Published in final edited form as:

Clin Exp Metastasis. 2010 October ; 27(7): 493–503. doi:10.1007/s10585-010-9338-8.

Alteration in Protein Expression in Estrogen Receptor Alpha Negative Human Breast Cancer Tissues Indicates an Malignant and Metastatic Phenotype

Ziad J. Sahab, Ph.D.¹, Yan-Gao Man, M.D., Ph.D.^{2,3}, and Qing-Xiang Amy Sang, Ph.D.^{1,*}

¹Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306-4390, USA

²Department of Gynecologic and Breast Pathology, Armed Forces Institute of Pathology and American Registry of Pathology, Washington, DC, USA

³Jilin University, Changchun, Jilin, PRC

Abstract

Ductal carcinoma *in situ* represents an earliest identifiable breast cancer lesion. The disruption of the myoepithelial cell layer and basement membrane is a prerequisite for DCIS to initiate invasion into the stroma. The majority of epithelial cells overlying a focally disrupted myoepithelial cell layer are estrogen receptor-alpha negative, or ER(-); however, adjacent cells within the same duct confined by an intact myoepithelial cell layer express high levels of ER. These ER (+) and ER (-) cells were microdissected from the same ducts of breast cancer patients. Differential proteins expressed by ER(+) and ER(-) cells were identified using two-dimensional gel electrophoresis followed by mass spectrometry and Western blot analysis. ER(-) cells express lower levels of superoxide dismutase, Ral A protein, galectin-1, uridine phosphorylase, cellular retinoic acid-binding protein 1, S100 calcium binding protein A11, and nucleoside diphosphate kinase A or non-metastasis protein 23-H1 (nm23-H1). The upregulated protein, Rho GDP-Dissociation Inhibitor 1 alpha, may induce chemotherapy resistance. The significant findings are that the microdissected ER(-) cells express 13.5 times less cellular retinoic acid-binding protein 1, a protein involved in cellular differentiation, and 34.2 times less nucleoside diphosphate kinase A or (nm23-H1), a metastasis suppressor, and express fewer proteins than adjacent ER(+) cells. The collective role of the alteration of protein expression in ER(-) cells may be promoting a more malignant phenotype than adjacent ER(+) cells, including decreased ability to undergo apoptosis and differentiation, and increased potential to damage DNA, metastasize, and resist chemotherapy.

Keywords

Apoptosis; Cancer Heterogeneity; Estrogen Receptor; Human Breast Cancer; Invasion; Metastasis Suppressor; Mass Spectrometry; Protein Biomarker; Proteomics; Tumor Progression

*Correspondence: Prof. Qing-Xiang Amy SANG, Ph.D., Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, 102 Varsity Way, Tallahassee, FL 32306-4390, USA; Tel: +1-850-644-8683; Fax: +1-850-644-8281; sang@chem.fsu.edu.

Introduction

Human ductal carcinoma *in situ* (DCIS) is the earliest identifiable breast cancer lesion, in which tumor cells are confined to the duct and surrounded by a layer of myoepithelial (ME) cells and the basement membrane, with no evidence of invasion into the surrounding stroma [1]. While approximately half of DCIS cases may eventually progress to an invasive cancer [2], many are likely to remain non-threatening lesions, which complicates determination of the best treatment regimen for breast cancer patients. In human breast ducts, the ME cell layer and basement membrane form a physical barrier between the stroma and the luminal epithelium or *in situ* breast tumors. The ME cells themselves are joined by intercellular junctions and adhesion molecules, forming a continuous layer that encircles the entire duct system, and a discontinuous layer or a basket-like structure that covers a vast majority of lobules and terminal duct-lobular units [35]. The basement membrane is composed of laminins, type IV collagen, entactin, heparan sulfate proteoglycans, and glycosaminoglycans, forming a continuous lining surrounding and attaching to ME cells via hemidesmosomes and focal adhesion complexes [35]. These structural features confer two essential functions upon the ME cells and the basement membrane. First, as the epithelium is normally devoid of lymph and blood vessels, and is therefore dependent upon the stroma for its metabolic and survival needs, the ME cells and basement membrane function as gate-keepers, directly regulating the communication between these two compartments. Second, due to the physical interposition of the ME cell layer and basement membrane between the stroma and epithelium, ductal tumor cells must first break their way through the ME cell layer, then the basement membrane, to reach the stroma and initiate invasion and subsequent metastases [6].

Estrogens play a major role in both the development of sexual glands and in the reproductive cycle [7], and the biological effects of estrogen are mediated through the estrogen receptor (ER). ER α , cloned in 1986 [8; 9], was believed to be the sole form of this receptor until 1996, when a second ER, called ER β , was also cloned [10; 11]. The exact roles of ER α and ER β in breast cancer are not fully understood, though it has been reported that estrogens are involved in the promotion of human breast cancer, possibly by way of their mitogenic activity [12].

Immunohistochemical staining for ER β in normal human breast tissues, DCIS, invasive cancers, and lymph node metastases has revealed a gradual reduction in ER β expression during the transition from normal tissue to preinvasive lesions to invasive cancers, and ER β was lost in 21% of the invasive cancers studied [13]. Another study utilizing similar techniques revealed that the percentage of cells positive for ER β was high in normal mammary glands and in nonproliferative benign breast disease, but the percentage of positive cells was decreased significantly in proliferative benign breast disease and in carcinoma *in situ*. These results are in agreement with results obtained when analyzing mRNA levels of ER β in normal mammary, benign breast disease, breast cancer, and metastatic breast cancer in lymph nodes [14], where it was shown that ER β mRNA expression was significantly decreased in breast cancer and lymph node metastatic tissues when compared with normal mammary and benign breast disease tissues. All of these results suggest that ER β exerts a protective effect against the mitogenic activity of estrogens

mediated by ER α , and may function as a tumor suppressor, as the loss of ER β expression seems to correlate with the progression of breast carcinomas.

To identify potential precursors of invasive lesions, we have carried out studies focusing on the correlation between the structural integrity in ME cell layer and the immunohistochemical and genetic profiles in adjacent epithelial cells. In 5,698 duct cross-sections from 220 patients with ER α (+), non-invasive breast tumors, we detected a total of 405 focal ME cell layer disruptions, defined as the absence of ME cells resulting in a gap equal to or greater than the combined size of 3 ME or epithelial cells [15]. Of these focal disruptions, 350 (86.4%) were overlaid by epithelial cell clusters with no or substantially reduced ER α expression, in contrast to adjacent cells within the same duct, which expressed high levels of ER α and overlaid an intact ME cell layer [15]. Additional studies revealed that cell clusters overlying focally disrupted ME layers generally showed a substantially higher proliferation rate when compared to adjacent cells within the same duct, as assessed by immunohistochemical staining for Ki-67 [16; 17]. These features potentially signal the formation of a biologically more aggressive cell clone, with a protein expression profile that has yet to be fully examined, while focal breakdowns in the ME cell layer are likely to serve as portals for tumor progression and early invasion.

To identify new breast cancer biomarkers, the protein profiles of nipple aspirate fluids [18], or the serum of breast cancer patients [19], have been compared to that of healthy donors. Identifying biomarkers by these less invasive methods is of great importance, but questions remain as to what early differences in protein profiles of these samples might manifest to detectable levels, as the low abundance of proteins secreted in the serum and other biological fluids as a result of the disease is often masked by the high abundance of albumin and other highly abundant proteins [20]. The protein profiles of breast cancer cell lines have also been compared to the profiles of normal breast cell lines [21], and while tumor-derived cell lines can be useful for initial studies [22], each line displays a unique evolution that may not truly mimic real *in vivo* conditions [23]. Comparisons have also been made between microdissected breast tissues composed of either normal ductal epithelium or ductal epithelium containing DCIS lesions [24]. However, the samples collected for this study, composed of 50,000-100,000 cells, were unlikely to have been homogeneous, and were likely composed of multiple cell types with divergent expression profiles

Many diseases manifest themselves through severe changes in human pathophysiology, which forms the basis for clinical chemistry and provides its value in diagnoses and subsequent therapeutic interventions [25]. Proteomics includes the global analysis of proteins expressed by the genome of an organism, an organ, a tissue, or a group of cells with the typical aim being the evaluation of quantitative changes that occur as a function of disease, treatment, or environment [26]. Proteomics strategies are used to identify cancer-specific protein markers that could provide the basis for the development of new diagnostic methodologies, early disease detection, and monitoring the efficacy of treatments [26; 27]. Marker detection and correlation with tumor growth is less obstinate for advanced tumors, but the true value of these developing applications lies in early tumor detection and the prompt identification of invasive phenotypes [25].

To identify candidate markers specific to the initiation of invasion by DCIS cells, we here identify proteins that are differentially expressed by the ER α (-) cell clusters overlying focally disrupted ME cell layer when compared to adjacent ER α (+) cells overlying non-disrupted ME cell layer within the same duct. Proteins extracted from ER α (+) and ER α (-) tissue samples were subjected to two-dimensional gel electrophoresis (2-DE) followed by MALDI-TOF-MS analysis of trypsin-digested spots. Differentially-expressed proteins that were identified by this method were investigated in ER α (+) and ER α (-) tissues microdissected from the ducts of the same patients (Figure 1) using Western blot. We report the differential expression of proteins of ER α (-) cells overlying a focally disrupted ME layer compared to adjacent ER α (+) cells bound by an intact ME layer.

Materials and Methods

Research involving human subjects was performed in accordance with the principles outlined in the declaration of Helsinki (1964). All the research projects involved in using human tissues and cells were approved by the Institutional Human Subject Review Board or Committees. Freshly-frozen DCIS breast tissue microdissected samples and corresponding slide-mounted, serial-sectioned paraffin-embedded tissues were provided by our co-author Yan-Gao Man, M.D., Ph.D., Director of the Gynecologic and Breast Research Laboratory at the Armed Forces Institute of Pathology and American Registry of Pathology in Washington, D.C.

Tissue Microdissection and Protein Extraction

Serial sections of 5 to 7 μ m thickness were cut and placed on positively-charged microscopic slides and rehydrated by ethanol gradient. Sections were immunohistochemically stained for ER α and different proliferation markers. Nonspecific antibody binding was blocked with blocking buffer (0.2% Triton X-100, 5% normal goat serum, and 3% bovine serum albumin in Tris-buffered saline) for 1 h at room temperature prior to overnight incubation at 4 °C with a monoclonal mouse anti-human ER α antibody (NCL-ER-6F11, Vector, Burlingame, CA) diluted into the same buffer (10 μ g/mL). Samples were then incubated with an alkaline phosphatase- conjugated secondary antibody diluted 1:5000 in blocking buffer for 4 h at room temperature, and positive signals were detected using Fast-Red (Sigma). Clusters of cells overlying myoepithelial disruptions, composed primarily of ER α (-) cells, were excised by laser-capture microdissection from adjacent, unstained serial sections. Clusters of ER α (+) cells from the same duct, but bound by an intact ME layer, were similarly obtained. Microdissected tissues were then homogenized in a tissue lysis buffer composed of 10 mM Tris, 130 mM NaCl, 1% octylglucopyranoside (OG), 1% CHAPS, 10 mM sodium phosphate, and 10 mM sodium fluoride (pH 7.5). The ratio of the volume of tissue lysis buffer added to the weight of the tissue was 1/1 (v/v). This was followed by vortexing for 10 min and then by centrifugation at 17000 $\times$ g for 20 min. This resulted in the formation of 3 layers: The top layer was composed primarily of non-soluble lipids, the middle layer contained the soluble proteins, and the bottom layer was composed of cell debris. The middle layer was recovered and used for subsequent analyses.

Protein Extraction from Whole Human Breast Tissue Samples

ER α (+) and ER α (-) tissues samples were minced using a scalpel, and proteins were extracted using the procedure outlined above.

Protein Quantitation

Protein concentrations of the tissue lysates were determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard according to the manufacturer's instructions. The concentrations of proteins in the tissue lysates from the microdissected cells and the tissue samples were determined using the standard curve generated by the absorbance of the standards at 562 nm in the Y axis *versus* the known concentration of the standards in the X axis.

Two-Dimensional Gel Electrophoresis

2-DE was performed as described previously [28]. Briefly, proteins were reconstituted in a rehydration buffer composed of 5 M urea, 2 M thiourea, 1% OG, 0.25% v/v Biolyte 3-10 ampholyte, 12.5% water saturated isobutanol, 10% isopropanol, 5% glycerol, and 50 mM dithiothreitol (DTT). The samples were rehydrated at 50 V for 16 hours at 20 °C using 11 cm Immobilized pH Gradient (IPG) strips, pH 4 to 7 (Bio-Rad). Proteins were focused at 250 V for 15 minutes, and then 8000 V was maintained for a total of 60,000 Vh per gel. The strips were then equilibrated for 10 minutes in 2.5 mL of a solution composed of 375 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, and 2% DTT. After this first equilibration, the strips were equilibrated for another 10 minutes in 2.5 mL of a second 6 M urea, 2% SDS, and 2% iodoacetamide. The equilibrated IPG strips were washed with cathode buffer (0.1 M Tricine, 0.1 M Tris-HCl pH 8.2, and 0.1% SDS) and placed onto a 10% Tris-HCl polyacrylamide gel. The anode buffer consisted of 0.2 M Tris-HCl pH 8.9. Gels were electrophoresed at 50 V for 30 min, then at 100 mA/gel until the end of the separation. Gels were stained with SYPRO Ruby (Bio-Rad) according to the following procedure: Gels were fixed in 50% methanol (v/v) and 12% acetic acid (v/v) for 2 hours, then incubated with SYPRO Ruby in the dark overnight. Gels were then washed with ddH₂O for 3 hours. Gels were scanned using Typhoon 9410 Scanning Systems (GE Healthcare). The excitation wavelength was 457 nm and the signals were detected at 610 nm. Spot detection and densitometry were performed using PDQuest 8.0 (Bio-Rad, Hercules, CA) and ImageJ (<http://rsb.info.nih.gov/ij>).

Maldi-Tof-Ms

Proteins were identified utilizing Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) [29–33] as described previously [34]. Briefly, after four iterations of 2-DE for each sample, protein spots were excised under UV light, combined, and incubated with 0.5 μ g/mL TPCK-treated trypsin. Peptides generated from the trypsin digestion were then mixed with a matrix (α -cyano-hydroxycinnamic acid) [35; 36] at a 100–1000-fold molar excess over the analyte [37]. This mixture was then spotted onto a target plate and allowed to evaporate until crystals were formed. The MALDI-TOF used for this analysis was a Kratos PC Axima CFR+ (Shimadzu Biotech) that operated in the reflectron positive ion mode with an operating voltage of 20 kV, an extraction voltage of

19.98 kV, and a pulse voltage of 2000 V. Spectra were acquired over the mass range of 500 to 3000 Da. MASCOT software was used to identify the proteins. The search was performed against the SwissProt database for the *Homo sapiens* taxonomy. The following parameters were used: one missed cleavage, mass tolerance of 1.3 Da, oxidation of methionine (variable modification), and carbamidomethylation (fixed modification).

Western Blotting

Proteins were reconstituted with SDS-PAGE buffer and 10 µg of each sample were loaded onto a 10% polyacrylamide gel. The gel was electrophoresed at 30 V for 30 min, then at 50 mA until the end of the separation. Proteins contained within the gel were then electroblotted onto a nitrocellulose membrane (50 V for 50 min). Western blot analyses were accomplished utilizing a 1 µg/mL dilution of primary antibody followed by incubation with a horseradish peroxidase-conjugated secondary antibody against the appropriate species. Visualization of the bands was then accomplished by the addition of a 1 to 1 ratio of Super Signal West Pico-Stable Peroxidase Solution and Luminol/Enhancer Solution (Pierce, Rockford, IL) and by developing the chemiluminescent signal in the dark using Kodak Scientific Imaging Film (Kodak cat. 1651496), Fixer and Replenisher/Developer and Replenisher (Kodak cat. 1901859) according to the manufacturer's instructions.

Results and Discussions

2-DE of microdissected ERα (+) and ERα (-) tissues

2-DE of proteins extracted from 150,000 microdissected ERα (+) and ERα (-) breast cancer cells shows that ERα (-) cells have reduced protein expression (Figure 2A), although there are 3 proteins exclusively expressed by the ERα (-) cells (Figure 2B *Blue spots*). PDQuest analysis of the images revealed the presence of 28 common proteins differentially-expressed between these two cell clusters (Figure 2B *Green spots*). ERα (+) cells express 83 proteins that are not expressed by ERα (-) cells (Figure 2B *Red spots*).

Differential protein expression between ERα (+) and ERα (-) breast cancer tissue samples

2-DE of ERα (+) invasive breast cancer tissue samples *versus* ERα (-) samples (Figure 3A) followed by MALDI-TOF analysis of tryptic digests (Figure 3B) revealed the differential expression of 247 proteins, 22 of which were identified (Table 1). Eight of these proteins were selected to be analyzed in the microdissected ERα (+) and ERα (-) tissues by Western blotting: Superoxide Dismutase (Mr 16154; pI 5.7), RalA protein 1 variant (Mr 53893; pI 4.92), Rho GDP-Dissociation Inhibitor 1 (RhoGDIα) (Mr 23250; pI 5.02) Galectin-1 (Mr 15048; pI 5.34), Uridine Phosphorylase 2 (UPase2) (Mr 36245; pI 6.21), Cellular Retionic Acid-binding Protein 1 (CRABP1) (Mr 15727; pI 5.3), S100 calcium binding protein A11 (S100-A11) (Mr 11487; pI 6.56), and Nucleoside Diphosphate Kinase A (NDPK-A) (Mr 17309; pI 5.83). The selection was based primarily on the role these proteins play in cellular distribution, apoptosis, phosphorolysis, tumorigenesis, invasion, or metastasis, as well as the availability of commercial antibodies.

Differential protein expression between Microdissected ER α (+) and ER α (-) Cells

Western Blotting was performed on lysates of the ER α (+) and ER α (-) tissues microdissected from the same duct of DCIS microinvasive cancer patients (Figure 4). The expression levels of Superoxide Dismutase, Ral A protein, Galectin-1, UPase, CRABP1, S100-A11, and NDPK-A were lower in ER α (-) cell clusters than their ER α (+) counterparts. Rho GDP-Dissociation inhibitor 1 (RhoGDI α) was the only protein upregulated in the ER α (-) cells (Table 2).

Microdissected tissues versus tissue samples

The low amount of proteins extracted from the ER α (+) and ER α (-) microdissected tissues did not allow for the identification of differentially-expressed proteins between these two cell clusters, and we therefore proceeded by analyzing differential protein expression from tissue samples composed exclusively of ER α (+) or ER α (-) invasive breast cancer cells as described in the pathology reports of these tissues. More than 1 mg of proteins was extracted from each tissue sample to allow for the protein identification using MALDI-TOF analysis on trypsin-digested spots. Western blotting was then performed to validate differential protein expression in five ER α (+) and ER α (-) tissue pairs microdissected from the same patients.

Differential protein expression between microdissected ER α (+) and ER α (-) cells

Superoxide dismutase plays a key anti-oxidant role in the cell by converting superoxide radical to hydrogen peroxide and oxygen [38]. Its cellular role is to provide a defense against the potentially damaging activities of superoxide radicals; diminished superoxide dismutase enzyme activity leads to many of the observed properties in cancer cells such as damaging mitochondrial and plasma membrane, modulating key enzyme activities by oxidizing sulfhydryl groups to disulfides, and causing DNA double-strand breakage and possibly mutation and genomic instability. Downregulation of this enzyme has been found in many cancers [39]. Superoxide dismutase protein expression level was 2.32 fold higher in ER α (+) than ER α (-) epithelial cells belonging to the same patient. Ral A protein is a Ras-related small GTPase. The Ral GTPase family of proteins plays a major role in intracellular membrane trafficking, as well as tumorigenesis, invasion, and metastasis [40; 41]. RalA binding protein was found to be 1.5 times higher in ER α (+) than ER α (-). RhoGDI α is a cellular protein that controls the cellular distribution and activity of RhoGTPases [42; 43]. Rho-GDI α is elevated in breast cancer cell lines and breast tumor tissues [44]. RhoGDI α has also been reported to promote the resistance of cancer cells to drug-induced toxicity, thus playing an anti-apoptotic role [45]. RhoGDI α was the only protein that was identified as elevated in ER α (-) cells when compared to its ER α (+) cells. Galectin-1 can induce apoptosis in breast cancer cells by blocking the cell cycle at the S/G2 transition [46]. It has been shown to be involved in cancer invasion and metastasis by regulating cell adhesion and migration [47]. Galectin-1 is thought to be synthesized by the carcinoma cells and secreted into the stroma. A recent study demonstrated increased expression of Galectin-1 in human breast cancer cells and cancer-associated stroma, and hypothesized that Galectin-1 plays a major role in tumor-stroma interaction in breast cancer [48]. Galectin-1 protein level was 8.7 times higher in ER α (+) when compared to the ER α (-) expression. Uridine phosphorylase

(UPase) catalyzes the phosphorolysis of uridine to uracil. It is also involved in fluoropyrimidine metabolism, playing a role in the intracellular activation of 5-fluorouracil [49]. UPase is elevated in various tumor tissues including breast cancer tissues. This elevation contributes to the therapeutic efficacy of fluoropyrimidines in cancer patients [50], and it has been demonstrated that elevated levels of UPase are correlated with favorable prognoses in cancer [51]. UPase was 11 times higher in ER α (+) than in ER α (-). Cellular retinoic acid binding proteins are thought to play a role in shuttling retinoic acid (RA) from the cytoplasm to the nucleus, therefore playing a role in cell growth and differentiation regulation in fetal and adult tissues [52]. CRABP1 regulates RA activity by increasing its degradation rate by enhancing the production of RA metabolizing enzymes [53]. CRABP1 levels were 13 times higher in ER α (+) than in neighboring ER α (-) cells. S100-A11 is involved in tumorigenesis [54], although it has been shown to be upregulated in some cancers [55] and downregulated in others [56]. S100-A11 levels in ER α (+) samples were twice more than that in ER α (-) samples. Nucleoside diphosphate kinase-A (NDPK-A or nm23-H1) is a metastasis suppressor gene identified in 1988 [57]. Restoration of NDPK-A expression reduces metastasis of breast cancer [58]. NDPK-A protein level was 34.23 times higher in ER α (+) cells than in nearby ER α (-) counterparts. The loss of ER α expression, signaling the early signs of breast cancer invasion, was accompanied by the downregulation of superoxide dismutase that plays an anti-oxidant role, galectin-1 known to induce apoptosis, uridine phosphorylase that contributes to drug efficacy, cellular retinoic acid-binding protein 1 that plays a role in cell growth and differentiation, S100-A11 involved in tumorigenesis, and a metastasis suppressor nucleoside diphosphate kinase A. RhoGDI α , known to induce the resistance of cancer cells to drug-induced toxicity, was the only protein that was more elevated in ER α (-) cells than in adjacent ER α (+) cells. The level of RalA binding protein that is involved in tumorigenesis and metastasis was 1.5 times higher in the microdissected ER α (+) cells than in adjacent ER α (-) ones. This change is not major when compared to the levels of metastasis suppressor NDPK-A, which was 34.23 higher in ER α (+) cells than in ER α (-) counterparts. The physiological and pathological roles of these differentiated expressed proteins are complicated, sometimes, may be conflicting to each other. Although the specific role of alteration of each of these identified proteins remains to be further investigated, the collective role may promote a malignant phenotype for ER α (-) cells, including decreased ability to undergo programmed cell death and differentiate, and increased potential to damage DNA and generate genomic instability, resist chemotherapy drugs, and metastasize. Disruption of both the ME cell layer and the basement membrane is a prerequisite for tumorigenic DCIS cells to initiate invasion into the stroma. The majority of epithelial cells overlying focally disrupted ME cell layers are ER α (-), while adjacent cells within the same duct confined by an intact ME cell layer express high levels of ER α . In this study, ER α (+) cells bound by an intact ME cell layer and ER α (-) cells overlying myoepithelial disruptions were microdissected from the same ducts of breast cancer patients and proteomic analyses revealed great discrepancies in protein expression within the same breast cancer duct accompanied by non-expression of this hormonal receptor.

Conclusions

Our study shows that substantial alteration in protein expression was observed between these adjacent epithelial cells. We propose two hypotheses to be further tested in the future. The first is that an ER α (-) cell cluster in DCIS with microinvasion may represent a clone from a genetically-altered cancer stem cell, that is why many of the differentiation makers are not expressed or expressed at much lower level than those of adjacent ER α (+) cells. Second, an ER α (-) cell cluster may be from dedifferentiated proliferating ER α (+) cells. The most significant facts are that the microdissected ER α (-) cells are pathologically more aggressive, overlay a focally-disrupted myoepithelial layer, express 13.5 times less cellular retinoic acid-binding protein 1, a protein involved in cellular differentiation, and 34.23 times less metastasis suppressor nucleoside diphosphate kinase A or nm23-H1, and express fewer proteins than adjacent ER α (+) cells in DCIS with microinvasion. Further research is required to test these two hypotheses and find out which one is the predominate case in the evolution of human breast DCIS to invasive carcinoma.

Acknowledgments

This work was supported in part by grant BCTR0504465 from the Susan G. Komen for the Cure Breast Cancer Foundation, grants from the Florida Breast Cancer Coalition Research Foundation, the Elsa U. Pardee Foundation, and the Florida State University to Dr. Q.-X. Sang, and a grant BCTR0706983 from the Susan G. Komen Breast Cancer Foundation, grant 05AA from the AFIP/ARP joint research initiative project, and grant 2006CB910505 from the Ministry of Chinese Science and Technology to Dr. Y.-G. Man. We appreciate the assistance of Suzan M. Semaan with the 2-D gel and Mass spectrometry experiments and Robert G. Newcomer for scientific editing of the manuscript.

References

1. Skinner KA, Silverstein MJ. The management of ductal carcinoma in situ of the breast. *Endocrine-Related Cancer*. 2001; 8(1):33–45. [PubMed: 11350725]
2. Breast Cancer Facts And Figures, American Cancer Society. 1999-2000
3. Jolicoeur F, Seemayer TA, Gabbiani G, et al. Multifocal, nascent, and invasive myoepithelial carcinoma (Malignant myoepithelioma) of the breast: An immunohistochemical and ultrastructural study. *International Journal of Surgical Pathology*. 2002; 10(4):281–291. [PubMed: 12490978]
4. Nerlich AG, Haraida S, Hagedorn H, et al. Morphological Aspects of Basement-Membranes and Their Receptors in Benign and Malignant Neoplasms (Review). *International Journal of Oncology*. 1995; 6(6):1193–1202. [PubMed: 21556658]
5. Tsubura A, Shikata N, Inui T, et al. Immunohistochemical Localization of Myoepithelial Cells and Basement-Membrane in Normal, Benign and Malignant Human-Breast Lesions. *Virchows Archiv a-Pathological Anatomy and Histopathology*. 1988; 413(2):133–139.
6. Man YG, Sang QXA. The significance of focal myoepithelial cell layer disruptions in human breast tumor invasion: a paradigm shift from the “protease-centered” hypothesis. *Experimental Cell Research*. 2004; 301(2):103–118. [PubMed: 15530847]
7. Gustafsson JA. Estrogen receptor beta - a new dimension in estrogen mechanism of action. *Journal of Endocrinology*. 1999; 163(3):379–383. [PubMed: 10588810]
8. Green S, Walter P, Kumar V, et al. Human Estrogen-Receptor Cdna - Sequence, Expression and Homology to V-Erb-A. *Nature*. 1986; 320(6058):134–139. [PubMed: 3754034]
9. Greene GL, Gilna P, Waterfield M, et al. Sequence and Expression of Human Estrogen-Receptor Complementary-DNA. *Science*. 1986; 231(4742):1150–1154. [PubMed: 3753802]
10. Kuiper G, Enmark E, PelttoHuikko M, et al. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93(12):5925–5930. [PubMed: 8650195]

11. Mosselman S, Polman J, Dijkema R. ER beta: Identification and characterization of a novel human estrogen receptor. *Febs Letters*. 1996; 392(1):49–53. [PubMed: 8769313]
12. Anderson E, Clarke R, Laidlaw I, et al. Experiments on proliferation of normal human breast tissue in nude mice do not show that progesterone does not stimulate breast cells: Reply. *Endocrinology*. 1996; 137(4):1505–1506. [PubMed: 8625931]
13. Skliris GP, Munot K, Bell SM, et al. Reduced expression of oestrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. *Journal of Pathology*. 2003; 201(2):213–220. [PubMed: 14517838]
14. Park BW, Kim KS, Heo MK, et al. Expression of estrogen receptor-beta in normal mammary and tumor tissues: is it protective in breast carcinogenesis? *Breast Cancer Research and Treatment*. 2003; 80(1):79–85. [PubMed: 12889601]
15. Man YG, Tai L, Barner R, et al. Cell clusters overlying focally disrupted mammary myoepithelial cell layers and adjacent cells within the same duct display different immunohistochemical and genetic features: implications for tumor progression and invasion. *Breast Cancer Research*. 2003; 5(6):R231–R241. [PubMed: 14580259]
16. Man YG, Shekitka KM, Bratthauer GL, et al. Immunohistochemical and genetic alterations in mammary epithelial cells immediately overlying focally disrupted myoepithelial cell layers. *Breast Cancer Research and Treatment*. 2002; 76:S143–S143.
17. Yousefi M, Mattu R, Gao CL, et al. Mammary ducts with and without focal myoepithelial cell layer disruptions show a different frequency of white blood cell infiltration and growth pattern: Implications for tumor progression and invasion. *Applied Immunohistochemistry & Molecular Morphology*. 2005; 13(1):30–37. [PubMed: 15722791]
18. Sauter ER, Zhu W, Fan XJ, et al. Proteomic analysis of nipple aspirate fluid to detect biologic markers of breast cancer. *British Journal of Cancer*. 2002; 86(9):1440–1443. [PubMed: 11986778]
19. Li JN, Zhang Z, Rosenzweig J, et al. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. *Clinical Chemistry*. 2002; 48(8):1296–1304. [PubMed: 12142387]
20. Sahab ZJ, Semaan SM, Sang QXA. Methodology and Applications of Disease Biomarker Identification in Human Serum. *Biomarker Insights*. 2007; 2:21–43. [PubMed: 19662190]
21. O'Neill KA, Miller FR, Barder TJ, et al. Profiling the progression of cancer: Separation of microsomal proteins in MCF10 breast epithelial cell lines using nonporous chromatophoresis. *Proteomics*. 2003; 3(7):1256–1269. [PubMed: 12872226]
22. Wu WG, Tang XM, Hu W, et al. Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clinical & Experimental Metastasis*. 2002; 19(4):319–326. [PubMed: 12090472]
23. Ornstein DK, Gillespie JW, Paweletz CP, et al. Proteomic analysis of laser capture microdissected human prostate cancer and in vitro prostate cell lines. *Electrophoresis*. 2000; 21(11):2235–2242. [PubMed: 10892734]
24. Wulfschuhle JD, Sgroi DC, Krutzsch H, et al. Proteomics of human breast ductal carcinoma in situ. *Cancer Research*. 2002; 62(22):6740–6749. [PubMed: 12438275]
25. Bischoff R, Luider TM. Methodological advances in the discovery of protein and peptide disease markers. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. 2004; 803(1):27–40.
26. Somiari RI, Sullivan A, Russell S, et al. High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. *Proteomics*. 2003; 3(10):1863–1873. [PubMed: 14625848]
27. Hanash SM. Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: Current status. *Electrophoresis*. 2000; 21(6):1202–1209. [PubMed: 10786892]
28. Sahab ZJ, Suh Y, Sang QXA. Isoelectric point-based prefractionation of proteins from crude biological samples prior to two-dimensional gel electrophoresis. *Journal of Proteome Research*. 2005; 4(6):2266–2272. [PubMed: 16335975]
29. Binz PA, Hochstrasser DF, Appel RD. Mass spectrometry-based proteomics: Current status and potential use in clinical chemistry. *Clinical Chemistry and Laboratory Medicine*. 2003; 41(12):1540–1551. [PubMed: 14708878]

30. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422(6928):198–207. [PubMed: 12634793]
31. Ferguson PL, Smith RD. Proteome analysis by mass spectrometry. *Annual Review of Biophysics and Biomolecular Structure*. 2003; 32:399–424.
32. Heck AJR, Krijgsveld J. Mass spectrometry-based quantitative proteomics. *Expert Review of Proteomics*. 2004; 1(3):317–326. [PubMed: 15966828]
33. Feuerstein I, Rainer M, Bernardo K, et al. Derivatized cellulose combined with MALDI-TOF MS: A new tool for serum protein profiling. *Journal of Proteome Research*. 2005; 4(6):2320–2326. [PubMed: 16335981]
34. Sahab ZJ, Iczkowski KA, Sang QXA. Anion exchange fractionation of serum proteins versus albumin elimination. *Analytical Biochemistry*. 2007; 368(1):24–32. [PubMed: 17618595]
35. Karas M, Hillenkamp F. Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10000 Daltons. *Analytical Chemistry*. 1988; 60(20):2299–2301. [PubMed: 3239801]
36. Tanaka K, Waki H, Ido Y, et al. Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*. 1988; 2(8):151–3.
37. Schiller J, Arnhold J, Benard S, et al. Lipid analysis by matrix-assisted laser desorption and ionization mass spectrometry: A methodological approach. *Analytical Biochemistry*. 1999; 267(1): 46–56. [PubMed: 9918654]
38. Briehl MM, Baker AF, Siemankowski LM, et al. Modulation of antioxidant defenses during apoptosis. *Oncology Research*. 1997; 9(6-7):281–285. [PubMed: 9406233]
39. Oberley LW, Buettner GR. Role of Superoxide-Dismutase in Cancer-Review. *Cancer Research*. 1979; 39(4):1141–1149. [PubMed: 217531]
40. Tchekina E, Agapova L, Dyakova N, et al. The small G-protein RalA stimulates metastasis of transformed cells. *Oncogene*. 2005; 24(3):329–335. [PubMed: 15467745]
41. Ward Y, Wang W, Woodhouse E, et al. Signal pathways which promote invasion and metastasis: Critical and distinct contributions of extracellular signal-regulated kinase and Ral-specific guanine exchange factor pathways. *Molecular and Cellular Biology*. 2001; 21(17):5958–5969. [PubMed: 11486034]
42. Sasaki T, Takai Y. The Rho small G protein family Rho GDI system as a temporal and spatial determinant for cytoskeletal control. *Biochemical and Biophysical Research Communications*. 1998; 245(3):641–645. [PubMed: 9588168]
43. Olofsson B. Rho guanine dissociation inhibitors: Pivotal molecules in cellular signalling. *Cellular Signalling*. 1999; 11(8):545–554. [PubMed: 10433515]
44. Fritz G, Brachetti C, Bahlmann F, et al. Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *British Journal of Cancer*. 2002; 87(6): 635–644. [PubMed: 12237774]
45. Zhang BL, Zhang YQ, Dagher MC, et al. Rho GDP dissociation inhibitor protects cancer cells against drug-induced apoptosis. *Cancer Research*. 2005; 65(14):6054–6062. [PubMed: 16024605]
46. Wells V, Davies D, Mallucci L. Cell cycle arrest and induction of apoptosis by beta galactoside binding protein (beta GBP) in human mammary cancer cells. A potential new approach to cancer control. *European Journal of Cancer*. 1999; 35(6):978–983. [PubMed: 10533482]
47. Rabinovich GA. Galectin-1 as a potential cancer target. *British Journal of Cancer*. 2005; 92(7): 1188–1192. [PubMed: 15785741]
48. Jung EJ, Moon HG, Cho BI, et al. Galectin-1 expression in cancer-associated stromal cells correlates tumor invasiveness and tumor progression in breast cancer. *International Journal of Cancer*. 2007; 120(11):2331–2338.
49. Cao DL, Russell RL, Zhang DK, et al. Uridine phosphorylase (-/-) murine embryonic stem cells clarify the key role of this enzyme in the regulation of the pyrimidine salvage pathway and in the activation of fluoropyrimidines. *Cancer Research*. 2002; 62(8):2313–2317. [PubMed: 11956089]
50. Yan R, Wan LX, Pizzorno G, et al. Uridine phosphorylase in breast cancer: a new prognostic factor? *Frontiers in Bioscience*. 2006; 11:2759–2766. [PubMed: 16720348]

51. Kawamura K, Takiguchi N, Wada A, et al. Up-regulated expression of the uridine phosphorylase gene in human gastric tumors is correlated with a favorable prognosis. *Anticancer Research*. 2006; 26(6C):4647–4651. [PubMed: 17214321]
52. Gorry P, Lufkin T, Dierich A, et al. The Cellular Retinoic Acid-Binding Protein-I Is Dispensable. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91(19): 9032–9036. [PubMed: 8090764]
53. Dong D, Ruuska SE, Levinthal DJ, et al. Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. *Journal of Biological Chemistry*. 1999; 274(34): 23695–23698. [PubMed: 10446126]
54. Kondo A, Sakaguchi M, Makino E, et al. Localization of S100C immunoreactivity in various human tissues. *Acta Medica Okayama*. 2002; 56(1):31–34. [PubMed: 11873942]
55. Oue N, Hamai Y, Mitani Y, et al. Gene expression profile of gastric carcinoma: Identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. *Cancer Research*. 2004; 64(7):2397–2405. [PubMed: 15059891]
56. Memon AA, Sorensen BS, Meldgaard P, et al. Down-regulation of S100C is associated with bladder cancer progression and poor survival. *Clinical Cancer Research*. 2005; 11(2):606–611. [PubMed: 15701847]
57. Steeg PS, Bevilacqua G, Kopper L, et al. Evidence for a Novel Gene Associated with Low Tumor Metastatic Potential. *Journal of the National Cancer Institute*. 1988; 80(3):200–204. [PubMed: 3346912]
58. Leone A, Flatow U, King CR, et al. Reduced Tumor-Incidence, Metastatic Potential, and Cytokine Responsiveness of Nm23-Transfected Melanoma-Cells. *Cell*. 1991; 65(1):25–35. [PubMed: 2013093]

Abbreviations

CRABP1	cellular retinoic acid-binding protein 1
DCIS	Ductal carcinoma <i>in situ</i>
2-DE	two-dimensional gel electrophoresis
DTT	Dithiothreitol
ERα	Estrogen Receptor alpha
IPG	immobilized pH gradient
MALDI-TOF-MS	Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry
ME cells	myoepithelial cells
M_r	molecular mass
NDPK-A (or nm23-H1)	nucleoside diphosphate kinase A (non-metastasis protein 23-H1)
OG	Octylglucopyranoside
pI	isoelectric point
RhoGDIα	Rho GDP-Dissociation Inhibitor 1 alpha
S100A11	S100 calcium binding protein A11
UPase	Uridine phosphorylase

Figure 1. ER α expression status in cell clusters overlying focally-disrupted myoepithelial cell layers. Human breast tissue sections from DCIS (a), hyperplastic (b), and normal-appearing (c) ducts were double-immunostained for ER α (brown) and SMA (red). Arrows identify myoepithelial cell layers. Circles identify ER α -negative cell clusters overlying focal myoepithelial cell layer disruptions (300 $\times$).

Figure 2.

(A) 2-DE of proteins extracted from 150,000 ER α (+) and ER α (-) cells, respectively, microdissected from human DCIS tissues originating from 3 patients. Proteins were rehydrated at 50 V for 16 hours at 20 °C using 11 cm Immobilized pH Gradient (IPG) strips, pH 4 to 7. A 10% polyacrylamide gel was used in the second dimension. Proteins were stained by SYPRO Ruby and visualized using an excitation wavelength of 457 nm. The signals were detected at 610 nm. (B) PDQuest imaging analysis of 2-DE representing proteins extracted from 150 thousand ER α (+) and ER α (-) cells microdissected from human DCIS tissues originating from 3 patients shows 28 differentially-expressed proteins.

Figure 3.

(A) 2-DE of proteins extracted from entire ER α (+) and ER α (-) tissue samples. Proteins were rehydrated at 50 V for 16 hours at 20 °C using 11 cm Immobilized pH Gradient (IPG) strips, pH 4 to 7. A 10% polyacrylamide gel was used in the second dimension. Proteins were stained by SYPRO Ruby and visualized using an excitation wavelength of 457 nm. The signals were detected at 610 nm. Proteins were identified using MALDI-TOF-MS. (B) MALDI-TOF mass spectra of tryptic digests obtained on a Kratos PC Axima CFR+ (Shimadzu Biotech) operated in the reflectron positive ion mode with an operating voltage of 20 kV, an extraction voltage of 19.98 kV, and a pulse voltage of 2000 V. Spectra were acquired over the mass range of 500 to 3000 Da.

Figure 4.

Western Blots performed on ERα (+) and ERα (-) cells that were microdissected from the same ducts of 5 different patients (A) superoxide dismutase, (B) Ral A protein, (C) RhoGDIα, (D) Galectin-1, (E) Uridine Phosphorylase, (F) cellular retinoic acid-binding protein 1, (G) S100-A11, and (H) nucleoside diphosphate kinase A. Lanes represent 10 µg of total protein. Western blot analyses were accomplished utilizing a 1 µg/mL dilution of primary antibody followed by incubation with a horseradish peroxidase-conjugated secondary antibody against the appropriate species with positive signals detected by chemiluminescence.

Table 1

Differentially expressed proteins between ER α (+) and ER α (-) tissue samples. Index numbers correspond to figure 3A.

Index no.	Accession	Mr	pI	Cov.(%)	Peptide Match	MOWSE	Protein Description	ER+/ER- Ratio
1	SODC_HUMAN	16154	5.7	72	11	104	Superoxide Dismutase	2.47
2	Q59E87_HUMAN	53893	4.92	45	36	71	Ral A Binding Protein	14.5
3	GDIR_HUMAN	23250	5.02	34	16	120	Rho GDP-Dissociation inhibitor 1	0.7
4	LEG1_HUMAN	15048	5.34	42	6	59	Galectin-1	2.21
5	UPP2_HUMAN	36245	6.21	26	10	94	Uridine Phosphorylase 2	5.92
6	RABP1_HUMAN	15727	5.3	39	5	55	Cellular retionic acid-binding protein 1	24
7	S10AB_HUMAN	11487	6.56	49	5	56	Protein S100-A11	3.01
8	NDKA_HUMAN	17309	5.83	31	6	68	Nucleoside Diphosphate Kinase A	3.26
9	METL5_HUMAN	24046	6.23	65	25	258	Methyl transferase-like protein 5	4.3
10	DIXC1_HUMAN	77886	5.85	34	33	134	Dixin	0.36
11	AIAG2_HUMAN	23873	5.03	54	18	99	Alpha-1-acid glycoprotein precursor	2.84
12	ALBU_HUMAN	71317	5.92	36	21	144	Serum albumin precursor	0.17
13	GRP75_HUMAN	73920	5.87	28	17	85	Stress-70 protein, mitochondrial precursor	2.21
14	ALBU_HUMAN	71317	5.92	20	13	66	Serum albumin precursor	0.23
15	EGLN1_HUMAN	46847	8.83	28	11	91	Egl nine homolog 1	0.32
16	APOA1_HUMAN	30759	5.56	42	12	96	Apolipoprotein A-1 precursor	0.87
17	FBXL8_HUMAN	41403	6.96	42	14	95	F-box/LRR-repeat protein 8	1.31
18	ITM2A_HUMAN	30121	5.65	23	6	72	Integral membrane protein 2A	0.73
19	ACTB_HUMAN	42052	5.29	26	9	83	Actin, cytoplasmic 1	1.64
20	TUSC4_HUMAN	44429	6.1	20	9	66	Tumor suppressor candidate 4	1.75
21	SSX2_HUMAN	21721	5.61	23	6	58	Protein SSX2	0.46
22	TPM4_HUMAN	28619	4.67	26	8	73	Tropomyosin alpha-4 chain	3.27

Table 2Differentially expressed proteins between microdissected ER α (+) and ER α (-) cells.

Protein Description	ER+/ER- Ratio	Protein Role
Superoxide Dismutase	2.32	Anti-oxidant (37)
Ral A Binding Protein	1.5	Tumorigenesis – Metastasis (39, 40)
Rho GDP-Dissociation inhibitor 1	0.67	Resistance to drugs (44)
Galectin-1	8.7	Apoptosis (45)
Uridine Phosphorylase 2	11	Contributes to drug efficacy (49)
Cellular retionic acid-binding protein 1	13.5	Cell growth and differentiation (51)
Protein S100-A11	2	Tumorigenesis (53)
Nucleoside Diphosphate Kinase A	34.23	Metastasis suppressor (56, 57)