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Review

Modeling embryogenesis and cancer: an approach based on an equilibrium between the autostabilization of stochastic gene expression and the interdependence of cells for proliferation

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Abstract

A large amount of data demonstrating the stochastic nature of gene expression and cell differentiation has accumulated during the last 40 years. These data suggest that a gene in a cell always has a certain probability of being activated at any time and that instead of leading to on and off switches in an all-or-nothing fashion, the concentration of transcriptional regulators increases or decreases this probability. In order to integrate these data in an appropriate theoretical frame, we have tested the relevance of the selective model of cell differentiation by computer simulation experiments. This model is based on stochastic gene expression controlled by cellular interactions. Our results show that it is readily able to produce tissue organization. A model involving only two cells generated a bi-layer cellular structure of finite growth. Cell death was not a drawback but an advantage because it improved the viability of this bi-layer structure. However, our results also show that cellular interactions cannot be simply based on raw selection between cells. Instead, tissue coordination includes at least two basic components: phenotypic autostabilization (differentiated cells stabilize their own phenotype) and interdependence for proliferation (differentiated cells stimulate the proliferation of alien phenotypes). In this modified autostabilization-

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selection model, cellular organization and growth arrest result from a quantitative equilibrium between the parameters controlling these two processes. An imbalance leads to tissue disorganization and invasive cancer-like growth. These findings suggest that cancer does not result solely from mutations in the cancerous cell but from the progressive addition of several small alterations of the equilibrium between autostabilization and interdependence for proliferation. In this frame, it is not solely the cancerous cell that is abnormal. The whole organism is involved. Tumor growth is a local effect of an imbalance between all the factors involved in tissue organization.

Keywords: Cell differentiation; Tissue organization; Cell proliferation; Cell death; Stochastic gene expression; Cancer; Selective model; Computer simulation

Contents

1. Introduction

Embryogenesis results in the ordered emergence of an adult organism made up of a multitude of differentiated tissues. Understanding the rules that govern this phenomenon remains a major challenge. For this purpose, molecular mechanisms controlling basic cell processes such as gene expression, cell division, cell differentiation and apoptosis have been widely studied. Because these studies have been conducted in the context of the paradigm of a determinist genetic program, stochastic aspects of cell physiology have been generally neglected. However, a large amount of data have now been obtained either at the cellular or molecular levels, suggesting an important role for stochasticity in gene expression and cellular differentiation. Taking into account these data, both experimentally and theoretically, could greatly increase our understanding of cellular physiology. These observations are all based on the same kind of experiments. If cell differentiation is a determinist mechanism, the behavior of all cells belonging to the same population should be homogeneous. In this frame, one does not predict variability between the kinetics of differentiation of single cells, apart from experimental noise or minor fluctuations. In contrast, in the frame of a stochastic mechanism, variability is expected to occur on a larger scale. To our knowledge, Till et al. (1964) were the first to use this strategy. Because of the variability observed, they suggested that the differentiation of cloned hematopoietic stem cells is a stochastic phenomenon. Since this pioneer work, a similar observation has been made in other experimental systems. In melanoma and leukemia cultured cells, the kinetics of expression of differentiation markers better fit models in which cells are assigned a probability of becoming differentiated, either after each mitosis, or as a continuous function of time (Gusella et al., 1976; Tarella et al., 1982; Bennett, 1983; review: Levenson and Housman, 1981). In a variety of cell types including myoblasts (Lin et al., 1994), goblet intestinal crypt cells (Paulus et al., 1993), hepatocytes (Michaelson, 1993), lymphocytes (Davis et al., 1993), neural crest cells (Baroffio and Blot, 1992), the gonadal cells in *Caenorhabditis elegans* (Greenwald and Rubin, 1992) and in vivo mice hematopoietic cells (Abkowitz et al., 1996), the analyses of cell fate determination also support a probabilistic model of cell differentiation.

At the molecular level, cells expressing the same phenotype and placed in homogeneous environments should always express the same genes if they are controlled by a tight determinist mechanism. In contrast, a large variability in gene expression has been reported between single cells from numerous cell lines, both in vivo and in cultured cells.

In cells stably transfected with a lacZ plasmid under control of either the IL-2 promoter, or the κB and NFAT-1 *cis*-activator elements (Fiering et al., 1990), or with target sequences of steroïd hormones (Ko et al., 1990), only a fraction of cells express lacZ. Similar results have been obtained with an LTR–HIV–lacZ plasmid (Ross et al., 1994). In rat neuroblastoma cells,

heterogeneous expression of the insulin receptor gene has been shown by direct RT-PCR analysis in single cells (Heams and Kupiec, 2003). In cells expressing luciferase genes under the control of HIV–LTR, cytomegalovirus or prolactin promoters (White et al., 1995; Takasuka et al., 1998), transcriptional activity has been reported to vary not only between single cells, but also to be discontinuous in time within the same cell. In multinucleated muscular cells of transgenic mice, different nuclei sharing the same cytoplasm differentially express lacZ placed under the control of the actin, troponin I or HMG6CoA reductase promoters. Heterogeneous expression of these genes has also been directly observed using in situ hybridization with actin and troponin probes (Newlands et al., 1998). Results have even been obtained demonstrating a heterogeneity of expression between the two chromosomes from the same pair in a diploïd organism. Indeed, in situ analyses with specific probes allowing to discriminate the different alleles of a multiallelic locus show that it is not always the same allele that is expressed, at a given time on the two chromosomes for immunoglobulin (Nemazee, 2000), olfactive receptors (Chess et al., 1994), globin (Wijgerde et al., 1995), T and NK receptors (Held et al., 1999) and cytokins IL-2 and IL-4 genes (Riviere et al., 1998; Hollander, 1999). According to the classical theory, the state of activation of a gene is determined by the composition of its nuclear environment in transcription factors. These experiments demonstrate that it is not sufficient and that there must be another important parameter involved in the control of gene expression. Globally, when quantitative analyses of transcription levels are made for series of genes within single cells, such a large variability is observed that the very notion of an "average cell", representing a cell type, that can be questioned (Levsky and Singer, 2003). In fact, all these data suggest a probabilist model, put forth by most of the authors. Within this new paradigm that departs from the classical view of a genetic program, each gene of a cell has a probability of being activated at any time. Instead of leading to on and off switches of genes in an all-or-nothing fashion, the concentration of transcriptional regulators increases or decreases this probability (reviews: Hume, 2000; Fiering et al., 2000; Paldi, 2003). In order to explain stochastic gene expression, it has been proposed that diffusion of chromatin molecules causes random local fluctuations in transcription regulators concentrations along DNA resulting in stochastic variability of gene expression (Kupiec, 1983, 1989, 1997; Ko, 1991; McAdams and Arkin, 1997, 1999; Misteli, 2001). Finally, a series of experiments showing how random fluctuations of gene expression in a gene network could lead to bi-stable states have demonstrated the potential importance of stochastic gene expression in cell differentiation (Becksei and Serrano, 2000; Becksei et al., 2001; Isaacs et al., 2003; Blake et al., 2003).

Taken altogether, these cellular and molecular data support a Darwinian (selective) model of cell differentiation. In the classical instructive model, cells differentiate because they receive signals that direct them to a particular lineage (Fig. 1A). Each signal corresponds to a "command" of the genetic program. In this determinist frame, all cells are expected to react identically to the stimulus and therefore variability is not expected. Instead, in the selective model, cells differentiate primarily because of internal and stochastic events such as the stochastic activation of genes. Cellular interactions act secondarily to coordinate the differentiation of the different cell lines by stabilizing their phenotypes (Fig. 1B; Till, 1981; Kupiec, 1983, 1997; Michaelson, 1987, 1993). Thus, the Darwinian model seems to be in better agreement with the data because it predicts stochasticity and variability in cellular behavior. However, in spite of the accumulating evidence, it is not yet acknowledged as a predominant mechanism, but rather as an

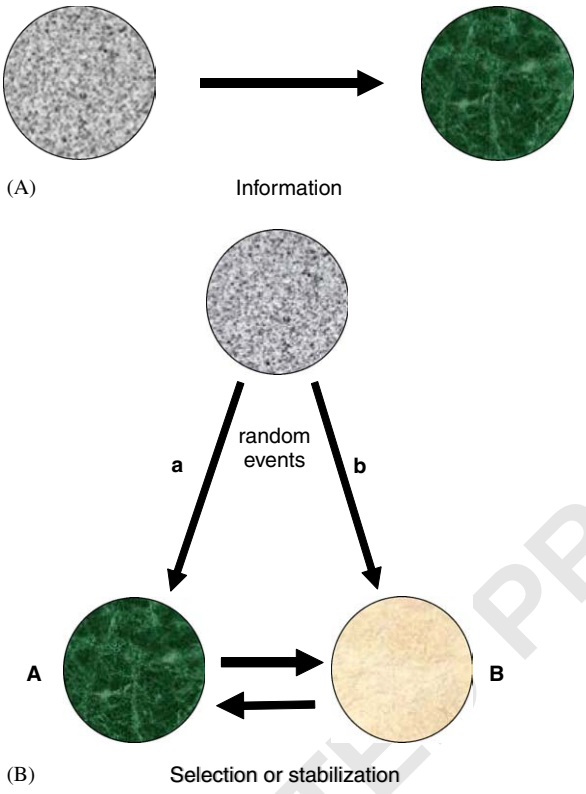

Fig. 1. (A) Instructive (or determinist) model of cell differentiation. (B) Selective (or Darwinian) model of cell differentiation. According to whether the random event *a* or *b* occurs, the cell differentiates into type *A* or *B*.

exception to the general instructive rule. Darwinian theories have been proposed, and are well accepted, in immunology and neurosciences (Jerne, 1955; Lederberg, 1959; Changeux et al., 1973; Edelman and Mountcastle, 1978). But, as regards the cell differentiation during embryo development, it is still believed to be deterministic in nature. The main reason in support of this opinion seems to be the highly precise and reproducible kinetics of this phenomenon. A stochastic process would be expected to be more chaotic. However, it is well known that stochastic processes at the molecular level can lead to organized macroscopic structures. Statistical physics gives many such examples. Another reason for the reluctance in accepting the selective model stems from its imprecision as regards the actual nature of the selective or stabilizing cellular interactions it relies on. Although epigenetic modifications of transcriptional regulators could be the molecular basis for stabilization of gene expression and thereby of cellular phenotypes (Kupiec, 1997; Misteli, 2001; Paldi, 2003), there is still a need to give a deeper insight into this mechanism. In order to address these questions and to evaluate the general relevance of the selective model, it is necessary to investigate further its capability to build organized structures. Finally, only in vivo experiments will elucidate the rules governing cell differentiation. But, computer simulation allows one to explore rapidly and globally the properties of a theory. For this reason, we have simulated the selective model of cell differentiation and conducted in silico computer experiments to test its

relevance. Our analysis shows that the selective model displays the main properties expected from a theory of embryogenesis. It is able to generate a stable cellular structure of finite growth. However, the simulation also demonstrates that the Darwinian model must be modified. Coordination in the development of differentiated tissues is not achieved merely by cellular selection (or stabilization) operating on cells differentiating stochastically, as shown in Fig. 1B. Instead, tissue coordination includes at least two basic components: phenotypic autostabilization (differentiated cells must stabilize their own phenotype) and interdependence for proliferation (differentiated cells stimulate the proliferation of alien phenotypes). Cellular organization is the result of a balance between these two processes. An imbalance leads to tissue disorganization and invasive cancer-like growth.

2. Models

Two versions of the model depicted in Fig. 1B have been studied.

2.1. Model 1

2.1.1. Cells and molecules

The cell population consists of two cell types, A and B. Each cell synthesizes S molecules at a certain rate Rs. In practice, the quantity of molecules synthesized at each simulation step is taken in a Gaussian distribution whose average is Rs and standard deviation σRs. These molecules could be signaling molecules involved in embryogenesis such as differentiation or growth factors. There are two types of S molecules: A cells synthesize a molecules and B cells synthesize b molecules. They are degraded at a certain rate (Rd). This parameter Rd includes catalysis after interaction with a cellular receptor. Because S molecules diffuse within the cell population, each individual cell is situated in an environment characterized by their concentrations. Biological molecules do not diffuse by a three-dimensional random walk as simple solutes do in water because of intracellular structuration and molecular overcrowding in the cytoplasm. Nevertheless, they still move by passive diffusion and random walk in restricted cellular compartments (for reviews see: Berg and von Hippel, 1985; Pederson, 2000) and, as experimentally demonstrated, establish gradients (Tabata and Takei, 2004). This phenomenon is documented but its complete and detailed representation in a cell is impossible to achieve at this time and would be cumbersome to simulate. For these reasons, we used Fick's laws. Therefore, there might be distortions in our models as regards the concentrations of S molecules within the cell populations. However, they do not modify the overall significance of our results. Thus, diffusion of S molecules occurs according to

$$\delta n/\delta t = D\Delta n, \tag{1}$$

where n is the local concentration of molecules and D the diffusion coefficient. If one assumes that at $t = 0$ no molecules stand at $r = 0$ (r = radius), then after a time t, the number of molecules at distance r is given by

$$N(r,t) = \frac{N_0}{4\pi Dt} e^{-r^2/4Dt}, \tag{2}$$

which is the integral solution we implemented in the simulation softwares. As a consequence after a duration t the mean free path traveled by molecules is

$$L = \sqrt{2Dt}. \tag{3}$$

In practice, the algorithm uses formula (2) where t is taken to be the simulation time step. In the simulation network associated to the concentration matrix, this integral diffusion formula is applied square by square using a superposition principle.

2.1.2. Stochastic differentiation

At any time, a cell has a certain probability to switch its phenotype. *A* cells can switch to *B*, *B* cells can switch to *A*. This phenotype switching probability (P) is a function (F) of the concentration of *S* molecules. *S* molecules stabilize cellular phenotypes as described in Fig. 1B. An increase of their concentrations in the immediate environment of a cell decreases its P. This aspect of the model is based on the following biological assumption. It is well established that signal transduction in cells leads to modifications in the phosphorylation of chromatin molecules and thereby in the equilibrium constants of chromatin molecular complexes. In turn, these modifications could result in either the stabilization or destabilization of chromatine structure and of stochastic gene expression (Kupiec, 1997). Similarly, any other epigenetic modification such as DNA methylation or protein acetylation could be involved in phenotype stabilization. However, in various experimental systems that have been studied, cells may be involved in two sorts of interactions. Either a cell acts on cells expressing a different phenotype or on cells expressing their own phenotype. For example, during the stochastic differentiation of gonadal cells in *C. elegans*, the anchor cell (AC) interact with the vulval cell (VU) (Greenwald and Rubin, 1992), whereas a mammalian muscle cell phenotype is achieved by positive auto-regulation (Edmondson et al., 1992). Consequently, in our model, the effect of an *S* molecule can be of two kinds. Either it stabilizes cells with the same phenotype as the cell it was produced from (autostabilization): *a* molecules stabilize *A* cells and *b* molecules stabilize *B* cells; or, it stabilizes cells with the other phenotype (interstabilization): *a* molecules stabilize *B* cells and *b* molecules stabilize *A* cells. In the simulation, both effects can be combined according to

$$P(T \to T^{-}) = q_{\text{auto}} F(NT) + q_{\text{inter}} F(NT^{-}),$$

where $P(T \to T^{-})$ is the probability for a cell of switching from one type to the other, T and T^{-} being the two cell types (if $T = A$, $T^{-} = B$; if $T = B$, $T^{-} = A$); NT and NT^{-} are the quantities of the corresponding *S* molecules (*a* and *b*) in the immediate environment of the cell; q_{auto} and q_{inter} are the relative proportions of auto- and interstabilization ($q_{\text{auto}} + q_{\text{inter}} = 1$); if $q_{\text{auto}} = 1$, the model works according to a purely autostabilization mode, conversely if $q_{\text{inter}} = 1$, the model works according to a purely interstabilization mode; F is a Fermi–Dirac function such as

$$F(x) = \frac{1 + e^{-\beta C_0}}{1 + e^{\beta(x - C_0)}},$$

where x is either NT or NT^{-}; C_0 is the value for x corresponding to the inflexion point of the function where $F(x) \approx 1/2$ for a large range of βC_0 values; β is a coefficient determining the steepness of the function; when β is big, the slope is abrupt with an almost direct transition between $F(x) = 1$ and 0, when β is small, the slope is gentle with a progressive transition between

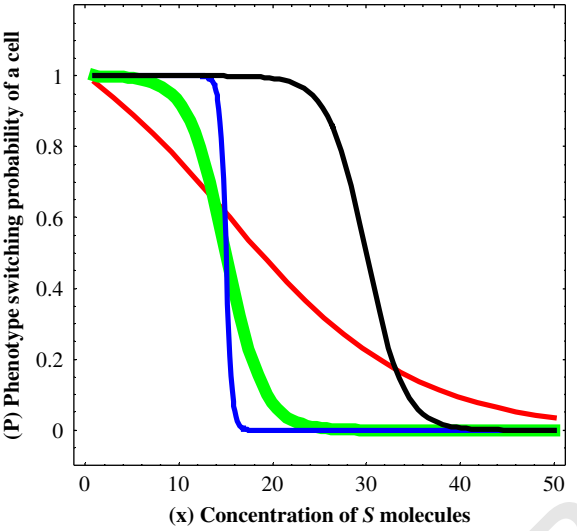

Fig. 2. Phenotype switching probability of a cell is a Fermi–Dirac-like function $F[x, C_0, \beta]$ of the concentrations of S molecules. x = concentration of S molecules, C_0 = inflexion point of the function, β = coefficient determining the slope of the function. Several examples are shown: $F[x,15.0,0.1]$ = red, $F[x,15.0,0.5]$ = green (these were the standard values used in our experiments), $F[x,15.0,3.0]$ = blue, $F[x,30.0,0.5]$ = black.

$F(x) = 1$ and 0. This function was chosen because by varying the values of C_0 and β it can describe a wide range of situations. Fig. 2 shows several examples of $F(x)$.

At the beginning of each simulation experiment the whole matrix representing the cell population (see Section 3) was filled with cells whose type, A or B, was chosen at random.

2.2. Model 2

Model 1 was useful in determining the respective effects of auto- and interstabilization but it did not generate organized cellular structures (see Section 4). Thus, we designed another version including other fundamental features of eukaryotic cell physiology. In this second model, in addition to their role in the stabilization of cell differentiation, S molecules are also necessary for cell division and cell survival.

Model 2 works according to the same rules and with the same parameters as Model 1 but cells are always in the autostabilization mode ($q_{\text{auto}} = 1$). In addition, in order to survive and proliferate, a cell of a given type needs S molecules produced by the other cell type (interdependence for proliferation). Indeed, it is well known that signaling molecules cause various effects on different target cells. According to the context, a growth factor may either be a proliferative factor, a differentiation factor or a survival factor (see for example: Fortunel et al., 2000; Tjwa et al., 2003). Thus, these molecules have also pleiotropic effects in Model 2. Moreover, S molecules may either be a signal or a trophic factor. As shown by the interesting work of Atamas (1996), in the frame of a selective model, there is a functional equivalence between a signal in a cellular system and food in an ecosystem. In both cases metabolization of the signal, or the

resource, leads to the multiplication of cells, or predators, and to organization of the system. Therefore, signals exchanged between cells can also be viewed as resources.

2.2.1. Cell survival

Cells A consume b molecules and similarly cells B consume a molecules. At each simulation step, the quantity Cc consumed by a cell is randomly taken from a Gaussian distribution whose average and standard deviation are Cs and σCs, respectively. If the quantity of adequate S molecules present in the environment is smaller than Cc, the cell dies.

2.2.2. Cell proliferation

Cells do not move in the matrix, but they can proliferate. In order to enter mitosis, cells need to consume, on average, a certain quantity of S molecules (Cp) produced by cells of the other type. In order to account for the variability of cell cycle duration (Liu et al., 2004 and references therein), for each cell and for each cell cycle, the actual quantity Cd of S molecules needed to enter mitosis is taken from a Gaussian distribution whose average and standard deviation are Cp and σCp, respectively. The quantities of S molecules consumed by a cell (Cc) are added at each simulation step until Cd is reached. At that step the cell divides and the new cell occupies an empty neighboring square chosen at random. If all squares are occupied, the cell does not divide. This simplification, which avoids additional computing, might introduce a bias in cell growths kinetics but it cannot change the overall signification of our results. Indeed, if cells were allowed to divide within the cell layers, these layers would grow faster, but it would not change their size which is determined by a balance between the parameters of diffusion and autostabilization as demonstrated by all our results (see Section 4).

Two alternative versions of Model 2 were studied. When a cell switched its phenotype during a cell cycle and therefore started to consume a different type of S molecules, either the counting of consumption was carried on with the new type of consumed molecules (Model 2a) either it was started again from zero (Model 2b). Model 2a integrates the fact that molecular recognition is degenerate in biological systems (see for example: Edelman and Gally, 2001; Moggs and Orphanides, 2001). Only a few ubiquitous intracellular signaling pathways exist that can be activated by many different degenerate signals. Therefore, it is very plausible that the effects of S molecules are not specific and can be added.

With either Model 2a or 2b, all cells died rapidly and the simulations failed if started with only one cell. It occurred because S molecules, which are necessary for cells to survive and proliferate, were not present at the beginning of the simulation. In fact, in actual organisms, fertilized ovum rely on their reserves during the initial hours of embryonic development. Instead of supplying the first cell with a reserve of S molecules, we skipped the first cell divisions. Therefore, in all the experiments, the matrix was filled at the beginning of the simulation with 16 cells whose type, A or B, was chosen at random. This ensured that S molecules were immediately produced in a quantity allowing the cell population to grow.

In summary, in each cell of the simulation step: (1) S molecules are synthesized; (2) S molecules are degraded; (3) S molecules diffuse; (4) the cell identity is determined stochastically according to S molecules concentrations; (5) the cell consumes S molecules. It either dies, or divides, or remains the same until the next simulation step. Table 1 summarizes the parameters of Models 1 and 2.

Table 1
Summary of simulation parameters

Parameters	Definition	Units
Rs	Rate of S molecules synthesis	Molecules/time simulation step
σRs	Standard deviation of Rs Gaussian distribution	Molecules/time simulation step
Rd	Rate of S molecules degradation	% Of molecules/time simulation step
L	Mean free path of S molecules	Distance per time simulation step
q_{auto}	Ratio of autostabilization	—
q_{inter}	Ratio of interstabilization	—
C_0	Inflexion of the Fermi–Dirac-like function (concentration of S molecules for which the phenotype switching probability is $\approx 1/2$)	Number of molecules/square
β	Slope of the Fermi–Dirac-like function	βC_0 is dimensionless
Cs	Average quantity of S molecules consumed by a cell during one simulation step	Number of molecules/square
σCs	Standard deviation of Cs Gaussian distribution	Number of molecules/square
Cp	Average quantity of S molecules for a cell to enter mitosis	Number of molecules/square
σCp	Standard deviation of Cp Gaussian distribution	Number of molecules/square

3. Material and methods

3.1. General features of the softwares

The cell population grows and differentiates in a two-dimensional matrix whose size can be determined in the command line of the software. Actual organisms are three-dimensional but this simplification allowed to spare a lot of computing time. Each square of this matrix may either be empty or filled with a cell. This matrix is a torus in which a square situated at one extremity is contiguous to the symmetrical square situated on the other side of the matrix. For example, square with coordinates [1, 1] is contiguous to square [1, 50] and [50, 1]. The software runs two other matrices identical to the first one but in which each square is a real number corresponding to the quantity of S molecules present at this spot. Thus, the central algorithm for molecular diffusion works with these matrices. Time is discretized and run as a succession of elementary intervals corresponding to simulations steps. At each step, molecular diffusion and cellular events (differentiation, division or death) are handled by the software.

The software was written in C^{++} and we used the ROOT framework developed at the Centre Européen de la Recherche Nucléaire (CERN) to generate a user-friendly interface made of four windows. One window is used for setting the values of the different parameters of the model. Another window shows the matrix filled with cells A (red squares) and B (green squares). Two other windows show separately the concentrations of each S molecule on the matrix.

3.2. Computation of failure rates

For a given set of parameters, the simulation was run at least 100 times for 100 simulation steps. The number of failures (the death of the entire cell population or in some rare occurrences a disorganized cell population, see Section 4) was recorded and counted manually.

4. Results

4.1. Inter- and autostabilization of cellular phenotypes exert different effects on tissue formation

We first wanted to test the respective effects of inter- and autostabilization on tissue formation. In a series of experiments, a matrix (50×50) was filled with cells whose phenotype (A or B) was chosen at random, each cell type having a probability $p = 1/2$. The cell population was then allowed to evolve according to rules of Model 1. A first visual examination showed obvious differences between the results of simulations run under either the inter- or autostabilization mode. With the interstabilization mode, small interspaced clusters of cells of both types were always generated (Fig. 3A), whereas large homogeneous areas corresponding to one cell type resulted from the autostabilization mode (Fig. 3B). However, in both cases, no organized structures involving the two cell types could be reproducibly detected. A quantitative analysis of variables including the time needed for stabilization and form (defined by the average radius, maximum radius, perimeter, filling in), made from a series of simulations run with an extensive range of values for the different parameters of the model did not uncover other hidden characteristics (data not shown). Therefore, we concluded that interstabilization might be needed to produce small cellular structures whereas autostabilization seems necessary to produce large

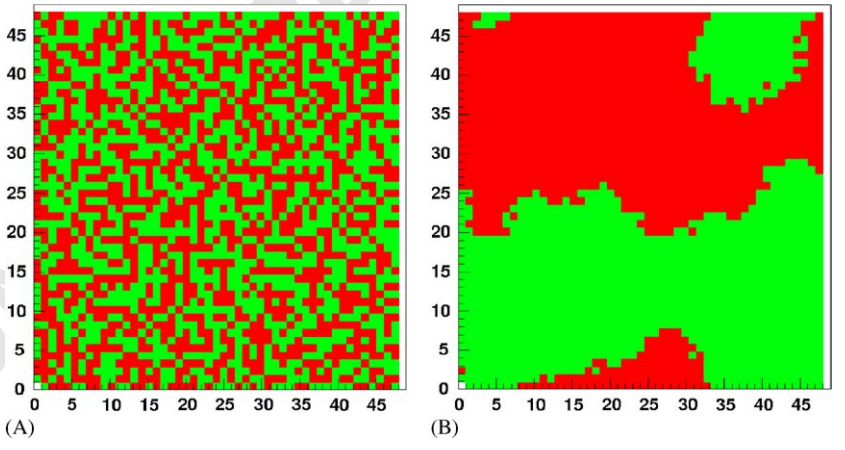

Fig. 3. Simulation of Model 1. Here and in all the following experiments presented in this article, the values of the parameters were identical for both cell types and S molecules. It has been checked that all the A/B cells symmetric structures are statistically equally distributed for all the results. (A) Rd = 0.15, Rs = 5, Qauto = 0, $C_0 = 4$, $L = 1.4$, $\beta = 1.1$; (B) Rd = 0.15, Rs = 5, Qauto = 1, $C_0 = 4$, $L = 1.4$, $\beta = 1.1$.

homogeneous areas of cells. But, neither of them is sufficient by itself, at least in our conditions, to produce an organization involving several tissues.

4.2. *The combined action of cell phenotype autostabilization and interdependence for proliferation generates an organized cellular structure of finite growth*

Since Model 1 did not produce reproducible structures, we added dynamic features of cell physiology, notably cell division and cell death. In addition, Model 2 combines stochastic differentiation controlled by autostabilization and interdependence for proliferation. Visual examination of the results obtained with several sets of values for the different parameters (Fig. 4A–G) immediately showed a striking difference between Models 1 and 2. The simulations, run

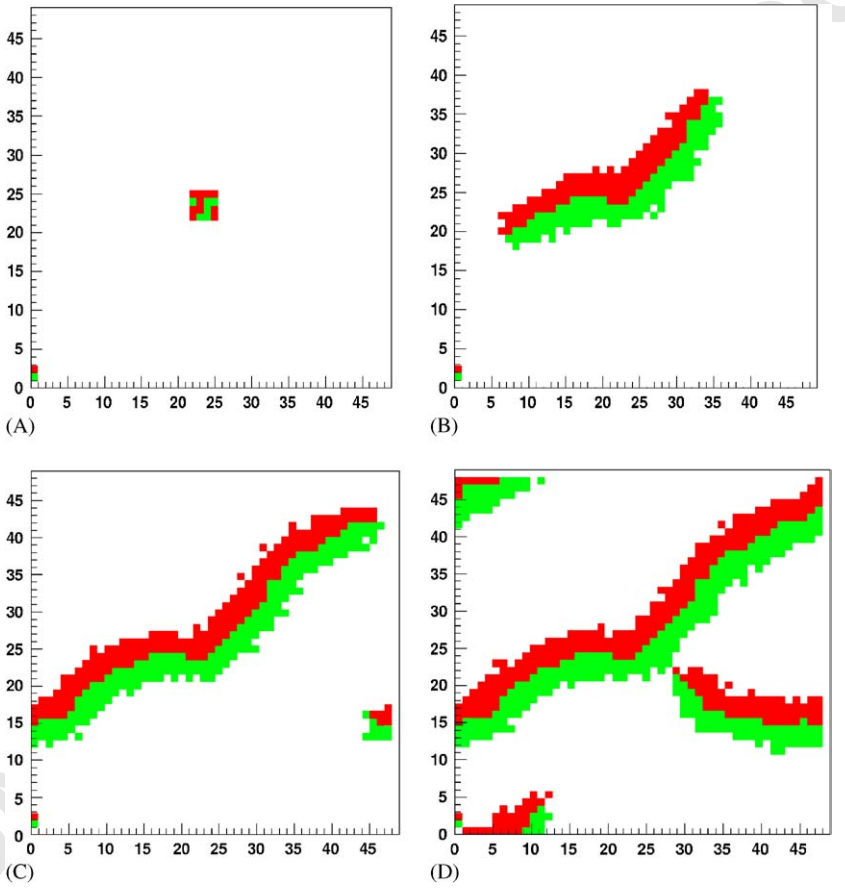

Fig. 4. Simulation of Model 2a. (A–F and G) Rs = 14, σRs = 0.1, Rd = 0.08, $L = 0.6$, $C_0 = 15$, $\beta = 0.5$, Cs = 2.4, σCs = 0.05, Cp = 6, σCp = 0.15. These parameters are the standard parameters we used in all the following experiments described in this article. Simulation steps: A = 0, B = 52, C = 105, D = 223, E = 552, F = 1021. H corresponds to another simulation with the same parameters: (H) Rs = 4, σRs = 0.1, Rd = 0.08, $L = 0.3$, $C_0 = 5$, $\beta = 0.5$, Cs = 0.2, σCs = 0.05, Cp = 1, σCp = 0.15. (I) Concentrations of a and b molecules across a typical bi-layer structure. Green and red boxes represent zones occupied by A or B cells.

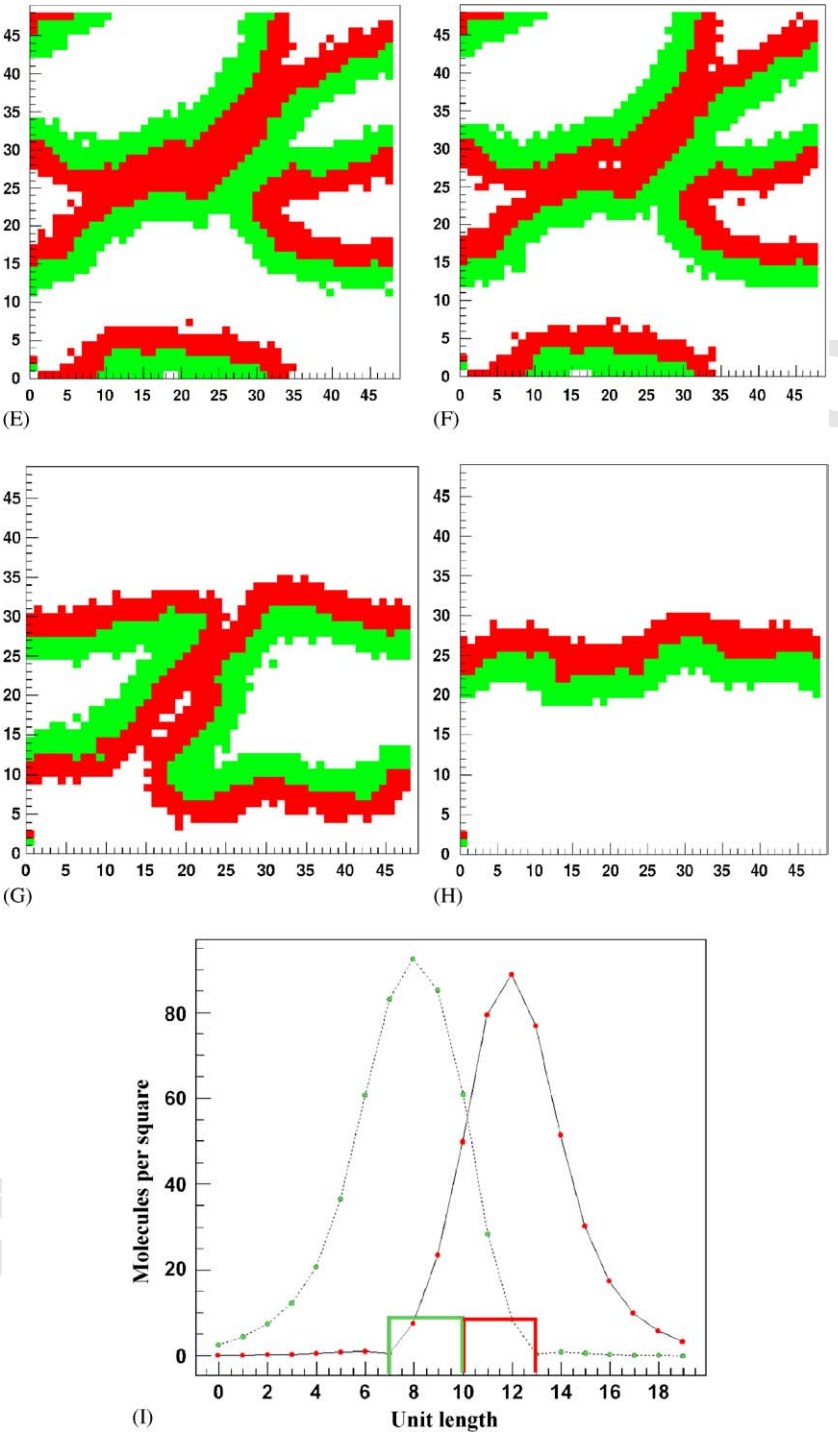

Fig. 4. (*Continued*)

either with Model 2a or 2b, produced a clearly recognizable organization. Starting from the initial 16 random cells (Fig. 4A), a cellular structure formed, made of two layers corresponding to the two cell types *A* and *B* (Fig. 4B). As this bi-layer structure continued growing longitudinally, its shape was not determined. It might fold up and vary each time the simulation was run again. When it reached one side of the matrix, it continued growing on the other side because of the toric structure (see Section 3). But, laterally, the cell layers were always adjacent, regular in width and separated by a well-defined interface (Fig. 4C and D). Unexpectedly, there was no infinite growth of these "virtual organisms". It reached a maximum development and then became stabilized even if the simulation was carried on (compare Figs. 4E and F). Fig. 4G shows another stabilized bi-layer structure obtained with the same set of parameters. Its overall shape is different but the bi-layer structure is conserved. A similar output was obtained each time the simulation was run again. The bi-layer structure remained invariant. Interestingly, this structure could be generated with different sets of parameter values (Fig. 4H). Fig. 4I shows the concentration profiles of *a* and *b* molecules across a lateral section of a typical bi-layer structure. Two symmetrical gradients of these molecules spread across the structure. Its lateral growth arrest as well as its composition of only one layer of each cell type suggested that this cellular organization does not result solely from interdependence for proliferation. Indeed, if this was the case, one could expect alternative growth of each cell type because of the nutriment availability. Cells *A* should proliferate on the external side of cell layer *B* because of the presence of *b* molecules, and similarly, cells *B* should proliferate on the external side of cell layer *A*. This phenomenon seemed important since we did not program in the model any condition that would cause such a cellular growth arrest. To explain it, we hypothesized that, because of concentrations of *S* molecules in these areas, phenotype autostabilization prevents cells from switching their phenotype in the external sides of the structure and consequently, to resume growth. Thereby, we tested this possibility by suppressing the cell phenotype autostabilization. For this purpose, C_0 was set to a very high value corresponding to a concentration of *S* molecules that could not be reached during the simulation (see legend of Fig. 5). As can be seen in Fig. 5, with Model 2a, the bi-layer structure was no longer produced. Large interspaced clusters of cells *A* and *B* grew progressively and invaded the whole

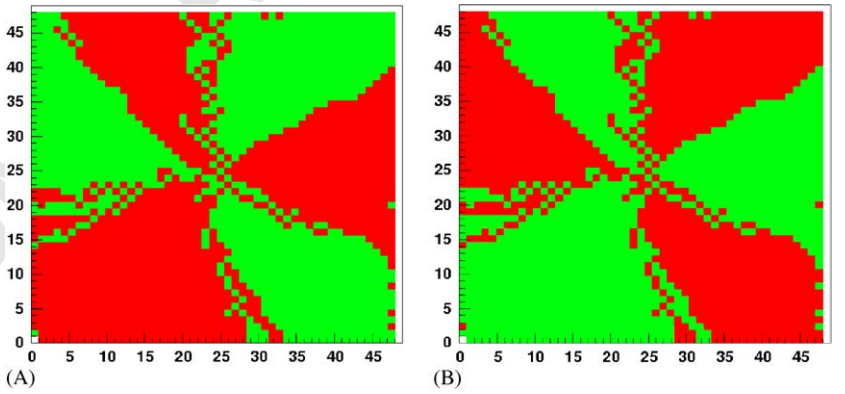

Fig. 5. Suppression of autostabilization (Model 2a). Standard parameters except $C_0 = 10{,}000$. Simulation steps: A = 300, B = 301.

matrix. Because of the absence of autostabilization, the cell clusters identity was unstable and they changed their type at each simulation step (compare Fig. 5A and B). With Model 2b, the bi-layer could not form either. However, in this case Cd could not be reached since cells changed their phenotype at each simulation step and the cell population simply stopped growing (data not shown). Similarly, we evaluated the necessity of interdependence for proliferation by preventing it. Rs and D were set to values that would make both kinds of S molecules overabundant in and outside the whole cell population (see legend of Fig. 6), therefore always available for both cell types in concentrations sufficient for proliferation. As can be seen in Fig. 6, it also resulted in the progressive growth of large clusters of cells A and B. However, in this case, due to autostabilization, the identity of these clusters remained stable. A similar bi-layer structure was obtained whatever Model 2 (a or b) was used. Altogether, these data indicate that both cell phenotype autostabilization and interdependence for proliferation are both necessary for generating the bi-layer structure (see Section 5).

We have also observed that asymmetric cellular structures with layers of different sizes could be formed by affecting different values for β and C_0 to A and B cells (data not shown). Finally, it should be noted that, due to the stochastic nature of the model, failures could occur in the formation of the bi-layer structure. It occurred in the initial steps of the simulations because of an imbalance in the ratio between the two cell types, leading to the death of the entire cell population. However, the failure rate depended on the values of the different parameters of the model. For certain sets of values it was low, compatible with biological reality in which embryonic mortality is a widespread phenomena (Ayalon, 1978). For Model 2a, with the standard set of parameters corresponding to Fig. 4A–D, failures occurred only 23 times out of 139 simulations, corresponding to a viability (V) of 79%. For Model 2b, with the same set of parameters, failures occurred 46 times out of 132 simulations ($V = 65\%$).

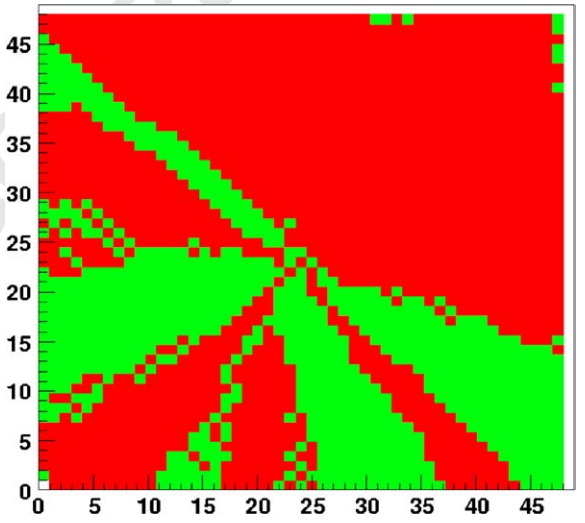

Fig. 6. Suppression of interdependence for proliferation. Standard parameters except Rs = 10,000, $L = 2$.

4.3. Cell death improves the bi-layer cellular structure formation

In order to evaluate the role of cell death in the bi-layer structure formation we modified the software of Model 2a. In this experiment, cells did not die, even if the quantity Cc of S molecules needed for survival was not present. However, they consumed all the remaining S molecules and divided if Cd was reached. The absence of cell death had an obvious but unexpected consequence. Although the bi-layer structure could still form, this happened at a much lower rate than when cell death was integrated in the model. Out of 100 simulations, failures occurred 50 times corresponding to $V = 50\%$ instead of 79%. In the first steps of simulation, all cells became converted into one of the two cell types and stopped growing (Fig. 7). From this result, cell death appears to play a positive role in the formation of the bi-layer structure. It removes cells that are not adjusted to their environment and this causes an imbalance in the ratio between the two cell types. In turn, this imbalance causes the autostabilization of only one cell type.

4.4. A distortion in any parameter can impair the bi-layer structure formation

To get more insight into the relative influence of the parameters of Model 2 on the bi-layer formation. For this purpose, we have proceeded to a separate distortion of each individual parameter, starting from the our standard set of values corresponding to Fig. 4A–G.

4.4.1. Length of diffusion (L)

As can be seen in Figs. 8A and B, increasing L from 0.4 to 0.8 allows the formation of the bi-layer structure with layers of increasing width. However, there are limits that cannot be exceeded. When the value of L becomes too small (= 0.1), small interspaced clusters of both cell types grow across the whole matrix (Fig. 8C). When it is too high (= 1), S molecules diffuse at long distances from the source cells; their local concentrations become too small, leading to death of the entire

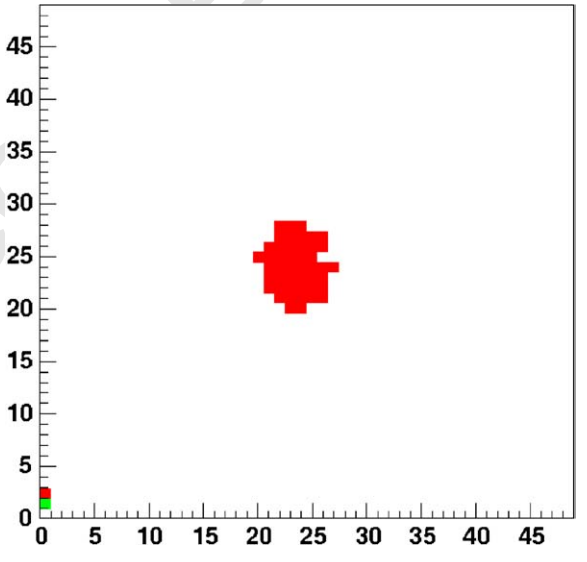

Fig. 7. Suppression of cell death. Standard parameters but cells could not die.

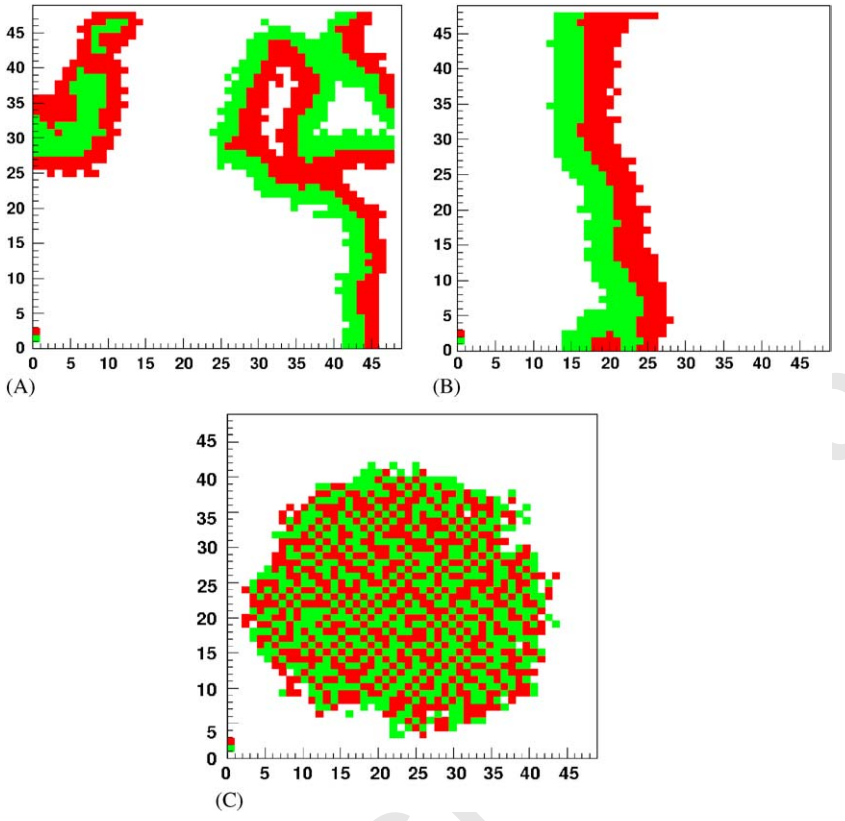

Fig. 8. Influence of diffusion. Standard parameters except (A) $L = 0.4$, (B) $L = 0.8$ and (C) $L = 0.1$.

cell population within the first steps of simulation. Similar results were obtained with either Model 2a or 2b.

4.4.2. Autostabilization (C_0 and β)

The suppression of autostabilization prevents the formation of the bi-layer structure (see Fig. 5). Its intensification leads to the same result. When the value of C_0 is too small (= 1), cells stabilize at low concentrations of S molecules. Consequently, large interspaced clusters of both cell types invade the whole matrix (Fig. 9A). Autostabilization is also dependent on the coefficient β. When its value is very small (= 0.01), cells cannot get fully stabilized. Small clusters of cells with unstable phenotypes invade the whole matrix (Fig. 9B). When the value of β is high (= 15), the transition from an unstable phenotype ($P = 1$) to a stabilized one ($P = 0$) occurs without intermediate stochastic states (see Fig. 2). However, the bi-layer formation is not prevented. Similar results concerning C_0 and β were obtained with either Model 2a or 2b.

4.4.3. Cell proliferation (Cs and Cp)

The rate of cell division depends on Cs and Cp. On average, the number of simulation steps needed for a cell to divide is equal to the ratio Cp/Cs. With Model 2a, when cell proliferation was slowed down (Cp = 10 and Cs = 1) compared to the standard parameters we used (see legend of

Fig. 4), the bi-layer could still form but failures occurred more frequently leading to death of all cells during the first simulation steps. This happened 95 times out of 148 simulations ($V = 35\%$). Model 2b produced similar results. With Model 2a, when cell proliferation was speeded up

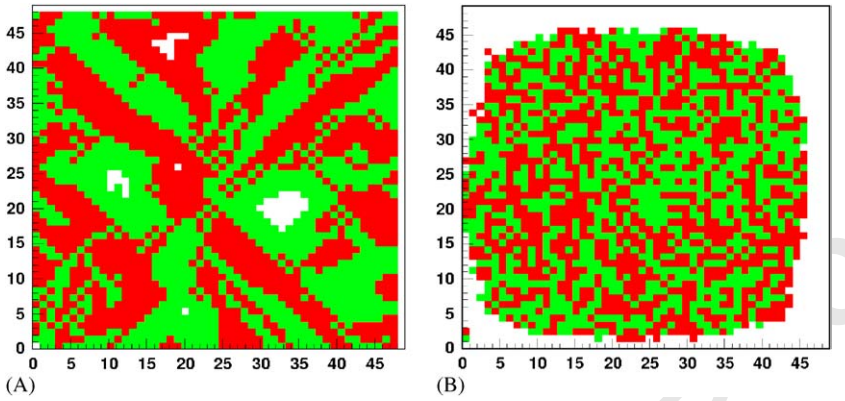

Fig. 9. Influence of autostabilization. Standard parameters except (A) $C_0 = 1$ and (B) $\beta = 0.01$.

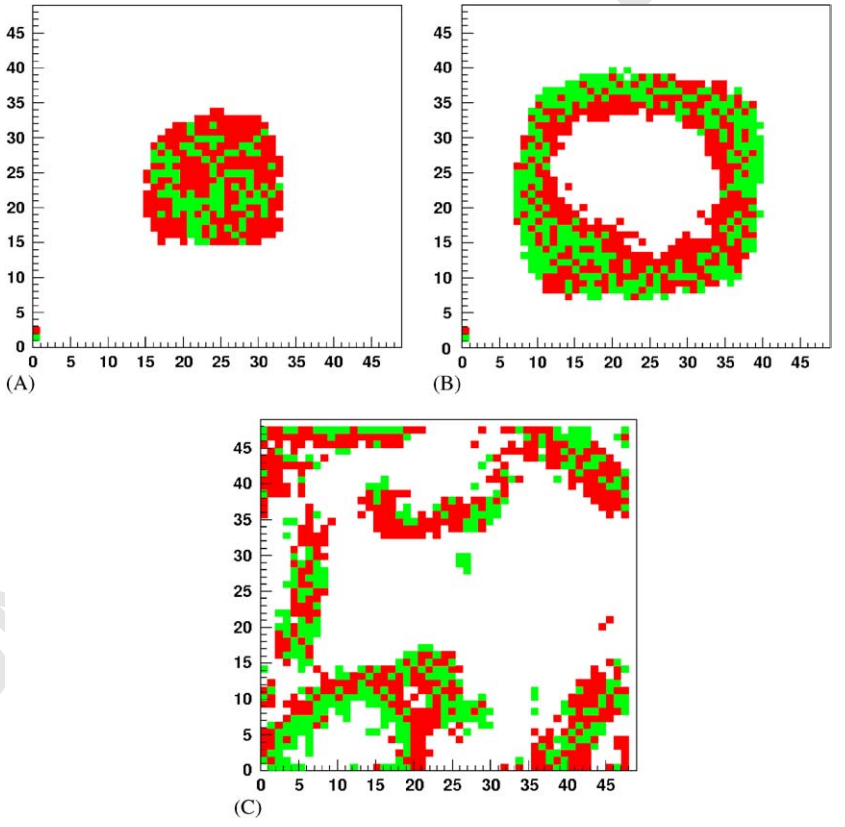

Fig. 10. Influence of the rate of synthesis of S molecules. Standard parameters except Rs = 5. Simulations steps: A = 45, B = 70, C = 150.

(Cs = Cp = 2), the bi-layer formed with a better efficiency. Failures were observed only 11 times out of 110 simulations ($V = 90\%$). With Model 2b, failures occurred 63 times out of 117 ($V = 46\%$). In these latter experiments, a few cells survived in the matrix after 100 simulation steps, when they were stopped. Otherwise the bi-layer was produced normally.

4.4.4. *Concentrations of S molecules (Rs)*

With either Model 2a or 2b, when *S* molecules were scarce (Rs = 1) compared to the standard parameters we used (see legend of Fig. 4), all cells always died in the first steps of simulation. Interestingly, when *S* molecules were synthesized at an intermediate rate (Rs = 5), a ring of mixed cells was produced transiently before it broke up (Fig. 10). In fact, the ring expanded until the torus geometry of the space made its edges to collide. The circular structure and its development were reproducible. When *S* molecules were overabundant (Rs = 100), large interspaced areas corresponding to the two cellular phenotypes invaded the matrix, as shown in Fig. 6.

4.5. *An imbalance between autostabilization and interdependence for proliferation leads to tissue disorganization and cancer-like growth*

Although we have not proceeded to a totally exhaustive analysis, our results are sufficient to show that the bi-layer formation depends on an equilibrium between the different parameters of the model. Since finite growth is an important feature of this process with considerable biological relevance, we were intrigued to see what would happen to an already formed bi-layer structure of the parameters controlling autostabilization and interdependence for proliferation were modified. In the first experiment, the simulation was run with our standard parameters (see legends of the values in Fig. 4) until the bi-layer formed and stopped growing (Fig. 11A). C_0 was then increased to a value of 45 instead of 15. This value corresponds to the concentration of *S* molecules on the external side of the bi-layer. In an actual organism, such a modification could result either from the mutation of a transcriptional regulator controlling a gene involved in autostabilization or its interaction with a carcinogenic product. In both cases, its affinity with DNA target sequences would be modified and consequently more molecules would be needed to achieve the same effect on gene expression. As can be seen in Fig. 11B, the bi-layer was not destroyed but cell

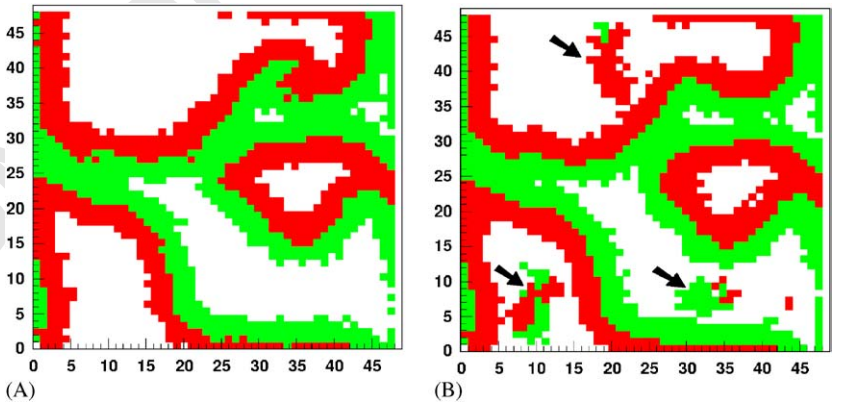

Fig. 11. A modification of C_0 causes cancer-like growth. Standard parameters except (B): $C_0 = 45$.

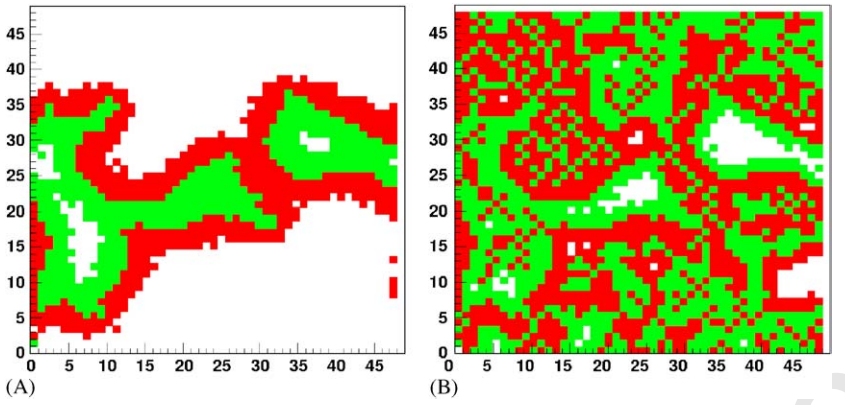

Fig. 12. A modification of L causes cancer-like growth. Standard parameters except (B): $L = 0.2$.

proliferation resumed locally on its external sides, producing masses of cells of both types that were continuously released in the environment and died after a variable period of time. In the second experiment, the bi-layer was also formed using the default parameters until it stopped growing (Fig. 12A). L was then set to 0.2 for both S molecules. In an actual organism, this could result from either a mutation of S molecules or from their interaction with a toxic substance modifying their diffusion properties. As can be seen in Fig. 12B, this led to a disorganized cellular growth that invaded the whole matrix.

These experiments demonstrate that, in the frame of our model, a quantitative modification of the parameters of either autostabilization or diffusion of S molecules leads to uncontrolled cellular growth.

5. Discussion

5.1. An autostabilization-selection model of tissue organization

The results reported in this article show that a modified selective model of cell differentiation integrating stochastic gene expression displays the main properties expected from a theory of embryogenesis. In fact, the generation of order at the macroscopic level from stochastic disorder at the molecular level has been acknowledged as an important principle in many areas of science for a long time. It lies, for example, at the very root of statistical physics. But, until now it has been rejected by mainstream biology. In his influential essay, "What is life", Schrödinger (1944) suggested, instead, what he called the "order from order" principle applying to biological systems. According to this principle, in living organisms, macroscopic order does not stem from stochastic molecular disorder caused by Brownian motion, but from the transformation of the information encoded in DNA into a three-dimensional cellular structure by a genetic program. Until recently, this genetic program has been envisioned as a totally deterministic mechanism in which genes are regulated by on and off switches (Jacob and Monod, 1961; Britten and Davidson, 1969; Oliveri and Davidson, 2004). This view is still widely accepted. However, it is now challenged by the accumulating evidence showing the importance of stochastic gene expression (see Section 1).

Indeed, if gene expression, considered as the basic step of the deterministic genetic program, is a stochastic event, there is a contradiction between the data and theory and one has to explain how these data can be reintegrated into a coherent theoretical frame. For this reason, we aimed to investigate the relevance of the selective model. As expected, the results we obtained demonstrate that a mechanism based on stochasticity in cell fate choice can produce an organized cellular structure. But, our results also show that the selective model should be modified, as regards the modalities by which cellular selection operates to coordinate the development of differentiated tissues. The simulations with Model 1 show that an interaction between cells, based simply on interstabilization, as was previously imagined (Fig. 1B) is insufficient. It leads to the formation of interspaced cell clusters. Although this pattern sometimes occurs during the development of organs such as the Drosophila brain (Urbach et al., 2003), this is not a general theme in embryogenesis. In contrast, autostabilization, which is not a property usually included in the selective model, produces large homogeneous areas of cells. But, these structures are not reproducible. These two complementary behaviors suggest that in order to generate an organized cellular structure both types of interactions are needed: an interaction linking the different cell types in order to create a pattern and autostabilization to give tissues extension in size. Model 2 works according to this principle. The growths of *A* and *B* cells are linked by interdependence for proliferation, combined with phenotypic autostabilization. As a result, this model is readily able to generate an organized cellular structure characterized by a bi-layer organization of finite growth. These findings can be integrated into a modified model shown in Fig. 13. This autostabilization-selection model might miss some important feature of living systems that would make it more accurate. It should integrate other types of interactions to produce various sorts of structures encountered in living beings. However, some argue that it is already relevant as a general theoretical frame. Indeed, cell layers are very common in a biological organization and finite growth is a major characteristic of development. By simply varying the values of the parameters of the model, a ring of cells with an internal cavity could also be produced. Cavities are a widespread feature of tissue organization and more specifically, this structure is reminiscent of a blastula with its blastocoel. Moreover, the simulations suggest that tissue formation is not the result of a single sort of cell interaction but involves multiple complementary molecules exerting

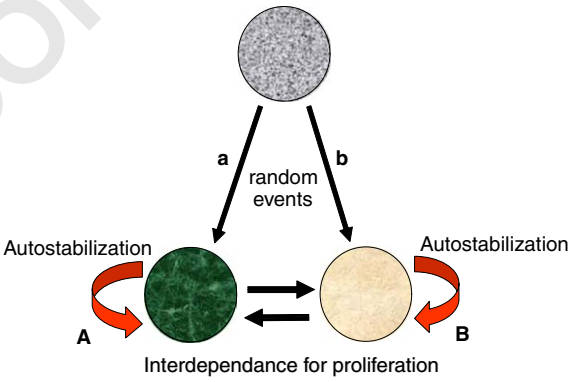

Fig. 13. Autostabilization-selection model of cell differentiation. Cell differentiation and tissue organization result from stochastic gene expression, interdependence for proliferation and autostabilization of cell phenotypes.

various effects. This is in agreement with basic knowledge in cellular physiology. Many different kinds of molecules such as differentiation, proliferation, survival or apoptotic factors are involved in the development. As is the case in actual experimental systems, in the autostabilization-selection model these molecules are ubiquitous and affect either cell differentiation, cell survival or cell proliferation on different cell types. These different effects could also be carried by multiple sets of molecules resulting in a more complex model. Nevertheless, our results show that a simple model involving only two cell types and two sorts of interaction is already sufficient to generate a basic cellular organization. Therefore, we suggest that a complementary action of autostabilization and interdependence for proliferation is an important requirement for tissue organization. Of course, a major difference between the autostabilization-selection model and the classical understanding of how morphogenetic factors act upon cells remains. In the usual instructive model, these molecules act as signals to promote a change in the cell state. In the autostabilization-selection model, they only stabilize a state previously achieved by a stochastic mechanism.

Although sufficient to show that the autostabilization-selection model displays the main characteristics of embryogenesis, our results do not constitute an exhaustive study of its properties. In order to simulate more complex experimental systems, the simple two-cell model presented here should be improved. It should be made three-dimensional and the number of cell types should be increased. This work is now under progress with a three-cell model. Our preliminary results show the formation of a three-layer structure (data not shown). Since we have understood the basic rules for the control of cell proliferation, it will be possible to create a model in which cells stop growing not only laterally but also longitudinally. As is often the case in computer simulation experiments, our models are two-dimensional but they should be made three-dimensional.

In the future, it will also be interesting to get insight into the dynamics leading to the formation of the bi-layer structure. Indeed, it is generally acknowledged that cells differentiate according to their position inside morphogen gradients (Gurdon and Bourillot, 2001; Wolpert, 1989). However, the dynamic aspect of the process is usually underestimated. The morphogen gradients are considered as prepatterns of the embryo in which morphogen concentrations act as information causing cell differentiation. It is probably different in the autostabilization-selection model. Both the concentration patterns and the bi-layer cellular structure seem to result from the same dynamics and their formations to be completely entangled.

5.2. *Cell differentiation and Natural Selection*

Since phenotypic autostabilization is not a concept that usually forms part of a selective model, a question arises from our results: to what extent is the autostabilization-selection model Darwinian and how can it be integrated within an evolutionary perspective?

A selective model explains a biological process by a mechanism combining random variations with selection. In this respect, it is conceptually analogous to Natural Selection. But, in order to explain the evolutionary origin of multicellularity, it has also been postulated that Natural Selection actually enters the organisms. In this frame, metabolic cooperation between cells is considered as the source of order during embryogenesis. Cellular differentiation is explained as an adaptation of cells to their microenvironment within the organisms. Each cell adjusts its

metabolism in order to use the available resources transferred from its neighboring cells (Kupiec, 1997; Blackstone, 2000; Furusawa and Kaneko, 2000; Pfeiffer et al., 2001; Schlichting, 2003). However, this theory only draws a general frame that does not exclude the appearance of additional mechanisms in the course of evolution. Our findings can easily fit within this scenario. Starting from a mechanism in which the interdependence for proliferation was based on raw metabolic selection of cells, the efficacy of cellular differentiation could be improved: firstly, by incorporating the action of molecules such as growth factors or hormones; secondly, by adding new mechanisms such as autostabilization. Because of these additional mechanisms, an organism cannot be viewed as a simple "cellular ecosystem" in which trophic interdependence for proliferation between cells leads to cellular self-organization. As we have demonstrated, the suppression of phenotypic autostabilization prevents cellular organization. Of course, this theory still needs to get more experimental support. The evidence for stochastic gene expression relies on its variability among single cells (see Section 1). However, in spite of the mass of data that has accumulated two interpretations are still competing to explain its existence. Either it is a fundamental process underlying cell physiology, or it is an unavoidable background noise of gene expression. In order to establish it experimentally as a meaningful biological parameter, it should be correlated with a cell process. In this regard, the autostabilization-selection model allows one to make predictions. For example, according to the autostabilization-selection model, when a cell is subject to a physiological change such as cell differentiation, variability in gene expression allows for the subsequent selection and amplification of adequate expression profiles. Therefore, in the course of differentiation kinetics, the cell-to-cell variability in gene expression is expected to decrease whereas a background noise is not expected to be correlated with cell differentiation. Another complementary prediction can also be made. In the frame of this model signal transduction, or metabolism, controls gene expression variability via phosphate flux. Therefore, its experimental alteration, for example by inhibiting or overexpressing a kinase or a phosphatase, should also alter the restriction of variability in gene expression and disturb the normal course of cell differentiation.

5.3. *Cell death and Natural Selection*

In multicellar organisms, cell death, not only occurs during development but also is an active process known as apoptosis with important consequences in many areas of physiology (Ameisen, 2002). However, the origin of its widespread occurrence remains somewhat enigmatic. Our results suggest a strong explanation for its existence and maintenance in the course of evolution. Indeed, in the frame of the autostabilization-selection model, cell death improves the viability of organisms and therefore there is no reason for it to be suppressed. This result further supports the hypothesis that cell differentiation originated from Natural Selection operating directly at the cellular level. Indeed, this theory predicts the death of cells that cannot adjust to their microenvironment as well as the differentiation of those that succeed. Therefore, in this frame, cell death and cell differentiation are two effects of the same process.

5.4. Cancer

According to the current theory of molecular biology, the genetic program is not only responsible for the control of cell differentiation, but also cell proliferation. Cells are thought to receive signals that determine them either to rest or to proliferate. In consequence, in the Somatic Mutation Theory of cancer, tissue disorganization and neoplasia result from mutations in oncogenes coding for these signals (Hahn and Weinberg, 2002). Dozens of these genes have been reported. However, a clear and rational understanding of neoplasia has not been achieved yet. Our results suggest another explanation. Tissue organization and cell proliferation are not controlled by a genetic program made of qualitative signals but result from a quantitative equilibrium between different parameters. Indeed, the bi-layer structure produced by the autostabilization-selection model displays finite growth, but this feature was produced without being programmed by an inhibitory signal. On the contrary, in this model, it is implicitly assumed that there is a constant supply of nutriments allowing for the synthesis of S molecules and for cell proliferation. Growth arrest results from the balanced effects of autostabilization and interdependence for proliferation and we have shown that a quantitative modification of one of these two processes leads to uncontrolled cellular growth. Consequently, these results suggest an alternative mechanism to the classical Somatic Mutation Theory of cancer. Because tissue organization and control of cell proliferation result from an equilibrium between autostabilization and interdependence for proliferation, any modification within cells that would modify the balance between their effects could lead to disorganization. As in the classical theory, it could be a mutation of a protein involved in autostabilization of phenotypes. It could also be a mutation preventing the diffusion of a trophic factor and thereby impairing interdependence for proliferation. But, it could even not be a mutation. The direct fixation of a carcinogenic product on a growth factor or the alteration of cell membranes could have the same effects. Moreover, if tissue organization arises from the combined action of several causes, the etiology of cancer could be diffuse because neoplasia would result from the progressive addition of several small alterations of these causes. Therefore, in this frame, it is not solely the cancerous cell that is abnormal. The whole organism is involved. Tumor growth is a local effect of an imbalance between all the factors involved in tissue organization. This conclusion of the simulations is in agreement with recent data showing the importance of cellular microenvironment and growth factor signaling in neoplasia (van Kempen et al., 2003; Kenny and Bissell, 2003; Bhowmick et al., 2004; Maffini et al., 2004; Hede, 2004). It is also in agreement with theoretical proposals that stress the importance of the tissue structure over the genome (Bissell et al., 1999; Sonnenschein and Soto, 1999).

Finally, the autostabilization-selection model might also enable us to make non-trivial predictions for a strategy against cancer. Indeed, the simulations suggest that tissue organization does not result from some "normal" values of the parameters, but from a ratio that has to be respected between these values in order to maintain an equilibrium. As a consequence, if one parameter is changed and the ratio is no longer respected, it should be possible to restore it by modifying another parameter. Under the current dominant paradigm if a protein affecting autostabilization is mutated and provokes cell proliferation, one will try to restore a "normal" autostabilization. For example, one will try to reinstate the wild-type gene-by-gene therapy. Instead, the autostabilization-selection model suggests that it should also be possible to restore the

adequate ratio between autostabilization and interdependence for proliferation by changing the parameters of diffusion of this same protein. This could be achieved either by adding other mutations to it or by affecting the medium in which it diffuses. For example, by mutating a membrane protein. Of course, to be able to do that one requires uncovering in more detail the relations between stochastic gene expression, autostabilization and interdependence for proliferation. This will be the task of a new research program.

5.5. Conclusion

The results reported here do not provide a fully elaborated theory of embryogenesis and tissue organization. But, the autostabilization-selection model of cell differentiation gives an example of a new approach to biological systems through which the phenotype is not the direct expression of information encoded in the DNA. The importance of the genome is not denied but it is no longer considered as governing the organism. This latter view that can be traced back to the foundation of genetics with Weisman's (1892) germ-plasm theory has been the dominant paradigm of molecular biology up to now. It underlies research programs focusing on gene analysis. This research has been fruitful but it now needs to be enriched by integrating all the components of biological organization (Crampin et al., 2004). The approach put forth in this article fits within this frame. Tissue organization results from an equilibrium between the influences coming from both the genome and cellular interactions. Embryogenesis is the evolution of the embryo toward this equilibrium and cancer the destruction of the equilibrium.

6. Uncited reference

Wieser et al., 1990.

Acknowledgements

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