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Review

Physical forces and non linear dynamics mould fractal cell shape. Quantitative morphological parameters and cell phenotype

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Summary. Cell shape is mainly determined by biophysical constraints, interacting according to non-linear dynamics upon the basic units provided by the genome. In turn, the specific configuration a cell acquires plays a fundamental, permissive role in modulating gene expression and many other complex biological functions. Cell shape is tightly connected to cell activity and can be considered the most critical determinant of cell function. As a consequence, measurable parameters describing shape could be considered as 'omics' descriptors of the specific level of observation represented by the cell-stroma system. Such an approach promises to formalize some of the underlying basic mechanisms and, ultimately, provide a holistic understanding of the biological processes.

Key words: Cell shape, Fractals, Biophysical constraints, Cytoskeleton, Non-linear dynamics

How relevant is the 'form' a cell acquires?

As far back as ancient Greek natural philosophers, the form of an organism has been thought to carry a relevant meaning, from both a philosophical and biological point of view (Shields, 1990). As pointed out by D'Arcy Thompson, the morphogenetic process represents, for many embryologists, "the 'final cause' - the Aristotelian τελοζ, of its own processes of generation and development". Even if such processes need to be understood like a "teleology without a τελοζ, [...] an adaptation without "design," a teleology in which the final cause becomes little more, if anything, than the mere expression or resultant of a sifting out of the good from the bad, or of the better from the worse, in short of a process of mechanism" (D'Arcy, 1917). According to Waddington "the whole science of biology has its origin in the study of the form" (Waddington, 1968), as its task is to understand why molecules, organelles, cells, tissue and organs have the form they do, and how they get that way. Moreover, understanding the morphogenetic process is essential not only for developmental biology, but it would also deepen our knowledge of reparative processes, cancer emergence and aging (Levin, 2011a).

A huge body of literature has been produced in the field. We have facts in abundance, but few general relationships with which to weave the particulars into a comprehensible pattern. However, data in themselves will not suffice: no new principle will declare itself from beneath a heap of facts. It is timely, even now, to distil from the avalanche of observations whatever general principles can be discerned.

The questions addressed by D'Arcy Thompson are still awaiting a satisfactory answer, though. What do these forms mean: are they products of natural selection, frozen accidents of biological history, or expressions of higher-order morphogenetic laws? These riddles define the scope of the field; we have no satisfactory solution to any one of them, and to find the answers we shall plainly require both guiding principles and much experimental information that are not now available.

In the meantime, data has been progressively gathered, highlighting *how relevant the form might be in ensuring the proper function*. Indeed, it has long been recognized that cell shape influences significantly many

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cellular functions (Watson, 1991). The complex structure of the living cell is critical for cellular function, and it has recently been argued that the spatial organization of the cell is even more important for cellular properties than is its genetic, epigenetic, or physiological state (Harrison and Brugge, 2010). Numerous cellular behaviours - including proliferation, metabolism, stem cell commitment and many others - have been found to be determined by cellular geometry (Bissel et al., 1977; Singhvi et al., 1994; McBeath et al., 2004). So far, it is not surprising that shapes and structures of cells and tissues are as diverse as the functions ascribed to them.

For a long time, before the onset of molecular biology, comparative studies of cell morphologies have been a matter of extensive investigation. The outstanding advances in the understanding of genetic and biochemical processes over the last half-century have progressively shifted the focus of cell biology from the structural features of cells towards its underlying molecular constituents, according to a reductionist paradigm (Schock and Perrimon, 2002). However, a framework based on a set of linear gene-protein interactions has proven to be unable to explain the emergence of complex forms living organisms acquire, as morphogenesis is likely to involve biochemical and biophysical cues provided by the three-dimensional (3D) organization of cells, tissues and organs (Gilbert and Sarkar, 2000; Carroll, 2005). The fact is that a cell is not a bag of enzymes and metabolites interacting each other according to a simplified, linear kinetic model, operating in an equilibrium regimen. In a large part cell processes function in a solid state: signalling molecules, membrane receptors, regulatory enzymes, are often coated and immobilized on the cytoskeleton and on other insoluble structural elements, distributed within the cytosol and the nucleus. Several processes are shown to be spatially ordered and therefore are tightly linked to the geometrical ('topological') configuration the cells assume, with respect to their internal components, as well as to the overall organization (the 'form') they acquire. Significant examples include the co-localization of tyrosine kinases with focal adhesions complex, some signalling pathways and certain mRNAs associated to cytoskeleton microfilaments (Vogel and Sheetz, 2006). In the nucleus, specific transcription factors, growth regulatory proteins, splicing factors and DNA-repair enzymes are also associated with nuclear scaffold. Those data highlight the relevance of cell architecture in modulating and driving biochemical processes as well as gene expression. Therefore, local sensing of force or topological (geometrical) relationships with the extracellular matrix (ECM), enable cells to translate those cues into biochemical signals that result in biological responses.

Unfortunately, for a long time, current working models have not properly considered the structural context and thereby both mechanical influences and normal macromolecular scaffolds were not included in the theoretical and experimental framework (Ingber, 1993). Yet such influences must be taken into account in order to understand how cells and tissues support biological functions. In order to do so, novel technologies have been developed to extensively investigate the inner cell's architecture and to correlate modification in structure to changes in biochemical processes (Théry, 2010). Those attempts have lead biophysicists, biologist and engineers to unite their efforts, therefore ensuring the take-off of molecular cell engineering as a novel scientific discipline.

This kind of investigation has shown how morphogenetic features emerge stochastically, according to a *self-organizing process*, so they cannot be explicitly encoded into the developmental-genetic "program", even if the genetic program is tuned to exploit them (Gibson and Gibson, 2009). Evidence in support of that framework has been provided by 3D-cultures of normal and malignant cells. Indeed, normal epithelial cells rapidly lose many features of their differentiated state upon dissociation and culture on plastic or glass substrata (Bissell et al., 1973; Bissell, 1981). On the contrary, morphological characteristics and functional properties are largely recovered by growing cells in a three-dimensional structure which provides the essential bio-mechanical cues that enable the cells to behave as a coherent, topologically organised, complex system (Barcellos-Hoff et al., 1989; Pageau et al., 2011). Such results demonstrated that 'signals' from the 'proper' surrounding microenvironment are required for the establishment and the maintenance of tissue organization, as well as of differentiating cell functions and morphology (Krause et al., 2010, 2012).

Cell shape acquisition is mainly driven by biophysical forces

Undoubtedly, the specificity of each individual cell shape is determined by both external as well as internal (mechanical, genetic and biochemical) cues. However, genes do not determine biological shape: they only participate providing basic units that are henceforth assembled into a 3D-architecture through a self-organizing process (Honda, 1999). Indeed, across the animal kingdom there is no one-to-one correspondence between homologous genes and morphological structures, and therefore processes leading to acquisition of a specific form depend mainly on where that particular cell is in the body and at what point of development time it is (Wray and Abouheif, 1998). Morphogenesis and phenotypic differentiation are time- and space-dependent processes (Nelson and Bissell, 2006): morphological plasticity, rather than being the result of genetic adaptation, reflects the influence of external physicochemical parameters on any material system and is therefore an inherent, inevitable property of organisms (Newman et al., 2006). As a consequence, the same cell type may adopt different shapes in response to different microenvironments. As an example, chondrocyte morphology is polygonal on

plasma fibronectin, but elongated on cellular fibronectin (West et al., 1984). Fibronectin in the micro-environmental medium promotes cell spreading of mammary epithelia (Hynes and Yamada, 1982), while basement membrane and laminin foster rounding of breast cells (Roskelley et al., 1994; Streuli et al., 1995).

The physical milieu integrating the different chemical and physical signals that drive cells and tissues towards differentiation is generally known as the morphogenetic field (Gilbert et al., 1996; Belousov et al., 1997; Beloussov, 2001). Within the field, morphogenetic cues exert short- and long-range influences by affecting gradients of morphogens and physico-mechanical stresses, through a non-local control of pattern formation. Moreover, morphogenesis is strongly dependent on the "substrate geometry" of the field: the geometrical configuration of the extracellular matrix on which cells are cultured determines anisotropy in physical forces acting on cells, and consequently influences differentially cell-to-cell interaction (Nelson et al., 2005).

According to this framework, the geometric form a cell acquires (i.e., its shape) represents the integrated endpoint of the morphogenetic cues acting on the living system: morphogenesis is indeed the process through which a population of cells rearranges into a distinctive shape. According to D'Arcy Thompson, "the form, then, of any portion of matter, whether it be living or dead, and the changes of form which are apparent in its movements and in its growth, may in all cases alike be described as due to the action of force. In short, the form of an object is a 'diagram of forces', in this sense, at least, that from it we can judge of or deduce the forces that are acting or have acted upon it: in this strict and particular sense, it is a diagram-in the case of a solid of the forces which have been impressed upon it when its conformation was produced, together with those which enable it to retain its conformation" (D'Arcy, 1917). In turn, cellular and tissue geometries act as both a template and instructive cue for further morphogenesis (Nelson, 2009).

These data evidence that cell shape cannot be understood when keeping cells isolated from their tissue. Indeed, single, isolated cells, by analogy with bubbles or liquid droplets, tend spontaneously to acquire a spherical form, thus minimizing both their surface area, that is to say, they reach the lowest energy level (Sackmann, 1994). Therefore, despite the complexity of cell architecture, the shape of isolated cells is determined to a large extent by surface tension. However, cells in tissues are only rarely rounded, thus outlining how important cell-to-cell and cell-ECM interactions are in shaping the form a cell ultimately acquires. That is to say, in living tissues surface tension is modulated by the opposite action of cortical tension and adhesion, both of which participate in remodelling the cell shape and so forth, conditioning their functions (Lecuit and Lenne, 2007). Cells 'adapt' their shape to different substrate's stiffness, and the spatial distribution of adhesion sites, as well as the geometrical configuration of their adhesive environment, determine the forces acting at sites of adhesion (Bischofs et al., 2009). The resulting balance of forces shapes cells form, and so modifies their spreading area, their motility, their contractile forces on adhesion sites and, eventually, their fate itself (Discher et al., 2009). It is worth noting that such a process behaves according to a non-linear dynamics, characterized by bistability and threshold values. A bifurcation analysis of cellular contractility as a function of substrate stiffness reveals a bistable response, thus defining a lower threshold of stiffness, below which cells are not able to build up contractile forces, and an upper threshold of stiffness, above which cells are always in a strongly contracted state (Besser and Schwarz, 2010). At breaking symmetry points, cells undergo abrupt changes in their morphology and in contractile forces, suggesting that different substrate stiffness induces different morphological responses.

Indeed, with the only remarkable exception of cells circulating in the blood, cells rely on their reciprocal adhesion structures and on their attachment to the ECM for proper modelling, growth, and function. Removing cells from their microenvironment may lead to programmed cell death ("anoikosis") (Taddei et al., 2012), inhibition of replication or differentiating processes (Ruoslahti and Reed, 1994). In some instances, putting cells into an "inappropriate" microenvironmental context can otherwise trigger pathological issues, and even neoplastic transformation (Biskind and Biskind, 1944). As a matter of fact cells will react to both the geometrical configuration and the biomechanical features of their environment, by modifying their structure as well as their behaviour (Dalby et al., 2004), according to the "contact guidance", a complex phenomenon earlier described by Weiss and Garber (Weiss and Garber, 1952). Indeed, external physical cues are likely to exert their regulatory role by influencing both ECM stiffness as well as the architecture of the cytoskeleton (CSK). The CSK, a complex framework of interconnected microfilaments and microtubules, provides the inner structure embedded into the cytosol of eukariotic cells (Fey et al., 1984). Cytoskeletal filaments both generate and resist mechanical loads. In addition, cytoskeleton components contribute in shaping cell form and in assuring resilience to shape distortion. The CSK is a network of three major structural elements: microtubules, intermediate filaments, and microfilaments, each consisting of polymers of protein subunits (Rodriguez et al., 2004). Cells generate mechanical tension in their actin CSK and exert tractional forces on their adhesion to ECM. Changes in the balance of forces between cells and ECM induce modifications in surface tension and in matrix properties (flexibility, stiffness, adhesivity). In this way physical cues can change cell shape and switch cells towards different phenotypic fates (Chen et al., 1997; Mammoto and Ingber, 2009). Protein complexes at the ECM-membrane-actin cytoskeleton junctions (e.g., focal

adhesions and fibrillar adhesions) provide physical connections between the extracellular and intracellular compartments, foster cell-to-cell connectivity, and eventually orchestrate actin cytoskeleton organization, cell shape modulation and other fundamental cellular processes (Ingber, 1997). Early events in force detection mechanically induce cytoskeletal changes that result in biochemical signals to mechano-responsive pathways than ultimately regulate cell form (Giannone and Sheetz, 2006).

Components of the CSK play a key role in motility, transport and cell division, providing essential scaffolding on which metabolic processes occur. Therefore, cytoskeletal morphology is thought to be a valuable indicator of cell injury and functionality (Fumarola et al., 2005). Inner cytoskeleton structure also provides 'privileged' pathways along which enzymes and substrates are coherently organized and oriented, in order to optimise their interactions (Ingber, 1993). Those biochemical activities are in turn deeply affected by shape changes and mechanical stresses that interact with the cytoskeleton architecture (Chicurel et al., 1998). A key feature of the cytoskeleton is that it is in a state of isometric tension, which ensures that various molecular-scale mechanochemical transduction mechanisms proceed simultaneously and produce a concerted response. It is likely that at least one pivotal mechanism through which these complex behaviours are modulated is mediated by shape control on focal adhesion structures. Indeed, focal adhesion, formation and organization are governed by both internal cytoskeletal and mechanics that result from large-scale changes in cell shape (Chen et al., 2003) (Fig. 1).

Mechano-transduction of physical forces along the cytoskeleton and the adhesion structures involves the nucleus organization as well. Because extracellular forces are transmitted to the nucleus, where they cause substantial deformations, it should be no surprise if these forces could directly or indirectly contribute to changes in chromatin structure, transcriptional activity and nuclear organization, given that the 3D- architecture of chromatin is a critical component of nuclear gene regulation (Le Beyec et al., 2007). "These features of living architecture are the same principles that govern tensegrity (tensional integrity) architecture, and mathematical models based on tensegrity are beginning to provide new and useful descriptions of living materials" (Ingber, 2008).

In addition, cell and tissue shapes are deeply sensitive to higher-level cues, namely, those exerted by the tissue as a whole. A neglected but meaningful experiment on the pronephric duct of polyploid salamander, made by Fankhauser (Fankhauser, 1945), and recently reviewed by Marshall (2011), provided relevant insight in the field. That paper found that "as ploidy increased, cell size increased without any increase in the diameter of the duct, so that the number of cells seen in a cross-section dropped from five to eight in haploids to three to five in diploids, and went down to one to three in pentaploids. In pentaploids, even though there was just a single cell, that one cell folded over to create a duct lumen within itself. This argues that the shape of a single cell can be greatly altered in order to produce a specified form for the overall tissue [...] These results provide a tantalizing hint that there is a fundamental tendency for a tissue to form a particular overall structure, and that the same structure will tend to form regardless of how its living material is partitioned into cells" (Marshall, 2011).

In turn, spatial patterning of individual cells generates global changes in tissue architecture that drive morphogenesis and the pattern of localized proliferation (Nelson et al., 2005). Cells of expanding epithelial buds proliferate more rapidly than cells located only micrometers away in the clefts of the same gland: reiteration of this simple rule over time and space leads to the fractal patterns of each tissue. It is harsh to ascribe that behaviour to a soluble morphogens gradient, as it emerges looking over the length scale of a single cell. Alternatively, that local growth differential suggests a kind of 'positional information', entangled within a geometric, ordered field resulting from the dynamic equilibrium of several physical forces (Gibson et al., 2006). The mechanisms underlying such processes are probably complex, and could arise, among others, from interactions with neighbouring cells (Blankenship et al., 2006; Farhadifar et al., 2007) and extracellular matrix constituents (Théry et al., 2006), tightly linked to the cell topology, i.e. the position a cell has in a 3D-microenvironment. In fact, by constraining cells to growth on microfabricated ECM islands of different shapes, Nelson and Bissel (2006) demonstrated a differential proliferation pattern, linked to the position

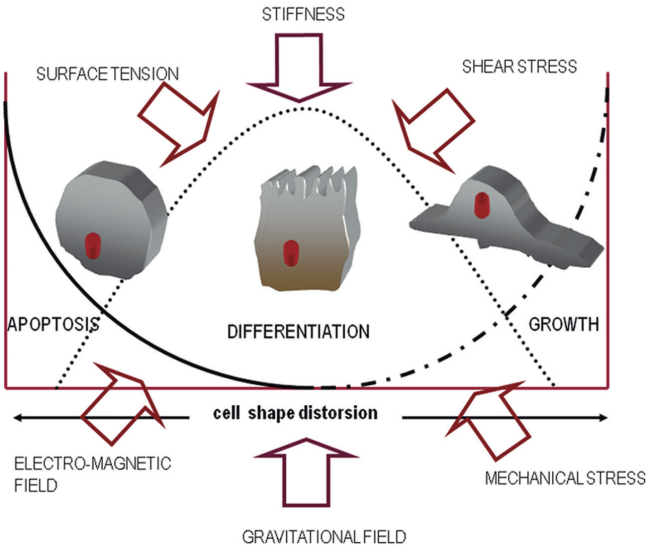

Fig. 1. Biophysical constraints and interactions with the extra-cellualr matrix shape cellular forms. In turn, modification of the cell shape triggers different biological pathways, leading to differential cell fate.

the cells acquire. High proliferating cells were found to be located at the periphery (edges of rectangles and outer boundary of circles and rings) of the islands, while DNA labelling indices dropped to undetectable levels in the centre of the aggregate. Computational modelling studies revealed that the positions of sustained growth corresponded to sites where mechanical stresses were highly concentrated. These forces are transmitted by both cytoskeleton and cell-cell adhesions, as proliferation gradients were significantly hampered after disruption of cell adhesion and cytoskeleton structure. These results indicate clearly that the geometry of the tissue dictates both cell morphogenesis and the growth pattern, providing positional information (Jaeger et al., 2008), which enables cells and tissues to recognize their location relative to each other within a complex 3D-structure.

In addition to chemical gradients and physical forces transmitted across the cytoskeleton, bioelectric properties of cells and tissues provide positional cues also. Experimental manipulation of transmembrane potentials can induce growth, phenotype reprogramming, reversing of the left-right asymmetry of internal organs, and cell differentiation (Adams et al., 2007; Levin, 2009, 2011b; Pai et al., 2012). In addition, exposure to a static magnetic field induces F-actin rearrangement and profound shape modification in lymphocytes (Chionna et al., 2003).

The relevance of physical forces in shaping cell form is dramatically highlighted by studies performed on cells growing in microgravity (Fig.2). The disruption of the normal gravity-dependent equilibrium of physical forces acting on a tissue may easily produce mutations and/or induce relevant changes in a gene's function: this is precisely what happens when cells and tissue are exposed to microgravity (Han et al., 1999; Hammond et al., 2000). However, it is noteworthy that such modifications are anticipated by dramatic changes in cell morphology, as well as in the fractal values of their form. Previous studies have documented both cytoskeleton disruption and morphological changes in a wide set of living cells exposed to microgravity, suggesting that cell shape change might be considered a paramount parameter of response to gravitational changes (Carmeliet et al., 1998; Sytkowki and Davis, 2001; Gaboyard et al., 2002). Indeed, microgravity affects microtubule self-assembly and thus hinders the correct organization of intermediate filaments and cell's adhesion sites (Papaseit et al., 2000). Simulated hypogravity induces in murine osteoblasts a significant decrease in fractal values, already after the first hours. Fractal dimension becomes afterward relatively stable and does not change significantly again, thus indicating that cells quickly acquire a new "morphological" equilibrium. This trend is accompanied by a parallel slow-down of cell proliferation with a concomitant loss in structural complexity (unpublished data).

Function follows form

Modification in cell morphology is an often under-appreciated aspect of experimental methodology.

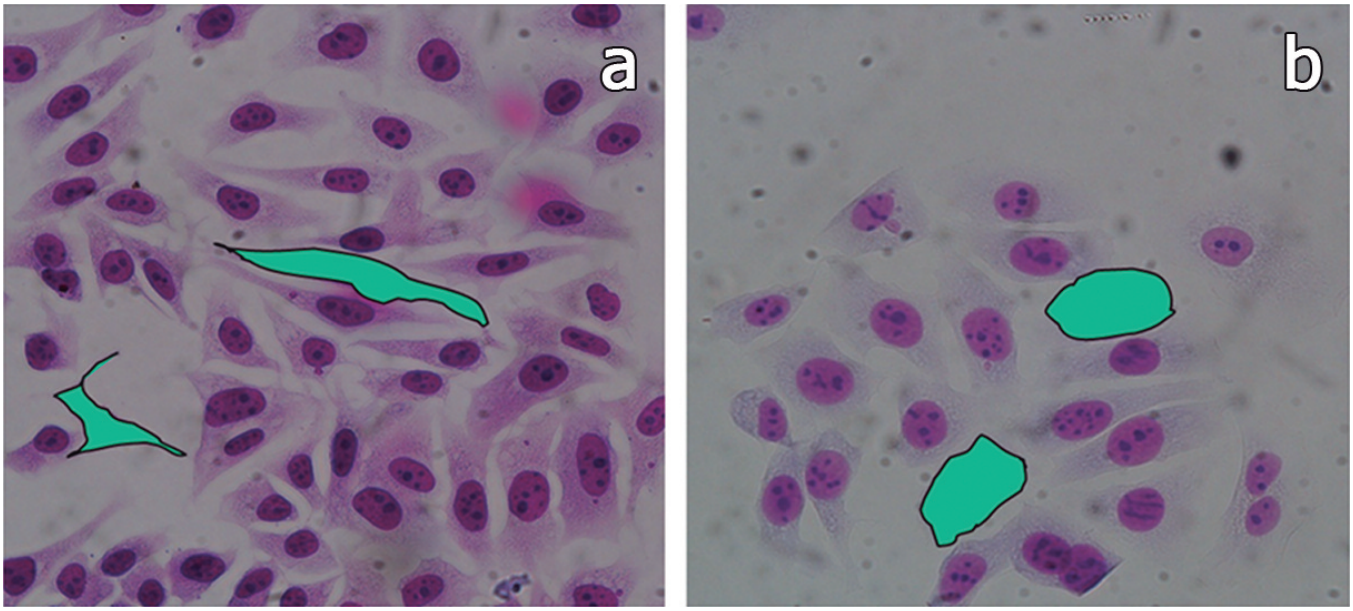

Fig. 2. Shape modification under microgravity. MCF-7 cells seeded on ground **(a)** and on simulated-microgravity on the Random Positioning Machine **(b)**, after 72 hours (personal communication).

However, a compelling set of results highlight the relevance of shape-phenotype relationships that over two decades ago motivated Folkman and Moscona (1978) to ask, "How important is cell shape?" Indeed, early evidence that cell shape changes have a functional significance came from experiments designed to force cell shape changes, tacking cells "into isolation" from the influences exerted by the extracellular matrix: morphological changes caused by depriving cells of substratum contact were shown to directly and dramatically influence both cellular growth and differentiation (Allan and Harrison, 1980; Shannon and Pitelka, 1981; Glowacki et al., 1983). Any mechanical factor able to induce change in cell shape and cytoskeleton will likely lead to relevant modification in biological functions. Indeed, highly stretched (distorted) cells exhibit enhanced sensitivity to mitogens, while cells with more rounded shape are prone to apoptosis (Chen et al., 1997; Ingber, 2005). Extracellular-dependent cell spreading was shown to control the growth of endothelial and hepatic cells (Ingber, 1990; Mooney et al., 1992), whereas the transcription of hepatocyte-specific genes was specifically promoted by cell rounding (DiPersio et al., 1991). Acquisition of a round shape leads cells to trigger specific gene expression in keratinocytes, steroidogenic cells, retinal pigmented epithelial cells and osteoblasts (Opas, 1989; Bidwell et al., 1993; Roskelley and Auersperg, 1993). As a general rule, cells spreading over a large surface, survive better and proliferate faster than round cells. On the contrary, cells constrained to grow on a restricted substratum contact area acquire a round shape and are committed to terminal differentiation and DNA-synthesis inhibition (Watt et al., 1988). In addition, changes in cell morphology are sufficient to induce epithelial-mesenchimal transition in breast and kidney cells (Ben-Ze'ev, 1984; Nelson et al., 2008). Considering the relevance of such processes during cancer development, it is tempting to think that microenvironmental-driven changes in shape morphology can significantly contribute to neoplastic transformation.

How can a modification in the form a cell acquire lead to such a relevant change in biological function? A provocative answer to the question has been provided by D.E. Ingber, who evidenced how cell shape represents a visual manifestation of an underlying balance of mechanical forces, which in turn convey critical regulatory information to the cell (Ingber, 1997, 2008). In turn, cell distortion significantly modifies both cytoskeleton and cell adhesion to ECM, leading then to significant modifications in several biochemical and genetic processes. In this way, cell shape changes influence complex, biological functions, like cell-to-cell adhesion, apoptosis, differentiation and proliferation (Britland et al., 1996; Chen et al., 1997). Cells growing on different microenvironments are submitted to different physical constraints (stiffness, surface tension and so forth) and consequently they acquire different morphologies and different pattern of gene expression (Dalby et al., 2005). As a consequence, cells acquire selective functional and differentiating features (Kenny et al., 2007). Indeed, functional differentiation depends upon the degree of complexity of the tissue architecture, thus indicating that the "ultimate regulator" of cell function is the tissue architecture itself (Bissel et al., 2003).

Quantitative measure of shape: fractal analysis

It has been claimed as "at present we have no rigorous way to define the level of organization in a cell. We are thus left to our subjective visual impression to say that the cell type X is more organized than cell type Y [….] For cellular complexity, we currently lack a good way to quantify polarization and organization" (Marshall, 2011). Indeed, despite many years of research, a method to precisely and quantitatively assess cell and tissue morphology remains elusive. Current practice for characterizing solid tumours, for example, involves the use of varying systems of tumour grading and staging and thus leaves diagnosis and clinical staging dependent on the experience and skill of the physicians involved. Moreover, although numerous disease markers have been identified, no combination of them has yet been found that produces a quantifiable and reliable measure of disease state. Therefore, more compelling quantitative methods to investigate morphological features are warranted. That goal would allow the assessment of the morphological complexity of cells and tissues using a "systemic approach".

Several attempts have been made in this field in order to minimize variability (Andrion et al., 1995) in histopathological evaluation and ensure eventually automated, highly reproducible image processing (Rohrschneider, 2007; Demir and Yener, 2009). Despite some promising results (Street et al., 1905; Heckman, 1990; Heckman and Jamasbi, 1999), a new conceptual framework, as well as image-analyser tools are warranted.

Fractal studies starting in the course of the last two decades may likely provide such an opportunity, as fractal analysis shows promise as an objective measure of seemingly random structures.

A fractal (from the Latin 'fractus', 'broken') is an object with a non-integer dimension that looks exactly the same at every scale. Fractals are irregular objects that display self-similarity or scale-invariance. Fractal patterns with various degrees of self-similarity have been studied in images, structures and found in nature and technology (Falconer, 2003). Euclidean descriptions are not adequate for complex irregular-shaped objects that occur in nature. These "non-Euclidean" objects are better described by fractal geometry, which has the ability to quantify the irregularity and complexity of objects with a measurable value called the fractal dimension (Mandelbrot, 1985).

A geometrically intuitive notion of dimension is as

an exponent that expresses the scaling of an object's bulk with its size:

$$\text{bulk(N)} = \text{size}^{\text{dimension}} \quad (1)$$

Here, bulk may correspond to a volume, a mass, or even a measure of information content, and size is a linear distance. For example, the area (bulk) of a plane figure scales quadratically with its side (size), and so it is two-dimensional, while a volume is related to the cube of its side. By transforming such relationship through the use of logarithms, we obtain a general equation of the form:

$$\text{Dimension} = \lim_{size \to 0} \frac{\log bulk}{\log size} \quad (2)$$

where size is generally expressed as a fraction of the entire bulk: 1/N = K. This ratio is generally known as homothetic, meaning the operation able to geometrically transform the space without changing its form, i.e. preserving the pattern between its constitutive elements. Bulk can be divided into N fractions (similar to the entire bulk), and each of those fractions has a length equal to 1/N=K. So, we obtain

$$D_{\text{segment}} = \frac{\log N}{\log N} = 1$$

while for an area we will have

$$D_{\text{area}} = \frac{\log N^2}{\log N} = 2$$

For a fractal object, like the Koch snowflake we have in the example reported in Figs. 3a, 4 segments similar to the entire bulk, each one equal to 1/3 of the entire length. Thus the (fractal) dimension of that object can be calculated as

$$D = \frac{\log 4}{\log 3} = 1,262$$

Its fractal dimension (1.262) therefore exceeds its topological dimension '1', providing a quantitative measure of the space-filling capacity of a pattern that tells how a fractal scales differently from the space in which it is embedded in (Sagan, 1994). Mathematically, dimension is expressed by so-called "power laws", since the equation (1) shows that some quantity, N, can be expressed as some power of another quantity, s:

$$N_{(S)} = S^{-\tau}$$

Taking the logarithm on both sides of the equation we find a relationhip indistinguishible from (2). By plotting log N(s) versus log s we obtain a straight line (the signature of the power law), being τ (a non integer number) the slope of the straight line. The scale invariance can be seen from the fact that the straight line looks the same everywhere (Fig. 3b).

Since the discovery of the fractal geometry of nature, fractals have become essential components in the modelling and simulation of a wide range of physical and biological phenomena. Indeed, as outlined by G.A. Losa, fractal science "has provided an innovative paradigm, a novel epistemological approach for interpreting the natural world and a more intelligent vision of life itself (in the etymological sense of the Latin word intelligere)" (Losa, 2009). Many living processes display fractal behaviour and/or a fractal structure: bronchial tree architecture (Shlesinger and West, 1991), heartbeat dynamics (Goldberger et al., 1985), protein surfaces (Goetze and Brickman 1992),

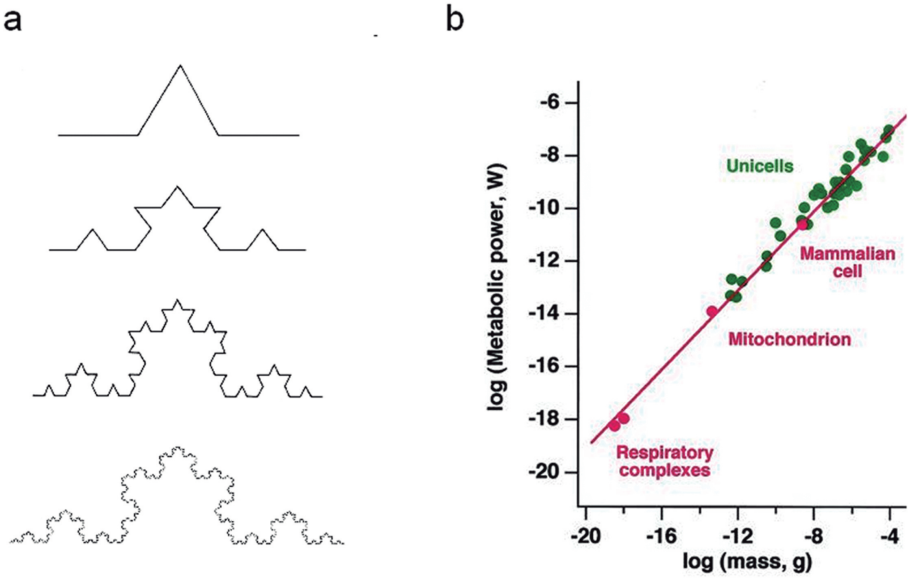

Fig. 3. a. Koch snowflake. Four segments similar to the entire bulk, each one equal to 1/3 of the entire length. Thus the (fractal) dimension of that object can be calculated as

$$D = \frac{\log 4}{\log 3} = 1,262$$

Its fractal dimension (1.262) exceeds its topological dimension '1', providing a quantitative measurement of the space-filling capacity of a pattern that tells how a fractal scales differently from the space that it is embedded in. **b.** Example of scale invariance according to a power law. The measurements of body mass versus metabolic rate shows that the metabolic rate R for all organisms follows exactly the 3/4 power-law of the body mass, i.e., $R \sim M^{3/4}$. It holds equally well from the smallest bacterium to the largest animal. The relation remains valid even down to the individual components of a single cell, such as the mitochondrion, and the respiratory complexes.

cells (Keough et al., 1991; Vilela et al., 1995), nuclear membranes (Landini and Rippin, 1993), chromatin structure (Einstein et al., 1998), fetal breathing dynamics (Szeto et al., 1992), microbial growth pattern (Obert et al., 1990), metabolic network pathways (Sernetz et al., 1985), fetal heart rate (Gough, 1993), convoluted surface of mammalian brain (Hofman, 1991), neural networks (Goldberger and West, 1987), long-range power-law correlation in DNA (Peng et al., 1992), neuronal shape (Caserta et al., 1990), pattern in human retinal vessels (Family et al., 1989), structure of biomembranes (Nonnenmacher, 1989), blood vessel systems (Zamir, 1999).

Fractal analysis also applies to complex phenomena that lack a single time scale (Goldberger, 1996). Fractal processes are characterized by generating irregular as well as non-stationary fluctuations across multiple scales (Peng et al., 1995). It is noteworthy that this fractal organization breaks down with aging and disease (Losa et al., 2002), suggesting that such processes are characterized by a loss of complexity (Goldberger, 1997). Indeed, with aging and pathology, fractal anatomic structures, as well as physiological rhythms, may show degradation or modification in their structural complexity.

Classical models of control are framed according to Cannon's concept of 'homeostasis', by which physiologic equilibrium lies in a constant 'steady state'. However, this approach is unable to reflect the overwhelming complexity underlying the regulation of physiological rhythms. Stasis is not a healthy condition at all. Health is maintained by a state of dynamic equilibrium, which allows the body to adapt and respond while maintaining stability, and thus the notion of 'homeostasis' is a misnomer. Physiologic stability is related to complex patterns of variability that incorporate long-range correlations, together with distinct classes of non-linear interactions. Indeed, "fractal physiology, exemplified by long-range correlations in heartbeat and breathing dynamics, may be adaptive for at least two reasons: (1) long-range correlations serve as an organizing mechanism for highly complex processes that generate fluctuations across a wide range of time scales and (2) the absence of a characteristic scale may inhibit the emergence of very periodic behaviours that greatly narrow system responsiveness" (Golberger, 2006). Therefore, the paradoxical appearance of highly ordered dynamics with pathologic states ("disorders") exemplifies the concept of complexity loss ('de-complexification') in aging and disease.

Physical meaning of fractals

The enormous success of linear, analytical mathematics since Newton is in large part responsible for the reductionist attitude of most twentieth century science, the belief in absolute control arising from detailed knowledge. Chaos science, i.e. non-linearity analysis, is the rediscovery that linear, integrable equations do not have infinite power as it outlines the relevance of a wide variety of scientific and engineering problems which do not respond to calculus (Yoshida, 2010). Non-linear theory has much to do with complex systems and fractals. Indeed, an object, which is chaotic in space, is called a "fractal" part. Similarly, a system displaying non-linear dynamics in time will likely have a fractal profile: indeed, its trajectories converge towards one or more 'fractal attractors', when they are represented in a phase-space. The specific feature of such a system is represented by the sensitivity to initial conditions. Let us consider two points in phase space, extremely close to each other: the two ensuing trajectories, though close to each other at the beginning, will eventually diverge exponentially away from each other, leading to different stable states, even for the same parameter values (a property known as bistability). That behaviour, in complex non-linear systems is the rule rather than the exception, and it says that any small uncertainty that may exist in the initial conditions will grow exponentially with time, and eventually it will become so large that we will lose all useful knowledge of the state of the system. Sensitivity to initial conditions is therefore the death of reductionism (Prigogine, 1997). Moreover, non-linearity is at the root of fractal emergence. Take a non-linear dynamical system. Pick a simple region in its phase space, such as a sphere or a cube or any other simple volume. Consider this region as a locus of possible initial conditions. Then let time flow. As each point of the region follows its trajectory, the region itself moves and changes shape. In the course of its evolution, slowly but surely the region will turn into a fractal. The fractal builds up as time progresses and becomes complete at infinite time. Therefore, every non-linear dynamical system is a fractal-manufacturing machine. Conversely, every fractal can be seen as the possible result of the prolonged action of non-linear dynamics. As such, a fractal can be thought of as a stable structure arising from non-linear dynamics, counteracting, in some way, the 'chaotic' processes entangled within the phase space. That is because within that phase space the system can reach different configurations (attractors), some of which are sufficiently stable and robust (resilient to external perturbations) to allow the emergence of (fractal) structure (Golberger, 2006). Such characteristics are a specific feature of complex systems.

A complex system possesses a structure spanning several scales and at every scale we find a structure, which leads to emergent behaviour. Emergence happens when you switch the focus of attention from one scale to the coarser scale above it. A certain behaviour, observed at a certain scale, is said to be emergent if it cannot be understood when you study, separately and one by one, every constituent of this scale, each of which may also be a complex system made up of finer scales. Thus, the emerging behaviour is a new phenomenon special to the scale considered, and it results from global interactions between the scale's constituents. The combination of

structure and emergence leads to self-organization, which is what happens when an emerging behaviour has the effect of changing the structure or creating a new structure. As non-linear systems are chaotic some of the time, this means that complex systems might be exposed to the 'risk' of chaotic processes (which are likely to drive the system towards disorganization and death). However, complex systems are only rarely truly chaotic, as they counteract chaos through self-organization and dissipative processes, reaching thermodynamically stable configurations. Indeed, a complex system always has several scales. While chaos may reign on scale n, the coarser scale above it (scale n ? 1) may be self-organizing, which in a sense is the opposite of chaos. By this way order comes form disorder. The resulting self-organizing structure shows itself to be more complex than the previous one. Therefore, complexity *involves interplay between chaos and non-chaos that ultimately leads to self-organization into fractal structures*.

Shape is produced by a self-organizing process

Self-organization processes lead to the emergence of a 'form' (shape) in space. That visible representation of the dynamic process represents a reliable index of complexity, as it refers to the effective system's degrees of freedom. Even if we still await a reliable definition of complexity, the number of degrees of freedom that the system possesses might measure that characteristic. However, even if there may be many nominal degrees of freedom available, the physics of the system may organize the motion as well as its shape into only a few effective degrees of freedom, represented in the phase space by a discrete number of attractors (Ricard, 1999). That property explains why a complex system displays only a *few discrete numbers* of spatial configurations or chemical processes. For that reason, organisms present only a small fraction of all possible forms. Like any other hierarchical construct, biological entities might potentially adopt an undefined possibility of configurations ("forms"). In living organisms, from a theoretical point of view, as the number of regulatory interactions grows, so does the number of phenotypes that can potentially be sampled. However, only a limited number of them is actually observed: an expansion in genotypic space makes the phenotypic space shrink owing to increased canalisation (or genetic robustness), which itself is probably a result of a larger number of non-genetic interactions. So, although increasing regulatory diversity allows a system to explore novel phenotypes, it also allows it to become more robust in specific areas of phenotypic space. In the case of protein structures, the number of folds is much lower than that expected when referring to the transfinite number of possible dispositions of N residues in space; different sequences may give rise to the same fold or, in turn, the same sequence can fold into the same three-dimensional form (Cordes et al., 2000). A similar behaviour has been documented for RNA polymers (Schultes and Bartel, 2000). This implies some sort of 'energy minimization' drastically constraining the number of allowable stable states (Denton and Marshall, 2001). As the 'form' is how self-organization manifests itself at the 3D-level, the fractal dimension can be considered a parameter of the 'true' (as opposed to the theoretically available number of configurations) complexity (Cutting and Garvin, 1987; Nonnenmacher et al., 1994), and as such can be thought of "in much the same way that thermodynamics might view intensive measures as temperature" (Smith et al., 1996). In other words, fractal dimension is a system property, and, as such, together with one or more independent variables, it could enable the construction of a diagram of phase transitions (like that relying on temperature, pressure and volume for gases) aimed at describing the evolution of a living system (Dinicola et al., 2011). In turn, it is not surprising that changes in the fractal dimensionality of cell membrane could mirror parallel changes in biophysics of the biological systems (Wang et al., 2010).

The process leading to an organized form is an extraordinary example of self-organization (Kushner, 1969; Goodwin, 1994). Indeed, one of the most astonishing properties of a self-organizing process is how they induce recognizable changes in the form the system acquires as a result of a phase-transition, involving both local and long-range rules. Self-organization apparently defies classic thermodynamics, as the emergence of new structures is associated with decreased entropy. Prigogine demonstrated how that paradox is only apparent, given that open thermodynamic systems (like living organisms) are dissipative: the apparent decrease in entropy is achieved by dissipation into the microenvironment, so that the total entropy of the system increases, and the second law of thermodynamics is satisfied) (Nicolis and Prigogine, 1977).

It is of utmost importance that self-organization arises in dissipative dynamical systems after symmetry breaking (Goldenfeld and Woese, 2011; Longo and Montévil, 2011), whose post-transient behaviour involves fewer degrees of freedom than are nominally available. The rupture of the symmetry gives the system a historical dimension, a sort of memory of the past event that took place at a critical moment, and which will affect the next evolution. Let us now highlight some relevant consequences linked to the rupture of symmetry, generally addressed by the physics of criticality (Binney et al., 1992). Transition to the critical point allows the system to acquire relevant features, such as long-range correlations and scale invariance. Long range correlations means that the determination of the system is thereafter global and not local. Scale invariance indicates that after symmetry breaking, the system presents the same behaviour at each scale. Scale invariance allows the system to acquire a 'structure of coherence', that is to say that local process are 'globally' determined, expressing a 'wholeness', and a complexity that "cannot be understood as "the sum of many local

behaviours by adding more and more local, possibly hidden, variables" (Longo and Montévil, 2011). The system is attracted to a lower-dimensional phase space, and the dimension of this reduced phase space represents the number of active degrees of freedom in the self-organized system. In this way the system relaxes to a state in which chaos has a low probability to emerge (low-dimensionality allows dramatic reduction of the system's stochasticity, constraining it into the defined boundaries of an attractor).

These attractors are stable configurations, characterized by fractal dimension and scale invariance (Chang et al., 2008). Thus, estimating dimension from a time series is one way to detect and quantify the self-organizational properties of natural and artificial complex systems. Given that such ordered organization relies only on the non-linear dynamics of the system, it is likely that the emerging structure will mainly depend on interacting biophysical cues. That is to say "much (and perhaps most) of the order that we see in living nature is an expression of properties intrinsic to complex dynamic systems organized by simple rules of interaction among large number of elements" (Goodwin, 2000). Indeed, a substantial amount of order is given for free by physics and not by the genetic code, thus "providing what we believe is the first convincing challenge to the Darwinian claim that cumulative selection for biological function is the major or sole generator of all organic form" (Denton et al., 2003). Turing (Turing, 1952) first described how simple non-equilibrium reactions could spontaneously produce morphological patterns in time and space, a finding further substantiate by the Belousov-Zhabotinsky experiment (Zaikin and Zabothinski, 1970). These self-organizing patterns are truly emergent properties, which are controlled by global parameters that determine the characteristics of both long-range activation and short-range inhibition. "Yet, molecular biologists are either unaware or overlook these elegant theoretical approaches to pattern formation in biological morphogenesis" (Weiss et al., 2003).

Fractals in biology

Mathematical fractal objects and models of dynamical systems characterized by non-linearity and multistability display self-similarity at all scales (Peitgen et al., 1992). However, for any real object in nature, the power-law property holds only for a limited range of scales. Therefore, efforts must be made to ensure that the experiment is performed within the proper interval of the independent variable. Naturally fractal objects are statistically self-similar only within a narrow range of scale, defined by upper and lower cut-off values (Cross, 1994). The dimension of a naturally occurring fractal is therefore associated with self-similarity over some regions of space or interval of time. Such fractals are referred to as 'truncated fractals'. A truncated fractal pattern is a common feature of attractors and basin boundaries belonging to the phase-space of dissipative systems, characterized by a non-linear dynamics (Paar et al., 2001). In such systems, the appearance of fractality at a certain range of scale can be associated with a higher tolerance in physiological functions, which is important for the adaptability of biological systems (West and Deering, 1994). The aforementioned considerations shed light on the preference shown by nature for truncated fractal structures: biological networks and living functions display higher robustness and ability to cope with external perturbations when they are organized into a truncated fractal structure (West, 1990). Thus, fractal geometry may eventually provide an evolutionary advantage to fractal processes, over those having classical structures and processes.

The aforementioned considerations are supported by the relevance the cell shape has in pathology and histopathology. A clear-cut link between cell geometry and cell function was firmly established a long time ago by means of simple morphological observations and yet, today, morphology constitutes the basis for anatomopathological diagnosis (Rosai, 2001). Indeed, significant relationships have been made between cell shape and modifications in phenotype, as well as between cell shape and several diseases such as cancer (Lelièvre et al., 1998). Furthermore, fractal dimension analysis is a useful method for quantitatively describing the process of cell differentiation. Oligodendrocyte-type 2 astrocyte lineage was allowed to differentiate in vitro and its fractal dimensions were measured over time. The fractal dimensions of the maturing cells correlated with perceived complexity; cells with elaborate process branching had larger fractal dimensions than cells with a simpler morphology (Behar, 2001).

Fractal analysis provides a useful tool to decipher the CSK structure and dynamics. Indeed, the structured (fractal) organization of the cytoplasmic milieu, as well as of CSK, can deeply influence enzymatic kinetics and biological functions. Cytosol is not a homogeneous colloidal soup in which processes behave according to classical diffusion and kinetic laws: it is not a "simple Newtonian fluid" (Clegg, 1984). Cytoplasm is compartmentalized by spatial and temporal variation of its internal organization, quantitatively described as fractals of the type of percolation clusters (Rabouille et al., 1992). Processes structured in percolation clusters and belonging to a fractal milieu display astonishing properties. Below a "percolation threshold pc the spreading process is confined to a finite region […] below this value the cluster behaves as locally connected while above pc the connection extend indefinitely […] Near the critical probability pc […] the percolation process undergoes a transition from a state of local connectedness to one where the connections extend indefinitely"; thus, "local cytoplasmic behaviour when subjected to fluctuations or perturbations may extend and globally impose that behaviour to far remote regions in the cellular cytoplasm" (Aon and Cortassa, 1994). Enzymatic reactions can be influenced by topological

segregation of the reactants, or because a volume may fractally evolve into an area by fractal folding. Thus, a biological system can greatly enhance the targeting of a molecule through modification of its dimensionality (Dewey, 1997): "a cell, by regulating the geometry or architecture of its CSK, in turn regulates the level of its percolation threshold, pc. Different pc levels will determine when the local level of a messenger or the product of an enzymatic reaction may extend to the whole cellular field" (Aon et al., 2000). The fractal organization of the cytoplasm is mainly supported by the architecture of CSK (Aon and Cortassa, 1993), and so it is not surprising that the polymerisation-depolymerization of CSK components may lead to relevant changes in cytosol fractality and, consequently, in enzymatic kinetics. This meaningful example highlights how the shift from local, microscopic disorder to macroscopic coherence (characterized by long-range correlations) would be given by a change in the inner fractal structure of the cell. In this way, the non-linear dynamics of a 'shape' parameter (the 'form' the CSK acquire), may affect a higher level of organization and functionality, giving rise to self-organised behaviours and synchronized processes (Cortassa et al., 1994). Furthermore, promising insights into how a cell's nucleus holds molecules that interact with DNA in their right location have been recently provided by fractal analysis. The movement of molecules within cells has been tracked in a lab dish, and then compared with the pattern of movement against mathematical models (Bancaud et al., 2009). The same rules have been demonstrated to apply to both large and small molecules, thus suggesting that the environment in which they move was truly fractal. In this way, molecules crowded together in different areas of the nucleus can change the way they interact with each other. Crowding is mainly determined by the chromatin profile itself, as it looks like a coastline displaying fractal structure, with holes allowing or blocking access to the different-sized molecules. Therefore, both small and large molecules are obstructed as they are facing the same crowded environment, regardless of scale. On the other hand, the fractal architecture differs between heterochromatin and euchromatin, and predicts that chromatin proteins use different target-search strategies in the two compartments. Euchromatin possesses a larger fractal dimension, and therefore it offers more exposed DNA at its surfaces. More accessible DNA surfaces can be scanned more efficiently by nuclear factors than in heterochromatin, thus favouring active transcription. In contrast, heterochromatin, which fills space more compactly (by having a reduced fractal dimensionality), is likely to present less exposed DNA at its surface, which is therefore less accessible (Cortassa et al., 1994). That is to say that the dynamics of soluble nuclear proteins are affected independently of their size by the fractal model of chromatin organization. The bulky fractal structure of chromatin could encourage proteins to hop around over large stretches of DNA, making it easier for them to scan for their target sequences. "Proteins that help to keep genes inactive, by contrast, often do so by altering histones — and because histones are plentiful, the inactivating proteins need to move more systematically. The flatter fractal structure of heterochromatin should encourage them to stick close to be able to do this. Therefore, the nucleus might be able to switch the behaviour of different areas of DNA simply by altering the fractal structure of chromatin" (Ainsworth, 2009).

The extracellular matrix and the microenvironment, to which cells of an organised tissue belong, also display a fractal dimensionality. Tissue structure provides a particular spatial organization allowing interactions between its various components, modulating the diffusion of regulatory cytokines and other signalling molecules (Meakin, 1988). Anomalies in the fractal tissue structure emerge as a consequence of complex cell-stroma interactions, and the loss of the proper fractal structure is associated with several pathological processes, such as cancer (Naeim et al., 1996; Eid and Landini, 2003). In studying tissue architecture a specific, fractal measurement which is supposed to measure the 'differences in texture', is provided by lacunarity. Lacunarity is a measure of the non-uniformity (heterogeneity) of structure or the degree of structural variance within an object and not the 'self-similarity' that is typical for a structure with fractal properties (Smith et al., 1996).

A quantitative method particularly useful for characterizing the fractality of irregular structures is bending energy analysis (Bowie and Young, 1977). The Normalized Bending Energy (NBE) is defined as the amount of energy required to transform the contour of a specific shape into its minimum-energy state, namely a circle ('curvegram') with the same perimeter as the original object (Young et al., 1974). A curvegram, representing the spectral decomposition of NBE at different spatial scales, can be accurately obtained by using digital signal processing techniques, and it provides multi-scale representation of the curvature. These kind of approaches have received increasing attention given that NBE has a thermodynamic meaning and may be viewed as an intensive measure of morphologic complexity (Bohr and Tél, 1988).

Cell shape and cancer

Cancer cells show several morphological differences compared to their normal counterpart, thus providing the basic rationale for their diagnostic identification (Fox et al., 1976). It is worth noting that shape undergoes progressive changes during neoplastic transformation, and that such feature might provide not only reliable diagnostic information, but also provide useful insight into carcinogenic mechanisms (Heckman et al., 1987; Marchok et al., 1978). These modifications involve both cytoskeletal integrity and cellular adhesive properties that are progressively altered in cells with increased

malignant potential, even in advance of any sign of tumour "progression" (Heckman and Olson, 1979).

Cancer cells are characterized by a large nucleus, having an irregular size and shape, the nucleoli are prominent, and the cytoplasm is scarce and intensely coloured or, on the contrary, is pale (Abercrombie et al., 1957). The nucleus of neoplastic cells plays, through its changes, a main role in the assessment of tumour malignancy. Changes concern its surface, volume, the nucleus/cytoplasm ratio, shape and density, as well as structure and homogeneity. Ultrastructural characteristics are related to nucleus segmentation, invaginations, changes in chromatin, such as heterochromatin reduction, increase of interchromatin and perichromatin granules, increase of nuclear membrane pores, formation of inclusions, etc. The cytoplasm also undergoes changes: new structures appear or normal structures disappear. The accumulation of ribosomal and RNA-messenger in the cytoplasm makes it basophilic. Malignant cells have a small cytoplasmic amount, frequently with vacuoles. At the same time, the cell membrane undergoes several important modifications, resulting in an irregular, spindled and flattened profile. Many tumour cells show immaturity of the cell surface, a higher degree of cell lability, and have a propensity for more modification, all features not seen in most differentiated adult cells. On the surface of malignant cells, atypical microvilli, pseudopods and vesicles with extremely active enzymatic equipment appear (Mareel and De Mets, 1984). Major changes involve the overall shape configuration and the underlying CSK architecture. Cyto-architectural changes are indeed common in transformed epithelial cells and they are thought to play a relevant role in both shape change as well as in the acquisition of new biological functions (Manger and Heckman, 1982).

However, although nearly a century of research has sought to identify a "simple" characteristic possessed by all tumour cells, the result of such inquiry has been recognition of the constantly evolving and complex character of all neoplastic cells. The achievable diagnostic accuracy is still tightly linked to the experience of the pathologist. This experience is gained from years of examining surgical biopsies, studying and comparing morphologic features, and relating these images to eventual case outcomes. However, even highly experienced pathologists examining the same biopsies may disagree on the diagnosis, suggesting fundamental differences in perception, visualization, and utilization of their recalled images (Connolly et al., 2000).

These limitations require for a different approach, aimed at 'quantifying' the complexity underlying the cancer cell profile. In seeking to apply measures of complexity to cancer in order to determine the correlation of those measures with the disease state, fractal analysis has been proven to be particularly useful, as documented by a continuously growing body of evidence (Baish and Jain, 2000; Spillman et al., 2004). However, despite the extensive effort made up to now and the huge body of scientific papers published so far, we are still waiting for a satisfactory definition of 'biological complexity'. The classical Shannon's communication theory is not ideally suited for describing a biological network information. Therefore, numerous articles have suggested alternative definitions (Prigogine, 1997; Albert et al., 2000). However, some theoretical approaches have little practical interest, when considering that parameters and variables thought to measure complexity are difficult to compute and translate into biologically meaningful features (Adami, 2002). A suitable, even if not exhaustive, conceptualisation of complexity should be addressed in terms of the required information for system description, as proposed by Chaitin (1974). Accordingly, a system could be considered ''maximally complex" when the rate of change of the irreducible amount of information required describing that system is an extremum. Such a definition fits well with one of the measures of complexity we shall consider, that is, the fractal dimension of cell shape.

These considerations shed light on basic biological mechanisms, and are also important from a clinical perspective. Indeed, fractal analysis can lead to a remarkable improvement in cyto-histological and radiographic diagnostic accuracy (Rangayyan et al., 1997; Rangayyan and Nguyen, 2007), providing reliable and unsuspected information (Cross, 1997). Entropy-based fractal image modelling, together with advanced image processing and pattern recognition, has been used in order to increase the diagnostic accuracy of mammographic detection of breast tumour (Sankar and Thomas, 2009). Mammographic density correlates with the fractal dimension of breast cancer and eventually with higher growth rate: therefore, tumour masses with higher fractal values show increased aggressiveness and poor prognosis (Norton, 2005). Fractal analysis has helped in discriminating benign from malignant tissues (Cross et al., 1995), nevi from melanomas (Carbonetto and Lew, 2010), and low- from high-grade tumours (Claridge et al., 1992). Furthermore, fractal studies allow the discovery of new markers, providing useful insights into cancer identification and prognosis (Adam et al., 2006; Ferreira et al., 2006; Nielsen and Danielsen, 2006).

Fractal studies elucidated some aspects of the complex interplay between cancer cells and their stroma by suggesting how tumour vascular architecture is determined by heterogeneity in the cellular interaction with the ECM rather than by simple gradients of diffusible angiogenic factors (Gazit et al., 1995). Moreover, fractal analysis of the interface between cancer and normal cells helps in understanding how cell detachment from the primary mass and infiltration into adjacent tissue occurs through a non-gene-based mechanism (Michaelson et al., 2005). Furthermore, changes in the epithelia/stroma interface following transition from benign to malignant disease are highly

correlated with the surface irregularity measured by fractal dimension (from 1.0 of normal epithelium to 1.62 for invasive tumour) (Landini and Rippin, 1996): both the global and local fractal dimension of the epithelium-stroma interface increases from normal through premalignant to malignant oral epithelium, implying that the involvement of the epithelium-stroma interface is not merely a consequence of tumour development, but instead is an intrinsic feature of the carcinogenic process: "thus, the landmark of tumour aggressiveness would not be solely a localized tumour cell proliferation [...] but a global switch or bifurcation between smooth margin and .fingering protrusions surface patterns [...] tumour growth occurs if surface tension or cell-cell adhesion is strong enough, but irregular, infiltrative structures resembling irregular outer boundaries of tumours emerge if these parameters are smaller" (Tracqui, 2009).

Moreover, studies focusing on nuclear shape revealed strong correlations between shape change and modifications in cellular phenotype (Lelièvre et al., 1998). Microenvironmental-induced shape changes in chondrocyte nuclei correlate with changes in collagen synthesis (Thomas et al., 2002) as well as in cartilage composition and density (Guilak, 1995). Changes in nuclear morphology are frequently associated with the acquisition of higher stiffness, and the latter is considered a prerequisite of the increased motility observed in metastatic cancer cells (Wolf, and Friedl, 2006). In turn, changes in nuclear shape may interfere with chromatin structure, and modulate gene accessibility and nuclear elasticity required for translocation, leading to a large-scale reorganization of genes within the nucleus (Dahl et al., 2008). On the other hand, nuclear morphology correlated significantly with survival time of patients with glioblastomas. This correlation was independent of other prognostic factors: total surgical resection, patients' age, and classification of the tumour as a primary or secondary glioblastoma (Nafe et al., 2005).

Fractal analysis has been proven useful in discriminating between high and low grade tumours, through the study of their respective epithelial-stroma architecture. Indeed, significant differences exist between the mean fractal dimensions corresponding to different tumour grades, given that the mean fractal dimension increases with increasing tumour grade (Tambasco and Magliocco, 2008). These results further outline the relevance of cancer-stroma relationships in supporting neoplastic behaviour.

Tumours have a very irregular, non-smooth boundary. These irregularities are usually present over a range of length scales, implying that the tumour boundary has a fractal nature, and could be characterized by a fractal dimension (Losa, 1995; Coffey, 1998). This means that the tumour invades the available space in a non-uniform way, which can be reliably described by its fractal value. Indeed, the roughness of the interface

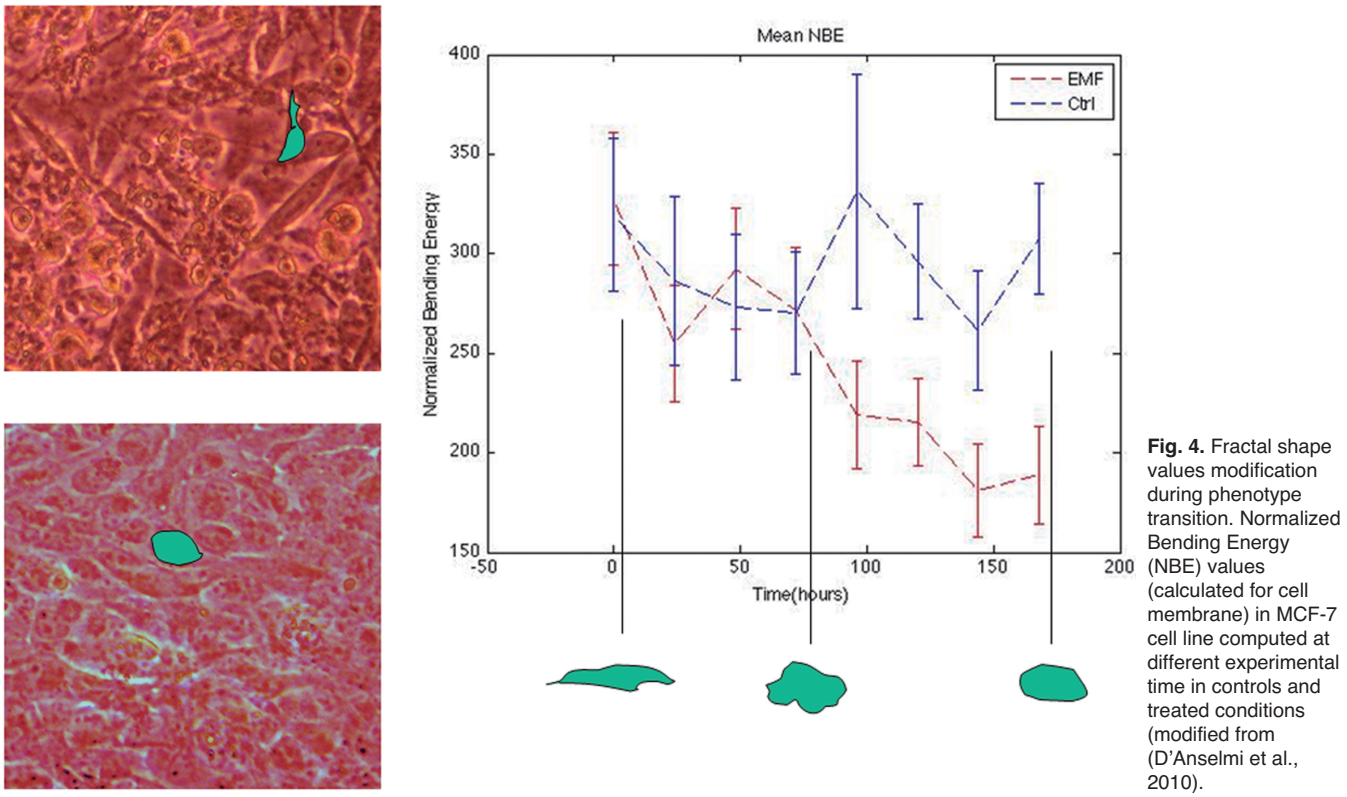

Fig. 4. Fractal shape values modification during phenotype transition. Normalized Bending Energy (NBE) values (calculated for cell membrane) in MCF-7 cell line computed at different experimental time in controls and treated conditions (modified from (D'Anselmi et al., 2010).

between the tumour and its surrounding environment is an indicator of whether the tumour is likely to become infiltrative or not. Tumours whose interfaces are very rough are seen to be more aggressive, while ones with relatively smooth boundaries are less likely to be highly infiltrative. Therefore, as the fractal dimension is closely related to the 'roughness' of cancer boundaries, it might provide useful information on the tumour invasiveness and, in a more general sense, into its behavioural features (Franze et al., 2008).

Indeed, some fractal studies have been performed to quantify the invasiveness, migratory property and treatment-responsiveness of cancer: high malignant, invasive and resistant cancer cells display higher fractal values (Sullivan et al., 2010). Similarly, chemo-resistant colon cancer cells have been demonstrated to display higher fractal dimension than their non-resistant counterparts. In invasive, chemo-resistant colon tumour cells, NBE values have been found to be significantly greater than those recorded in wild-type, non-resistant cancer cells (Pasqualato et al., 2012). High fractal dimension values are associated to a ''diffusive'' shape, the form the cell acquires when it displays an invasive pattern, the stage that precedes metastatic spreading (Rohrschneider et al., 2007). Indeed, the fractal dimension increases along with the proliferation index (according to a correlation factor of about R=1) (Izquierdo-Kulich et al., 2009). On the basis of these experimental data a clear-cut relationship between the fractal dimension of the cell pattern, the fractal value of the tumour-host interface, and the ratio between mitosis and apoptosis rate has been established on the basis of a mesoscopic model able to predict tumour cell dynamics and behaviour. These data highlighted that the more invasive the cancer cells, the higher fractal values are. On the contrary, tumour cells undergoing phenotypic reversion under treatment showed a significant reduction in their shape-related fractal values (Bizzarri et al., 2011). Whereas neoplastic transformation is characterized by a progressive increase in cell fractality, the reversion of breast cancer phenotype is followed by an impressive change in both the form and the fractal dimension of the cell profile (Fig.4) (D'Anselmi et al., 2010). Accordingly, carcinoma cells P19 undergoing differentiation when treated with all-trans retinoic acid show a significant reduction in their fractal dimension (Waliszewski and Konarski 2002). Thus, fractal analysis of tumour colonies demonstrated to be able to discriminate between treated and not treated samples with higher accuracy with respect to standard procedures (Sungkaworn et al., 2006). Fractal measurements enable one to follow modifications induced in cancer cells by drug or endocrine treatment as well (Losa et al., 1998). Therefore, reversion into a more ''physiological'' fractal-dimension implies reduced morphologic instability and an increase of cells connectivity. These data emphasize the relevance of shape fractal parameters as descriptors of cell transition from one phenotype to another.

Shape as a system's property

Changes in cell morphology have been extensively studied as they are universally correlated with eukariotic cell differentiation. It is now increasingly clear that the specific configuration a cell acquires plays a fundamental role in modulating gene expression and complex biological functions. Cell shape is tightly connected to cell activity, and can be considered as "the most critical determinant of cell function (…) cell shape per se appears to govern how individual cells will respond to chemical signals in their local microenvironment" (Ingber, 1999). As a consequence, measurable parameters describing shape could be considered as 'omics' descriptors of the specific level of observation represented by the cell-stroma system. Within that framework shape can reliably be considered a "system's property", reflecting the thermodynamics and the non-linear dynamics underlying biological processes. Such an approach promises to formalize some of basic mechanisms and, ultimately, provide a holistic understanding of biological processes (Anderson and Quaranta, 2008).

The current renewed increased interest in shape studies is moving from research highlighting how important morphostasis is, i.e. the maintenance of appropriate form. As keenly outlined by M. Levin, "morphostasis allows organisms to resist aging and tumourigenesis for decades while individual cells senesce or undergo DNA damage". Therefore, it turns out that "the restoration of shape is a central goal of regenerative medicine [indeed] maintenance of correct patterning of tissues and organs is the cornerstone of health: many biochemical interventions ultimately entail an attempt to restore the body's 'goal state' with respect to shape" (Levin, 2011b).

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