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Interplay of bistable kinetics of gene expression during cellular growth

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Abstract

In cells, the bistable kinetics of gene expression can be observed on the level of (i) one gene with positive feedback between protein and mRNA production, (ii) two genes with negative mutual feedback between protein and mRNA production, or (iii) in more complex cases. We analyse the interplay of two genes of type (ii) governed by a gene of type (i) during cellular growth. In particular, using kinetic Monte Carlo simulations, we show that in the case where gene 1, operating in the bistable regime, regulates mutually inhibiting genes 2 and 3, also operating in the bistable regime, the latter genes may eventually be trapped either to the state with high transcriptional activity of gene 2 and low activity of gene 3 or to the state with high transcriptional activity of gene 3 and low activity of gene 2. The probability to get to one of these states depends on the values of the model parameters. If genes 2 and 3 are kinetically equivalent, the probability is equal to 0.5. Thus, our model illustrates how different intracellular states can be chosen at random with predetermined probabilities. This type of kinetics of gene expression may be behind complex processes occurring in cells, e.g., behind the choice of the fate by stem cells.

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(Some figures in this article are in colour only in the electronic version)

1. Introduction

The expression of the information encoded in genes is known to occur via a templated polymerization called transcription, in which the genes are used as templates to guide the synthesis of shorter molecules of RNA [1]. Later on, many RNAs, or, more specifically,

messenger RNAs (mRNA) serve to direct the synthesis of proteins by ribosomes. Another large class of RNA includes non-coding RNAs (ncRNA) [2, 3]. The functions of the latter RNAs are based on their abilities to bind to and modulate the activity of mRNAs and/or proteins [3]. The whole process of gene expression can be regulated at all the steps. Specifically, the gene transcription, performed by RNA polymerase during its association with DNA, is often controlled by master regulatory proteins. Such proteins associate with DNA and either facilitate or suppress the RNA synthesis.

The positive and negative feedbacks between RNA and protein formation may result in complex kinetic features. In particular, the gene-expression kinetics may exhibit bistability in a certain range of governing parameters [4–7]. Practically, this means that by changing a governing parameter, e.g., signal amplitude, one can observe a stepwise transition or, in other words, switch from one regime of gene expression to another regime. Such switches often play a key role in regulation of cellular processes. For this reason, the bistable kinetics of gene expression have long attracted attention, and now the understanding of general factors which may result in bistability is relatively complete. The numerous models available in this field are focused on the expression of one or two genes or more complex genetic networks including mRNAs and proteins and exhibiting bistability under steady-state conditions [4–7]. The first bistable models including ncRNAs were recently proposed in [8, 9]. There are also models describing ensembles of bistable cells under steady-state conditions [10]. The effect of cellular growth on the simplest bistable kinetics of gene expression, including a gene with positive feedback between the protein and mRNA production, was simulated in [11, 12]. The interplay of bistable kinetics of gene expression during cellular growth has not been analysed so far. In this work, we show what may happen in this case.

To motivate our work in more detail, we may refer to stem cells (for some other real examples, see review [13]). Such cells are remarkable due to their ability for self-renewal as well as generation of more specialized cells via symmetric and asymmetric divisions, $S \to 2S$ and $S \to S + D$, respectively (D is a differentiated cell) [14]. With this potential, stem cells play a key role during the first steps of the development of biological species. By adulthood, the relative number of stem cells becomes low and their presence is nearly invisible. Nevertheless, they remain crucial for reproduction of species and also for maintenance and repair of various tissues. For these reasons and also due to potential applications in medicine, the stem-cell research is of high current interest (for the introduction into the state of the art in this interdisciplinary field, see a few reviews in Nature Insight [15]). Despite the wide front of activity in this area, the understanding of the mechanisms of functions of stem cells is still far from complete.

Conceptually, the ability of stem cells to divide symmetrically and asymmetrically is often believed to be related to the bistability in gene expression (for a review, see [16, 17]). In the corresponding models, the states of a cell are identified with the steady states (or attractors) of bistable or multistable genetic networks (see, e.g., [7, 18–23]). The models of this type make it easily possible to describe deterministic choice of the cell fate or, more specifically, to fix one of the states under the influence of cell–cell communication or external signals (e.g., in stem-cell niches [24, 25]).

In some cases, stem cells choose however one or another pathway stochastically without apparent regard to the environment. Since the seminal studies by Till *et al* [26], the corresponding kinetics are often phenomenologically described by introducing the division rate constants and probabilities corresponding to different pathways (see, e.g., recent simulations of proliferation and differentiation of cells in a stem-cell niche [27]). The mechanism determining these probabilities is now uncertain. The corresponding experimental data indicate that the differentiation of stem cells is accompanied by the changes of the transcriptional activity

of a multitude of genes [28, 29]. For this reason, the identification of the key steps is still a challenge. Physically or mathematically, the probabilistic choice between the division channels is expected to be somehow related either to chaotic kinetics, predicted by deterministic equations, or to stochastic kinetics arising due to a small number of reactants participating in some of the steps. Following the latter line, we have recently suggested [17] that the stochastic behaviour of a single stem cell can be explained by (i) the existence of a short stage of decision whether it will divide symmetrically or asymmetrically and (ii) control of this stage by stochastic bistability in gene expression. However, a full model of this scheme has not been constructed. In this work, we propose such a model. In particular, we show that assumptions (i) and (ii) can be realized via the interplay of bistable kinetics of gene expression during cellular growth.

2. Model

The available models of bistable gene-expression kinetics include (i) a gene with positive feedback between protein and mRNA production, (ii) two genes with negative mutual feedback between protein and mRNA production or (iii) more complex networks. Here, we treat the situation when the protein produced via the activity of gene 1 of category (i) controls the activities of genes 2 and 3 of category (ii). The activity of genes 2 and 3 is first low. During cellular growth, the state of gene 1 changes and it results in the transition of one of the other genes (2 or 3) to a highly active state. The high activity of gene 2 or 3 is identified with cell differentiation. Thus, in our model, the differentiation is related to cellular growth. This scenario of cell differentiation is novel.

The simplest generic bistable kinetic gene-transcription model belonging to category (i) implies that the rate of the synthesis of mRNA (R_1) is high provided that n regulatory sites are occupied by protein (P_1) produced via R_1 translation and that the P_1 association with and dissociation from the gene are rapid so that these steps are at equilibrium. In this case, the probability that a regulatory site is occupied by P_1 is $k_a c_{P_1}/(k_d + k_a c_{P_1})$, where k_a and k_d are the association and dissociation rate constants, and c_{P_1} is the P_1 concentration. Taking into account that the P_1 concentration is related to the number of P_1 copies and cell volume as $c_{P_1} = N_{P_1}/v$, the probability that a regulatory site is occupied by P_1 can be represented as $N_{P_1}/(K_1 v(t)/v_0 + N_{P_1})$, where v_0 is the volume corresponding to the beginning of the cell cycle, and $K_1 = k_d v_0/k_a$ is the constant characterizing the P_1-gene association–dissociation equilibrium.

With the specification above and $n = 4$, the mean-field kinetic equations for the numbers of R_1 and P_1 copies in a cell during its growth are as follows [11]:

$$\frac{\mathrm{d}N_{R_1}}{\mathrm{d}t} = k_b + k_{r1}\left(\frac{N_{P_1}}{K_1 v(t)/v_0 + N_{P_1}}\right)^4 - k_{R_1} N_{R_1}, \tag{1}$$

$$\frac{\mathrm{d}N_{P_1}}{\mathrm{d}t} = k_{s1} N_{R_1} - k_{P_1} N_P, \tag{2}$$

where k_b and k_r ($k_b \ll k_r$) are the rate constants of the basal and P_1-regulated gene transcription, $[N_{P_1}/(K_1 v(t)/v_0 + N_P)]^4$ is the probability that four regulatory sites are occupied by P_1, $v(t) = v_0 \exp(k_g t)$ is the cellular volume ($k_g \equiv (\ln 2)/t_c$ is the growth rate constant and t_c is the cell-cycle duration), k_{s1} is the P_1-synthesis rate constant, and k_{R_1} and k_{P_1} are the degradation rate constants.

Using equations (1) and (2), we assume that all the rate constants are independent of cellular growth. Depending on the details, this approximation may or may not be valid (see

the discussion in [11]). For example, the protein synthesis is performed by ribosomes, and k_{s1} is independent of time provided that the number of ribosomes linearly increases with the cellular volume. In reality, the deviations from the linear growth cannot be excluded. This effect is however not expected to be crucial.

In our model, gene 1 is described by equations (1) and (2). As time progresses and the cell grows, the concentration of proteins becomes more dilute. This causes fewer proteins to bind to the DNA and the gene becomes less active. As a result, with increasing time, these equations predict (provided that the parameters are suitable) a sharp transition from the state with high R_1 and P_1 populations to the state with low R_1 and P_1 populations. In our scheme, this transition or, more specifically, the drop in the P_1 population is associated with a short stage determining the fate of the evolution of two other genes. In this relation, it is appropriate to note that equations (1) and (2) do not take duplication of genes into account. In our model, the duplication is considered to occur after the decision stage and accordingly we do not need to explicitly incorporate it into the analysis.

The P_1-regulated kinetics of the transcription of genes 2 and 3 are described as

$$\frac{\mathrm{d}N_{R_2}}{\mathrm{d}t} = k_{r2}\left(\frac{\mathcal{K}_2}{\mathcal{K}_2 + N_{P_1}v_0/v(t)}\right)^2\left(\frac{K_2}{K_2 + N_{P_3}v_0/v(t)}\right)^2 - k_{R_2}N_{R_2}, \tag{3}$$

$$\frac{\mathrm{d}N_{P_2}}{\mathrm{d}t} = k_{s2}N_{R_2} - k_{P_2}N_P, \tag{4}$$

$$\frac{\mathrm{d}N_{R_3}}{\mathrm{d}t} = k_{r3}\left(\frac{\mathcal{K}_3}{\mathcal{K}_3 + N_{P_1}v_0/v(t)}\right)^2\left(\frac{K_3}{K_3 + N_{P_2}v_0/v(t)}\right)^2 - k_{R_3}N_{R_3}, \tag{5}$$

$$\frac{\mathrm{d}N_{P_3}}{\mathrm{d}t} = k_{s3}N_{R_3} - k_{P_3}N_P, \tag{6}$$

where N_{R_2}, N_{P_2}, N_{R_3} and N_{P_3} are the numbers of R_2, P_2, R_3 and P_3 copies, $\kappa_{r2}, \kappa_{r3}, k_{s2}$ and k_{s3} are the synthesis rate constants, and $k_{R_2}, k_{P_2}, k_{R_3}$ and k_{P_3} are the degradation rate constants. Equation (3) implies that the R_2 synthesis is suppressed by P_1 and P_3. In particular, the synthesis is considered to occur provided that two regulatory sites are free of P_1 and other two regulatory sites are free of P_3 ($\mathcal{K}_2$ and K_2 are the constants characterizing protein–gene association–dissociation equilibrium). In turn, equation (5) implies that the R_3 synthesis is suppressed by P_1 and P_2 ($\mathcal{K}_3$ and K_3 are the corresponding association–dissociation constants).

In the absence of suppression of the R_2 and R_3 synthesis by P_1 (at $N_{P_1} \to 0$), equations (3)–(6) predict bistability in the expression of genes 2 and 3 provided that the rate constants of the R_2, P_2, R_3 and P_3 synthesis are sufficiently high. During one of the stable steady states, the R_2 and P_2 populations are high and the R_3 and P_3 populations are low. During another steady state, the situation is opposite. The probability of trapping into one of these states depends on the initial conditions in the beginning of the cell cycle. In reality, the initial conditions in the beginning of the cell cycle are however poorly defined. In addition, there are experimental indications that the decision stage of differentiation is inside the cell cycle, not in the beginning (for example, the differentiation of mammalian cells seems to be controlled by regulating the progression through the G1 phase and entry into the S phase [30]). For these reasons, genes 2 and 3 alone are not sufficient in order to describe spontaneous differentiation. In our model, the decision stage is replaced inside the cell cycle due to the regulation of the performance of genes 2 and 3 by gene 1. Below, we illustrate this scenario in detail.

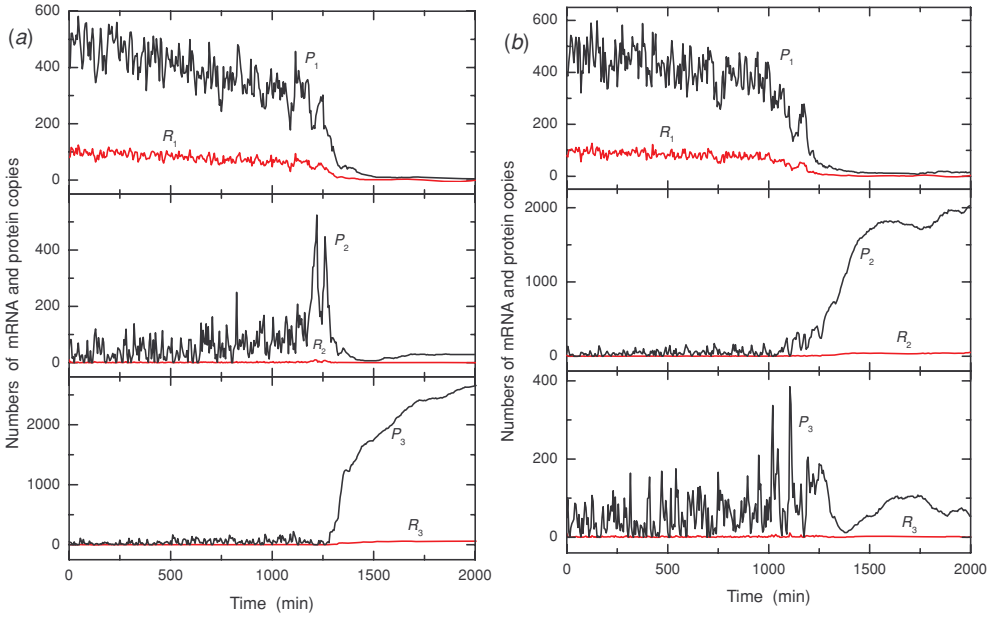

Figure 1. ((*a*) and (*b*)) Numbers of mRNA and protein copies as a function of time during two Monte Carlo runs with $k_{r2} = k_{r3} = 100\ \text{min}^{-1}$ and $t_c = 2000$ (for the other parameters, see the text).

3. Results of calculations

To illustrate the kinetics predicted by equations (1)–(6), we use typical biologically reasonable parameters (see, e.g., review [4]). In particular, the parameters for gene 1 are as follows: $k_b = 1\ \text{min}^{-1}$, $k_{r1} = 100\ \text{min}^{-1}$, $K_{P_1} = 50$, $k_{R_1} = 0.7\ \text{min}^{-1}$, $k_{s1} = 5\ \text{min}^{-1}$ and $k_{P_1} = 1\ \text{min}^{-1}$. In this case, the maximum numbers of R_1 and P_1 copies are $N_{R_1} \simeq k_{r1}/k_{R_1} = 140$ and $N_{P_1} \simeq k_{s1}k_{r1}/(k_{P_1}k_{R_1}) = 700$.

Genes 2 and 3 are first considered to be kinetically equivalent. Specifically, we employ $k_{r2} = k_{r3} = 100\ \text{min}^{-1}$, $\mathcal{K}_2 = \mathcal{K}_3 = 50$, $K_2 = K_3 = 100$, $k_{s2} = k_{s3} = 50\ \text{min}^{-1}$ and $k_{R_2} = k_{R_3} = k_{P_2} = k_{P_3} = 1\ \text{min}^{-1}$. With these parameters, the maximum numbers of R_2 and P_2 (or R_3 and P_3) copies are $N_{R_2} \simeq k_{r2}/k_{R_2} = 100$ and $N_{P_2} \simeq k_{s2}k_{r2}/(k_{P_2}k_{R_2}) = 5000$.

The duration of the cell cycle is assumed to be $t_c = 2000$ or 4000 min.

To include fluctuations into the kinetics under consideration, we have performed the corresponding kinetic Monte Carlo simulations by using the standard Gillespie algorithm [31] (for the model-specific details, see [11]). Typical stochastic kinetics calculated with the parameters above are shown in figure 1.

With increasing time, as already noted in the previous section, the model predicts that gene 1 exhibits a sharp transition from the state with high R_1 and P_1 populations to the state with low R_1 and P_1 populations. During the first stage, the high P_1 population suppresses the rate of R_2 and R_3 synthesis, and the R_2, R_3, P_2 and P_3 populations are low. After the transition to the second stage, the low P_1 population does not suppress the rate of R_2 and R_3 synthesis, and the R_2, R_3, P_2 and P_3 populations begin to grow rapidly. With increasing P_2 and P_3 populations, these species start to suppress the R_3 and R_2 synthesis, respectively. For this reason, the R_2, R_3, P_2 and P_3 populations cannot become high simultaneously.

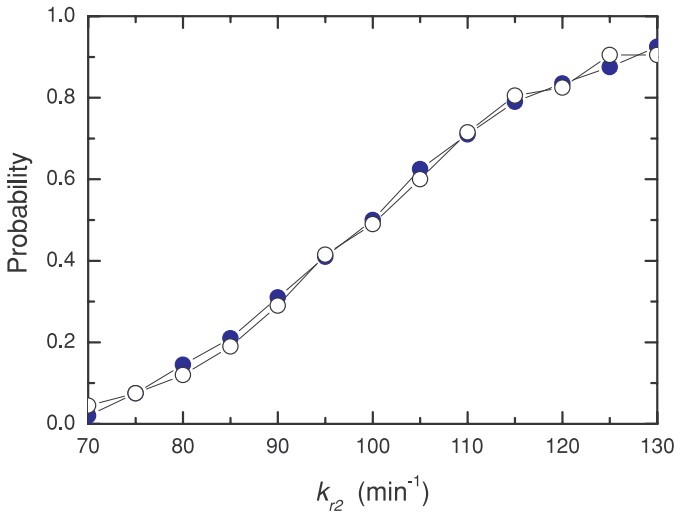

Figure 2. Probability that the cell reaches the state with high R_2 and P_2 populations as a function of k_{r2} for $k_{r3} = 100$ min^{-1} and $t_c = 2000$ min (filled circles) and 4000 min (open circles). Each data point was calculated by using 100 Monte Carlo runs.

Due to fluctuations, one of the genes (2 or 3) eventually dominates and the cell is trapped either to the state with low R_2 and P_2 populations and high R_3 and P_3 populations (figure 1(*a*)) or to the state with high R_2 and P_2 populations and low R_3 and P_3 populations (figure 1(*b*)). In this example, genes 2 and 3 are kinetically equivalent. For this reason, the probability to get to one of these states should be 0.5. The Monte Carlo simulations confirm that this is the case.

If genes 2 and 3 are not kinetically equivalent, then the probability to be trapped into one state described above is usually lower than 0.5, and the probability to be trapped into the other state is higher than 0.5. Our experience indicates that the variation of the parameters can easily result in appreciable preference of one of the states. This effect is illustrated in figure 2 showing the dependence of the probability to be trapped into the state with high R_2 and P_2 populations and low R_3 and P_3 populations on k_{r2} for $t_c = 2000$ and 4000 min (all the other parameters are fixed as described above). As one could expect, this probability is lower than 0.5 at $k_{r2} < k_{r3}$ and higher than 0.5 at $k_{r2} > k_{r3}$.

In general, the bistable kinetics are well known to be sensitive to kinetic parameters. For the models under consideration, this is also the case as shown in figure 2 by varying k_{r2}. On the other hand, the kinetics presented are insensitive to the growth rate. In particular, the results obtained for $t_c = 2000$ min are nearly the same as those for 4000 min (figure 2), because the cellular growth is slow and during the growth the gene expression is close to that predicted by using the steady-state approximation (with respect to the change of the cellular volume).

4. Conclusion

In summary, we have analysed the interplay of bistable kinetics of gene expression during cellular growth. In particular, we have shown that in the case where gene 1, operating in the bistable regime, regulates mutually inhibiting genes 2 and 3, also operating in the bistable regime, the latter genes may eventually be trapped either to the state with high transcriptional

activity of gene 2 and low activity of gene 3 or to the state with high transcriptional activity of gene 3 and low activity of gene 2. The probability to get to one of these states depends on the values of the model parameters. If genes 2 and 3 are kinetically equivalent, the probability is equal to 0.5. Thus, basically, the model shows how the genes can play dice during cellular growth. Specifically, the model illustrates how different intracellular states can be chosen at random with predetermined probabilities. This type of kinetics of gene expression may be behind complex processes in cells, e.g., behind the choice of the fate by stem cells.

Concerning cell differentiation, one might argue that gene 1 in our model could be replaced by time dependence introduced into the rate constants related to genes 2 and 3. Such perturbations of gene 2 or 3 typically switch these genes away from the bistable regime and usually are not efficient if the goal is to describe spontaneous differentiation occurring with certain probabilities. Nevertheless, this strategy may work if one focuses on the perturbation of the rate of the formation of mRNA 2 and 3 (as has been done in our treatment above), use appreciable amplitude of this perturbation, and extend the length of its period. In this case, in analogy with our simulations, the results will depend on the ratio of the rate constants of k_{r2} and k_{r3}. In the symmetric case with $k_{r2} = k_{r3}$, the probability of one of the outcomes will obviously be 0.5 irrespective of the length of the perturbation period. If $k_{r2} \neq k_{r3}$, the probability of one of the outcomes will depend on the length of the perturbation period, and this dependence is expected to be weak. Concerning biology, it is worth however articulating two points. First, it is desirable to have a perturbation with a large amplitude. In figure 1, for example, N_{P1} drops from about 500 to 20. Such changes can hardly be realized without bistability of gene 1. Second, the duration of the decision stage should be relatively short, because as already mentioned in section 2 there are indications that the differentiation of cells is controlled by regulating a short stage of the progression through the G1 phase and entry into the S phase. The fastest switch can be performed by using a stepwise perturbation (like in our model). In this case, the duration of the decision stage is about 15% of the duration of the cell cycle (see, e.g., figure 1). This value is biologically reasonable. The extension of the length of the perturbation period will result in a longer decision stage, and it will not be biologically reasonable.

Finally, we may note that the problem treated in this paper is interdisciplinary and can be viewed from very different perspectives. The model, results and discussions presented help to understand the situations in the related areas.

References

[1] Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P 2002 *Molecular Biology of the Cell* (New York: Garland)

[2] Amaral P P, Dinger M E, Mercer T R and Mattick J S 2008 The eucariotic genome as an RNA machine *Science* **319** 1787–9

[3] Goodrich J A and Kugel J F 2006 Non-coding-RNA regulators of RNA polymerase II transcription *Nat. Rev. Mol. Cell Biol.* **7** 612–6

[4] Kaern M, Elston T C, Blake W J and Collins J J 2005 Stochasticity in gene expression: from theories to phenotypes *Nat. Rev. Genet.* **6** 451–64

[5] Paulsson J 2005 Models of stochastic gene expression *Phys. Life Sci.* **2** 157–75

[6] Kaufmann B B and van Oudenaarden A 2007 Stochastic gene expression: from single molecules to the proteome *Curr. Opin. Gen. Dev.* **17** 107–12

[7] Mariani L, Löhning M, Radbruch A and Höfer T 2004 Transcriptional control networks of cell differentiation: insights from helper T lymphocytes *Prog. Biophys. Mol. Biol.* **86** 45–76

[8] Zhdanov V P 2008 Bifurcations in the interplay of messenger RNA, protein, and nonprotein coding RNA *J. Phys. A: Math. Theor.* **41** 285101

[9] Zhdanov V P 2009 Bistability in gene transcription: Interplay of messenger RNA, protein, and nonprotein coding RNA *BioSystems* **95** 75–81
[10] Nakajima A and Kaneko K 2008 Regulative differentiation as bifurcation of interacting cell population *J. Theor. Biol.* **253** 779–87
[11] Zhdanov V P 2006 Transient stochastic bistable kinetics of gene transcription during the cellular growth *Chem. Phys. Lett.* **424** 394–8
[12] Zhdanov V P 2007 Stochastic bistable kinetics of gene transcription during the cell cycle *JETP Lett.* **84** 632–4
[13] Losick R and Desplan C 2008 Stochasticity and cell fate *Science* **320** 65–8
[14] Morrison S J and Kimble J 2006 Asymmetric and symmetric stem-cell divisions in development and cancer *Nature* **441** 1068–74
[15] Insight: Stem cells 2006 *Nature* **441** 1059–102
[16] Viswanathan S and Zandstra P W 2003 Towards predictive models of stem cell fate *Cytotechnology* **41** 75–92
[17] Zhdanov V P 2007 Stem cell proliferation and differentiation and stochastic bistability in gene expression. *J. Exp. Theor. Phys.* **104** 162–9
[18] Xiong W and Ferrell J E 2003 A positive-feedback-based bistable 'memory module' that governs a cell fate decision *Nature* **426** 460–5
[19] Lai K, Robertson M J and Schaffer D V 2004 The sonic hedgehog signaling system as a bistable genetic switch *Biophys. J.* **86** 2748–57
[20] Chang H H, Oh P Y, Ingber D E and Huang S 2006 Multistable and multistep dynamics in neutrophil differentiation *BMC Cell Biol.* **7** 11
[21] Chickarmane V, Troein C, Nuber U A, Sauro H M and Peterson C 2006 Transcriptional dynamics of the embryonic stem cell switch *PLOS Comput. Biol.* **2** 1080–92
[22] Callard R E 2007 Decision-making by the immune response *Immunol. Cell Biol.* **85** 300–5
[23] Huang S, Guo Y P, May G and Enver T 2007 Bifurcation dynamics in lineage-commitment in bipotent progenitor cells *Dev. Biol.* **305** 695–713
[24] Moore K A and Lemischka I R 2006 Stem cells and their niches *Science* **311** 1880–5
[25] Scadden D T 2006 The stem-cell niche as an entity of action *Nature* **441** 1075–9
[26] Till J E, Siminovitch L and McCulloch E A 1964 Stochastic model of stem cell proliferation based on growth of spleen colony-forming cells *Proc. Natl. Acad. Sci. USA* **51** 29–36
[27] Zhdanov V P 2008 Simulation of proliferation and differentiation of cells in a stem-cell niche *Physica* A **387** 6126–36
[28] Gurok U, Steinhoff C, Lipkovitz B, Ropers H-H, Scharff C and Nuber U A 2004 Gene expression changes in the course of neural progenitor cell differentiation *J. Neurosci.* **24** 5982–6002
[29] Palmqvist L, Glover C H, Hsu L, Lu M, Bossen B, Piret J M, Humphries R K and Helgason C D 2005 Correlation of murine embryonic stem cell gene expression profiles with functional measures of pluripotency *Stem Cells* **23** 663–80
[30] Burdon T, Smith A and Savatier P 2002 Signalling, cell cycle and pluripotency in embryonic stem cells *Trends Cell Biol.* **12** 432–8
[31] Gillespie D T 1977 Exact stochastic simulation of coupled chemical reactions *J. Phys. Chem.* **81** 2340–61