Identifying differentially secreted proteins using metabolic incorporation of stable isotopes (SILAC)

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Secreted proteins—autocrines, growth factors, and receptor fragments among them—affect cell cycle, migration, and repair.[1,2] They are shed upon the stimulation of specific proteases[3] or through concerted exocytosis pathways.[4] Receptors for cytokines and autocrines are promising therapeutic targets, but the autocrines themselves are present at nanomolar concentrations in a background of extracellular matrix (ECM) proteins.[5] Thus, even though changes in autocrine concentrations may mark receptor stimulatory events, they are difficult to detect. The ECM is an aggregate of prior secretions that have coalesced enzymatically into a complex and dynamic scaffold upon which the cells communicate and survive.[6] We describe a means to disentangle the relative contributions of normal and malignant primary epithelial breast cells from the same patient to secreted proteome differences measured in the extracellular milieu. To do so, we make use of stable isotope labeling by amino acids in cell culture (SILAC), an unbiased metabolic labeling strategy. SILAC provides the means to quantify differential expression among hundreds of gene products. In our own study, ~80% of the proteins characterized were classified as secreted in protein databases or in recent literature reports. Interestingly, protease inhibitors, including serpins, tissue inhibitor of metalloproteases 1 and 2, and plasminogen activator inhibitor 1, as well as ligands suppressive to angiogenesis receptors, were significantly downregulated in breast cancer cells. ECM proteins such as collagens and osteoblast-specific factor 2 are upregulated in cancer cells. We have validated several markers using western blotting and immunohistochemistry. We believe that relatively modest changes in the protocol would allow its adaptation to the study of other primary cell lines or even stem cells undergoing transformation.

INTRODUCTION

Cytokines, autocrines, hormones, and components of the extracellular matrix (ECM) are secreted either via a concerted exocytosis pathway [4] or following stimulated shedding events driven by newly activated proteases.[3] Secreted or shed proteins often play a pivotal role in biological transformations of neighboring cells and are potential targets for protein therapeutics.[5]

Secreted proteins are often glycosylated and commonly have an amino acid signal located at the N-terminus of the nascent protein.[7] The initial stages of glycosylation are conducted as the protein enters the lumen of the endoplasmic reticulum, where the export signal sequence is also trimmed. The mature glycosylation pattern evolves as the protein transits the Golgi apparatus and, ultimately, joins budding vesicles that carry the cargo and merge with the plasma membrane.[8] Some shed proteins become integrally bound to the ECM or act as paracrines to influence the behavior of remote cells. Within the ECM, secreted proteins are diluted by six or more orders of magnitude relative to cellular vesicles and are subject to oxidation, proteolysis, glycosylation, crosslinking, and other forms of posttranslational modification.

Conventional proteomic techniques may be hampered by the complexity and broad dynamic range of such samples. Without a favorable strategy, the odds of identifying minute changes in the secretion pattern of autocrines, growth factors, and receptor fragments are diminished by the abundance of serum and ECM proteins.[6]

SILAC strategy. Stable isotope labeling with amino acids in cell culture (SILAC) allows clear quantification of cellular aspects that differ between two phenotypes.[9–11] SILAC labels proteins with either normal light or isotopically heavy amino acids using the natural metabolic machinery of the cell. Stable isotope incorporation occurs as the proteins are assembled in the cell, so that the light and heavy forms are effectively labeled at every site of arginine or lysine incorporation. Moreover, the resulting polypeptides are chemically identical and comigrate in physical methods (such as SDS-PAGE, isoelectric focusing, or liquid chromatography (LC)). This eliminates quantification error due to unequal sampling in analytical separations.[12–15] Still, the peptides are isotopically distinct, so that light and heavy forms are easy to distinguish in mass spectrometry (MS) experiments. Though cell populations (or equimolar lysates of them) are mixed at harvest, the relative intensity of sequence-

matched peptide pairs can be used to disentangle differential protein expression or modifications resulting from stimulus. The correlation between peptides and their aboriginal protein is determined from the dissociation pattern of mass-selected, activated peptides.

Other quantitative strategies such as isotope-coded affinity tags (ICAT), iTRAQ™ reagents (Applied Biosystems), and difference in-gel electrophoresis (DIGE) apply chemical tags to cell lysates. By contrast, SILAC is simple to use because metabolic labeling is (a) passive and complete, such that cellular proteins are stoichiometrically enriched in the heavy or light amino acid supplied to the respective growth media and (b) compatible with any lysis buffer or workflow.[16] Though SILAC is primarily applicable to cell cultures, experiments have been performed in tissues,[17,18] parasites,[19] and animals.[20] SILAC has also been employed to characterize the relative expression of secretomes between two human pancreatic cell lines.[21] A general review of quantitative isotope labeling strategies applied to diverse organisms is available.[22]

Herein, we describe the use of SILAC to distinguish differential secreted protein expression from normal and malignant primary epithelial breast cells obtained from the same patient. This is possible because, despite their low copy number, secreted proteins from SILAC experiments bear exact-mass correlated labels even before they are diluted into the ECM. Even deeper proteome coverage can be obtained with affinity fractionation steps [6] or glycopeptide enrichment procedures,[23] which are beyond the scope of this discussion.

MATERIALS AND METHODS

Materials. NuPAGE® gels and premade buffers, SimplyBlue™ SafeStain, the SILAC™ Protein ID and Quantitation Media Kit Lysine (DMEM-Flex), dialyzed fetal bovine serum (FBS), and epidermal growth factor (EGF) were obtained from Invitrogen. Ultracentrifugal filter units with 5,000 Da cutoff were purchased from Millipore. Aprotinin, leupeptin hemisulfate, phenylmethanesulfonyl fluoride, and insulin were purchased from Sigma. Trypsin was obtained from Promega. Normal (HTB-125™) and malignant (HTB-126™) primary endothelial breast cells, isolated from a 74-year-old female with breast carcinoma, were purchased from the American Type Culture Collection.

Cell labeling. SILAC culturing and analysis of crude membrane fractions of this particular cell line has recently been described.[24] Briefly, normal breast cells were maintained in DMEM medium containing dialyzed 10% FBS and 30 ng/ml EGF light L-lysine and light L-arginine (light medium), whereas malignant cells were maintained in DMEM medium supplemented with dialyzed 10% FBS, heavy [U-$^{13}C_6$] L-lysine, and heavy [U-$^{13}C_6$, $^{15}N_4$] L-arginine (heavy medium) from the kit. The cells were allowed to grow for six doublings to achieve maximum incorporation of exogenous amino acids into proteins.

To verify full incorporation of light or heavy amino acids, aliquots of normal and malignant breast cells (approximately 10^5 cells each) were harvested separately for matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS analysis. A detailed protocol can be found at www.invitrogen.com/content/sfs/brochures/SILAC-ApplicationNote-2.1.pdf.

Sample preparation. When analyzing differential expression, the density of normal and malignant cell populations is determined empirically and the cell mixtures normalized by direct count. Two 100 mm plates each of SILAC-labeled normal (~10^6 cells/100 mm dish) and malignant (~10^6 cells/100 mm dish) breast cells were washed three times with phosphate-buffered saline and incubated for 24 to 48 hours with serum-free SILAC DMEM media supplemented with either light or heavy amino acids (see www.invitrogen.com/content/sfs/brochures/SILAC-ApplicationNote-2.1.pdf for more details). Culture media (20 ml each) from normal and malignant cells were mixed and centrifuged at 2,500 × g for 10 minutes to remove cell debris. The supernatant was concentrated to approximately 0.5–1 ml with a centrifugal filter device (5,000 Da cutoff) and then dried using a centrifugal vacuum concentrator. Pellets were dissolved in 100 µl of 2X SDS sample buffer containing 50 mM DTT and heated at 95°C for 15 minutes, followed by SDS-PAGE fractionation and Coomassie staining. An entire gel lane was excised into approximately 40 sections and digested with trypsin as described previously.[24] Tryptic peptides were extracted from the gel and dried with a centrifugal vacuum concentrator.

Mass spectrometry and data analysis. Dry tryptic peptides were resuspended in 20 µl of 10% acetonitrile in 0.1% formic acid and then analyzed by nanoelectrospray LC-MS on a Q-Tof™ API-US or Q-Tof Premier™ instrument (Waters Corporation). An Atlantis™ dC_{18}, 3 µm, 100 µm × 100 mm column (Waters Corporation) was used for peptide separation. A gradient of 5–45% (v/v) acetonitrile in 0.1% formic acid over 45 minutes, then 45–95% acetonitrile in 0.1% formic acid over 5 minutes, was used. Each single survey scan was followed by up to four component MS/MS scans to acquire MS/MS data with a 1.9 second scan time.

For database searching, tandem mass spectra were extracted, charge state deconvolved, and deisotoped by Mascot Distiller version 2.1.7. All MS/MS samples were analyzed using Mascot (Matrix Science; version 2.2.03) and X! Tandem (www.thegpm.org; version 2007.01.01.1),[25] which were set up to search the December 12, 2007, version of the IPI database, which may be accessed by FTP (ftp://ftp.ebi.ac.uk/pub/databases/IPI/msipi/current/). The search was restricted to *Homo sapiens* (69,832 entries), assuming the digestion enzyme trypsin and up to one missed cleavage. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 200 ppm. $^{13}C_6$-labeled lysine, $^{13}C_6$- and $^{15}N_4$-labeled arginine, and oxidation of methionine were specified in Mascot and X! Tandem as variable modifications. Mascot and X! Tandem output showed peptides labeled with light or heavy lysine, arginine, or both. A total of 370 proteins were assigned, and a complete compilation has been submitted elsewhere. Here, we illustrate expression analysis for very low–abundance proteins, such as growth factors, with a few examples from this larger study.

Scaffold software (version Scaffold-01_07_00, Proteome Software Inc.) was used to validate MS/MS-based peptide and protein identifications. Peptide sequence assignments were accepted only if they earned a confidence ranking of >95.0% as established by the Peptide Prophet algorithm.[26] Protein identifications were accepted if they could be established at >90.0% probability and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.[27] These criteria were sufficient to restrict the False Discovery Rate to <1% overall as determined by simultaneous searches against a randomized database of equal size. Homologous proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Quantification of peptide pairs was performed individually by examining both MS and MS/MS spectra.

RESULTS AND DISCUSSION

SILAC protocol for secreted factors. In SILAC, duplicate cell cultures are maintained in culture media containing light (normal) or heavy isotope–labeled (^{13}C and ^{15}N) amino acids, which are incorporated using the normal metabolic machinery of the cells. Within six doublings, the amino acids are fully incorporated throughout the sequence of every protein in growing cells. At this point, a stimulus may be applied to either the light or heavy culture and, following an incubation period, the cells are mixed 1:1 or may be lysed immediately and mixed after normalizing for the abundance of common housekeeping proteins. After mixing, the proteins may be cleaved, sorted, or selectively enriched using a variety of methods. The peptides, which

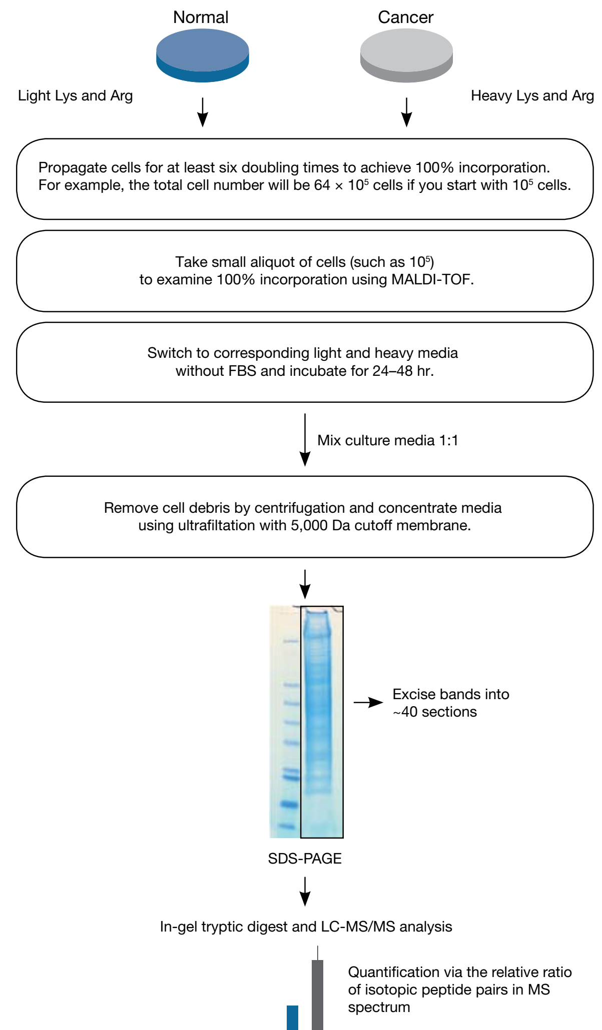

Figure 1—Experimental outline for quantifying differential secreted protein expression using the SILAC approach. Normal and cancer cells were grown for at least six doubling times in media supplemented with dialyzed FBS plus either light (normal) lysine and arginine, or heavy isotope–labeled lysine and arginine, and then switched to corresponding light and heavy media without FBS. Culture media were mixed 1:1 and centrifuged to remove cell debris, concentrated using ultrafiltration devices, and the proteins in the media separated by SDS-PAGE. The entire gel lane was divided into approximately 40 sections and subjected to tryptic digestion. The tryptic peptides were analyzed by nanoelectrospray LC-MS/MS, and the protein precursors were identified using Mascot Server. The quantification of relative protein expression was determined by the chromatographic response observed for each isotopic peptide pair in the mass spectrum.

are paired by sequence, cannot be resolved by biological assays or analytical migration. However, because they are isotopically distinct (the heavy isotopic forms of lysine and arginine differ by 6 Da and 10 Da, respectively), they are readily distinguished using conventional MS methods, and tandem MS methods can definitively assign the parent proteins. Hence, one may disentangle complex networks that alter their expression pattern or become reorganized as a specific response to a stimulus.[28]

Figure 1 illustrates the use of the SILAC procedure to study secreted or shed proteins in normal and malignant cells. Because all proteins, regardless of their copy number, are labeled at multiple sites, we can inspect the total ion current recorded by the mass spectrometer (the ion chromatogram) to identify sequence-matched peptides arising from the two mixed cell states (or phenotypes). From the relative abundance of their constituent peptides, we can identify protein expression changes that accumulated during serum starvation. Peptides from unaffected proteins will be present in the spectrum at equal abundance, while the affected proteins can be distinguished by the relative peak areas of their constituent peptide pairs.

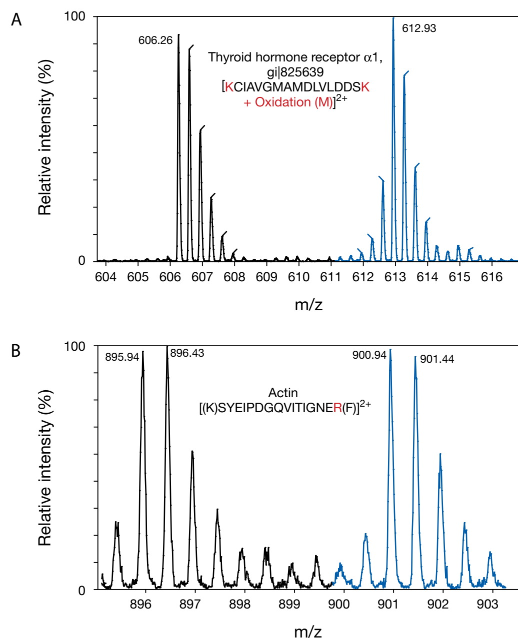

Figure 2—Secreted proteins in equal abundance in light and heavy labeled media. Peptides appear as sequence-matched pairs separated by a fixed mass offset. If protein expression does not differ between the samples grown in light and heavy media (**A**), or if the peptide is due to contamination by an abundant cytosolic protein such as actin (**B**), recovered peptides appear at a 1:1 ratio.

This approach to SILAC is designed to minimize the effect of contaminating serum components (which would introduce a light labeled background to both cell states) by including a step of careful washing and overnight serum starvation. The media may be supplemented with defined growth factors (e.g., insulin, selenium, and T3) during this time to keep the cells healthy as their secretions accumulate in the absence of serum.[29,30]

SILAC differentiates affected targets from background. As described earlier, target proteins are easily identified by examining the total ion chromatogram while looking for mass-paired peak ratios that differ between treated and control samples. Metabolic labeling experiments are ideal for identifying proteins differentially secreted in culture because their peptides appear as sequence-matched pairs separated by a fixed mass offset. Recovered peptides appear at a 1:1 ratio if expression of the precursor protein is not affected by the phenotype change caused by the stimulus. Figure 2A illustrates a doubly-charged tryptic peptide from thyroid hormone receptor α1, KCIAVGMAMDLVLDDSK. The matched response demonstrates that this particular protein does not vary in expression between the tumor and epithelial cells. Figure 2B shows a peptide pair from actin, SYEIPDGQVITIGNER, representative of abundant cytosolic (or housekeeping) proteins that can contribute to background contamination. A matched response here demonstrates that media volumes were carefully controlled prior to mixing. It is also worth noting that common contaminants from media and cytosol (albumin, heat shock proteins, tubulin, actin, etc.) represent <9% of the 53,404 MS/MS spectra acquired during five replicate experiments. The procedure therefore indeed appears to yield predominantly secreted proteins.

Metabolic labels increase reproducibility. The achievable precision is a major concern for any method that is used to identify relevant biomarkers against an overwhelming background of ECM proteins. SILAC metabolic labeling offers a major advantage in that every protein incorporates the amino acid label (heavy lysine or arginine) at multiple sites along the protein backbone. It has also been noted that the interconversion of arginine and proline during creatine synthesis results in the presence of an additional satellite peak that shadows proline-containing peptides arising from heavy arginine–labeled media. Proline-containing peptides from heavy arginine–labeled media appear in the spectra with a series of heavy isotopic satellites positioned toward higher mass at 3 and 2 m/z units above

the double and triple charge states, respectively. This is because proline intermediates along the citric acid cycle retain five ^{13}C atoms and one ^{15}N atom from each arginine. Accordingly, the protein expression ratio determined from proline-containing peptides may be brought into line with the sibling peptides from the same protein by summing the peak intensities of these mass channels. This improves the assignment confidence and allows precision estimates from several independent measurements of all tryptic peptides that may be assigned to the same protein.

For example, Figure 3 illustrates three peptides from transketolase (P29401), a vitamin B1–dependent enzyme linking the pentose phosphate pathway with the glycolytic pathway. It plays a key role in the alternative salvage pathway of pyrimidine synthesis in tissues under acute stress. It has been noted that total transketolase (TK1 and TK2) levels in the serum of breast cancer patients demonstrate a significant positive correlation with cancer stage.[31] Moreover, elevated levels of circulating transketolase are associated significantly ($P < 0.003$) with recurrence of breast cancer in tamoxifen-managed patients.[32] Figure 3 also illustrates the advantage of having multiple measures of expression derived from the sibling peptides of a single protein. The average fold change (calculated with inclusion of the proline satellites) was 3.3 with a relative standard deviation of 8%. These standard deviations are not out of the ordinary provided the protein is represented with at least three peptides observed across replicate experiments.

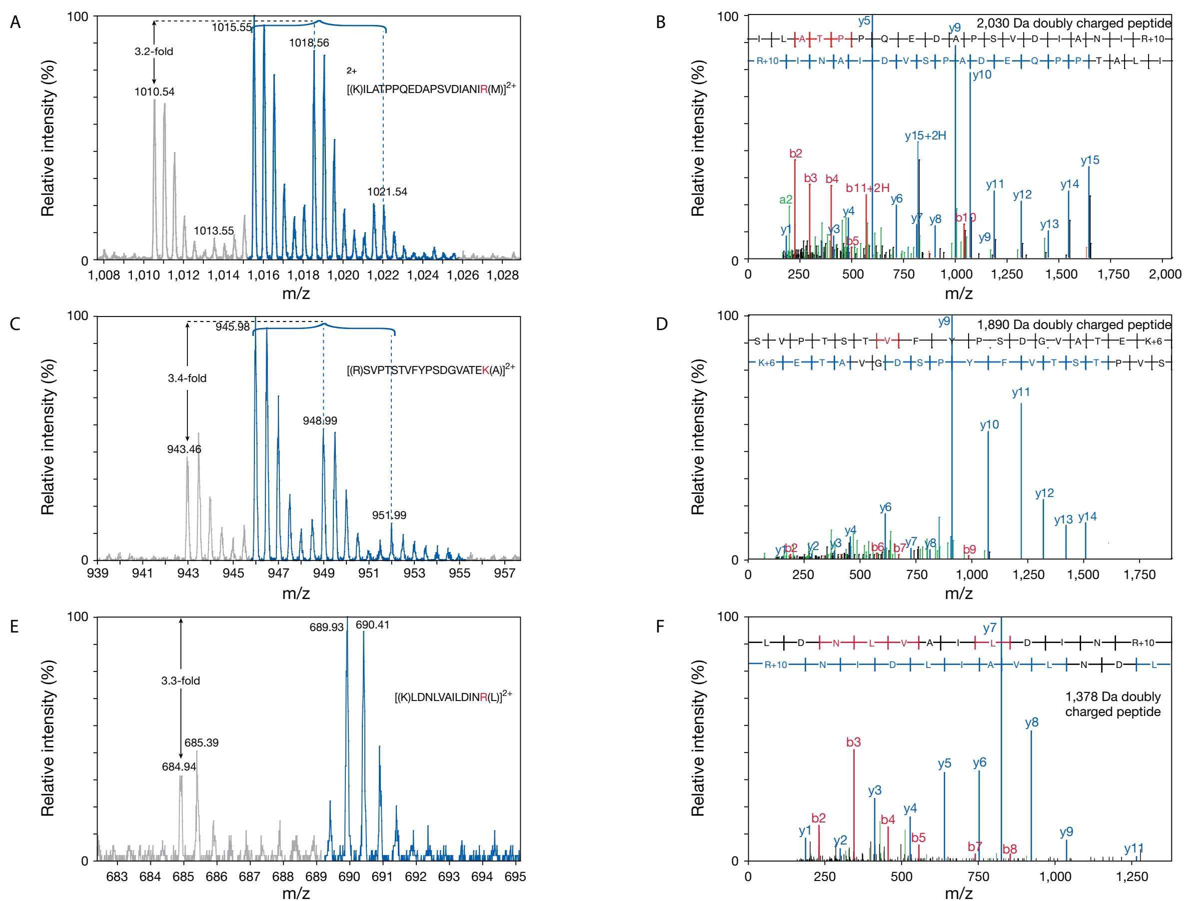

Figure 3—Reproducibility of analyzing secreted proteins using SILAC. Three of the tryptic peptide pairs from transketolase secreted from light and heavy labeled normal and malignant breast cells were analyzed by LC-MS/MS. (**A**,**C**,**E**) The mass spectra were summed across the duration of peak elution. (**B**,**D**,**F**) The corresponding mass-selected fragmentation patterns for the parent ions. Ion fragments labeled in red are due to peptide cleavage at a given amide bond numbered from the amino terminus (so-called b-ions), whereas ions indicated in blue are due to cleavage at a given amide bond numbered from the carboxyl terminus (so-called y-ions). Overlapping ion assignments often extend from both ends of the peptide to confirm identification. The precision of quantification was determined by averaging the relative peak intensities (including peaks due to heavy proline satellites) across peptide pairs from each protein, three examples of which are shown. The results indicate that the protein is downregulated 3.3 ± 0.28 fold (95% confidence using a one-sided *t*-test).

Examples of differential protein expression. The majority (77%) of the assigned proteins were classified as secreted in protein databases and literature reports, while 11% were unknown or hypothetical proteins. Figures 4A–D show the total ion chromatogram traces for some peptides that are associated with significantly increased or decreased protein expression levels in malignant cells.

Figure 4A shows increased expression (6.4-fold) of nephroblastoma overexpressed gene (NovH, accession number P48745), which encodes a cysteine-rich growth factor that is overexpressed in nephroblastomas. It is a member of the CCN family of proteins that includes connective tissue growth factor. NovH increases cell adhesion and migration of glioblastoma cells via matrix metalloprotease 3 expression and a platelet-derived growth factor receptor α–dependent mechanism.[33] The CCN proteins encoded by this set of genes are known as immediate-early genes, because they are expressed after induction by mitogen growth factors or certain oncogenes.

The upregulation (~>10-fold) of CA-125 (accession number Q8WX17) is illustrated in Figure 4B. The CA-125 protein is a membrane-localized proteoglycan that makes a single pass through the plasma membrane. The shedding of this protein's enormous extracellular mass is thought to be triggered by phosphorylation of the cytosolic C-terminus,[34] and its specific binding to mesothelin has been implicated in cell–cell adherence, while the shedding of its proximal domain may increase the motility of the cell.[35] The CA-125 protein is known as a marker for ovarian cancer, but it is also elevated in metastatic breast cancers.[36]

The downregulation (>12-fold) of brain-specific angiogenesis inhibitor 2 (BAI2) is depicted in Figure 4C. BAI2 (accession number O60241) is a transmembrane protein involved in the inhibition of blood capillary growth. Isoform 1 is expressed principally in the brain, but the other homologs are distributed in several tissues. The GA-binding protein γ chain (GABPγ) associates with the cytoplasmic domain of BAI2, while GABPα/γ or GABPα/β work as transcriptional repressors of vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis. Decreased expression of BAI2 is therefore accompanied by decreased free GABPα and GABPγ, which elicits an increase in VEGF expression and subsequent angiogenesis.[37,38]

The downregulation (6.8-fold) of pigment epithelium-derived factor (PEDF) (accession number P36955), also known as serpin F1, is shown in Figure 4D. PEDF is a secreted neurotrophic protein that induces extensive neuronal

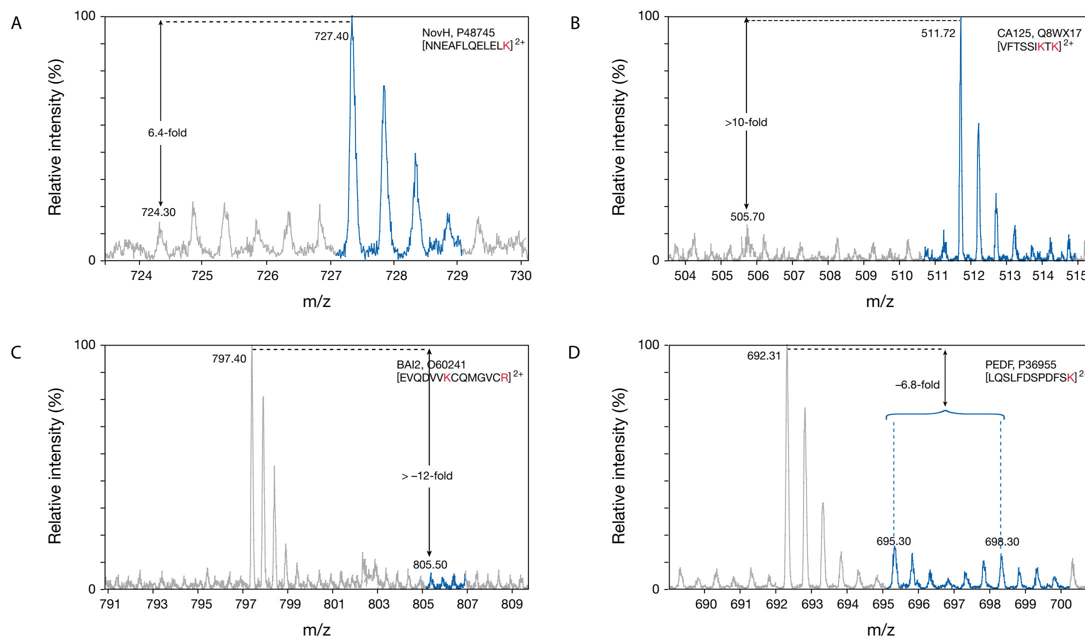

Figure 4—Examples of differential expression of secreted proteins. Normal and malignant breast cells were analyzed for differential secreted protein expression using SILAC and MS analysis as described. Based on the MS analysis, NovH protein (**A**) and CA-125 protein (**B**) are upregulated, while BAI2 (**C**) and PEDF (**D**) are downregulated in the malignant cells.

differentiation in retinoblastoma cells. Recently, investigators have shown that protein kinase A and the protein kinase CK2 regulate the balance between neurotrophic and potent angiostatic effectors via phosphorylation of PEDF.[39,40] Interestingly, recent reports suggest that unlike the transcriptional control of VEGF exerted by BAI2, PEDF inhibits angiogenesis via regulated intracellular proteolysis of the C-terminal region of VEGF receptor 1.[41] Moreover, *in vivo* gene transfer of PEDF inhibits tumor growth in syngeneic murine models of thoracic malignancies[42] so that its suppression in malignant epithelia is consistent with activation of an invasive phenotype. This study also characterized the significant downregulation of another secreted serpin, serpin E2, which is a protease inhibitor with activity toward trypsin, thrombin, plasmin, urokinase-type plasminogen activator, and other serine proteases. It has been reported that serpin E2 promotes ECM production and local invasion of pancreatic tumors *in vivo*.[43]

Validating differential protein expression. Once differential protein expression is detected using SILAC, it is extremely important to validate the results. One approach is to propagate the malignant cells from our example in culture medium containing the light label, and the normal cells in heavy label media, and reacquire the data set. Significant regulatory events should appear with relative peak areas inversely correlated to the original data set. Another approach is to verify significant targets by western blotting, reference to publicly available literature, or, preferably, immunohistochemical staining in human tissue sections. These approaches to validation are only touched on here.

The Secreted Protein Database (http://spd.cbi.pku.edu.cn) is a curated collection of secreted human, mouse, and rat proteins. Of the 370 proteins assigned in this study, 62% were classified as secreted according to this particular database. Since the size of the database is still relatively small, a literature search was carried out for the rest of the proteins. We discovered that an additional 25% of the proteins are reported as secreted in the literature. It should be noted that not all the proteins identified are known to be secreted. The major contaminants are cytoskeletal and cytosolic proteins, which probably originated from dead cells. Based on trypan blue staining, less than 8% cell death was observed after 48 hour incubation in serum-free media.

Approximately 37% of proteins identified in our study are ECM and cytoskeletal proteins, such as collagens, vimentin, laminin, fibronectin, extracellular matrix protein 1, annexin, filamin, lumican, plectin, and osteoblast-specific factor 2. Extracellular proteins are known to modulate integrin-ECM interactions and to regulate proliferation, adhesion, migration, and invasion of cancer cells.[44] A number of the proteins we identified are involved in metabolism and signal transduction, such as the aforementioned NovH and BIA2, as well as adrenomedullin, Notch4 inhibin βA chain precursor, and galectin-3. Approximately 4% of the proteins we identified belong to growth factor, cytokine, or chemokine families, including bone-derived growth factors, CYR61 protein, colony-stimulating factor, growth arrest-specific protein 6, stem cell growth factor, tumor necrosis factor, transforming growth factor-β, migration inhibitory factor, migration stimulation factor, motility accessory factor, PEDF, and proliferation-inducing gene 2. Transforming growth factor-β signaling is involved in many cellular processes including cell growth, cell differentiation, apoptosis, and cellular homeostasis.

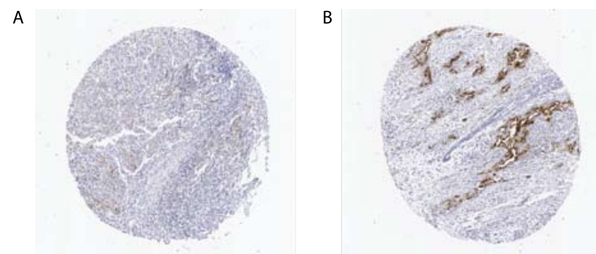

Figure 5—Validation of differential protein expression using immunohistochemistry. In this experiment, formalin-fixed paraffin-embedded sections from normal (**A**) and malignant (**B**) human breast tissues was subjected to a immunohistochemical staining protocol using monoclonal mouse anti–human CA-125 antibody. Compared to the normal tissue section, the lumen of milk ducts of the malignant tissue section shows strong positive staining. Figure obtained with permission from the Human Protein Atlas website (www.proteinatlas.org).[45]

An example of immunohistochemical validation from the Human Protein Atlas website (www.proteinatlas.org) is shown in Figure 5, which illustrates immunohistochemical staining for CA-125 protein, which is upregulated in breast cancer cells (Figure 4B). Strong positive immunohistochemical staining for CA-125 protein is observed on the lumen of milk ducts in malignant breast tissue sections, compared to normal tissue sections taken from a 50-year-old female.[45]

CONCLUSION

We have demonstrated a simple and effective SILAC scheme for profiling nanomolar amounts of secreted and shed proteins that are present in an abundance of background proteins. Using the SILAC labeling method, a total of 370 proteins were identified and quantified from normal and

malignant cells. The majority (~80%) of the identified proteins were classified as secreted in protein databases and literature reports, while 11% were unknown or hypothetical proteins. Interestingly, protease inhibitors such as plasminogen activator inhibitor 1 were significantly downregulated, whereas ECM proteins such as collagens were upregulated in breast cancer cells, consistent with an elementary phenotype of tumor invasion and metastasis. Several of the targets can be validated by western blotting or by reference to publicly available literature and databases. These results indicate that the SILAC approach described here may provide a general means for the identification of diagnostic biomarkers and prove to be an effective first step in defining disease biomarkers in a rational and targeted fashion.

In summary, SILAC metabolic labeling creates structurally matched peptide pairs that flag expression changes by their relative abundance. A further stage of simplification, such as immunoprecipitation or affinity enrichment, should yield even more comprehensive coverage of targeted complexes.

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