Gene regulatory networks: a new conceptual framework to analyse breast cancer behaviour

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Received 13 May 2010; revised 3 August 2010; accepted 3 August 2010

The study of complex systems has clearly evidenced that a few overall behavioural properties cannot be inferred from the properties of their single parts and are rather determined by their architecture. Such an approach has been recently proposed in biology to understand genome functioning and in oncology to endeavour a more consistent explanation of the variegated cancer behaviours. In the present perspective, we summarise the basic concepts of the proposed global approach and then we reconsider, in this new context, tumour dormancy and primary tumour removal effects, which recently emerged as critical points for breast cancer understanding.

Key words: breast cancer, cancer attractors, gene regulatory networks, recurrence dynamics, tumour dormancy

introduction

Breast cancer is the second most commonly diagnosed cancer in the world [1] and is characterised by a high heterogeneity in terms of risk of developing the disease [2], histological morphology, biological traits and clinical behaviour. Breast cancer is one of the most investigated areas of oncology, with >100 000 published papers during the past decade, reporting studies on the disease at molecular, cellular, tissue and clinical levels. In spite of this tremendous effort, the disease is still an ambiguous neoplasm. The main difficulty in developing an exhaustive picture of breast cancer (and of other neoplasms, as well) results from the failure of the current conceptual framework to link knowledge acquired from these different yet complementary areas of investigation.

methods

The still prevailing multistep progression model of tumorigenesis, assuming random mutations and/or epigenetic DNA and chromatin alterations driving selectively favourable traits of the neoplastic cell, appears to be inadequate to suitably explain the process, in spite of several ad hoc added hypotheses aimed to address its drawbacks [3, 4]. Indeed, the number of oncogenes and tumour suppressor genes, putatively involved in the process of oncogenesis, goes on increasing and covering a wide variety of cellular functions without providing, nonetheless, a consistent picture of the process. In particular, the multistep progression model cannot easily explain the sophisticated switch of entire intracellular machineries that underlies the acquisition of the metastatic mesenchymal phenotype without assuming an extremely strong and oriented selection pressure into the tissue, which is neither plausible nor supported by observations.

Furthermore, at a philosophical level, the methodological mindset underlying most experimental and clinical investigations is a reductionist approach to cell functioning. Indeed, in the presence of new technologies providing a huge amount of biomolecular details, the current approach seeks comprehensive descriptions of all constituent parts, with the implicit notion that extension of the knowledge about the individual pathways will suffice for understanding how the whole living organism works. Yet, a more global approach adopting the principles utilised in the study of complex systems, which acknowledges that an understanding of a higher order quality may arise from the collective action of the individual parts, has been recently proposed [5]. Although such a new network-based approach is still quite abstract, its general principles may provide a new intellectual framework that can hopefully guide the integration of existing data and new paradigm-challenging observations.

In the present review, we will summarise the basic concepts of the proposed network-based approach and we will use it to reconsider two hot issues, namely tumour dormancy and primary tumour removal effects.

gene regulatory networks

Molecular biology has been greatly permeated, during its development, by the concept that a particular cell phenotype is the result of a linear chain of causal relationships among regulatory pathways. In particular, transcription factors are considered key intrinsic regulators of cell fate through direct control of the expression of given genes. However, this conceptual framework is challenged by the findings that there are significant cross-talks among pathways at almost every level of the signalling cascade, that the same transcription factor may control the expression of up to hundred genes and that, conversely, a single gene may be controlled by several regulatory molecules including the recently discovered class of
microRNA [6]. For example, a transcription factor such as Ras, Myc or HIF-1 can have multiple disparate, if not opposite, outcomes (cell proliferation rather than cell differentiation/quiescence or apoptosis), depending on the cellular context [7]. The advent of genomic and proteomic technologies has further emphasised the idea that molecular pathways are just parts of a complex network and arisen the need for a new conceptual approach. Furthermore, the hypothesis that the genome may act as a highly integrated device is suggested by the evidence that genes and chromosomes are non-randomly localised within the nucleus. Several gene loci are located in the peripheral area of the nucleus when inactive, whereas they are repositioned centrally upon activation [8, 9]. At least in some situations, the non-random organisation of the genome and the coordinate gene regulation are correlated [10]. All these considerations strongly suggest to consider molecular pathways as partial components of a higher order structure, i.e. a gene regulatory network (GRN).

The notion of network has proven to be a powerful tool for the analysis of several different systems, such as social [11] and ecological [12] systems and the World Wide Web [13]. In particular, scale-free networks (Figure 1), where a small but significant fraction of hub elements are highly connected, whereas the majority of elements are poorly connected, were found to be a common architecture of large intracellular networks [14, 15]. Living cells are able to conserve cell functionality under random perturbations from the microenvironment and also to undergo specific adaptive changes in certain situations. These properties may just emerge from the network architecture. In particular, scale-free networks are intrinsically stable against variations of their internal parameters with enough adaptability due to the sensitivity of the highly connected hub elements to perturbations [15]. Furthermore, there is evidence that GRNs operate close to a crucial condition for living organisms, namely in a condition of valuable compromise between stability and adaptability [16–18]. They are neither too stiff to maintain the actual state in spite of major perturbations nor too flexible to display a chaotic behaviour in response to every minimal perturbation.

**attractors and basins of attraction in GRNs**

Let us examine a few general properties of the GRN ensuing from the fact that each gene expression is regulated by the expression of other genes and by additional epigenetic factors. Accordingly, genes cannot change their activity freely because of the multiple regulatory interactions they undergo, which introduce constraints to the possible gene expressions. Therefore, only given gene expression profiles (i.e. cell phenotypes) are permitted (i.e. are stable). The GRN status, however, is not inalterable, as it faces several disturbing conditions that tend to shift the initial network state. Two possible outcomes may arise. The first one implies that the GRN may display small transitory changes in gene expressions and then return to the initial state. This means that the system behaves as attracted by the initial configuration, at least while it remains within a ‘basin of attraction’, corresponding to all possible configurations from which the system comes back to the starting point. By contrast, the second possibility implies that under the perturbation spur, the GRN may undergo an avalanche of significant changes in gene expressions and, following a transient phase, it may reach a new ‘attractor’. The novel self-stabilising discrete state, determined by new mutual interactions of the network components, will result in a different phenotype with a definite gene expression profile. An intuitive representation of these behaviours that makes use of the stability state analysis in a gravitational field is reported in Figure 2. The provocative idea that the genome is a self-stabilising entity has been recently supported by an analysis of the entropy changes during differentiation of haematopoietic progenitors to erythroid and neutrophil cell types [19]. During an initial transitory phase the entropy value increases, whereas,

**Figure 1.** Representative structure of a simple scale-free network (A). In such a network, a few highly connected nodes, or hubs (red circles), play an important role in keeping the whole network together. The network maintains its basic structural organisation when the number of connected nodes increases, while achieving high grade of complexity (B).
The notions of attractor and basin of attraction are illustrated by elementary mechanical knowledge. In a two-dimensional representation, a marble on a shaped profile in the gravitational field may keep still in a few equilibrium points (A, I, M) that are not equivalent, however. Points A1 and A2 correspond to stable equilibrium (equilibrium attractors), points I1 and I2 correspond to unstable equilibrium and point M corresponds to a meta-stable equilibrium. Minimal external perturbations will move the marble away from points I1 and I2, while the marble will generally return to its attractor within its basin of attraction (A1) even under the action of more important perturbations. Major energy transfers will be able to induce a marble jumping out of its basin of attraction (A2) and being trapped by another attractor. The meta-stable state (M) is a weak attractor from which the marble may relatively easily reach a neighbouring stable attractor.

Upon cell commitment, it gradually declines concurrently to the differentiate cell types reaching a higher ordered state than that of their progenitors.

According to the GRN approach, the global architecture of the network creates a landscape of mutually exclusive attractors surrounded by their basin of attraction and separated by areas of unstable states, an intuitive representation of which is reported in Figure 3. Attractors are corresponding to distinct cell phenotypes (the word is used with a broad meaning of any observable characteristic of the cell such as morphology, biochemical or physiological properties, or behaviour, e.g. proliferation, quiescence, differentiated state), while unstable states can only transiently be occupied. In such a context, cell fate may be imagined as a path through a land with valleys and crests or plateaux separating them. In this attractor landscape, cells with a given phenotype usually reside in the corresponding valley, within which they can limitedly move without significant phenotypic changes. However, some factors may drive the cell out from the valley in an unstable region, from where it may reach another more or less neighbouring attractor, corresponding to a new phenotype. This model explains why cell-type-specific genome-wide expression profiles, defined by the value of thousands of variables, are so reliably established during differentiation, as if orchestrated by an invisible hand. In particular, this model has recently been proposed to explain the regulation of erythroid and myeloid differentiation from a haematopoietic progenitor and even for the analysis of embryonic stem cell systems [20].

It should be noted that in the GRN approach cell fates do not result from ‘instructive’ processes due to signals inducing up- or down-regulation of particular genes in order to produce a particular behaviour. On the contrary, regulatory signals destabilise the present status inducing a ‘selection’ of a further given attractor among a finite number of others. Indeed, attractors are ‘pre-existing’ as potential stable states of the GRN. According to this view, the new phenotype is neither necessarily related to the old one nor the obligatory result of a specific regulatory pathway, although the attractor landscape makes certain transitions easier than others. This model gives a reasonable explanation of the observed finding that even non-specific signals (e.g. small chemicals like dimethyl sulfoxide or mechanical distortion) may produce distinct outcomes, such as differentiation, proliferation and quiescence, each of them involving the differential expression of thousands of genes [21, 22]. A remarkable support to the model comes from a recent report [23] providing evidence that activation of endothelial cells is primarily controlled by breast epithelial cell polarity and tissue architecture. Thus, the activation of the angiogenic switch would not simply be caused by genetic changes within cells but rather could be linked to how cells sense their reciprocal architecture and how they interact with the microenvironment. Also, the GRN approach assumes that some early attractors, including embryonic developmental programs, persist in the attractor landscape of the cells residing in adult-stage attractors. This feature provides a reasonable explanation to a few recent reports on reprogramming of differentiated cells to induced pluripotent stem (iPS) cells under the action of a small number of inducing genes [24, 25]. The few inducing factors, indeed, are not required to re-instruct the cell to reach the iPS status but only to destabilise the present status and to make accessible the iPS attractor.

Finally, it should be emphasised that the attractor landscape is more than a metaphor: the mental image of the attractor landscape is submitted to the basic principles of system
dynamics, which describe how elements of a complex system interact with each other to produce collective behaviour, and has a formal basis that may support computational modelling.

cancer attractors

All the above concepts have implications for a new integrative understanding of tumour formation and metastatic progression [26]. Terminally differentiated normal cells result from a number of orderly phenotypic transformations starting from stem cells. Stem cells require both stability, to support self-renewal and maintenance of uncommitted state, and flexibility in the cell fate choice, to permit cell-type diversification and differentiation. On the contrary, terminally differentiated cells should be in a stable and irreversible state under homeostatic or physiological conditions. In the conceptual frame of GRN dynamics, this process can be conceptualised by assuming that stem cells occupy considerably less stable attractors than terminally differentiate cells and that during differentiation, definite signals drive a given path from the former to the latter state. During all phases of the differentiation process, however, a rewiring of the network architecture may reshape the attractor landscape, hence allowing the cell to acquire new, self-stabilising atypical gene expression programs. Cancer would correspond to such emerging cell states, i.e. to 'cancer attractors', namely unused attractors with a viable proliferative phenotype that become accessible as a consequence of genetic- and epigenetic-related changes of the ordinary landscape. This model can easily account for many aspects of both tumorigenic processes and cancer behaviours [27]. In particular, the characteristic traits of tumour phenotype (proliferation, migration, invasion, angiogenesis etc.) would not be de novo inventions but derived from early self-organising attractors, inaccessible in the normal mature tissue and made re-accessible because of the altered attractor landscape. For example, the metastasis-related epithelial to mesenchymal transition and the opposite mesenchymal to epithelial transition would correspond to switching between pre-existing attractor states that may be induced by a number of microenvironmental signals [28].

The concept that most of the tumour hallmarks could be considered normal-like yet decontextualised processes is supported by experimental findings indicating that normal cells may display cancer-like behaviour and, conversely, cancer cells may regain normal cell traits. Indeed, it has been observed that untransformed mouse mammary cells injected into the bloodstream may establish residence in the lung for extended time, yet conserving their ability of supporting full mammary development when retrieved, thus indicating that normal cell traits are sufficient for supporting a substantial portion of the metastatic cascade [29]. Of note, if they are engineered to express inducible oncogenic transgenes, they may assume malignant growth in the lungs upon immediate or delayed oncogene induction, thus supporting the concept that the metastatic ability can be, at least in part, disconnected from the neoplastic phenotype. Conversely, it has been observed that some carcinoma cells can revert to normal phenotype and behaviour when placed in appropriate tissue milieu [30, 31] and, in the case of teratocarcinoma cells transferred into blastocyst [32, 33], even preserve pluripotency and contribute to multiple normal tissues, in spite of their still persistent transformed genetic pattern.

tumour dormancy

Let us reconsider tumour dormancy in the light of the proposed GRN approach. At least two different types of tumour dormancy may occur during the complex metastatic process of cancers [34]. Cellular dormancy labels the condition of single cells showing lack of proliferation or apoptosis that may remain quiescent in different tissues [35, 36]. Putatively dormant single cells may be detected in the bone marrow and circulatory system of breast cancer patients [37, 38]. Micrometastatic dormancy identifies a phase where, concomitant to the failure in recruiting a blood supply, balanced apoptosis and proliferation result in no growth [39, 40]. The occurrence of these two dormant phases during the metastatic process is well established in animal models, whereas the inherent difficulty of finding, imaging and analysing dormant tumour cells in patients has till now prevented from directly studying them in humans [41].

A remarkable model of cellular dormancy suggests that the mechanism may result from tumour cell–microenvironment cross-talks [42]. In one set of studies, the induction of dormancy following extravasation has been found associated to the modulation of extracellular signal-regulated kinase (ERK)/p38 signalling ratio, via a β-1 integrin pathway: high ERK/p38 signalling ratio is apparently correlated to proliferation, whereas the opposite occurs in cellular dormancy [43, 44]. In other experimental settings (both in animals and in reconstituted basement membrane assays), the β-1 integrin pathway proved to be involved in the cellular dormancy [45, 46]. Finally, it has been recently demonstrated, by direct inspection, that single-tumour cells may reside in metastasis-free organs of mice harbouring growing metastases in other organs and that they may resume the same proliferative and metastatic capability as their ancestors after rescue and reseeding [36]. Therefore, the fate of disseminated tumour cells would be fundamentally determined by non-permissive and conducive microenvironmental signals, the nature and the source of which is still poorly understood.

Mechanisms underlying micrometastatic dormancy basically involve angiogenesis, an apparently irreversible switch [47]. Following early reports about the role of neovascularisation on tumour growth rate [48], spontaneous transitions from avascular tumour foci to vascularised growing metastases have been identified and conceptualised as ‘angiogenic switch’ [49]. Later on, several reports from animal models provided additional basic details. Human tumours contain tumour cell subpopulations with different angiogenic potential, ranging from complete inability to high angiogenic competence [50]. The steady state of non-angiogenic micrometastases results from the balance between proliferating cells and cells undergoing apoptosis [51, 52]. While angiogenic tumours expand rapidly after implantation, dormant non-angiogenic tumours remain initially microscopic, switch to angiogenic phenotype following a predictable cancer-specific time and grow as rapidly as angiogenic fast-growing tumours [53].
Normal stroma can have a suppressive role, whereas, by contrast, activated stroma (i.e. tumour stroma or wound-healing stroma) may have an enhancing role [54]. Altogether, these findings support a model of micrometastatic dormancy where the threshold separating tumour growth and tumour dormancy depends on the local balance of angiogenesis inhibitors and promoters.

Looking at tumour dormancy from the GRN viewpoint, the question is as follows: what signal(s) may drive the cell inside or outside the dormancy basins of attraction? In a transcriptional analysis carried out to distinguish dormant versus fast-growing tumours, an extensive gene expression rearrangement with activation and regulation of several pathways not linked to dormancy, at least hitherto, was observed [55]. Therefore, the angiogenic switch that drives the end of micrometastasis dormancy might not result from a change in the balance of angiogenesis inhibitors and promoters only, as till now believed. Other not necessarily specific factors could be involved, as it is even suggested by the above reported role of cell polarity and tissue architecture on endothelial cell activation [23]. Furthermore, a crucial question remains still unanswered: is the transition primarily depending on the interactions between cells and microenvironment that drive regulatory adjustments, or rather is it a cell-centred process towards a new self-stabilising configuration? The predictability of the cancer-specific times to angiogenic switch observed in a few animal models [47, 53] apparently supports the latter process, whereas a number of other experimental findings suggest a role for extracellular factors [51, 54]. Transitions from and to dormancy are crucial points in the clinical history of patients (see below) and presumably important targets for therapy. Future investigations should clarify the transition mechanisms.

**primary tumour surgical removal effects**

For many years, tumour growth was considered desperately continuous and relentless. This concept, however, is not supported by the analysis of local recurrence dynamics [56] that, in agreement with the bimodal risk pattern for distant relapse and/or mortality [57], suggests the occurrence of phases of growth interruption. The bimodal risk pattern has been extensively validated in 12 independent databases and also is identifiable in at least eight other studies [58]. It is similar in all analysed subsets [by tumour size, nodal status, estrogen receptor (ER) content, menopausal status] of patients [59, 60] and, of note, it does not depend on the site of metastasis [61]. Within the common recurrence rhythm, the level of recurrence risk at a given time is apparently related to both tumour factors (e.g. tumour size, nodal status, ER status) and host factors (e.g. menopausal status, age).

The reported clinical findings suggest and support a paradigm of metastasis development assuming both cellular and micrometastatic tumour dormancy, with orderly transitions through these two quiescent states, eventually resulting in clinically evident tumour growth, and, moreover, a transient phase of acceleration of metastatic growth following surgical excision of the primary tumour [59, 62, 63]. Such a model was supported by a computer simulation, as well [64]. The main concept underlying the growth enhancing effect of primary tumour surgical removal is the occurrence of a homeostatic equilibrium relating primary tumour and its metastases, which should be broken down by surgery. Although tumour homeostasis was recognised and studied in animals for over a century [63], it was not well recognised and accepted by most clinicians.

Tumour homeostasis may be well understood in a network approach. The concept of stable states emerging from interacting elements may be extended to explain architectural features at the tissue level, i.e. when interacting elements are whole cells and not subcellular components. Interactions, embodied by molecular or mechanical cell–cell communications, would accordingly generate tissue-level attractors, ultimately sustaining tissue and organ homeostasis [26]. Experimental evidence of such interactions was provided by studies carried out in three-dimensional cell culture systems consisting of collagen I gel [65], where myoepithelial cells derived from normal mammary gland of mice were co-cultured with luminal epithelial cells. Cells ‘spontaneously’ organised into acini-like structures where double-layered configurations very similar to true acini traits were observable, displaying an external ‘basal’ membrane, a myoepithelial cell layer surrounding a luminal layer and an inner lumen. In an attractor landscape, tissues and organs, which are usually in their typical stable state, may move to a different attractor involving new traits, when an appropriate perturbing stimulus takes action, as in the case of normal liver that may undergo considerable organ rearrangements when changes in some significant parameter occur. Neoplastic tissue would not escape this general behaviour and a main perturbation like primary tumour surgical removal may imply a rewiring of the neoplastic tissue network architecture, resulting in a dormant metastasis switch to a growing phase [59, 62]. The bimodal pattern of clinical recurrence is very likely an indicator of this phenomenon, which generates a time frame for recurrences.

It is within this common time cadence that specific clinically relevant host–tumour interactions emerge in the form of the different risk levels that we associate to particular tumour and host traits. Hence, both the subclinical metastatic pattern existing at the time of diagnosis and the susceptibility of dormant single cells or micrometastases to awaken concomitantly to the primary tumour removal have main prognostic value. Unravelling factors and mechanisms underlying these two features may have significant clinical consequence and should be considered a research priority.

**conclusions**

In conclusion, the GRN dynamics provides a conceptual framework allowing the integration of several segments of the present knowledge about breast cancer. In particular, it provides a theoretical support to the breast cancer model empirically devised to explain clinical findings and based on the concepts of tumour homeostasis, tumour dormancy and surgery-related enhancement of metastasis development. It is not substitutive of the current approach of most investigations stressing the importance of detailed pathway analysis. At
present, it is usually assumed that genes differently expressed in normal and in neoplastic cells are cancer-related genes that might provide information about the intricate mechanisms causing tumour performance. The new conceptual framework emphasises the limitations of such an approach, even if it does not disregard the importance of pathway analyses. Indeed, it does not consider the sum of all the pathways a ‘complicated’ network, but rather it puts emphasis upon the ‘emergent’ information harboured by such a ‘complex’ network as an entity.

The GRN approach inspires radical changes in both the intellectual attitude of researchers and the focal points of researches. Assuming that tumours make use of normal-like processes, it is crucial to distinguish whether the observed molecular changes associated to a given tumour behaviour are part of the tumour-inducing mechanism or reflect recovered standard traits of the self-stabilising process. Under this light, more attention should be paid to the relationships between cells than to the cell itself, a notion that is also in agreement with definite experimental and clinical findings [63, 66, 67]. From a clinical point of view, revealing details of the processes underlying dormancy induction and dormancy interruption may be crucial for a significant improvement in breast cancer treatment and cure. It is evident, nevertheless, that the complementary views rising from detailed pathway analyses at the level of nominal genes and proteins and from the search for additional information provided by networks as entities are both indispensable.

disclosure
The authors declare no conflict of interest.

references
The authors declare no conflict of interest.


