Microdialysis Combined with Proteomics for Protein Identification in Breast Tumor Microenvironment In Vivo

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Abstract Tumor microenvironment constitutes a reservoir for proteins released from tumor cells and the host, which can contribute significantly to tumor growth and invasion. This study aims to apply a method of combining in vivo microdialysis and proteomics to identify proteins in mammary tumor interstitial fluids, a major component of tumor microenvironment. In vivo microdialysis was performed in polyomavirus middle T antigen (PyVmT) transgenic mouse mammary tumors and age-matched control wild-type mammary glands. Over four hundred proteins were identified from the microdialysis perfusates, using the Multidimensional Protein Identification Technology. Osteopontin (OPN) is one of the proteins overexpressed in breast tumor perfusates, as confirmed with immunoassays. OPN was also found to be present in tumor-associated stroma in

both PyVmT and human breast tumors, using immuno-histochemistry. Specifically, fibroblasts were further shown to express OPN at both mRNA and protein levels. In vitro assays showed that OPN can stimulate PyVmT breast carcinoma cell proliferation and migration. Finally, the expression of OPN was significantly higher in the peripheral blood of mice bearing breast tumors, compared to wild-type mice. Overall, microdialysis combined with proteomics is a unique technique for identifying proteins in a tumor microenvironment in vivo. Mammary fibroblasts can secrete OPN, and its overexpression in mammary tumor microenvironment may contribute significantly to mammary tumor progression.

Keywords Microdialysis · Tumor microenvironment · Proteomics · Osteopontin · Fibroblasts

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Abbreviations

CM	Conditioned media
ECM	Extracellular matrix
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
MIF	Macrophage migration inhibitory factor
MMTV-PyVmT	Mouse mammary tumor virus-polyomavirus middle T antigen
MudPIT	Multidimensional protein identification technology
MW	Molecular weight
OPN	Osteopontin
SPARC	Secreted acidic cysteine rich glycoprotein
TIMP2	Tissue inhibitor of metalloproteinase 2
TSP-1	Thrombospondin 1
uPA	Urokinase-type plasminogen activator

Introduction

Various cells in the tumor microenvironment—such as fibroblasts, inflammatory cells, and vascular cells—contribute significantly to tumor growth, invasion, and metastasis [1, 2]. Tumor associated stromal cells show increased angiogenesis, accelerated proliferation, altered extracellular matrix (ECM), and amplified inflammatory cell recruitment [3]. A better understanding of the proteins presented in tumor microenvironment will advance our understanding of tumor-host interactions. Furthermore, various proteins and cells present in the tumor microenvironment have been suggested as drug targets for cancer treatment [4, 5]. However, the complex tumor microenvironment is nearly impossible to reproduce *in vitro*. A method that can systematically characterize the protein profiles in a tumor microenvironment *in vivo* will significantly improve our understanding of roles that tumor microenvironment exerts in tumor cell progression.

In vivo Microdialysis is an attractive technique for collecting soluble proteins from tumor interstitial fluids, a major component of tumor microenvironment. Microdialysis was introduced as a technique for obtaining molecules from interstitial fluid in the extracellular space of tissues [6]. A microdialysis probe mimics a blood vessel. A perfusion fluid, such as physiological saline solution, is pumped through the inlet lumen and transported through the outlet lumen, carrying the substance collected in the extracellular space. The probe consists of a double lumen catheter and a semipermeable membrane. Proteins present in the tissue interstitial fluid diffuse into the probe due to osmotic pressure. The dialysate leaving the probe reflects the chemical composition of the interstitial space of the tissue. Traditionally, *in vivo* microdialysis has been primarily used

to analyze brain functions and neurotransmitters [7]. It also plays important roles in the study of pharmacokinetic-pharmacodynamic properties of drugs [8]. With the development of probes that can collect large molecules, microdialysis has been used to collect proteins from tissue interstitial fluid, in both live animals and humans, at various organ sites [9]. Probe implantation into peripheral tissues is a minimally invasive procedure, typically with no evidence of tissue edema [10]. In breast cancer studies, microdialysis has been used to measure changes in VEGF expression in the breast tumor extracellular space, and in response to treatments with estradiol [11]. Similarly, lysosomal protease Cathepsin D expression levels in the breast tumor environment were measured, and expression was shown to be inhibited by introduction of tamoxifen [12]. Recently, IL-8 was successfully collected from human breast tissues, using microdialysis, and findings suggested that estradiol increases IL-8 secretion *in vivo* [13].

In addition to examining individual target protein changes in tumor microdialysis perfusates, high throughput identification of the tumor microenvironment protein profile using proteomic techniques is possible. This potentially provides a means of identifying proteins not previously known to have important functions in a tumor microenvironment. Liquid chromatography coupled with mass spectrometry offers a powerful technique for high-throughput protein identification. Multidimensional protein identification technology (MudPIT) [14], which involves peptide separation by strong cation exchange followed by reverse phase chromatography, has been successfully used in various proteomics studies to identify the proteins in complex biological mixtures [15, 16].

In this study, our goal is to combine microdialysis and MudPIT proteomic technologies to identify the proteins in the mammary tumor microenvironment that may promote tumor cells progression. *In vivo* microdialysis experiments were performed in mouse mammary tumor virus-polyomavirus middle T antigen (MMTV-PyVmT) transgenic mouse mammary tumors and wild-type control mouse mammary glands. MudPIT proteomics and immunoassays were used to identify and verify the proteins present in the microdialysis perfusates. Osteopontin (OPN) was further analyzed to study its roles in tumor progression and potential translational applications.

Method

In Vivo Microdialysis in Mouse Mammary Gland and Tumor

In vivo microdialysis experiments were performed on 12 week-old MMTV-PyVmT transgenic mice [17] and

age-matched wild-type control mice in FVB background. The mice were anesthetized using isoflurane, and a heating pad was used to maintain body temperature. A small incision was made on the mammary tumor or normal mammary gland. A microdialysis probe with 100,000 Da molecular weight cut-off (CMA, Stockholm, Sweden) was inserted into the mouse normal mammary gland or mammary tumor, using a guided cannula. The length of the microdialysis probe used in this study was 4 mm. Once the probe was in position, the cannula was withdrawn leaving the probe completely embedded in either the tumor tissue or normal mammary gland. Sterile physiologic salt solution containing 147 mM Na⁺, 4 mM K⁺, 2.3 mM Ca²⁺, and 156 mM Cl⁻ was perfused through the probe at a flow rate of 1.5 µl/min. After a 30 min equilibration period, the outgoing perfusate was collected on ice for 4 h. After the experiment, the mice were sacrificed and sera were obtained. The normal mammary gland or mammary tumors bearing microdialysis probes were dissected and fixed for sectioning.

Analysis of the Microdialysis Perfusates Using MudPIT and Multiplex Immunoassay

The high-throughput protein identification technique, MudPIT, was used to analyze the microdialysis perfusates from both MMTV-PyVmT mammary tumors and control mammary glands. The protein concentrations of the microdialysis perfusates were determined using the micro BCA assay (Pierce, Rockford, IL) according to the manufacture's instruction. For MudPIT analysis, 50 µg of protein was obtained by combining the microdialysis perfusates from six mice in either PyVmT or Wild-type groups. (8.3 µg of protein was obtained from each individual mouse.) The protein mixtures were digested using trypsin as previously described [18]. The tryptic peptides were desalted using a reverse-phase C18 trap (Michrom Bioresources, Auburn, CA). The tryptic peptides were then loaded directly onto a microcapillary (100 µm i.d.) column that was packed with 3 cm of 5 µm strong cation exchange material (Partisphere, Whatman, UK) followed by 2 cm of C18 reverse-phase material (Synergi 4u Hydro RP80a, Phenomenex, Torrance, CA). Samples were eluted with a flow rate at 0.5 µl/min with an automated 6 cycle multidimensional chromatographic separation as previously described [19]. The eluted peptides were directly analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) using a linear ion trap LTQ mass spectrometer (Thermo Finnigan, San Jose, CA). During the gradient, eluting ions were analyzed by one full precursor MS scan (400–2,000 m/z) followed by five MS/MS scans on the five most abundant ions detected in the precursor MS scan while operating under dynamic exclusion.

The program *extractms2* was used to generate the ASCII peak list and identify +1 or multiply charged precursor ions from the native mass spectrometry data file. Tandem spectra were searched using SEQUEST-PVM algorithm [20, 21] against the Refseq mouse protein database (Released May 2005, 26,304 entries). Only fully tryptic peptides were considered as candidates. Due to the SEQUEST algorithm has an internal non-zero minimum fragment ion tolerance based on binning peaks to approximate unit Da bins, 0.0 Da fragment ion mass tolerance was selected. This 0.0 Da mass tolerance corresponded to a tolerance of 0.5 Da for fragment ion due to the build-in unit mass binning. Cysteine residues were set as fixed modification for carbamidomethylation, and methionine residues were set as a variable modification for oxidation. The SEQUEST output was consolidated into hypertext files and analyzed using the PeptideProphet (version TPP v4.2) [22] and ProteinProphet programs (version TPP v4.2) [23]. These programs calculate probability values to the peptide and protein identification, indicating the likelihood that the respective peptide or protein has been positively identified. A 95% identification probability cutoff corresponding to an estimated false positive rate of 0.5% was selected based on the ProteinProphet algorithm. A minimum of two unique peptides were further required for each protein identification.

In addition, the microdialysis perfusates and sera collected from MMTV-PyVmT mice and wild-type mice were analyzed using the Luminex-based multiplex immunoassay with RodentMAP® antigens by Rules-Based Medicine (RBM, Austin, Texas) as previously described [24, 25].

OPN Expression in Mammary Tumor Tissue and Cultured Fibroblast Cells

Immunohistochemistry of OPN was performed on mouse and human breast cancer tissues. Paraffin-embedded control normal mammary glands, PyVmT mouse mammary tumor and human breast carcinoma sections were cut at 5-µm, deparaffinized in xylene and processed in alcohol. Antigen retrieval was performed in citrate buffer, pH 6.0 using a microwave oven. The sections were treated with 3% H₂O₂ followed by 4°C overnight incubation with primary anti-mouse OPN antibody (1:50) (American Research Products, Belmont, MA) or primary anti-human OPN antibody (1:1000) (Sigma, St. Louis, MO). After application of secondary antibodies, the Vectastain ABC staining technique was performed as suggested by the manufacturer (Vector Laboratories, Burlingame, CA). The slides were counterstained with hematoxylin and coverslipped.

The expression of OPN was also analyzed in mouse and human mammary fibroblast and carcinoma cells. Human

breast tissue samples were obtained from women who had reduction mammoplasty. Approval to remove tissue from pathology samples was granted by the Vanderbilt University Medical Center Institutional Review Board. All patients provided written informed consent. In addition, normal mouse mammary glands were obtained. Fibroblast cultures were prepared by chopping tissues, followed by digestion in 1% collagenase plus 175 mg/ml hyaluronidase in DMEM (Dulbecco's Modified Eagle Media)/F12 for 2–4 h. Cells were centrifuged and enzyme was washed away by addition of normal media and repeated centrifugation. Human fibroblasts were cultured in DMEM plus 10% iron-rich calf serum. Mouse fibroblasts were cultured in DMEM/F12 plus 5% adult bovine serum and 10% fetal bovine serum. All cells used were from the first 10 passages after initial culture. Fibroblasts prepared by this method express the fibroblast markers vimentin, smooth muscle actin and N-cadherin, but lack expression of the epithelial markers cytokeratin 14, cytokeratin 18 and E-cadherin by RT-PCR analysis (data not shown). The human carcinoma cells used in this study were previously described elsewhere [26, 27].

Cells grown in their normal media were plated 20,000 cells per well in normal media in 24-well plates. After 12 h for cell attachment, media was replaced with serum-free DMEM/F12 for 24 h. Conditioned media was collected and frozen at -80°C . RNA was isolated from adherent cells after conditioned media was collected. The correlation of OPN expression at protein and mRNA level in mammary fibroblasts was first analyzed. After 24 h incubation, serum free conditioned media (CM) were obtained from the primary mammary fibroblast cell lines, which were isolated from FVB mice as previously described [28]. The CM was filtered with a $0.2\ \mu\text{m}$ filter and the expression levels of OPN were determined using OPN ELISA kit (R&D System, Minneapolis, MN). RNA was isolated from confluent cells using RNeasy mini kit (Qiagen, Valencia, CA) with optional on-column DNase digestion to eliminate contaminating DNA. One microgram of total RNA was reverse transcribed to generate cDNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Relative cDNA quantity was determined by real-time RT-PCR using iCycler instrumentation and software (BioRad, Hercules, CA) using Sybr green detection (Applied Biosystems, Foster City, CA). Primer sequences: mGAPDH-for AGAA CATCATCCCTGCATCC, mGAPDH-rev CACATTGGGG GTAGGAACAC, mOsteopontin-for TTCCAAGAGAGC CAGGAGA, mOsteopontin-rev TTGTGGCTCTGA TGTTCCAG, hGAPDH-for CCACATCGCTCAGACACC AT, hGAPDH-rev GCAACAATATCCACTTTACCAGAG TTAA, hOsteopontin-for TTGCAGTGATTGCTTTTGC, hOsteopontin-rev GCCACAGCATCTGGGTATTT. Primer sets whose names start with 'm' are mouse-specific, while

primer sets whose names start with 'h' are human-specific. All sequences are listed in 5' to 3' orientation. Reaction conditions: enzyme activation for 10 min at 95°C , followed by denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C repeated for 50 cycles.

PyVmT Tumor Cell Proliferation Assay and Wound Closure Assays with OPN Treatment

PyVmT carcinoma cells growing in DMEM supplemented with 10% FBS were plated at a density of 2×10^3 in 96-well plates. After reaching 80% confluency, the cells were washed with PBS, and the standard media was replaced with 100 μl of DMEM with 0.5% FBS containing recombinant OPN (R&D Systems) at concentrations of 1 ng/ml, 5 ng/ml, 10 ng/ml and 100 ng/ml. The cells were incubated in the presence of OPN for 48 h. WST-1 proliferation assays (Roche, Indianapolis, IN) were performed according to manufacturer instructions. PyVmT carcinoma cell migration assays were performed in 6-well plates. After the cells reached 100% confluency, scratch-wounds (1 mm width) were created. The cells were washed and incubated with 2 ml of serum-free culture medium with OPN at the following concentrations: 0 ng/mL, 1 ng/mL, 5 ng/mL, and 10 ng/mL. Wound closure was measured at 0 h, 8 h, 24 h, 30 h and 48 h time-points. The images of the wound closures were calculated at each time point. Two-sided Student's T test was performed to determine the differences in PyVmT carcinoma cell proliferation and migration with varying OPN dosage.

Bioinformatics Analysis

The cellular compartmental protein analysis was performed by searching the Gene Ontology database using the Gene Set Analysis Toolkit 2.0 [29] and the RESNET5 Database using Pathway Studio software (Ariadne, Rockville, MD) [30].

Results

Collection of Proteins from Tissue Interstitial Fluids Using In Vivo Microdialysis

Given the heterogeneity of mammary tumors, it is important to be able to examine the tumor regions where the microdialysis probe was inserted. Figure 1 shows microdialysis probes inserted into normal mammary gland (Fig. 1a) and mammary tumor (Fig. 1b). The probe in the normal mammary gland was embedded in the mammary fat pad, while care was taken to maintain the integrity of the peritoneal cavity. For the mammary tumor, the probe was

Fig. 1 The microdialysis probes embedded in tissue. **a–b** The microdialysis probes in the normal mammary fat pad and solid mammary tumor, respectively. **c–d** H & E stained sections demonstrate that microdialysis probes were adjacent to normal mammary gland adipose cells or mammary tumor cells, respectively

inserted into a solid tumor mass. In addition, hematoxylin and eosin stained sections were obtained to further verify that microdialysis probes were properly positioned within a normal mammary gland (Fig. 1c) or mammary tumor (Fig. 1d). The microdialysis probes' semipermeable membranes were found to be adjacent to either normal adipose cells or tumor cells.

Identification of the Proteins in Microdialysis Perfusates Using MudPIT

To further identify the proteins present in the microdialysis perfusates, MudPIT, which is based on multidimensional liquid chromatography—tandem mass spectrometry, was used to analyze the protein tryptic digests from microdialysis perfusates. A total of 99 proteins were identified in both groups, while 297 and 45 proteins were only identified in PyVmT mammary tumor and wild-type groups, respectively (Fig. 2a). The proteins identified in tumor or normal mammary gland microdialysis perfusates are listed in Supplementary Table 1 and 2, respectively. The proteins collected using microdialysis must pass through a semipermeable membrane and the protein sizes and charges play important roles. Therefore, the distribution of the theoretical molecular weight (MW) and charge of the identified proteins were examined. Figure 2b indicates the distribution

of the theoretical MWs for all of the identified proteins. The number of proteins decreases as the MW increases, consistent with the fact that the larger the proteins are, the more difficult it is for them to pass through the semipermeable membrane. However, the protein MW was calculated based on theoretical sequences for intact proteins. The actual protein MWs in tissue may be different due to protease cleavage and potential post-translational modifications. Figure 2c shows the distribution of theoretical isoelectric points of the identified proteins, indicating that proteins with pI between 5 and 10 can generally pass through the membrane. Bioinformatic analysis was performed to examine the cellular component of the proteins identified in the microdialysis samples. The cellular distribution for all of the identified proteins is shown in Fig. 2d. Although proteins from various cellular components were observed, extracellular proteins constitute a major component of the identified proteins. This result indicates that microdialysis technique may effectively collect soluble proteins in extracellular space.

To further confirm some of the differentially expressed proteins, Luminex-based immunoassays were performed. Clusterin, Cystatin-C, and OPN were only identified in the tumor microdialysis perfusates but not in the normal mammary gland perfusates, using the MudPIT approach. Using immunoassay, these three proteins were further

Fig. 2 Protein identification from microdialysis perfusates and related bioinformatic analyses. **a** the number of proteins identified from normal mammary gland and tumor microdialysis perfusates. **b** The distribution

of the theoretical molecular weight of the identified proteins. **c** The distribution of the theoretical isoelectrical points of the identified proteins. **d** The potential cellular distribution of the identified proteins

found to be presented at higher levels in the tumor microdialysis perfusates compared with normal mammary gland microdialysis perfusates ($p < 0.005$) (Fig. 3a–c), which is consistent with the MudPIT results.

OPN Expression in Mouse and Human Mammary Carcinoma and Fibroblast Cell Lines

As one of the proteins confirmed to be overexpressed in the tumor microdialysis perfusates, OPN was further analyzed to determine specifically which cells in mammary tumor microenvironment express it. Immunohistochemistry for OPN was performed in wild-type mammary gland, PyVmT breast tumor and human breast cancer. OPN was mainly found in the lumen of the ducts in normal mammary gland but not in the adipose tissue (Fig. 4a). OPN was found to be presented in both tumor cells and the tumor associated

stroma, in both PyVmT (Fig. 4b) and human breast tumor tissues (Fig. 4c), indicating that mammary fibroblasts may express OPN in addition to the tumor cells.

To further confirm that fibroblasts express OPN, various mouse and human mammary fibroblasts were analyzed. First, OPN mRNA expression and protein expression were found to be positively correlated, by analyzing ten different primary mouse fibroblasts isolated from mouse mammary glands (Fig. 5a). In addition, OPN mRNA expression was found in both primary mouse PyVmT carcinoma cell lines as well as in primary mouse fibroblast cell lines (Fig. 5b). OPN mRNA expression in human carcinoma cell lines as well as human fibroblast cell lines was also examined. Although both cell lines expressed OPN mRNA, the human fibroblasts expressed higher levels of OPN mRNA. Two different cell lines were tested in each group, as shown in Fig. 5b and c.

Fig. 3 Confirmation of the differentially expressed proteins, clusterin, cystatin-C and osteopontin, using Luminex based immunoassay. Microdialysis perfusates obtained from mammary tumor and normal

mammary glands were analyzed and significant differences in the protein expressions were observed ($p < 0.005$)

OPN Stimulates PyVmT Carcinoma Cells Proliferation and Migration

To investigate the effect of OPN on tumor progression, different dosages of recombinant OPN protein were used to treat PyVmT carcinoma cells. The proliferation and migration rates of PyVmT cells responding to different doses of OPN were analyzed using WST-1 proliferation assay and wound closure assay, respectively. OPN was found to directly stimulate PyVmT cell proliferation ($p < 0.001$) as well as migration ($p < 0.001$) (Fig. 6a–b). Both cell proliferation and migration showed consistent increase with escalating doses of OPN. As OPN is present in tumor extracellular microenvironment, it is likely to enter into blood circulation. OPN expression levels in sera from PyVmT and wild-type control mice were analyzed. Figure 6c shows that serum OPN levels were significantly higher in the PyVmT mice, compared with controls,

suggesting the potential of OPN as a blood-based protein biomarker candidate for breast cancer.

Discussion

In this study, the combination of in vivo microdialysis and proteomics is shown to be an effective method for identifying the proteins in a breast tumor microenvironment in a high throughput manner. Over 400 proteins were identified from breast tumor and normal mammary gland microdialysis perfusates. The expression levels of OPN in tissue and its effects on PyVmT carcinoma cell growth and migration were analyzed. Sera OPN levels were found to be significantly higher in PyVmT mice with mammary tumor, compared to non-tumor controls.

Availability of an effective method to analyze and characterize protein profiles in a tumor microenvironment

Fig. 4 Immunohistochemistry of OPN in wild-type mouse mammary gland, PyVmT breast tumor and human breast cancer. **a** OPN was mainly found in the lumen of the ducts in normal mammary gland but

not in the adipose tissue. **b** OPN was found to be presented in both PyVmT carcinoma cells and the tumor associated stroma. **c** OPN was found in human breast cancer associated stroma

Fig. 5 Examination of OPN mRNA in mouse and human breast cancer and fibroblast cell lines. **a** A strong correlation was observed between mRNA- and protein-level expression of OPN in multiple primary mouse fibroblasts. **b** Both primary mouse PyVmT ($N=2$) and

fibroblasts cell lines ($N=2$) express OPN mRNA. **c** OPN mRNA was expressed at higher levels in human breast fibroblast cell lines ($N=2$) compared with carcinoma cell lines ($N=2$)

in vivo will significantly improve our understanding of roles that tumor microenvironment exerts in tumor cells progression. In addition to microdialysis, other approaches for collecting soluble proteins from normal breast and breast tumor microenvironment have been reported, such as obtaining ductal lavage or nipple aspirate fluid samples [31–33]. Ductal lavage and nipple aspirate fluid samples have been routinely obtained from human subjects. The minimally invasive ductal lavage and non-invasive nipple aspirate fluid procedures allow analysis of molecular profiles from the local intraductal microenvironment in both healthy female and breast cancer patients [32, 34–37]. Compared to these two methods, microdialysis technique has the advantage of collecting proteins from specifically targeted tumor regions. It is well known that wide heterogeneity exists within mammary tumors. Different patterns of molecular profiles could be obtained by position-

ing microdialysis probes semipermeable membranes adjacent to different tumor regions, such as normal adipose cells or tumor cells. Different proteins that are involved in various functions in normal ECM or adipose tissue and breast tumor extracellular space, including ECM proteins, proteases, hormones, cytokines and signaling molecules, could be obtained using microdialysis technique.

Microdialysis is considered as a minimally invasive procedure and has also been successfully performed in a variety of human tissues, such as normal breast tissue and breast tumors [13], pelvic peritoneum [38], flap reconstructions [39], abdominal adipose tissue [40], brain tumors [41], forearm skin [42], and renal tissue [43]. Microdialysis combined with proteomics will likely be performed in human breast cancer in the future, yielding complementary molecular profiles comparing these results with those obtained from ductal lavage and nipple aspirate fluid

Fig. 6 **a** OPN stimulates PyVmT carcinoma cells proliferation using WST-1 assay. **b** OPN stimulates PyVmT carcinoma cells migration using wound closure assay. **c** The sera OPN protein expression in

PyVmT-tumor bearing mice ($N=5$) is significantly higher than its expression in the sera from wild-type mice ($N=5$)

samples. The identified up-regulated or down-regulated molecules may significantly improve our understanding of the paracrine and autocrine protein interactions in breast tumor microenvironment, not only in mouse models but also in vivo in human subjects.

Proteins involved in various functions in the breast tumor extracellular space were identified, such as extracellular matrix proteins, proteases, cytokines and signaling molecules. Among the identified ECM proteins, thrombospondin 1 (TSP-1) is known to be overexpressed in human breast cancer tissue [44], and TSP-1 has been shown to promote various cancer cells' adhesion, invasion, and metastasis [45–47]. Other identified ECM proteins, such as fibronectin 1, collagens, secreted acidic cysteine rich glycoprotein (SPARC), nidogen 2, and decorin, are also known to be associated with breast cancer development [48–54]. Multiple proteases and protease inhibitors were identified in the tumor perfusates, such as cathepsins B, D, L, and S and cystatin C. Cathepsin D expression is increased in human breast cancer and can increase breast cancer cell proliferation and metastasis [55, 56]. Cathepsin B and cystatin C are also expressed in human breast cancers [57], and cathepsin B expression is related to the invasiveness of transformed human breast epithelial cell lines [58]. Other proteases and protease inhibitors, such as urokinase-type plasminogen activator (uPA) and tissue inhibitor of metalloproteinase 2 (TIMP2), are known to regulate breast tumor invasion and metastasis, and are suggested to have prognostic value [59, 60]. Among cytokines identified in this study, macrophage migration inhibitory factor (MIF) is known to exert pleiotropic functions including control of cell proliferation and differentiation [61]. Overexpression of MIF is observed in human breast cancer and is suggested to induce angiogenesis [62]. Various anti-MIF approaches have been suggested for cancer treatment [63]. S100A8 and S100A9 have been shown to play important roles in the tumor microenvironment as chemo-attractants for recruiting myeloid cells in the pre-metastatic lung [64]. In addition, many proteins with previously less known functions in breast tumor growth and metastasis were also identified. Further study of these proteins may significantly improve our understanding of the paracrine and autocrine protein interactions in breast tumor microenvironment.

One of the identified proteins, OPN, was shown by both “shotgun” proteomics and immunoassay to be more highly expressed in mammary tumor. Our study further demonstrated that mammary fibroblasts express OPN, both in tissue and in primary fibroblast cells. However, the microdialysis fluid contains a pool of proteins originated from several sources, including epithelial cells, stromal cells, endothelial cells and resident white blood cells, and any or all of these sources may secrete OPN. In fact, it has been reported that breast epithelial cells and activated immune

cells could express OPN [65, 66]. Therefore, OPN from different components in the breast microenvironment may contribute to tumor growth and invasion via different autocrine and paracrine signaling pathways.

As a secreted phosphorylated glycoprotein, OPN is rich in aspartate and sialic-acid residues [67]. OPN binds to integrins, particularly $\alpha_v\beta_3$, and to CD44 to mediate its signaling pathways [68–70]. OPN was shown in our study to directly stimulate PyVmT breast carcinoma cell proliferation and migration, suggesting mammary fibroblasts can directly contribute to the mammary tumor cells' growth and invasion via this paracrine signaling pathway. Various methods for inhibiting OPN in order to control tumor growth have been proposed. For example, Agelastatin A, a naturally occurring oroidin alkaloid, has been shown to inhibit OPN-mediated adhesion, invasion, and colony formation for human breast cancer cells [71]. Reduced in vivo tumorigenicity of osteosarcoma cells has been observed by blocking OPN mRNA translation via antisense human OPN RNA expression vector [72]. The growth inhibitory effect of human OPN antibody has also been reported using human prostate cancer cell lines [73]. All of these reports are consistent with findings that OPN acts as a paracrine and autocrine mediator of cancer growth and progression, which warrants further identification of safe and effective OPN inhibitors in controlling tumor growth [74].

Microdialysis combined with proteomics may present a unique strategy for identification and detection of potential blood-based cancer protein biomarker candidates. In this study, OPN was initially identified as being overexpressed in tumor microdialysis perfusates. Accordingly, OPN was found to be significantly higher in the sera of PyVmT mice when compared to controls. For women with metastatic breast cancer, higher expression of plasma OPN (>123 ng/mL) was also found to be significantly associated with poor prognosis [75]. Furthermore, other proteins identified in the tumor microdialysis perfusates, such as cathepsin B and cystatin C, have been suggested as prognostic biomarkers in the sera of patients with early-stage and inflammatory breast cancer [76]. Another protein identified in the tumor microdialysis perfusates, TSP-1, was suggested as a plasma biomarker associated with breast cancer aggressiveness. Women with lymph node metastases from breast cancer had significantly higher levels of circulating TSP-1 in plasma than women with breast cancer who were node negative [77].

In conclusion, microdialysis combined with proteomics provides a unique approach to detecting the complex protein profiles found in breast tumor microenvironment in vivo. Proteins released from both tumor cells and the host microenvironment can be collected and identified in a high throughput manner, which will increase our

knowledge on tumor microenvironment and the potential roles it plays in tumor progression.

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