

Using SILAC to Quantitate Differential Secreted Protein Expression in Normal and Malignant Breast Cancer Cells

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Introduction

Proteins such as cytokines, autocrines, hormones, digestive enzymes, and components of the extracellular matrix (ECM) are secreted using a concerted exocytosis pathway (1) or are shed upon the stimulation of specific proteases (2). These secreted or shed proteins often play a pivotal role in biological transformations and are potential targets for protein therapeutics (3).

Most secreted proteins are predicted to have a 70 amino acid signal peptide located at the N-terminus of the nascent protein (4). The initial stages of glycosylation are conducted as the protein enters the lumen of the endoplasmic reticulum (ER), where the signal sequence is cleaved. The mature glycan pattern evolves as the protein transits through the Golgi apparatus and, ultimately, budding vesicles containing the glycosylated protein cargo merge with the plasma membrane (5). In this process, the secreted and shed proteins are released into the bloodstream or extracellular fluid where they are diluted by six or more orders of magnitude and subjected to proteolysis (6).

Secreted or shed proteins are present at nanomolar concentrations in an abundant background of ECM and serum proteins. Conventional proteomic techniques may be limited by the complexity and broad dynamic range of such samples (7). Without a more favorable strategy, the odds of identifying minute changes in the secretion pattern of autocrines or growth factors can be remote due to the abundance of serum proteins and ECM.

SILAC strategy

Stable isotope labeling by amino acids in cell culture (SILAC) is an emerging technology for quantitative proteomics that allows clear quantification of cellular aspects that differ between two phenotypes (8, 9, 10, 11). SILAC uses the normal metabolic machinery of the cell to label proteins with light (normal) or heavy (isotope-labeled) amino acids. Proteins and peptides containing the light or heavy amino acids are chemically identical and therefore comigrate in any separation method (such as SDS-PAGE, isoelectric focusing, or liquid chromatography), eliminating quantification errors due to unequal sampling (12). However, because the peptides are isotopically distinct, the light and heavy peptides are easily distinguishable by mass spectrometry (MS). Based on the relative intensity of the isotopic peptide pairs in MS, you can easily quantify differential protein expression and the status of posttranslational modification between two different samples (13). Despite their low copy number, secreted or shed proteins contain the isotopic labels even before they are diluted into the ECM. For example, if shedding of a particular ectodomain protein is repressed by a stimulus, the sequence-matched satellite peptide pairs produced will appear unequal in area. The correlation between a particular peptide and its aboriginal protein is determined from the fragmentation pattern (MS/MS profile).

Compared to other quantitative proteomic strategies such as ICAT[®], iTRAQ[™], and DIGE that apply chemical tags to the cell lysate, SILAC is simple and easy to use, compatible with any lysis buffer or separation method, and provides almost 100% labeling efficiency (14). Though SILAC is primarily applicable in cell culture, experiments have been performed in tissues (15), parasites (16), animals (17), and other biological systems (18).

This application note describes the use of SILAC to distinguish differential secreted protein expression within an abundance of background proteins from normal and malignant primary endothelial breast cells obtained from the same patient. Proteome coverage can be extended with affinity fractionation steps (7) or glycopeptide procedures (19).

Materials and methods

Materials

NuPAGE[®] gels and premade buffers, SimplyBlue[™] SafeStain, SILAC[™] Protein ID and Quantitation Media Kit Lysine (DMEM-Flex), dialyzed FBS, and epidermal growth factor (EGF) were obtained from Invitrogen. Ultracentrifugal filter units with 5,000 Da molecular weight cutoff were purchased from Millipore. Aprotinin, leupeptin hemisulfate, phenylmethanesulfonyl fluoride (PMSF), 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 female with breast carcinoma, were purchased from the American Type Culture Collection.

Cell labeling

SILAC culturing and analysis of crude membrane fractions for normal (HTB-125[™]) and malignant (HTB-126[™]) breast cells has recently been described (20). Briefly, normal breast cells were maintained in DMEM medium containing 10% dialyzed 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 10% dialyzed FBS, heavy [U-¹³C₆] L-lysine, and heavy [U-¹³C₆, ¹⁵N₄] L-arginine (heavy medium). The cells were allowed to grow for six doubling times to achieve maximum incorporation of heavy amino acids into proteins.

To verify incorporation of light or heavy amino acids, aliquots of normal and malignant breast cells (approximate 10⁵ cells each) were harvested separately. See the appendix for a detailed protocol.

Sample preparation

When analyzing differential expression, the density of normal and malignant cell populations is determined empirically and the cell mixtures are normalized by direct count. Two sets of 100 mm plates, each containing SILAC-labeled normal (~10⁶ cells/plate) or malignant (~10⁶ cells/plate) breast cells, were washed three times with phosphate-buffered saline and incubated for 24–48 hours with serum-free SILAC DMEM Labeling Media supplemented with light or heavy amino acids (see appendix for more detail). Twenty milliliters each of culture media 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 molecular weight 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 and Coomassie staining. An entire gel lane was excised into approximately 40 sections and digested with trypsin as described previously (20). 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 analyzed by nanoelectrospray LC-MS on a Q-TOF instrument (Waters Corporation). Peptide separation was performed on the Atlantis[™] dC18 (3 mm, 100 mm × 100 mm) column (Waters Corporation) using a gradient of 5–45% (v/v) acetonitrile in 0.1% formic acid over 45 minutes, and then 45–95% acetonitrile in 0.1% formic acid over 5 minutes. Four components were used to acquire MS/MS data with 1.9 s scan time.

Raw data files from the Q-TOF instrument were processed with Daemon (Version 2.1.7, Matrix Science, London) and searched against the NCBI database using the Mascot search algorithm (21). No smoothing or baseline subtraction was applied to the raw spectra. The peptides were constrained to be tryptic with one missed cleavage allowed. Oxidation of methionine resi-

due, heavy Lys, and heavy Arg with delta mass of 6 Da and 10 Da, respectively, were selected for variable modification. The mass tolerance of the precursor peptide ion was set at 50 ppm, and the mass tolerance of the MS/MS fragment ions was set at 0.2 Da. The Mascot output showed peptides labeled with light or heavy Lys and/or Arg. To compile the summary of identified proteins (data not shown), we employed the Protein Prophet and Peptide Prophet algorithms, as implemented in version 1.05 of Scaffold (Proteome Software, Portland, OR) (22). We required 95% confidence for individual peptides and a minimum protein confidence of 80%. A similar number of proteins were identified using a threshold model for Mascot scores. Quantitation of peptide pairs was done and validated manually by examining the MS and MS/MS spectra.

Results

SILAC protocol for secreted or shed proteins

SILAC is a systems biology approach to measuring differential protein expression induced by a stimulus. Using the normal metabolic machinery of the cells, duplicate cell cultures are labeled with light (normal) and heavy (isotope-labeled, ^{13}C and ^{15}N) amino acids introduced into the culture media. Within six doublings, the amino acids are fully incorporated throughout the sequences of every protein in growing cells. At this point, a stimulus may be applied to the light or heavy medium followed by an incubation period, and then the cells are mixed and lysed. The proteins can be processed together using any protein separation method, eliminating quantification errors due to unequal sampling. The peptides are chemically identical and cannot be resolved by biological assays or analytical migration. However, because they are isotopically distinct, they can be easily distinguished by mass using MS analysis (the heavy isotopic forms of Lys and Arg differ by 6 Da and 10 Da, respectively, from normal Lys and Arg). This analysis enables disentanglement of networks that reorganize as a specific response to a stimulus (23).

Figure 1 illustrates the use of the SILAC procedure to study secreted or shed proteins in normal and malignant cells. Since all proteins are labeled at multiple sites regardless of their copy number, 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). Also, from the relative abundance of their constituent peptides, we can identify protein expression changes derived from cellular adaptation. Unaffected proteins will be present in the spectrum at equal abundance, while the affected proteins are distinguished by the differences in their peak areas.

The SILAC procedure is designed to minimize the effect of interfering serum components by including a step of careful washing and overnight serum starvation. The media may be supplemented with defined growth factors (e.g., insulin, sele-

nium) during this time to keep the cells healthy as their secretions accumulate in the absence of serum (24). The following day, culture medium is harvested, and the secreted or shed proteins are concentrated using a low molecular weight cutoff membrane as described in the appendix.

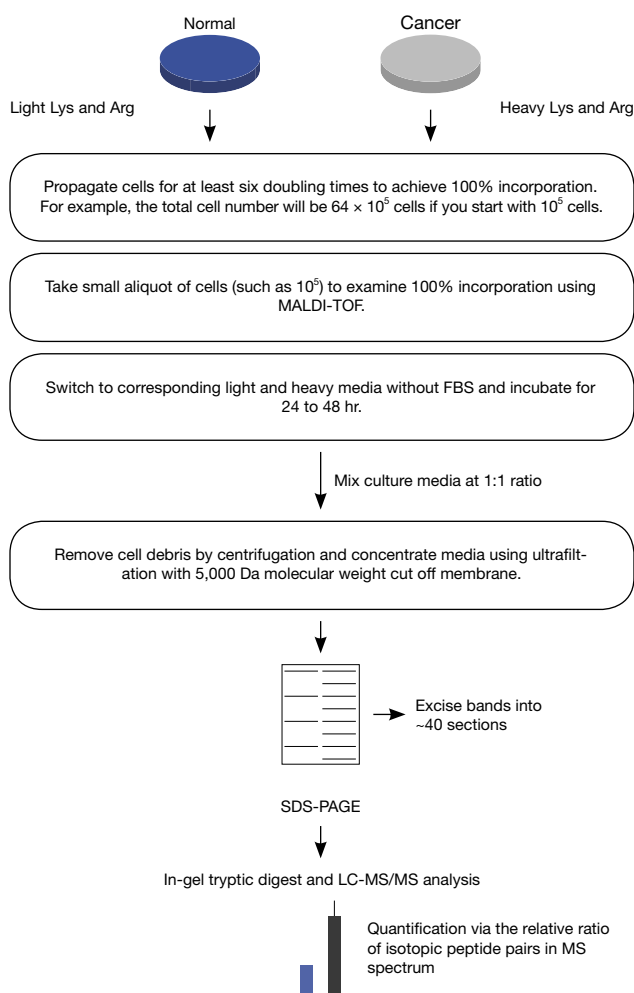


Figure 1—Experimental outline for differential secreted protein expression using the SILAC approach. Normal and cancer cells were grown for at least six doubling times in media supplemented with dialyzed FBS plus either light (normal) Lys and Arg, or heavy (isotope-labeled) Lys and Arg, and then switched to corresponding light and heavy media without FBS. Culture media were mixed 1:1 and centrifuged to remove cell debris. Media were concentrated using ultrafiltration devices, and samples were analyzed 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 MS.

SILAC distinguishes 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 especially advantageous in identifying proteins secreted in culture because peptides naturally expressed appear as sequence-matched pairs separated by a fixed mass offset. If protein expression does not differ between the two states, recovered peptides appear at a 1:1 ratio. Figure 2 shows a peptide pair, KCIAVGMAMDLVLDDSK (light and heavy forms) derived from the thyroid hormone receptor alpha 1 protein from human, representative of the unaffected background. The peptide pairs do not vary in their expression ratios between the two states.

Metabolic labeling increases 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 Lys or Arg) at multiple sites along the protein backbone. Following trypsin digestion, most peptides carry a single label at their C-terminus, allowing identification of several peptides from the same protein. This improves the coverage confidence and allows precision estimates from several independent measurements. For example, Figure 3 illustrates three peptides from pregnancy-specific beta-1-glycoprotein 4, a member of the CEA (carcinoembryonic antigen) family. Our results show that the protein is down-regulated in this lineage of malignant breast cells by 7.7 ± 0.8 fold. Pregnancy-specific beta-1-glycoprotein is a secreted protein that has been reported to be expressed by placental syncytiotrophoblasts, but its precise function is unknown (25).

As illustrated in Figure 3, when transcriptional turnover is associated with proteins secreted from the malignant state, a signature of differential abundance is reflected in the chromatographic MS trace.

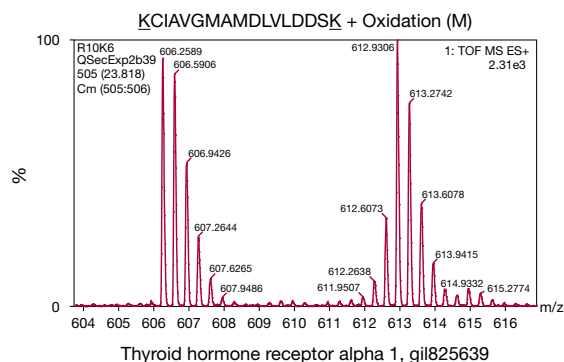


Figure 2—Equal abundance of secreted proteins from light and heavy labeled media. Metabolic labeling experiments are especially advantageous in identifying proteins secreted in culture because peptides naturally expressed appear as sequence-matched pairs separated by a fixed mass offset. If protein expression does not differ between the two states, recovered peptides appear at a 1:1 ratio. Hence, SILAC “tags” readily distinguish interesting targets from background.

Examples of differential protein expression

Using SILAC for quantitation of differential protein expression in normal and malignant cells, we have identified and quantified a total of 428 proteins. The majority (85%) of the identified proteins were classified as of secreted type 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 (7.4-fold) of NOV (nephroblastoma overexpressed gene) protein (accession number P48745). The NOV protein is a member of the CCN family of proteins and is a cysteine-rich secreted protein that is over-expressed in nephroblastomas. The NOV protein increases cell adhesion and migration of glioblastoma cells via matrix metallo-protease 3 expression and a PDGFR-alpha dependent mechanism (26). The CCN proteins are encoded by a set of immediate-early

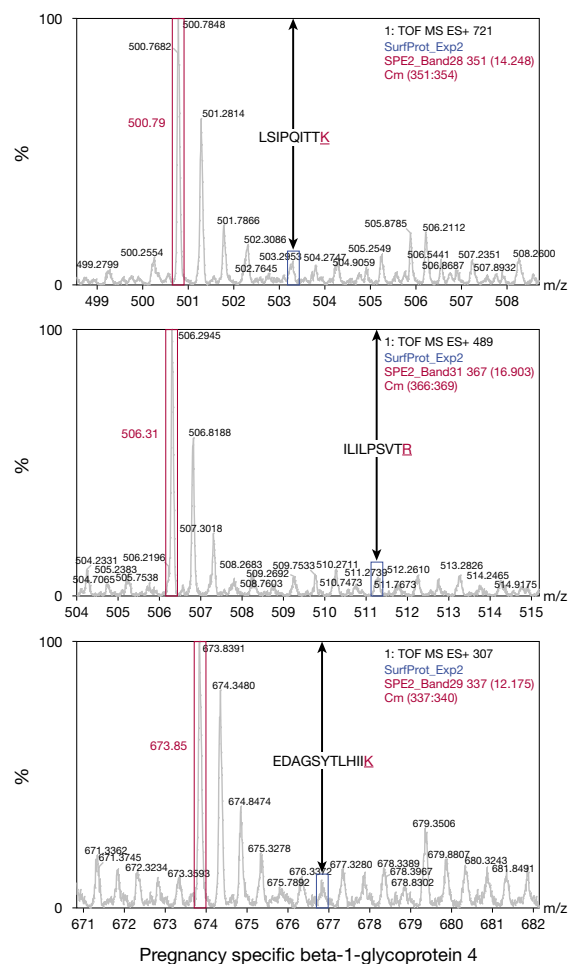


Figure 3—Reproducibility of analyzing secreted proteins using SILAC. Four tryptic peptide pairs from pregnancy-specific beta-1-glycoprotein 4 secreted from light and heavy labeled normal and diseased cells were analyzed by LC-MS/MS. The reproducibility of quantitation was determined by averaging the relative peak intensities for all peptide pairs, three examples of which are shown. The results indicate that the protein is down-regulated 7.7 ± 0.8 fold in malignant breast cells studied.

genes and are expressed after induction by mitogen growth factors or certain oncogenes.

The up-regulation (9.2-fold) of CA-125 protein (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 and is a product of the MUC16 gene. The shedding of the protein's enormous extracellular mass is thought to be triggered by phosphorylation of the cytosolic C-terminus (27), 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 (28). The CA-125 protein is known as a marker for ovarian cancer, but is also known to be elevated in metastatic breast cancers (29).

The down-regulation (12.7-fold) of brain-specific angiogenesis inhibitor 2 (BAI-2) is depicted in Figure 4C. The brain-specific angiogenesis inhibitor 2 (accession number O60241) is a trans-membrane 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 gamma chain (GABPγ) associates with the cytoplasmic domain of BAI-2, while GABPα/γ or GABPα/β work as transcriptional repressors of VEGF, a potent stimulator of angiogenesis. Decreased expression of BAI-2 is therefore accompanied

by decreased free GABPα and GABPγ which elicits an increase in VEGF expression and subsequent angiogenesis (30, 31).

The down-regulation (6.8-fold) of serpin F1 (accession number P36955), also known as pigment epithelium-derived factor (PEDF), is shown in Figure 4D.

Serpin F1 is a secreted, neurotrophic protein; which induces extensive neuronal differentiation in retinoblastoma cells. Recently, investigators have shown that PKA and CK2 regulate the balance between neurotrophic and potent anti-angiogenesis effects via phosphorylation of PEDF (32). Interestingly, recent reports suggest, that unlike the transcriptional control of VEGF exerted by BAI-2, PEDF inhibits angiogenesis via regulated intracellular proteolysis of the C-terminal region of vascular endothelial growth factor receptor 1 (33). Moreover, *in vivo* gene transfer of PEDF inhibits tumor growth in syngeneic murine models of thoracic malignancies (34) so that its suppression in malignant epithelia is not inconsistent with activation of an invasive phenotype. This study also characterized the significant down-regulation (12) of another secreted serpin, serpin E2, which is a protease inhibitor with activity towards trypsin, thrombin, plasmin, uPA, and other serine proteases. It has been reported that serpin E2 promotes extracellular matrix production and local invasion of pancreatic tumors *in vivo* (35).

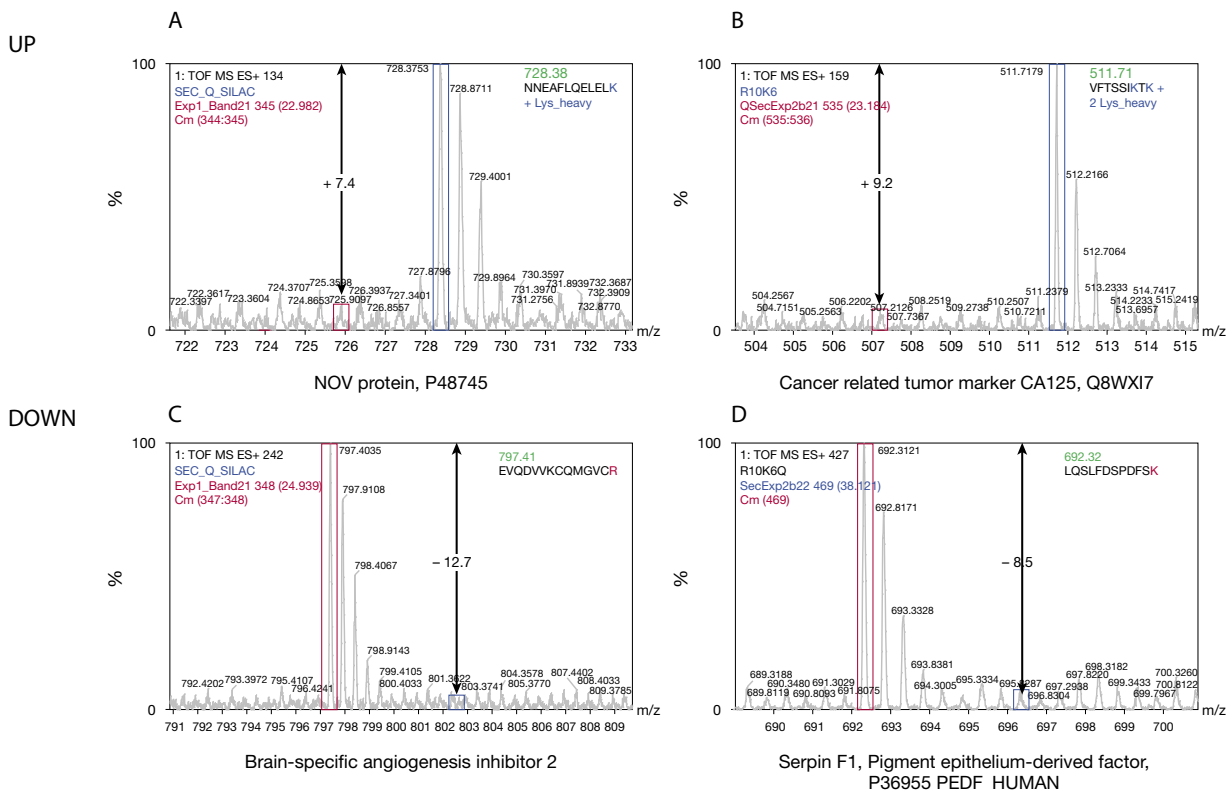


Figure 4—Examples of differential expression of secreted proteins. Normal and malignant cells were analyzed for differential secreted protein expression using SILAC and MS analysis as described in the Appendix. Based on the MS analysis, NOV protein (A) and CA-125 protein (B) are up-regulated, while BAI-2 (C) and PEDF (D) are down-regulated in breast cancer cells.

Validating differential protein expression

Once differential protein expression is detected using SILAC, it is extremely important to validate the results using various approaches. One approach demonstrated here is to propagate the malignant cells from our example in culture medium containing the light label and reacquire the data set. Significant regulatory events should appear with relative peak areas inversely correlated to the original data set (data not shown). Another approach is to verify significant targets by western blotting or verify selected targets by references to publicly available literature and databases. An example of the latter from the ProteinAtlas website (www.proteinatlas.org) is shown in Figure 5, which illustrates immunohistochemical staining for CA-125 protein, which is up-regulated 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 (36).

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 428 proteins were identified and quantitated from normal and malignant cells. The majority (85%) 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 and pregnancy zone protein precursor, among others, were significantly down-regulated, whereas extracellular matrix proteins such as

collagens were up-regulated in breast cancer cells suggesting a basic model for tumor invasion and metastasis.

Several of the targets were validated by western blotting (data not shown) or by references 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 a more comprehensive coverage of targeted complexes (14,23).



Figure 5—Validation of differential protein expression using immunohistochemistry. In this experiment, formalin-fixed paraffin-embedded human breast tissue sections from normal and malignant tissues obtained from a 50-year-old female was subjected to the 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 ProteinAtlas website (www.proteinatlas.org), Persson, A. et al. (2006) A human protein atlas based on antibody proteomics. *Curr Opin Mol Ther* 8(3):185-90.

Ordering information

Product	Quantity	Cat. no.
SILAC™ Protein Identification and Quantitation Media Kit with [U- ¹³ C ₆]-L-Lysine (*Lys) and DMEM-Flex	1 kit	MS10030
with [U- ¹³ C ₆]-L-Lysine (*Lys) and RPMI-Flex	1 kit	MS10031
with [U- ¹³ C ₆]-L-Lysine (*Lys) and IMDM-Flex	1 kit	MS10032
with [U- ¹³ C ₆]-L-Lysine (*Lys) and Advanced DMEM/F-12-Flex	1 kit	MS10033
SILAC™ Membrane Protein Identification and Quantitation Kit with [U- ¹³ C ₆]-L-Lysine (*Lys) and DMEM	1 kit	SM10002
with [U- ¹³ C ₆]-L-Lysine (*Lys) and RPMI 1640	1 kit	SM10006
SILAC™ [U- ¹³ C ₆ , ¹⁵ N ₂]-L-Arginine (*Arg)	100 mg	MS10009
SILAC™ [U- ¹³ C ₆]-L-Arginine (*Arg)	100 mg	MS10011

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Appendix

SILAC protocol to study secreted proteins (secretome) or metabolomics

Use the modified SILAC protocol below to study secreted proteins or metabolomics.

Serum-Free SILAC DMEM Labeling Medium and SILAC DMEM Growth Medium

You will need 3 x 1 L SILAC depleted DMEM basal medium for this application. Prepare 2 x 1 L serum-free SILAC Light or Heavy Labeling Media (contains SILAC-depleted DMEM basal medium supplied with each SILAC™ kit supplemented with 100 mg/ml L-lysine, 100 mg/ml L-arginine, and 100 X L-glutamine) as described below. Perform all steps in a tissue culture hood under sterile conditions.

1. Set aside 1 L of SILAC depleted DMEM basal medium for washing cells prior to switching cells to serum-free medium.
2. Resuspend 100 mg L-lysine HCl and 100 mg [U-¹³C₆]-L-lysine (*Lys) each in 1 ml basal, unsupplemented DMEM medium supplied with the kit. Mix well until completely dissolved.
3. Resuspend 100 mg L-arginine from each vial (2 vials are supplied in the kit) in 1 ml basal, unsupplemented DMEM medium each supplied with the kit. Mix well until completely dissolved.
Note: If you are using double labeled arginine (available separately from Invitrogen, see ordering information), resuspend 100 mg [U-¹³C₆, ¹⁵N_{2]-L-arginine (*Arg) or 100 mg [U-¹³C₆]-L-arginine (*Arg) in 1 ml basal, unsupplemented DMEM supplied with the kit. Mix well until completely dissolved.}
4. To one 1 L bottle of DMEM from Step 3, add L-lysine HCl (100 mg/ml) from Step 1 and L-arginine (100 mg/ml) from Step 2 to prepare light DMEM medium supplemented with Light (normal) lysine and arginine. Mix well and mark the bottle appropriately.

- To the second 1 L bottle of DMEM from Step 3, add *Lys (100 mg/ml) from Step 1 and L-arginine (100 mg/ml) from Step 2 to prepare DMEM single labeling medium supplemented with light arginine and heavy (isotope labeled) lysine. Mix well and mark the bottle appropriately.
Optional: If you are preparing double labeled medium, add *Lys (100 mg/ml) from Step 1 and *Arg (100 mg/ml) from Step 2 to prepare DMEM double labeling medium supplemented with heavy (isotope labeled) arginine and lysine. Mix well and mark the bottle appropriately.
- To each 1 L medium bottle, add 10 ml 100X L-glutamine supplied with the kit.
- Optional:** Add 10 ml 100X Penicillin-Streptomycin, if needed (highly recommended). You may supplement the medium with additional growth factors or cytokines, if needed for your specific cell line.
- Transfer 400 ml of each Serum-free SILAC Light and Heavy Labeling Media to 0.22 µm filtration device and filter sterilize each medium.
- To the remaining 600 ml of each Serum-free SILAC Light and Heavy Labeling Media, add 70 ml dialyzed FBS and 0.7 ml SILAC™ Phenol Red Solution (10 g/L) to prepare SILAC Light and Heavy Growth Media. Filter-sterilize each medium using 0.22 µm filtration device and mark the Light and Heavy Growth Media.
- Store each medium at 2 to 8°C, protected from light until use. The medium is stable for 6 months when properly stored (avoid introducing any contamination into the medium).

Labeling and cell culture

- Harvest the normal and malignant cells separately and transfer an appropriate volume of cell suspension in two separate sterile 15 ml conical tubes to obtain 1×10^5 cells per tube.
- Centrifuge the cells at $1,000 \times g$ for 5 minutes at room temperature.
- Aspirate the medium and resuspend the cells as follows:
 - Tube 1: Resuspend the cells in SILAC Growth Medium containing light lysine and grow in 60 mm culture dishes for adherent cells or 10 ml culture flasks.
 - Tube 2: Resuspend the cells in 3 ml SILAC Growth Medium containing heavy lysine and grow in 60 mm culture dishes for adherent cells or 10 ml culture flasks.
- Incubate the flasks or dishes in a 37°C incubator containing a humidified atmosphere of 8% CO₂.
- Change the medium or split the cells every 3–4 days (depending on the cell line) using the appropriate SILAC Light or Heavy Growth Medium. Cells grow at a similar rate in each media.
- Expand each cell population for at least six doubling times to achieve >95% incorporation of labeled amino acid into the proteins.
- After six doublings, harvest a small aliquot of cells ($\sim 1 \times 10^6$ cells) from each cell population to determine the efficiency of incorporation (10).
 - Lyse each cell pellet separately in 500 µl 1X NuPAGE® LDS Sample Buffer and 50 µl NuPAGE® Reducing Agent (10X). Heat the samples at 70°C for 8–10 minutes.
 - Load the samples from light and heavy medium side by side on a NuPAGE® Novex® 4–12% Bis-Tris Gel and perform electrophoresis.
 - Stain the gel with Coomassie® R-250 Stain.
 - Excise 3–4 side-by-side protein bands from each lane.
 - Perform in-gel trypsin digestion.
 - Perform MS analysis. The MS analysis should show an increase in mass by 6 Da for peptides labeled with isotope-labeled Lys when compared to peptides labeled with normal Lys. If you used double labeling with isotope-labeled Arg and isotope-labeled Lys, the MS analysis should show an increase in mass by 6 and 10 Da for peptides labeled with heavy Lys and Arg, respectively, when compared to peptides labeled with normal (light) Lys and Arg.
- At the end of six doublings, you will have 6.4×10^6 cells for each cell population.

Secretome analysis

After verifying 100% incorporation of heavy amino acids into proteins, perform quantitative experiments between two stages (normal and diseased) of cells.

- For secretome analysis, use two 100 mm plates each of normal and diseased cells.
- When cells reach 80% confluency, wash cells 2–3 times with SILAC depleted DMEM basal medium (third bottle of media) to remove all the dialyzed FBS.
- Incubate the normal and diseased cells in the corresponding Serum-free SILAC Light or Heavy Labeling Media for 24–48 hr.
Most immortalized cell lines grow properly in serum-free media for 24–48 hr. For primary cell culture, you can add Insulin-Transferrin-Selenium Supplement (Cat. no. 51500-056) at 1:1,000 dilution to Serum-free Labeling Media to enhance cell survival.
- Harvest Serum-free SILAC Light and Heavy Labeling Media (20 ml each) and mix the media at 1:1 ratio.
- Remove any cell debris by centrifugation at $2,500 \times g$ and concentrate the supernatant to about 0.5–1.0 ml using ultrafiltration device using a 5,000 Da molecular weight cutoff membrane.
- Dry the concentrated media using centrifugal vacuum concentrator.
- Dissolve the protein sample in 2X NuPAGE® LDS Sample Buffer containing 50 mM DTT, heat at 95°C for 30 minutes (longer time is needed to completely dissolve the pellets).
- Analyze the samples by NuPAGE® SDS-PAGE. Protein bands are stained with SimplyBlue™ SafeStain, excised, and digested with trypsin. Tryptic peptides are analyzed by LC-MS/MS (see methods).
- Analyze the relative ratio of isotopic peptide pairs in the MS spectrum to quantitate differential protein expression. Quantification is performed manually at this point as software for quantification is not well established yet.

Note: Add the following text (including the " character) at the end of the Mascot Modification File to enable identification of proteins and isotopic peptide pairs for SILAC.

```
"
Title: Lys_light
Residues: K 128.09497 128.1741
"
```

```
"
Title: Lys_heavy
Residues: K 134.09497 134.1741
"
```

```
"
Title: Arg_light
Residues: R 156.10112 156.1875
"
```

```
"
Title: Arg_heavy
Residues: R 166.10112 166.1875
"
```

This will show isotope labeled lysine, [U-¹³C₆]-L-lysine, 6 Da larger than normal lysine (light lysine) and isotope labeled arginine, [U-¹³C₆, ¹⁵N₄]-L-arginine, 10 Da larger than normal arginine (light arginine).