

Invited Review

Network-based drugs and biomarkers

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Abstract

The structure and dynamics of protein signalling networks governs cell decision processes and the formation of tissue boundaries. Complex diseases such as cancer and diabetes are diseases of such networks. Therefore approaches that can give insight into how these networks change during disease progression are crucial for better understanding, detection and intervention. The era of network medicine has begun; however, there are fundamental principles associated with molecular networks that are essential to consider for this field to succeed. Here, we introduce network biology and some of its associated technologies. We then focus on the multivariate nature of cellular networks and how this has implications for biomarker and drug discovery using cancer metastasis as an example.

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Introduction

Molecular, phenotypic and patient tracing of disease initiation and progression before, during and after therapeutic intervention is paramount for the successful treatment of (complex) diseases [1]. In the pursuit of personalized medicine, this puts even more demand on the fine-tuned monitoring of molecular entities that can give insight into the specific state of a diseased patient or tumour. Traditionally, molecular pathology has been performed by analysing well-characterized individual genes, proteins or other molecules identified by targeted studies. Subsequently, this was expanded to systematic interrogation of mRNA levels using gene expression microarrays. This technology has since been upgraded with full-genome, deep- and transcriptome-sequencing platforms that, for example, enables mRNA or non-coding RNA absolute quantitation, detection of gene copy-numbers and genomic sequencing [2]. Similarly, recent advances in mass spectrometry-based analysis now enable detection and quantitation of selected small compounds, proteins and other biomolecules [3]. As a consequence, much hope has been placed in so-called 'biomarker discovery', which aims to identify and assess individual molecular entities (proteins, in particular) to drive molecular pathology towards higher-throughput and clinical applications, using technologies such as serum mass spectrometry. However, these efforts make assumptions that might conflict with our current understanding of how molecular networks and systems become disease driving. Cellular signalling networks

are highly dynamic, in both structure and utilization [4] and in both cell- and tissue-specific contexts [5,6] (Figure 1).

In this review we discuss important aspects of molecular networks that should be considered in the design of biomarker assays. An essential aspect of cellular signalling networks is that at any given time they are in a given state that impacts directly on the cellular response to an environmental stimulation (or cue) [4–8]. This multivariate nature enables cells to respond to multiple input cues in an integrative and quantitative manner [7] (Figure 2). We will argue that failing to describe network states and biological context for molecular biomarkers can have potentially damaging consequences for the patient.

The cue, signal and response model of cell signalling

Any given cell in a physiological (or non-physiological) environment receives numerous simultaneous input cues that must be processed and integrated to determine changes in cellular behaviour, such as migration, proliferation, apoptosis and differentiation [4,7] (Figure 1). Reversible protein modifications are one of the underlying mechanisms for cellular information processing in signalling networks (Figure 2). Protein phosphorylation in particular has proved to be a key media for cellular signal propagation. Through the ability to control protein–protein

Cells process multiple simultaneous *inputs* to yield biological *outputs*
- this process is governed by multivariate signalling networks

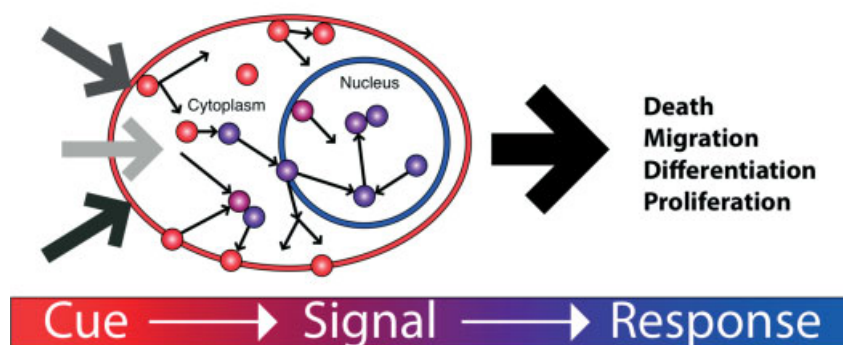


Figure 1. Cue, signal and response model of cell behaviour. Cell behaviour is governed by multivariate network states. To process multiple cues from the environment and/or internal changes, cells integrate information in signalling networks in order to respond to these constantly varying inputs. Thus, to describe and construct predictive models of cell behaviour, quantitative measurements of these network states and simultaneous quantitation of phenotype and multiple cues are essential

**Dynamic protein signalling networks
integratively link genome, proteome and
environmental cues to disease/phenotype**

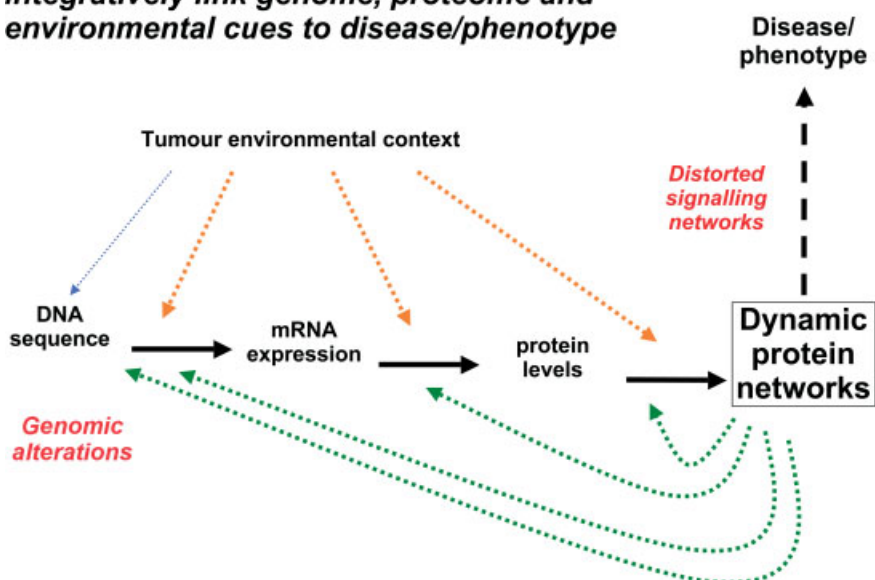


Figure 2. Control hierarchies important for phenotype and disease. The control networks that directly modulate or define phenotype and cell/tissue behaviour are dynamic protein operations, in particular signalling networks. This cellular network layer is in constant change and flux, due to integration of inputs from other cellular control layers, eg. changes to the genome, mRNA expressions or environmental cues. It is the interplay of these integrative cellular information-processing systems that can converge towards diseased states and ultimately can initiate and drive complex regulatory disease such as cancer

interactions, protein–phospholipid interactions, structural complex nucleation, allosteric structural reorganization, enzyme activity and degradation and translocation, phosphorylation impacts on every aspect of cellular biology [4,9]. Cell behaviour and phenotype are directly controlled by dynamic networks of protein interactions in response to external or internal cues/stimuli (Figures 1, 2). Signalling networks, such as those regulated by kinases and phospho-binding domains (eg the SH2 domains) are essential for the integrative response a cell must make at any given point in time to extra- or intracellular cues [4–11].

Integrative network biology

While studying molecular interactions has been a research focus for many years and has provided much insight into biology, the new age has come for integrative network biology. The aim with integrative network biology is to provide models of cellular networks based on integration of a large and heterogeneous dataset, eg originating from proteomics and high-throughput functional genomics studies [4,10–14]. We, and others, have shown that the network models pertaining to cellular signalling can also be utilized for fine-grained prediction of human disease

[15–18]. Through quantitative systematic measurements of these networks and integration of multiple types of data, it is now possible to define dynamic changes to these networks and perform computational modelling of the networks that enables predictions to be made about specific responses such systems should elicit [4,7,8,13]. In particular, we have recently shown that, integrating systems genetics data [19] with phospho-proteomics data and computational models of cellular kinase specificity [11], we could derive an integrative network model of JNK regulation in the fruit fly [12]. This network can now serve as a framework for future targeted proteomics studies [20,21] in cancer cells (*in vitro* and *in vivo*) to define how the network evolves and changes during cancer progression. We are now actively pursuing the integration of data from deep sequencing, functional genomics and extreme-throughput microscopy and mass spectrometry to perform network medicine and systems-level modelling of cancer metastasis [4,18].

A potential concern with systems-level data and derived models relates to the fact that these datasets most often are incomplete and often associated with significant errors. There are numerous ways to address this; here we will simply make the point that one advantage of integrating data from different sources is that the amount of false-positive observations will be reduced, as the independent observations from the various data sources contribute to the overall probability of a network edge (or similar) being true. At the modelling level these issues must always be dealt with in two independent ways: first, any model should be experimentally validated and predictive of phenotype or cellular behaviour; second, a computational model should always be ‘benchmarked’ through ‘*in silico*’ validation. The latter requires golden datasets and is not always possible, but both approaches can significantly enhance the power of a model [10]. A related issue is that of so-called ‘biological noise’: eg not all phosphorylation events or protein–protein interactions in a cell necessarily result in phenotypic change [19]; thus, how does integrative network biology assist in analysing such events? As above, the integration of, for example, systems genetic data with physical interaction data will very likely unravel such cases, as likely only one type of association will be observed. However, many such interactions might actually have a function although they do not lead to an ‘observable’ cellular or higher phenotype, and we stress that one needs to be careful about excluding data based on hypothetical noise models. Finally, because integration of data relies on quantitative measurements, it is key that samples (such as a proteomics samples) are analysed in biological and technical triplicates.

Multivariate nature of cellular networks

A fundamental and essential aspect to cellular networks is that they are multivariate (Figure 1). As cells

must be able to receive and respond to many hundreds or even thousands of concurrent external and internal cues, these are processed by signalling networks in an integrative fashion. In other words, the cell in a sense computes its response by taking into account not one but many inputs, and in particular it takes into account the current state of its signalling networks. This aspect of cell signalling (and biological networks) was shown in a seminal paper from the laboratories of Lauffenburger and Yaffe [7]. This paper showed that while the activating phosphorylation of JNK kinase had been previously debated to be either anti- or pro-apoptotic, it can indeed be both, as this entirely depends on the state of the network when it receives either a TNF or EGF cue. A systems model for JNK signalling was established and it was shown that the model provided stronger predictive power when the multivariate analysis was used as foundation. In a similar study, the MIT labs showed how common information processing mediates cell-specific responses to stimuli [5]. Therefore, to fully understand a cellular response to a perturbation (such as to a drug), it is essential to analyse this in the context of the cell’s multivariate state [5–7]. This observation does not just have implications for biomarker discovery, as discussed below, but also for how analysis of biological systems should be performed. For example, whereas treating a cell line with a ligand and performing global phospho-proteomics might be informative, it is intrinsically a single-dimensional approach that ignores the multivariate nature of the system under study. A titration study where both ligand and receptor are utilized would not only be more informative but also would enable predictive models to be created.

The multivariate nature of cellular networks is also important for genetic studies; whereas the knockdown or deletion of a single gene can provide insight into the relationship between the gene and the phenotype, this approach often misses the minor contributions of many genes to a phenotype. Image-based assays that provide high-dimensional read-outs can be used to classify many genes simultaneously into distinct functional categories. For example, we have previously demonstrated that quantitative measurement of kinase activity can be used in tandem with systematic large-scale combination RNAi knockdown and proteomics to unravel the regulatory networks of JNK kinase [12]. Similarly, Bakal *et al* used large-scale quantitative morphological signatures to classify hundreds of genes into local groups of genes associated for specific aspects of morphology, eg protrusions [20]. The morphology of cells has proved to be a powerful and very sensitive read-out of the involvement of a gene or genetic relationship in biological processes, likely due to the fact that most of these influence the cytoskeleton [20]. In the future, such assays can be combined with mutations or over-expressed genes identified from deep-sequencing efforts to further shine light on complex phenotypes and how they relate to genotype across cell lines.

Future biomarkers — network signatures

The principle of multivariate signalling integration provides a framework for future systems biology-driven studies because it emphasizes the requirement for multi-drug/multi-stimulus studies. However, most current biomarker discovery is not taking this principle into consideration, which could have potentially dangerous implications. For example, should the phosphorylation site on JNK be used as a biomarker, it could have damaging consequences for the patient, as the context-dependent or multivariate nature of the regulation of this site would not be immediately apparent [13,22,23]. It could very well be that in a given patient or tumour context this site would be anti-apoptotic at a specific stage of disease progression. Thus, using this site as a primary biomarker to determine patient treatment could result in responses to therapeutic agents opposite to those desired. Therefore, we would suggest that caution be taken in using individual nodes or modulations (eg a phosphorylation site) outside the context of a network as indicative or predictive markers. Instead we encourage that the network models themselves should be deployed directly as markers. As we are moving towards multi-dimensional and integrative network models of cell behaviour, we expect that such models will be useful not just for determining network structures that can be targeted but also as predictive markers of disease emergence or progression [4,22–25]. This will require robust quantitative network assays to become more widespread and user-friendly [21]. As mass-spectrometry proteomics continues to be a challenging technology to master, it is likely that network models established by quantitative mass spectrometry in the first instance will be easiest to implement in the clinic as affinity-based microarrays [4]. However, this will also require the development of new computational algorithms to analyse such data [4,8,13].

Networks as drug targets

We previously introduced the concept of network medicine [18] by describing how protein-signalling networks, their structure and dynamics are not only associated with and responsible for driving complex diseases such as cancers but indeed might also be powerful drug targets. Recently, two groundbreaking studies have partly validated this suggestion. In the first study, Forest White and Paul Huang utilized quantitative mass spectrometry to model *EGFR–Met* signalling networks and could suggest potential combination-treatment options for glioblastoma [22]. In a more recent paper, Birgit Schorble *et al* used computational network models to identify *ErbB3* as a therapeutic target, despite it not being an over-expressed or mutated oncogene in most cancers [23]. In both studies, quantitative network analysis resulted

in some of the fastest-ever clinical trials for new treatments.

The treatment of HIV provides a powerful example of how targeting the network can be used to control disease. The reason for the success of highly active antiretroviral therapy (HAART) is that several antiviral drugs (typically three or four) are taken in combination. Side-effects and viral resistance are limited by following a complex and specific treatment regime. Intensive study of the HIV retrovirus life cycle has led to the identification of critical protein networks and enabled the development of drugs that target these networks. HAART has prolonged the life and improved the quality of life for millions of patients globally. This therapeutic approach clearly demonstrates the clinical benefit of targeting the network instead of single molecular targets.

We predict that in future studies, network models will not be limited to those of signalling molecules but will also include integrative patterns/signatures from disease–disease correlations and social networks (Figure 3). Recent studies of the relationships between diseases have suggested that drug side-effects can be used to associate diseases [26] or that this can be done through analysis of changes to protein expression [15,27,28]. In addition to this, we have recently shown that multiple diseases converge in a nexus of networks when considering evolutionary conserved regulatory networks [16,17]. We propose that such networks might be crucial multi-node drug targets [29] in multiple diseases and new trials with combination drugs should be encouraged and attempted.

Implications for cancer metastasis research

Here, we use metastasis as an example of how network biology may be applied to tackle a complex and important challenge in the treatment of cancer. Metastasis, the spread of cancer through the body, is a multi-step process consisting of a series of discrete biological processes [30]. Each step is regulated by a complex interplay between cell–cell and cell–matrix interactions and is strongly influenced by microenvironmental factors [31]. As tumours grow, space, nutrients and oxygen become limiting and the cancer cells acquire the ability to invade into neighbouring tissues and spread through the body to seek out new terrain [32].

There is a propensity for tumours to seed in particular organs, which cannot be fully explained by blood flow. Stephen Paget put forth the ‘seed and soil’ theory in 1889 to explain the patterns of metastases [33]. Paget described tumour cells as ‘seeds’ and the host environment as the ‘soil’, and proposed that their interaction determines metastatic outcome. Hence, without the correct seed–soil interactions, metastasis to this site will not take place.

Metastatic progression is thus completely dependent upon cellular context [34]. It is the dynamic

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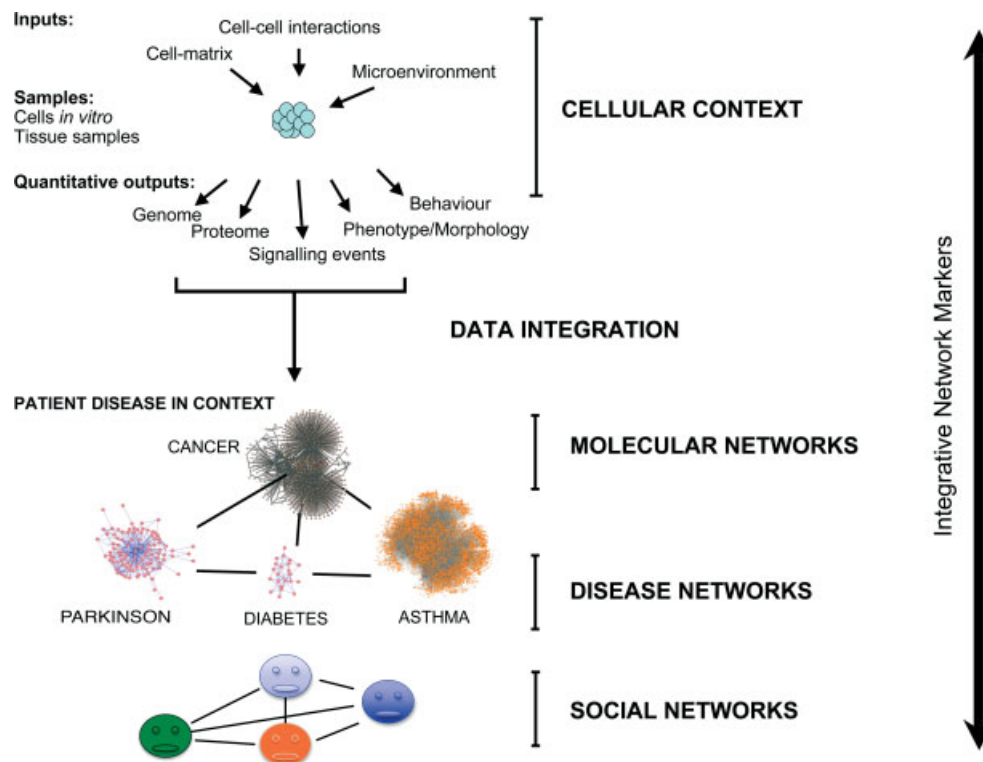


Figure 3. Integrative network biology-based biomarkers. Through integration of quantitative molecular and behavioural data collected from cells grown in conditions that mimic cellular contexts important to disease (cell interactions and micro-environmental factors), and quantitative data acquired from *in vivo* models and human patient data, we can define networks associated with disease progression and identify network-based biomarkers, thus understanding patient disease in context. The goal of network medicine and network-based biomarkers is to correlate network dynamics and states to phenotype and patient disease data [18]. The knowledge of social networks might also be useful [45]. Based on integrative network models, potential multi-node signatures can be defined as markers and/or drug targets. An iterative process can be undertaken to test these networks in tumours and in combination drug trials. Targeting protein–protein interactions and non-obvious nodes [23,47] in the network will be increasingly important. Disease networks and social networks can be compared

interactions between tumour cells and their microenvironment that determine the metastatic ability of the tumour. This is clearly demonstrated when one alters the cellular environment to enable invasion and metastatic dissemination. For example, hypoxia (low oxygen) is known to be a potent driving force for metastatic progression, and there is a plethora of experimental studies to show that oxygen deprivation of cancer cells enhances their invasive and metastatic potential [35–38]. These studies have led to the identification of promising anti-metastatic targets, such as lysyl oxidase (LOX) [37,38]. Cellular context also determines the response of cancer cells to drug treatment, as recently demonstrated by 3D breast cancer cell culture studies using Her2-targeting agents [39].

Defining the molecular networks for the metastatic process presents a major challenge, due to the multitude of interactions and influencing factors. However, without an understanding of these networks, we will never be able to identify effective therapeutic strategies to reduce the number of cancer patient deaths due to metastases (currently > 90%). In order to tackle the complex process of metastasis, it is important to isolate key steps in progression and focus on defining the molecular signatures relating to the specific processes involved. This can be done through the acquisition of

cell behaviour and molecular data, and has classically resulted in the generation of cross-comparative lists that have provided insight into tumour progression. For example, Kenny *et al* demonstrated the strength of performing gene expression and morphology studies in parallel to investigate breast cancer cell invasion [40]. In this new era of integrative network biology, we can now attempt to construct predictive models of cell behaviour and disease progression through algorithmic integration of systems-level quantitative cell behaviour and molecular data [4].

The influence of key interactions can be measured *in vitro* using cells by mimicking ‘real-life’ situations and providing the conditions that create the context conducive for metastatic progression, eg by changing the microenvironment to which these cells are exposed or performing cell mixing experiments to study cell–cell interactions. Studies may also be performed *in vivo* using whole organisms, such as mouse models of cancer. Intravital imaging can be employed to investigate cell–cell/cell–matrix interactions using differentially labelled tumour/stromal cells and matrix components [41]. Current technologies available enable the quantitation of cell behaviour both *in vitro* and *in vivo*. Cell migration, invasion and phenotype can be quantified in a high-throughput manner

in vitro, using commercially available kits or confocal microscopy [42], and can be assessed *in vivo* through intravital imaging and analysis of pathology [43]. Metastatic growth can be assessed *in vivo* through non-invasive imaging of labelled tumour cells (such as those expressing luciferase using the IVIS system), ultrasound, PET and CT scanning. In addition, some of these techniques enable quantification of aspects of the tumour microenvironment, eg tumour oxygenation and metabolism. PET, CT and MRI scans from human cancer patients provide vital clinically relevant data, and analysis of the pathology of human patient samples enables the quantitation of certain cellular phenotypes and behaviour [44].

Quantitative analysis of molecular entities, such as gene expression studies, deep sequencing and mass spectrometry proteomics, can be used to understand the networks associated with metastatic progression. Molecular, phenotypic and behavioural data can be collected together in a quantitative systematic manner and integrated computationally to define the networks for the specific steps in the metastatic process. These networks can be perturbed using genetic, drug or other inhibitory methods to confirm predictive ability. Through the integration of data collected from cells *in vitro*, tissue samples from mouse models *in vivo* and from human cancer patients, networks can be defined, ultimately helping us to understand patient disease in context (Figure 3). These studies will enable us to understand where promising anti-metastatic targets, such as LOX, sit in the network and thus how best to deliver and time therapeutic agents that will disrupt the network. Without information on the network, single-target agents will likely result in network compensation and drug resistance, and efficacy in only a small percentage of patients, as clearly demonstrated by the majority of clinical trials to date.

Perspectives

In this way, network biology studies will enable us to address complex biological processes with clinical implications [18,45]. Network biology is maturing and will revolutionize our understanding of metastasis and our approach to drug design, ultimately reducing patient suffering and death. The focus of this new approach to cancer research is to elucidate at a systems level how the dynamic behaviour and function of signalling networks contributes to the process of cancer progression. To reach this goal, we and others have shown that computational modelling and quantitative measurements must be intimately intertwined [14]. The reason for this is that network mechanisms (eg feedback loops and evolutionary conservation) can only be discovered through integrative studies, and because these control cell behaviour they are inherently powerful drug targets [16–18,46,47]. It is therefore essential that biomarker definitions progress from individual nodes to network markers in order to

identify robust, predictive and quantitative markers or signatures for complex diseases.

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Teaching materials

PowerPoint slides of the figures from this review are supplied as supporting information in the online version of this article.

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