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Towards elucidation of functional molecular signatures of the adhesive-migratory phenotype of malignant cells

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ABSTRACT

Over the years, malignant transformation has been investigated on multiple levels, ranging from clinical pathology to the underlying molecular mechanisms. In "zooming in" on this process, cancer biologists have focused their attention on the molecular and cellular manifestations of the "transformed phenotype", including the genomic instability of cancer cells, their deregulated transcriptional activity, their aberrant morphology and dynamics, and the altered signaling networks activated in them. Attempts to elucidate the mechanisms underlying malignant and metastatic transformation are primarily motivated by the desire to identify specific molecules and signaling pathways that can serve as targets for novel therapies.

In recent years, such studies were reinforced by major technological and conceptual developments: novel and powerful tools for genomic and proteomic analysis have been developed, and advanced computational approaches offer "systems-level" integration of rich and complex biological datasets into meaningful functional networks. In this article, we consider the current and potential impact of these new experimental approaches and, in particular, the recent progress made in quantitative proteomics, to elucidate the mechanisms underlying the "transformed phenotype". We will primarily focus on the adhesion and migration of cancer cells, and their relationships to the deregulated growth, metastatic dissemination, and anchorage independence associated with malignant transformation.

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1. The diversity and complexity of the transformed phenotype

The search for characteristic features of transformed cells is largely motivated by the desire to identify diagnostic markers, as well as potential therapeutic targets, whose expression and activities are relevant to the clinical manifestations of a cancerous growth—namely, deregulated cell proliferation, invasiveness, and metastatic potential [1–3]. This search is conducted using a wide variety of biological systems, ranging from naturally occurring human cancers, via tumors grown in appropriate animal models, to cultured cells. At each of these "hierarchical levels", different cellular features are investigated, including: (I) the morphology and architecture of the tumor tissue, and diversity in appearance of the transformed cells; (II) the physiological characteristics of the cancer cells, including their altered metabolic properties [4–6], deregulated growth [7–9], refractoriness to physiological cell death [10,11], adhesive properties [12], and migratory potential [13,14]; (III) at the proteomic level, a search for specific molecular "biomarkers" with diagnostic and prognostic value, which in some cases could prove useful as therapeutic targets; (IV) at the molecular genetic level, whereby mutations in particular genes, as well as alterations in the transcriptional program, are associated with malignant transformation [15,16].

Studies based on these diverse approaches have shed considerable light on various aspects of the malignant process and, in a few cases, have pinpointed specific therapeutic targets from which small molecule-based and antibody-based "targeted therapies" (e.g., Gleevec®, Herceptin®, Velcade®) were developed. However, the overall success in developing new therapies, based on the identification of particular cancer-associated targets, is rather limited. This somewhat disappointing outcome can be attributed to the extraordinary molecular diversity of human cancer cells (both patient-to-patient variability and intra-tumoral cellular variability), and to the limited information available on the mechanistic relationships between the "clinical phenotype" and the "molecular phenotype". Possible approaches for bridging this gap will be discussed below.

2. Involvement of aberrant cell adhesion and migration in the acquisition of the transformed phenotype

It is well beyond the scope of this article to address the molecular underpinnings of the numerous clinical manifestations of the trans-

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formed phenotype. Rather, we will focus here on the characteristic effects of malignant transformation on two closely interrelated cellular processes: cell adhesion, and cell migration. Malignant transformation is frequently associated with altered cell-matrix adhesion [17–19], reduction in cell-to-cell cohesion [20,21] and an increase in cell migration [22,23]. This association is not surprising, in view of the invasive and metastatic potential of many aggressive cancers. In such cases, the pathological processes associated with cancer growth are based on the invasion of individual cells into neighboring tissues, thus perturbing the structural–functional integrity of the tissue, as well as affecting tissue innervation and obstructing blood and lymphatic vessels.

During metastasis, the loss of cell–cell cohesion, intravasation into the cancer-associated vasculature, migration-associated with the formation of metastatic cell growth, and extravasation into distant organs–are all highly dependent upon changes in the "adhesive-migratory" phenotype which, in and of itself, is highly complex (and poorly understood) at the molecular level [24,25]. For example, certain stages in the process of malignant transformation are often compared to the epithelial-to-mesenchymal transition (EMT), whereby cells acquire enhanced migratory capacity, invasive features, resistance to apoptosis, and an ability to alter the extracellular matrix (ECM) [26–28]. Key features of EMT include reduced cell–cell adhesion via loss of E-cadherin, ZO-1 and desmoplakin, concurrent with marked changes in the ECM, such as overexpression of fibronectin on the one hand, and overexpression of metalloproteinases (MMP9 and MMP2), which degrade the matrix, on the other. Thus, changes in the transcriptional program associated with malignant transformation may be involved, at least in part, in the changes exhibited in the adhesive and migratory behavior of the cells. One outstanding question is whether these changes are also regulated at the post-transcriptional and post-translational levels.

Adhesion-mediated environmental sensing, which takes place at molecularly- and structurally-defined adhesion sites (e.g., integrin-mediated ECM adhesions, or cadherin-mediated adherens junctions [29–31]) is integrated, processed and further stimulates a variety of signaling networks that trigger and coordinate multiple regulatory pathways within the cells. While the validity of adhesion-mediated signaling is widely accepted, the underlying mechanisms are still poorly understood. For example, what molecules provide the basis for such adhesive interactions? At what spatial and temporal resolutions does adhesion-mediated signaling occur? How are diverse molecular interactions, mediated via the same adhesion site, regulated? How do physical features of the adhesive surface (e.g., the ECM) activate specific signaling pathways? In particular, it is critical to determine if and how cancer-related deregulation of signaling processes affects the interactions of cells with their environment, and vice versa.

The networks of molecules that participate in cell adhesion with the ECM and neighboring cells are known collectively as the integrin– and cadherin–adhesomes. The proteins that interact within these sites consist of hundreds of known components whose mutation, deregulated expression, or reorganization could be responsible for some of the major characteristics of cellular transformation. In order to address the mechanisms underlying the changes in the adhesive and migratory behavior of cancer cells, it is essential to obtain a system-wide molecular view of the transformation process, which can be clarified at three major hierarchical levels: the genome, the transcriptome, and the proteome. These three approaches are highly complementary, shedding light on the levels themselves, and on the mechanisms underlying the malignant transformation.

Cancer development is commonly driven by genomic instability, and a tendency to accumulate mutations that affect cell behavior and fate [32]. The sequencing of the human genome at the beginning of the millennium [33,34] and the exponential development of sequencing technology, offer new strategies for surveying many other genomes. One of the largest international efforts currently underway involves the sequencing of cancer genomes, and is mainly conducted by the international cancer genome consortium (www.icgc.org) and by several other large centers. This project includes sequencing of large proportions of the genomes of different cancer types, to identify those somatic mutations that drive the transformation process. Due to the partially stochastic nature of mutagenesis, and the great variability in the number of mutations per tumor, it is unlikely that numerous mutations, common to many tumors, would be found. Rather, the intent is to find commonly mutated pathways, or processes that are altered by the somatic mutations in one or several of their components.

One such large-scale study involved the sequence and analysis of 518 protein kinase genes in 210 tumor samples [35]. The resulting data showed that several kinases (e.g., CDC42BPs, PAK proteins, ROCK1 and 2, PTK2B and MYO3B) that play a role in the adhesive-migratory machinery are mutated in these tumors. In other projects, all coding exons were sequenced, but in a smaller number of tumor samples: a study of breast and colon cancers included 11 tumors of each type [36], and a study of pancreatic cancer included 24 tumors [37]. In both of these analyses, mutated adhesion- and migration-related genes were clearly represented, and included multiple ECM proteins and their modifiers (collagens, fibronectin, laminins, and metalloproteinases from the MMP and ADAM families). Adhesion molecules such as integrins, plakoglobin and multiple cadherins were mutated as well, as were their intracellular binding partners, such as talin and catenins [36,37]. Another group of mutated genes were those encoding G-protein modifiers, among them multiple Rho GAPs and GEFs, which presumably affect actin organization, and thereby alter cell migration. Moreover, multiple mutations were also identified in intracellular matrix proteins such as myosins, titin, and keratins [36,37].

This powerful approach highlights the initial events in the transformation process, encompassing changes on the DNA level. From these findings, it is apparent that both intracellular and extracellular structural proteins are mutated, and may therefore trigger different cellular signals.

3. Large-scale genomic analysis of tumor samples: in search of transcriptomic signatures

The simplicity and robustness of microarrays make them a widely used, global analytical method in general, and specifically in cancer research. In several key studies [38–41], large numbers of tumors and expression profiles related to disease prognosis and progression were examined. One of the earliest large-scale functional genomics studies of tumor samples profiled metastatic adenocarcinoma, in comparison to primary adenocarcinoma of varying origins: a 128-gene profile was created to determine metastatic potential [38]. Examination of this signature in relation to adhesion and migration yielded only a few proteins involved in these processes. Collagen type I levels were high in the metastatic tumors, while myosin-related proteins, proto-cadherin and Ablim1 levels were high in the primary tumors.

Another large-scale study revealed a molecular signature that discriminates between breast tumors with good and bad prognoses [40]. A signature of 231 genes was first created, and then minimized to 70 genes. This molecular signature formed the basis of the MammaPrint test that was approved by the FDA in 2007 [42,43]. A search for adhesion and migration-related genes in this signature revealed only three proteins. Collagen type IV and matrix metalloproteinase 9 (MMP9) were both associated with a poor prognosis, reflecting changes in the ECM that can enable invasiveness. Myosin

light chain-interacting protein was associated with a good prognosis, and coincides with higher levels of myosin-related genes in the primary tumor signature described above.

The broad use of microarrays in cancer studies has enabled researchers to perform meta-analyses of multiple microarray experiments. These studies aim at finding common signatures, independent of the type of microarray used, the research laboratory performing the analysis, and even the cancer type being analyzed. One such meta-analysis, which included 40 microarray datasets, compared cancer to healthy tissue, low- vs. high-grade tumors, or tumor prognosis and outcome [39]. Based on this analysis, the authors created a cancer vs. normal signature of 67 genes, and a differentiated vs. non-differentiated cell signature of 69 genes. Both signatures mainly include genes involved in cell cycle and transcriptional regulation. The only adhesion/migration-related genes were COL1A2 and MMP9, both reflecting ECM-related changes. Another meta-analysis included 26 datasets corresponding to approximately 1,500 microarray experiments involving various cancer types [41]. A 46-gene signature was thus created to distinguish between normal and cancerous tissue. In this meta-analysis as well, only two genes were related to the adhesive-migratory phenotype; the first, myosin heavy chain (MYH11), and the second, a subunit of the ARP2/3 complex (ARPC1B).

These large-scale studies clearly show that the adhesive-migratory changes are not prominent in the genetic signatures characteristic of cancer development and prognosis. Changes in collagens, MMPs and myosins are apparent on the transcriptional level; however, for a global view of the adhesive-migratory state, other analytical methods must be used.

4. Deregulated adhesion-mediated signaling in cancer

The aforementioned experimental approaches, based on advanced molecular genetic technologies, provide – and will surely continue to provide – important information on the molecular processes underlying malignant transformation; yet their capacity to provide insights into signaling processes, or the molecular reorganization of cellular architecture, is limited. These technological limitations are noteworthy, in view of the involvement of deregulated cross-talk between the cellular signaling machinery and the adhesion/migration-related cytoskeleton, which affects the cell adhesion and migration typical of cancer. The bi-directional nature of adhesion-mediated signaling is supported by evidence showing that: (I) cell adhesion, in addition to its role in inducing and maintaining tissue integrity, also enables "environmental signaling" that helps to regulate cell proliferation, survival and fate [44–47] and (II) post-translational modification of structural and regulatory components of cell–cell and cell–matrix adhesions can dramatically affect the organization of the adhesion sites [48,49].

Bioinformatic examination of the integrin adhesome reveals its incredible molecular complexity: ~180 different molecules are associated with the adhesion sites either constitutively or transiently, encompassing a huge number of potential interactions (~750 reported links) ([50,51]; see also: http://adhesome.org). This complex network involves a wide variety of actin-associated proteins, which regulate the organization and stability of the actin-based microfilament network; and multiple adaptor proteins, which populate the submembrane "adhesion plaque" to which actin filaments are anchored (Fig. 1). Remarkably, nearly 40% of the reported components of the integrin adhesome constitute signaling molecules. Besides modulating the adhesion sites themselves, and affecting cellular interactions and cell migration, changes in adhesion-mediated signaling can also alter other features of the transformed phenotype. Indeed, examination of either the integrin adhesome, involved in cell-ECM adhesion [50], or the cadherin adhesome, associated with cell–cell contacts [52], reveals multiple signaling proteins that are known to be directly associated with cancerous processes (for a list of selected molecules, see Table 1). Both of these adhesion networks contain multiple Ser/Thr as well as Tyr kinases and phosphatases. They also include receptor tyrosine kinases and their adaptor proteins, through which they regulate such signaling pathways as the PI3K-Akt pathway and the Ras-

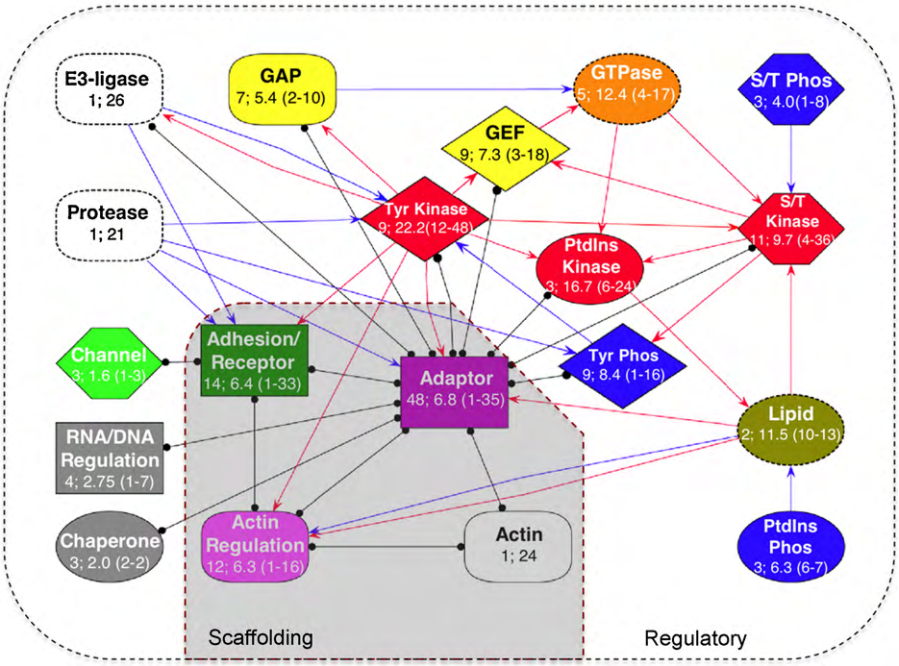

Fig. 1. Interactions between functional families of adhesome components. Each protein in the adhesome was categorized into one of 20 groups, according to its known biological activity. The families are shown in unique combinations of color and shape, indicating the number of family members, followed by the average number of their interactions. In addition, the dominating interactions between families (red arrows, activating interactions; blue arrows, inhibiting interactions; black lines, binding interactions) are shown (modified from Zaidel-Bar et al., 2007 [51]).

Table 1
Signaling components in focal adhesion and cell–cell junctions.[a].

Protein function/pathway	Focal adhesion proteins[b]	Cell–cell junction proteins[c]
Tyrosine kinase	CSK, FAK, FYN, YES, LYN, SRC, SYK, ABL	FER, FYN, SRC, YES1
Ser/Thr kinase	ILK, LIMK1, PAK1, PRKACA, PRKCA, ROCK1	CSNK1A1, CSNK2A1, GSK3B, MARK3, PRKACA, PRKCA, PRKCI, STK24, WNK4
Receptor tyrosine kinase	IR	EGFR, ERBB2, MET
TGFbeta receptor		TGFBR1, TGFBR2
Tyrosine phosphatase	LAR-PTP, PTP-PEST, PTPRO, RPTP-alpha, SAP1, SHP1, SHP2	ACP1, PTPN1, PTPN13, PTPN14, PTPN6, PTPRF, PTPRJ, PTPRK, PTPRM, PTPRU, PTPRZ1
Ser/Thr phosphatase	ILKAP, PPM1M, PPP2CA	PPP2R5
Non-signaling tumor suppressors	VCL	CDH1
Non-signaling oncogene	CTTN, VAV1-3, STAT3	CTTN
MAPK pathway	HRAS, p120GAP, SOS1, MAPK1, MAPK8	IQGAP, KRAS, MAPK1, ZAK
PI3K-Akt pathway	PTEN, PI3K, AKT, PDPK1	PIK3R1, PTEN
Adaptor protein	CAS, GAB1, GRB2, GRB7, IRS1, NCK2, SHC,	GRB2, CTTNB1, APC

[a] Proteins are represented by their gene name. Each protein in the table appears once per junction type.
[b] Focal adhesion data are based on: Zaidel-Bar and Geiger [50].
[c] Cell–cell adhesion data are based on: Paris and Bazzoni [52].

MAPK pathway. It is conceivable that through these and other proteins, integrin and cadherin adhesions can transmit survival and proliferative signals, hallmarks of the transformed state. Furthermore, adhesome networks also contain known oncogenes (e.g., beta-catenin, Stat3, Akt) and tumor suppressors (e.g., E-cadherin, APC), which directly drive cellular transformation.

Another level of complexity arises from the ability of the signaling machinery to "switch on" or "switch off" many of the molecular interactions that take place in the adhesome. For example, tyrosine phosphorylation may affect the molecular dynamics and organization of adhesion sites [53,54]. It can modulate enzymatic activity or binding affinities, thereby creating global changes in the adhesion architecture. As an example, expression of constitutively active $pp60^{src}$, a highly potent adhesome-associated tyrosine kinase, abolishes focal adhesions and stress fibers (Fig. 2), which are then replaced by highly dynamic dot-like adhesion sites, known as podosomes, which cluster together, forming ring-like adhesion structures. These changes are attributable to the enzymatic activity of $pp60^{src}$, since the addition of an inhibitor that blocks the kinase activity restores the original fibroblastic morphology (Fig. 2). Similarly, tyrosine phosphorylation of the adaptor protein CRK by Abl [55], locks the molecule in a folded structure, while its dephosphorylation by a protein tyrosine phosphatase, PTPN1, opens up CRK, rendering its SH2 domain available for binding to one of several phospho-proteins (e.g., paxillin, cbl, shc), and thus affecting the adhesome network.

Based on the strong association between signaling and adhesive sites, several screens were conducted, in which the effects of knockdown or overexpression of particular scaffold or signaling proteins on the formation of matrix adhesions or on cell migration, were examined (e.g., http://www.cellmigration.org/resource/discovery). Using siRNA targeting adhesion/migration-associated molecules, protein/lipid kinases, and phosphateses, Simpson et al. (2008) identified specific genes whose suppression affected the rate of closure on "wounds" introduced into a monolayer of epithelial cells [56]. Among the effective molecules discovered in this screen were kinases and phosphatases, many of which were not known to be *bona fide* adhesome components. Similarly, Winograd-Katz et al. [57] discovered a wide variety of signaling molecules whose knockdown suppressed the nucleation of focal adhesions, their rate of maturation, and the apparent stability of these sites. In

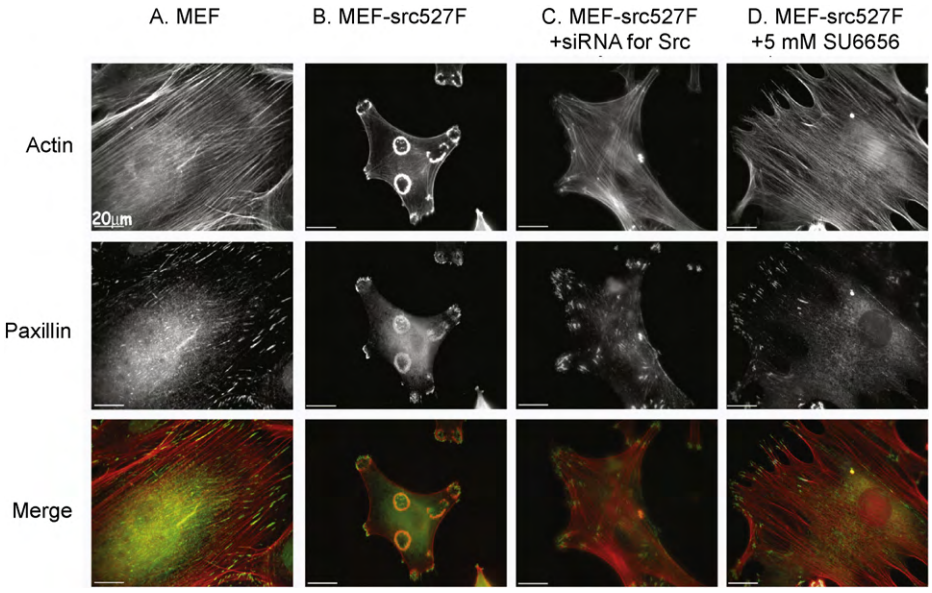

Fig. 2. Effect of deregulated $pp60^{src}$ (srcY527F) on the actin cytoskeleton and focal adhesions (paxillin) of mouse embryonic fibroblasts (A. MEF). Notice that expression of srcY527F (B) induces morphological changes in cell shape (reduced cell spreading), loss of stress fibers and focal adhesions, and formation of posodome-based dynamic rings. Suppression of srcY527F expression by siRNA (C), or inhibition of its enzymatic activity by su6656 (D), lead to partial restoration of stress fibers and focal adhesions. Scale bar = 20 μm (provided by Michal Brunner and Sabina Winograd-Katz).

this study, the prominence of positive hits was quite high (nearly 45% of the 1,080 siRNAs tested displayed a statistically significant effect: 86 of these were most prominent). Notably, different genes affected different parameters of focal adhesions, including their nucleation, distribution within the cells, and rate of maturation.

5. Mass spectrometry-based proteomics for tumor analysis: in search of functional proteomic signatures

Based on the strong involvement of signaling in cell adhesion and migration, it is clear that analysis of the proteins and their modification is essential, to understand adhesive-migratory machineries and their dynamics. One approach for detecting specific modified proteins is based on the use of conformation-specific or phospho-specific antibodies [58,59] but these approaches, powerful as they might be, are not quantitative when performed on a large-scale, do not enable discovery of novel components and their particular modifications and, in most cases, do not enable a global view of the changes. For unbiased analysis, global mass spectrometry (MS)-based proteomic methods must be employed. The classical proteomic approach of 2D gel-electrophoresis [60], despite its broad use, has never enabled the comprehensive analysis of complex samples. This method detects only a few hundreds of the most abundant proteins, and due to the overlap of spots, the method is not quantitative [61,62]. 'Shotgun' proteomics is the current mainstream method used for large-scale proteomics, enabling system-wide analysis and identification of new protein components [63–65]. With this method, proteins are digested into peptides, creating complex mixtures of tens of thousands of peptides that may then be analyzed in several chromatographic runs, followed by on-line electrospray ionization of peptides into the mass spectrometer. Recent developments in mass spectrometry introduced hybrid instruments that combine high resolution and high mass accuracy with high sequencing speed and sensitivity, in the form of the LTQ-FT, LTQ-Orbitrap and the LTQ-Orbitrap Velos (Thermo Fisher Scientific) [66,67]. These instruments have dramatically advanced 'shotgun' proteomics, enabling confident identification of thousands of proteins in complex mixtures.

Recent proteomic developments have also enabled analysis of sub-proteomes, such as post-translationally modified proteins, and protein complexes. For example, several methods were developed for enrichment of phosphopeptides, their identification, and the localization of the phosphosite [68–70]. Similarly, lysine-acetylated peptides can also be enriched, identified and localized [71]. Other methods were developed to analyze protein–protein interactions, and to isolate and identify protein complexes [72]. The use of stable isotopes, whereby several samples are differentially labeled, mixed and analyzed, has further improved quantitative mass spectrometry. The most commonly used quantitative proteomic methods are chemical labeling with iTRAQ (isotope-tagging reagents for relative and absolute quantification) [73,74], and metabolic labeling with SILAC (stable isotope labeling with amino acids in cell culture) [75,76]. New developments in SILAC-based proteomics have broadened its scope to include *in vivo* studies in mice [77], and analyses of human tumor tissue [78].

Mass spectrometry can be applied to analysis of protein extracted from a variety of cancer-related sample types—from cells in culture, animal models, tumor tissue and even from microdissected tissue sections (Fig. 3). After protein digestion, peptides can be fractionated or enriched to find modifications, followed by liquid chromatography and analysis in the mass spectrometer to yield identification of thousands of proteins. To date, no large-scale quantitative proteomic analyses of cancer progression have been published. Nevertheless, several key studies have shown the potential of these methods to analyze tumor samples, and to quantify modifications in cancer- and adhesion-related proteins. One of the first large-scale phospho-proteomic analyses tracked phosphorylation dynamics following stimulation of HeLa cells with EGF, and identified 6,600 phosphorylation sites [69]. Although not directly representing phosphorylation changes along the progression of transformation, this study demonstrated the potential to monitor changes in the phosphorylation of multiple actin modifiers and focal adhesion proteins in response to a pro-tumorigenic

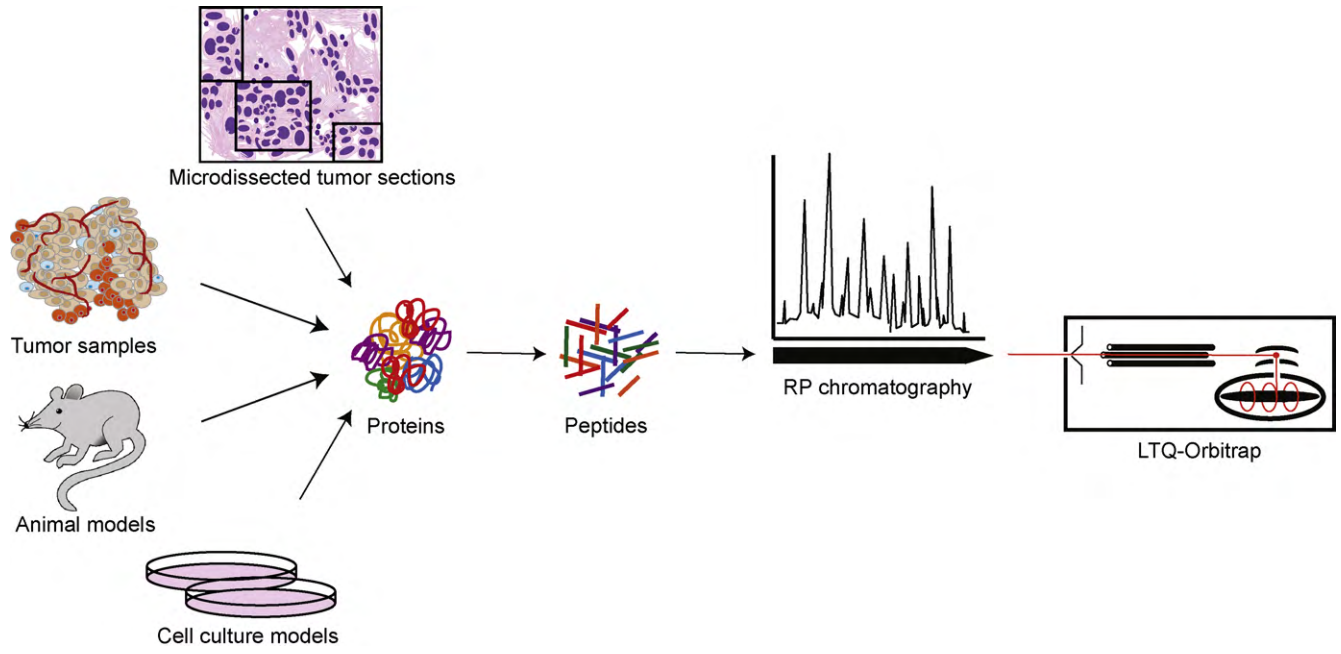

Fig. 3. Workflow of mass spectrometry-based proteomics for the analysis of tumor-associated samples. Proteins are extracted from cells in culture, from tissue of animal models, from tumor biopsies, or from microdissected tumor sections. Proteins are digested, and the resulting peptides are separated by reverse-phase chromatography, followed by electrospray into the mass spectrometer for analysis.

factor. Application of the same phospho-enrichment protocol to melanoma samples from a mouse melanoma model enabled identification of 5,600 phosphorylation sites [79]. In this study, hundreds of phosphosites were detected on focal adhesion, cytoskeletal, and actin-modifying proteins. Another large-scale study examined tyrosine phosphorylation on a large set (>150) of non-small cell lung cancer (NSCLC) tumor samples [80]. This study was mainly performed using low-resolution mass spectrometers, and was only semi-quantitative. The majority of the phosphorylation sites were identified on kinases, and on adhesion and cytoskeletal components. Its authors could further divide the tumors into five groups, one of which was characterized by high levels of focal adhesion kinase and Src activity. Combining this approach with stable isotope labeling and high resolution data would increase confidence in the accuracy of both phosphosite identification and quantification.

6. Future perspectives

With recent developments in mass spectrometry-based proteomics, many opportunities will soon enable a more comprehensive view of the adhesive and migratory machineries. Phospho-proteomic analysis can reveal adhesive changes in the course of transformation, in both animal models and in human tumor samples. Yet the question of whether the adhesive changes would appear throughout the tumor sample, or only within a part of it, remains unanswered. Previous studies that identified a genomic signature from whole tumors imply that the difference in the metastatic potential is apparent throughout the tumor. Nevertheless, it has yet to be investigated whether the entire tissue sample bears the same adhesion-related proteomic and phosphoproteomic signatures. New developments will soon enable detailed analysis of intra-tumoral heterogeneity by microdissection of various areas of tumors or metastatic lesions, and even allow for large-scale retrospective analyses of formalin-fixed tissue blocks. To make full use of such proteomic information for personalized, targeted therapy, another critical step must be developed; namely, integration of quantitative proteomic information with genomic and transcriptomic data, coupled with the development of powerful bioinformatic tools that would enable an accurate understanding of the particular deregulated signaling pathways whose therapeutic targeting appears to be most promising. Such technological developments might open up new avenues for the emergence of new and powerful approaches for personalized cancer diagnostics and therapy.

Conflict of interest statement

None declared.

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