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Identification of Differentially Secreted Biomarkers using LC-MS/MS in Isogenic Cell Lines Representing a Progression of Breast Cancer

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Abstract

Proteins secreted (the secretome) from cancer cells are potentially useful as biomarkers of the disease. Using LC-MS/MS, the secreted proteomes from a series of isogenic breast cancer cell lines varying in aggressiveness were analyzed by mass spectrometry: non-tumorigenic MCF10A, premalignant/tumorigenic MCF10AT, tumorigenic/locally invasive MCF10 DCIS.com and tumorigenic/metastatic MCF 10CA cl. D. Proteomes were obtained from conditioned serum-free media, partially fractionated using a small reverse phase C2 column and digested with trypsin for analysis by LC-MS/MS, using a method previously shown to give highly enriched secreted proteomes (Mbeunkui, et. al., J. Prot. Res. 5, 899–906 2006). The search files produced from 5 analyses (3 separate preparations) were combined for database searching (Mascot) which produced a list of over 250 proteins from each cell line. The aim was to discover highly secreted proteins which changed significantly in abundance corresponding with aggressiveness. The most apparent changes were observed for alpha-1-antichymotrypsin and galectin-3-binding protein which were highly secreted proteins from MCF10 DCIS.com and MCF10CA cl. D, yet undetected in the MCF10A and MCF10AT cell lines. Other proteins showing increasing abundance in the more aggressive cell lines included alpha-1-antitrypsin, cathepsin D and lysyl oxidase. The S100 proteins, often associated with metastasis, showed variable changes in abundance. While the cytosolic proteins were low (e.g. actin and tubulin), there was significant secretion of proteins often associated with the cytoplasm. These proteins were all predicted as products of non-classical secretion (SecretomeIP, Center for Biological Sequence Analysis). The LC-MS/MS results were verified for five selected proteins by western blot analysis, and the relevance of other significant proteins is discussed. Comparisons with two other aggressive breast cancer cell lines are included and the protein with consistent association with aggressiveness in all lines, and in unrelated cancer cells, was the galectin-3-binding protein which has been associated with breast, prostate and colon cancer earlier, supporting the approach and findings. This analysis of an isogenic series of cell lines suggests the potential usefulness of the secretome for identifying prospective markers for the early detection and aggressiveness/progression of cancer.

Keywords

secretome; proteomics; secreted proteins; breast cancer; MCF10; alpha-1-antichymotrypsin; galectin-3-binding protein; mass spectrometry; LC-MS/MS; non-classical secretion

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1. Introduction

Breast cancer is one of the leading causes of cancer-related death in women throughout the world [1]. Early detection and prevention of this disease is urgently needed because many patients succumb to advanced disease as the primary tumor metastasizes to other organs. The prediction of the biological behavior of a tumor and the eradication of the disseminated malignancy remain among the most important clinical challenges in oncology. During breast cancer metastasis, cell-cell interactions are decreased leading to cell detachment from the primary tumor, whereas cell-extracellular matrix interactions are increased, thus facilitating migration and invasion [2]. Therefore, proteins secreted into the extracellular microenvironment by the tumor cell play key roles in cell adhesion, intercellular communication, motility and invasion. In our previous work [3], we developed a method for secreted protein analysis that we applied to three different cancer cell types. We anticipate that the profile of secreted proteins may aid as tumor markers to provide information for assessing disease risk, prognosis, metastatic potential and evaluation of response to therapy. Currently, none of the routinely used clinically validated tumor markers have sufficient diagnostic or accurate predictive capabilities across all categories and stages of breast cancer. Consequently, there is a great need for the identification of additional tumor markers for breast cancer.

Molecular biomarkers such as clusterin and galectin-3-binding protein (LGALS3BP) [3] and tyrosine kinases [4] may have prognostic significance in breast cancer, but most studies involving these biomarkers have compared cells derived from unrelated individuals. Differences among the cancer cell donors, their origin, the passage number and culture conditions create huge variations that may be unrelated to normal and malignant behaviors. A comparison of malignant and non-malignant cell line variants from the same lineage will avoid this problem. Dwek and Alaiya demonstrated that it is possible to distinguish between normal, benign, and cancerous breast tissues on the basis of the protein profile by analyzing the proteomes of healthy tissue and those that represent different stages of the disease [5].

Advanced molecular technology methods have allowed the analysis of global gene expression or protein profiles in cancerous *versus* normal cells. The purpose of these studies is to identify mRNA or protein markers that are differentially expressed between benign and malignant cells [6,7]. However, these methods have limitations as the amount of active proteins in a cell [8] and post-translational modifications, that may be essential for protein function and activity, are undetectable at the level of mRNA expression. Therefore, the analysis of cellular proteins has become a key field of developing research in the post-genome era [9]. It provides new opportunities to clarify disease mechanisms and to discover new diagnostic biomarkers and therapeutic targets.

Here, we report the analysis of secreted proteins from the MCF10 isogenic series of breast cancer cell lines by LC-MS/MS. Specifically, we have analyzed the secreted proteins from MCF10A (non-tumorigenic), MCF10AT (premalignant; tumorigenic), MCF10 DCIS.com (tumorigenic and locally invasive) and MCF 10CA cl. d (tumorigenic and metastatic). These cell lines represent a stepwise progression of breast epithelial cells towards a metastatic phenotype. Hence, identification of the secreted proteome of these cells may yield insights into the alterations in the profile of secreted proteins associated with the acquisition of an aggressive phenotype. The aim was to identify major secreted proteins which undergo significant changes in abundance across the cell line series, rather than to provide an intensive proteomic coverage. To this end, a rather simple fractionation of the proteome was possible with estimation of proteins concentrations based on their relative search scores (Mascot), with the most abundant having the highest score [10]. The results gained by mass spectrometry analysis were reproducibly consistent, compared with two other metastatic breast cancer cell lines, and the differential expression of some proteins was validated by Western blot analysis.

2. Materials and Methods

Chemicals

Water and acetonitrile (ACN) were obtained from Honeywell Burdick & Jackson, Muskegon, MI. Urea, ammonium bicarbonate, trifluoroacetic acid (TFA), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St Louis, MO).

Cell culture conditions and collection of conditioned medium

MCF10A (non-tumorigenic, non-metastatic), MCF10AT (tumorigenic, non-metastatic), MCF10 DCIS.com (tumorigenic; locally invasive, non-metastatic) and MCF10CA cl. D (metastatic) were obtained from the Barbara Ann Karmanos Cancer Institute's Cell Research Core. Cells were cultured in DMEM-F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% horse serum, epidermal growth factor (25 ng/ml), cholera toxin (100 ng/ml), insulin (10 μg/ml) and cortisone (0.5 μg/ml) [11–13]. Cells were grown to approximately 60% confluence (approximately 2×10^6 cells) in 100 mm culture dishes (Corning Inc., Corning, NY). The cell monolayer was rinsed with serum free medium at 37°C for 15-minutes (3 times). The cells were then incubated in the serum-free medium at 37°C for 16-hours. Comparison data discussed from the MA11 cell line was previously published [3] and the secretome was also similarly collected from MT-1 cells. This line was established from human tumor xenografts grown in nude mice and is estradiol and progesterone receptor negative [14].

Preparation of the secreted proteins

After incubation, the conditioned medium from 2 plates (4×10^6 cells) was carefully removed and centrifuged 800 × g at 4 °C for 10 minutes to remove suspended cells. TFA (0.1%) was added to acidify the medium and minimize enzymatic activity, and the medium was stored at −80 °C until analysis. The cells are still 90–100% viable after the serum free growth as determined by trypan blue exclusion counting. To illustrate the reproducibility of the results, 3 independent preparations of conditioned media were prepared from each MCF10 cell line, spaced apart by approximately 2 weeks. Secreted proteins were isolated and fractionated (tC2 reversed-phase column, C2 SepPak, Waters, Milford, MA) as described previously [3]. Proteins fractions were obtained using 3 mL of solvents containing increasing amounts of ACN in 0.1% TFA from 30 to 70% (30%, 40%, 50%, 60% and 70%) at 1 mL/min, and then concentrated to dryness using a speed vac concentrator (Savant Instruments Inc., Holbrook, NY) as outlined in Scheme 1. Approximately 25μg of total protein was present in each fraction.

Protein digestion

The dried protein fractions were dissolved and reduced in a solution (10 μL) containing urea (8 M), EDTA (5 mM) and TCEP (10 mM), and then incubated at room temperature for about 2 hours. To avoid complications with over alkylation [15], cysteines were not "capped" and reformation of disulfides was prevented by the continued presence of the stable TCEP. The reduced proteins were then diluted to 40 μL with 50 mM ammonium bicarbonate pH 7.8 to give a final urea concentration of 2 M. Sequencing grade trypsin (20 pmol) (V5111, Promega, Madison, WI) was added to the protein solution and the digestion performed overnight at 37 °C.

Capillary LC-MS/MS Analysis

The tryptic peptide digests of the secreted proteins were analyzed using a Waters (Milford, MA) capillary liquid chromatograph coupled to the nanoflow electrospray source of a Waters Q-TOF Ultima API-US mass spectrometer. Peptides were separated on a C18 reverse phase column (0.15mm × 150mm, VC-10-C18-150; Micro-Tech Scientific, Vista CA) using a binary

solvent system made up of 98.8% water, 1% acetonitrile and 0.2% formic acid (solvent A), and 94.8% acetonitrile, 5% water and 0.2% formic acid (solvent B). The peptides were eluted from the column with a linear gradient program that changed the composition of solvent from 5% B to 90% B over 100 min at a constant flow rate of 1 μL/min. Data from the eluted peptides were acquired using the Waters MassLynx 4.0 software. An initial 40 min MS/MS analysis (1μL injected of each fraction) was performed to check the quality and the concentration of the secretome. Final data were acquired using 180 minute analyses (approximately 2μ g protein injected based on the intensity of the initial run) in a data-dependent mode where each full MS scan was followed by MS/MS of the four most intense ions. To optimize peptide coverage, a mass/charge exclusion list was maintained so that the same peptide was not selected for MS/MS within a period of one minute. To confirm the reliability of the MS results, the analysis of two preparations from each cell line was duplicated.

Database Searching

Data from the LC-MS/MS runs were converted to search files ("pkl" files) using MassLynx and poor data rejected below a "QA threshold" of 20. All initial run (40-minute) data was searched using an in-house Mascot search engine (version 2.1.03, Matrix Science Ltd., London, U.K.) using the following parameters: database NCBInr (August 23, 2006 revision; approximately 153,000 human sequences), mammalian species, enzyme trypsin with up to two missed cuts, no modifications, and a mass accuracy of 0.2 Da for both precursor ions and MS/MS data. Samples showing serum proteins or cell lysis were discarded. All pkl files from the final 180-minute LC-MS/MS analyses for a cell line were appended and final searching of these cell line specific pkl files was performed against the human subset of the NCBI RefSeq (reference sequencing) database (26571 entries) to ensure reproducible naming across cells lines and duplicate analyses, and to provide signal sequences of proteins where they exist. Only proteins matching at least 3 peptides in Mascot were automatically accepted. Proteins identified by one or two ions were hand-sequenced if they had an individual score of over 50. For these, following MaxEnt III conversion of the data to a noise reduced +1 spectrum, manual sequencing was performed without prior knowledge and the peptide mass and partial sequence searched against the RefSeq database. Only those peptides showing a unique identification were accepted. The identified secreted proteins were characterized and their location/function assigned as described previously [3] by searches of the signal peptide and the transmembrane sequences.

Western blot analysis

Conditioned medium was collected as described above. The conditioned serum free media samples were spun at 800 × g at 4°C for 10-minutes to remove any cells from the media. Samples were then concentrated using a Millipore Amicon Ultra 4 Centrifugal Filter Unit (Billerica, MA) with a 10 kDa cut-off. Samples were added to the columns and centrifuged at 7500 × g for 8-minutes at 4°C until a final volume of 250 μl was obtained. The column was washed with 3 mL of dH_2O and was centrifuged again at 7500 × g for 8-minutes at 4°C until a final volume of 250 μl was obtained. Total protein concentration from each sample was measured using Micro Pyrogallol Red reagent (Sigma, St. Louis, MI) for equal loading onto protein gels. For serum containing media (5% FBS), the ProteoSeek Albumin/IgG removal kit (Pierce, Rockford, IL, USA) was used to remove albumin. Due to the increase in volume as a result of this procedure, samples again concentrated using a 10 KDa cut-off filter.

Approximately 8 μg of total protein was resolved on a 10% SDS-polyacrylamide gel and transferred onto PVDF membrane. Membranes were blocked overnight in 5% skim milk in PBS. Proteins of interest were probed by using the following primary antibodies: mouse anti-osteonectin 1:1000 (Haematologic Technologies Inc., Essex Junction, VT), rabbit anti-alpha-1-antitrypsin 1:2000 (DakoCytomation, Carpinteria, CA), rabbit anti-alpha-1-antichymotrypsin

1:5000 (DakoCytomation), mouse anti-mesothelin 1:500 (Zymed [Invitrogen], Carlsbad, CA) or rabbit anti-galectin-3-binding protein 1:2500 (gift from S. Laferté, Department of Biochemistry, University of Saskatchewan, Canada). Respective horseradish peroxidase-labeled secondary antibodies were used, and reactive bands were visualized with enhanced chemiluminescence detection reagent Luminol (Santa Cruz Biotechnology, Santa Cruz, CA).

3. Results

Sample preparation and quality control

Cell confluence on plates and the incubation time in serum-free medium were optimized as described previously [3], largely using the initial 40-minute MS/MS runs to identify any cell lysis and serum proteins. For protein identification, cells were incubated in serum-free medium at 60% confluence for 16-hours in order to maximize recovery of the secreted proteins while minimizing cell lysis. The medium was removed and the proteins concentrated and fractionated using our C2 reversed phase approach [3]. Three independent secreted proteome preparations (each with 5 factions from the tC2 column) of each cancer cell line were analyzed on the LC-MS/MS, two in duplicate. Within repeat preparations and analyses minor differences were observed in the number of peptides identified and the order of the proteins in the Mascot results, especially for those with very few peptides assigned. As proof of consistency, the peptides identified from five proteins in each preparation, and in the duplicate injection of the third preparation, are shown in Table 1. These proteins were selected due to the significant change in expression across the cell lines. As expected by the MS/MS process, small changes are observed in the numbers of peptides “hit” during the analysis, although the trend is still clearly apparent. The total amount of protein produced in the 16-hour serum free medium was lower in the less aggressive cells lines as was indicated by the height of the peptide signals in the LC-MS/MS analyses and consistent with the highest Mascot protein scores in each (10A: 2078; 10AT: 2286; CA cl.d: 3179 and DCIS 5013). This agrees with the growth rate of these cells and suggests that the secretion of these proteins may be associated with the cell cycle. The collection of the serum free media was limited to 16-hours as there was a risk of cell lysis during longer incubations.

Final Protein Characterization

Based on the consistency of the search results obtained across preparations and duplicate analyses, the search data (pkl files) from all fractions and analyses of each cell line were appended, and searched against the human subset of the RefSeq database from NCBI. Over 250 proteins were identified per cell line and secreted protein abundance was based on the Mascot score and archived as the position in the results list (1 is the highest scoring protein, i.e. the most abundant protein) [10]. Many proteins showed differential expression in the various cell lines. Proteins such as serpin peptidase inhibitor (AACT; alpha-1-antichymotrypsin; SERPINA3), serine (or cysteine) proteinase inhibitor, clade A (AAT; alpha-1-antitrypsin; SERPINA1), galectin-3-binding protein (LGALS3BP; lectin, galactoside-binding, soluble, 3 binding protein; 90K) and secreted protein, acidic, rich in cysteine (SPARC; osteonectin) were more highly secreted from the tumorigenic cell lines MCF10 DCIS.com and MCF10CA cl. D. Conversely, mesothelin isoform 1 (MSLN) was more expressed in the non-tumorigenic and non-metastatic cell line MCF10A. As the aim of this research was to identify highly secreted proteins which changed in abundance across the series, emphasis was largely placed on the top ten proteins in each cell line. A list of these more significant proteins is provided as Table 2, showing their ranking in the list (1 = most abundant) along with the number of peptide sequence hits mapped to each within the combined search files. A search of the data sets was performed using “no enzyme” (non-specific cutting) to identify if other enzymatic activity was present or if the proteins were present in pieces. There was minimal evidence of this (less than 3% of peptide identifications) and many were attributable to chymotryptic cut

at one end of the peptide. In most cases there was an overlapping tryptic peptide which suggests, but does not prove, that proteins were present in full length form. However, proteins were eluted in discrete fractions and were not spread out across the C2 reversed phase separation profile suggesting that they were not present in highly fractionated forms. This also agreed with the Western blot analyses which showed single bands at the expected molecular weight, except for a pair of closely chromatographing peaks for AAT. This was supported by the over 90% coverage of proteins high on the secreted proteome lists in the combined data sets, after making allowance for signal sequence, and glycopeptide areas which will not be detected by MS/MS without a deglycosylation step. Proteins that are membrane associated like mesothelin, a shed protein [16], had few peptides identified during this research (Table 1) but these were still spaced over both the megakaryocyte potentiating factor (n-terminal) and "mature" mesothelin c-terminal sequences.

Validation of the differential protein expression

The differential expression of proteins observed in the LC-MS/MS analysis was validated by western blotting analysis of five selected proteins, LGALS3BP, AACT, AAT, SPARC and MSLN. The first four of these were more expressed in the cell lines (MCF10CA cl. D and MCF10 DCIS) and MSLN in the less aggressive cell lines (MCF10A and MCF10AT), according to the MS/MS data. These observations were confirmed by western blotting analysis of the selected proteins as shown in Figure 1. Thus, significant levels of LGALS3BP, AACT, AAT and SPARC were clearly present in the serum free media of the invasive and metastatic cell lines MCF10 DCIS.com and MCF10CA cl. D, while MSLN was more highly expressed in the non-tumorigenic MCF10A cell line. The secretion of SPARC can be seen in the MCF10 DCIS.com and MCF10CA cl. D cells, but appears to be more pronounced in the MCF10 DCIS.com cells. This is totally consistent with the results obtained by the LC-MS/MS analysis. Since we are analyzing secreted proteins, we present a non-specific band (NS) that was obtained during immunoblotting as a proof of equal loading across all lanes. While the absence of serum could possibly change the cells and their secreted proteome, a Western blot analysis of the serum-free and serum-containing secreted proteomes showed no significant difference for galectin-3-binding protein (Figure 2).

Comparison with two other aggressive breast cancer cell lines

We have previously published the secretome from the MA11 [3] and have also analyzed the secreted proteome from MT-1 breast cancer cell lines. Data from both were searched using pooled search (pkl) files. Interestingly, while the alpha-1-antitrypsin and alpha-1-antichymotrypsin were correlated with aggressiveness in the MCF10 cell lines, they were not within the top 100 proteins identified from either MA11 or MT-1 cells. However, galectin-3-binding protein, peptidylprolyl isomerase isoform 1 and enolase 1 remained consistently highly secreted. A limited selection of proteins for these two cell lines compared to those of MCF10CA cl. D is shown in Table 3.

4. Discussion

In the past few years, new multi-step pathways of breast cancer progression have been delineated through genotypic-phenotypic correlations. A few biological markers including the cyclins, components of the urokinase plasminogen system, and the presence of circulating epithelial cells have been associated with patient outcome. Despite this, there remain a number of unmet clinical needs, and more sensitive and specific indicators of prognosis are required to identify those patients at greatest risk for disease progression. The ready identification of target biomarkers representative of the various stages of breast cancer would help improve early detection, diagnosis and prognosis of breast cancer, and such markers could then be

analyzed on clinical samples, offering an individual interpretation of disease status and likely progression.

Secreted proteins play a critical role in tumor metastasis as they are involved in cell-cell communications, cell adhesion, motility and invasion. We analyzed secreted proteins from a series of MCF10 breast cancer cell lines, following our previously described method for secreted protein preparation and analysis [3]. The results presented were obtained using isogenic cell lines that represent progression from non-tumorigenic breast epithelial cells (MCF10A) to the tumorigenic and metastatic MCF10CA cl. D cells. This research was designed to exploit large scale secreted protein profiles with up- or down-regulated expression associated with aggressive versus non-aggressive phenotypes. The methods for secreted protein preparation and analysis following the described approach were reproducible and consistent within all the cell lines investigated.

Mass spectrometry-based proteomics has the capability of identifying hundreds of proteins in a single experiment [17]. Within complex LC-MS/MS analyses, major peptides may be hit uniformly. However, less abundant peptide ions may be missed in one run and hit during another and the use of combined data from repeat runs may lead to the identification of different peptides, and increased coverage. To assist in these analyses and to provide reliable proteomic profiling, three independent secreted protein preparations from each cancer cell line were prepared and analyzed by MS/MS, two of these in duplicate. Each proteome preparation was fractionated using an increased acetonitrile gradient on a reverse phase C2 column to provide better MS/MS coverage of the peptides in each fraction's digest, followed by 180 minute LC-MS/MS analyses. While a 2-dimensional LC analysis or enhanced fractionation by LC may have improved the coverage and number of proteins identified, the approach used repeatedly identified the major secreted proteins and their change in abundance across the cell lines, while minimizing instrument time. As the objective of this research was to identify significantly secreted proteins which may serve as biomarkers, a more in-depth coverage of the proteome was not necessary. Within the major proteins identified, the numbers of peptides hit were relatively constant and certainly indicated significant changes in abundance between the cell lines (e.g. see Table 1). Minor changes were observed, but these did not alter the trend observed. As different peptides may be identified in each run, to provide the highest reliability in the data and profiling of the changes in abundance, the data from all analyses of each cell line were combined into a single search file. This was then searched with Mascot against the NCBI reference sequence (RefSeq, restricted to human) database. This database contains validated gene sequence derived proteins, and has approximately 26,600 human sequences, and provides signal sequences for functional prediciton. Because of the limited size of the database and the absence of variable names, a direct comparison of the proteins found in each proteome was enabled. A partial list of proteins is shown in Table 2, largely selected based on high secretion in one of the cell lines.

Validation of the LC-MS data

To validate the MS/MS results, we selected five proteins for examination based on their position in the Mascot score list, their change in expression across cell lines and the vital role they play in many cancers. Western blot analysis (Figure 2) was performed on these five proteins and the results confirmed the MS-based analyses (Figure 1). These proteins are discussed below:

Galectin-3-binding protein (LGALS3BP; 90K) was strongly expressed in the aggressive cell lines MCF10 DCIS.com and MCF10CA cl. D, and is one of the most significant metastatic markers detected in these analyses. LGALS3BP is a large oligomeric glycoprotein first identified by Iacobelli et al. as a tumor-associated antigen in breast cancer [18]. Although its functions are not well clarified, it was recently demonstrated to induce promatrilysin (matrix

metalloproteinase 7) expression in prostate [19] and colon cancers [20]. LGALS3BP was also highly significant when we examined the secreted proteome of three unrelated metastatic cell lines, MA11 in breast cancer, OHS in osteosarcoma and WM266-4 in melanoma [3], and in the secretome of the unrelated MT-1 breast cancer cell line (Table 3).

Alpha-1-antichymotrypsin (AACT) and **alpha-1-antitrypsin** (AAT) were elevated in the aggressive cell lines, in concordance with their association with tumorigenicity in patients with various types of cancer [21,22]. Both proteins are serine proteinase inhibitors produced by various tumor cells. While AAT is primarily secreted by the liver [23], it is also secreted by monocytes and certain types of tumors [24–26], and is associated with inflammation, infection and other malignant diseases. Its C-terminal fragment can potentiate proliferation, invasiveness and NF-κB (nuclear factor kappa B) activity in breast cancer cells [27]. Kuvibidila and Rayford [28] demonstrated a correlation between AAT and the prostate cancer marker prostate-specific antigen (PSA), and both AAT and AACT bind PSA [29]. AAT is known to down regulate tumor necrosis factor-induced apoptosis, elevated levels may therefore promote cell survival and growth of tumors [30]. AACT expression in lung adenocarcinomas was significantly higher in advanced tumors with a higher rate of mitosis [31], and was inversely correlated with shorter disease free survival (DFS) and prognosis. Both Higashiyama, et al. [31], and Laursen and Lykkesfeldt [32] concluded that AACT acts as a minor growth factor-like substance in breast cancer, in concordance with its association of poor prognosis in breast cancer patients. Somewhat contrasting to these reports are the clinical use of chymotryptic protease inhibitors (e.g. AACT) as cancer suppressing agents [33].

SPARC or osteonectin (secreted protein, acidic, rich in Cysteine; BM40) is a 32 kDa glycoprotein that interacts with extracellular matrix (ECM) proteins to promote adhesion of cells to the matrix, thereby inducing a biological state conducive to cell migration. SPARC is also thought to play an important role in tissue remodelling, angiogenesis, embryonic development and tumorigenesis. The levels of SPARC in breast tumor tissues were significantly higher compared to normal background breast tissue, which has been previously reported in advanced breast cancers and node-positive tumors [34]. Likewise, our analysis revealed that secretion of SPARC was elevated in the relatively aggressive MCF10 DCIS.com and MCF10CA cl. D cell lines. High expression of SPARC has also been associated with frequent microcalcification deposition in the breast cancer lesion [35]. Functionally, SPARC promotes invasiveness [36], motility [37] and activity of matrix metalloproteinases [38] in breast and prostate cancer cells. SPARC was previously observed in our secreted proteome analysis of a melanoma and an osteocarcoma cell line [3].

Mesothelin (MSLN) is a differentiation glycoprotein antigen present on the cell surface of normal mesothelium and is highly expressed in mesothelia-derived tumors, including epithelioid-type mesotheliomas, most ovarian cancers and pancreatic cancer [39–45]. Proteolytic cleavage of the 69-kDa mesothelin precursor protein yields MSLN, a cell membrane–associated 40-kDa glycosylphosphatidylinositol-linked glycoprotein [46]. Another cleavage product is megakaryocyte potentiation factor (MPF), a 33-kDa soluble protein originally identified as a polypeptide from the culture supernatant of a pancreatic cancer cell line [47]. We have validated that the expression of MSLN is the highest in the non-tumorigenic MCF10A cells. The other three cell lines showed significantly reduced levels of MSLN by both mass spectrometry and immunoblotting (Table 2;Figure 1). The biological functions of MSLN and MPF are not clear. MSLN binds CA125 (MUC16) that is present on many ovarian cancer cells [48,49], eluding to the suggestion that it might have a role in the metastatic spread of ovarian cancer in the peritoneal cavity [50]. Yen et al. [51] recently provided new evidence that diffuse MSLN expression is associated with prolonged patient survival.

Proteomic analysis revealed other secreted proteins of relevance in cancer that fluctuated, or were expected to flucuate with metastatic potential. While these were not validated by Western blot, they were selected for discussion based on their correlation with disease progression, and/or their appearance in our previous secreted proteome analyses [3]. They validate the secreted proteome profiles determined during this research.

Lysyl oxidase (LOX) is an extracellular matrix protein upregulated in highly invasive, metastatic breast cancer cells [52], and promotes migration and invasion of invasive breast cancer cell lines [53]. Based on the MS analysis, the non-tumorigenic MCF10A cells do not secreted appreciable LOX, whereas the more aggressive cells do. LOX may be also required to create a niche permissive for metastatic growth. Functionally, inhibition of LOX eliminated metastasis in mice with orthotopically grown breast tumors. Clinically, elevated LOX levels correlate with metastatic disease [54]. LOX expression is upregulated in distant metastatic breast cancer tissues compared with primary cancer tissues. Patients with high LOX-expressing tumors have poor distant metastasis-free and overall survivals [55].

Zinc alpha-2-glycoprotein 1 (ZAG) is secreted by a variety of normal epithelia. Serum levels of ZAG are elevated in men with ZAG-expressing prostate tumors. Interestingly, high-grade prostate tumors expressed significantly less ZAG than moderate grade tumors [56]. In contrast, in patients with node positive breast cancer, serum concentrations of ZAG were significantly higher than controls [57]. Further, women with advanced breast cancer had higher serum ZAG levels than those with earlier disease. In our study, the most aggressive MCF10CA cl. D line did not secrete appreciable ZAG. It is possible that we may encounter a different scenario in clinical samples.

Cathepsin D is an aspartic endo-protease that is ubiquitously distributed in lysosomes [58]. In addition to its classical role as a major protein-degrading enzyme in lysosomes and phagosomes, cathepsin D also activates precursors of biologically active proteins in pre-lysosomal compartments of specialized cells [59]. In most breast cancers cathepsin D is over-expressed from 2- to 50-fold compared to its concentration in other cell types such as fibroblasts or normal mammary glands [60] and several independent clinical studies have shown that the level in primary breast cancer cytosol is an independent prognostic parameter correlated with the incidence of clinical metastasis and shorter survival times [61,62]. Our profiling reveals that cathepsin D becomes more abundant in the aggressive cell lines. While it functionally stimulates metastasis, it also acts as a mitogen for cancer cells and promotes angiogenesis [63], and induces stromal proliferation in tumors. In turn, the stromal fibroblasts produce proteases that aid in the degradation of the extracellular matrix [64–66].

S100 Proteins

The S100 family of calcium binding proteins has been shown to be involved in a variety of physiological function, such as cell proliferation, extracellular signal transduction, intercellular adhesion, motility as well as cancer metastasis [67,68]. Molecular analysis of breast tumors has revealed that several S100s exhibit altered expression levels during breast tumorigenesis and/or progression. From our analyses, we see that the S100 proteins did not show a consistent increase in abundance with the acquisition of an aggressive phenotype.

Non-classical secretion

While actin and tubulin proteins (freely available in lysed cells) were low on the Mascot list, during these analyses several proteins, which may be considered to be cytosolic due to the absence of a signal sequence, were near the top of the list. These include annexin A2, enolase 1 and peptidylprolyl isomerase isoform 1, which were actually 3 of the top 5 proteins in the secreted proteome of MCF10A cells. These proteins do not contain a traditional signal sequence

and when analyzed using the CBS SignalP software (http://www.cbs.dtu.dk/services/SignalP/), they are not considered as secreted by the normal pathways. However, these proteins are all predicted to undergo non-classical protein secretion as predicted by (http://www.cbs.dtu.dk/services/SecretomeP/), and it is therefore likely that these proteins are secreted by non-traditional mechanisms and not arise from cell lysis, and this has been confirmed for annexin A2 [69–71]. Enolase 1 (α-enolase) also lacks a signal sequence and is an enzyme that converts 2-phospho-D-glycerate to phosphoenol-pyruvate in the glycolysis pathway, and is overexpressed in breast cancer cells that constitutively express the anti-apoptotic protein, Bcl-xL [72]. It is a multifunctional enzyme that also plays a role in various processes such as growth control, tolerance to hypoxia, and allergic responses [73]. α-enolase is a plasminogen-binding protein and also appears to play a major role in the promotion of plasminogen activation by leukocytic cells [74]. Peptidylprolyl isomerase (cyclophilin A) has been associated with a number of cancer histiotypes including breast [75] and pancrease [76,77], and has been implicated in cellular resistance to hypoxia- and Cisplatin-induced cell death [78] and resistance to aplidin [79]. Its secretion from regressive tumor masses has been previously been studied by MS [80] and it has been profiled as one of a number of glycolytic enzymes increased in pancreatic cancerous tissues [76].

To our knowledge this is the first report of identifying potential secreted biomarkers of breast cancer progression using an isogenic series of cell lines, although cell lysates were recently profiled using gel-based analyses [81]. Our results have shown that there are significant changes in the secreted proteome associated with cell aggressiveness within the MCF10 cell series, and such changes may be useful in breast cancer for predicting the aggressiveness of a patient's tumor. Most significant was the increased secretion of alpha-1-antichymotrypsin and galectin-3-binding protein in the more aggressive cell lines. Secreted proteins have the highest potential of being found in the bloodstream, leading to the potential use of these markers being used for the early detection of cancer, monitoring for reoccurrence, and an estimation of its aggressiveness. The need to perform these analyses on a number of cell types was indicated by the absence of the alpha-1-antichymotrypsin in the aggressing MA11 and MT-1 cell lines. In contrast, galectin-3-binding protein was consistently highly secreted and was detected previously as a major secreted protein from melanoma and osteosarcoma cells [3]. It has also been identified in colon tumors [20]. Proteins were likely largely present in their intact form (minus any signal sequences) based on the coverage of the major proteins and the Western blot analyses performed. The secretome is thus a good indicator of target proteins for biomarker analysis and validates their sampling in the blood stream or in the proximity of a tumor, such as with a fine needle aspirate or ductal lavage. With several options possible in the treatment of an early discovered breast lesion, histological studies directed by the results of secretome analyses may provide rapid identification of highly specific markers for the disease.

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REFERENCES

1. Flagg EW, et al. Validation of the American Cancer Society Cancer Prevention Study II Nutrition Survey Cohort Food Frequency Questionnaire. Epidemiology 2000;11(4):462–468. [PubMed: 10874556]
2. Ruoslahti E. Fibronectin and its integrin receptors in cancer. Adv Cancer Res 1999;76:1–20. [PubMed: 10218097]

3. Mbeunkui F, Fodstad O, Pannell LK. Secretory protein enrichment and analysis: an optimized approach applied on cancer cell lines using 2D LC-MS/MS. J Proteome Res 2006;5(4):899–906. [PubMed: 16602697]
4. Hunter T. Oncoprotein networks. Cell 1997;88(3):333–346. [PubMed: 9039260]
5. Dwek MV, Alaiya AA. Proteome analysis enables separate clustering of normal breast, benign breast and breast cancer tissues. Br J Cancer 2003;89(2):305–307. [PubMed: 12865921]
6. Porter DA, et al. A SAGE (serial analysis of gene expression) view of breast tumor progression. Cancer Res 2001;61(15):5697–5702. [PubMed: 11479200]
7. Porter D, et al. Molecular markers in ductal carcinoma in situ of the breast. Mol Cancer Res 2003;1 (5):362–375. [PubMed: 12651909]
8. Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 1997;18(3–4):533–537. [PubMed: 9150937]
9. Chambers G, et al. Proteomics: a new approach to the study of disease. J Pathol 2000;192(3):280–288. [PubMed: 11054709]
10. Liu H, Sadygov RG, Yates JR 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 2004;76(14):4193–4201. [PubMed: 15253663]
11. Miller FR, et al. MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. J Natl Cancer Inst 2000;92(14):1185–1186. [PubMed: 10904098]
12. Santner SJ, et al. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat 2001;65(2):101–110. [PubMed: 11261825]
13. Strickland LB, et al. Progression of premalignant MCF10AT generates heterogeneous malignant variants with characteristic histologic types and immunohistochemical markers. Breast Cancer Res Treat 2000;64(3):235–240. [PubMed: 11200773]
14. Naundorf H, et al. Characterization of two human mammary carcinomas, MT-1 and MT-3, suitable for in vivo testing of ether lipids and their derivatives. Breast Cancer Res Treat 1992;23(1–2):87–95. [PubMed: 1446057]
15. Boja ES, Fales HM. Overalkylation of a protein digest with iodoacetamide. Anal Chem 2001;73(15): 3576–3582. [PubMed: 11510821]
16. Ho M, et al. Mesothelin is shed from tumor cells. Cancer Epidemiol Biomarkers Prev 2006;15(9): 1751. [PubMed: 16985043]
17. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003;422(6928):198–207. [PubMed: 12634793]
18. Iacobelli S, et al. Detection of antigens recognized by a novel monoclonal antibody in tissue and serum from patients with breast cancer. Cancer Res 1986;46(6):3005–3010. [PubMed: 3516389]
19. Bair EL, et al. 90K/Mac-2 binding protein is expressed in prostate cancer and induces promatrilysin expression. Prostate 2006;66(3):283–293. [PubMed: 16245278]
20. Ulmer TA, et al. Tumor-associated antigen 90K/Mac-2-binding protein: possible role in colon cancer. J Cell Biochem 2006;98(5):1351–1366. [PubMed: 16518858]
21. Chawla RK, et al. Serum alpha-1 proteinase inhibitor in advanced cancer: mass variants and functionally inert forms. Cancer Res 1987;47(4):1179–1184. [PubMed: 3492270]
22. Tountas Y, et al. Alpha 1-antitrypsin and cancer of the pancreas. Digestion 1985;31(1):37–40. [PubMed: 3872237]
23. Travis J, Salvesen GS. Human plasma proteinase inhibitors. Annu Rev Biochem 1983;52:655–709. [PubMed: 6193754]
24. Isaacson P, et al. Alpha-1-antitrypsin in human macrophages. J Clin Pathol 1981;34(9):982–990. [PubMed: 6792245]
25. Kataoka H, et al. Properties of alpha 1-antitrypsin secreted by human adenocarcinoma cell lines. FEBS Lett 1993;328(3):291–295. [PubMed: 8348978]
26. Perlmutter DH, et al. Expression of the alpha 1-proteinase inhibitor gene in human monocytes and macrophages. Proc Natl Acad Sci U S A 1985;82(3):795–799. [PubMed: 3871944]
27. Zelvyte I, Lindgren S, Janciauskiene S. Multiple effects of alpha1-antitrypsin on breast carcinoma MDA-MB 468 cell growth and invasiveness. Eur J Cancer Prev 2003;12(2):117–124. [PubMed: 12671535]

28. Kuvibidila S, Rayford W. Correlation between serum prostate-specific antigen and alpha-1-antitrypsin in men without and with prostate cancer. J Lab Clin Med 2006;147(4):174–181. [PubMed: 16581345]
29. Zhang WM, et al. Measurement of the complex between prostate-specific antigen and alpha1-protease inhibitor in serum. Clin Chem 1999;45(6 Pt 1):814–821. [PubMed: 10351990]
30. Van Molle W, et al. Alpha 1-acid glycoprotein and alpha 1-antitrypsin inhibit TNF-induced but not anti-Fas-induced apoptosis of hepatocytes in mice. J Immunol 1997;159(7):3555–3564. [PubMed: 9317155]
31. Higashiyama M, et al. Alpha-1-antichymotrypsin expression in lung adenocarcinoma and its possible association with tumor progression. Cancer 1995;76(8):1368–1376. [PubMed: 8620411]
32. Laursen I, Lykkesfeldt AE. Purification and characterization of an alpha 1-antichymotrypsin-like 66 kDa protein from the human breast cancer cell line, MCF-7. Biochim Biophys Acta 1992;1121(1–2):119–129. [PubMed: 1599933]
33. Kennedy AR. Chemopreventive agents: protease inhibitors. Pharmacol Ther 1998;78(3):167–209. [PubMed: 9690817]
34. Watkins G, et al. Increased levels of SPARC (osteonectin) in human breast cancer tissues and its association with clinical outcomes. Prostaglandins Leukot Essent Fatty Acids 2005;72(4):267–272. [PubMed: 15763438]
35. Bellahcene A, Castronovo V. Expression of bone matrix proteins in human breast cancer: potential roles in microcalcification formation and in the genesis of bone metastases. Bull Cancer 1997;84(1):17–24. [PubMed: 9180854]
36. Jacob K, et al. Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. Cancer Res 1999;59(17):4453–4457. [PubMed: 10485497]
37. Campo McKnight DA, et al. Roles of osteonectin in the migration of breast cancer cells into bone. J Cell Biochem 2006;97(2):288–302. [PubMed: 16173048]
38. Gilles C, et al. SPARC/osteonectin induces matrix metalloproteinase 2 activation in human breast cancer cell lines. Cancer Res 1998;58(23):5529–5536. [PubMed: 9850090]
39. Argani P, et al. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). Clin Cancer Res 2001;7(12):3862–3828. [PubMed: 11751476]
40. Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. Int J Cancer 1992;50(3):373–381. [PubMed: 1735605]
41. Hassan R, et al. Localization of mesothelin in epithelial ovarian cancer. Appl Immunohistochem Mol Morphol 2005;13(3):243–247. [PubMed: 16082249]
42. Hassan R, et al. Mesothelin is overexpressed in pancreaticobiliary adenocarcinomas but not in normal pancreas and chronic pancreatitis. Am J Clin Pathol 2005;124(6):838–845. [PubMed: 16416732]
43. Ordonez NG. Application of mesothelin immunostaining in tumor diagnosis. Am J Surg Pathol 2003;27(11):1418–1428. [PubMed: 14576474]
44. Thomas AM, et al. Mesothelin-specific CD8(+) T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. J Exp Med 2004;200(3):297–306. [PubMed: 15289501]
45. Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. Clin Cancer Res 2004;10(12 Pt 1):3937–3942. [PubMed: 15217923]
46. Onda M, et al. Megakaryocyte potentiation factor cleaved from mesothelin precursor is a useful tumor marker in the serum of patients with mesothelioma. Clin Cancer Res 2006;12(14 Pt 1):4225–4231. [PubMed: 16857795]
47. Yamaguchi N, et al. A novel cytokine exhibiting megakaryocyte potentiating activity from a human pancreatic tumor cell line HPC-Y5. J Biol Chem 1994;269(2):805–808. [PubMed: 8288629]
48. Lloyd KO, Yin BW. Synthesis and secretion of the ovarian cancer antigen CA 125 by the human cancer cell line NIH:OVCAR-3. Tumour Biol 2001;22(2):77–82. [PubMed: 11125279]
49. Yin BW, Lloyd KO. Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. J Biol Chem 2001;276(29):27371–27375. [PubMed: 11369781]

50. Hassan R, et al. Detection and quantitation of serum mesothelin, a tumor marker for patients with mesothelioma and ovarian cancer. Clin Cancer Res 2006;12(2):447–453. [PubMed: 16428485]
51. Yen MJ, et al. Diffuse mesothelin expression correlates with prolonged patient survival in ovarian serous carcinoma. Clin Cancer Res 2006;12(3 Pt 1):827–831. [PubMed: 16467095]
52. Kirschmann DA, et al. A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res 2002;62(15):4478–4483. [PubMed: 12154058]
53. Payne SL, et al. Lysyl oxidase regulates breast cancer cell migration and adhesion through a hydrogen peroxide-mediated mechanism. Cancer Res 2005;65(24):11429–11436. [PubMed: 16357151]
54. Erler JT, Giaccia AJ. Lysyl oxidase mediates hypoxic control of metastasis. Cancer Res 2006;66(21): 10238–10241. [PubMed: 17079439]
55. Erler JT, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 2006;440(7088): 1222–1226. [PubMed: 16642001]
56. Hale LP, et al. Zinc alpha-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. Clin Cancer Res 2001;7(4):846–853. [PubMed: 11309332]
57. Bundred NJ, et al. Zinc alpha-2 glycoprotein levels in serum and breast fluids: a potential marker of apocrine activity. Eur J Cancer 1991;27(5):549–552. [PubMed: 1828959]
58. Barrett AJ. Cathepsin D. Purification of isoenzymes from human and chicken liver. Biochem J 1970;117(3):601–607. [PubMed: 5419752]
59. Diment S, Martin KJ, Stahl PD. Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processing of proteins. J Biol Chem 1989;264(23):13403–13406. [PubMed: 2760027]
60. Capony F, et al. Increased secretion, altered processing, and glycosylation of pro-cathepsin D in human mammary cancer cells. Cancer Res 1989;49(14):3904–3909. [PubMed: 2736531]
61. Rochefort H. Cathepsin D in breast cancer: a tissue marker associated with metastasis. Eur J Cancer 1992;28A(11):1780–1783. [PubMed: 1389510]
62. Westley BR, May FE. Prognostic value of cathepsin D in breast cancer. Br J Cancer 1999;79(2):189–190. [PubMed: 9888456]
63. Liaudet-Coopman E, et al. Cathepsin D: newly discovered functions of a long-standing aspartic protease in cancer and apoptosis. Cancer Lett 2006;237(2):167–179. [PubMed: 16046058]
64. Basset P, et al. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 1990;348(6303):699–704. [PubMed: 1701851]
65. Uria JA, et al. Regulation of collagenase-3 expression in human breast carcinomas is mediated by stromal-epithelial cell interactions. Cancer Res 1997;57(21):4882–4888. [PubMed: 9354453]
66. Christensen L, et al. Immunohistochemical localization of urokinase-type plasminogen activator, type-1 plasminogen-activator inhibitor, urokinase receptor and alpha(2)-macroglobulin receptor in human breast carcinomas. Int J Cancer 1996;66(4):441–452. [PubMed: 8635858]
67. Emberley ED, Murphy LC, Watson PH. S100A7 and the progression of breast cancer. Breast Cancer Res 2004;6(4):153–159. [PubMed: 15217486]
68. Sherbet GV, Lakshmi MS. S100A4 (MTS1) calcium binding protein in cancer growth, invasion and metastasis. Anticancer Res 1998;18(4A):2415–2421. [PubMed: 9703888]
69. Danielsen EM, van Deurs B, Hansen GH. "Nonclassical" secretion of annexin A2 to the lumenal side of the enterocyte brush border membrane. Biochemistry 2003;42(49):14670–14676. [PubMed: 14661980]
70. Deora AB, et al. An annexin 2 phosphorylation switch mediates p11-dependent translocation of annexin 2 to the cell surface. J Biol Chem 2004;279(42):43411–43418. [PubMed: 15302870]
71. Chasserot-Golaz S, et al. Annexin 2 promotes the formation of lipid microdomains required for calcium-regulated exocytosis of dense-core vesicles. Mol Biol Cell 2005;16(3):1108–1119. [PubMed: 15635098]
72. Espana L, et al. Bcl-x(L)-mediated changes in metabolic pathways of breast cancer cells: from survival in the blood stream to organ-specific metastasis. Am J Pathol 2005;167(4):1125–1137. [PubMed: 16192647]

73. Pancholi V. Multifunctional alpha-enolase: its role in diseases. Cell Mol Life Sci 2001;58(7):902–920. [PubMed: 11497239]

74. Lopez-Alemany R, et al. Inhibition of cell surface mediated plasminogen activation by a monoclonal antibody against alpha-Enolase. Am J Hematol 2003;72(4):234–242. [PubMed: 12666133]

75. Neubauer H, et al. Breast cancer proteomics by laser capture microdissection, sample pooling, 54-cm IPG IEF, and differential iodine radioisotope detection. Electrophoresis 2006;27(9):1840–1852. [PubMed: 16645950]

76. Mikuriya K, et al. Expression of glycolytic enzymes is increased in pancreatic cancerous tissues as evidenced by proteomic profiling by two-dimensional electrophoresis and liquid chromatography-mass spectrometry/mass spectrometry. Int J Oncol 2007;30(4):849–855. [PubMed: 17332923]

77. Li M, et al. Cyclophilin A is overexpressed in human pancreatic cancer cells and stimulates cell proliferation through CD147. Cancer 2006;106(10):2284–2294. [PubMed: 16604531]

78. Choi KJ, et al. Overexpressed cyclophilin a in cancer cells renders resistance to hypoxia- and Cisplatin-induced cell death. Cancer Res 2007;67(8):3654–3662. [PubMed: 17440077]

79. Gonzalez-Santiago L, et al. Proteomic analysis of the resistance to aplidin in human cancer cells. J Proteome Res 2007;6(4):1286–1294. [PubMed: 17338558]

80. Huang CM, et al. Mass spectrometric proteomics profiles of in vivo tumor secretomes: capillary ultrafiltration sampling of regressive tumor masses. Proteomics 2006;6(22):6107–6116. [PubMed: 17051643]

81. Li DQ, et al. Identification of breast cancer metastasis-associated proteins in an isogenic tumor metastasis model using two-dimensional gel electrophoresis and liquid chromatographyion trap-mass spectrometry. Proteomics 2006;6(11):3352–3368. [PubMed: 16637015]

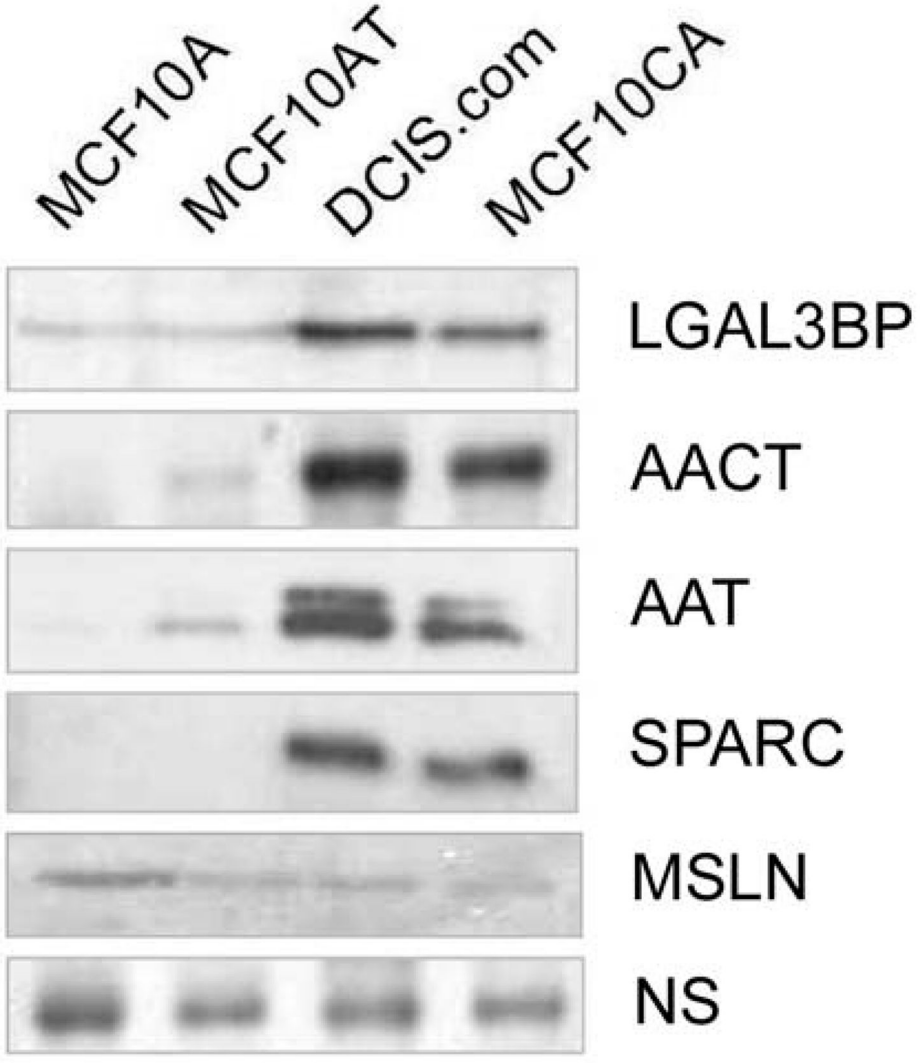


Figure 1.
The profile of the secreted proteome changes as the cells become progressively more aggressive. Conditioned serum free media from the cell lines was concentrated and the total protein estimated. Eight micrograms of total protein was resolved by SDS-PAGE and immunoblotted for LGAL3BP (galectin-3-binding protein), AACT (alpha-1-chymoantitrypsin), AAT (alpha-1-antitrypsin), SPARC (osteonectin) and MSLN (mesothelin). NS represents a non-specific band used to show equal loading.

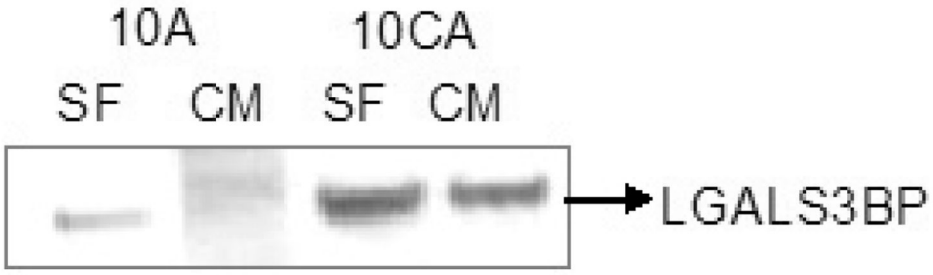
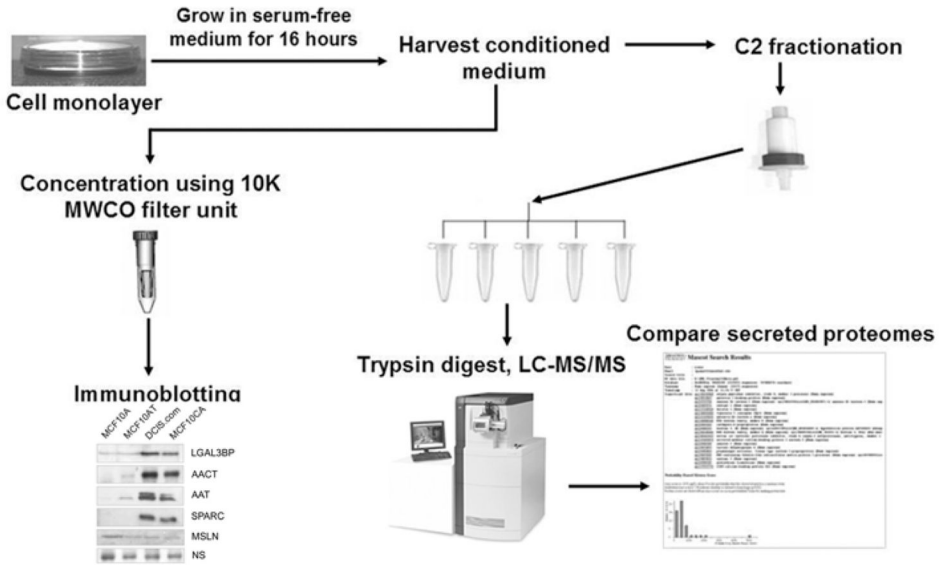


Figure 2.

The profile of the secreted proteome remains unchanged even in the absence of any serum in the growth medium for 16–18 hours. Conditioned media (containing 5% FBS (CM) as well as serum free (SF) media) from the cell lines was concentrated eight-fold. Albumin was depleted from the serum-containing samples. Eight micrograms of total protein was resolved by SDS-PAGE and immunoblotted for galectin-3-binding protein (LGALS3BP). 10A corresponds to MCF10A cells and 10CA to MCF10CA cl. D cells.



Scheme 1.

View of the overall strategy for secreted proteome comparisons by LC-MS/MS with Mascot searching and verification by western blotting.

Table 1

Repeatability of the secreted proteome of five selected proteins from three preparations, one analyzed in duplicate.

Protein name[a]	NCBI gi #	MCF10A			MCF10AT			MCF10 DCIS.Com			MCF10CA cl. D		
		Peptides matched[b]			Peptides matched			Peptides matched			Peptides matched		
Alpha-1-antichymotrypsin	50659080	0	0	0 0	0	3	0 0	16	19	14 15	13	10	10 10
Alpha-1-antitrypsin	50363217	0	0	0 0	0	0	0 0	11	9	6 7	6	4	4 4
Galectin-3-binding protein	5031863	0	0	0 0	3	3	3 3	13	11	14 13	10	10	8 8
Mesothelin isoform 1 preproprotein	53988378	4	3	4 3	3	2	3 2	0	0	0 0	0	0	0 0
Secreted protein, acidic, cysteine-rich	4507171	0	0	0 0	0	0	0 0	4	6	5 6	0	0	0 0

[a] Proteins were selected based on those that showed a significant increase in abundance in the more aggressive cell lines.

[b] MS/MS spectra of peptides were searched against the RefSeq NCBI database using the Mascot search engine. The numbers represent the peptides detected in each preparation for the protein. The number of peptides approximates the amount of protein present.

Table 2

List of selected[a] secreted proteins observed across the MCF10 cell lines, documented by their position in the Mascot search list and the combined number of peptide matched in the combined search data [b].

	NCBInr gi #	MCF10A [e]		MCF10AT		MCF10 DCIS.com [c]		MCF10CA cl. D	
		Mascot position	Sequences matched	Mascot position	Sequences matched	Mascot position	Sequences matched	Mascot position	Sequences matched
	4502101	10	42	6	46	14	32	3	83
rm 2 [c]	4757756	3	63	3	80	3	114	2	134
rotein	4503143	88	13	21	47	9	66	19	93
1 [c]	42716297	197	3	5	65				
	4503117	70	9	169	4	151	4	79	24
sor	4503107			25	22	25	21	87	17
ecursor	4758116	189	1	96	5	39	16	90	11
	4503571	1	94	2	100	4	85	1	188
g protein (LGAL3BP)	5031863			76	18	2	133	7	75
ferase [c]	4504183	4	34	4	42	19	22	23	40
y, member E [c]	10800144	6	51	16	57	8	48	5	111
	38455402					6	76		
proprotein (LOX)	20149540			118	4	116	7	332	2
n 1 preproprotein	53988378	46	24	77	16				
isoform 1 [c]	10835063	22	37	10	62	24	21	8	105
merase isoform 1	10863927	5	61	14	58	22	34	9	119
	32455266	37	21	34	19	44	16	47	35
ator inhibitor type 1,	24307907					167	9	161	10
	4826898	26	29	11	36	30	23	4	97
rm a	13325075			175	2	7	56	42	19
ling protein A13	5174659	231	4					202	3
ling protein A14	10190712	210	2			45	18	66	13
ling protein A16	17933772	156	2	140	1	20	22	68	13
ling protein A2	5174661	110	12			75	28	46	32
ling protein A6	7657532	84	25	91	11	119	11	112	17
ling protein P	5174663	16	21	170	6			113	12
related protein 1	56117838					113	15	286	9
calcium-binding 2	11545873					13	29	45	27
acidic, cysteine-rich RC) [d]	4507171					31	40		2
) proteinase inhibitor,	50363217					12	55	81	14
) proteinase inhibitor, n)	4505789	238	5	213	3			231	13
nhibitor (AACT)	50659080			65	8	1	323	6	87
rsor	55749480			89	3	68	7	162	6
1 precursor	40317626	118	5	93	8	34	23	136	15
f metalloproteinase 1	4507509	201	2	46	10	46	11	53	23
wth factor	4507467	176	10	1	176	126	1	41	34
protein 1 (ZAG)	4502337	24	28	94	5	61	8		

re selected from over 200 from each proteome. The selection was based on their appearance high on the Mascot list and/or their significant change across cell lines.

[b] Data from all search files from each secreted proteome (5 fractions each preparation, 3 preparations with 2 analyzed in duplicate) were combined and searched with Mascot against the RefSeq database from NCBI. The "most abundant" protein with the highest score is listed as Mascot position #1. The number of peptides include those that matched across all LC-MS/MS analyses. If a protein was not detected above the threshold score, a blank entry is used.

[c] These proteins, while considered cytosolic, are predicted to be secreted by non-classical mechanisms using SecretomeP.

[d] The SPARC in MCF10CA gave a Mascot score one point below the threshold and thus did not receive a position. It did have two peptides, one of which was manually sequenced and uniquely identified the protein.

[e] The second most abundant protein in MCF10A and fifth in MCF10 DCIS.com was a keratin. This was consistently present in the three preparations of these cell lines.

Table 3

Comparative data from the aggressive MA11 and MT-1 cell lines of major secreted proteins observed in the MCF10 isogenic series. For comparison, the data from the aggressive MCF10CA cl. D cell line are appended. Clearly the galectin-3-binding protein is most consistently secreted in all three aggressive cell lines. SPARC was very weak in all three lines and is thus not presented.

Protein name	NCBI gi #	MA11	MT-1	MCF10CA cl. D
		Mascot position	Mascot position	Mascot position
Alpha-1-antichymotrypsin	50659080	>100	>100	6
Alpha-1-antitrypsin	50363217	>100	>100	81
Galectin-3-binding protein	5031863	6	1	7
Mesothelin isoform 1	53988378	10	23	>100
Enolase 1	4503571	1	3	1
Peptidylprolyl isomerase isoform 1	10863927	3	2	9