNF)- $\alpha$ , and monocyte chemoattractant protein (MCP)-1 and IL-6.<sup>35</sup> Online coupling of *in vivo* mi-

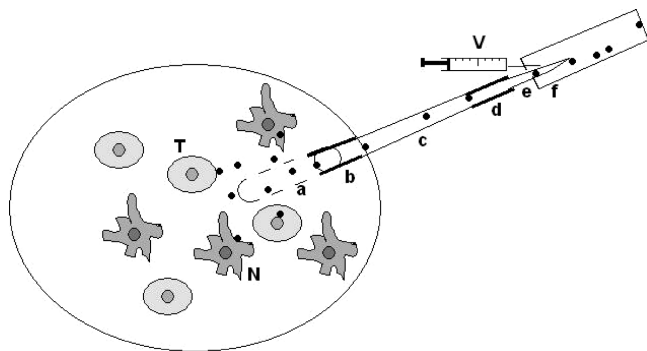
**Table 1.** Ultrafiltration Sampling

substances	references
Proteins/Peptides	6–9 and 11
Cytokines	108 and 109
Ions	32 and 42
Drugs and metabolites	110 and 111

cro-dialysis with mass spectrometry has been also demonstrated.<sup>36</sup> Jakubowski and co-workers developed a miniature hollow-fiber microdialysis device optimized for desalting small-volume neuronal samples online, with the device directly linked to a dynamic nanoelectrospray ionization assembly interfaced with an ion trap mass spectrometer.<sup>39</sup> The device using online microdialysis-dynamic nanoelectrospray mass spectrometry was able to detect acidic peptide, acidic peptide 1–24, and delta-bag cell peptide that were secreted from the peptidergic bag cell neurons of the marine mollusk, *Aplysia californica*. Although the microdialysis method has been extensively applied for *in vivo* sampling of proteins, there are still several concerns when it was employed for sampling. Primarily, fluids collected by microdialysis did not directly reflect the tissue concentration. Since perfusion fluid was injected when microdialysis was performed, the tissue concentration of the samples taken by microdialysis has to be calculated by taking into account the diffusive characteristics of the membranes, the composition of the perfusion fluid, the flow rate of fluid through the membranes, and the recovery of the samples. Furthermore, since microdialysis sampling is mainly based on diffusion of the analyte into the dialysate, sampling of proteins with larger molecular weights is always a challenge.

Like microdialysis, ultrafiltration techniques also employ semipermeable membranes to separate substances.<sup>40</sup> Substances pass through the ultrafiltration membrane by convection together with the fluids in which they are dissolved. Thus, sample concentration in the ultrafiltration-collected fluids directly reflects the tissue concentration. The ultrafiltration sampling technique applies a vacuum to semipermeable membranes to extract fluids containing secretome from the extracellular space.<sup>41–44</sup> There are several advantages of ultrafiltration sampling in comparison with *in vivo* sampling with microdialysis. First, the ultrafiltration collects a small volume sample and thus allows samples to be taken more frequently. Second, it is potentially better for *in vivo* monitoring because no dilution factor has to be considered.<sup>32</sup> In the past, ultrafiltration sampling has been utilized to collect various ions<sup>32</sup> such as calcium, potassium, and sodium to monitor glucose from subcutaneous tissue, blood, and saliva as well as to detect cytokines and drug metabolites in various biological fluids (Table 1). Recently, our laboratory linked this technology to mass spectrometry for *in vivo* sampling of peptides and proteins.<sup>6–9</sup>

A CUF probe has been newly developed in our laboratory based on the ultrafiltration technology. A semipermeable hollow membrane fiber, a key component of CUF probe, is positioned at the front of CUF probe (Figure 1) and connected to a polytetrafluoroethylene (PTFE) tube. The semipermeable hollow membrane fibers with various molecular weight cutoffs (MWCs) can be made with different surface-charged materials. One end of the semipermeable hollow membrane fiber was attached to the PTFE tubing through a small section of fused silica capillary, while the other end was sealed with epoxy. A sharpened needle was attached to the end of the PTFE



**Figure 1.** Sampling tumor secretome *in vivo* via a CUF probe. A CUF probe can be implanted into a growing tumor mass. The implanted probe collects the *in vivo* tumor secretome released either from tumor cells (T) or nontumor cells (N). The CUF probe extracts tumor secretome by using a vacuum applied to semipermeable hollow membrane fiber (a). The vacuum can be created by withdrawing a syringe from the vacutainer (V). The extraction efficiency of the CUF probe relies on the various pore sizes and surface charges of membrane fibers as well as the nature of proteins/peptides. As a general rule, large sizes of proteins/peptides are retained, while small proteins/peptides pass through the membrane. The CUF probes can be fabricated to fit the various sizes of tumor masses. The semipermeable hollow membrane fiber at the front end of the probe is entirely covered after implantation of CUF probe into a tumor mass. One end of the semipermeable hollow membrane fiber is glued with a small section of fused silica capillary (b) and attached to a PTFE tubing (c), while the other end is completely sealed with epoxy. A sharpened needle (e) is attached to the end of the PTFE ultra-microbore tube (d) and inserted into the vacutainer. *Solid dots:* tumor secretome.

ultramicrobore tube. The probe was connected to a vacutainer so that negative pressure would drive the ultrafiltration process to collect extracellular fluids. Distinct membrane fibers with a broad range of MWCO(s) and varying electro-negativities can be accommodated to CUF probes. We have implanted the CUF probes into mice and collected secretomes *in vivo* from ear skins,<sup>8</sup> skin wounds,<sup>7</sup> sodium lauryl sulfate-treated skins<sup>9</sup> and solid tumors.<sup>6</sup> In our laboratory, a regressive tumor model was used to examine the efficiency of CUF probe *in vivo* sampling. CUF probes were implanted into tumor masses 1 (progressive stage) and 3 weeks (regressive stage) after C3H/HeN mice were injected with ultraviolet (UV)-induced fibrosarcoma UV-2240 cell lines. The UV-2240 tumor cell line is highly antigenic and is routinely rejected when transplanted into normal syngeneic mice, but grows progressively in immune-deficient mice.<sup>6,45</sup> The CUF probe was implanted into the central part of tumor masses to collect *in vivo* tumor secretome for 6 h. During sample collection, the semipermeable membrane fiber in the front end of CUF probe was entirely enclosed by a tumor mass. Implantation of the CUF probe involved inserting a 22-gauge needle into the tumor mass as a guide and then feeding the probe through the needle. The needle was then removed. The CUF probe-collected secretome which contains a multiple protein/peptide mixture was digested with trypsin. Subsequently, the complex mixture of tryptic digests was directly subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and quadrupole time-of-flight (Q-TOF) MS/MS for protein identification. Five secreted proteins (cyclophilin-A, S100A4, profilin-1, thymosin beta 4 and 10), previously associated with tumor progression, were identified from tumor masses at the progressive stage.

Five secreted proteins including apolipoprotein A-I, apolipoprotein C-I, and three protease inhibitors (fetuin-A, alpha-1 antitrypsin 1–6, and contrapsin) were identified from tumor masses at the regressive stage. Although ultrafiltration separation has been applied for the *in vitro* preparation of breast tumor samples for proteomics studies,<sup>46</sup> this technology has never been used for sampling *in vivo* secretome in breast tumor microenvironments. Thus, the CUF probe may be an excellent alternative for sampling ductal lavage from breast tumors.<sup>47</sup>

The probe can be positioned in the different locations of tumor microenvironments in order to obtain a distinctive expression pattern of secretome. A tumor microenvironment is generally consisted of various types of cells and, as a whole, heterogeneous. Previous studies have revealed that the expression of many proteins was varied in the different distributions of tumor tissues.<sup>48,49</sup> Gathering CUF probe-collected secretomes from different portions of breast tumor microenvironment may be able to reflect the overall features of tumor secretions. Although various lengths of semipermeable membranes in CUF probes can be fabricated to fit in different sizes of tumor masses, the probes also can be implanted nearby tumor initiation sites where host cells interact with the early stage tumors. CUF probes have been connected to a freely moving arm system for long-term and dynamic sampling collection.<sup>9</sup> The connection will allow continuously sampling of the biomarkers at the early stages of tumor formation. The semipermeable membrane in the front end of CUF probe is a key component for probe sampling. It has been demonstrated that cellular debris and hemoglobin were predominantly collected by a CUF probe without a semipermeable membrane, leading to the failure in the protein identification by mass spectrometry.<sup>8</sup> Although implantation of CUF probes is a minimally invasive sampling method, control experiments to subtract the effect of implantation-induced minor tissue damages are required. Proteins collected by CUF probes are a complex mixture derived from secreted proteins, cell matrix proteins, as well as proteins from interstitial fluid or plasma. Incorporation of affinity-based trapping agents<sup>35</sup> into CUF probes may enhance the protein selectivity. Furthermore, the establishment of comprehensive proteomes of tumor and nontumor cells as well as plasma in the future will assist to distinguish the cell sources of secretomes collected by CUF probes.

#### 4. Tumor ECM in Microenvironment

The paradoxical role of immune system developing during tumorigenesis is increasingly clear but still needs more experimental confirmation that the neoplastic immune response consists of humoral B cells, chronic inflammatory cells, and that Th2 cells lead to tumor initiation and progression.<sup>28,50</sup> On the other hand, when cytotoxic T cells, Th1 cells, and natural killer (NK) cells are profoundly expressed in environment, it would protect against tumor development.<sup>51–53</sup> In addition, malignant areas can infiltrate regulatory T cells (Tregs) leading to immunosuppression effect on cytotoxic T cells.<sup>52,54</sup> IL-2 depleted Tregs can cause markedly immune-mediated rejection of breast cancers without concomitant lymphopenia.<sup>55,56</sup> The neoplastic cells have to avoid cytotoxic T lymphocyte rejection for survival.<sup>57</sup> This can be partially achieved by IL-10, known for blunting antitumor activity by cytotoxic T lymphocytes, secreted both by TAMs and tumor cells.

Hyaluronan, also called hyaluronic acid (HA), an abundant glycosaminoglycan in ECM, is highly overexpressed in malig-

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nant tumors and involved in enhancing these cells' motility and invasion.<sup>58</sup> HA interacts with tumor cells through its surface receptor, CD44, and hyaluronan-binding protein (RHAMM) by which it promotes tumor cell growth, survival, and migration. HA promotes tumor-associated angiogenesis likely by a mask that protects cancer cell from cytotoxic T cells and chemotherapeutic drugs.<sup>58</sup> Increased HA and hyaluronan synthase 2 expression levels can contribute to highly metastatic breast carcinomas.<sup>59</sup> In contrast, breast cancer metastasis is blocked through antisense-mediated suppression of hyaluronan synthase 2 and CD44.<sup>60,61</sup> More recently, the masking of the erythroblastic leukemia viral oncogene homologue 2 (ErbB2) in trastuzumab-resistant breast cancer are positively associated with CD44 and HA expressions. The hyaluronan synthase suppressor, 4-methylumbelliferone, greatly inhibited HA synthesis levels resulting in promotion of trastuzumab binding to ErbB2, and significant ErbB2 reduction.<sup>62</sup> Heparanase is a  $\beta$ -endoglucuronidase, which degrades heparin sulfate in the cancer cell ECM. Heparanase breaks down heparin sulfate leading to FGF2 and hepatocyte growth factor secretion, and increase breast tumor growth, invasion, and angiogenesis.<sup>63,64</sup> Osteopontin (OPN) is a phosphorylated, integrin-binding glycoprotein and commonly overexpressed in ECM of many types of cancers.<sup>65</sup> OPN, which can mediate cell–cell and cell–matrix communication promotes cell adhesion, angiogenesis, invasion, and metastasis. OPN mainly interacts with integrins through a specific RGD motif, and as well as nonintegrin receptor, CD44.<sup>66</sup> Overexpressed OPN can highly upregulate hyaluronan synthase 2 and stimulate HA production leading to metastatic breast cancer.<sup>67</sup> OPN also plays a role in recruiting macrophages, leukocytes to a tumor environment, and ECM remodeling. Overexpression of OPN is not only found in cancer cells relevant to breast, lung, colorectal, stomach, and ovary cancers and melanoma but also serum and plasma from such patients.<sup>68</sup> These lend OPN great potential to be a prognostic and therapeutic target for breast cancer and others. Tenascin-C is an ECM glycoprotein, highly expressed in most tumors and early stages of development, but much restricted in developed tissues.<sup>69,70</sup> Overexpression of tenascin-C has been correlated with tumorigenesis and angiogenesis through regulation of oncogenes, tumor suppressor genes, genome integrity genes, and evading of immune surveillance.<sup>71</sup> Tenascin-C may inactivate cell cycle checkpoint genes in tumor cell to facilitate neoplastic phenotype expansion.<sup>72</sup> Breast cancers are currently found to produce syncytin, an endogenous retroviral envelope glycoprotein, which is involved in fusions between placental trophoblasts.<sup>73,74</sup> It is generally accepted that breast cancer can spontaneously fuse with normal endothelial cells, and may abrogate the tumor or transit into more aggressive phenotype according to the net expression level of tumor suppressor genes.<sup>75</sup> Syncytin stimulates IL-1 beta and nitric oxide synthase expression, and may modulate host immune system to regulate cell apoptosis and metastasis. From current clinical data, syncytin presents a positive prognostic factor in breast cancer because it inhibits tumor cell penetration of endothelial cell hindrance.<sup>76</sup>

Because of the imbalance between cytotoxic, humoral immune system, and tumor microenvironment, the neoplastic cells could possibly evade immune-mediated elimination and may favor a more aggressive type. The ECM constituted of tumor cell is very different from the surrounding normal cell. The ECM-derivative glycoprotein may have immuno-modulating ability to make normal cell into tumor-prone type.<sup>77</sup> These

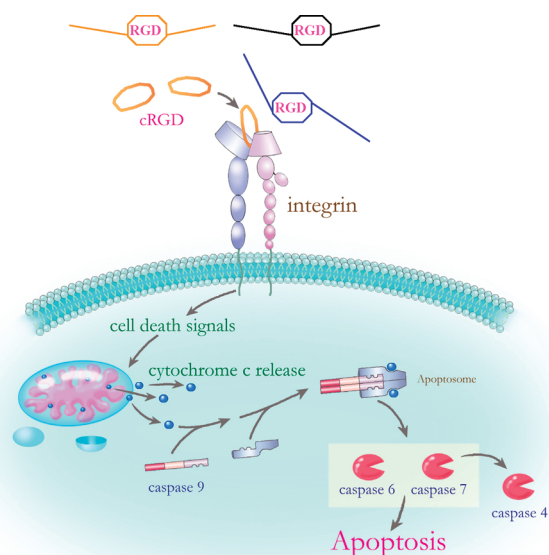
may imply that a crucial and urgent topic is how we globally understand the scientific impact of glycan or post-translational modification information of ECM protein on breast cancer tumorigenesis and metastasis.

### 5. Integrins as a Therapeutic Target in Breast Cancer Metastasis

Cell adhesive interactions play important roles during many normal physiological processes such as embryonic development and wound repair, and also during the progression of diseases such as cancer. Cell adhesion is mediated by the specific interactions of cell surface receptors with extracellular glycoproteins. The best characterized cell adhesion receptors are the integrins.<sup>78</sup> Integrins are heterodimeric cell surface receptors whose extracellular domains bind matrix proteins,<sup>79,80</sup> including laminin-1, laminin-5 (epiligrin), fibronectin, collagen, and entactin.<sup>81</sup> Functions of integrins have been shown to be involved in several important cellular processes, including cell differentiation, angiogenesis, apoptosis, cell migration, and tumor growth.<sup>82</sup> The best characterized integrin ligand is fibronectin which is a multifunctional glycoprotein comprised of three different types of homologous repeating units, type I, type II, and type III. Fibronectin has one cell adhesive region which is located near the center of the polypeptide chain in the ninth and tenth type III modules and binds to the alpha 5 beta 1 integrin. The biological function of the central cell adhesive region requires two critical amino acid sequences, an Arg-Gly-Asp (RGD) sequence and a Pro-His-Ser-Arg-Asn (PH-SRN) sequence.<sup>78</sup> Their function in synergy is for optimal binding to the alpha 5 beta 1 integrin.<sup>78</sup> Since integrin mediated proliferation,<sup>12,13,83</sup> adhesion,<sup>84</sup> migration,<sup>85</sup> and invasion<sup>86</sup> of breast cancer cells in response to ECM, targeting these integrins to modulate integrin–ECM interactions in tumor microenvironment may be a prospective approach to reduce the dissemination of breast carcinoma *in vivo*.

The tripeptide sequence RGD (Arg-Gly-Asp) is a common cell-recognition motif, which is part of integrin binding ligands, like fibronectin, fibrinogen, and vitronectin. This sequence has been used as a lead compound for developing different integrin antagonists.<sup>87</sup> RGD-containing peptides have been found to be the efficient inhibitors of integrin–ligand interactions in studies of cell adhesion, migration, growth, and differentiation. Recently, the RGD-containing peptides have been further discovered to be able to induce cell apoptosis mediated by activation of caspase-3 (an important molecule standing in the cell death pathway).<sup>88</sup> Therefore, it is highly worth to develop more potent RGD-containing peptides as drugs for cancer therapy.

In our recent research, we synthesized a cyclic RGD, (Tpa-RGDWPC, cRGD), which can mimic the RGD motif of integrin-binding ligands to bind to integrin. This binding could not induce the signal pathway involved in cell growth and proliferation as indicated by the observed growth factor receptor-bound protein 2 (GRB2) behavior. GRB2 is a component in the integrin signal cascade and was found to be down-regulated after cRGD treatment. As a result, the breast cancer cells, MCF-7, proceeded to apoptosis pathways. These findings showed that cRGD acts as an anticancer compound to decrease the proliferation of breast cancer MCF-7 cells.<sup>12</sup> On the other hand, the gene network results indicate that the cell death caused by cRGD may be triggered through activation of caspase pathway.<sup>13</sup> In Figure 2, we show the cRGD-induced apoptosis hypothetical pathway, proposed signaling pathway by which



**Figure 2.** The cRGD-induced apoptosis hypothetical pathway. The tripeptide RGD is a common domain in ECM ligands (such as fibronectin, fibrinogen, and vitronectin) for integrin binding. cRGD is a synthetic tripeptide with a similar structure with RGD in the integrin binding ligands. This is a proposed signaling pathway by which interaction of cRGD with integrin  $\alpha 5\beta 3$  in the plasma membrane leads to cancer cell apoptosis via caspase-9 activation.

interaction of cRGD with integrin  $\alpha 5\beta 3$  in the plasma membrane leads to cancer cell apoptosis via caspase-9 activation. Caspase-9, located at the upstream, activates downstream effector caspase-7. The activation of caspase-9 is believed to be a well-defined outcome of the release of mitochondrial cytochrome c into the cytoplasm and its subsequent association with the Apaf-1 protein. The assembly of cytochrome c, Apaf-1, and procaspase-9, called apoptosome, triggers the activation initiated by the Apaf-1–caspase-9 apoptosome.<sup>89</sup> These results show that RGD-containing peptides can be potential drugs for breast cancer therapy.

### 6. Drugs Targeting Tumor Microenvironment

Many studies proved the crucial role that ECM played in tumor development. Among the activation of PDGF,<sup>90</sup> TGF- $\beta$ ,<sup>91</sup> or other growth factors, precursor fibroblasts will differentiate into myofibroblasts which contribute to dense and rigid ECM network around tumors.<sup>18</sup> The remodeling microenvironment leads to the elevated interstitial fluid pressure (IFP) that reduces drug uptake of cancer cells and thus confers them chemoresistance. To enhance the efficiency of cancer therapy, some researchers designed drugs that can “normalize” IFP and increase the drug delivery to cancer cells (Table 2).<sup>92</sup> One of the strategies is blocking the activation of TAF. PDGF receptor tyrosine kinase is associated with the TAF activation and thus becomes the target of anticancer drug design.<sup>93</sup> Imatinib (ST1157),<sup>94,95</sup> the first generation drug with the PDGFR tyrosine kinase inhibitory effects, was found to decrease IFP around tumors. Imatinib-driven IFP decrease benefitted the elevated sensitivity to anticancer agents of subcutaneous growth thyroid carcinomas. Beside imatinib, other PDGF receptor inhibitors, like SU11248<sup>96</sup> and BAY43–9006,<sup>97</sup> have been developed and tested in clinic study. These drugs mentioned above have

**Table 2.** Anticancer Agents Targeting Tumor Microenvironment<sup>a</sup>

targeted cell	targeted molecule	agent
TAF	PDGFR	Imatinib (ST1157)
		SU11248
	FAP	BAY43-9006
		CDP860
TAM	VEGF	Sibrotuzumab
		DNA vaccine
	TGF- $\beta$	PT-100
		Anti-VEGF antibody
	Legumain	Fc:T $\beta$ R11
		DNA vaccine

<sup>a</sup> TAF, tumor-associated fibroblasts; TAM, tumor-associated macrophages; PDGFR, plated-derived growth factor receptors; FAP, fibroblast activation protein; VEGF, vascular endothelial growth factor; TGF- $\beta$ , transforming growth factor  $\beta$ .

multitargets, and can also block other target receptors, like c-kit. CDP860<sup>98</sup> is a polyethylene glycol conjugated Fab molecule, which binds to the PDGFR- $\beta$  more specifically and inhibits its activity. The other target of drug design is FAP.<sup>99</sup> FAP is overexpressed in tumor-associated fibroblasts including breast cancer but rarely occurs in health tissue. This property makes it an attractive candidate in cancer-directed prodrug design. Sibrotuzumab,<sup>100</sup> a monoclonal antibody directed against FAP, has been demonstrated to specifically accumulate in tumors but not in normal tissues. The conjugation of toxic agent with sibrotuzumab may enhance the efficiency of chemotherapy. Besides, FAP also functions as a serine protease which can cleave gelatin and collagen and contributes to ECM remodeling.<sup>99</sup> A DNA vaccine has been developed based on this concept. This vaccine successfully inhibited the growth of primary cancer cells and metastasis of murine colon and breast cancers.<sup>101</sup> On the other hand, the FAP protease inhibitor, (PT-100),<sup>102</sup> elevated the anticancer activity of immune system by enhancing the production of granulocyte-colony stimulating factor (G-CSF) in stromal cells. TAM also plays an important role in tumor development.<sup>25</sup> Cancer cells can make VEGF and enhance the infiltration of macrophage into tumor areas.<sup>103</sup> In cancer microenvironment, TGF- $\beta$ , secreted by cancer or stromal cells, will activate TAM to enhance the expression of VEGF, MMP-9,<sup>104</sup> and other cytokines (like IL-1 beta) and result in tumor progression. Prohibition of the migration or activation of TAM seems to be a novel approach to interfere with tumor development. Bevacizumab,<sup>103</sup> an anti-VEGF monoclonal antibody, could neutralize the tumor-derived VEGF and reduce macrophage density in xenograft human anaplastic thyroid carcinoma. Furthermore, tumor extracellular fluid volume, tumor-vessel leakage of plasma protein, and IFP were decreased concomitantly. Another agent targeted to TAM is soluble TGF- $\beta$  receptor type II-murine Fc:IgG2A chimeric protein (Fc:T $\beta$ R11).<sup>91</sup> Fc:T $\beta$ R11 could counteract TGF- $\beta$  and reverse IFP elevated by TAM. The growth inhibition effect of doxorubicin was increased significantly *in vivo*. The other strategy aims at TAM destruction. A DNA vaccine against legumain, an endopeptidase overexpressed by TAM, has been proven to emerge CD8<sup>+</sup> T cell responsive to TAM, and increase the survival rate in murine model.<sup>24</sup>

### 7. Conclusions and Perspectives

There is a dynamic interaction within the breast tumor microenvironment, where host and tumor cells compete with

each other to maximize their own survival.<sup>105</sup> Although many theories have been proposed, the mechanisms underlying the dynamic interaction between tumor and host cells has not been fully explored. The loss of surface antigens, such as major histocompatibility (MHC) class I, is an independent indicator of good prognosis in breast cancer.<sup>106</sup> It has also been well-documented that cancer patients' sera contain an impressive variety of immunosuppressive proteins, indicating that many immunosuppressive substances may be secreted from either tumor or host cells.<sup>107</sup> These substances, secreted in the tumor microenvironment, can be defined as part of the tumor secretome. Results from our current proteomics analysis provide a molecular explanation for the properties of ECM in breast cancer cells. ECM is one of components in the tumor secretome and can modulate the host immune system, thus, regulating tumor cell progression. Unraveling the tumor secretome will facilitate the development of antibreast cancer drugs specifically targeting secreted proteins/peptides. CUF probe sampling provides a promising method to dynamically obtain pure tumor secretome samples *in vivo*. More importantly, CUF probes locally sample the secretome directly in the tumor microenvironment. Mass spectrometry integrated with CUF probe sampling facilitates the secretome identification. Profiling the *in vivo* secretome within tumor microenvironments of breast cancers at different stages by CUF probes linked to mass spectrometry may be a powerful approach to identifying biomarkers for early detection.

**Abbreviations:** CUF, capillary ultrafiltration; ECM, extracellular matrix; EGF, epidermal growth factor; ELISAs, enzyme linked immunoabsorbent assays; ErbB2, erythroblastic leukemia viral oncogene homologue 2; FAP, fibroblast activation protein; FGF, fibroblast growth factor; HA, hyaluronic acid; 5-FU, 5-fluorouracil; G-CSF, granulocyte-colony stimulating factor; GRB2, growth factor receptor-bound protein 2; HGF, hepatocyte growth factor; IFP, interstitial fluid pressure; IL, interleukin; LC, liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight MS; MCP, monocyte chemoattractant protein; MHC, major histocompatibility; MMP, matrix metalloproteinases; MS, mass spectrometry; MWCs, molecular weight cutoffs; NK, natural killer; OPN, osteopontin; PDGF, platelet-derived growth factor; PTFE, polytetrafluoroethylene; Q-TOF MS/MS, quadrupole time of flight MS/MS; RHAMM, hyaluronan-binding protein; TAFs, tumor-associated fibroblasts; TAMs, tumor-associated macrophages; TGF, transforming growth factor; TIF, tumor interstitial fluid; Tregs, regulatory T cells; TNF, tumor necrosis factor; UV, ultraviolet; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

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