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ORIGINAL ARTICLE

Gravity constraint in cell phenotypic determination

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Abstract

Distinct phenotypes emerge spontaneously when mammalian cells are cultured under microgravity conditions. Such finding is explained by the interplay among the intrinsic stochasticity, which, in turn, is successively 'canalized' and sustained by the activation of a specific gene regulatory network. However, when the two cell subsets are reseeded into a normal gravity field the two phenotypes collapse into one. Gravity constraints the system in adopting only one phenotype. Cell fate commitment is achieved through a de novo reshaping of the overall cell morphological and functional organization, and cannot be explained as a 'selecting' effect. Those findings highlight how constraints – acting as global order factors – drive cell specification and behavior. These data cast on doubt the current explanatory bottom-up, molecular based models.

Keywords: microgravity, constraints, cell fate commitment, non-equilibrium thermodynamics, non-local control

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1. Differentiation: a complex issue

The current prevailing paradigm in biology posits that biological process can be exhaustively explained according to an 'instructive' molecular model where molecules drive the systems towards specific, irreversible, commitments, ultimately leading the unfolding of a 'program' already 'embedded' into genes. This model has been already questioned from both the experimental and theoretical point of view (Noble 2012).

According to the classical molecular paradigm, differentiation is viewed as the accomplishment of a 'genetic program' throughout the consequential activation of a multistep signaling cascade, supposed to be modulated by a set of genetic regulatory elements (Maniatis 1987). Despite receiving some confirmation, such model (Rieger 2009) is currently deemed inadequate in gasping the overwhelming complexity of the differentiation process (Orkin 2008; Robb 2007).

For instance, insertion of the erythropoietin receptor into macrophage precursors allows erythropoietin to stimulate macrophage colony formation, without promoting the de novo growth of red blood cells.

Conversely, insertion of the macrophage colony-stimulating factor receptor into erythroid precursors allows M-CSF to stimulate the development of erythroid clusters (McArthur 1994). Moreover, differentiation could occur even in the absence of both growth factors and cell division (McArthur 1994; McArthur 1995).

Therefore, cell fate specification cannot be longer viewed as the deployment of a strictly deterministic instructive program.

2. Stochasticity and Gene Regulatory Networks

Currently, cell-differentiating processes are interpreted by adopting a Waddington's landscape as an explicating framework (Waddington 1957). In this diagram stable and metastable states are recognized by calculating values of the Gene Regulatory Networks (GRNs) as ordinary differentiated equations (ODE) - which activation state define a sub-set of gene expression patterns. Mathematical formalization of the landscape in the last decades has led to the definition of the multidimensional dynamical systems framework (Kauffman 1969; Huang 2005).

Activity of GRNs is assumed highly sensitive to small changes in cell environment, thus 'capturing' stochastic events described at the molecular level. Besides this framework represents a true advancement in respect to the old-fashioned deterministic models – which posit a linear correspondence among genes, proteins, and cell functions – it is still insufficient in grasping the overwhelming complexity of cell regulation during cell fate commitment (Noble 2012).

Several experimental and modelling approaches based on different GRNs architecture provided compelling evidence that stochasticity in gene expression pattern shall likely allow to the emergence of a wide range of gene patterns that could account for the difference in phenotypes observed among even isogenic cell populations (Elowitz 2002; Kupiec 1983). The random pattern of gene expression produces probabilistic outcomes by activating switching mechanisms that select between alternative regulatory paths, ultimately partitioning an isogenic cell population into different phenotypes as the cells follow different paths (McAdams 1997). The transition from a single to a bi-stable phenotypic system is triggered by stochasticity coupled to the non-linear dynamics of the transcriptional regulatory network (Huang 2009).

Thus, intrinsic noise enables the phenotypic diversification of identical cells exposed to the same environment by amplifying the response of slight differences between cells when challenged by diverse kind of perturbations (Neildez-Nguyen 2008).

Indeed, the spontaneous emergence of phenotypic heterogeneity in isogenic cells is a frequently observed and reproducible phenomenon (Salazar-Ciudad 2001). Noise can modify the expression of genes fully involved in cell decisions making, and those changes can dramatically influence GRN state of activation, involving nested positive and negative feedback loops, thus giving rise to bi- and tri-stable systems, i.e. promoting the emergence of co-existing, differentiated phenotypic states within the same cell population (Lu 2013).

However, it would be expected that the intrinsic noise (stochasticity) in gene expression patterns would be reduced and finely 'canalized' to support the de-novo emerging phenotype. Instead, approaching a phase transition, the intrinsic noise in gene expression increases steadily, while the stochastic dynamics governing the switching of cells from one differentiation state to another shows a peak in gene expression variability at the point of fate commitment (Richard 2016). In fact, commitment of progenitor cells to a new phenotype is preceded by the destabilization of their high-dimensional attractor state, such that differentiating cells undergo a critical state transition (Mojtahedi 2016).

Indeed, the most challenging aspect of Waddington's framework arises at the bifurcation points, where cell fate decisions take place (Moris 2016). Stochasticity may destabilize an attractor state thus resulting (with different probabilities) in various transcriptional profiles, characterized by the emergence of gene expression patterns that would support independent cell 'identities'. In principle, such expression profiles are transient, given that intrinsic randomness continuously challenges their stability. This may suggest a typical state of « extended criticality », as described in Longo-Montevil (Longo 2014). The collection of these different transcriptional profiles is properly a 'transient state' and it represents a raw substrate for cell fate switching, but in its own cannot decide about the fate the cell will choose. An external, 'driving' factor is therefore required to 'push' cell fate into one well-defined direction, choosing among those provided by the branching tree.

Therefore, what makes the cell takes the irreversible decision to differentiate at a point when the system seems to be totally disorganized? How cells can be deterministically driven toward a specific state notwithstanding the intrinsic stochasticity of a complex system like a living cell? What kind of global cues act to provide an order (an organization) to a system apparently ruled only by stochasticity?

3. Constraints and global order cues

Without more form of active/changing forces or constraints, the GRN model is, metaphorically, a purely syntactic system, and, as such it is incomplete, and requires a semantic partner (Rosen 2000). In other words, a living system necessitates an environment with which it can interact.

Indeed, many environmental factors have been proven playing an invaluable role during stem cell fate specification and phenotypic determination (Guilak 2009). It should be stressed that the biological purpose of cell fate specification is to provide the constitution of different tissues and organs, a process that cannot be conceived as a transformation occurring in single, isolated cells. Instead, this process take place into a field – the morphogenetic field – integrating many different biochemical and physical cues (Bolker 2000). This preliminary, basic premise implies that the transition from a phenotype to another one should be investigated by considering the cross-talk occurring among cells and their biophysical environment.

However, hardly GRN-based models could translate for the changes occurring in the surrounding microenvironment or in the overall cell structure. Furthermore, GRN formalism is not suited to contemplate the influence of field-dependent physical factors, as gravity or electromagnetic fields.

Many of those microenvironment-dependent factors are currently recognized as 'constraints', and usually fall in the class of *physical* factors, including mechanical stress, stiffness, surface tension, shear stress (Clause 2010). Constraints may arise from the interaction dynamics of the elements of the system (cells and their molecular components), or may be generated by the system at higher levels (tissue, organ), while other very singular constraints depend on the field in which the system is located (gravitational and magnetic field).

Perturbations induced by these factors - even when they are applied locally, within a discrete region of the system – are propagated to the entire system, showing long range correlations. As such, they affect the overall system, without necessarily targeting single, discrete molecular pathways. In addition, when these physical factors are considered as 'control' parameters - by analogy with physical phase transitions - they may trigger an 'all-or-nothing' transition (a 'first' class transition from a configuration to another) when their values are trespassing some threshold values.

Constraints represent *additional forces on the system that contribute to shaping the system dynamical behavior*, but are usually neither described nor considered. This situation is the consequence of the general Lagrangian/Hamiltonian dynamical formalism currently adopted for systematically construct coherent dynamical models for systems that are free or at least that do not work on their constraints, the canonical form for reversible dynamics. It should be stressed that constraints may reshape the overall topology of the Waddington's landscape, as they modify the dynamical bifurcation tree (Hooker 2013).

Evaluating how pure physical constraints actually modulate cell fate specification is however a hard task, given that the physics governing events often changes with scale, so that the models themselves must change in structure as the ramifications of events pass from one scale to another (Green 2017).

Therefore, besides some fruitful attempts have been made by investigating the behavior of living matter under the influence of modified physical fields (like the bioelectromagnetic field (Levin 2012), scientific investigations are usually rare or uniquely based on computer modelling.

Studies in microgravity could help in settling this issue, as biological changes can be investigated by experimentally removing the constraint (gravity), without interfering *directly* with the intrinsic molecular cell machinery.

4. Spontaneous emergence of different phenotypes in microgravity

Different kind of mammalian cells cultured in real and simulated (weightlessness) conditions undergo deep changes in shape morphology (Masiello 2014; Testa 2014; Grimm 2014).

Shape changes occur even after six hours of microgravity conditioning, when two different morphologic phenotypes emerge and cells are partitioned almost equally into two phenotypically different populations. One population is represented by flat, spindle cells adhering to the substrate, while clumps or rounded cells constitute the second subset, floating in the culture medium (Chang 2006; Stockholm 2010).

Distinct modifications in CSK architecture, gene expression and biochemical/biophysical properties are associated with this morphological remodeling. Both populations underwent a large rearrangement of F-actin, α-tubulin, and vimentin compared to on ground control cells (see Figure 1a).

In microgravity conditions, F-actin filaments of adherent cells showed a disappearance of the complex cytosolic network, which appeared mostly localized on the cell border (see Figure 1b). In floating cell clumps, the actin meshwork appeared completely disrupted, and the filaments were mainly localized behind the cell border. Tubulin meshwork was also altered in both cell phenotypes, showing disruption of the radial pattern, with interrupted filaments disseminated throughout the entire cytoplasm (see Figure 1c).

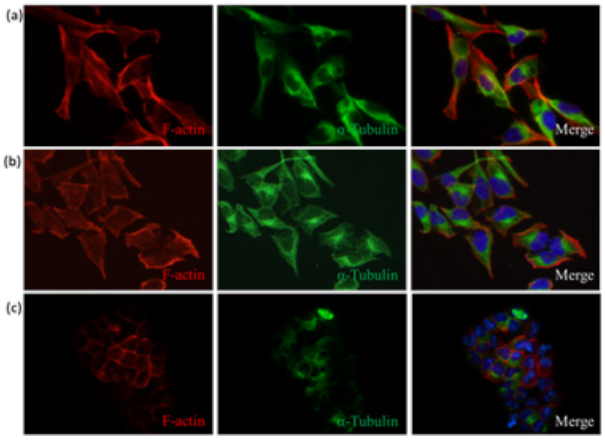

Figure 1. Immunofluorescence images of F-actin and α-tubulin in MDA-MB-231. Rhodamine-phalloidin staining of MDA-MB-231 showing F-actin distribution patterns (red color) and immunostaining of α-tubulin (green color) and HOECHST 33342 to stain nuclei (blue color) after 24 hours in on ground control cells (a), RPM adherent cells (b), and RPM cell clumps (c). Magnification ×200.

The emergence of these two populations is not a transient effect, given that the relative proportion of the two morphological classes remains invariant for the full period of observation (>7 days). Yet, after that period, both cell clusters obtained in microgravity can still recover in few hours their native phenotypic morphology when replaced into normal gravity. In fact, in 1g, the two cell phenotypes collapse into one, undistinguishable from the original, ground-based phenotype. What is even more surprising is that, by separately reseeding the two cell clusters previously obtained during a first-course culture in weightlessness again in the same microgravity field, two distinct phenotypes emerge once more from each cell phenotype. This is to say that each cell cluster always reproduces two distinct phenotypes (adherent and floating) when seeded in microgravity.

The fact that subpopulations of cells selected in microgravity for a given phenotypic state return towards equilibrium proportions when again reseeded into microgravitational conditions suggests that any subpopulation of cells will return to a fixed equilibrium of cell-state proportions over time when the gravity constraint is removed. Such a behavior implies the possibility of one or more interconversions to transition between any two states.

This simple experiment demonstrated that a field 'deprived' of the gravity constraint, does not provide a 'stable' environment for life, as it favors a never-ending transition among differently differentiated phenotype. Overall, the absence of gravity enables the emergence of a 'permanent transition state', where true extended criticality allows the system travelling across unlimited phase transitions. Furthermore, those data indicate that microgravity does not act by selecting a pre-existing phenotype, hidden in the primary population.

The physical constraint (gravity), 'constraints' the overall system in collapsing into only one attractor, i.e. a specific cell differentiated state. This effect occurs notwithstanding the GRN state of activation, and we can surmise it eventually constraints GRN to change its state of activity. Thus, constraint provides the system with a 'deterministic' output that would had be otherwise impossible to obtain. Such findings highlight the role played by constraints as 'organizing principles' (Mossio 2016) and could therefore shed lights in grasping key feature of cell differentiating processes.

5. How gravity constraint works

It is very unlikely that such microgravity driving effects on cell (anomalous) specification could occur through the release of selected, specific 'signaling molecules', unleashed by the removal of gravity given that gravity force is a too weak force for influencing single molecular mechanisms (Pollard 1965). Notwithstanding, living cells do experience relevant gravity effects, involving shape and cytoskeleton rearrangements, and proteomic and gene expression changes (Bizzarri 2014).

In fact, no 'gravity sensors' have been described in animal cells so far and thereby cells cannot directly 'sense' the changes that take place in the gravity field.

However, it should be kept in mind that cell 'insensitivity' to weak physical cues is a tenable assumption only for isolated systems, i.e. ideal systems governed by equilibrium thermodynamics. Such systems are in fact almost completely insensitive to weak electromagnetic or gravitational fields (Kondepudi 1981). The situation is drastically different for non-equilibrium systems (Kondepudi 1998) in which, during phase transitions, when the increased cooperativity among the system's elements increases, the field energy increases too – from mgl/kT to mgl/kT1/3, being mgl/kT1/3 » mgl/kT because of the non-linear dynamics of the system. Thus, the increase in the external energy field overcomes the intrinsic thermal fluctuation. As a result, noise-dependent effects are amplified and propagated along the entire system. In turn, the fluctuation in numerous order parameter values steadily increases as the system is approaching a bifurcation point (Nicolis 1981; Kaern 2005). In such conditions, a system far from equilibrium can form stationary spatial patterns

under the influence of the field constraint. This process involves a global determination of local phenomena, through canalization of the dynamics, which rules the molecular interactions at the local level. These conformational changes are, like phase transitions, cooperative, meaning that they involve interactions between all the component parts.

6. Physical cues actively shape the form the cell acquire through cytoskeleton remodeling

In absence of proper physical constraints – as gravity in our experiments – cells are unable to find a unique, specific differentiated fate. This happens because "gravity constrains development: typically, it canalizes cytoskeletal growth towards relatively flat structures as well as it selects negatively shapes that are unsuitable for subsistence or movement. When this constraint is reduced or disappears, descent with modification yields a larger variety of enabled structures. One may consider then the resulting forms as due to the plasticity of organismal development, as cytoskeleton seem shaped, not just selected, also by gravity (Bravi 2015) ["emphasis added"]. Indeed, the CSK architecture is actively shaped not by 'instructive' cues from the genome, but from environmental physical cues.

CSK remodeling is a self-assembling, non-linear process, highly sensitive to the gravity field. Noticeably, cytoskeletal proteins seem to be the first proteins influenced by microgravity given that the CSK network and its correlated networks of interaction are troubled in a few seconds when cells are exposed to microgravity (Corydon 2016), even before any change in gene expression pattern could be recorded (Gershovich 2012).

Microtubules, a major component of the cytoskeleton, are tubular polymers of two globular proteins (α and β tubulin). Microtubules are affected by a highly dynamic regimen, involving a continuous disassembly and assembly of their monomer constituents. That process is GTP-dependent and characterized by intrinsic instability, leading tubulin to be preferentially added to one extremity of a microtubule while being lost from the other. Ultimately, tubulin monomers self-organize to form stationary macroscopic patterns This process is highly sensitive to gravity (Papaseit 2000), as gravity triggers self-organization of tubulin fibers by 'canalizing' the higher fluctuations produced at the 'bifurcation' time by the partial overall disassembly of microtubules. This interaction causes a 'drift' term, which breaks the symmetry of the transport processes and therefore promotes microtubule growth along a specific direction, to minimize the free energy (Portet 2003). Under gravity, striped patterns of microtubules oriented consecutively at acute and obtuse angles appeared, whereas in weightlessness, no pattern formation arises and microtubules self-organize into an isotropic configuration without preferential orientation (Tabony 1994). Instabilities observed in microgravity-seeded microtubules represent bifurcation points, i.e. symmetry-breaking events leading towards different attractor states – i.e., different ''phenotype configurations' - given that changes in microtubule architecture will ultimately trigger profound modification in cell shape morphology (Ingber 1997). In turn, cell shape changes will significantly affect cell behavior and functions (Folkman 1978). Indeed, the complex dynamic cross talk among CSK and nucleoskeleton (NSK) can modify the chromatin architecture and consequently the gene expression pattern (Nishioka 2002). Early CSK remodeling has been demonstrated to critically affect longer-term differentiating processes (Guilak 2009), while a similar predictive value has been observed by tracking cell morphological changes through long-term, high-throughput time-lapse microscopy (Buggenthin 2017). In addition, CSK changes can modify some pivotal pathways involved in cell fate specification through a subtle modulation of the contractility forces acting on and inside the cells (McBeath 2004).

It is worth noting that CSK components behave as dissipative system, able in 'sense' and amplifying even minor changes in the local balance of forces (Mizuno 2007). Rearrangements of CSK configurations are hence transmitted inside the cell, leading to dramatic changes in histone acetylation and methylation patterns, therefore enacting profound chromatin remodeling (Rehfeldt 2007), thus paving the way for a selective gene transcription process (Li 2006).

7. Constraints allow the system accessing only a limited number of unpredictable phenotypes

In our breast cell model, microgravity enables the emergence of two distinct morphological phenotypes. Yet, we may argue that the 'phenotypic landscape' that the system can freely explore does not include infinite possibilities (configurations) for a set of given constraints that remain constant, i.e., invariant under the time the process is observed, as previously suggested by Waddington (Waddington 1957).

However, besides a system could access only a finite number of phenotypes, this statement does not implies that the number of these phenotypes is pre-given in a 'fixed' landscape. Thereby, a Waddington's landscape depicting phase-transitions as such observed in purely physical systems, it not applicable for living system given that the continual symmetry changes in biological dynamics do not allow applying an a priori formalization of the phase spaces, as happens in physical sciences (Sun 2012; Longo 2012). If constraints could change, hence cell-environment interactions will follows, and then the landscape evolve and changes accordingly (Montévil 2015).

Moreover, changes acquired by travelling along the landscape can be epigenetically retained, and eventually transmitted to the next generation. In this case, maintenance of the species-dependent phenotypic landscape (an 'invariant' feature of each organism within a species) is associated by a continuous addition of novelties inside the same phenotypic cluster. Thereby, "diversity is the result of historicized invariance, as the specificity of each organism depends on its phylo- and ontogenetic history" (Longo 2017).

Indeed, novelty 'emerges' as a result of reorganization of (phenotypic and epigenetic, in that case) traces of the past experience that, ultimately, will lead to reshaping the landscape. In other words, the geometry of the Waddington's landscape is historically designed across times, and it is continuously redrawn by the dynamic interplay among constraints, external cues and the genetic/epigenetic background of the living system. Overall, these data strongly support the notion that cell fate specification can hardly by explained by a gene/molecular-centered approach.

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