Breast Cancer

WHEN PROTEOMICS CHALLENGES BIOLOGICAL COMPLEXITY*

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Proteomics is now entering into the field of biomedicine with declared hopes for the identification of new pathological markers and therapeutic targets. Current proteomic tools allow large-scale, high-throughput analyses for the detection, identification, and functional investigation of low-abundant proteins. However, the major limitation of proteomic investigations remains the complexity of biological structures and physiological processes, rendering the path of exploration of related pathologies paved with various difficulties and pitfalls. The case of breast cancer illustrates the major challenge facing modern proteomics and more generally post-genomics: to tackle the complexity of life. *Molecular & Cellular Proteomics* 2:281–291, 2003.

During the last several years, the field of proteomics has evolved considerably. Traditional two-dimensional (2D)1 electrophoresis (2DE) has been improved and new off-gel methods have been developed. At the same time, mass spectrometry not only allows protein identification with increasing sensitivity, but also the precise exploration of protein-protein interactions and post-translational modifications, such as phosphorylation. A further major step has been large-scale, high-throughput analysis, rendering possible the description and functional investigation of proteomes. In biomedicine, this has led to considerable hope for the identification of potential pathological markers and therapeutic targets (1, 2), particularly in the field of cancer (3). However, the biggest impediment to proteomic analyses remains biology and its intrinsic complexity. After genome sequencing and now proteome analyses of breast cancer. The first is to discover new molecular markers for early diagnosis and profiling of breast tumors. The second is to decipher the intracellular signaling pathways leading to the initiation and progression of breast tumors. Such data should provide the knowledge base for the identification of new therapeutic targets and the development of innovative strategies against breast cancer.

Current methods used to detect breast tumors, either benign or malignant, are based on mammography. However, there are intrinsic limitations to mammography. First, there are suggestions that X-rays can potentially induced carcinogenesis, and second, it is clear that to be detected in mammography, a breast tumor should be at least a few millimeters in size. However, a tumor of this size already contains several hundred million cells. From the cellular point of view, given the fact that a single cell can lead to the development of a whole tumor (clonal origin of cancer), it is already late when a breast tumor is detected by mammography. Thus, this constitutes an important limitation to mammography. In clinical practice, after the surgical removal of a tumor, its characterization as a malignant or benign tumor is made by histology. Such parameters as tumor size and inflammation, histoprognostic grading, and node involvement are then used to decide treatment and prognosis. However, breast cancer is not an homogeneous disease and there are different types. Depending on the cellular and histological origin of the cancer cells and on the evolution of the disease, a broad range of breast tumor types have been described. Several studies have been performed in an attempt to identify factors associated with either the growth rate or the metastatic potential of breast tumors (i.e. prognostic factors) or factors related to sensitivity and/or resistance to therapeutic agents (i.e. predictive factors). The main biological markers recommended for routine use is the presence of estrogen (estradiol and progesterone) receptors for the selection of patients potentially responding to treatment with anti-estrogen such as tamoxifen (4).

On the therapeutic side, practical consequences for treatment, derived from a better understanding of the molecular basis of cancer cell growth, are now emerging. This is evidenced by the development of therapeutic strategies based, for example, on the inhibition of tyrosine kinase receptors.

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Received, April 29, 2003, and in revised form, May 29, 2003

Published, MCP Papers in Press, May 29, 2003, DOI 10.1074/mcp.R300003-MCP200

1 The abbreviations used are: 2D, two dimensional; 2DE, 2D electrophoresis; HSP, heat shock protein; FGF, fibroblast growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; MAP, mitogen-activated protein.

**CHALLENGES FACING RESEARCH ON BREAST CANCER**

There are two main expected outcomes from proteomic analyses of breast cancer. The first is to discover new molecular markers for early diagnosis and profiling of breast tumors. The second is to decipher the intracellular signaling pathways leading to the initiation and progression of breast tumors. Such data should provide the knowledge base for the identification of new therapeutic targets and the development of innovative strategies against breast cancer.

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On the therapeutic side, practical consequences for treatment, derived from a better understanding of the molecular basis of cancer cell growth, are now emerging. This is evidenced by the development of therapeutic strategies based, for example, on the inhibition of tyrosine kinase receptors.
This approach is well illustrated with ErbB2, a tyrosine kinase receptor overexpressed in more than 20% of breast tumors. Specific inhibition of ErbB2 using Herceptin, a truncated blocking antibody directed against it, has been successfully developed and has now entered into clinical practice (5). However, the growth of breast cancer cells can be regulated by other growth factors that either stimulate or inhibit their proliferation, migration, and differentiation, thus acting in concert to promote tumor growth and metastasis (6, 7). Probably related to this, the efficacy of Herceptin appears to be limited to a small proportion of breast cancers, and the identification of other targets, and corresponding drugs, remains a major interest for the development of targeted therapeutic strategies.

Clearly, there is a critical need to find new molecular parameters not only for detection, but also for typing and treatment of breast cancer. As proteomics provides a global approach for the identification of protein regulation during pathological processes, the discovery of new markers and therapeutic targets as well as the corresponding drugs is a highly anticipated outcome. However, both the mammary gland and breast tumors are complex and dynamic structures, and just as to report the course of a football game with a photo camera is not an easy task, it is, at present, still rather puzzling to comprehensively explore the process of breast carcinogenesis with genomic and proteomic tools.

MAMMARY GLAND AND BREAST TUMORS: CELLULAR ECOSYSTEMS

The breast contains two compartments: the glandular portion, which is involved in production and transportation of milk, and the stromal and connective tissues. The glandular part of the mammary gland has 15–20 lobes, and within each lobe there are many smaller lobules ending in dozens of tiny bulbs that can produce milk (Fig. 1). The lobules are all linked by thin tubes called ducts, and all ducts lead to the nipple. The cells forming the ducts and lobules are epithelial cells whose main function is to produce and to secrete the various constituents of milk. In addition, epithelial cells are surrounded by a layer of myoepithelial cells, attached on a basal membrane, whose role is to maintain the tubular structure of ducts and lobules. Surrounding the lobules and ducts, connective and fat tissues are composed of fibroblasts, with their abundant extracellular matrix, and adipocytes. In addition, both blood vessels and lymph vessels irrigate the mammary gland, and nerve fibers, mostly sensory and sympathetic, are also present.

The development of the mammary gland occurs predominantly after birth, and the mature breast undergoes cyclic changes regulated by both the menstrual cycle and the gestation/parturition processes (Fig. 2). To acquire their functionality, the epithelial cells must receive proper signals from hormones (estrogens, progesterone) as well as from nearby cells and components of its microenvironment (growth factors). Before epithelial cells can produce milk, they develop into lobuloalveoli (functional units) through morphogenesis involving cell proliferation, invasion, and differentiation. The cyclic development of the mammary gland reflects the fact that it is only needed during well-defined periods of life. Thus, mammary epithelium undergoes repeated cycles of growth, differentiation, and regression. Continuous cycling leads to the formation of a ductal tree. Multiplication of breast epithelial cells occurs constantly, stimulated by estrogens during menstrual cycle and pregnancy. After parturition, functional differentiation of the epithelial cells takes place. During lactation, these cells produce large amounts of milk, and after weaning, the gland regresses by a process of extensive cell
death and tissue remodeling. Therefore, the mammary gland is a cellular ecosystem in which each represented cell type is subject to a constant turnover. This is particularly the case for the epithelial cells, which are subject to various hormone and growth factor stimulation throughout their lifetime, with correlated changes in morphology and metabolism.

Most breast tumors are of epithelial origin, and therefore the large majority of malignant breast tumors are classified as carcinomas (malignant epithelial tumors). Sarcomas (malignant tumors arising from connective tissue) are rarely observed in the breast. The term “breast cancer” encompasses numerous types of tumors that are classified with respect to their origin and to their histological features. There are two main classes: \textit{in situ} carcinomas are characterized by tumor cells localized either in the ducts (ductal carcinomas \textit{in situ}) or in the lobules (lobular carcinomas \textit{in situ}), without invasion through the basement membrane into the surrounding stroma. In contrast, in invasive carcinomas, the basement membrane is partially or totally destroyed and cancer cells progressively invade surrounding tissues, eventually leading to metastatic cells. The group of invasive carcinomas includes more than 10 different types. The invasive ductal carcinomas (65–80% of all breast cancers) and the invasive lobular carcinomas (5–15% of the cases) constitute the two main types, while others forms such as mucinous, medullar, tubular, and apocrine carcinomas are less frequent (0.1–4%). Besides \textit{in situ} and invasive carcinomas, Paget’s disease of the nipple constitutes a particular form of malignant epithelial tumor, where malignant cells infiltrate the epidermis. Finally, a number of tumors are benign; numerous types have been described, and among them epithelial atypical hyperplasia is considered a precancerous form. It is generally considered that 6–8 years take place between the appearance of the first cellular modification leading to hyperplasia and the detection of breast tumor by mammography. It is clear that despite the clonal origin of breast tumors, the successive generations of transformed cells creates an intrinsic cellular heterogeneity. In addition, normal epithelial, myoepithelial, fibroblastic, and endothelial cells are also trapped in breast tumors and therefore continue their development in a modified environment. Moreover, local inflammation is eventually observed associated with breast tumors, indicating the activity of cells from the immune system and their interaction with the tumor.

Finally, breast tumors can be seen as cellular ecosystems in which cell populations are constantly renewed to give rise to a progressively more aggressive tumor. This selective process takes place under the influence of hormones and growth factors, and breast cancer cells are therefore affected by a network of intercellular interactions (Fig. 3). Interestingly, genetic instability can be observed in breast tumors as in the evolution of species; molecular modifications leading to changes in receptivity and response to hormones and growth factors are essential parameters that can confer a selective advantage to emerging cells. Altogether, breast cancer is a heterogeneous disease with different types of breast tumors that are cellularly heterogeneous and undergo a constant process of dynamic evolution.

**PROTEOMICS OF BREAST TUMOR BIOPSY: TRUE PITFALL?**

Differential proteomic analysis to detect and characterize polypeptides in disease versus normal tissue biopsies is generally presented as an appropriate way to identify markers of pathologies. However, in the case of breast cancer, but certainly not limited to it, the pertinence of this approach can seriously be questioned. As normal mammary gland tissues and breast tumors are dynamic and heterogeneous cellular structures, a global differential analysis of their proteomes refers finally to a comparative study of cell mixtures with different proportions of epithelial and of other cell types including myoepithelial, fibroblast, and endothelial cells, supplemented by adipocytes, nerve fibers, circulating cells, and macrophages. Several studies have reported differential proteomic analyses of breast tumor biopsies. As early as 1974, the use of 2DE
Proteomics of Breast Cancer

**Fig. 3.** Breast cancer cells are under a network of cellular interactions mediated by hormones and growth factors. +, indicates a stimulation of cellular growth, and -, an inhibition. IL, interleukin; MPP, metalloprotease; MDGI, mammary-derived growth inhibitor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; HGF/ SF, hepatocyte growth factor/scatter factor; TNF, tumor necrosis factor alpha; IGFBPs, IGF binding proteins; MDGI, mammary-derived growth factor I; NGF, nerve growth factor.

...to resolve serum proteins was reported, with differences being noted in the protein patterns of individuals suffering cancer (8); however, this study was essentially descriptive, and no protein identifications were made. In 1984, it was found that most polypeptides were consistently present in both malignant and nonmalignant breast tissues, as only 10 polypeptides differed out of 350 resolved (9). Subsequently, using better technology, it was shown that of ~1000 silver-stained cytosolic polypeptides observed, the 2DE patterns from normal and malignant tissue differed in only 6 places qualitatively and only 22 places quantitatively (10, 11). A more precise characterization of such polypeptide differences was published in the early 90s with the demonstration of a defect in tropomyosin 1, 2, and 3 expression in mammary carcinoma, suggesting that such abnormalities may play a role in breast neoplasia (12, 13). Differential distribution of heat shock protein (HSP) family members has also been described (14, 15). Recently, a comparison of normal ductal/lobular units versus ductal carcinomas has been published with a total of 57 proteins found to be differentially expressed (16). From all of these data, it appears that the set of differentially expressed proteins identified are different from one study to another, suggesting either a lack of experimental standardization or problems of heterogeneity between the biological material used in each study. Probably as a consequence of this, the relevance of these data for clinical practice has still to be established, and indeed none of the potential markers identified by proteomics of breast tumors so far are routinely used by clinicians for either diagnosis, treatment choice, or prognosis. In our laboratory, we have also experienced the limit of analyzing breast tumor biopsies as a whole. Indeed, we described some of the modifications reported above for tropomyosin or HSP (12–15), but these differences were only quantitative and no protein was found exclusively in cancer versus normal breast tissues. Consider the fact that such a global analysis is in fact describing the proteomes of about 10 different cell types, the lack of specificity found in cancer samples might not be considered so unusual. A classical 2D gel of a breast tumor biopsy is shown in Fig. 4 and reveals a further level of complication, namely the major protein that can be observed is serum albumin. Therefore, breast tumor biopsies not only contain a mixture of the different mammary cell types mentioned above, but they also contain blood compounds due to the vascular irrigation of breast tumors. As blood cannot be washed out of biopsies, proteomic analyses of breast tumors also measure proteins from circulating cells and from the plasma, further confounding an already complex situation. On the other hand, blood should also be regarded as an important point to consider in any differential analysis of pathological versus normal situation. In terms of biomedical applications, the detection of proteomic markers in the biological fluids such as plasma and urine is of promising interest. To be able to detect breast cancer, or any other cancer, from a simple blood analysis would represent a major breakthrough, and such an achievement provides impetus to the currently ongoing plasma project (17). However, even thought significant progress has already been made (18, 19), searching for tumor-released proteins in plasma or their fragments in urine is like looking for a needle in a bale of hay, and it might turn out to be more productive to identify markers from tumor cells themselves before secondarily assaying them in the plasma.

Considering the cellular complexity of mammary tumors, microdissection is now regarded as a reasonable alternative for selectively isolating cell types to be analyzed. Techniques such as laser capture microdissection (20) can be used for the isolation of malignant cells prior to sample preparation for proteomic analysis and might facilitate marker protein discovery. However, initial enthusiasm for microdissection has progressively been diminished by several limiting aspects. The first problem is quantitation; as no amplification technique can be applied to proteins (as PCR for DNA/RNA), it is necessary to start any proteomic analysis with a significant amount of material. In our hands, a minimum of 100,000 cells are nec-

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2 A. S. Vercoutter and H. Hondermarck, unpublished data.
...is not totally reliable to recognize cell types (specially cancer) since a huge quantity of work with a constant problem in the techniques, the preparation of such a number of cells requires that, samples can be frozen before performing proteomic analysis. When considering the set up of large-scale analyses of cancer proteomes, underway in several international programs, with tumor resection and protein extraction being performed in different clinical centers, the issue of standardization is obviously a sensitive point.

When comparing cancer tissues to normal tissues or the corresponding dissected cells, a fundamental physiological question also arises: what is a normal breast tissue? Due to the changing hormone concentration (estrogens and progesterone) during the course of menstrual cycle, the status of normal breast tissue is clearly also changing and therefore it is difficult to define a reference state. Hormonal stimulation of breast epithelial cells will be different depending on the hormonal status of the women at the time the sampling is made, and the same concern applies to resected breast tumor biopsies. The stimulation by hormones is known to modify gene expression in both normal and cancer cells and consequently the proteome of breast epithelial cells. Thus, the hormonal environment of biopsied tissue should ideally be evaluated, but this is practically very difficult if not impossible to implement. One way to avoid this intrinsic difficulty related to in situ endogenous hormonal stimulation of breast epithelial cells is to use cell cultures in which cells can be kept in standard medium, which can be regarded as a way to standardize the environment and the conditions applied to the cells.

Thus, despite its intrinsic promise, proteomic analysis of breast cancer is not so straightforward. Clearly, “the devil is in the details,” and differential proteomics of normal versus cancer breast biopsies is in fact associated with a series of difficult problems making the approach a potential pitfall. Unfortunately, this conclusion is probably not limited to breast cancer, and the suitability of differential proteomics performed from biopsies should be questioned in any attempt to identify markers or therapeutic targets.

FROM CELLULAR MODELS TO TUMOR BIOPSIES: A PRODUCTIVE WAY

In contrast to the studies of breast tumor biopsies, proteomic analysis starting from breast cancer cells in culture has already given significant results with the identification of proteins with clinical interest. In 1980, Westley and Rochefort identified a secreted 46-kDa glycoprotein, induced by estrogens in human breast cancer cell lines, that was identified with specific antibodies as being the protease cathepsin D (22). In 1989, a computer-based analysis of 2DE gels reported...
a total of eight polypeptide differences between cancerous and normal breast epithelial cells in tissue culture (23). More precise characterization of such polypeptide differences was published in the early 90s with the demonstration that normal breast epithelial cells produce keratins K5, K6, K7, and K17, whereas tumor cells produce mainly keratins K8, K18, and K19 (24). This distribution was secondarily confirmed in tumor samples (25), and cytokeratin immunodetection is now eventually used to help discriminate benign from malignant cells on histopathological slides. Finally, the first characterization of normal breast epithelial and myoepithelial cell proteins and of breast cancer cells using mass spectrometry was published more recently and revealed a limited number of differences (26). The study of cells, both normal and cancerous, in culture definitely offers the possibility to directly investigate proteins specifically produced, or not produced, by breast cancer cells. This has also been shown with proteinase inhibitors TIMP-1 and PAI-1, originally studied in breast cancer cells, which have recently been shown to be complementary in determining prognosis of breast tumors (27). Cell fractionation prior to proteomic analysis has also been explored, as recently illustrated for plasma membranes of breast cancer cells (28). Importantly, proteomic analysis from cell cultures can be realized in standardized medium conditions, providing a comparable and controllable status of hormone and growth factor stimulation. In addition, phenotype and behavior of the cells, i.e. proliferation/migration/differentiation/survival, can be experimentally studied. Interestingly, differences between the proteome of normal versus cancerous breast epithelial cells appear to be quite limited. In our laboratory, about 2000 proteins were analyzed in normal breast epithelial cells, compared with two cancer cell cultures placed in the same medium conditions. It can be noted that most detected proteins were present in similar quantities in the three samples. Importantly, only a limited number of proteins, about 20, differ between normal and cancerous samples and therefore have the potential to be relevant markers. These values were obtained after computer analysis of 2D gel protein separations, but similar results have been obtained using isotope-coded affinity tagging. The fact that only a limited number of individual protein modifications can be seen between normal and cancer cells is in fact not surprising as a similar situation has already been described at the genomic level. A limited number of molecular modifications, affecting oncogenes and suppressor genes, are required to transform normal cells into cancerous ones (29). This limited number of molecular modifications ultimately makes cancer cells quite similar to normal ones and probably accounts for several important consequences encountered with cancer cells. The first is the difficulty to detect and type cancer cells; so far, no one has been able to identify a universal cancer marker that would allow early detection of the pathology. It can also be postulated that for the same reason the immune system encounters difficulties in efficiently recognizing cancer cells. The second consequence is that the definition of drugs specifically targeting breast cancer cells has so far been difficult and current treatments have therefore numbers of heavy side effects. In this context, the identification of proteins specifically expressed by cancer cells would be essential for drug targeting. Alternatively, proteins downregulated in cancer cells would also be of interest as their level of expression could be used to discriminate and type breast cancer cells. In addition, if such a protein disappearance is germane to cancer cell growth and metastasis, its re-induction could potentially lead to a reversion of the cancerous phenotype. To date this is only a distant possibility, but normalizing cancer cells by re-expressing specific proteins controlling the normal state of growth would provide a theoretical basis for potential gene therapy of cancer.

A first step toward the identification of proteins downregulated in cancer cells has recently been provided by the molecular chaperone 14-3-3 sigma, and, interestingly, this is a good illustration of the usefulness of cellular models and of the complementarity of genomic- and proteomic-based approaches. 14-3-3 is a family of highly conserved protein forms (alpha, beta, delta, sigma, zeta) of 25–30 kDa, expressed in all eukaryotic cells, that play a role in the regulation of signal transduction pathways implicated in the control of cell proliferation, differentiation, and survival (30). 14-3-3 proteins are known to associate directly or indirectly with signaling proteins such as the insulin-like growth factor (IGF)-1 receptor, Raf, mitogen-activated protein (MAP)/extracellular signal-regulated kinase kinases, and phosphatidylinositol 3-kinase, but the precise molecular mechanism by which they activate or inhibit these elements remains unclear (30). We have shown that the sigma form of 14-3-3 is easily detectable in 2DE gels of normal breast epithelial cells using low-sensitivity Coomassie staining, whereas the spot was undetectable in breast cancer cell protein profiles (31). Nevertheless, 14-3-3 sigma was also present in breast cancer cells, but due to the very low levels, more sensitive silver staining was necessary for its detection. Therefore, 14-3-3 sigma down-regulation appears to be a major modification of the proteome associated with breast epithelial cell carcinogenesis, and we subsequently have investigated its distribution in breast cancer biopsies. Based on data gathered in cell culture, the analysis of breast tumors with 2DE was set up with a narrow-range pH gradient to provide an optimal view of the gel area containing 14-3-3 sigma. We showed that the level of 14-3-3 sigma is systematically down-regulated in tumor biopsies (31). This demonstrates that starting with a cellular proteome, which exhibits a lower level of complexity, facilitates the identification of relevant proteins that can secondarily be found and quantified in tumor biopsies. At the mRNA level, it was shown that gene expression of 14-3-3 sigma is 7–10 times lower in breast cancer cells than in normal breast due to the high frequency

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3 A. S. Vercoutter and H. Hondermarck, unpublished data.
of hypermethylation of the 14-3-3 sigma locus (32). Interestingly, the mRNA for 14-3-3 sigma was undetectable by Northern blot analyses in 45 of 48 primary breast carcinomas studied; in contrast, we have detected the 14-3-3 sigma protein in 30 of 35 primary tumor samples, indicating the high sensitivity provided by proteomic analysis as well as the complementarity of both strategies for identifying cancer markers. From the clinical point of view, it has been reported that down-regulation of 14-3-3 sigma gene expression is an early event in breast carcinogenesis (33), and we have recently observed that overexpression of 14-3-3 sigma in cancer cells reverses the proliferative phenotype. This emphasizes the invaluable use of cell cultures, not only for the detection of markers directly from cancer cells but also because in vitro models allow experimental work necessary for the understanding of protein function.

EXPLORING THE MECHANISM OF CARCINOGENESIS: FROM ONCOGENES TO GROWTH FACTOR SIGNALING

Molecular events leading to breast epithelial cell carcinogenesis involve modification in the structure and expression of both oncogenic and tumor suppressive genes (such as myc, ErbB2, and p53), leading to an unbalanced growth characterized by high rates of cell proliferation and eventually cell migration (29). These genetic modifications also confer an ability of cancer cells to survive environmental stresses, which would otherwise lead to apoptosis. Moreover, mammary tumors develop slowly, with an estimated time of 6–8 years for the attainment of a tumor 0.5 cm diameter, from one originating cell. This fact emphasizes the crucial importance played by endocrine and paracrine regulation of breast cancer cell growth in tumor development. Therefore, breast carcinogenesis is clearly based on both genetic and epigenetic parameters; interestingly, proteomics can provide useful information for the comprehension of these two aspects.

Mechanism of Oncogene Activity—Despite the fact that modifications of oncogenes and tumor suppressor gene expression are recognized to be central to cancer cell development and metastasis, the mechanism of several of these remains to be determined. This is the case for H19, an oncofetal gene, which encodes an untranslated mRNA (34) that has been shown to stimulate cancer cell growth (35). H19-transfected breast epithelial cells appear to grow faster (35), but no molecular target has been described. Recently, it was shown that growth factor stimulation of breast cancer cells, for example by fibroblast growth factor (FGF)-2, results in a strong and sustained stimulation of H19 gene transcription (36). In order to identify molecular events involved in H19 oncogenic activity, we have developed a proteomic-based strategy (37). Breast mammary cells were transfected with H19, and the resulting proteomic profile was established using 2DE. Changes in protein synthesis were determined by computerized analysis and revealed an essential modification in the intensity of one spot induced by H19 overexpression. Mass spectrometric analysis of this spot, performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and tandem mass spectrometry, allowed the identification of the protein up-regulated by the H19 gene as thioredoxin (37), one of the major proteins regulating the intracellular redox metabolism (38). The thioredoxin system is a general protein disulfide-reducing complex and includes the NADPH-dependent flavoprotein thioredoxin reductase (38). These data do not fully elucidate the mechanism of action of H19 but provide a first-identified target, demonstrating the value of proteomic analysis for the understanding of the molecular mechanisms involved in oncogene activity. More generally, this shows that the transfer of information is not only from genomics to proteomics, but also from proteomics to genomics. It is usually said that proteomics can be performed because the human genome was previously sequenced, allowing the possibility to perform protein identification through data base searching. On the other hand, as shown with the oncogene H19, proteomics can provide useful information for the understanding of the genome and its functioning in a physiopathological context.

Growth Factors Signaling—The growth of breast cancer cells is regulated not only by estrogenic hormones (estradiol and progesterone), but also by different growth factors (6, 7). For example, IGF-I or epidermal growth factor (EGF) stimulate the proliferation of breast cancer cells, whereas other factors like mammary-derived growth factor inhibitor inhibit their growth. Hepatocyte growth factor/scatter factor has been shown to stimulate the migration of breast cancer cells, and thus metastasis. We and others have shown that FGFs, which are pleiotropic polypeptides involved in the control of cell growth, are stimulators of both breast tumor growth and metastasis (39, 40). More recently, we have demonstrated that nerve growth factor (NGF), well known as the archetypical neurotrophin, is able to stimulate both proliferation and survival of breast cancer cells through distinct signaling pathways initiated by different NGF receptors (41–43).

Many growth factors initiate intracellular signaling through tyrosine kinase-membrane receptors, which in turn induce intracellular protein-protein interaction and phosphorylation cascades involving a variety of signaling proteins such as the MAP kinases. These cascades of protein phosphorylation ultimately induce changes in gene expression, with consequent modifications in protein synthesis, leading to either cell survival, proliferation, differentiation, or migration. The development of Herceptin, a truncated antibody directed against the tyrosine kinase receptor Erb-B2, and its successful use in clinical practice shows that deciphering growth signaling in breast cancer cells can potentially lead to the development of realistic therapeutic strategies. In addition, modifications of the proteome induced by expression of the oncogene Erb-B2 have also been described (44). Nevertheless, signaling pro-

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4 E. Adriaenssens and H. Hondermarck, unpublished data.
teins, such as kinases and phosphatases, are regulatory proteins present in low quantities in the cell, and their activation-deactivation, by phosphorylation-dephosphorylation, renders these difficult to study. Classical methods to investigate these processes are based on the use of specific antibodies to purify known signaling proteins, followed by SDS-PAGE separation and Western blot analysis of tyrosine phosphorylation. Alternatively, phosphotyrosine-containing proteins can be purified and signaling proteins immunodetected. However, the main drawback of these traditional protocols is their difficulty in identifying proteins with no previously described function in signal transduction. In contrast, proteomics is providing a way to de novo identify signaling proteins, and it is certainly one of the most exciting challenges of functional proteomics to shed new light on the signaling pathways leading to cancer cell development.

Before the word proteomics appeared, the use of 2DE for studying growth factors mechanism of action was initiated with pheochromocytoma PC12 cells (45). In these cells, early protein synthesis induced by the differentiating activity of NGF and FGF-2 has been compared with the mitogenic activity of EGF using 2DE separation of $^{35}$S metabolically-labeled amino acids. The results revealed an initial modulation of protein synthesis induced by the three growth factors and provided some of the early evidence for a specificity of the differentiative versus proliferative pathways induced by tyrosine kinase growth factor receptors. Several years after, proteomics has been used successfully for the study of protein phosphorylation cascades (46, 47). Recently, a major breakthrough in the use of proteomics for studying signal transduction has been achieved with phosphoaminoacid ion scanning, allowing the localization of phosphorylated amino acid residues in a protein and its first application for EGF and FGF-2 signaling (48, 49).

In breast cancer cells, the increase in tyrosine phosphorylation of several proteins induced by FGF-2 has been characterized and includes the FGF receptor, the FGF receptor substrate (FRS2), the oncogenic protein Src, and the MAP kinases p42/p44. Interestingly, it was also shown that such stimulation induces the tyrosine phosphorylation of cyclin D2 (50). Phosphorylation of cyclin D2 had not been previously described in growth factor signaling, although it was known that progression through the cell cycle is under the strict control of cyclins and their catalytic subunits, the cyclin-dependent kinases. Modifications in protein synthesis induced by FGF-2 have also been studied (51) and revealed that this growth factor induced an increase in the level of stress proteins HSP90 and HSP70 as well as of the proliferating cell nuclear antigen (a regulator of the polymerase delta) and the transcriptionally controlled tumor protein, a protein of unknown function that is found associated with cell proliferation.

Fig. 5 summarizes the signaling pathways of FGF-2 in breast cancer cells. Interestingly, the testing of existing pharmacological inhibitors or the development of new drugs can also be monitored by proteomic analysis. The application of such a protocol has, for example, led to the identification of HSP90 as a target in breast cancer. Previous studies using mammalian cells have shown that mitogens such as IGF and EGF increase cellular synthesis and accumulation of HSP90 and HSP70 (52). Using proteomic analysis, it has been shown that synthesis of HSP90 is up-regulated after FGF-2 stimulation in breast cancer cells (51). HSP90 is up-regulated in cancer cells and after stimulation by growth factors. HSP90 can form complexes with many components of growth factor signaling pathways (53), and these interactions permit their protection and correct conformational folding. Interestingly, geldanamycin, an inhibitor of HSP90 activity, totally blocks FGF-2-induced growth of breast cancer cells, suggesting that appropriate protein folding and trafficking are essential for the stimulation of breast cancer cell growth. Therefore, the overexpression of HSP90 appears to be essential to breast epithelial cell tumorigenesis, and this protein is a potential therapeutic target in breast cancer.

Toward Single-Cell Proteomics?—As described above, proteomics is now an efficient way to identify growth signaling proteins. Sensitivity of the methods of detection have been improved and functional aspects as protein-protein interaction as well as post-translational modifications can reliably be studied. However, the most challenging aspect of studying intracellular signaling has now shifted to the understanding of global signaling networks. A living cell, in a physiological or pathological situation, is under stimulation of different factors at the same time, and the activated signaling pathways are susceptible to interacting with one another. For example it is...
known that the activation of transforming growth factor b receptors leads to stimulation of Smad signaling proteins, which are translocated to the nucleus to modify gene expression; interestingly, Smad proteins can also lead to the phosphorylation of the MAP kinases leading to the inactivation of their translocation to the nucleus (54). Therefore, the signaling pathways of growth factors and hormones are definitely not independent ways that regulate cell behavior, and their functioning increasingly appears to depend on interrelations and cross-talks. In this regard, kinetics of signal activation is also subject of increasing interest, and to tackle these new challenges in the field of signal transduction further improvement of the proteomic technologies are necessary. Indeed, current analysis of protein-protein modifications and post-translational modifications requires a certain amount of proteins that therefore have to be prepared from whole-cell cultures. As mentioned before, tens of thousands cells are required for any proteomic analysis, and it is now well established that all individual cells in culture will have a different response profile to growth stimulators. For example, cancer cells are hardly synchronized in culture, and stimulation will be obtained only on cells in G0/G1 phase. Therefore, not all cells will respond with the same time course to growth factor stimulation, and the kinetics of signaling pathway activation will consequently vary from one cell to another. Ideally, a precise investigation of intracellular signaling would be required to work on a single cell, allowing the spatio-temporal integration of multiple pathways. The concept of single-cell proteomics and its implementation for the study of intracellular signaling would definitely be a major advancement to investigate the complexity of intracellular signaling in cancer cells, and consequently to define future therapeutic strategies.

CONCLUSION

Breast cancer is providing a good illustration of the issues facing proteomics, and more generally post-genomics, in the elucidation of the fundamental mechanism of pathologies and the definition of new tools for their detection and treatment. Despite the considerable technological improvements in genomics and proteomics, it seems that the complexity of biological situations is the main limitation to a precise understanding of the molecular mechanism leading to multifactorial diseases such as cancer. Therefore, we should probably be careful on the biological relevance of proteomic investigation. In this regard, “blind proteomics” leading to data base creation is certainly a valuable approach to define the basis for further work, but it should also be remembered that proteomic analysis does not generate, by itself, any biological mean. To define the entire human proteome, or specific proteomes of tissues or organs like the breast, is included in various research programs, but the description and annotation of proteomes is definitely not sufficient to uncover protein function and the complex regulation network involved in multifactorial diseases. This is clearly an issue that the field of proteomics will have to deal with. In a way, the genome project had somehow contributed, for a while, to a shortening of our vision of biology. Everything important was supposed to be contained in a single long molecule (DNA) and it was more or less believed that all in biology and medicine would become understandable after its sequencing. During the course of human genome sequencing, the scientific community and the public progressively realized that the accomplishment of this huge project was not going to revolutionize the field of biomedicine. As stated by the French philosopher René Descartes in the 17th century, “connaître n’est pas comprendre” (to know does not mean to understand), and this still does apply to our time. Simply because biology is much more complex than the structure and functioning of nucleic acids, the practical applications have been quite limited. Is a similar situation going to apply with the exploration of human proteome? Possibly, and careful examination of the present situation, based on lessons of the past, encourages humility in defining what the knowledge of the human proteome would lead to. At the end, there is a reasonable probability that the complexity of life might still prevail, limiting the generation of valuable data for practical outcomes in biomedicine. Therefore, rather than rediscovering the fundamental questions at the end of the project, it would probably be better to base any proteomic strategy on clearly defined biological questions in an integrative spirit based on hypothesis-driven approaches. Looking back, it seems that the major outcome of the human genome sequencing has finally been to open the way to the exploration of the proteome, transferring the goals, the hope, and the difficulties/pitfalls to this last one. This is clearly a challenging, but also a promising, heritage.

* This work is supported by the Ligue Nationale Contre le Cancer (Comité Departemental), the Fondation pour la Recherche Médicale (Comité du Nord), the Association pour la Recherche contre le Cancer (Contract 4339), the Ministère de la Recherche et de l’Education Nationale, the Genopole de Lille, and the Région Nord-pas-de-Calais. To whom correspondence should be addressed: UPRES-EA 1033, Batiment SN3, Université Lille 1, 59650 Villeneuve d’Ascq Cedex, France. Tel.: 33-3-20-43-40-97; Fax: 33-3-20-43-40-38; E-mail: hubert.hondermarck@univ-lille1.fr.

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