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Proteotranscriptomic Profiling of 231-BR Breast Cancer Cells: Identification of Potential Biomarkers and Therapeutic Targets for Brain Metastasis*

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Brain metastases are a devastating consequence of cancer and currently there are no specific biomarkers or therapeutic targets for risk prediction, diagnosis, and treatment. Here the proteome of the brain metastatic breast cancer cell line 231-BR has been compared with that of the parental cell line MDA-MB-231, which is also metastatic but has no organ selectivity. Using SILAC and nanoLC-MS/MS, 1575 proteins were identified in reciprocal labeling experiments and 1584 were quantified in the two cell lines. A total of 152 proteins were confidently determined to be up- or down-regulated by more than twofold in 231-BR. Of note, 112/152 proteins were decreased as compared with only 40/152 that were increased, suggesting that down-regulation of specific proteins is an important part of the mechanism underlying the ability of breast cancer cells to metastasize to the brain. When matched against transcriptomic data, 43% of individual protein changes were associated with corresponding changes in mRNA, indicating that the transcript level is a limited predictor of protein level. In addition, differential miRNA analyses showed that most miRNA changes in 231-BR were up- (36/45) as compared with down-regulations (9/45). Pathway analysis revealed that proteome changes were mostly related to cell signaling and cell cycle, metabolism and extracellular matrix remodeling. The major protein changes in 231-BR were confirmed by parallel reaction monitoring mass spectrometry and consisted in increases (by more than fivefold) in the matrix metalloproteinase-1, ephrin-B1, stomatin, myc target-1, and decreases (by more than 10-fold) in transglutaminase-2, the S100 calcium-binding protein A4, and L-plastin. The clinicopathological significance of these major proteomic changes to predict the occurrence of brain metastases, and their potential value as therapeutic targets, warrants further investigation. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.046110, 2316–2330, 2015.

Brain metastases affect 10–20% of cancer patients with disseminated disease (1). Even small lesions can cause neurological disability, and the median survival time of patients with brain metastases is short, with about 80% mortality within one year of diagnosis. The molecular basis of cancer metastases to the brain remains unknown and with advances in the control of systemic disease, the incidence of brain metastases is increasing (1, 2). In the case of breast cancer, brain relapse typically occurs years after primary tumor excision, suggesting that disseminated breast cancer cells must first acquire specialized functions to invade and grow in this organ (3). Retrospective studies of breast cancer patients with brain metastases found that a young age at diagnosis, primary tumors that are estrogen receptor negative or overexpressing the human epidermal growth factor receptor 2 (HER2)1 and/or epidermal growth factor receptor, and the

1 The abbreviations used are: HER2, human epidermal growth factor receptor-2; EFNBI, ephrin-B1; ER, estrogen receptor; FOXL1, forkhead box protein O1; KDM5B, Lysine (K)-Specific Demethylase 5B; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LCP1, lymphocyte cytotoxic protein 1 (L-plastin); miRNA, microRNA; MMP1, matrix metalloproteinase-1; MYCT1, myc target-1; PGR, progesterone receptor; PRM, parallel reaction monitoring; S100A4, S100 calcium-binding protein A4; SILAC, stable isotope labeling of amino acids in cell culture; STOM, stomatin; SYVN1, synovial apoptosis inhibitor 1; TGF-β, transforming growth factor-β; TGM2, transglutaminase-2; TNF-α, tumor necrosis factor-α; TERM1, triggering receptor expressed on myeloid cells 1; UAP1, UDP-N-acetyl-glucosamine pyrophosphorylase.
presence of lymph node or distant metastases were all associated with a higher risk of brain metastatic disease (4). However, at this stage there is no molecular marker, at the gene, mRNA or protein level that is clinically useful to predict, diagnose, or treat breast cancer-derived brain metastases (5). Therefore it is essential to better define the molecular basis for these phenomena and delineate predictive biomarkers and therapeutic targets for future innovative treatments.

In the present study, proteome changes associated to brain metastatic capabilities of breast cancer cells were explored. To that purpose, we have used the unique opportunity provided by the 231-BR/MDA-MB-231 cellular models. 231-BR cells (also named MDA-MB-231BR) have initially been established from the triple negative (no expression of estrogen receptor, progesterone receptor, and HER2) MDA-MB-231 cells, which are highly metastatic but have no organ specificity, being able to metastasize to many different sites. The brain metastatic 231-BR cell line has been derived from MDA-MB-231 cells after successive rounds of implantation, resection from the brain, and re-injection into mice, to produce a subline with selectivity for the brain as compared with other metastatic sites (6). 231-BR cells metastasize with 100% frequency to the brain and they have progressively emerged as an established preclinical model of brain metastatic breast cancer (2). For instance, they have been used to demonstrate that Her-2 overexpression increases the metastatic outgrowth of breast cancer cells in the brain (7), that the anti-HER2 drug lapatinib can inhibit the growth of brain metastatic cells (8), and that the blood–tumor barrier permeability determines drug efficacy in experimental brain metastases (9). They have also been used to analyze brain metastasis in magnetic resonance imaging (10) and to investigate brain damage induced by brain metastases (11).

Despite the considerable interest and use of 231-BR cells for studying the mechanisms underlying brain metastasis, the proteome of these cells has not been thoroughly explored. One study (12) has reported a 2D electrophoresis-based analysis, but no major proteome changes were identified. In the present report, stable isotope labeling of amino acids in cell culture (SILAC) reciprocal labeling was performed on both thebrain metastatic breast cancer cell line 231-BR and the parental cell line MDA-MB-231, which is also metastatic but has no organ selectivity. After extraction, mixing 1:1, and protein digestion with trypsin, LC-MS/MS analysis (using a Q-Exactive Plus from Thermo Fisher Scientific after liquid chromatography on a Nano-Acquity ultra performance column from Waters) was used to identify and quantify reciprocally labeled proteins. Peptide sequence, protein identification, and quantification were obtained using Protein Prospector and the SwissProt database. Major protein changes observed in SILAC were validated in parallel reaction monitoring (PRM) from nonlabeled proteins. Protein pathway analysis was performed with Ingenuity Pathway Analysis software. In parallel to proteomics, a transcriptomic analysis was conducted. mRNA and miRNA were extracted from both cell lines (three biological replicates). GeneChip Exon arrays analysis and GeneChip miRNA arrays were used for analyzing the expression of mRNA and miRNA respectively.

10% fetal calf serum (Sigma-Aldrich, St. Louis, MO) in 75 cm² tissue culture flasks in a humidified incubator at 37 °C with 5% CO₂. 231-BR and MDA-MB-231 cells were cultured in RPMI 1640 medium without arginine and lysine and supplemented with 10% dialyzed fetal bovine serum (Pierce SILAC Protein Quantitation Kit, Rockford, IL). Light condition medium was supplemented with L-lysine 2HCl (Lys0) l-arginine HCl (Arg0), labels dissolved in SILAC media (Thermo Fischer Scientific after liquid chromatography) were used for protein preparation before independent analyses by mass spectrometry. Specifically, there were two biological replicates for 231-BR cells (15C6 L-Lysine labeled-Heavy and 12C6 L-Lysine labeled-Light), and two biological replicates for MDA-MB-231 (15C6 L-Lysine labeled-Heavy and 12C6 L-Lysine labeled-Light). For mass spectrometric analyses (15C6 L-Lysine labeled-Heavy) 231-BR were analyzed versus (12C6 L-Lysine labeled-Light) MDA-MB-231, and (15C6 L-Lysine labeled-Light) 231-BR versus (13C6 L-Lysine labeled-Heavy) MDA-MB-231. The final results correspond to the mean of the two mass spectrometric analyses.

**Protein Preparation for Mass Spectrometry**—Membrane proteins were enriched from the soluble proteins of the SILAC heavy and light reciprocally labeled 231-BR and MDA-MB-231 cell pellets and unlabeled cell pellets for label-free parallel reaction monitoring (PRM) analysis (1 x 10⁵ cells) by dissolving each cell pellet in 1 ml of ice-cold 0.1 M Na₂CO₃ supplemented with protease inhibitor (Roche Complete EDTA Free), sonicated for 2 x 20 s and incubated for 1 h at 4 °C. The homogenates were then centrifuged at 100,000 x g for 90 min at 4 °C (13). The membrane pellets were redissolved in 500 mM triethylam-
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Protein Quantification Using SILAC—SILAC quantification measurements were extracted from the raw data by Search Compare in Protein Prospector. Search Compare averaged together MS scans from \(-10\) s to \(+20\) s from the time at which the MS/MS spectrum was acquired to produce measurements averaged over the elution of the peptide. Search Compare calculates a noise level in the averaged spectrum. Only peaks with a signal to noise of greater than 10 were used in quantification measurements. The raw data has been uploaded to the MassIVE public repository: ftp://MSV000078911@massive.ucsd.edu. If one of the SILAC pair is above this threshold and the other is below, then the ratio is reported with a \(>\) or \(<\) (see raw data for SILAC), indicating one value was below the noise level, so the ratio reported is a minimum estimate. The standard deviation of the log ratios for peptides matched to the same protein (where all ratios should be the same) was 0.11. The twofold threshold change employed as significant therefore corresponds to 2.74 \((\log(2)/0.11)\) standard deviations from the mean. Assuming a Gaussian distribution, this threshold would correspond to a 99.4\% confidence threshold that a twofold difference is nonrandom. This corresponds to a 0.6\% FDR. Furthermore it is necessary to emphasize here that multiple testing adjustment is not required in this study, as we are only testing one hypothesis; that protein \(x\) is changing in abundance in one cellular model. For these calculations the fact that one protein changes or not does not affect the probability of another protein changing.

**PRM Mass Spectrometry**—PRM was used to confirm the major protein changes in 231-BR versus MDA-MB-231 cells (proteins up-regulated by more than fivefold and down-regulated more than 10-fold) observed in the SILAC analyses. Peptides were extracted from 231-BR and MDA-MD-231 cells as described above (see Protein preparation for mass spectrometry) but from a different batch of cultured cells (biological replicate). These peptides were loaded on C18-containing stage tips prior to reversed phase chromatography on a SPE-LC (modified EASY-nLC 1000, Thermo, Odense, Denmark), as described in Falkenby et al. \((77)\). Short gradients ranging from 4 to 35\% in 5 min were used. In-house prepared 6 cm columns with pulled emitter and ReproSil-Pur 120 C18-AQ 3 \(\mu\)m material (Dr Maisch, Germany) were used in combination with a nano-ESI source (Proxeon, Odense, Denmark). PRM was performed using a Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). Methods optimized for collision energy, charge state, and retention times for the most significantly regulated peptides were generated experimentally using two unique peptides of high intensity and confidence for each target protein. Targeted MS2 spectra were acquired using a PRM approach \((18)\) at a resolution of 35,000, employing a high AGC target value of 3e6 ions and a maximum injection time of 100 ms. Scheduled acquisition in windows of up to 1 min was used to limit the number of concurrent targets to a maximum of four. The raw data has been uploaded to the MassIVE public repository: ftp://MSV000078911@massive.ucsd.edu. These data were analyzed using Skyline (MacCoss Lab, University of Washington) \((19)\) where signal intensities for individual peptide sequences for each of the significantly altered proteins were quantified relative to each sample and normalized to heat shock 60kDa protein 1 (chaperonin, HSPD1) that was found to be homogeneously expressed in each cell type (peptides for HSPD1 were optimized and shown to be high intensity and confident transitions). Quantification was performed by measuring the extracted ion chromatogram for each transition for each peptide in triplicate LC MS/MS scans and results were compared between cell types using a Student’s\’ t test.

**Exon-based Microarray Analysis of mRNA Expression**—Isolation of RNA was performed from three biological replicates of 231-BR and MDA-MB-231 cells using the illustra RNASpin Mini Isolation Kit \((GE Healthcare)\) according to the manufacturer’s instructions \((protocol 25\)–\(5050\)–\(70PC)\). The RNA concentration was measured using Nano...
photometer (Implen, Munchen, Germany) and the quality was determined using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). Microarray analysis of mRNA expression was performed at the Australian Genome Research Facility (AGRF, Melbourne, Australia). Briefly, a total of 3 μg was labeled using the Affymetrix WT cDNA Amplification kit (Millenium Science, Mulgrave, Australia). The subsequent cRNA was cleaned using the Affymetrix GeneChip Sample Cleanup kit (Millenium Sciences). Upon cleaning of the cRNA, dUTP was incorporated into the second cycle of the first strand cDNA synthesis step. The presence of the dUTP was used to facilitate fragmentation using the APE1 and UDG enzymes that specifically recognized dUTP. The fragmented cDNA was quality checked using the Agilent Bioanalyser 2100 with the NanoChip protocol. The fragmented single stranded cDNA was end-labeled using terminal deoxynucleotidyl transferase and the WT Terminal Labeling kit (Millenium Sciences). A total of 5 μg of labeled cDNA for each of the three biological replicates of 231-BR versus MDA-MB-231 was then hybridized to the HumanExon 1.0 ST Array GeneChip (Millenium Sciences) by preparing a probe mixture (labeled cDNA at 0.025 μg/μl) that includes 1× hybridization buffer (100 mM MES, 1 mM NaCl, 20 mM EDTA, 0.01% Tween-20), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml BSA, and 7% DMSO. A total hybridization volume of 220 μl was prepared for each sample and 200 μl loaded into a HumanExon 1.0 ST Array GeneChip. The chip was hybridized at 45 °C for 16 h in an oven with a rotating wheel at 60 rpm. After hybridization the chip was washed using the appropriate fluidics script in the Affymetrix Fluidics Station 450. Upon completion of the washing, the chips are then scanned using the Affymetrix GeneChip Scanner 3000. The scanner operating software, GCOS, converts the signal on the chip into a DAT file, which was used for generating the subsequent CEL file for analysis. The ratio of gene expression in 231-BR versus MDA-MB-231 cells was derived from Cel files, but is presented in terms of % of increase/decrease. The raw data has been uploaded to the MassIVE public repository: ftp://MSV000078911@massive.ucsd.edu. The data from each array was imported into Genespring GX v 12.1 (Agilent Technologies) as CEL files and robust multi-array analysis was used to identify genes with significantly altered expression (>2-fold, p < 0.05). To correct for false positive results, a Benjamini and Hochberg FDR of 5.0% was used for multiple testing.

MicroRNA Expression Profiling—Isolation of microRNA (miRNA) from three biological replicates of 231-BR and MDA-MB-231 cells was performed using the mirVana miRNA isolation kit (Life Technologies) according to the manufacturer’s instructions (protocol 1560 M Rev. C). The RNA concentration was determined using a Nanophotometer (Implen) and the quality was determined using Agilent 2100 Bioanalyser (Agilent Technologies). Total RNA (1 μg) from each replicate was biotinylated using the FlashTag Biotin HSR kit (Genisphere, Hatfield, PA) in triplicate according to the manufacturers’ instructions (protocol 877.888.3DNA). Labeled RNA was hybridized to GeneChip miRNA-2 arrays (Affymetrix, Santa Clara, CA) for 16 h before washing and staining the arrays using the GeneChip Hybridization (Affymetrix). Wash and Stain kit according to the manufacturers’ instructions (protocol P/N 702731 Rev. 3). Arrays were scanned on a GeneChip® Scanner 3000 7G (Affymetrix). Analysis GeneChip miRNA-2 arrays contain 15,644 probe features representing 1105 unique human mature miRNAs, 1105 unique human pre-miRNAs and 2334 human snoRNA and scaRNAs (Affymetrix). The data from each array was imported into Genespring GX v 12.1 (Agilent Technologies) and robust multi-array analysis was used to log-transform, background-correct, quantile normalize, and summarize the probe features resulting in a set of expression signal intensities. The signal intensities were then baseline transformed to the median signal intensity of all arrays. Unpaired moderated t tests were used to identify miRNAs with significantly altered expression (>2-fold, p < 0.05). To correct for false positive results, a Benjamini and Hochberg FDR of 5.0% was used for multiple testing. Supervised hierarchical cluster analysis was performed on miRNAs that were found to be significantly different (>2 fold, p < 0.05, FDR<0.05). Similarity in the average expression patterns between miRNAs was measured by Euclidian’s distance. The raw data has been uploaded to the MassIVE public repository: ftp://MSV000078911@massive.ucsd.edu. Biological targets of differentially expressed miRNAs were identified by searching for the presence of conserved eight-mer and seven-mer sites within genes that match the seed region of each miRNA. For miRNA families, conservation cutoffs were defined as described by Friedman et al. (20) as follows: broadly conserved (conserved across most vertebrates, usually to zebrafish); conserved (conserved across most mammals, but usually not beyond placental mammals), or poorly conserved (all others). For human miRNA, site conservation were defined by conserved branch length, with each site type having a different threshold for conservaion: eight-mer => = 0.8; seven-mer-m8 => = 1.3; seven-mer-m1A => = 1.6 as defined (20). The data were searched for miRNA potential targets using sRNA Target Base (starBase, http://starbase.sysu.edu.cn) (21), which integrates data from 21 Ago or TNRC6 CLIP-Seq sequence data sets with the target prediction programs Target Scan, Pictar and miRanda. The analysis was performed as previously described (22) and miRNA that were validated by all three target-prediction algorithms, and for which confidence is therefore high, have been selected.

Molecular Pathway Analysis—SILAC data was analyzed using Qia-gen Ingenuity Pathway Analysis (IPA®, Qiagen Redwood city, CA, www.qiagen.com/ingenuity). Proteins showing differential regulation (at least twofold expression changes) were uploaded and networks were generated using data sets containing gene identifiers and their corresponding expression values. Networks of these focus genes were then algorithmically generated based on their connectivity using stringent human filter options describing molecules and relationships. Graphical networks depicting significant activation or inhibition of molecular pathways were generated. Pathways with a p value <0.05 were considered to be significantly regulated. The p value<0.05 was used to identify pathways in the study that might explain the changes of protein expression observed between 231-BR and MDA-MD-231 cells. It suggests a statistically significant link between proteins showing significant regulation and genes that are regulated by a known transcription regulator. It was calculated, as part of Ingenuity Pathway analysis process, using Fisher’s Exact Test (significance of the association or contingency test).

RESULTS
Proteome Profiling of 231-BR versus MDA-MB-231 Cells—Using SILAC based LC-MS/MS analysis of 231-BR and MDA-MB-231 cells, 2266 and 2434 proteins, respectively, were identified in reciprocal labeling experiments. Together, 1957 proteins were reciprocally identified and 1584 were quantified in both experiments. A total of 152 proteins, representing 9.6% of all quantified proteins, were found to be regulated by at least twofold in 231-BR as compared with MDA-MB-231, with 40 up- and 112 down-regulations (Fig. 2A). The correlation plot of changes in protein quantification obtained in the two replicates of the SILAC analysis is presented (Fig. 2B). The Pearson coefficient of correlation (R square) was 0.66 (p < 0.0001), demonstrating the reliability of the list of regulated
Fig. 2. Distribution of protein, mRNA, miRNA changes in 231-BR compared with MDA-MB-231. A, The level of 1584 proteins were compared between 231-BR and MDA-MB-231 cells and 152 were found regulated by more than twofold (112 decreased and 40 increased). The number of proteins (y axis) is in Log scale. B, Correlation plot between SILAC replicates. Changes in protein levels obtained in replicate 1 versus replicate 2 are presented. The x and y axes are in Log scale. The Pearson correlation coefficient (R square) was 0.66 (p < 0.0001). C, 22,011 mRNA were quantified in 231-BR versus and MDA-MB-231. Three hundred and six mRNA were significantly regulated by at least twofold (70 decreased and 226 increased). D, Correlation plot between protein and mRNA level changes. The mRNA change was plotted for the 152 protein changes in 231-BR. The Pearson correlation coefficient (R square) was 0.67 (p < 0.0001). E, 1105 miRNA were quantified in 231-BR versus MDA-MB-231 and 45 were found regulated by at least twofold. Nine miRNA were decreased and 36 increased in 231-BR cells. F, Correlation plot between miRNA and their predicted protein targets. The Pearson correlation coefficient (R square) was 0.37 (p < 0.0459).
proteins. The characteristics (gene and protein name, accession number, number of unique peptides, protein fold changes, best peptide E-values, mRNA fold changes, and associated nominal p values) of the 152 proteins differentially regulated between 231-BR and MDA-MB-231 are shown in supplemental Table S1. For mRNA, test adjustments (Bonferroni, sidak, q-value) are in the raw data accessible on line (MassIVE public repository: ftp://MSV000078911@massive.ucsd.edu). Analysis of the localization (Fig. 3A and 3B) and function (Fig. 3C and 3D) of regulated proteins showed differences between up-regulated (Fig. 3A and 3C) and down-regulated (Fig. 3B and 3D) proteins. Statistical significances were calculated using Chi-square (two-tailed). The proportion of nuclear proteins was higher among up-regulated (45%) than down-regulated (24%) proteins (p < 0.001) and the proportion of cytoplasmic proteins was higher in the down-regulated proteins (56%) compared with the up-regulated proteins (40%) (p = 0.023). The proportion of membrane and extracellular proteins was not statistically different between up- and down-regulated proteins (p > 0.05).

The largest proteome changes in 231-BR were increases (by more than fivefold) in the matrix metalloproteinase MMP1, the growth factor ephrin-B1 (EFNB1), the membrane protein STOM, the N-acetyl-glucosamine pyrophosphorylase UAP1, the target of Myc MYCT1, and decreases (by more than 10-fold) in the transglutaminase TGM2, the metastasis associated protein S100A4 and the actin cross-linker LCP1 (or l-plastin). The MS/MS spectra of the most up-regulated and down-regulated proteins, MMP1 and TGM2, respectively, are presented in Fig. 4. Figs. 4B, 4C, 4E, and 4F demonstrate the reproducible quantification results obtained between the reciprocal labeling experiments.

PRM Analysis of the Major Protein Changes in 231-BR versus MDA-MB-231—The major protein changes in 231-BR (increases by more than fivefold for MMP1, EFNB1, STOM1, UAP1, MYCT1 and decreases by more than 10-fold for TGM2, S100A4, and LCP1) were analyzed by label-free PRM mass spectrometry. The analysis was performed from a different set of cell cultures than for the SILAC analyses (biological replicate). Product ions of selected peptides were monitored in parallel using one injection over a full mass range. Two unique peptides for each protein were used for quantification. Each PRM experiment was performed in triplicate and normalized to control peptides revealed by SILAC based LC-MS/MS analysis to be expressed 1:1 (HSPD1, further confirmed to be expressed 1:1 by PRM). Peptide ratios for the label-free PRM quantification were generated in Skyline measuring the area.
FIG. 4. LC-MS/MS based identification and quantification of MMP1 and TGM2. Matrix metallopeptidase-1 (MMP1) and transglutaminase-2 (TGM2) were differentially expressed between 231-BR and MDA-MB-231 cells. A, MS/MS spectrum of peptide MIAHDFPGIGHK from MMP1. B, Precursor intensity for this peptide in 231-BR cells is roughly 14-fold more intense than in the parent cell line, based on SILAC labeling; C, In the reciprocal labeling experiment, the heavy precursor is now significantly more intense. D, MS/MS spectrum of heavy-labeled peptide MDLLPLHMGLHK from TGM2. E, The light equivalent of this peptide was not detected in 231-BR sample; F, In the reciprocal labeling experiment signal for the 231-BR sample is in the noise level of the spectrum, indicating an at least 18-fold difference in expression level between cell lines.

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under the curve of three to eight transitions selected for each peptide (precursor ion/product ion). These were averaged for each protein and then compared with averaged reciprocal SILAC quantification values. All transition ions obtained for each of the nine analyzed proteins are presented (supplemental Table S2). The quantifications for each analyzed protein (using two peptides/protein) in 231-BR versus MDA-MB-231 are presented (supplemental Fig. S3). The results indicate that for 7/8 proteins analyzed, the PRM data validated the results obtained using SILAC. The increase in MMP1, EFN1, STOM1, MYCT1 in 231-BR, as well as the decrease in TGM2, S100A4, and LCP1 was confirmed by PRM. The only change that was not confirmed by PRM was the increase in UAP1. As shown in supplemental Fig. S3, the two peptides used for UAP1 provided contradictory results in PRM as one showed no change whereas the other indicated decrease in 231-BR. In the SILAC analyses, UAP1 was characterized with only one peptide. Therefore, change in the UAP1 level in 231-BR could not be confirmed. A correlation curve was generated (Fig. S5) between protein changes observed by PRM and by SILAC. The coefficient of correlation (coefficient of determination, R-square) was 0.86 ($p < 0.0003$), indicating a high level of correspondence in the results obtained by the two methodological approaches.

Expression of mRNA in 231-BR versus MDA-MB-231 Cells—Changes in mRNA levels in 231-BR compared with MDA-MB-231 cells were analyzed in exon-based microarray analysis. A total of 296 changes in individual mRNA levels of at least twofold were observed in 231-BR (supplemental Table S4). This included 226 up-regulations and 70 down-regulations of individual miRNAs. The distribution of changes in mRNA is reported in Fig. 2C and the relative mRNA levels for each of the proteins regulated in the SILAC proteomics experiment are reported in supplemental Table S1. Overall, only 43% of the individual changes in protein expression were associated with corresponding changes in the transcript levels. The correlation plot of changes in proteins versus mRNA (Fig. 2D) indicated a Pearson correlation coefficient (R square) of 0.67 ($p < 0.0001$). This shows that alterations in miRNAs are limited predictors of changes in the corresponding proteins, suggesting post-transcriptional regulation. Noticeably, there was a significant difference between up- and down-regulated proteins in regard to changes in mRNA levels. In the case of up-regulated proteins, 65% had a corresponding increase in mRNA, but for down-regulated proteins a corresponding change in mRNA level was found in only 35% of cases. Therefore 65% of the up-regulated proteins could be related, at least in part, to increases in mRNA level, whereas only 35% of the down-regulated proteins were accompanied by a decrease in mRNA, further supporting post-transcriptional regulation as being particularly relevant to the down-regulation of individual proteins in 231-BR.

miRNA Expression in 231-BR versus MDA-MB-231 Cells—The level of 1105 unique mature miRNA, 1105 pre-miRNAs, and 2334 snoRNA and scaRNAs was analyzed in 231-BR and MDA-MB-231 cells using microarrays. A total of 45 miRNAs were found to be differentially regulated by at least twofold in 231-BR cells (supplemental Table S5), including nine down-regulations and 36 up-regulations (Fig. 2E). The higher proportion of miRNA that were increased matched the predominant down-regulation of protein levels observed in the SILAC proteomic results (supplemental Table S1), suggesting that miRNAs are involved in protein level changes, and in particular in the down-regulations. However, using prediction of miRNA targets, we were able to identify only six of the proteins regulated in 231-BR as potential targets of four regulated miRNA (supplemental Table S6). To be more specific, three increases in miRNA (miR-195, miR-182, miR-34a) potentially corresponded to down-regulation at the protein levels (for VAMP8, IGF2R, HDGF, ACTR2, ANLN), and 1 miRNA decrease (miR-424–5p) corresponded to up-regulation at the protein level (SLC4A7). Overall, five out of the 112 down-regulated proteins were targets of certain miRNAs, whereas it

$y = 1.3x - 0.14$
was 1/40 for up-regulated proteins; this was not statistically different (p > 0.05 using Chi-square two-tailed). Also, the correlation plot between changes in miRNA and their predicted protein targets (Fig. 2F) indicated a Pearson correlation coefficient (R square) of only 0.37 (p < 0.0459).

Integrated Overview of the Proteome/Transcriptome/miRNA Changes in 231-BR versus MDA-MB-231 Cells and Molecular Pathway Analysis—An overview of changes observed at the three expression levels (protein - mRNA - miRNA) is presented in Table I. Ingenuity pathway analysis revealed that most proteome changes in 231-BR (Fig. 6A) were related to cell death and survival, cell growth and differentiation, cellular movement, cell cycle and cell-to-cell interaction. In terms of prediction of upstream regulatory pathways, the pathways regulated by tumor necrosis factor α (TNF-α), extracellularly regulated kinases, the histone demethylase KDM5B (Lysine (K)-Specific Demethylase 5B), the estrogen receptor and the cyclin dependent kinase 1A (CDKN1A) were activated in 231-BR (Fig. 6C) were predicted to be related to transforming growth factor β (TGF-β), the E3 ubiquitin-protein ligase SYVN1 (synovial apoptosis inhibitor 1 or synoviolin), the transcription factor FOXO1 (Forkhead box protein O1), the receptor TREM1 (triggering receptor expressed on myeloid cells 1), and the progesterone receptor. The TNF-α and TREM1 pathways were found with 5% FDR and p < 0.05, whereas all the other pathways were found with 1% FDR and p < 0.01 (Fig. 6B and 6C).

DISCUSSION

In this study, the differential protein/mRNA/miRNA content of a brain-colonizing breast cancer cell line (231-BR) was compared with the parental nonspecific metastatic cell line (MDA-MB-231), with the view of determining the molecular features that could account for the differing phenotypes of these cells. There has already been many studies comparing the proteome of metastatic versus nonmetastatic breast cancer cells (23–27); this study focused on a unique genetically homogenous cellular model to delineate proteome changes associated to a brain-seeking phenotype of breast cancer cells. The seed and soil theory of metastasis that was originally established in breast cancer by Paget in 1889 (28), described metastasis in terms of an interaction between a tumor cell and a local environment. This concept has been extended to other types of cancer and although it is constantly revisited (29), there is an agreement that adequate molecular determinants are necessary, on both the cancer cells and the target organ, to permit the establishment of metastases. The proteomic signature identified in this study provides a framework for a better understanding of the mechanisms triggering brain metastasis, and for delineating future biomarkers and therapeutic targets of clinical interest.

Overall Changes in Proteins versus mRNA and miRNA—Comparing changes in 231-BR at the protein versus the mRNA level indicates that only 43% of the individual protein changes were associated with concomitant changes in the respective mRNA. This confirms the already reported fact that the transcript level is a limited predictor of the protein level (30, 31). Currently, breast tumors are classified based on transcriptomics/gene expression into four main classes: luminal A, luminal B, HER2+,-, and triple negative; however, this classification is constantly being refined with the addition of new subclasses (32). The limited association between changes at the protein versus mRNA level that are reported herein points to the need to further explore the proteome of breast tumors, and ultimately define the proteogenomic profile of this disease. This study represents an exploratory step in this direction. Significantly, most of the proteome changes in 231-BR were decreases in individual protein levels (112/152), suggesting that down-regulation of specific proteins may be an important part of the mechanism underlying the ability of breast cancer cells to metastasize specifically to the brain. Alternatively, it is also possible that the proteins are down-regulated simply because they are no longer needed in brain metastasis and they have been selected against during the multiple cycles of injection and recovery from the mice. Interestingly, differential microRNA analyses indicated that most miRNA changes in 231-BR were up-regulations (36/45), suggesting that the trend toward protein down-regulation could be at least partially controlled by opposite changes in levels of regulatory miRNAs. However, target prediction of miRNA, using three different target prediction tools, identified only four regulated miRNA potentially corresponding to six regulated proteins. Together, these results suggest that changes in miRNAs are part of the molecular profile of brain metastatic breast cancer cells, but the correlation between specific up-regulated miRNAs, putative down-regulated protein targets and brain metastatic phenotype was not tested experimentally. Further functional investigation will be needed to clearly link specific changes in miRNA to changes in protein levels.

Pathway Activation/Inactivation—It is important to emphasize the predictive nature of pathway analysis. The pathways presented here indicate predicted activation and inhibition of

| Regulated in 231-BR | Total numbers quantified |
|---------------------|-------------------------|
| Proteins            | 112                     |
| mRNA                | 70                      |
| miRNA               | 9                       |
signaling networks based on the up- and down-regulations determined in the SILAC analysis. However, it does not constitute a graphical representation of SILAC results and there is no direct evidence in our study for those hub proteins. However, as described below, the literature indicates that these hubs are expressed in breast cancer cells. Significantly inhibited pathways in 231-BR were those related to TGF-β, the E3 ubiquitin-protein ligase SYVNV1 and the transcription factor FOXO1, whereas the pathways associated to TNF-α, and the histone demethylase KDM5B were activated. TGF-β has been shown to be produced by both glial cells and neurons, and to participate in the development and maintenance of the brain.

Fig. 6. Protein pathways regulated in 231-BR. Pathway analysis of proteins differentially regulated in 231-BR versus MB-MDA-231 was performed using Ingenuity Pathway Analysis. A, Protein pathways activated in 231-BR were found related to cell death and survival, protein synthesis, cell cycle, cell-to-cell interaction, and molecular transport (number of proteins for each pathway are indicated in ordinate, p values are indicated on top of each column). B, Predicted upstream pathways activated in 231-BR. The p values for activated pathways were CDKN1A, 4.1 × 10^{-10}; extracellularly regulated kinases, 2.9 × 10^{-13}; KDM5B, 7 × 10^{-10}; TNF-α, 4.3 × 10^{-10}; ER, 2.9 × 10^{-15}. C, Predicted upstream pathways inhibited in 231-BR. The p values obtained for inhibited pathways were TGF-β, 2. × 10^{-10}; SYVNV1, 6.25 × 10^{-7}; FOXO1, 4.69 × 10^{-7}; TREM1, 3.73 × 10^{-7}; PGR, 9.44 × 10^{-7}. For B and C, the TNF-α and TREM1 pathways were found with 5% FDR and p < 0.01. Networks are presented in the centroid of connecting lines in bold font with rectangle icons. Blue fill indicates a significant inhibition in the 231-BR cells and B orange indicates significant activation. Proteins are represented by circle or oval icons with a green shading corresponding to a twofold or greater decreased expression, and a red shading correspond to a twofold or greater increased expression in the 231-BR cells. Intensity of each color represents the level of expression. Solid lines suggest direct interactions and dashed lines suggest indirect ones. Orange lines lead to activation and blue lines lead to inhibition. Yellow lines are for inconsistency in state (up or down) of downstream molecule and gray are for effect yet to be predicted. Protein names are reported in supplemental Table S1.
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microenvironment mainly through its neuroprotective effect (33). TGF-β has also been shown to inhibit the anchorage-independent growth of 231-BR (6); therefore it is conceivable that the down-regulation of the TGF-β signaling pathway could be a way to escape growth inhibition by TGF-β normally present in the breast microenvironment. The pathway controlled by SYVN1 was also down-regulated in 231-BR. SYVN1 is involved in endoplasmic reticulum (ER)-associated degradation and removes unfolded proteins accumulated during ER stress by retrograde transport to the cytosol from the ER. This protein also uses the ubiquitin-proteasome system for additional degradation of unfolded proteins. The potential role of SYVN1 in carcinogenesis is, at this stage, limited to the targeting of the tumor suppressor p53 for ubiquitination (34) leading to its degradation in the cytoplasm, but it is unclear how this relates to brain metastasis. The third down-regulated pathway identified is related to the transcription factor FOXO1. Suppression of FOXO1 activity has recently been associated with increased tumorigenicity of breast cancer cells (35) and FOXO1 is involved in the control of E2F1 transcriptional specificity and apoptotic function (36). Therefore the down-regulation of FOXO1 is coherent with increased tumor aggressiveness and in return may also participate in the regulation of the TGF-β, MMP1, and SYVN1 pathways. In contrast to inhibited pathways, this study also points to the significant activation of pathways associated to TNF-α and the histone demethylase KDM5B. TNF-α is a major pro-inflammatory cytokine involved in growth, differentiation, and survival of many cell types. In breast cancer cells, TNF-α participates in the epithelial–mesenchymal transition phenotype (37) and its targeting, using blocking antibodies, suppresses breast cancer growth (38). Our study therefore emphasizes the value of targeting the TNF-α pathway in breast cancer. Similar to genetic alterations, epigenetic aberrations contribute significantly to tumor initiation and progression. The pathway related to the histone demethylase KDM5 was found to be increased in 231-BR cells. The KDM5 family of histone demethylases is capable of removing tri- and dimethyl groups from lysine 4 on histone H3, a modification that occurs at the start site of transcription in actively transcribed genes (39). Preclinical studies suggest that the inhibition of these enzymes can suppress tumorigenesis (40), and this study points to a possible involvement in breast metastasis. Interestingly, the pathways controlled by progesterone and estrogen receptors were found differentially regulated, despite the fact that 231-BR and MDA-MB-231 cells are triple negative breast cancer cells that do not express these receptors. However, the pathway analysis provides clues for alternate regulation. Indeed, the regulation of NT5E, ITGB1, and ITGA3, which can be controlled by ER, can also occur through the pathways mediated by TREM1 and TGF-β (as shown in Fig. 6C). Also, the regulation of ETS1 can be induced through the TNF-α pathway (as shown in Fig. 6B). Similarly, the up-regulation of F3 that can be controlled by PR can also be regulated through the TNF-α pathway (as shown in Fig. 6B).

Each of these alternate potential effectors (TREM1, TGF-β, and TNF-α) has been identified previously in breast cancer cells (41–43).

Proteins Up-regulated in the Brain Metastatic 231-BR Cells—Of particular interest was the up-regulation (by ~14-fold) of the matrix metalloproteinase MMP1, which promotes collagen degradation. MMP1 overexpression has been associated with metastatic capacities of breast cancer cells by favoring extracellular matrix degradation and thus facilitating invasion and extravasation of cancer cells (44). Of note, MMP1 expression is under the control of the transcription factor ETS1 that was also found to be up-regulated (although to a lesser extent than MMP1) in 231-BR. MMP1 overexpression is associated with poor patient outcome (45) and in a mouse xenograft model it has been shown that MMP1 inhibition decreases local growth and brain metastasis of breast cancer cells (46). Therefore the results presented here confirm the involvement of MMP1 in brain metastasis by showing its increased protein level in 231-BR cells. A few recent studies have suggested that MMP1 plays an important role in the regulation of neuronal apoptosis and astrocyte proliferation (47), suggesting that MMP1 up-regulation in breast cancer cells could lead to a remodeling of brain extracellular matrix, making it favorable for their implantation into the brain microenvironment. In terms of therapeutic targeting, inhibitors of MMPs, despite considerable excitement during the last two decades, have failed to enter the clinic because of their unwanted musculoskeletal side effects (48, 49) and it is therefore unlikely that targeting MMP1 would be a viable therapeutic option for metastatic breast cancer. Interestingly, other enzymes involved in extracellular matrix remodeling were also found among the regulated proteins.

Another major change in 231-BR cells was the up-regulation (by ninefold) of ephrin-B1. Ephrins are plasma membrane-bound growth factors that act by stimulation of Eph tyrosine kinase receptors on juxtaposed cells, initiating multiple intracellular phosphorylation cascades (50). The ensuing signals are bidirectional as ephrins can also transduce signals (known as reverse signals) following their interaction with Eph receptors. The biological functions of ephrins range from cell growth, adhesion, and migration to axon guidance and angiogenesis, resulting in critical regulatory roles in embryonic development and carcinogenesis (50). Eph receptors require direct cell-to-cell interaction for activation and they are divided into EphA and EphB receptor classes, depending on their preferential binding affinity for ephrinA or ephrinB ligands. Eph receptors have been documented in cancer (51), but the ligands have not been thoroughly investigated and in particular there is limited data on ephrinB1. However, a recent study has shown that enhanced expression of ephrinB1 is associated with lymph node metastasis and poor prognosis in breast cancer (52). The authors did not propose a mechanism to explain the association between ephrinB1 and poor patient
survival, but another recent study has shown that ephrinB1 is a substrate for PTEN and interacts with the tyrosine kinase receptor HER2 (or ErbB2) (63). Interestingly, breast tumors overexpressing HER2 are prone to metastasize to the brain (7) and therefore an association of HER2 with ephrinB1 could be a driver of brain metastasis. In terms of therapeutic potential, ephrinB1 is expressed at the cell surface and could potentially be targeted. Although there are no inhibitors of ephrins in clinical use, a number of peptides and chemical compounds that target Eph receptors and inhibit ephrin binding or downstream kinase activation have been identified (56). These molecules show promise as probes to study Eph receptor/ephrin biology, as lead compounds for drug development and as targeting agents to deliver drugs or imaging agents to tumors. The data presented herein clearly support that the potential use of ephrin inhibitors against brain metastasis should be further considered.

Stomatin (STOM) and MYCT1 were also found to be strongly up-regulated (by at least fivefold) in 231-BR. Stomatin is an integral membrane protein, the absence of which is associated with a form of hemolytic anemia known as hereditary stomatocytosis. It is reported that stomatin regulates the gating of acid-sensing ion channels in mammalian neurons, but the function of stomatin is not fully understood (57). MYCT1 (or MTMC1) is a helix-loop-helix leucine zipper transcription factor, which is a direct target of c-Myc. Its overexpression recapitulates multiple c-Myc phenotypes, including cell transformation (61). Deregression of c-Myc is a hallmark of many human cancers, as the c-Myc oncoprotein directly regulates the expression of >1500 genes controlled by RNA polymerases I, II, and III (62). Although a role for MYCT1 in brain metastasis has not been reported, this study suggests that it could be related to brain metastasis.

Proteins Down-regulated in 231-BR Cells—The most dramatically down-regulated protein was tissue transglutaminase TGM2, which was decreased by ~50-fold in 231-BR. Interestingly the mRNA of TGM2 was also strongly decreased (by ~16-fold), suggesting a regulation at the transcriptional level. TGM2 is a thiol enzyme that catalyzes a crosslinking reaction between a specific γ-glutamyl containing peptide substrate and either a ε-amino group from a peptide-bound Lys residue or a free primary amine (63). These reactions result in post-translational modifications of proteins that can alter their solubility, structure, and function. The γ-glutamyl-ε-lysine (iso-peptide) bonds that can be catalyzed by TGM2 result in the formation of either an inter- or intra-isopeptide bond. Many intra- and extracellular proteins have been identified as TGM2 substrates (63). TGM2 acts at both intracellular and extracellular levels (49), as TGM2 is localized at the cell surface, cytoplasmic, and nuclear levels. The intracellular substrates of TGM2 noticeably include the transcription factor NFκB, which is involved in breast cancer cell growth and survival (64). TGM2 activates NFκB by cross-linking and polymerizing the inhibitor of NFκB, IkappaB, leading to its proteosomal degradation (64). In addition, protein crosslinking is important for extracellular matrix stabilization and while at the cell surface TGM2 interacts with a variety of ECM proteins including integrins (65) and fibronectin (66). Cell surface TGM2 is involved in stabilizing tissues and in particular, lower TGM2 level leads to less cross-linked collagen (67) that can be more efficiently digested by metalloproteinases such as MMP1. Thus, the strong up-regulation of MMP1 in 231-BR and the down-regulation of TGM2 may synergistically contribute to destabilizing the brain microenvironment and facilitate metastatic implantation and growth.

Another strongly down-regulated protein (by 26-fold) was the calcium binding and metastasis-associated protein, S100A4. It is well documented that S100A4 is expressed in cancer cells and contributes to tumor cell motility and metastatic progression, as well as angiogenesis (68). An important characteristic feature of S100 proteins is their dual function, inside and outside the cell, which may explain how it is able to participate in a phenotype characteristic of cancer metastasis. However, the exact underlying mechanisms remain unresolved (69). It is not known in this study whether the change in S100A4 occurs at the intra- or extracellular level, or both, but the strong decrease in 231-BR suggests a relationship to the brain metastatic capability of breast cancer cells. In addition, LCP1 (or α-plastin) was found down-regulated by more than 20-fold. This protein is an actin filament cross-linker that has been shown to contribute to the fine-tuning of actin turnover in breast cancer cells, and its phosphorylation by PKC-delta has recently been shown to induce actin polymerization and tumor cell invasion (72). The expression of LCP1 enhances metastatic properties in both prostate cancer and melanoma cells (73) and this study indicates a potential link with brain metastasis as well.

**CONCLUSION**

This exploratory proteotranscriptomic study provides a knowledgebase for better understanding the molecular mechanisms leading to brain metastasis and for delineating future biomarkers and therapeutic targets of clinical interest. The primary purpose of the study was to identify proteins regulated in the human MDA-MB-231/231-BR model of brain metastasis, which is unique because of its syngeneic nature. The PRM analysis has been used as a further step to validate the more dramatic changes observed in SILAC. In this regard, the major up-regulated proteins (MMP1, EFN1, STOM, MYCT1) certainly represent the most valuable candidates for further functional and clinical investigations by brain metastasis specialized groups. However, the limitation of using a single parent-descendent cell culture system dictates caution in the interpretation of the results and suggests that a conservative approach toward describing broad applications is appropriate. In future studies, xenografts may be used to examine the proteome in the context of animal model to see
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If the cell line differences persist in the microenvironment. In addition, functional investigations will have to be conducted to evaluate the impact of the identified proteins in brain metastasis and to define the clinical relevance. For instance using shRNAi against the up-regulated proteins of interest (or conversely overexpressing a down-regulated protein) in 231-BR could be used in functional brain metastasis assays. However, there is no in vitro assay for brain metastasis, and in vivo animal models have to be used as previously reported (7, 8). At the clinical level, it would be valuable to correlate the expression of these proteins with the occurrence of brain metastases and the survival of breast cancer patients. However, most data available, like in the Tumor Cancer Genome Atlas (TCGA) (74), are at the gene/mRNA levels, and TCGA breast cancer does not record brain metastasis. In this context, proteogenomics, a globalized approach encompassing gene/mRNA/protein levels together (75), represents a promising perspective. A first proteogenomic analysis of colon cancer has recently been published (76) and the completion of the same kind of study in breast cancer would provide a powerful means to explore the functional and clinical involvement of the proteins we describe here. Finally, the clinical ramifications of this study may go beyond breast cancer. Brain metastases commonly arise from primary cancers of the lung and skin (melanoma), as well as at a lower frequency in patients with other cancer types (1) and the candidate biomarkers identified in this study could eventually be relevant for brain metastases in other types of cancer. However, it is impossible with only one cellular model to determine how generalizable the observations will be, and this hypothesis will have to be experimentally tested by investigating brain metastasis models in other cancers.

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