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Large-Scale Analysis of Breast Cancer-Related Conformational Changes in Proteins using SILAC-SPROX

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Abstract

Proteomic methods for disease state characterization and biomarker discovery have traditionally utilized quantitative mass spectrometry methods to identify proteins with altered expression levels in disease states. Here we report on the large-scale use of protein folding stability measurements to characterize different subtypes of breast cancer using the Stable Isotope Labeling with Amino Acids in Cell Culture and Stability of Proteins from Rates of Oxidation (SILAC-SPROX) technique. Protein folding stability differences were studied in a comparison of two luminal breast cancer subtypes, luminal-A and -B (i.e., MCF-7 and BT-474 cells, respectively), and in a comparison of a luminal-A and basal subtype of the disease (i.e., MCF-7 and MDA-MB-468 cells, respectively). The 242 and 445 protein hits identified with altered stabilities in these comparative analyses, included a large fraction with no significant expression level changes. This suggests thermodynamic stability measurements create a new avenue for protein biomarker discovery. A number of the identified protein hits are known from other biochemical studies to play a role in tumorigenesis and cancer progression. This not only substantiates the biological significance of the protein hits identified using the SILAC-SPROX approach, but it also helps elucidate the molecular basis for their dysregulation and/or dysfunction in cancer.

TOC

Keywords

MCF-7; BT-474; MDA-MB-468; protein folding; chemical denaturation

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INTRODUCTION

Protein biomarker discover efforts in breast cancer and other disease states have typically relied on the large-scale protein expression level profiling strategies using various mass spectroscopy-based proteomic approaches. Such protein biomarker studies of breast cancer have included analyses of cell culture models^{1–8} and clinical specimens of the disease (e.g., breast tissues,^{6, 9–13} and various types of biofluids^{14–19}). The results of these studies have helped provide a better understanding of the biology of breast cancer, but they have not been as useful as expected at identifying disease-related proteins that can be exploited for diagnostic and therapeutic purposes. One possible reason for this may be the indirect link between a protein's expression level and its function, i.e., disease-related changes in protein function may not always be reflected in the changes in their abundance. Therefore, global profiling of a more functionally relevant property of proteins derived from disease-related samples has the potential to provide better insight into the molecular basis of the disease and to produce more useful biomarkers.

Protein folding stability is a useful property for such high-throughput profiling because changes in protein folding stability can result from a number of biologically significant phenomena such as mutations, modifications and altered interactions with cellular targets. Recently, this property has been utilized to identify age-related differences in mouse brain proteins²⁰ and to uncover novel molecular signatures of breast cancer using several well-established cell culture models of the disease.^{21–22} Using the SILAC-SPROX approach it was possible to differentiate the MCF-7, MCF-10A and MDA-MB-231 cell lines based on the global/sub-global unfolding properties of the proteins in these cell lines.²¹ Similarly, a SILAC-LiP approach successfully differentiated the MCF-7 and MCF-10A cell lines based on the protease susceptibility of the proteins in these two cell lines.²²

Like other cancers, breast cancer is highly heterogeneous in that it can exhibit a number of different biological characteristics. Thus, there are a number of different subtypes of the disease. For example, breast cancer has been classified into five subtypes based on molecular profiling using DNA microarrays: luminal-A, luminal-B, basal-like, HER2-enriched, and normal breast-like groups.^{23–24} As part of this work, we extend our initial SILAC-SPROX study to several additional cancer subtypes in order to identify subtype specific thermodynamic stability profiles. Reported here are the results of SILAC-SPROX analyses performed on the proteins from two cell culture models of the luminal subtypes (i.e., MCF-7 and BT-474 cells) and from a basal subtype (i.e., MDA-MB-468 cells). The luminal subtypes, which are the most common subtypes among breast cancers,²⁵ display a high expression of hormone receptors (estrogen receptor/progesterone receptor, ER/PR) and associated genes, and generally carry a good prognosis. Compared to the luminal-A subtype, the luminal-B subtype exhibits relatively high proliferation rate and less favorable prognosis.²⁶ The basal-like subtype has high expression of basal markers (such as cytokeratins 5, 6, 14, 17) and proliferation related genes and typically no expression of hormone receptors or HER2, a member of the epidermal growth factor family of receptors. Although the basal subtype is only found in ~15% of breast cancer diagnoses, it has been associated with aggressive behavior and poor prognosis.²⁵

The differential stability profiles generated here using the SILAC-SPROX technique²⁷ enabled the analysis of nearly 1000 proteins for breast cancer-related thermodynamic stability differences. Ultimately, 242 and 445 protein hits were identified in the two comparative analyses performed here, which included a comparison of the MCF-7 versus BT474 cell lines and a comparison of the MCF-7 versus MDA-MB-468 cell lines. The results of this work indicate that thermodynamic stability measurements can be used to differentiate breast cancer subtypes and uncover unique and shared molecular features in these subtypes. This work also included the use of a new automated data analysis pipeline for hit identification in SILAC-SPROX experiments.

EXPERIMENTAL

Cell Culture and Cell Lysate Preparation

The breast cancer cell lines BT-474 and MDA-MB-468 were cultured following the American Type Culture Collection (ATCC) guidelines. Heavy SILAC-labeled breast cancer cell line MCF-7 was cultured using heavy-labeled lysine and arginine according to the established protocols (see Supporting Information for additional details).²⁸ The cells were lysed by mechanical disruption in the presence of 1 mm diameter zirconia/silica beads (Biospec) using a Disruptor Genie (Scientific Industries). This involved 10 cycles consisting of 20s of disruption and 1 min of cooling on ice. The lysis buffer was 20 mM phosphate buffer (pH 7.4) containing a cocktail of protease inhibitors that included the following: 1 mM 4-(2-Aminoethyl) benzenesulfonyl uoride hydrochloride (AEBSF), 500 μ M Bestatin, 15 μ M E-64, 20 μ M Leupeptin, and 10 μ M Pepstatin A (Thermo Pierce). Lysed cells were centrifuged at $15,000 \times g$ for 15 min at 4°C and the supernatant was used for subsequent analyses.

SILAC-SPROX Analyses

The SILAC-SPROX technique was used in two different comparative studies (including one involving heavy-labeled MCF-7 and light-labeled BT-474 cell lines and one involving heavy-labeled MCF-7 and light-labeled MDA-MB-468 cell lines). Each comparative analysis was performed in triplicate as previously described.²⁷ Briefly, in each comparison, the total protein concentrations in the light- and heavy- labeled cell lysate samples were determined using a Bradford assay and normalized to the same concentration (4–6 mg/mL total protein depending on the experiment). Aliquots (20 μ L) of the light- and heavy- labeled cell lysate samples were each mixed with 75 μ L of a series of 20 mM phosphate buffers (pH 7.4) containing increasing concentrations of urea (0–9 M). The final urea concentrations ranged from 0 to 7 M. Each mixture was equilibrated for 16– 18 hours, and 5 μ L of 30% (w/w) hydrogen peroxide (Sigma) was added to each of the protein samples in the denaturant-containing buffers. After 6 min, the reaction in each protein sample was quenched with 734 μ L of 375 mM L-methionine (Sigma). The light- and heavy- labeled cell lysate samples that corresponded to the same denaturant concentration were combined, and the proteins were precipitated after addition of 318 μ L of trichloroacetic acid (1g/mL) (Sigma) and an overnight equilibration at 0 °C. The resulting protein pellets were subjected to a quantitative, bottom-up proteomics analysis using SILAC quantitation.

Proteomic Sample Preparation

The protein pellets from each denaturant-containing buffer were dissolved in 60 μ L of 0.5 M triethylammonium bicarbonate (TEAB) buffer (Sigma) containing 0.1% sodium dodecylsulfate (SDS). The disulfide bonds were reduced with 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Thermo Fisher, Inc.) for 1 hour at 60 °C. The samples were then treated with 10 mM methylmethane thiosulfonate (MMTS) (Sigma) for 15 min at room temperature. Ultimately, the protein samples were digested with trypsin using an enzyme/substrate ratio of 1/50 (w/w) at 37 °C with overnight incubation. The proteolytic digestion reaction was quenched upon acidification (pH ~ 2–3) with trifluoroacetic acid (TFA) (Sigma).

LC-MS/MS Analyses

The peptide mixtures were desalted using C18 resin (The Nest Group) according to the manufacturer's protocol. LC-MS/MS analyses were performed on a Q-Exactive Plus high-resolution mass spectrometer (Thermo Scientific, Inc.) with a nanoAcquity UPLC system (Waters Corp.) and a nano-electrospray ionization source. Samples were trapped on a Symmetry C18 300 mm $\times$ 180 μ m trapping column for 3 min at 5 μ L/min (99.9/0.1 v/v water/acetonitrile 0.1% formic acid), and separated on a 75 μ m $\times$ 250 mm column packed with 1.7 μ m Acquity HSST3 C18 stationary phase (Waters Corp.). Peptides were separated using a gradient of 3 to 30% acetonitrile with 0.1% formic acid over 90 min at a flow rate of 0.4 μ L/min. Data collection was performed in a data-dependent acquisition (DDA) mode with a resolution of 70,000 (at m/z 200) for full MS scan from m/z 375–1600 with a target AGC value of 1×10^6 ions, followed by 20 product ion scans at a resolution of 17,500 (at m/z 200), using an AGC target value of 1×10^5 ions, a max fill time of 60 ms, and normalized collision energy of 30 V.

Proteomic Data Analysis

The raw LC-MS/MS data files were searched using MaxQuant 1.5.2.8²⁹ against the 20265 human proteins in the 2014-04 release of the UniProt Knowledgebase downloaded on 5/16/2014. Searches were performed with fixed MMTS modification on cysteine and SILAC labeling of lysine and arginine, variable oxidation of methionine, deamidation of asparagine and glutamine, and acetylation of the protein N-terminus. Trypsin was set as enzyme and up to two missed cleavages allowed. The mass tolerance for precursor ions was set to 20 ppm for the first search where initial mass recalibration was performed, and a 4.5 ppm precursor mass tolerance was used for the main search. The mass tolerance for fragment ions was set to 0.02 Da. Also included were a match between runs and re-quantification of the searched peptides. The rest of the parameters were set at the default settings. In cases where a given peptide was matched to multiple protein isoforms or multiple members of a protein family, the peptide was assigned to the leading razor protein listed by MaxQuant algorithm. The false discovery rate for peptides and proteins identification was set at < 1%. The mass spectrometry proteomics data will be deposited to the Proteomexchange (<http://www.proteomexchange.org>) via the PRIDE partner repository³⁰ with the data set identifiers to be released after publication.

Data analysis was accomplished using a Mathematica 11.0 script that was developed in-house and designed with a data analysis workflow similar to that described in our earlier SILAC-SPROX work.^{21, 27} Briefly, in each biological replicate, only peptides identified with heavy to light (H/L) ratios > 0 were used for the following analysis. To normalize protein expression level differences in the cell lines, the H/L ratios of non-methionine-containing peptides from a given protein were divided by the median H/L ratio of all non-methionine-containing peptides from that protein. The H/L ratios of the methionine-containing peptides were also normalized by the corresponding protein medians. The normalized H/L ratios measured for a given methionine-containing peptide and charge state at a given denaturant concentration were averaged and log 2 transformed. Only methionine-containing peptides identified in 7 or more denaturant concentrations were considered for hit selection, which initially involved identification of methionine-containing peptides with 2 or more consecutive log₂(normalized H/L) values that were significantly different from 0. Log₂(normalized H/L) values that were significantly different from 0 were considered to be those that fell below or above the 5th and 95th percentiles, respectively, of log₂(normalized H/L) values of all the non-methionine-containing peptides detected across all the denaturant concentrations (see Figure S-3). The 5th and 95th percentiles for the log₂(normalized H/L) values varied slightly depending on the biological replicate (see Figure S-3), but were generally close to -1 and 1, respectively. Subsequently, the log₂(normalized H/L) values versus denaturant concentration data obtained for oxidized and wild type methionine-containing peptides with 2 or more consecutive log₂(normalized H/L) values that were significantly different from 0 were fitted to Equations 1 and 2, respectively.

$$\text{Log}_2(H/L) = \text{Log}_2 \left(\frac{1 - A * e^{\frac{-k_{ox}t}{1 + e^{-\frac{\Delta G + \Delta \Delta G + m_1[D]}{RT}}}}}{1 - A * e^{\frac{-k_{ox}t}{1 + e^{-\frac{\Delta G + m_2[D]}{RT}}}}} \right) \quad (1)$$

$$\text{Log}_2(H/L) = \frac{1.44k_{ox}t}{1 + e^{-\frac{\Delta G + m_2[D]}{RT}}} - \frac{1.44k_{ox}t}{1 + e^{-\frac{\Delta G + \Delta \Delta G + m_1[D]}{RT}}} \quad (2)$$

Each equation represents the ratio of the two SPROX curves expected for the heavy- and light- labeled peptide. In each case, A is the extent of the oxidation (i.e., the amplitude of the SPROX curve), k_{ox} is the pseudo-first order rate constant associated with the oxidation reaction of an unprotected methionine residue, which has been previously determined to be 0.0065 s^{-1} when the concentration of H_2O_2 is 0.5 M ,³¹ t is the oxidation reaction time (360 s), G is the protein folding free energy, ΔG is the change in the folding free energy, m_1

and m_2 are $\frac{\delta \Delta G}{\delta [\text{Urea}]}$, representing the steepness of the transition regions of the two SPROX curves, R is the ideal gas constant, and T is temperature. Constraints were applied in order to avoid overfitting and ensure physical significance of the parameters (see Supporting Information).

The goodness of fitting was evaluated by the R^2 extracted from the fitting and a customized deviation using Equation 3.

$$CDev = \frac{\sqrt{\frac{\sum_{i=1}^n (A_{Fit,i} - A_{Exp,i})^2}{n-1}}}{\text{Max}(A_{Exp,i}) - \text{Min}(A_{Exp,i})} \quad (3)$$

In Equation 3, n is the total number of denaturant concentrations identified, $A_{Exp,i}$ is the H/L ratio for the i^{th} data point from experiment, $A_{Fit,i}$ is the H/L ratio for the i^{th} data point calculated from the fitted model. Smaller $CDev$ indicates better fitting. Data sets with $R^2 > 0.8$ and $CDev < 0.2$ were classified as good fitting.

In order to achieve optimal fitting results, the data was fitted an additional n times with the systematic removal of one point, where n is the total number of denaturant concentrations identified. The best regression was selected by the highest R^2 value.

After fitting the data for each methionine-containing peptide with 2 or more consecutive normalized H/L ratios different from 1 in each biological replicate, a single data set was generated for each of the assayed peptides by averaging the well-fit data ($R^2 > 0.8$ and $CDev < 0.2$) from all the biological replicates for that peptide. This averaged data set was again fitted to Equations 1 or 2, as described above. Ultimately, hit peptides were selected as those with averaged data sets that were well-fit to Equations 1 or 2. Transition midpoints (i.e., $C_{1/2}$ values) were calculated from the fitted parameters using Equations 4 and 5 for heavy- and light- labeled peptides, respectively.

$$C_{1/2, \text{Heavy}} = \frac{-0.28 - \Delta G - \Delta \Delta G}{m_1} \quad (4)$$

$$C_{1/2, \text{Light}} = \frac{-0.28 - \Delta G}{m_2} \quad (5)$$

The final output of the Mathematica script consists of an Excel spreadsheet with all the assayed peptides and proteins (Tables S-1, S-2), an Excel spreadsheet with all the identified hits and their respective ΔG , $C_{1/2}$ values (Tables S-3, S-4), and the fitted SILAC-SPROX curves for all the peptide hits (Figures S-1, S-2)

RESULTS

Experimental Design

The experimental workflow used in this study is shown in Figure 1. The cell culture models of breast cancer studied here included the MCF-7, BT-474 and MDA-MB-468 breast cancer cell lines. Each cell line represents a different tumor subtype and has different molecular

features. The MCF-7 cell line is ER+/PR+/HER2- and reflective of the luminal-A subtype of breast cancer. The BT-474 cell line is ER+/PR+/HER2+ and is representative of the luminal-B subtype of breast cancer. The MDA-MB-468 cell line is a basal-like subtype and triple negative (ER-/PR-/HER2-).

Two different comparative analyses were conducted using the SILAC-SPROX protocol²⁷ (Figure 1). In one analysis, the thermodynamic stabilities of proteins in the BT-474 cell lysate were compared to those in the MCF-7 cell lysate. In a second analysis, the thermodynamic stabilities of proteins in the MCF-7 cell lysate were compared to those in the MDA-MB-468 cell lysate. The MCF-7 cell line was heavy-labeled in both comparative analyses. Hit proteins with different stabilities in the two cell lines were identified based on the H/L ratios of the methionine-containing peptides identified and quantified in SILAC-SPROX experiment (Figure 1).

Proteomic Coverage

Summarized in Table 1 is the proteomic coverage obtained in the SILAC-SPROX experiments performed in this work. In total, approximately 3500 methionine-containing peptides from close to 1000 proteins were assayed in the MCF-7 versus BT-474 cell line comparison, and about 3000 methionine-containing peptides from close to 800 proteins were assayed in the MCF-7 versus MDA-MB-468 cell line comparison (Tables S-1, S-2). To be assayed for protein folding stability changes in the SILAC-SPROX experiment, a protein must be consistently identified with a same methionine-containing peptide from at least seven denaturant concentrations in the bottom-up shotgun proteomics analysis. Due to this limitation, the number of peptides and proteins assayed in the SILAC-SPROX experiment is significantly smaller (i.e., ~8-fold lower) than the total number of peptides and proteins successfully identified and quantified in the LC-MS/MS readout (Table 1).

The frequency of methionine residues in protein sequences is about 2.5%. Therefore, a typical protein generally contains multiple methionine residues. In the case of small, single domain proteins with highly cooperative protein unfolding/folding reactions, the different methionine-containing peptide probes detected in the SILAC-SPROX experiment provide redundant biophysical information (i.e., report on the same chemical denaturation behavior). However, in the case of large, multi-domain proteins, the different methionine-containing peptide probes detected in SILAC-SPROX provide unique biophysical information about the specific protein domains to which they map (i.e., report on domain specific chemical denaturation behavior).

Hit Identification

In total, 413 methionine-containing peptides from 257 proteins were identified as hits in the MCF-7 versus BT-474 cell line comparison, and 1012 methionine-containing peptides from 450 proteins were identified as hits in the MCF-7 versus MDA-MB-468 cell line comparison. Representative peptide hits are shown in Figure 2. The peptide hits accounted for 12% and 34% of the assayed methionine-containing peptides in each comparison, respectively. The false positive rate of peptide hit discovery in the current study can be estimated by using the hit selection criteria described here to select hits among the non-

methionine-containing peptides. Such an analysis of the non-methionine-containing peptide data from the two cell line comparisons revealed peptide hit rates of ~5%. This estimate of the false positive rate of peptide hit discovery is similar to the 3.5% false positive rate of peptide hit discovery that was previously determined in a control experiment involving light- and heavy- labeled MCF-7 cells.²⁷ The 12 and 34% peptide hit rates observed in the two cell line comparisons described here are significantly above these estimated false positive rates of peptide hit discovery using the SILAC-SPROX protocol. The hit rate in the MCF-7 versus BT-474 cell line comparison is also similar to that observed in previous SILAC-SPROX analyses of the MCF-10A, MCF-7 and MDA-MB-231 breast cancer cell lines (i.e., 10–12%).²¹ The hit rate in the MCF-7 versus MDA-MB-468 cell line comparison is relatively high.

The hit identification strategy used in this study is identical to that described in reference 21, with the exception that it utilized a regression analysis instead of a visual inspection of the data to select hits. Application of the hit identification strategy described here to the SILAC-SPROX data reported in reference 21 resulted in a large overlap with the peptide hits reported therein (i.e., ~50%, data not shown). The peptide hits in reference 21 that were not identified as hits with the new hit identification strategy primarily included: 1) those with too few data points (i.e., the new regression analysis was only applied to peptides with H/L data from at least seven (instead of six) denaturant concentrations) and 2) those with poor quality data sets (i.e., the data was not well-fit to Equations 1 or 2).

The confidence level associated with a peptide hit identified in SILAC-SPROX is raised when both wild type and oxidized forms of the same peptide are consistently identified as hits (i.e., both forms of the peptide show the same stabilizing (or destabilizing) SILAC-SPROX behavior) (Figures 2A and 2B). The MCF-7 versus BT-474 and MCF-7 versus MDA-MB-468 cell line comparisons performed here identified 39 and 121 such hit peptide pairs (respectively), among which 4 and 103 pairs (respectively) showed consistent behaviors. These consistent peptide pairs act as “internal controls” for the quantitative changes in their thermodynamic stabilities and help substantiate the results reported here. The hit peptide pairs with inconsistent data are likely false positives, and the peptides in these pairs were excluded from the final hit lists used in the following discussion. The final hit lists included 341 peptides from 242 proteins in the MCF-7 versus BT-474 cell line comparison, and 972 peptides from 445 proteins in the MCF-7 versus MDA-MB-468 cell line comparison (Tables S-3, S-4).

The confidence level associated with a peptide hit identified in SILAC-SPROX is also raised when it is identified in multiple biological replicates or with multiple peptide probes that include the same methionine residue. In both of the cell line comparisons performed here, approximately 15% of the methionine-containing peptide hits were identified as hits in two or more biological replicates (Tables S-3, S-4). Unfortunately, the remaining 85% of the methionine-containing peptide hits were only assayed in one biological replicate. However, 22% and 30% of the protein hits from the MCF-7 versus BT-474 and MCF-7 versus MDA-MB-468 cell line comparisons (respectively) were identified with multiple methionine-containing peptide probes, even though the methionine-containing peptides were only identified as a hit in one biological replicate (Tables S-3, S-4). In some cases, multiple

unique peptide sequences sharing the same methionine residue (in the same wild type (or oxidized) form) were assayed. A total of 5 of the 21 and 23 of the 43 sets of overlapping methionine-containing peptides in the MCF-7 versus BT-474 and MCF-7 versus MDA-MB-468 cell line comparisons (respectively) yielded consistent results (Tables S-3, S-4). Approximately, 50% of the protein hits in each comparison were identified with a single peptide hit in one replicate.

Classification of Hit Behavior

The peptide hits identified in this work displayed the same three types of SILAC-SPROX behaviors as those observed in the previous SILAC-SPROX analyses of the MCF-10A, MCF-7 and MDA-MB-231 breast cancer cell lines.²¹ The first type of behavior results when a methionine-containing peptide is globally protected in both cell lines, but the transition midpoints are shifted (see Figures 2A and 2B). The second type of behavior results when a methionine-containing peptide is globally protected in one cell line and it is only locally (or not at all) protected in the other cell line (Figure 2C). The third type of behavior results when a methionine-containing peptide undergoes different degrees of local protection between the two cell lines, or a transition from local protection in one cell line to no protection in the other cell line (Figure 2D). These different types of peptide hits all indicate differential stabilizations of the proteins from which the peptides are derived.

The differential stabilizations in these proteins can result in shifts in the transition midpoints ($C_{1/2}$) and/or changes in the cooperativity of their folding/unfolding reactions (m -values). We note that $C_{1/2}$ and corresponding ΔG values can be determined only for those peptides that come from globally protected regions in proteins (Figures 2A and 2B). Also of note is that the accuracies of the m -value determinations in this work were limited due to the number of data points in each chemical denaturation set.

A total of 106 of the 341 peptide hits identified in the MCF-7 versus BT-474 cell line comparison and 467 of the 972 peptide hits identified in the MCF-7 versus MDA-MB-468 cell line comparison showed the first type of SILAC-SPROX behavior (Figures 2A and 2B) and had sufficient data points for protein stability changes quantification (Tables S-3, S-4). Two-thirds of the 106 peptide hits with quantifiable protein stability changes in the MCF-7 versus BT-474 cell line comparison were the result of a protein stabilization in the BT-474 cell line. This is similar to that observed in the previous SILAC-SPROX analyses of the MCF-10A, MCF-7 and MDA-MB-231 breast cancer cell lines in which ~50% of the protein hits were the result of stabilizations.²¹ Approximately 90% of the protein stabilizations and destabilizations detected in the MCF-7 versus BT-474 cell line comparison were the result of $C_{1/2}$ value shifts of 1.2 to 5.6 M. The median $C_{1/2}$ and ΔG values were 2.7 M and 1.7 kcal/mol, respectively. In contrast, 445 (95%) of the 467 peptide hits with quantifiable protein stability changes in the MCF-7 versus MDA-MB-468 cell line comparison resulted from protein stabilizations in the MDA-MB-468 cells. In general, the magnitudes of the detected stability changes for the MCF-7 versus MDA-MB-468 cell line comparison were similar to those above.

Protein Expression Level Analysis

The median H/L ratio of all non-methionine-containing peptides from a given protein can be used to evaluate the relative protein expression levels between the light- and heavy- labeled cell lines (Tables S-5, S-6). We compared the expression level data generated in this work with that previously reported in the literature for the cell lines in this study¹ and found that they were in general agreement (Figure 3A). We further examined proteins with expression level changes > 2-fold in this work and in reference 1 and also found a large degree of overlap (~35%) between our data and the data in reference 1 (Figures 3B and 3C).

In both of the comparative analyses performed here, ~70% of the protein hits identified with thermodynamic stability differences between the two cell lines did not have significant (i.e., less than 2-fold) changes in their expression levels. This is similar to that observed in the previous SILAC-SPROX analyses of the MCF-10A, MCF-7 and MDA-MB-231 breast cancer cell lines where it was also noted that a large fraction (~45%) of the differentially stabilized proteins identified therein did not have significant changes in their expression levels. These results suggest that protein folding stability can provide information about disease states that is orthogonal to that obtained in protein expression level analyses, and thus may be a useful protein property to exploit in protein biomarker discovery studies.

DISCUSSION

Comparison Between Luminal-A and -B Subtypes

The differentially stabilized proteins identified in the BT-474 versus MCF-7 comparison were subjected to bioinformatics analyses using PANTHER to investigate their potential biological context.^{32–33} The glycolysis pathway was significantly overrepresented (i.e., 2.9-fold) in the protein hits (p-value < 0.05). Increased glycolysis is observed in cancer cells where they use this metabolic pathway for ATP generation (i.e., the Warburg effect). Notably, seven glycolytic enzymes that play important roles in the glycolytic metabolism (i.e., ALDOA, GAPDH, PGK1, PGAM1, ENO1, PKM, and LDHB) were identified with altered thermodynamic stabilities in the BT-474 versus MCF-7 comparison. Considering the close link between protein folding and function, this may indicate an altered glycolytic activity between the two cell lines, which can result from the higher proliferation rate observed in the luminal-B subtype of breast cancer. Indeed, it has been shown that phosphatidylinositol 3-kinase (PI3K) is often activated in ErbB2-overexpressing breast cancer cells and results in an overactive PI3K/AKT/mTOR pathway, which plays a critical role in controlling cell cycle, growth and survival. Such an overactive PI3K/AKT/mTOR pathway has been shown to increase glucose uptake and lead to a switch from mitochondrial respiration to lactate production.³⁴ A previous study has also shown that the PI3K/mTOR/p70^{S6K} signaling pathway plays an enhanced role in the anchorage-independent growth of ErbB2 overexpressing breast cancer cells.³⁵ Inhibition of glycolysis has been suggested to be a promising therapeutic strategy for cancer treatment particularly in the case of cancers showing a high dependence on this bioenergetic metabolism.³⁶

A gene ontology term analysis of the protein hits identified in the BT-474 versus MCF-7 comparison reveals about a 2-fold increase in the fraction of cytoskeletal proteins as

compared to those assayed (Figure 4A). These included 22 of the protein hits described here such as tubulins (e.g., TUBA1B, TUBB4B, TUBB2A and TUBB6) as well as many actin related proteins (e.g., ACTG1, MYL3, MYH9, etc., see Table S-3). These cytoskeleton proteins are highly versatile in the regulation of cell cycle, cellular morphogenesis and cell migration. They are responsible for driving chromosomal separation and cell division in the cell cycle, promoting stable cell-cell adhesions through interactions with cadherins during cellular morphogenesis, and forming protrusions to disseminate to the surrounding tissue during cell migration.³⁷ The observation of cytoskeleton proteins being differentially stabilized in the two luminal subtypes may reflect a greater involvement of this protein class to meet the higher proliferation requirement in the luminal-B subtype.

Proteins with chaperone activity were also enriched in the hits identified in the BT-474 versus MCF-7 comparison (Figure 4A). These included a total of 18 protein hits (see Table S-3). Increased chaperone activity has been shown to be characteristic of tumor growth due to their primary function in facilitating protein folding. HSPs have been shown to have critical functions associated with the cell cycle and proliferative response.^{38–39} It is also interesting to note that six of the eight subunits of T-complex protein 1, which mediates the folding of the major cytoskeletal proteins tubulins and actins and has been implicated in the cancer cell proliferation, were identified as hits in this work.⁴⁰ Our work suggests that the functions of these proteins may be disease state specific and result from differential stabilizations.

Considering the different expression levels of HER2 in the MCF-7 (HER2-) and BT-474 (HER2+) breast cancer cell lines, the protein hits identified in the comparison were interrogated for potential direct and/or indirect interactions with HER2 using the STRING database analysis.⁴¹ Two protein hits (plakoglobin and α -catenin), which are both in the catenin family, are known to have interactions with HER2. The catenin family is responsible for connecting cadherin receptor cytoplasmic domains to actin filaments in adherens junctions of epithelial cells. It includes β -catenin that binds to cytoplasmic domain of cadherin, α -catenin that binds to β -catenin and actin, δ -catenin and γ -catenin (plakoglobin) that is homologous to β -catenin. The cadherin-catenin complex (known as adherens junctions) plays an important role in stabilizing adhesion between cells and regulating cell growth. Direct association of HER2 with β -catenin and plakoglobin has been demonstrated in human cancer cells where the authors suggested that the HER2-mediated signaling might regulate the cell adhesion and invasive growth of cancers.⁴² Additionally, the actin binding protein vinculin was also identified with altered stability in the MCF-7 versus BT-474 cell line comparison. Thus, these protein hits together suggest changes in cell-cell adhesion between the two cell lines, which may be the result of the distinct proliferation signature in the MCF-7 and BT-474 cell lines.

Comparison Between Luminal A and Basal Subtypes

The MCF-7 versus MDA-MB-468 cell line comparison yielded a high hit rate (34%) and a large fraction of protein stabilizations (95%) in the MDA-MB-468 cell line compared to the MCF-7 cell line. This is in contrast to the other cell line comparison performed here as well as to the previous SILAC-SPROX analyses of the MCF-10A, MCF-7 and MDA-MB-231

breast cancer cell lines in reference 21 where approximately equal distribution between protein stabilizations and destabilizations was observed. One explanation for the large number of destabilizations observed in the MCF-7 versus MDA-MB-468 cell line comparison is the more extreme phenotypic differences between the two cell lines. The basal subtype is proliferative, high-grade and associated with a poor prognosis, while the luminal-A subtype is usually low-grade and displays a relatively favorable survival. Our work suggests that this thermodynamic stability signature of destabilizations may be a major biophysical distinction between the luminal-A and basal subtypes that the two cell lines represent. However, additional experiments on more cell lines and tumor tissue samples are needed to further investigate this hypothesis. The protein hits with altered thermodynamic stabilities identified in the MDA-MB-468 versus MCF-7 comparison covered a wide range of protein classes including nucleic acid binding proteins (21%), hydrolase (10%), transferase (9%), enzyme modulator proteins (8%), cytoskeletal proteins (8%), oxidoreductase (7%) and chaperone proteins (7%) (Figure 4B). However, no one class appeared to be overrepresented in the hits.

Notably, fourteen of the protein hits (see Table S-4) identified in the MDA-MB-468 versus MCF-7 comparison are indicated in the ubiquitin proteasome pathway (UPP) as revealed by the PANTHER pathway analysis. The ubiquitin proteasome system is responsible for the degradation of most intracellular proteins and therefore is an important regulator of many critical cellular processes including cell cycle progression, proliferation, differentiation and apoptosis. Since cancer is characterized by uncontrolled cellular proliferation or by a failure of cells to undergo apoptosis, the alteration of proteasome activity has been implicated in several cancers. Increasing evidence has shown that cancer cells exhibit high proteasome activity to support their uncontrolled cell division by getting rid of tumor suppressors such as p53, pro-apoptosis proteins such as Bax and cell cycle inhibitors such as p27.⁴³⁻⁴⁴ Up-regulation of proteins involved in UPP has also been observed in cell transformation and tumorigenesis.⁴⁵ It is interesting to note that only two proteins were annotated with association to the UPP among the differentially stabilized proteins identified in the BT-474 versus MCF-7 comparison. This suggests a similar degree of involvement for UPP in the two luminal subtypes and a very different degree of involvement for UPP between the luminal and basal subtypes of breast cancer.

A major difference between the MCF-7 and MDA-MB-468 cells is that the MCF-7 cells have high expression of hormone receptors while the triple negative MDA-MB-468 cells lack specific cell surface receptors. This difference is likely to contribute to the protein hits observed in our work. Indeed, we found six protein hits that have previously known direct interactions with ER including chaperone proteins (HSPA4, HSPA8, HSP90AA1), 26S proteasome (PSMC5, PSMD1) and MAPK1. These proteins are involved in many different pathways such as those that regulate cell proliferation (the MAPK signal pathway). Changes in the thermodynamic stabilities of these proteins may induce a series of alterations in their downstream targets and the associated pathways, resulting in the high hit rate observed here.

CONCLUSIONS

This study extends the use of thermodynamic stability profiling strategy to more breast cancer subtypes (i.e., luminal-A, luminal-B and basal-like). The use of well-established cell lines in this study provides not only a reliable source of highly homogeneous cells, but also an opportunity to validate the findings in this proof-of-principle work with previous studies involving these cell lines. Indeed, the thermodynamic stability measurements described here successfully differentiated the different cancer subtypes studied here and identified a subset of protein hits, a number of which have been previously found to be biologically relevant to the disease. These protein hits included many that are associated with cell proliferation processes such as cell cycle, growth factor signaling and metabolism, which is one of the major differences among the breast cancer subtypes in this study. Therefore, this study demonstrates the potential of using differential thermodynamic stability profiling as an unbiased approach for the discovery of disease-related biomarkers and the elucidation of biologically important aspects of diseases.

Notably, a significant fraction (~70%) of the protein hits did not have altered protein expression levels. This makes the differential thermodynamic profiling strategy especially attractive because such strategy can not only establish a potential biophysical link between the differentially stabilized proteins and the specific disease states, but also provide information about disease states that is orthogonal to that obtained in protein expression level analyses. Therefore, it can be used to complement protein expression level studies especially when protein expression profiles fail to discriminate some disease states. However, the protein hits identified using such a thermodynamic profiling strategy do need further validation in clinical samples before their translation into diagnostic or therapeutic practice. A promising validation strategy is the recently developed targeted mass spectrometry-based SPROX approach for the detection and quantitation of target methionine-containing peptides,⁴⁶ which will help validate the changes of protein folding stability observed in the cell culture models of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of the experimental workflow used in this work. Shown at the bottom are the protein unfolding curves extracted from the corresponding SILAC-SPROX behaviors shown for a wild type methionine-containing peptide.

Figure 2.

Representative SILAC-SPROX data and associated protein unfolding curves obtained in this work. (A) Data obtained on the peptide AMEVDERPTEQYSDIGGLDK from 26S protease regulatory subunit 6A in the MCF-7 versus MDA-MB-468 cell line comparison. (B) Data obtained on the peptide AM(ox)EVDERPTEQYSDIGGLDK from 26S protease regulatory subunit 6A in the MCF-7 versus MDA-MB-468 cell line comparison. (C) Data obtained on the peptide DHASIQM(ox)NVAEVDKVTGR from 40S ribosomal protein S21 in the MCF-7 versus BT-474 cell line comparison. (D) Data obtained on the peptide NPEEALEDTLNQVMVFK from Cullin-1 in the MCF-7 versus BT-474 cell line

comparison. (E) Data obtained on a subset of the non-methionine-containing peptides from 26S protease regulatory subunit 6A in the MCF-7 versus MDA-MB-468 cell line comparison including: DSYLILETLPTDYDSR (●), EKAPSIIFIDELDAIGTK (■), LKPGDLVGVNK (◆), QTYFLPVIGLVDAEK (▲), VDILDPALLR (▼). In (A) – (D), the solid lines represent the best fit of the data to Equations 1 or 2; the dashed curves represent the extracted SPROX curves; the vertical lines indicate the transition midpoints of the SPROX curves; ‘X’ indicates a data point that was not included in the regression analysis.

Figure 3.

Evaluation of the expression level data generated in this work. (A) Global distribution of the differences between the expression level data generated in this work and those reported in reference 1 for the MCF-7 versus BT-474 (solid line) and MCF-7 versus MDA-MB-468 (dashed line) cell line comparisons. Expression level data reported in reference 1 were processed to generate ratios of protein abundance in the MCF-7 cell line versus the other cell line (BT-474 or MDA-MB-468). (B) and (C) Venn diagrams showing the overlaps between proteins with expression level changes > 2-fold identified in this work and those in reference

1 for the MCF-7 versus BT-474 (B) and MCF-7 versus MDA-MB-468 (C) cell line comparisons.

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Figure 4.

Protein classes observed in the proteins assayed and identified as hits with altered thermodynamic stabilities in the (A) BT-474 versus MCF-7 and (B) MDA-MB-468 versus MCF-7 cell line comparisons. Each protein class contained at least 4 proteins. Protein classes enriched in the hits are indicated with a “*”.

Table 1

Summary of the Proteomic Data Obtained in the SILAC-SPROX Experiments for Differential Thermodynamic Stability Analysis in the MCF-7 vs BT-474 and MCF-7 vs MDA-MB-468 Cell Line Comparisons

Experiment	Biological Replicate	Identified Peptides (Proteins)	Assayed Peptides (Proteins) ^a	Hit Peptides (Proteins) ^b
MCF-7 versus BT-474	1	12388(1954)	1172(417)	
	2	16903(2511)	1572(554)	
	3	22096(2581)	2914(765)	
	Total	28098 (3705)	3464 (957)	413(257)
MCF-7 versus MDA-MB-468	1	13089(1981)	629 (249)	
	2	10507(1704)	796 (302)	
	3	23999 (2642)	2833(710)	
	Total	26571 (3460)	2980 (790)	1012(450)

^a Assayed peptides are the methionine-containing peptides that were identified from seven or more denaturant concentrations in the SILAC-SPROX experiment.

^b Hit peptides are the methionine-containing peptides with averaged data sets (among biological replicates) that are well-fit to Equations 1 or 2 and have altered H/L ratios at two or more consecutive denaturant concentrations in the SILAC-SPROX experiment.