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Growth signaling in breast cancer cells: outcomes and promises of proteomics

Eric Adriaenssens[a], Jérôme Lemoine[b], Ikram El Yazidi-Belkoura[a], Hubert Hondermarck[a,*]

[a]*Laboratoire de Biologie du Développement UPRES-EA 1033, Université de Lille1, Bâtiment SN3, 59650 Villeneuve d'Ascq Cedex, France*
[b]*Laboratoire de Chimie Biologique, UMR 8576 CNRS, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, France*

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Abstract

Methods in functional proteomics are now used to study the intracellular signaling pathways that underlie the development of breast cancer. As shown with fibroblast growth factor-2, the oncogenic/non-coding mRNA *H19* and 14-3-3 proteins, proteomics is a powerful approach to identify signaling proteins and to decipher the complex signaling circuitry involved in growth of breast cancer cells. Together with genomics, proteomics is now providing a way to define molecular processes involved in breast cancerogenesis and to identify new therapeutic targets.

Keywords: Growth factors; Oncogenes; Breast cancer; Signaling pathways; Proteomics

1. Introduction

The proteome, first formalized in 1995, designs all the proteins expressed by the genome of a cell, tissue, or organism. Complementing the field of genomics, proteomics is designed to elucidate both protein levels and post-translational modifications in different cell types under different physiological conditions [1]. Cellular protein can now be analyzed using high resolution two-dimensional electrophoresis (2DE) and proteins of interest can then be characterized by mass spectrometry. The progress in genomic sequencing and in the availability of Internet databases, as well as the development of mass spectrometry-based strategies for protein identification, heralded the use of proteomic analysis in biomedicine [2]. The use of proteomics for analyzing signal transduction pathways that lead to breast cancer cell development has now become a reality and provides the knowledge base for the identification of therapeutic targets and the development of new anti-cancer strategies [3].

* Corresponding author. Tel.: +33-3-20-43-40-97; fax: +33-3-20-43-40-38.
E-mail address: hubert.hondermarck@univ-lille1.fr (H. Hondermarck).

Abbreviations: 2DE, two-dimensional electrophoresis; FGF, fibroblast growth factor; HSP, heat shock protein; MALDI–TOF, matrix-assisted laser desorption/ionization–time of flight; MAP-kinase, mitogenic activated protein-kinase; MEK, MAP-kinase–kinase; NGF, nerve growth factor.

2. Proteomics to decipher intracellular signaling pathways

Proteomics was first mooted as a way to map all cellular proteins using 2DE gels. This aspect, designated expression proteomics, is now complemented by an emerging field named functional proteomics. This concerns itself with protein interactions and activations within functional contexts [4]. Recent publications have demonstrated the value of proteomics for the study of intracellular signaling, especially in the case of growth factor stimulation, opening a way of understanding the molecular mechanisms involved in tumor growth [3,5,6]. Growth factors, essential polypeptides required for eukaryotic cell growth, initiate their stimulation through specific, tyrosine kinase–membrane receptors, which in turn induce intracellular protein-phosphorylation cascades. Classical methods to study these processes were 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 were purified and signaling proteins immunodetected. Similarly, early changes in gene expression are usually studied by Northern blotting or quantitative RT–PCR assays for specific mRNAs after cell stimulation. The main limitation of these traditional protocols is their difficulty in identifying proteins with no previously described function in signal transduction.

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The use of 2DE for studying the mechanism of action of growth factors was initiated for pheochromocytoma PC12 cells [7]. In these cells, early protein synthesis induced by the differentiating activity of nerve growth factor (NGF) and fibroblast growth factor-2 (FGF-2) has been compared to the mitogenic activity of epidermal growth factor (EGF) using 2DE separation of ^{35}S metabolically-labeled amino acids. This study demonstrated the value of 2DE technologies to investigate changes in protein synthesis induced by growth factors, opening new way for the detection of signaling involved in cellular growth. More recently, proteomics has been successfully used for the study of protein phosphorylation cascades. This has been demonstrated for platelet-derived growth factor and EGF stimulation of mouse fibroblasts [8,9]. NIH 3T3 cells were exposed to the growth factors and the phosphotyrosine containing proteins immunoprecipitated before separation either on SDS–PAGE or 2DE. Mass spectrometry analysis allowed the identification of several known signaling proteins, and of other proteins not previously reported to be activated by growth factors, including a plexin-like protein and the guanine nucleotide exchange factor Vav-2. Therefore, proteomic analysis can now be used to study the successive steps of growth factor signaling (Fig. 1).

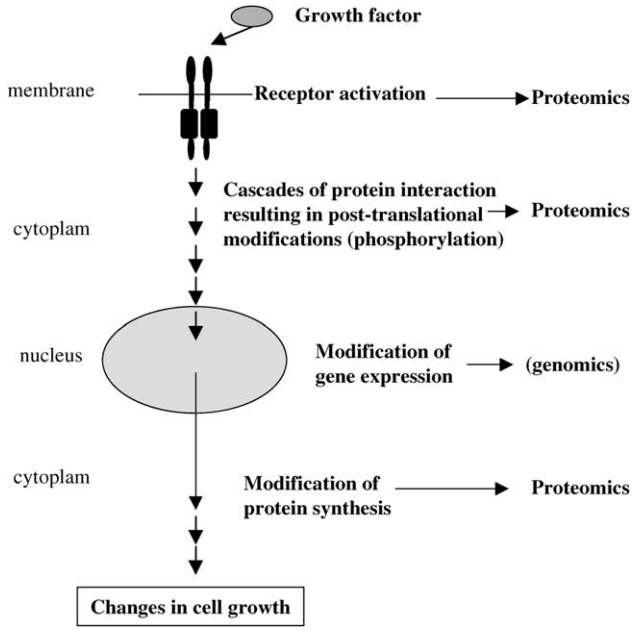

Fig. 1. Proteomics for studying growth factor signaling pathways.

3. Growth of breast cancer cells

Molecular events leading to breast epithelial cell carcinogenesis [10] involve modification in the structure and expression of both oncogenic and suppressive genes (such as *ras* and *p53*, respectively), leading to an unbalanced growth characterized by high rates of proliferation and eventually migration, as well as a tendency to better survive environmental stress which would otherwise lead to apoptosis (programmed cellular death). As illustrated in Fig. 2, the growth of breast cancer cells is under the control of estrogenic hormones (estradiol and progesterone) and growth factors. Estrogens are necessary for the development of normal cells and some cancer cells, which has led to the development of anti-estrogen strategies, best represented by the use of tamoxifen [11]. In addition to estro-

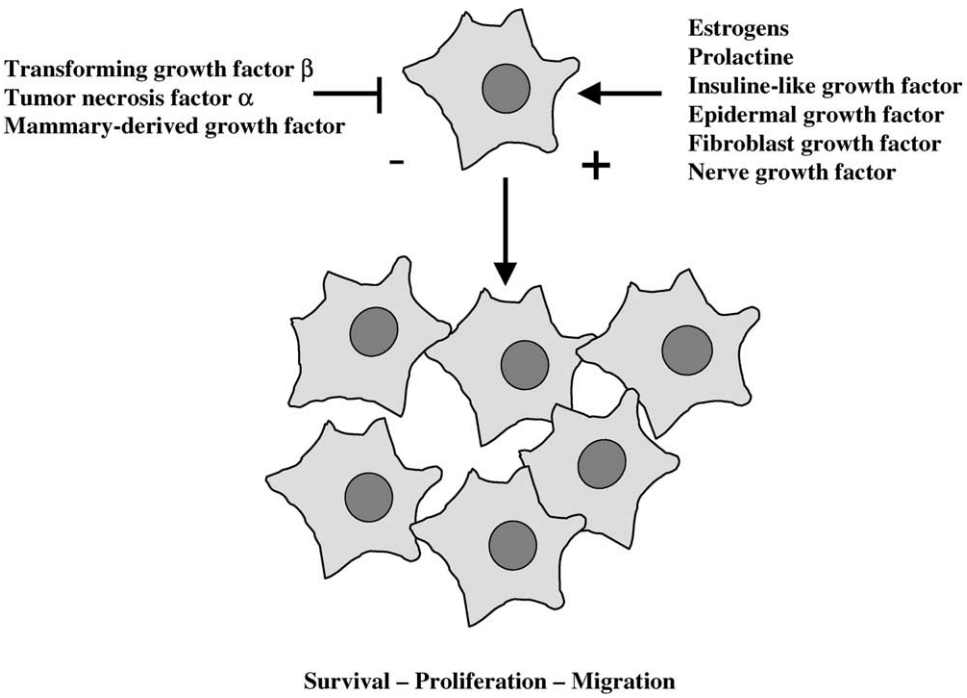

Fig. 2. Growth regulators of breast cancer cells.

genic hormones, the growth of breast cancer cells can be regulated by various growth factors that control proliferation, migration, and apoptosis [12,13]. For example, insulin-like growth factor I or EGF stimulate proliferation of breast cancer cells whereas other factors like mammary-derived growth factor inhibitor or transforming growth factor beta inhibit their growth. Hepatocyte growth factor/scatter factor has been shown to stimulate the migration of breast cancer cells, and thus metastasis [14]. Interestingly, FGF-2 has been shown to stimulate both proliferation and migration of breast cancer cells [15]. Recently, we have also demonstrated that NGF, better known as the archetypal neurotrophin, is able to stimulate both proliferation and survival of breast cancer cells [16,17]. Interestingly, these two biological effects are mediated through distinct signaling pathways initiated by different NGF receptors [17]. NGF stimulation of the tyrosine kinase receptor trkA leads to the activation of the MAP-kinase (p42/p44) pathway and cell proliferation whereas the activation of p75NTR (a TNF receptor family member) induces a nuclear translocation of the transcription factor NF-κB resulting in a better survival of breast cancer cells.

4. Proteomic analysis of FGF-2 signaling pathway

FGFs are pleiotropic polypeptides involved in the control of cell proliferation, differentiation, and survival [18]. The prototypic FGF family member, FGF-2, has been shown to be involved in many forms of cancer cell growth and metastasis. In breast cancer, the overexpression of FGF-2, as well as its receptors, has been reported in a significant percentage of breast tumors [19]. We and others have found that FGF-2 is a strong activator of breast cancer cell proliferation and migration [15,20,21], indicating a key role for this growth factor in breast tumor growth and metastasis. Classical methods to study intracellular signaling have been applied to FGF-2 activation of breast cancer cells; they have uncovered a complex signaling network involving activation of the FGF-receptor tyrosine kinase and MAP kinase p42/p44, as well as the signaling proteins FAK (Focal adhesion kinase), Src, Rac-1, and Nck [15,22]. The proteomic protocol that we used to investigate FGF-2 signaling is summarized in Fig. 3. We studied both the rapid changes (occurring during the first minutes) in protein tyrosine phosphorylation, and the modifications in protein synthesis induced by FGF-2. Using phosphotyrosine immunoprecipitation and SDS–PAGE, we were able to detect an increase in tyrosine phosphorylation of several proteins which have been characterized by mass spectrometry as being the FGF receptor, the FGF Receptor Substrate (FRS2), the oncogenic protein Src, and the MAP kinases (p42/p44). Interestingly, we also found that FGF-2 stimulation induces the tyrosine phosphorylation of cyclin D2 [23]. Phosphorylation of cyclin D2 was not been previously described in growth factor signaling, although it is known that progression through the cell cycle is strictly under the control of cyclins and their catalytic subunits CDKs (cyclin-dependent kinases). We showed, using pharmacological inhibition of the activities of the oncoprotein

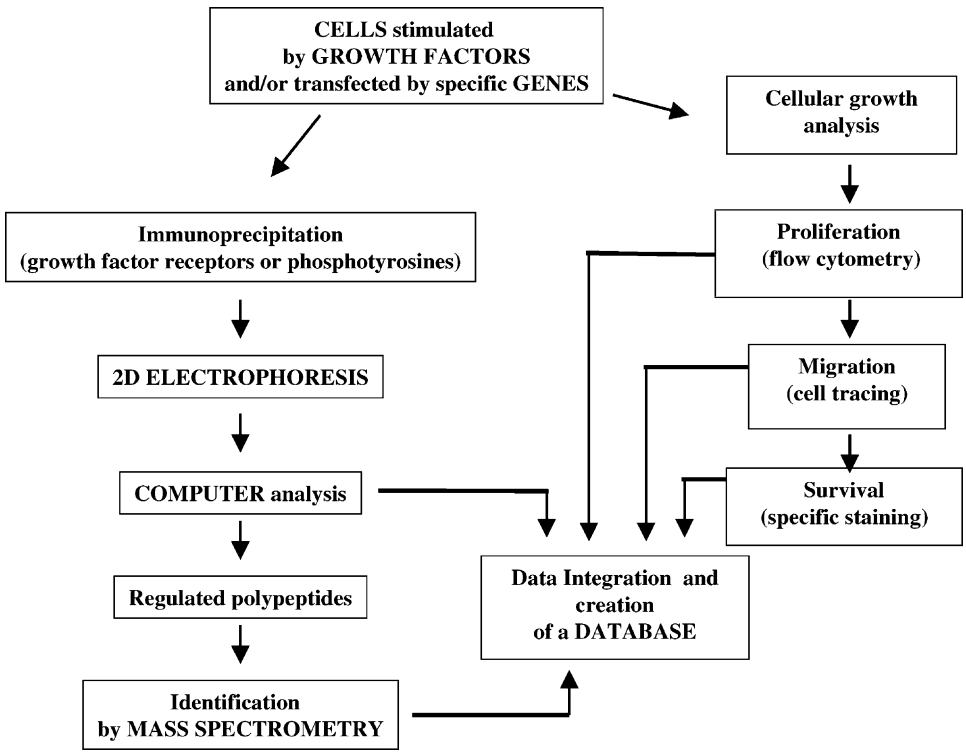

Fig. 3. Protocol for proteomic analysis of growth signaling.

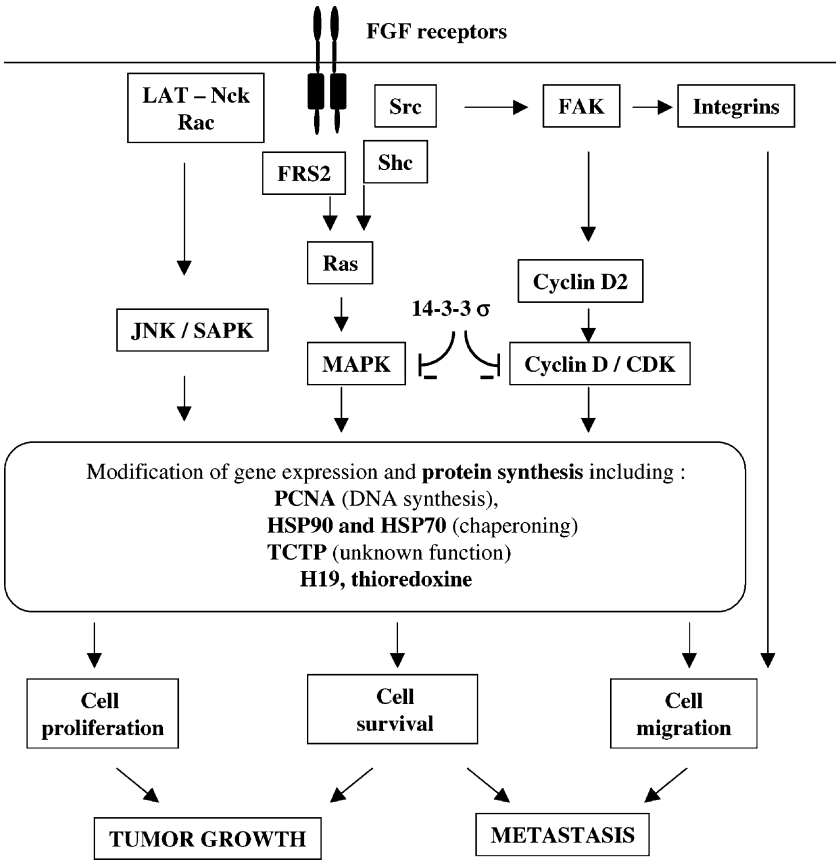

Fig. 4. FGF signaling in breast cancer cells.

Src and of p42/p44, that Src activity is required for the FGF-2-induced phosphorylation of cyclin D2, whereas MAP-kinases are not.

After metabolic labeling of MCF-7 cells with ^{35}S-amino-acid proteins and computerized analysis of 2DE autoradiograms, several proteins were found up-regulated during FGF-2 stimulation [24]. Two of the FGF-2 regulated proteins belong to the heat shock protein family (HSP). Previous studies using mammalian cells have shown that mitogens such as insulin-like growth factor and EGF increase cellular synthesis and accumulation of HSP90 and HSP70 [25]. We demonstrated here that synthesis of HSP90 and HSP70 are both up-regulated after FGF-2 stimulation in breast cancer cells, suggesting that appropriate protein folding and trafficking are essential processes for the regulation of breast cancer cell growth. In addition, we have shown that geldanamycin, an inhibitor of HSP90 activity, totally blocks the FGF-2-induced proliferation and general growth of breast cancer cells. HSP90 can exist in a complex with many components of the growth factor signaling pathway: tyrosine kinase receptors, Src, Raf, or MAP-kinase–kinase [26], and its interaction with these signaling proteins allows for their protection and correct conformational folding. These data suggest that the expression of this protein is part of breast epithelial cell tumorigenesis, and thus HSP90 constitutes a potential therapeutic target for breast cancer treatment. Finally, we also found that the transcriptionaly controlled tumor protein (TCTP) is up-regulated by FGF-2. TCTP was initially described as a tumor-related polypeptide, but it has more recently been found in normal human cells. Despite its ubiquity and high level of conservation, the physiological role of TCTP remains to be determined, but the up-regulation that we observed in breast cancer cells suggests a role in breast tumorigenesis. Fig. 4 summarizes the signaling pathway of FGF-2 in breast cancer cells.

5. Proteomic identification of a molecular target for the oncogenic mRNA *H19*

H19 is an oncofetal gene, which encodes an untranslated mRNA [27] and has been shown to regulate cancer cell growth [28] but no molecular target has been assigned to *H19* and its mechanism of action remained unknown. We have developed a proteomic-based strategy to identify molecular targets of *H19* in breast cancer cells [29]. Breast mammary cells have been transfected with *H19* and the proteomic profile established using 2D electrophoresis of ^{35}S-methionine metabolically labeled proteins. Changes in protein synthesis have been studied by computerized analysis and revealed essentially one modification in spot

intensity induced by *H19* transfection. Mass spectrometry analysis of this spot performed by MALDI–TOF and MS–MS allows the identification of the protein up-regulated by *H19* gene as the thioredoxin, one of the major proteins regulating the intracellular redox metabolism [29]. The thioredoxin system is a general protein disulfide reducing complex, which includes the NADPH-dependent flavoprotein Thioredoxin reductase. Thioredoxin is activated in response to oxidative stress caused by UV or X-ray irradiation or inflammatory cytokines. Following threshold of the oxidative stress it can cause either a positive response of the cell such as proliferation or a negative response such as apoptosis [30]. Thioredoxin scavenges the reactive oxygen species damages produced by oxidative stress but there is growing evidence that Thioredoxin modulates expression of various kinds of genes in regulating the DNA binding of several transcription factors. More particularly, it is well demonstrated that Thioredoxin increases transcription activity of NF-κB in enhancing its DNA binding in the nucleus [31]. Recently, we have shown that growth factor stimulation of breast cancer cells, specially by FGF-2, results in a strong and sustained stimulation of *H19* gene transcription [32]. Altogether our data demonstrates the value of proteomic analysis in deciphering the signaling pathways involved in the mechanism of oncogenesis.

6. 14-3-3 sigma: a potential therapeutic target

14-3-3 is a family of highly conserved protein forms (alpha, beta, delta, sigma, tau, zeta) of 25- to 30-kDa expressed in all eukaryotic cells, which play a role in the regulation of signal transduction pathways implicated in the control of cell proliferation, differentiation, and survival [33]. 14-3-3 proteins are known to associate directly or indirectly with signaling proteins such as the insulin-like growth factor I receptor, Raf, MEK kinases, PI3-kinase, but the precise molecular mechanism by which they activate or inhibit these elements remains unclear [33]. Transcription analysis has confirmed that gene expression of 14-3-3 sigma is 7–10-fold lower in breast cancer cells than in normal breast cells due to the high frequency of hypermethylation of the 14-3-3 sigma locus [34]. We found, using proteomics, that the 14-3-3 sigma protein is present in breast cancer biopsies at levels which average 10-fold lower than normal breast epithelial cells [35]. Our data show that the protein 14-3-3 sigma can be used to discriminate cancer from non-cancerous breast epithelial cells.

Interestingly, NGF and FGF-2 activate the Ras/Raf/MAP-kinase pathway in breast cancer cells, resulting in stimulation of the cell proliferation [15,16], but neither NGF nor FGF-2 has a mitogenic effect on normal breast epithelial cells [16,36]. Paradoxically, therefore, normal breast epithelial cells express both NGF and FGF receptors but do not proliferate in response to either cognate factor. The reason for the lack of sensitivity to these mitogens for normal breast epithelial cells is not understood, but the growing body of evidence implicating 14-3-3 sigma in cell cycle progression suggests that its high level in normal cells may block the mitogenic effect of growth factors. The sigma form of 14-3-3 is a p53-regulated inhibitor of G2/M progression and its overexpression can cause cell cycle arrest [37]. In addition, 14-3-3 is able to regulate cdc phosphorylation and activities, so controlling cell proliferation [33]. Importantly, in breast cancer cells it was shown that 14-3-3 sigma directly associates with cyclin-dependent kinases to negatively regulate cell growth [38]. The higher level of 14-3-3 sigma in normal breast epithelial cells may therefore contribute to the blocking of high cellular proliferation and it can be reasonably proposed that restoring higher levels of 14-3-3 sigma to breast cancer cells might lead to their decreased proliferation.

7. Conclusion

The proteomic data obtained to date in deciphering growth signaling pathways and searching therapeutic targets for breast cancer are clearly only a first step. Future developments in the field are likely to depend on further technological innovations in proteomic analysis. A typical 2DE gel can reliably separate and detect 2000 proteins. The human genome may contain as many as 30,000 genes; as a result of mRNA splicing, post-translational modifications and proteolysis, the number of proteins is clearly higher than the number of spots detectable on a 2DE gel. Although 2DE provides the highest resolution in protein separation [39], some proteins cannot be studied. Hydrophobic proteins, for example, do not dissolve in the solvents used for isoelectric focusing, and the development of detergents with enhanced solubilization capabilities are needed to improve the analysis of plasma membrane proteins. The use of the isotope affinity tagging of cysteine residues (I-CAT) method in conjunction with LC–MS–MS is also a promising way to access less soluble proteins, since peptides issued from protein digestion (which are more likely to be soluble than entire proteins) are separated and quantified [40].

Based on a concept developed for the study of nucleic acids, the possibility of using protein chip technologies has emerged as a way for by-passing the intensive, time-consuming method of 2DE. The principle of using protein chips for the identification of new markers depends on being able to flush potential ligands over arrays impregnated with different types of affinity probes, and subsequent analysis of any differential protein binding [41]. Mass spectrometry would then allow for the identification of proteins differentially expressed or post-translationaly modified.

Protein identification is now currently performed after proteolysis, by searching genomic and proteomic databases with accurate peptide mass data. Mass spectrometry and advanced bioinformatics have made possible sensitive

identification of spots in 2DE gels, and have been central to the creation of proteomics. For biologists, it can be predicted that mass spectrometry will engage the next set of challenges: (i) to lower the protein quantities needed for identification, (ii) to characterize post-translational modifications directly from a spot in a gel, and (iii) to study non-covalent protein interactions. The intrinsic sensitivity of improved MALDI–TOF mass spectrometers, such as the MALDI QqTOF, are well beyond the currently used range of sensitivity. Indeed, attomole ranges have been recently reported for peptide standards. This indicates that it is both the biological origin and chemical treatments of samples, and not the instruments, which are more likely to be responsible for limitations in sensitivity [42]. However, great promise is offered by the current development in LC–MS and LC–MS–MS for handling protein mixtures, especially when associated with quantitative methods such as the isotope affinity tagging of cysteine residues [40].

Protein phosphorylation has been shown to be a very unstable and labile modification, making its analysis in a large number of breast tumor biopsies uncertain. Finally, mass spectrometry is now offering the possibility of directly studying the non-covalent interactions of proteins with other proteins, or with drug molecules, thus opening a way to study intracellular signaling networks [43]. Electrospray ionization mass spectrometry has been used to study protein interactions driven by non-covalent forces [44]. The gentleness of the electrospray ionization process allows intact protein complexes to be directly detected by mass spectrometry, and stoichiometry of the complex can be easily obtained from the resulting mass spectrum, because the molecular weight of the complex is directly measured. In conclusion, it is becoming clear, that mass spectrometry, in combination with other biophysical methods such as NMR, is promising new directions for the study of signaling networks and for the design of new anti-cancer drugs.

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