CANCER GENOMICS & PROTEOMICS 7: 253-260 (2010)

Tyrosine Kinase-Deficient EphB6 Receptor-dependent Alterations in Proteomic Profiles of Invasive Breast Carcinoma Cells as Determined by Difference Gel Electrophoresis

RAJ P. KANDPAL

Department of Basic Medical Sciences, Western University of Health Sciences, Pomona, CA 91766, U.S.A.

Abstract. *The expression profiles of the erythropoietin producing hepatocellular carcinoma (Eph) receptor family of tyrosine kinases have been previously shown to provide molecular signatures of normal breast cells, breast tumor cells and invasive breast carcinoma cells. In particular, the expression of EphB6 receptor is lost in invasive breast carcinoma cell line MDA-MB-231. The comparative proteomic profiles of native and EphB6-expressing MDA-MB-231 cells using difference gel electrophoresis (DIGE) and liquid chromatography–mass spectrometry of selected proteins are presented in this study. The expression of more than 70 proteins was significantly altered in EphB6-transfected MDA-MB-231 cells. These altered proteins are involved in glycolysis, cell cycle regulation, tumor suppression, cell proliferation, mitochondrial metabolism, mRNA splicing, DNA replication and repair. Although the majority of these proteins have been implicated in tumorigenesis, the impairment of energy homeostasis and altered regulation of signaling pathways appear to be noteworthy targets of EphB6. Based on the identities of altered proteins and the pathways regulated by these proteins, this study suggests that the interactions of EphB6 with a wide variety of proteins lead to altered proteomic profile of EphB6-transfected MDA-MB-231 cells.*

The erythropoietin producing hepatocellular carcinoma (Eph) receptors are the largest family of receptor tyrosine kinases (1). These receptors are activated by their cognate ephrin ligands. Eph receptors and ephrin ligands are classified into A and B classes based on their sequence similarities. There are 14 Eph receptors and 9 ephrin ligands known in mammalian cells. These receptors have been well characterized for their involvement in brain development, axon guidance and angiogenesis (2). The role of these receptors and ligands is becoming increasingly important in cancer initiation and progression. While Eph receptors and ephrin ligands are known to be up-regulated in some cancers (3-5), EphB6 is lost in more aggressive/invasive breast cancers, melanomas and neuroblastomas (4, 6, 7). It has been previously shown that down-regulation of EphB6 is related to epigenetic silencing of this gene by promoter methylation (8), as confirmed by detection of the methylated promoter using methylation sensitive PCR (8, 9). It is interesting to note that, unlike other members of the Eph receptor family, EphA10 and EphB6 are kinase-deficient. To address the biological role of EphB6, its interacting partners were characterized by using a yeast two-hybrid system. The results of that study indicated the interaction of its cytoplasmic domain with intracellular proteins such as aldolaseA, dynactin, clusterin and plekstrin homology domain-containing family B member 1 protein (10). Based on the cellular functions of these interacting partners, it was suggested that EphB6 directly or indirectly influences a variety of cellular pathways. These results have led to the speculation that proteomic profiles of mammalian cells with or without EphB6 will be significantly altered in the presence of EphB6 in mammalian cells.

Based on the unique and interesting EphB6 interactors, its knase-deficient status and involvement in cancer invasiveness, the downstream effects of EphB6 signaling are likely to illuminate the importance of proteins that are possibly influenced by EphB6 signaling. Although EphB6 interactors are largely indicated by the effect they have on other signaling proteins, the study in (10) was the first to show their direct interaction. A recent elegant study also demonstrated direct interactions of Eph receptors with other proteins (11).

The present study has focused on determining the downstream proteomic consequences of EphB6 signaling in MDA-MB-231 breast carcinoma cell line. These cells are highly invasive as evidenced by *in vitro* assay and *in vivo* animal experimentation. The invasiveness of MDA-MB-231 cells is attributed to the lack of EphB6 protein (12). Thus, the

Correspondence to: Dr Raj Kandpal, Department of Basic Medical Sciences, Western University of Health Sciences, 309 E. Second Street, Pomona, CA 91766, U.S.A. Tel: +1 9097063520, Fax: +1 9094695698, e-mail: rkandpal@westernu.edu

Key Words: Mass spectrometry, difference gel electrophoresis, DIGE, proteomic profile, EPHB6, breast cancer invasiveness, metastasis.

proteomic profiles of native and *EphB6*-transfected cells will help confirm the direct as well as the indirect involvement of EphB6 in cellular pathways.

A variety of approaches have been used to investigate the global effects of a specific protein or a specific pathway. These methods include gene profiling, micro RNA profiling, evaluation of cellular phenotype *in vitro* and *in vivo* effects of specific proteins during animal development. While the latter strategies related to *in vivo* phenotypic effects require several months to be performed, microarray profiling of mRNA and miRNAs are relatively straightforward. However, the profiling results do not always correspond to proteins. Such methodology-specific shortcomings can be overcome by determining the proteomic profiles of cells that are phenotypically different or made different by the presence or absence of a specific protein. Difference gel electrophoresis (DIGE) is an informative methodology for large-scale proteomic investigations (13), and it was applied in this investigation for qualitative and quantitative proteomic differences between native and *EphB6*-transfected MDA-MB-231 cells.

Table I. *Summary of altered spots*.

Spot characteristics	
Spots altered in fluorescence yield	250
Spots excised for sequencing	250
Total number of unique sequences identified	858
Total number of sequences with multiple peptides	76
Least fold change used as a cutoff	1.3

Table II. *Altered proteins in native and EphB6 transfected MDA-MB-231 cells*.

Proteins/Fold changes	Up-regulated	Down-regulated
Total number of proteins	46	30
1.3-1.5 Fold	18	8
1.5-2.0 Fold	23	15
2.0-3.0 Fold	3	6
>3.0 Fold	2	1

Materials and Methods

Cell culture. MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37˚C in the presence of 7% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA), 2.0 mM L-glutamine (Gibco), 25 units/ml penicillin (Gibco) and 25 μg/ml streptomycin (Gibco).

Transfection of MDA-MB-231 with EphB6 construct. MDA-MB-231 cells were stably transfected with a full-length *EphB6* cDNA cloned in pCDNA3.1+, as described previously (12).

Briefly, MDA-MB-231 cells were transfected with *EphB6* cDNA construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. The transfected cells were grown in media containing G418 (Invitrogen) at a concentration of 500 μg/ml and the surviving clones were analyzed for the levels of *EphB6* transcript and protein. As a control, MDA-MB-231 cells were transfected with pCDNA3.1+ (empty vector) and G418-resistant clones were isolated.

Total RNA isolation and PCR amplification of EphB6. Approximately 2×10^6 native MDA-MB-231 cells or *EphB6*-transfected cells were lysed with Trizol reagent, and RNA was isolated as described previously (12). An aliquot of RNA was reverse transcribed and amplified by PCR using *EphB6* primers (12).

Western blot analysis. The cells were grown to the logarithmic phase in appropriate media, the medium was removed and the plates were washed with ice-cold PBS. The cells were lysed by incubating with an aliquot (500 μl) of cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing 1 mM phenylmethylsulphonyl fluoride (Sigma, St Louis, MO, USA) for 5 minutes on ice, and the cell lysate was collected. The lysate was sonicated, the homogenate centrifuged at 16,000×g in a microfuge at 4˚C for 10 minutes and the supernatant was collected.

Approximately 50 μg of protein (in a volume of 20 μl), as determined by the Bradford reagent (Sigma), were combined with 10 μl of 3× loading buffer (Cell Signaling Technology) supplemented with 125 mM dithiothreitol. The samples were loaded onto a 10% SDS-polyacrylamide gel and electrophoresed. The proteins were transferred from the gel to a PVDF membrane (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA) and processed for the detection of EphB6 or β-actin.

Two-dimensional DIGE. The conventional two-dimensional (2D) gel electrophoresis restricted the analysis of one sample at a time and thus made comparative analysis of two samples relatively labor-intensive. However, the use of fluorescent dyes and the availability of gel scanners have made simultaneous analysis of two samples in the same gel convenient by employing the DIGE strategy (13). This approach was used to distinguish between the protein complements of MDA-MB-231 cells in the presence and absence of EphB6 protein.

To isolate proteins, the culture medium containing MDA-MB-231 cells was centrifuged and the cell pellet was collected. The pellet was mixed with 0.5 ml ice-cold protein lysis solution (BioRad, Hercules, CA, USA) in the presence of a protease inhibitor cocktail (Roche, Nutley, NJ, USA), vortexed, passed repeatedly through a 20 gauge syringe needle and placed on ice for 30 min. The particulate material was removed by centrifugation at 40˚C for 10 min, and the protein concentration in the supernatant was determined by using 2D Quant kit (Amersham Biosciences). The samples were cleaned using 2D Clean up kit (BioRad) before loading them on isoelectric focusing (IEF) gel strips.

The protein lysates were labeled by incubating on ice for 30 min in the dark with either Cy3 or Cy5 at a concentration of 400 pmol dye/50 μg of protein. The labeling reaction was terminated by incubating with 10 mM lysine for 10 min. The labeled protein was mixed with 150 μg of unlabeled protein and used for isoelectric focusing. The isoelectric pH gradient (IPG) strips (pH 3-10, GE Healthcare, Piscataway, NJ, USA) were rehydrated in IPGphor IEF system (GE Healthcare) by incubating them with protein samples in a

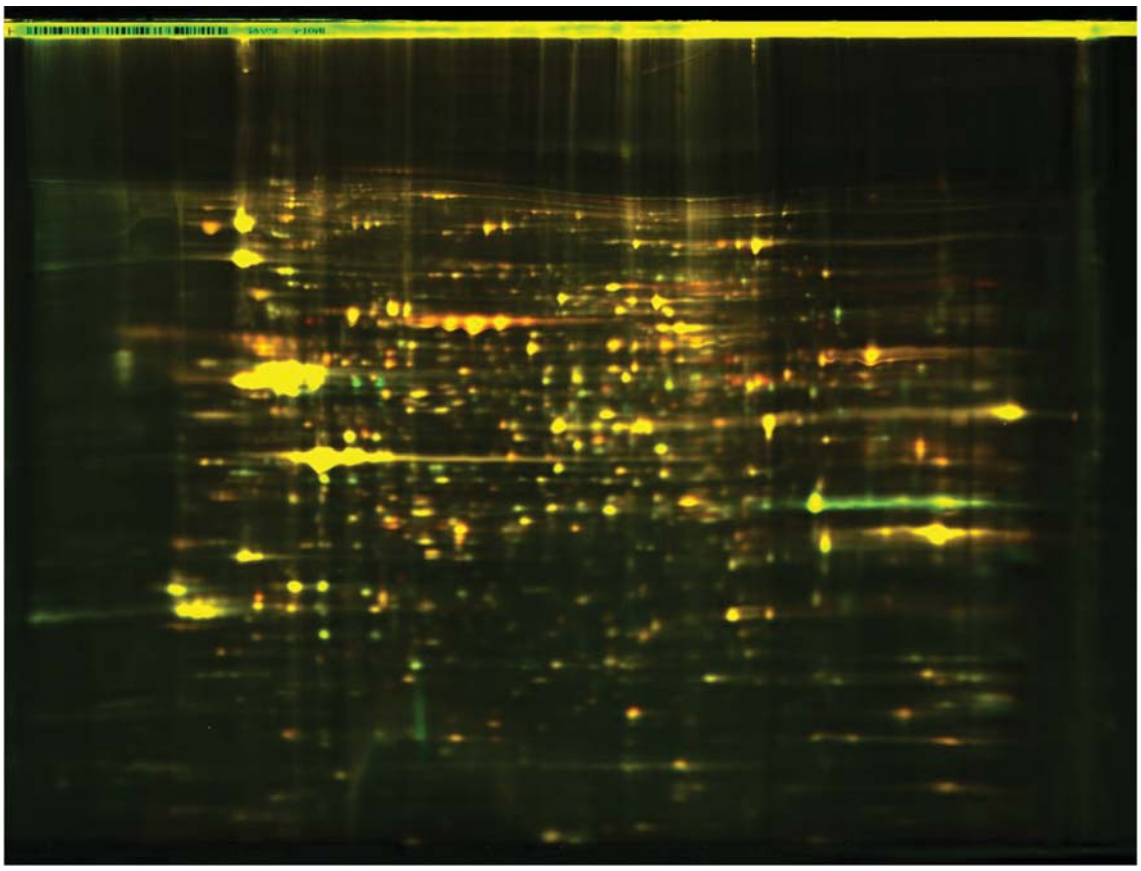

Figure 1. *Superimposed image of proteins labeled with green and red fluorescent dyes. Two-dimensional gel electrophoresis of a mixture of vector transfected MDA-MB-231 cell lysate labeled with Cy3 (green) and EphB6 transfected MDA-MB-231 cell lysate labeled with Cy5 (red) was performed as described under Materials and Methods and the gel was imaged at appropriate wavelengths. The green and red fluorescent spots indicate the altered abundance of specific proteins.*

buffer containing 8 M urea, 4% CHAPS, bromophenol blue, 1% Phamlyte and 2 mg/ml DTT. Isoelectric focusing was performed in IPGphor II (GE Healthcare) for a period of 65.5 kVh. The strips were equilibrated for 15 min with a solution of 50 mM Tris-Cl buffer, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue and 1% DTT (w/v). The equilibration was carried out for another 15 min in the mixture that contained 2.5% iodoacetamide instead of DTT. These equilibration steps led to reduction of proteins and subsequently carbamidomethylation of cysteine sulfhydryl groups. The IPG strip was carefully placed on top of a 12.5% SDS-polyacrylamide gel cast on low-fluorescence glass plates. The electrophoresis was carried out at 1W for 2 hours followed by 2W of constant power until the blue dye had run off the gel.

Visualization of fluorescent protein spots on gel and analysis of gel image. A Typhoon 9410 imager (GE Healthcare) was used to visualize protein spots. The excitation and emission wavelength combinations of 532 nm/580 nm and 633 nm/670 nm were used to capture the images of proteins labeled with Cy3 and Cy5, respectively. The spot detection, matching and normalization were carried out by using DeCyder software (GE Healthcare). The quantitative comparison of individual spots was performed by recording relative fluorescence of Cy3 and Cy5 at desired excitation/emission wavelengths. The spot intensities were compared and the fold change for each protein spot determined using PROGENESIS software (Nonlinear USA Inc., Durham, NC, USA). Protein spots showing significant changes were marked for spot picking and further analysis.

Spot excision mass spectrometry, analyses and database searches. The proteins in the gels were immobilized by incubation for 10 min in a mixture of 10% methanol and 7% acetic acid. The unlabeled portion of the proteins was visualized by incubating with deep purple stain. The gel was scanned in a Typhoon scanner and spots of interest were excised using an Ettan Spot Picker (GE Healthcare) and then digested with sequencing-grade modified trypsin (Promega Corporation, Madison, WI, USA). The digested peptides were identified based on their mass spectra using a Fourier Transform Linear Trap Quadrupole (FT-LTQ) mass spectrometer (Finnigan, San Jose, CA, USA). Briefly, the protein digests were loaded onto a trapping column for concentrating and removing salts and then separated on a reverse phase column. Subsequently peptides were infused and tandem mass spectroscopy (MS) spectra were acquired in the positive ion mode. Protein identification was carried out by searching against non-redundant NIH's NCBI databases (www.ncbi.nlm.nih.gov) using MASCOT (Matrix Science Inc., Boston, MA). The search criteria

Table III. *Identities of specific altered proteins.*

Up-regulated proteins
Plastin-2
Insulin-like growth factor 2 mRNA-binding protein 2
Protein FAM71A
Neuroblastoma breakpoint family member 20
Serine hydroxymethyltransferase, mitochondrial
Proton-associated sugar transporter A
Serum albumin
SH2 domain-containing protein 3C
Serum albumin
Prefoldin subunit 5
Mitogen-activated protein kinase 8
Rho GDP-dissociation inhibitor 1
SH2 domain-containing protein 3C
Coiled-coil domain-containing protein 105
Proteasome subunit beta type-8
Dihydrolipoyl dehydrogenase, mitochondrial
Glutamate dehydrogenase 1, mitochondrial
Teneurin-4
Forkhead box protein P3
Down-regulated Proteins
Tubulin alpha-1C chain
RuvB-like 2
Calmodulin
Adenomatous polyposis coli protein 2
Protein S100-A13
Annexin A2
Glyceraldehyde-3-phosphate dehydrogenase
Serpin B9
60S acidic ribosomal protein P2
Uncharacterized protein HSPC234
Actin, cytoplasmic 2
Serine/threonine-protein kinase PRP4 homolog

Down-regulated proteins (Continued)
Growth factor receptor-bound protein 2
Transketolase
Serpin B6
Plasmolipin
Annexin A2
Endophilin-B1
Pre-mRNA cleavage complex 2 protein Pcf11
Protein NPAT
Zinc finger protein 616
DNA topoisomerase 2-binding protein 1
Heterogeneous nuclear ribonucleoprotein H
Beta-centractin
Proton-associated sugar transporter A
Galectin-8
DNA topoisomerase 2-beta
Nucleoside diphosphate kinase A
Estradiol 17-beta-dehydrogenase 12
60 kDa Heat-shock protein, mitochondrial
Zinc finger E-box-binding homeobox 1
Rho-related GTP-binding protein RhoJ
V-set and transmembrane domain-containing protein 2A
Pericentrin
Adenine phosphoribosyltransferase
Nesprin-1
Phosphoglycerate kinase 1
T-complex protein 1 subunit theta
Serine/threonine-protein kinase TAO3
Pyruvate kinase isozymes M1/M2
Putative uncharacterized protein LOC285679
Transgelin-2
Tubulin alpha-1B chain
Far upstream element-binding protein 2
Zinc finger FYVE domain-containing protein 16
Microsomal triglyceride transfer protein large subunit
Tubulin alpha-1B chain
Peptidyl-prolyl cis-trans isomerase B

accounted for carbamidomethylation of cysteine, partial oxidation of methionine, and with one missed cleavage allowed. The mass tolerance limits for MS and MS/MS were set at 15 ppm and 0.8 Da, respectively for FT-LTQ. For LTQ, mass tolerance of 1.5 Da and 0.8 Da were set for MS and MS/MS, respectively.

Results

Stable expression of EphB6 in transfected MDA-MB-231 cells. The native and *EphB6*-transfected MDA-MB-231 cells were compared for the levels of *EphB6* transcript and protein. As expected, the transcript and EphB6 protein were not detectable in the native cells, whereas abundant amounts of transcript and protein were observed in MDA-MB-231 cells stably transfected with an *EphB6* expression construct. These observations indicate that the above cell line-pair is appropriate for investigating EphB6-dependent proteomic profiles of MDA-MB-231 cells.

Proteomic profile of native and transfected MDA-MB-231 cells. The protein lysates of native and *EphB6*-transfected MDA-MB-231 cells were analyzed by DIGE. Conventional methods of comparative proteomics often result in considerable variations between technical replicates. In contrast, DIGE allows simultaneous analysis of two samples in the same gel after normalization with an internal loading control (13). The fluorescence scanning of the gel indicated over 1000 spots labeled with Cy3 (Figure 1, green fluorescence of vector transfected MDA-MB-231 cell lysate) or Cy5 (Figure 1, red fluorescence of lysate from *EphB6*-transfected MDA-MB231 cells). The relative abundance of individual spots was assessed and quantification was performed as described in the Materials and Methods.

The quantification of spots revealed changes in the fluorescence of over 250 protein spots (Table I) that were up- or down-regulated in MDA-MB-231 cells transfected with

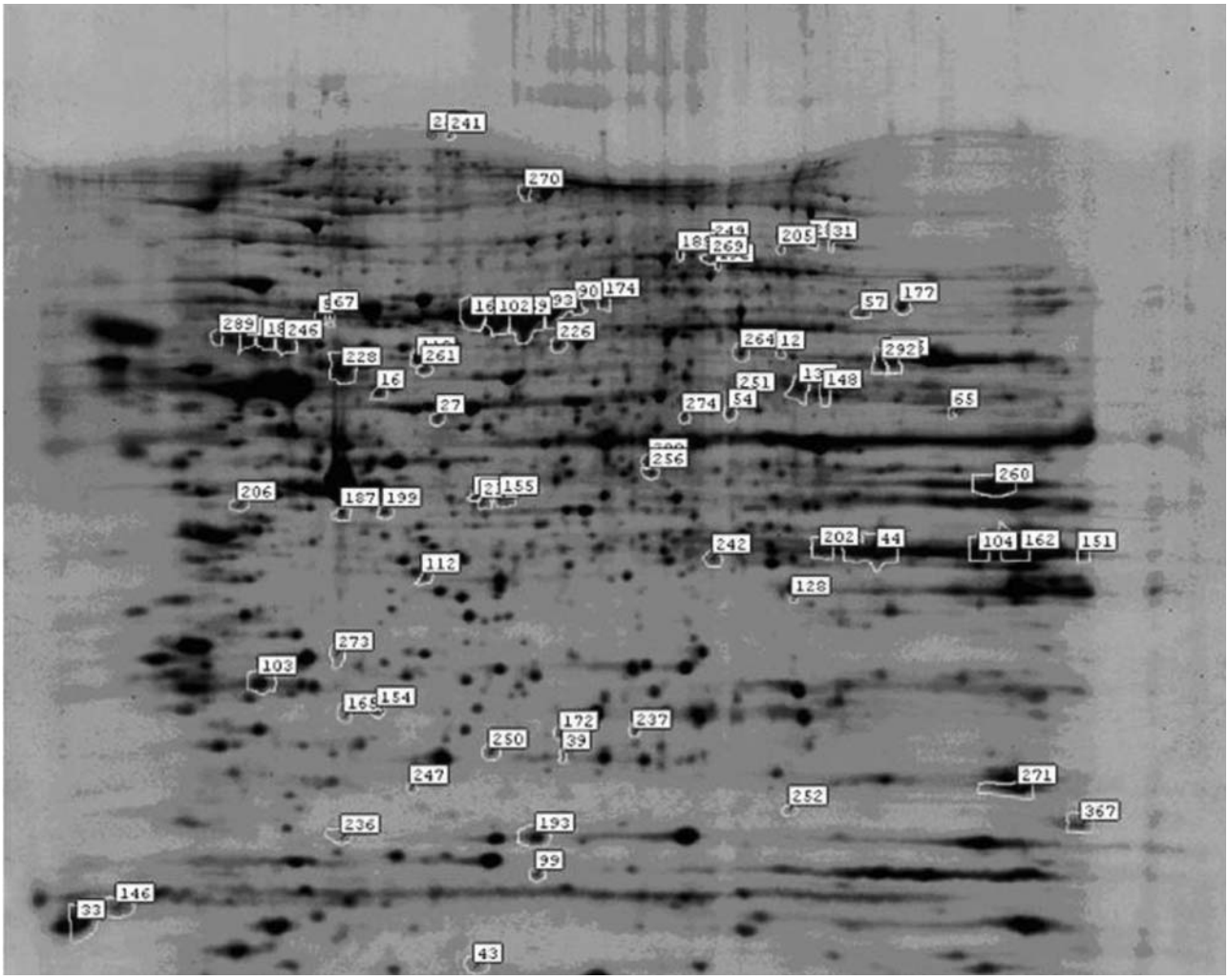

Figure 2. *Stained gel for spot excision and sequencing. The preparative gel was visualized after incubating with a deep purple stain. The spots were marked by comparing with the corresponding coordinates in the fluorescent image shown in Figure 1. The spots were excised and processed as described in the Materials and Methods.*

EphB6. These 250 spots yielded 858 sequences with matches in the database. These protein spots were visualized with a deep purple stain and marked for extraction and sequence determination (Figure 2). Each spot marked in Figure 2 represented more than one protein. Therefore, the abundance of actual protein undergoing the change was reflected in the number of peptide fragments that matched to that protein. These proteins were classified by the degree of changes varying by at least 1.3-fold and greater (Table II). The proteins fell into four groups based on 1.3- to 1.5-fold change, 1.5- to 2.0-fold change, 2.0- to 3.0-fold change and >3.0-fold change. The majority of these alterations were 1.5- to 2.0-fold changes. Among these proteins, plastin-2 and insulin-like growth factor 2 mRNA-binding protein 2 were up-regulated by 4.3- and 3.7-fold, respectively, in MDA-MB-231 cells transfected with *EphB6*. In contrast, tubulin alpha-1C chain protein was down-regulated by 3.3-fold. A summary of up- and down-regulated proteins is presented in Table III.

The altered proteins were classified into categories based on their biological function (Table IV and Figure 3). These proteins were involved in processes such as cell shape and actin cytoskeleton, radiosensitivity, helicases, signal transduction, mitochondrial proteins, metabolism, transport, chaperones for protein folding, proteasome, mRNA processing, transcription factors, replication/repair, cell cycle and centrosome. The maximum numbers of genes altered in *EphB6*-expressing MDA-MB-231 cells belonged to signal transduction and metabolism. The miscellaneous category consisted of proteins such as galectin, transgelin, V-set and transmembrane domain containing protein, coiled-coil domain protein and some uncharacterized protein coding sequences (Table V).

Table IV. *Functional classification of altered proteins.*

Process	Gene count
Actin/cell shape	4
ER stress	1
Helicases	2
Signaling	12
Mitochondria	3
Metabolism	12
Transport	5
Folding/chaperone	2
mRNA processing	4
Transcription factor	3
Replication/repair	2
Cell cycle	1
Centrosome	1
Miscellaneous	8

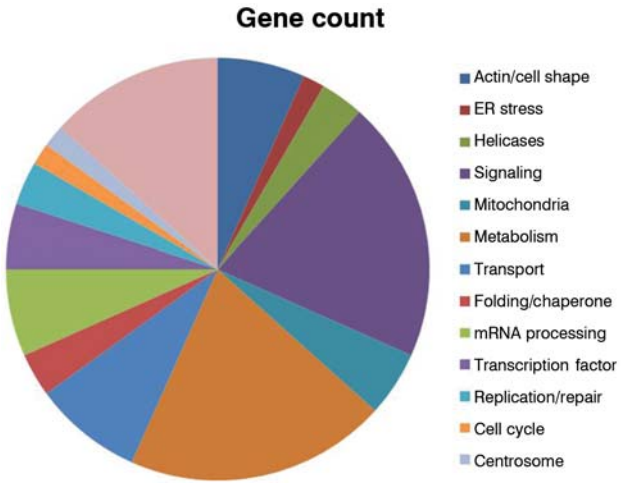

Figure 3. *Pie diagram of various functional classes of proteins presented in Table IV.*

Among the larger categories of signal transduction and metabolism, interestingly, all genes were altered between 1.5- and 2.0-fold. Noteworthy among the proteins affecting metabolism were enzymes of glycolysis, nucleotide synthesis, protein synthesis/turnover and estrogen synthesis. While the amounts of glyceraldehyde-3-phosphate dehydrogenase decreased significantly in *EphB6*-transfected cells, a notable decrease in the amounts of pyruvate kinase and phosphoglycerate kinase was also observed. Other notable proteins included plastin, cytoplasmic actin, RhoJ, nesprin 1, prefoldin subunit 5, T-complex protein 1 subunit theta, proteasome subunit beta type B, S100A3, protein assisted sugar transporter A, plasmolipin, tubulin-alpha-1 B chain and zinc finger FYVE domain containing protein-16.

Discussion

The altered transcript profiles determined by gene microarrays have frequently been implicated in cellular phenotypes. Such conclusions are based on the assumption that the abundance of transcripts relate to changes in the corresponding proteins. However, the changes in transcript levels may not always match with the amounts of proteins (Kandpal, unpublished observations). It was therefore reasoned that a direct proteomic profile may shed light on specific proteins as molecular determinants of overt phenotypes. It has been previously shown that MDA-MB-231 cells do not have any detectable expression of EphB6 (4) and forced expression of EphB6 in MDA-MB-231 cells leads to altered invasiveness and other phenotypic characteristics of these cells (12). Furthermore, EphB6 interacts with a variety of proteins such as aldolaseA, clusterin, dynactin, plekstrin homology domain-containing family B member 1 protein and transmembrane protein 25 (10). Such a diverse set of proteins interacting with EphB6 suggests that its presence may affect a wide variety of proteins. To identify the affected proteins, a comparative proteomic profile of native and *EphB6*-transfected MDA-MB-231 cells was performed in the present study.

The effects of EphB6 on glycolysis were predicted on the basis of EphB6 interaction with aldolase A, a glycolytic enzyme (10). It is known that energy homeostasis is altered in tumor cells (14), and if expression of a specific protein is affecting some tumorigenic processes then it is not surprising that energy balance or homeostasis in transfected cells would be altered. Such energy homeostasis was reflected in the change of enzymes such as glyceraldehyde-3-phosphate dehydrogenase (NADH production), phosphoglycerate kinase (ATP production) and pyruvate kinase (ATP production). These results indicate that *EphB6* transfection is likely to decrease the availability of NADH for ATP production *via* oxidative phosphorylation.

An important enzyme related to estrogen hormone that was altered is estradiol 17 β-dehydrogenase. Interestingly, estradiol 17 β-dehydrogenase was down-regulated in EphB6 transfected cells. It warrants mention that MDA-MB-231 cells are estrogen receptor (ER) negative. It is tempting to speculate that EphB6 or its interactors/targets may influence non-classical receptor mediated non-genomic effects of estrogen (15). It has been previously reported that Eph receptors may affect the pathways leading to cell proliferation mediated by JNK proteins (16). Such changes are in conformity with the altered abundance of SH2 domain containing 3C protein (SH2DC3). Interestingly, SH2DC3, also known as SHEP1, has been shown to interact with EphB2 in yeast two-hybrid assay (17) and its binding to the conserved tyrosine-phosphorylated motif in the juxtamembrane region of EphB2 has been confirmed. These associations provide a confirmation of relevant proteins altered in *EphB6*-transfected cells.

Among other signaling proteins, the change in the amounts of proteins involved in signaling pathways mediated

Table V. *Identities of altered proteins in various functional categories.*

Actin-related/ cell shape/motility	**Metabolism**	**Transcription factors**
Plastin	60S acidic ribosomal protein	Fork head box protein P3
Actin cytoplasmic 2	Transketolase	Zn finger E box binding homeobox
RhoJ	Serpin B6	Zn finger protein 616
Nesprin 1	Nucleoside diphosphate kinase	
	Estradiol 17-β dehydrogenase 12	**Replication/repair**
ER stress/ radiosensitivity	60 kD Heat-shock protein	
	Adenine phosphoribosyl transferase	DNA topoisomerase 2 beta
IGF2PBP2	Phospho glycerate kinase	DNA topoisomerase 2 binding protein 1
	Pyruvate kinase M1/M2	
Signal transduction		**Cell cycle**
	Transport	
Calmodulin		NPAT
APC2	S100A3	
Annexin A2	Proton-assisted sugar transporter	**Centrosome**
Serum Albumin	Plasmoloipn	
SH2 domain containing 3C (Eph-binding)	Tubulin alpha-1B chain	Pericentrin
MAPK-8	Zn Finger FYVE domain containing	
RhoGDP dissociation inhibitor 1		**Helicase**
Teneurin 4	**Chaperone**	
Growth factor receptor bound protein-2		RUV2
NDP Kinase	Prefoldin subunit 5	RNA Helicase
Ser/Thr protein kinase-TAO3	T-complex protein 1 subunit theta	
Zn Finger FYVE		**Uncharacterized micc.**
	Proteasome	
Mitochondria		FAM71A
	Proteasome subunit beta type B	Neuroblastoma breakpoint family member 20
Dihydrolipoyl dehydrogenase		Coiled-coil domain
Glutamate dehydrogenase	**Splicing/ mRNA processing**	HSPC 234
Endophilin B1		Galectin
	Ser-thr protein kinase PRP4	V-set transmembrane domain containing protein 2A
Metabolism	mRNA cleavage complex 2	LOC 285679
	Heterogeneous nuclear ribonuclear protein-H	Transgelin
Serine hydroxy methyl transferase	Far upstream element binding protein 2	
Glyceraldehyde-3-phosphate dehydrogenase		
Serpin B9		

by calcium (calmodulin and annexin), wnt (APC2), ras (growth factor receptor bound protein-2), and rho proteins have been conclusively shown to participate in various tumor phenotypes (17-22). The changes in the levels of these proteins in *EphB6*-transfected MDA-MB-231 cells may partly be explained by some yet uncharacterized interactions between the intermediates of various pathways.

Although direct effects of EphB6 on apoptosis were not investigated in this study, the altered abundance of RUVB2, proteasome subunit b-type 8, serpin B9, endophilin B1 and DNA topoisomerase-2 binding protein 1 suggest that expression of EphB6 may influence apoptosis. Such apoptotic functionality is consistent with the proposed tumor suppressor action of EphB6 (23).

Transgelin is reported to be down-regulated in transformed cells, thus being an early indicator of tumorigenesis (24). When *EphB6*-transfected cells were compared to native MDA-MB-231 cells, the abundance of transgelin was higher in the transfected cells, suggesting a trend towards normal cell profile. Based on the interaction of transgelin with actin fibres (25) and the alterations in the abundance of plastin, actin, RhoJ, nesprin 1 and Rho GDP dissociation inhibitor, it was likely that the effects of EphB6 on actin cytoskeleton are responsible for the phenotypic changes observed in *EphB6*-transfected MDA-MB-231 cells. The alterations in proteins such as pericentrin, NPAT, DNA topoisomerase 2- β, DNA topoisomerase binding protein 1 and RUV2 indicated that the transfected cells are possibly affected at the level of cell cycle and cytokinesis. It is, therefore, reasonable to predict that EphB6 transfected cells may have altered characteristics of various phases of cell cycle.

An important set of proteins involved in transcription were also altered in *EphB6*-transfected MDA-MB-231 cells. These regulatory proteins are either transcription factors or participate in splicing reactions. It is interesting to note that transfection

of *EphB6* caused a change in the abundance of a variety of proteins. Such multitude of changes is expected due to the greater availability or lack of a general transcription factor(s). However, the altered levels of these transcription factors may not be directly attributed to EphB6.

In conclusion, *EphB6*-transfected MDA-MB-231 cells showed molecular profiles that were different than the native cell line. In particular, the metabolic enzymes involved in energy homeostasis, cytoskeleton regulatory proteins, a variety of signaling pathways and transcription factors were noteworthy. The observations on enzymes of glycolysis supported the reports that metabolic therapy to offset aberrant energy status may be a beneficial mode of breast cancer intervention. It warrants mention that the altered protein profiles reported here need to be validated with additional cell lines and tumor specimens that lack the expression of EphB6 receptor. The results of the present study suggest that EphB6 and its interactors influence a variety of pathways in the cell that are partly responsible for the observed cellular phenotypes.

Acknowledgements

The Author wishes to thank Drs Anand Swaroop and Rong-Fong Shen for their support, and Drs Jacob Nellissery, Brian Fox and Ganachari Nagaraja for technical assistance.

References

1 Manning G, Whyte DB, Martinez R, Hunter T and Sudarsanam S: The protein kinase complement of the human genome. Science *298*: 1912-1934, 2002.

2 Gale NW, Holland SJ, Valenzuela DM, Flenniken A, Pan L, Ryan TE, Henkemeyer M, Strebhardt K, Hirai H, Wilkinson DG, Pawson T, Davis S and Yancopoulos GD: Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. Neuron *17*: 9-19, 1996.

3 Liu W, Ahmad SA, Jung YD, Reinmuth N, Fan F, Bucana CD and Ellis LM: Coexpression of ephrin-Bs and their receptors in colon carcinoma. Cancer *94*: 934-939, 2002.

4 Fox BP and Kandpal RP: Invasiveness of breast carcinoma cells and transcript profile: Eph receptors and ephrin ligands as molecular markers of potential diagnostic and prognostic application. Biochem Biophys Res Commun *318*: 882-892, 2004.

5 Kinch MS, Moore MB and Harpole DH, Jr.: Predictive value of the EphA2 receptor tyrosine kinase in lung cancer recurrence and survival. Clin Cancer Res *9*: 613-618, 2003.

6 Easty DJ, Herlyn M and Bennett DC: Abnormal protein tyrosine kinase gene expression during melanoma progression and metastasis. Int J Cancer *60*: 129-136, 1995.

7 Tang XX, Evans AE, Zhao H, Cnaan A, London W, Cohn SL, Brodeur GM and Ikegaki N: High-level expression of EPHB6, EFNB2, and EFNB3 is associated with low tumor stage and high TrkA expression in human neuroblastomas. Clin Cancer Res *5*: 1491-1496, 1999.

8 Fox BP and Kandpal RP: Transcriptional silencing of EphB6 receptor tyrosine kinase in invasive breast carcinoma cells and detection of methylated promoter by methylation-specific PCR. Biochem Biophys Res Commun *340*: 268-276, 2006.

9 Fox BP and Kandpal RP: DNA-based assay for EPHB6 expression in breast carcinoma cells as a potential diagnostic test for detecting tumor cells in circulation. Cancer Genomics Proteomics *7*: 9-16, 2010.

10 Fox BP and Kandpal RP: Yeast two-hybrid assay reveals several interactors of EphB6 receptor. Open Proteomics J *1*: 79-85, 2008.

11 Jørgensen C, Sherman A, Chen GI, Pasculescu A, Poliakov A, Hsiung M, Larsen B, Wilkinson DG, Linding R and Pawson T: Cell-specific information processing in segregating populations of Eph receptor ephrin-expressing cells. Science *326*: 1502-1509, 2009.

12 Fox BP and Kandpal RP: EphB6 receptor significantly alters invasiveness and other phenotypic characteristics of human breast carcinoma cells. Oncogene *28*: 1706-1713, 2009.

13 Minden JS, Dowd SR, Meyer HE and Stuhler K: Difference gel electrophoresis. Electrophoresis 30 Suppl *1*: S156-S161, 2009.

14 Gatenby RA and Gillies RJ: Why do cancers have high aerobic glycolysis? Nat Rev Cancer *4*: 891-899, 2004.

15 Meldrum DR: G-Protein-coupled receptor 30 mediates estrogen's nongenomic effects after hemorrhagic shock and trauma. Am J Pathol *170*: 1148-1151, 2007.

16 Cheng N and Chen J: Tumor necrosis factor-alpha induction of endothelial ephrin A1 expression is mediated by a p38 MAPK- and SAPK/JNK-dependent but nuclear factor-kappa B-independent mechanism. J Biol Chem *276*: 13771-13777, 2001.

17 Dodelet VC, Pazzagli C, Zisch AH, Hauser CA and Pasquale EB: A novel signaling intermediate, SHEP1, directly couples Eph receptors to R-Ras and Rap1A. J Biol Chem *274*: 31941-31946, 1999.

18 Means AR: The year in basic science: calmodulin kinase cascades. Mol Endocrinol *22*: 2759-65, 2008.

19 Hayes MJ, Longbottom RE, Evans MA and Moss SE: Annexinopathies. Subcell Biochem *45*: 1-28, 2007.

20 Jarrett CR, Blancato J, Cao T, Bressette DS, Cepeda M, Young PE, King CR and Byers SW: Human APC2 localization and allelic imbalance. Cancer Res *61*: 7978-7984, 2001.

21 Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D and Schlessinger J: The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell *70*: 431-442, 1992.

22 Karlsson R, Pedersen ED, Wang Z and Brakebusch C: Rho GTPase function in tumorigenesis. Biochim Biophys Acta *1796*: 91-98, 2009.

23 Yu J, Bulk E, Ji P, Hascher A, Tang M, Metzger R, Marra A, Serve H, Berdel WE, Wiewroth R, Koschmieder S and Müller-Tidow C: The EPHB6 receptor tyrosine kinase is a metastasis suppressor that is frequently silenced by promoter DNA hypermethylation in non-small cell lung cancer. Clin Cancer Res *16*: 2275-2283, 2010.

24 Zhao L, Wang H, Deng YJ, Wang S, Liu C, Jin H and Ding YQ: Transgelin as a suppressor is associated with poor prognosis in colorectal carcinoma patients. Mod Pathol *22*: 786-796, 2009.

25 Goodman A, Goode BL, Matsudaira P and Fink GR: The Saccharomyces cerevisiae calponin/transgelin homolog Scp1 functions with fimbrin to regulate stability and organization of the actin cytoskeleton. Mol Biol Cell *14*: 2617-2629, 2003.

Received May 26, 2010
Revised July 17, 2010
Accepted July 16, 2010