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REGULAR ARTICLE

Beyond the Oncogene Paradigm: Understanding Complexity in Cancerogenesis

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Abstract In the past decades, an enormous amount of precious information has been collected about molecular and genetic characteristics of cancer. This knowledge is mainly based on a reductionistic approach, meanwhile cancer is widely recognized to be a 'system biology disease'. The behavior of complex physiological processes cannot be understood simply by knowing how the parts work in isolation. There is not solely a matter how to integrate all available knowledge in such a way that we can still deal with complexity, but we must be aware that a deeply transformation of the currently accepted oncologic paradigm is urgently needed. We have to think in terms of biological networks: understanding of complex functions may in fact be impossible without taking into consideration influences (rules and constraints) outside of the genome. Systems Biology involves connecting experimental unsupervised multivariate data to mathematical and computational approach than can simulate biologic systems for hypothesis testing or that can account for what it is not known from high-throughput data sets. Metabolomics could establish the requested link between genotype and phenotype, providing informations that ensure an integrated understanding of pathogenic mechanisms and metabolic phenotypes and provide a screening tool for new targeted drug.

Keywords Reductionistic paradigm · Reversibility of cancer · Aneuploidy · Morphogenetic field · Complexity · Chaotic behavior · Systems biology disease

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1 The Central Dogma in Oncology

Cancer is believed to be a disease that begins at the cellular level. The first step is the initiation of cancer in a single somatic cell, which must then transfer its acquired abnormality to its progeny, bypassing the immune regulatory control system (somatic mutation theory of cancer). The initiation of cancer is mainly conceived as a mutation that involves a set of regulatory genes, with either enhancing (proto-oncogenes) malignant properties, first discovered in the 70s (Stehelin et al. 1976), or inhibiting (tumor-suppressor genes) malignant properties (Fearon and Vogelstein 1990).

However, intensive research on cancer, along the lines of this currently accepted paradigm, has led to the emergence of several anomalies and contradictions that cannot be fully explained within such a reductionistic paradigm (Baker and Kramer 2007; Marcum 2005). Carcinogenesis is thought to start from a genetic mutation in one, or a set, of regulatory genes (proto-oncogenes), which then become oncogenes. Paradoxically, tumor progression is also explained as a sort of micro-evolutionary Darwinian process, through which the single mutated cell leads to a population of clonally-derived cells, which means we would expect to find "mutated" oncogenes in all transformed cells. However, oncogenes such as H-ras, N-ras and K-ras are non-clonal in prostate (Konishi et al. 1995), colon (Baisse et al. 2001) and melanoma (Van Elsas et al. 1995) cancers, and mutant Her/EGFR/neu/Erbb-2 is non-clonal in gliomas (Park et al. 1995) and bladder (Sauter et al. 1993) and breast (Szollosi et al. 1995) cancers. Moreover, genetic alterations believed to be associated with malignant tumors have also been described in normal tissues (Whashington et al. 2000). Furthermore, ras-positive melanoma initially metastasizes without *ras* genes (Albino et al. 1984), whereas cancer cells retain tumorigenicity after spontaneous loss of the *ras* gene (Plattner et al. 1996; Van't Veer et al. 2002). Thus, a single mutation does not suffice to cause cancer (Land et al. 1983): several stepwise genetic alterations are required for full tumor development (Vogelstein and Kinzler 1993). Analysis of somatic mutations in different cancers reveals that, contrary to the "dogma", mutation of both oncogenes and tumor-suppressor genes involves a fraction of such genes and seldom occurs in a late stage of metastatic progression. Mutations of the key-oncogene epidermal growth factor receptor (EGFR) have been found in but a minority of spontaneous human cancers (Paez et al. 2004). Moreover, though a CDC4 mutated-phenotype is claimed to be a chief cause of chromosome instability in cancer, only 22 out of 190 examined cancers share this mutation (Rajagopolan et al. 2004). Cancer based on oncogene-mediated transformation has often been induced in animal cells, rarely in human cells because human cells are known to be more resistant to oncogenic transformation (Holliday 1996). Whereas normal rodent cells can easily be transformed by a combination of two or more oncogenes, normal human cells have never been reproducibly transformed by such combinations. It is noteworthy that significant results have only been achieved in the transformation of embryonic or adult fibroblasts with a high aneuploidy rate (Akagi et al. 2003). Thus, differences in susceptibility to oncogenic transformation are to be attributed to as yet unknown host factors: while recognising the role played by oncogenes, Chin et al. raised questions as to whether experimental cancer-promoting mutations remain relevant during tumor

maintenance and whether oncogene-independent mechanisms contribute significantly to established tumors (Chin et al. 1999). 'Simple' inactivation of *c-myc* (Jain et al. 2002) or inhibited expression of other oncogenes (like BCR-ABL1, *ras*) (Huettner et al. 2000) has not actually been observed in tumor regression, and the role of "transgenic" oncogenes in inducing tumors in transgenic animals remains unclear, or, at best, presumptive (Duesberg 2003).

2 Do Mutations Beget Cancers or Do Cancers Beget Mutations?

In a noteworthy paper, Hahn et al. (1999) demonstrated that artificial ectopic overexpression of at least four genes suffices to convert human normal cells into tumor cells. These results were not reproduced in another experiment (Morales et al. 1999) and have been criticized (Li et al. 2000), it being suggested that the observed transformation was due to the induced genome-wide instability, which lead to aneuploidy. As the spontaneous mutation rates of somatic (as well as of cancer) (Loeb 1997) cells are very low (Friedberg 1985), a combination of four mutations is unlikely to occur spontaneously within a single cell in the appropriate order and fashion. A simple estimate, based on the known somatic cell mutation rate of around 10^{-12} per nucleotide per generation, suggests that a stepwise accumulation of four independent mutations within a single cell during the life span of a human being is impossible. Moreover, there is as yet no evidence that any of the so-called oncogenes is expressed at a higher rate in a primary cancer than in its normal tissue counterpart (Duesberg 1995). Upon analyzing several genes of colon and pancreas cancer cells, Zhang et al. were surprised to find that widely studied oncogenes, such as *c-fos* and *c-erbb3*, were expressed at much higher levels in normal colon epithelium than in colorectal cancers (Zhang et al. 1997). It is highly unlikely that the alteration of a single key cellular factor may transform a normal cell into a cancer cell under non-artificial circumstances (Duesberg and Rasnick 2000). As stated by Prehn in the 90 s, "the hypothesis that cancer is usually the result of genomic mutations may be wrong" (Prehn 1994, 2005), despite the plethora of oncogenes and tumor suppressor genes continually being discovered. Compelling evidence suggests that it is unlikely that one, or several, genes can exert pleiotropic powers over so many others, thereby leading to the complex array of cancer phenotypes. Nor is there any independent genetic evidence that the mutations of these genes have such powers. Simple mathematical approaches, based on metabolic control analysis, have outlined how, as the number of gene products required to produce a particular phenotype increases, the effect of varying any one of these products becomes quite small; similar considerations can be made for genetic mutations (Kacser and Burns 1981; Heinrich and Rapoport 1974). Consequently, alterations in a few 'gatekeeper' or 'caretaker' genes are unlikely to suffice to 'cause' cancer (Duesberg et al. 2004). Cancers may exhibit mutations primarily because replicative errors are repaired either slowly or not at all owing to the impairment of epigenetic mechanisms or complex regulatory pathways (mainly metabolic, proliferating and apoptotic); nevertheless, any mutations produced in this way may be "irrelevant to issues of tumor development" (Boland and Ricciardiello

1999), or may have limited biological significance and "are not likely to play a dominant part in cancer" (Hua et al. 1997).

This hypothesis is supported by the fact that several well-known carcinogenic compounds are not genotoxic, i.e. they induce cancer *without inducing any mutation* in DNA genes (Ashby and Purchase 1988; Lijinsky 1989). Furthermore, according to the standard carcinogenic model based on somatic mutation, exposure to a carcinogenic compound would predict rapid transformation as the carcinogen would mediate mutation. This, however, is in contrast to the exceedingly long latent period between carcinogen exposure and cancer initiation documented by both epidemiological and experimental data.

3 Reversibility of Cancer Transformation

If cancer onset were truly an event due to the accumulation of mutations in a few key-genes, once the threshold has been crossed there would be no way back towards normality. This however is not true, as experimental studies reporting spontaneous clinical regression or dramatic phenotypic reversal from in vitro cancer cell cultures demonstrate (Challis and Stam 1990).

A growing body of evidence has suggested that the re-establishment of appropriate interactions between human cancer cells and the surrounding microenvironment (i.e. stromal cells and tissues) can reverse the neoplastic phenotype. Indeed, cancer cell/stromal cell interaction plays a crucial role in tumor growth and evolution, affecting gene transcription, differentiating and apoptotic pathways (Mauro et al. 1994; Inouc et al. 2001; Dong-Le Bourhis et al. 1997; Lin et al. 1998), though little is known about the mechanism through which host cells interfere with cancer growth; cell-to-cell communication-mediated tumor growth inhibition appears to be very tissue-specific, and it should also be borne in mind that host-tumor interaction is not a one-way process (Krutovskikh 2002). Normal cells located in the wrong tissue degenerate into cancer cells (Biskind and Biskind 1944), whereas neoplastic cells introduced into a blastocyst (Brinster 1974; Mintz and Illmensee 1975; Hochedlinger et al. 2004), co-cultured with normal cells (Mizrachi et al. 1990), implanted into a normal microenvironment (Mc Cullogh et al. 1997) or subjected to differentiating (Missale et al. 1998; Bizzarri et al. 2003; Sell and Pierce 1994; Wang et al. 2002) and embryonic signals (Lee and Herlyn 2006; Lee et al. 2005; Cucina et al. 2006), either undergo apoptosis or become normal, thereafter contributing to the development of organised "normal" bodily structure. Moreover, some preliminary data have shown that differentiating-based treatment responds significantly both in vivo (Yu and Tsai 2001) and in clinical trials (Livraghi et al. 2005; Sell 2004).

These results indicate that "cancer can be epigenetically re-programmed into normal cell types" (Li et al. 2003). One set of data cast doubts on the "irreversibility" of cancer transformation by showing that heritable normal behaviour or phenotype can be restored by appropriate signals from the environment (Kenny and Bissell 2003) i.e. by restoring a normal, strong morphogenetic field, which ultimately leads to differentiation (Sell 2004) or programmed cell death

(Hendrix et al. 2007). In other words, "re-establishing appropriate interactions of human cancer cells with the substratum can reverse neoplastic behavior even in the presence of grossly abnormal genetic damage" (Barcellos-Hoff 2001; Zutter et al. 1995; Bissell et al. 2002). These results have questioned the view that cancer was caused by DNA mutations, "hence the dictum: 'once a cancer cell, always a cancer cell' was invalidated and the data instead suggested an epigenetic control of the expression of neoplastic phenotypes" (Soto and Sonnenschein 2004).

4 Cancer and the Morphogenetic Field

It is highly unlikely that this phenotypic reversal is due to the reversion of multiple point mutations, which are claimed to be the cause of cancer: "in other words, cells may show a neoplastic phenotype in the absence of mutations" (Sonnenscheim and Soto 2000). Indeed, cell growth and proliferation control cannot be considered an exclusively internal cellular issue when the cell belongs to an organism. A growing body of evidence indicates that more general chromosome abnormalities may play a critical role in the transformation of normal human cells into cancer cells (Hooth et al. 1998) and constitute the 'Achilles heel' of cancer (Lengauer et al. 1998; Prasad and Lengauer 2001). It is the breakdown in space-temporal organization (a morphofunctional entity called "morphogenetic field") (Webster and Goodwin 1996), and the resulting disruption of the normal signalling mechanism, that is likely to disrupt the *coherence* between the cell and organism, thereby contributing to the onset of cancer (Fogarthy et al. 2005; Aranda-Anzaldo 2001). Disruption of the 'morphogenetic field' is indeed evidenced in cancers by the loss of fractal structure and by the drift toward geometrical homogenisation (Naeim et al. 1996).

It has been proposed that this could happen when the genome of a cell drives the cell to aneuploidy (Duesberg et al. 2001). A link between chromosomal aberrations and the pathogenesis of cancer was first described by Von Hanssemann (1890) and Boveri (1914). Some recent studies (Meijer 2005; Breivik 2005) have proposed a revival of aneuploidy as a consequence of genomic instability and as a major determinant of cancer (Duesberg et al. 2005; Weaver and Cleveland 2006; Pellman 2007; Wolf et al. 2004). It is noteworthy that inactivation of oncogenes after the onset of aneuploidy and the consequent tumor transformation does not result in tumor regression (Felsher and Bishop 1999); moreover, in mutant-p53 mice that retain a diploid chromosome number, early onset of spontaneous tumors is efficiently suppressed, which is in sharp contrast to what occurs in p53-defective animals with aneuploidy-bearing tumors (Liu et al. 2004). Nevertheless the aneuploidy-based theory of carcinogenesis seems to be no more than a sophisticated variation of the somatic mutation theory and did not account for several paradoxes of cancer biology.

It has been suggested that cancer arises as a result of several genomic events that occur in a requisite epigenetic context (Laird and Jaenisch 1996; Lotem and Sachs 2002; Suzuki and Miyata 2006; Brown and Strathdee 2002), characterized by 'defective' differentiating signaling networks and chromosomal instability (Storchova and Pellman 2004; Duelli et al. 2007), which in turn provides the permissive context in which oncogene activation can produce a neoplastic

phenotype (Shima et al. 2007; Huettner et al. 2000); this statement explains why "it may be more difficult to induce the regression of cancers through oncogene inactivation" (Gorre et al. 2001).

Within a more general perspective, the appearance of a tumor might signal that the properties of the 'field' are gradually lost when the living structure is beyond its prime i.e. when its morphogenetic field is weakened (Needham 1950; Potter 2001; Rubin 1985). The weakening or disruption of the original morphogenetic field also makes room for the influence of aberrant or artificial "pregnant entities" i.e. morphogenetic driving forces, which may overwhelm it or superpose it to the "original" field (Hamburger 1988): just as some malformations (e.g. cyclopia, polydactyly) can be produced experimentally or by mutation, cancer can be induced without a mutation (Opitz 1985). Dramatic changes in the metabolic environment (related to diet, genome instability i.e. aneuploidy, or epigenetic modifications), an altered structure in the overall neuroimmunoendocrine network or disruption of the signaling-regulatory pathways originating from the stroma (Sell 2004; Arnold et al. 2002) and surrounding tissues, may markedly affect the physiologic structure and genomic functioning of the cell (Barcellos-Hoff and Rafani 2000). Some intriguing experimental data show that tumors and morphological transformation may arise as a response to constraints in physiological growth or metabolism *in the absence of carcinogens*, and that this neoplastic process can be reversed by lifting these constraints (Rubin et al. 1990). This behavior is typical of an "adaptational response" and, when combined with other evidence, demonstrates that tumorigenesis does not require conventional genetic alterations (Rubin et al. 1992). As Radisky et al. (2001) stated, it is "the altered communication within the tumor, rather than mutations per se, that is the defining characteristic of cancer: any attempts made to restore a "normal signalling context" may effectively revert the neoplastic transformation". Therefore, cancer, and any transformation in cellular organization, should be viewed as a result of a conflict between an organised morphology and the emergence of a new unpredictable *teleion*—defined as a *'strange attractor'* (Aranda-Anzaldo 2002)—towards which the biological system drifts, thereby leading to a new and aberrant amorphous state.

Three decades ago Pierce et al. (1978) suggested that cancer was a problem of "developmental biology", stating that *how the genome is controlled* in tumor cells is as important as genomic aberrations. Watson recognized that cancers are characterized to a large extent by uncontrolled growth. We are thus dealing with a problem of cell division *control* (Watson 1976), which includes both apoptosis and cell differentiation (Corn and El-Deiry 2002). "Nevertheless, the difference between cancer and normal cells is not just a matter of rate but of control, and the control may be a matter of degree" (Watson 1976), the only useful distinction thus being that "the cancer cell is less subject to the normal devices which tell a cell not to divide". Some conclusions have been drawn from these statements. Pitot stated that "the unique characteristic of neoplasia at the biological level involves derangement of control mechanisms […] The most significant distinctions defining the neoplastic phenomenon are the abnormalities seen in the regulation of gene expression" (Pitot 1975). However, the challenge of defining cancer cells persists despite the remarkable progress made by both genomic and proteomic research; as

Becker says: "we are unable to define the malignant cell, in that the biochemical diversity between two different cancers is so great that no two tumors are exactly alike" (Becker 1975). After 20 years of experimentation, Becker may be right insofar as no malignant biochemical "archetype", particularly from a genetic point of view, has yet been documented, while Pitot is right in saying that all malignant cells have one feature in common: they are no longer subject to the orchestrated control network that enables each cell to respond to both intrinsic and environmental stimuli by regulating gene expression or metabolic processes properly. In this respect, the central issue in oncology is not simply the digital information encoded in DNA, but the signaling network and the complexity that arises from different hierarchical levels: gene, transcriptome, proteome. According to the "central dogma of molecular biology" (Crick 1970), DNA is the only source of biological information, which flows from DNA to RNA and to proteins. However, a growing number of phenomena cannot be explained by the "central dogma", starting with the process of morphogenesis that enables the genome of cells to be differently expressed. This occurs mainly through a set of regulatory pathways, including metabolic, post-translational factors, environmental inputs of both a chemical and physical nature, and, last but not least, through epigenetic heritable mechanisms, i.e. DNA methylation and histone acetylation (Wolffe and Matzke 1999). Attention has focused prevalently on discrete abnormalities in digital encoding units (i.e. the single-point gene mutation), resulting in "the search for oncogenes and tumor-suppressor genes, which is simply the search for the rate-determining molecular steps in carcinogenesis. However, the results of biochemical experiments of the last 25 years […] have shown that complex systems are not controlled by slow or rate-determining steps" (Rasnick and Duesberg 1999).

5 Limits of the Reductionistic Paradigm

The current carcinogenic paradigm is based on the assumption that all biological information is embedded in the DNA sequences insofar as any modification/mutation of the gene is thought to be linearly and automatically translated into a well-defined cellular abnormality. Although the biological information stored in DNA sequences is digital (Hood and Galas 2003), the information does not flow from DNA to mRNA and subsequently to proteins (enzymes) linearly because the digital data are converted into analogical data. This means that the information must be processed, is partly lost and is significantly modified by post-translation processes (Heffner 2005). The power of DNA derives from the fact that it is a digital information carrier, which means the information is highly stable and reproducible. However, the lack of a digital receiver in most biological and physiological systems prevents digital information processing: if the processing includes even only one analog step "the perfection of the digital steps is wiped out. The processing then becomes effectively analog". This means that biological information is not embedded exclusively in DNA sequences, but is also contained in epigenetic and regulatory signal (both external and internal) networks (Laird 2005).

Interconnected metabolic processes act together as an integrated unit that affects gene expression and the enzymatic processes. Genomic expression is governed by biochemical mechanisms that perceive the bioenergetics of the cell. Moreover, biochemical processes require the production of entropy as a driving force. But how does one explain the increasing order of developing organisms? How does one resolve the paradox, first expressed by Schrödinger, that self-organization processes minimize chaos, whereas entropy, i.e. chaos in the thermodynamic sense, must be increased? As first pointed out by Onsager (1931), and subsequently demonstrated by non-equilibrium thermodynamics (Prigogine 1962), the two processes must be coupled: close to equilibrium, dependence of entropy-producing processes on the 'driving force' of the 'self-organizing' field should equal the dependence of the process rate of the latter on the driving force of the former. In this regard, it is noteworthy that even in simple non-organic systems, such as the Beloussov-Zhabotinsky reaction (Zaichin and Zhabotinsky 1970), highly ordered, complex structures arise from processes that do not interact linearly, thereby demonstrating that 'self-organizing' information is inherently linked to the biochemical network 'context' (Bhalla and Yvengar 1999).

Indeed, in living cells, a set of several gradients of morphogens (specified maternally), which were first referred to by Paul Weiss as a 'morphogenetic field' (Weiss 1969) and provide supplemental information not previously encoded in the cell genome, are required to ensure appropriate development (Gilbert et al. 1996; Beloussov et al. 1997; Gilbert 1997; Lawrence 1992). Further advances, above all in network thermodynamics, served to reinterpret and develop Onsager's previous statements, introducing new, more "holistic" concepts regarding systems and building thermodynamics equations in a way that allowed information on the system's organization to be identified in the equations' structure (Mikulecky 2001). These new approaches clearly highlighted the shortcomings of the reductionistic and mechanistic thought, according to which even more complex biochemical pathways (such as glycolysis) are governed by a single pacemaker gene or enzyme. Oscillations in biochemical processes are instead simultaneously controlled by several steps in the intracellular network and the oscillations actively synchronized. 'Desynchronization' of the regulatory pathways in cancer probably reflects the genome 'energy overload', which is due to several internal and external factors (Dimitrov 1993). As suggested by S. Hauptman, there may be a direct link between the information entropy of the genome (which increases over time, according to the second law of thermodynamics) and its instability (Streheler 1986). Thermal noise acts as a modulator of genetic instability (Johnson 1987). Genetic instability therefore increases in the event of an 'energy overload', leading to random, DNA-wide alterations that are directly linked to the onset of aneuploidy. Within this perspective, tumors are 'dissipative structures', thermodynamically speaking (Glansdorff and Prigogine 1971): "cancer is a special kind of adaptation to energetic overload [and] […] the genetic alterations are probably secondary changes. Cancer serves to dissipate energy in a type of developmental process […] an entropic devolution" (Hauptman 2002). Moreover, cancer growth is characterized by "loss of information" and unpredictable genetic pathways: "thus, fixed, linear sequences leading to cancer will likely be uncommon in human cancers" (Gatenby and Frieden 2002).

These elementary observations thus cast serious doubts on the simplistic, linear, causal relationship "gene – protein – function". This presumptive relationship can only be applied to a limited number of phenomena, in which the components involved can be added or subtracted from the system, and in which the temporal order of events is clear. In a cyclically reinforcing system, the removal of any component may affect all the other elements, not those downstream alone, and temporal relationships become more confused as all the elements change in concert. The reductionistic paradigm is unable to solve some questions arising from the pleiotropic functions sustained by the interplay between different enzymes and proteins. How can the same pathway determine different biological responses? And how are genomic, transcriptomic and proteomic signals regulated to produce a data set of metabolites (metabolome)? We now know that simple, quantitative measurements of some parameters are not sufficient to ensure a comprehensive understanding of complex system behavior. Pathways are traditionally drawn as separate linear entities for the sake of clarity, though this does not reflect the reality. The amplitude and duration of the signal flux through a pathway may actually determine the biological outcome. A classic example are the PC12 cells, in which continuous ERK activation triggers neuronal differentiation, while transient ERK activity induces proliferation (Vaudry et al. 2002). Biochemical pathways are extensively connected and embedded in networks, frequently being characterized by non-linear dynamics. Thus, when faced with the complexity of both external and internal stimuli (i.e. modifications in gene expression and translational and post-translational protein variability), the specificity of biological responses is largely generated by the combinatorial integration of pathway crosstalk and the versatility of component function. Thus, the digital information encoded in DNA sequences might be efficiently and "creatively" transduced into the analogical information displayed by complex biological systems, often structured in a tightly hierarchical regulatory pathway and characterized by non-linear dynamics. As stated by Waliszewski, genotypes and phenotype are interconnected in a non-bijective manner, i.e. the "relationship is neither "surjective", because not all the elements in a given set of genes possess a counterpart in the phenotype set, nor is it "injective", because two or more genes may contribute to the emergence of the same phenotype trait […] indeed, the same or very similar phenotypes may be associated with distinct patterns of gene expression" (Waliszewski et al. 1998). Moreover, over-expression of even a single gene, due to genetic manipulations, may result in an unexpected, marked 'holistic' change in both metabolome and transcriptome, involving an up-regulation both of paralogue genes and of genes with an unknown function (Manetti et al. 2004; Tohge et al. 2005). Therefore, no simplistic linear correlations can be drawn from raw gene expression profiles to cellular metabolic phenotypes. The classical genetic analysis has taught us to expect every genotype to determine a single phenotype. However, even in cases in which there is a single allelic difference between two strains, certain phenotypic traits cannot merely be considered as the result of the additive effect of independently acting genes. As demonstrated by Kacser and Small (1996), some biochemical systems, such as those containing positive feedback or those more generally embedded in complex networks, display bistability or multistability. In other words, a single genotype can

display two or more distinct "stable" phenotypes; which of these is realized will depend on where the system starts (i.e. the "previous history" of the system dramatically influences its evolution) or what environmental perturbation it has experienced. Thus, the specificity of biological responses is largely generated by the "combinatorial integration of pathway crosstalk and the versatility of component function, two regulatory motifs that generate a multitude of complex behavior" (Kolch et al. 2005). The system is deeply affected by the so-called "initial conditions", as would be expected in complex systems, and its property cannot be assigned to single elements. We are thus faced with systemic, "emerging" properties that originate at the metabolic level, not the genetic level. Moreover, such properties can be inherited.

One promise made by molecular genetics, and which was widely accepted by the mass media, was that a detailed knowledge of the human genome would permit the prognosis to be predicted accurately and targeted therapies to be discovered more easily, not only for Mendelian disorders, but even for cancer, a naïve assumption that was based on a reductionistic view of genotype-phenotype correlations. We must bear in mind that phenotypes of even "simple" Mendelian disorders are complex traits (Weatherall 2001; Scriver and Waters 1999): "an individual with a clinical disorder is not the product of the single gene that is disrupted, but the genetic disruption is embedded within the context of the individual entire genome and environmental experience" (Dipple and McCabe 2000). This statement represents "a logical extension of concepts developed by metabolic-control analysis": as the activity of biochemical pathways is influenced by non-allelic polymorphisms or additional independent mutations, individuals within the population will differ in flux through the various steps of the pathway, thereby imposing an additional magnitude of complexity (Dipple et al. 2001).

6 Complexity and Chaotic Behavior

Owing to the non-linear dynamics of biological systems, the intrinsic structure of biochemical networks is more complicated than that found in systems with linear dynamics, which means the network may go through a number of chaotic states. Chaos is intimately linked to the non-linear dynamics of living systems and may be synthetically defined by three fundamental characteristics. Firstly, chaos is an irregular (aperiodic) oscillatory process, i.e. subtle changes in one or a number of parameters can easily modify tumor behavior in the "space of the phases" towards different oscillation areas (attractor basins). Secondly, chaos is neither a wild nor "random" (stochastic) process: chaos is "deterministic", displaying a "quasiperiodicity" in the short term and evolving thereafter toward increasing complexity and unpredictability. Thirdly, chaotic systems are highly responsive to the initial conditions. Normal cells belong "on the edge of chaos" in order to ensure the high degree of plasticity that is required in epigenetic control mechanisms (Ohlsson et al. 2003).

A non-linear biological system may actually afford finer, more rapid and better controllability of the coefficient of energy than a linear system (Skinner 1994); it is

therefore not surprising that a number of disease conditions are characterized by marked periodic, non-chaotic states (Walleczek 2000). Moreover, methods and analytical tools derived from chaos theory (catastrophe theory) are now increasingly being used in image analysis systems, in the detection of coding DNA-regions, in assessing the space-filling properties of tumors and, more generally, in biological research modeling (Cross 1997).

Several reports suggest that tumorigenesis (Malins et al. 1998) as well as cancer behavior (Coffey 1998; Rew 1999), particularly in solid tumors (Calin et al. 2003), are an entirely deterministic chaotic process characterized by reduced complexity, as is indicated by a loss of the golden mean and by the disappearance of self-similarity (fractality) (Sedivy 1999). Fractal dimension changes during the transition from the proliferation to the differentiation phase: tumor progression leads the primary cancer population to the 'degenerated' stationary state characterized by altered dynamics of gene expression, loss of connectivity and collectivity (Waliszewski et al. 2001). These particular features lead to increased instability and loss of "ordered heterogeneity" at the genetic, structural, temporal and functional levels (Posadas et al. 1996; Sedivy and Mader 1997; Rubin 2007). Chaotic processes in cancer may give rise to some relevant consequences and the marked sensitivity to their initial conditions might explain the unpredictability of tumor development that has been documented both in vitro (Pescarmona et al. 1999) and in vivo (Schipper et al. 1996; Nathanson 1976; Hirshberg and Barasch 1995). The efficacy of "classic" therapeutic strategies, even if specifically targeted, may be severely hampered by chaotic processes owing to the overlapping, redundant functions of altered pathways and marked genetic or karyotypic instability: "until we understand the intricacies of these interactions, attempts at therapeutic interventions can only be expected to have a modest impact" (Marshall 2000). Moreover, the study of cancer through chaos theory-based models may provide a new rationale and relevant applications in pharmacodynamics (Dokoumetzidis et al. 2001) as well as in modeling the antitumor activity of drugs (Liang and Sha 2004). Cancer systems seem to be "robust" systems (Kitano 2004) that are apparently refractory to major environmental (dietary availability, tumor–host interactions, drugs) perturbations. However, a set of experimental data indicate that by interfering with the non-linear dynamics of the tumor itself and *synchronizing* tumor cells within the "chaotic" regimen, it should be possible to induce an unexpected "fragility" in the cancer system, which would in turn promote dormancy or apoptosis. This strategy would lead to unexpected results: "when the parameters of the tumor are in the 'chaotic' region, very low doses of drug can be effective in preventing growth. This leaves hope for the future application of much less toxic therapies than the ones currently in use" (Delsanto et al. 2000). Indeed, it may be possible to control chaotic processes by introducing small, critically timed perturbations (Weiss et al. 1994); although 'external' interference would be minimal, the efficacy of this process would lie in its ability to deviate a very sensitive system from its spontaneous kinetics while keeping it bound. Therefore, "if chaotic functioning were found to be common in tumor cells, it may be possible to use this phenomenon clinically, as it has only a very small toxicity cost" (Guerroui et al. 2005).

7 Cancer as a Systems Biology Disease

Recent proposals to sequence cancer genomes held scientific promise of personalized cures for each type of cancer. Various investigators claimed that this achievement would transform our understanding of cancer, so, as E. Lander hoped, "knowing the defects of the cancer cell points you to the Achilles' heel of tumor" (Pollack 2005). The Human Cancer Genome Project aims to catalog all somatic mutations from primary tumors as the basis for designer drugs. Success is predicated on the assumption that cancer pathogenesis is linearly linked to mutated genes and that drugs can be targeted at very specific mutated regions of gene products. However, the first clinical attempts based on this reductionistic promise have been contradictory and discouraging, and it is highly questionable whether "mechanistic" genome-based therapeutic programs will be successful in the future (Horrobin 2000). As pointed out by G. Miklos in a recent commentary, "despite the glacial progress in treatment and the advent of 'molecularly targeted' therapy, cancer research continues to focus myopically on individual oncogenes, tumor suppressor and repair genes, with little effort devoted to alternative mechanisms and targets" (Miklos 2005). Cancers with mutated oncogenes, which are not generally transcriptionally active, are unlikely targets for therapies based on oncogene control: the presence, amplification and abnormal expression of erbB-2 escape detection in almost 80% of breast cancers (Pollack et al. 2002); similarly, activated *ras* and other oncogenes are involved in but a minority of natural colon cancers (Duesberg and Rasnick 2000). In other words, as Duesberg et al. stated and Weinstein (2002) had initially suggested, "transcriptionally inactive oncogenes are no Achilles heel of cancer". Molecularly targeted therapies have had very limited success against solid tumors and results are disappointing. Not only have these new drugs frequently had severe toxic effects (Ozcelik et al. 2002) and low objective response rates (Marx 2004), they have also resulted in but minimal improvements in both the disease-free interval and survival (Hofmann et al. 2002). These findings are hardly surprising. The "Achilles heel of tumor" is not a single gene-based event, but involves several regulatory pathways and the complex interplay of genetic, transcriptomic and metabolomic networks. Changes in signaling pathways, influenced by both external and internal stimuli and characterized mainly by non-linear biochemical dynamics, can occur "completely independently of oncogenic or tumor suppressor or mismatch repair mutations and would not be detected by a human cancer genome sequencing effort. [….] A good place to start would be to dismiss the fallacious notion that single mutations in primary tumors are the optimal starting point for research" (Esteller 2000).

The paradigm "one genotype–one phenotype" has provided the basis not only for classical genetic analysis but also for the dominant "oncogene paradigm". There is however growing evidence that a complex biological system may have two, or more than two, phenotypic outputs for a given set of genetic parameters: "this means that a single genotype can display two distinct [stable] phenotypes. Which of these is realized will then depend on where the system starts from or what environmental perturbation it has experienced" (Kolch et al. 2005). This fascinating property cannot be assigned to a single element: it is a systemic property, it can be inherited

and it originates at the metabolic and not the genetic level. These considerations imply that cancer—like other "stable states" of chronic illness—may be better understood through an "integrated global profiling strategy" (Hanash 2004), taking into consideration the principles that govern complex, non-linear systems. Indeed, the somatic mutation theory of carcinogenesis has been criticized for a number of reasons, with alternative paradigms to this theory being based above all on the "aneuploidy hypothesis" or "tissue field theory of carcinogenesis", which have been thoroughly discussed in peer-reviewed journals (Rubin 2006; Capp 2005a). It is highly likely that the specific properties of cancer cells result from aneuploidy or deregulation in the extracellular signaling pathways rather than from specific mutations: malignancy behavior seems to be the stochastic consequence of a global deregulation of gene expression following the disruption by the carcinogens of the cellular interactions that normally stabilize gene expression and maintain a well-differentiated state (Capp 2005b).

8 New Paradigm and Different Methodologies

Paradoxically, it is the overwhelming amount of data yielded by classic molecular biology that has highlighted the need for a new paradigm (Strohman 1997). Moreover, understanding cancer complexity is not only a matter of alternative theories, but requires, first and foremost, a "systemic" methodological approach.

Over the past decades, an enormous amount of valuable information has been collected on the molecular and genetic characteristics of cancer. This information was obtained above all by means of a reductionistic approach, even though cancer is widely held to be a 'systems biology disease' (Rew 2000). Bloom (2001) recognized the post-genome sequencing era as the end of "naïve reductionism", defining reductionism as "the attempt to explain complex phenomena by defining the functional properties of the individual components" of a system. However "organisms are clearly much more than the sum of their parts, and the behavior of complex physiological processes cannot be understood simply by knowing how the parts work in isolation" (Strange 2004). Thus, despite the outstanding progress made in cancer cell molecular biology, "the emerging complexity of the entire 'cancer system' overwhelms us, leaving an enormous gap in our understanding and predictive power" (Hornberg et al. 2006). Shortfalls "in reductionism are increasingly apparent. Mostly these arise from information overload […] another problem is oversimplification" (Gallagher and Appenzeller 1999). The "failure" of reductionism can be ascribed to both "ontological" (i.e. lack of information) and epistemological (i.e. inability to evaluate or understand the information embedded in the complex biological matrix) reasons (Bray 1997). It is not solely a problem of how to integrate all the information available in such a way as to be able to still cope with the complexity; bearing in mind that systems biology is likely to *revolutionize* our understanding of cell and disease mechanisms, we must recognize that a thorough transformation of our scientific *weltanschauung* is urgently needed. We need to think in terms of networks (Sweetlove and Fernie 2005): "understanding of complex function may in fact be impossible without recourse to influence—

generally referred to as non-linearities -outside of the genome: "the directions for reading the maps are not included in the [DNA] package. And the real secrets of life are obviously in those missing directions, in the rules and constraints that organize genetic agents into functional arrays. These rules and constraints are more than likely embedded in the organization of life rather than in the catalogue (i.e. the genome) of the organization's agents, and we have mistaken the former for the latter" (Rew 2000).

In this respect, our biological and mathematical models should be structured assuming that living processes as well as disease mechanisms are governed by non-linear oscillations of complex signaling networks, operating on the edge of chaos. So far, our predicting power will be ineluctably a *probabilistic one* (Zhou et al. 2004). The road map of how we get there involves connecting experimental unsupervised multivariate data to a mathematical and computational approach than can simulate known biologic systems for hypothesis testing or that can account for what is not known from high-throughput data sets (Phelps et al. 2002; Khalil and Hill 2005; Ge et al. 2003).

9 System Thinking

Although rediscovered only recently (Barabasi and Oltvai 2004), "network thinking" has long pervaded biology research (Von Bertalanffy 1969; Polany 1968), but tools that allow the assessment of pathway function in a system context have been lacking until now.

Within the context of systems biology, metabolic engineering has emerged as the scientific field whose aim it is to gain an integrated knowledge of the biochemical cellular pathways. As in systems biology, the focus in metabolic engineering is no longer on the analysis of individual, isolated reactions, but on integrated networks of metabolic pathways. This approach addresses the limitations of the one-gene/one-function paradigm and provides a framework for understanding general biological properties, such as adaptability and homeostasis, as well as testable hypotheses that serve to explain, and not merely describe, cellular events such as apoptosis and differentiation (Cusik et al. 2005; Vidal 2005; Lazebnik 2002).

The main concern of metabolic engineering is the overall metabolic system, insofar as information, whether it is digital or analogical, on intracellular regulatory mechanisms can only be obtained by studying the interaction between the various parts of a network (Friboulet and Thomas 2005). In this perspective, metabolomics was defined by Steve Oliver (Nielsen and Oliver 2005) as the discipline that describes "the complete set of metabolites/low-molecular-weight intermediates [the metabolome] that are context dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism". This statement accounts for steady-state analysis, whereas "metabonomics", a term proposed by Nicholson and Wilson (2003), outlines the need for a more dynamic understanding and was designed to assess "the quantitative measurement of the multivariate metabolic responses of multicellular systems to pathophysiological stimuli or genetic modifications". Indeed, the metabolome of a given cell represents

the integrated end-point of many regulatory signalling events, elicited by the spatial and temporal interplay of genetic, epigenetic and environmental factors, and is highly context-dependent. In contrast to the classical molecular approach, in which control is exerted along a hierarchical flux from the genome to the metabolic pathways, metabolomics is concerned with both horizontal and vertical networks.

Metabolome analysis measures changes in the relative concentrations of metabolites following the deletion or overexpression of a gene and may allows the target of a novel gene product to be located in the metabolic map. The individual components in the transcriptome, the proteome and the metabolome are complex functions of many cellular components. Thus, the transcription of a given gene is a function of the concentration of transcription factors, and of the activities of upstream kinases and receptors. Similarly, the concentration of a given protein is determined by the concentrations of its corresponding mRNA and by the activity of the translational apparatus. However, since metabolite concentrations are determined by the activities of many enzymes, the individual components of the metabolome are generally far more complex functions of other cellular components than either proteins or mRNAs. Thus, the metabolic network is a complex network of reactions that are closely connected; since different reactions in the metabolic network are coupled, even small perturbations in the proteome or in the functional activity of the genome may lead to significant changes in the concentrations of many metabolites. Moreover, metabolome analysis might allow the phenotype of silent mutations to be mapped; more generally, it might reveal whether any given modification in gene activity (deletion, mutation, over-expression etc.) revealed by functional genome analysis will lead to significant metabolic changes and, lastly, whether a different phenotypic conformation and behaviour may result from this complex interplay. The mapping of measurements of several metabolites onto metabolic charts demonstrates how metabolic profiling can be combined with transcriptome and genome analysis to depict cellular responses when cells are exposed to different environmental conditions. This dynamic, integrated approach to cell (metabolomics) or whole organism (metabonomics) studies may provide information confirming that the carcinogenetic phenomenon is a tightly connected process resulting from the interplay of genomic, transcriptomics, proteomic and metabolomic networks. Within this complex framework, oncogenesis should no longer be viewed as a merely "deterministic" consequence of altered gene function, but rather as a non-linear end-point of the network-structured interactions in response to several different environmental and endogenous stimuli.

Metabolic regulatory pathways are rarely totally hierarchical, i.e. the flux through the steps in a metabolic pathway is not proportionate to the concentrations of corresponding enzymes or related-mRNAs, and even strategic pathways such as glycolysis are rarely regulated by gene expression alone (Westerhoff and Palsson 2004). There may be an incomplete correlation even when regulation is mainly hierarchical, which indicates that the ultimate biochemical output of a biochemical pathway is influenced to a greater extent by the internal network structure than by classical biochemical parameters such as enzyme kinetics or substrate and protein concentrations (Kuile and Westerhoff 2001). Indeed, from a classical point of view, biochemical reactions are reported to come under the control of a "rate-limiting

step", the flux through the related pathway ultimately being determined by the kinetics of the "rate-limiting step". In the 1970s metabolic control analysis (MCA) challenged this reductionistic approach, focusing on the complex, dynamic structure of metabolic control (Kacser and Burns 1973; Cornsih-Bowden and Cardenas 2000). Metabolite concentrations are determined by the activities of a number of enzymes and are influenced by numerous factors that may be either intracellular or extracellular. Thus, "both transcriptome and proteome may be vastly incomplete monitors of regulation of cell function". This accounts for the disappointing results achieved by means of targeted gene therapies: there have been few reports (Sthephanopoulos and Valin 1991; Bailey 1999) of successful metabolic flux alterations following the manipulation of gene expression (i.e. gene therapies) because of the complex, non-linear nature of metabolic control architecture.

Metabolomics may provide the missing link between genotype and phenotype (Fiehn 2002), yielding information that is complementary to genomic, transcriptomic and proteomic analysis and that would advance our understanding of pathogenic mechanisms and metabolic phenotype (Miccheli et al. 2006), improve diagnosis (Yang et al. 2004; Odunsi et al. 2005), help monitor treatment regimens (Lehtimaki et al. 2003) and serve as a screening tool for new targeted drugs (Cascante et al. 2002; Miccheli et al. 2003; Colafranceschi et al. 2007). Metabolomics provides a straightforward investigative approach which, as stressed by Bailey, we urgently need to be able to tackle the cell as a 'complex system', identify novel drug targets and, ultimately, revert the current stagnation in the rate of pharmaceutical discovery (Bailey 2001). Furthermore, metabolome analysis can be used to study functional genomics. As stated by Oliver, "the measurement of the change in the relative concentrations of metabolites as the result of the deletion or overexpression of a gene […] should allow the target of a novel gene product (or, indeed, inhibitor) to be located in the metabolic map". Moreover, metabolomics can be used to unravel functions of "silent" genes, i.e. genes that can be deleted without apparently altering protein levels or mRNA synthesis (Raamsdonk et al. 2001).

The metabolic network is a complex network of tightly connected reactions. This assumption has led several scientists to consider a new rational approach in "metabolic engineering" that can provide a dynamic representation of the network characteristics, thereby allowing work on biochemical manipulation of metabolic networks to progress (Sthephanopoulos and Sinskey 1993). As pointed out by Nicholson in a recent paper, "if we are to capture the full power of systems biology and new genomic tools for drug discovery, we need to measure and model the *whole system*, which includes environmental factors. This is 'global' system biology; simple pathway analysis alone is unlikely to suffice to explain many disease processes" (Nicholson and Wilson 2003; Goh et al. 2007). We are, unfortunately, a long way from achieving this goal. Currently available bioinformatics tools, i.e. multivariate statistical analysis and mathematical modelling, cannot easily incorporate data obtained from different analytical technologies into complex non-linear dynamic systems. Indeed, even if an increasing effort is being made to elaborate new modelling paradigms, considerable difficulties, of both a 'scientific' and 'non-scientific' nature (Strohman 2002), are being encountered. This, however, is the challenge and the new perspective facing us; the time has come to change the

dominant paradigm in both molecular biology and carcinogenesis, even if "it is now becoming evident how much more complex the task is than was thought not long ago" (Sonnenschein and Soto 2005).

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