Characterization of stem cells and cancer cells on the basis of gene expression profile stability, plasticity, and robustness

Dynamical systems theory of gene expressions under cell-cell interaction explains mutational robustness of differentiated cells and suggests how cancer cells emerge

*Kunihiko Kaneko**

Here I present and discuss a model that, among other things, appears able to describe the dynamics of cancer cell origin from the perspective of stable and unstable gene expression profiles. In identifying such *aberrant* gene expression profiles as lying outside the normal stable states attracted through development and normal cell differentiation, the hypothesis explains why cancer cells accumulate mutations, to which they are not robust, and why these mutations create a new stable state far from the normal gene expression profile space. Such cells are in strong contrast with normal cell types that appeared as an attractor state in the gene expression dynamical system under cell-cell interaction and achieved robustness to noise through evolution, which in turn also conferred robustness to mutation. In complex gene regulation networks, other *aberrant* cellular states lacking such high robustness are expected to remain, which would correspond to cancer cells.

Keywords:
attractor; cell-cell interaction; evolution; robustness; stem cell

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Department of Basic Science and Research Center for Complex Systems Biology, University of Tokyo, Komaba, Meguro, Tokyo, Japan

***Corresponding author:**
Kunihiko Kaneko
E-mail: kaneko@complex.c.u-tokyo.ac.jp

Abbreviation:
GRN, gene regulation network.

Introduction

Robustness and plasticity are essential features of all biological systems [1–8]. Robustness describes the insensitivity of a system to external perturbations, and plasticity describes the changeability of a system in response to changes in external conditions. In this sense, robustness and plasticity tend to compete with each other if they work at the same dimension. Biological systems, however, somehow strike a balance between the two, or adopt each of the two separately depending on the situation.

Sources of external perturbations have distinct time scales. On a faster time scale, external perturbations of noise occur in developmental processes. On a much slower time scale, changes in genetic sequence may perturb developmental dynamics. Biological organisms somehow achieve robustness at these two different scales, developmental and evolutionary [9–12].

In multicellular organisms, the developmental process typically has an intermediate timescale – intracellular gene expression dynamics and a multicellular process involving the increase in cell number [12–14]. Cells differentiate into several types, while an organism or tissue is shaped over a time scale

based on the increase in the cell number, which is much slower. Multicellular organisms achieve robustness to external perturbations for both the scales, that is, cell-type-specific stability in gene expression and stability against a change in the number distribution of cells. Also, the path taken to reach the final cellular state is fairly stable.

Furthermore, in multicellular organisms, a multipotent cell generally exists that has the potentiality to differentiate into many cell types, depending on conditions. In this sense, such cells have plasticity, while robustness of an organism at a global scale is achieved. Understanding multipotency and robustness in developmental processes remains one of the basic problems in developmental biology.

In this essay, I discuss the issue of robustness and plasticity in terms of dynamical systems theory [15, 16]. Concepts in dynamical systems are central to understanding this mathematical formalization of cell behavior, so that I give a brief intuitive account on these:

The concept of an attractor: Waddington put forward an image of development as a ball rolling down along a landscape with peaks and valleys [1]. The ball will finally come to rest at the bottom of a valley, a local minimum of the particular landscape. If there are many valleys and peaks, the final position may depend on the starting point of the ball. In the theory of dynamical systems, the final destination of the ball is the 'attractor', and each of the domains of the valley separated by peaks is known as the ''basin'' of the respective attractor.

Now imagine some perturbations kick the ball. If the valley is deep, the final position of the ball is still at the same bottom of the valley. Hence the attractor concept is important to understand the *robustness* of a system.

Essentially this attractor concept can be applied to gene expression levels resulting from a network of interacting proteins – in this case, gene/protein expression levels being the ball. With time, the expression levels result in an equilibrium between the components, which corresponds to the bottom of the valley in the afore-mentioned landscape. Perturbation on the ''ball'' – i.e. changes in one or more protein levels, caused by environmental change or noise – may shift the equilibrium to a new one, corresponding to the ball being jostled out of one valley, and finding its way to a nearby depression in the landscape.

Sometimes, the nature of interactions between proteins can generate a bistable system where two very different equilibria – or attractors – exist, for example in the case of a two-protein network where protein A inhibits the synthesis of protein B and protein B likewise inhibits the synthesis of protein A.

So far, I have talked about simple, static, attractors. There are also attractors in which the ball (i.e. the expression level) keeps changing position with time. These correspond, for example, to an oscillating state of protein levels, such as that manifested by the following simple network: protein A activates transcription of protein B and itself, and protein B inhibits transcription of protein A. Under appropriate setting and parameter values, the very oscillatory behavior of the system is a stable state, and hence can be considered as an attractor. If more proteins are involved, the oscillation may be complex, without periodic repetition.

Flow in state space: Mathematically, the above picture is represented by a *flow* in the state space. For simple illustration, consider a system consisting of two proteins X and Y. Then the 'state' of this two-protein system is plotted as a point in a two-dimensional space, with x and y axes as the expression levels (concentration) of X and Y, respectively. In general, each protein influences the synthesis or degradation of the other protein. Hence, depending on concentrations of the two proteins, their increase or decrease in time is determined (by the gene network, i.e. by the rule of chemical reactions). In other words, 'flow' arises in the two-dimensional x–y plane, represented by arrows in the plane. The expression levels (concentrations) change in time along this flow, and this trail of points in the plane is a *trajectory*. Now, with time they may settle on a point where flow vanishes, i.e. the reactions are still occurring, but they balance each other out, and there is no net directionality.

This point corresponds to the bottom of the valley, where the flow to roll down the ball vanishes. To compare the landscape picture, 'height' is assigned at each point in this state space, and the flow is assigned to the direction of decreasing height. However, the 'flow' picture in the 'state space' is more general than the landscape, and it can cover the case that the concentrations do not settle to fixed values, but keep on oscillating. For example, if x and y settle to simple sinusoidal oscillation, given by $x(t) = r\cos(t) + x_0, y(t) = r\sin(t) + y_0$, the trajectory forms a circle, with a radius r, centered at (x_0, y_0). In this case the attractor is a circle, whereas in general the attractor corresponding to oscillatory state is a closed curve in the state space (Fig. 1).

This picture is straightforwardly extended to the case in which more genes (proteins) are involved. If three are involved, the state space is three dimensional, and if k proteins are involved, the state space is k-dimensional (see Fig. 1, and Box 1).

In general, there is deviation in the concentration change from the original flow by gene regulation dynamics, since all the chemical reactions are due to random collisions of molecules. This random perturbation is called ''noise'' (see Box 1).

Now, consider the effect of perturbation by noise on the state variables (concentrations) at an attractor. Once the cell state is on an attractor, its state variables return to the original values by the dynamics even if perturbed by noise, as long as the state stays within the basin of attraction. This means that the protein concentrations stay at a certain range of values, determined by an attractor, even under the noise. Hence, the attractor concept is essential to understanding *robustness against noise* [12, 16–18, 23].

Still, the notion of attractors does not solve all the questions related to robustness. First, whether the cell state reaches an attractor globally from various initial values depends on the size of basin. During the dynamics to reach an attractor, the trajectory may be perturbed by noise and the state may not reach the given attractor. Hence, the robustness depends on gene expression dynamics. How are gene-expression dynamical systems evolved to increase robustness?

Second, stem cells in multicellular organisms can both proliferate and differentiate. For proliferation, the cell state

Box 1

Terminology in dynamical systems with applications to biological systems

Terminology in Dynamical Systems applied to cell behavior (see Fig. 1)

Consider a cell state that is specified in terms of the concentrations of its components. For example, if there are k proteins, the cell state is described by the concentrations of those proteins, i.e. $(p_1(t), p_2(t), p_3(t), \ldots, p_k(t))$ which may change through time, t. These are *state variables*, whose number, k, is the *degree of freedom* of the system. These state variables influence each other, and change across time. This gives rise to a *flow* in the state space. Accordingly, the time course of the change in state variables is represented as a *trajectory* in the state space. For example, expression of a particular gene is either activated or repressed by expression of other genes, and the concentrations of the resulting proteins change in time in accordance with the gene regulatory network.

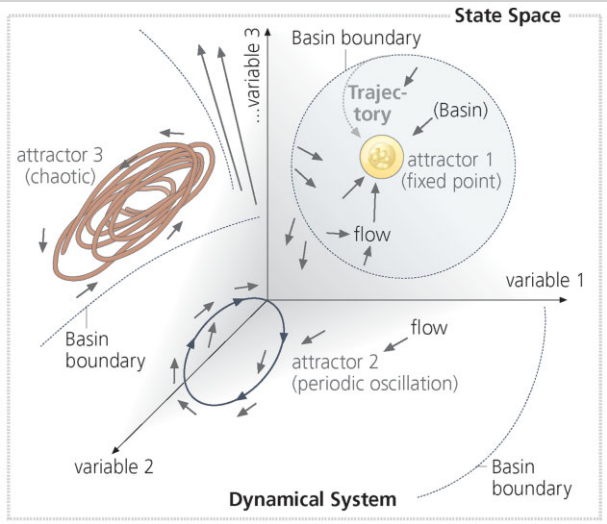

Figure 1. Schematic description of dynamical systems corresponding to the terms in Box 1.

With time, the state variables reach, and stay at, a certain range of values. This region of state space in which the state variables can occupy after sufficient time is called an *attractor*. The attractors can be stationary states (fixed points), periodic oscillation (closed curve in the state space), aperiodic irregular oscillation (chaotic behavior).

There can be several attractors that are reached depending on the initial values of the variables. The region of such initial values that from which the system will "gravitate" towards each attractor is called its *basin* of attraction. The boundary of the basin divides the state space, with regards to which attractor the flow of state variables reaches.

Consider N cells, each of which has k state variables that interact with each other. Then the flow (temporal change) of k variables of each cell is modified following the states of other cells. With this cell-cell interaction, the state variables may be kicked away from the original attractor, while novel attracting states that did not exist in the single-cell dynamics may be generated (*stability induced by interaction*).

Terminology in Developmental Biology used in the present text

Noise (in gene expression dynamics)

On average, change in concentration of proteins is given by concentrations of chemicals that constitute the reaction. However, the reaction is due to molecular collisions, and there are deviations from the average. This fluctuation is regarded as a noise term added to the dynamical system. Note that this particular use of the term 'noise' is adopted in physics and also in recent literature in systems biology, but in evolutionary biology it is used in a different context.

Robustness to noise

Insensitivity of the phenotype (state variables of the attractor) to perturbation by noise during the (developmental) dynamics.

Robustness to mutation (mutational robustness)

Insensitivity of the phenotype (state variables of the attractor) to the change of the rule of (developmental) dynamics introduced by genetic change.

should be stable, while for differentiation it should be plastic. How are robustness and plasticity compatible in stem cells?

Here I discuss these issues from a dynamical-systems perspective, based on my group's earlier numerical simulations of cell differentiation and evolution of cells over a decade. Following the discussion, we propose a hypothesis on cancer state as an attractor that is not visited in the normal course of development and lacks *mutational robustness*.

Robustness to noise arises naturally when evolution of gene networks is modeled on a background of moderate noise

Let us begin the discussion on robustness at the single-cell level. As surveyed in the introduction, I represent the phenotype of a cell by state variables that are a set of protein concentrations. The temporal change in cell state is determined by complex gene expression dynamics governed by a GRN. These dynamics are subjected to noise due to molecular fluctuations in chemical reactions [24–28]. Under noise, as shown in Fig. 2A, the *orbit* in the state space, i.e. the time course of protein concentrations, required to reach the relevant phenotype might be easily diverted to phenotypes that lose the desired function.

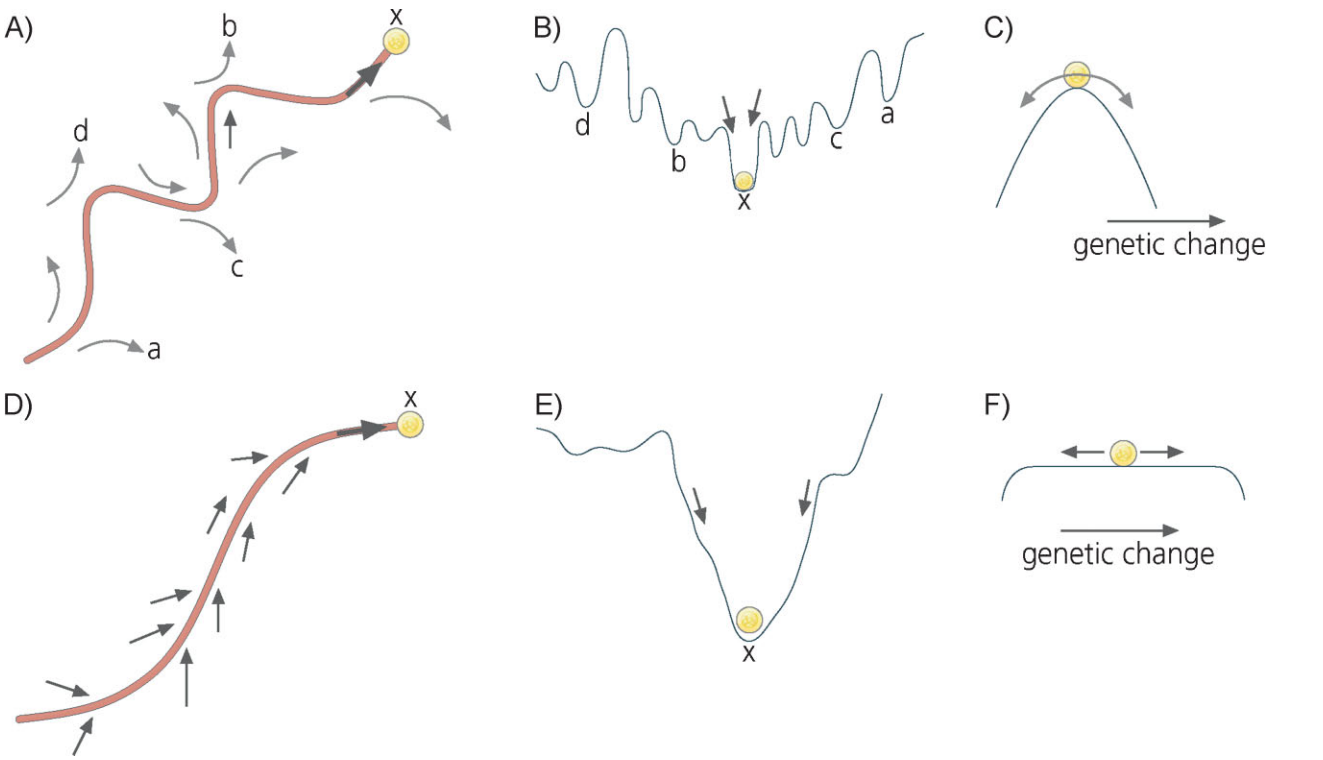

Figure 2. Schematic representation of dynamics of a cellular state. **A–C:** Non-robust case. **D–F:** Robust case evolved under a sufficient noise level in gene expression dynamics. **A,D:** Flow structure of the cellular state. **B,E:** Illustration of gene expression dynamics as the falling motion of a ball in a landscape. **C,F:** Robustness against mutational change.

The author studied gene expression dynamics with randomly chosen regulation networks that control activation and repression among proteins, and found that most dynamics have such weakness against perturbations [11, 12]. Even if a network happened to result in the phenotype concerned, only a very *narrow* path existed to reach the functional phenotype ("x" in Fig. 2A and B). GRNs were then evolved numerically by using a genetic algorithm to increase a fitness function [11, 29–31] that was defined to achieve functional phenotypes, i.e. to generate a given gene expression pattern. When gene expression dynamics were subjected to a sufficient level of noise, it could not generate the functional phenotype stably if the dynamics were of the type given in Fig. 2A and B. Rather, the orbit of the dynamics would be kicked away from the original trajectory, and the generated phenotype would be different from it, as shown as a, b, c,... in Fig. 2A and B. However, when the network evolved, they were found to attain robustness to noise, as shown in Fig. 2D and E. The orbit required to generate the requested phenotype was not easily perturbed by noise, and even from perturbed gene expression states, the orbits as well as the final expression pattern converged to the original phenotype. Hence, robustness to noise was evolved naturally (see also [32, 33]).

Robustness to noise results in robustness to mutation in gene expression patterns

Let us represent the attraction to the final gene expression pattern as the motion of a ball in a landscape. From numerical simulations, it was found that gene expression dynamics from most GRNs chosen randomly was represented by a rugged landscape as in Fig. 2B, whereas, after evolution under a sufficiently high noise level, it exhibited a rather smooth landscape with global attraction to the phenotype, as in Fig. 2E. In contrast, when the gene expression dynamics evolved under a lower noise level (or without noise), the GRN remained in the form of Fig. 2A and B.

Now, consider mutation to the GRN. (Here I use the term *mutation* rather naively, to include any genetic change to modify the GRN.) Now there are two distinct concepts on robustness, that to noise and to mutation (genetic change). The former concerns the stability of a cellular state against noise in gene expression dynamics, for cells sharing the same genes, while the latter concerns the stability against genetic change that slightly alters dynamical systems itself, by modification of GRN (see Box 1).

From extensive simulations, it was found that robustness of cellular state to mutation differs distinctly between the above two types of dynamics given in Fig. 2A and D [29–31]: the GRN without robustness to noise (Fig. 2A and B) also lacked robustness to mutation (Fig. 2C). Genetic changes perturbed GRNs and the flow in the state space would be perturbed. Then, the generated gene expression pattern no longer gave rise to the requested phenotype. In dynamics with a rugged landscape as in Fig. 2A and B, we can naturally expect that a

slight change easily destroys the attraction to the original valley. Hence, deleterious mutations would often appear.

In contrast, it was found that the GRN evolved under a sufficiently high noise level (Fig. 2D and E) had robustness to mutation (Fig. 2F). The attraction to the original path in Fig. 2D was so strong that most perturbations in flow caused by mutations in GRNs still gave rise to the original phenotype. Hence, most mutations were neutral. In a smooth landscape with a global basin of attraction, changes in dynamics caused by mutations are expected not to damage the attraction to the original gene expression pattern, which explains the robustness to mutation.

Different robustness to mutation between the above two cases were numerically confirmed [29–31]. In the GRN evolved under noise, orbits from different initial values took a similar path, as postulated by Waddington as *homeorhesis* [1]. Evolution of robustness to noise and mutation was also confirmed in catalytic reaction networks [34] and a toy protein-folding model [35]. Such converging orbits were also observed in the gene expression dynamics of yeast [33].

To summarize, evolution to achieve robustness to noise was found to introduce robustness to mutation. Hence, cells under selection pressure for some function are expected to achieve these two types of robustness.

Multipotency and differentiation can be explained by modeling gene expression dynamics and cell-cell contacts together

Cell-cell interactions channel differentiation towards stable types

In a multicellular system, developmental dynamics are more complex; they involve both intracellular gene expression dynamics and cell-cell interaction. The latter influences the internal expression dynamics and depends on the surrounding cells, whose number increases over development. The importance of cell-cell interaction was experimentally demonstrated as the community effect [36], and discussed for differentiation from stem cells [37, 38]. During the developmental process, differentiation occurs from stem cells to several cell types. All the cells converge to one of these types, while stem cells can remain, and have potentiality both for proliferation and differentiation [39, 40].

Waddington's epigenetic landscape accounts for this channeling

To understand robustness in these cell types as well as over the temporal course of development, Waddington adopted the landscape picture as a metaphorical picture for developmental processes [1]. We could interpret this picture as follows (Fig. 3). At each time point there are several valleys, which correspond to each cell type; in terms of dynamical systems as applied to gene expression, each is regarded as an attractor, as discussed in the last section (Fig. 2). In fact, several attempts have been made to represent such a landscape from the gene expression dynamics [41] and experimental data [42]. Note, to support several cell types, gene expression dynamics must have several attractors that correspond to several valleys, as also demonstrated in several GRN models [18, 23].

However, the landscape must change over time to explain fundamental features of tissues

If the landscape is temporally fixed, however, we cannot answer the following three basic questions. First, why are some cells located in a basin of one valley, and others in a different valley? How are such *initial conditions* for each cell type determined? Second, the number distribution of cells at each valley should not be arbitrary, but rather kept within some range, for a tissue or a multicellular organism to function normally. Indeed, the organism or tissue consists of several cell types whose number ratios are determined to some degree. How is such proportional regulation achieved? Third, how is the temporal course of differentiation, i.e. at what stage each cell type appears, determined in a robust manner?

Indeed, in the landscape drawn by Waddington, the hills and valleys are not fixed, but also develop over time. Hence, two courses of events exist. Over

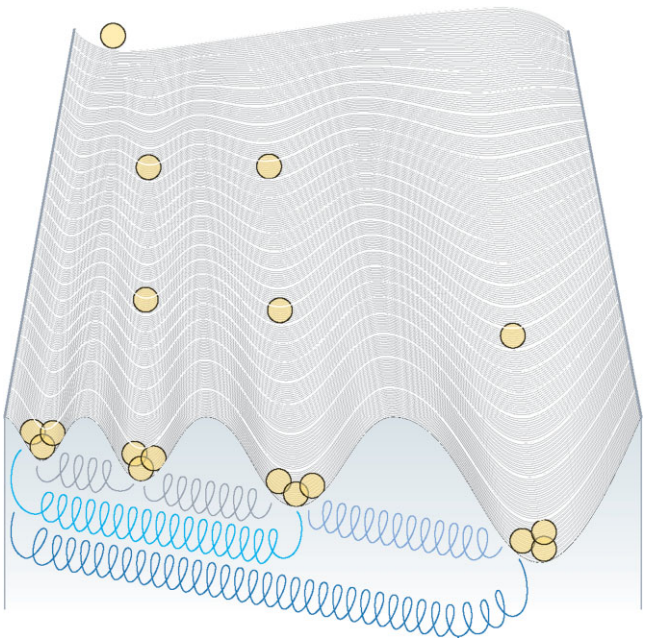

Figure 3. *Epigenetic* landscape shaped with the increase in cell number and cell-cell interaction. Schematic drawing. The *springs* between cells at different valleys are schematic representations of cell-cell interaction. (In the present biology, epigenetics often refer to histone modification and DNA methylation. The term epigenetics, however, was originally coined by Waddington, as a developmental process by which interaction among genes as well as with the environment produce a phenotype.)

a faster scale, cells are attracted into each valley, while the landscape itself grows over a slower time scale of development. This change in the landscape occurs through the increase in cell number. With this increase, the gene expression dynamics of each cell can be modified by the changes in cell-cell interactions. This change will introduce the change of the landscape (Fig. 3).

Modeling gene expression dynamics in concert with cell-cell interactions recapitulates stem cell and differentiated cell identities

Based on the above picture, Furusawa and the author studied a class of models [14, 43–46], in which a cell state was represented by a set of concentrations of proteins (gene expression levels). The model consisted of (i) intracellular reaction (gene expression) dynamics; (ii) cell-cell interactions mediated by the diffusion (or transport) of some chemicals; and (iii) increase in cell number over time as a result of cell division. Protein concentrations changed due to intracellular reactions and were also influenced by the cell-cell interaction,

Box 2

A bit more on intra-inter-cellular dynamics theory for cell differentiations

(a) Differentiation of phases of oscillations

Furusawa and the author chose a gene regulation network (GRN) that showed temporal oscillation in concentrations of some proteins at the single-cell level (Fig. 4A). With the increase in cell number by divisions, the degree of cell-cell interaction increases, which causes these oscillations to desynchronize among cells. This is because only some chemical species diffuse, and homogenization of concentrations works only for some chemicals. With intracellular feedback reactions [13, 16–19], cell-to-cell difference in the concentration for other non-diffusible chemicals is not reduced but is amplified. Thus, the phase of oscillations differs across cells. This difference in phases, under the influence of the cell-cell interactions, leads to irregularity in oscillation, which causes differences in protein concentrations between cells to be amplified.

(b) Differentiation in composition of proteins

This amplification reaches a stage at which the composition of proteins is distinct between cells, leading to differentiation to a new cell type (Fig. 4B). Some proteins in differentiated cells have much lower or higher concentrations than in the original cell type. The cell state leaves the basin of attraction of the original cell type: a gate opens, allowing a cell to exit from the original valley. Here, the instability for differentiation is not directly coupled with the cell division event, but occurs through the increase in cell number.

(c) Stabilization of a differentiated state by cell-cell interaction

The new stationary cell state is stabilized by the interaction with the original cell type. In other words, a new *valley* is generated. In this differentiated cell type, the concentration oscillation that exists in the original cell type is lost or weakened (Fig. 4B).

These dynamics of differentiation can be understood as follows. The trajectory of the original cell state is modulated from the single-cell attractor by the cell-cell interaction, so that it goes across the basin boundary of the original attractor. Then, the state of such cells moves to a new state, implying cell differentiation into another type. The stability of the differentiated type depends on the cell-cell interaction, and accordingly, on the number ratio of the two cell types.

(d) Regulation between proliferation and differentiation leading to macroscopic robustness

In the above it is found that stem cells in the model lose their stability to sustain their original state, due to the cell-cell interaction. However, the increase in the number of differentiated cells changes the interaction, and the original cell state restores stability; thus, the attractor and its basin boundary no longer touch (Fig. 4C). With the proliferation of the original cell type, its attractor and its basin boundary touch again, and the differentiation occurs. In this way, stem cells in the model keep on either proliferating or differentiating, depending on the number ratio of the cell types. If the ratio of stem cell is too large (small), the ratio of differentiation (proliferation) increases, so that the number distribution of two cell types remains within a certain range, implying (macroscopic) robustness of the number ratio of cells of different types (see [20] for a demonstration of such regulation of cell number and [21] for possible relationship with the present theory. Also see [22] for a bifurcation analysis on the stabilization of a new cell type by cell-cell interaction.).

(e) Generality and limitations

Thus, characterization of stem cells, as well as the loss of potentiality of further differentiation as a result of cell-differentiation, is represented in terms of dynamical systems of interacting cells. As long as such gene expression dynamics allows for oscillation between *on* and *off* states of gene expression, this course of differentiation appears universally.

Of course, there will be limitations to the theory here. When isolated and put into the original culture, differentiated cells in the model investigated here often revert to the original stem cell state. The differentiation does not reach the stage of rigid fixation, for which molecular processes such as DNA methylation might be necessary.

which changed with the increase in cell number. Furusawa and the author carried out extensive simulations of these models and confirmed the following picture (see Box 2 for details):

(1) Let us first assume that concentrations of some proteins at the single-cell level showed temporal oscillation (Fig. 4A). As the number of cells increased, the influence of cell-cell interaction increased, and accordingly, these oscillations lost synchrony among cells. With intracellular feedback reactions [13, 16–19], cell-to-cell difference in the concentration was amplified, until some proteins were highly expressed in some cells, whose state deviated from the original attractor (Fig. 4B).

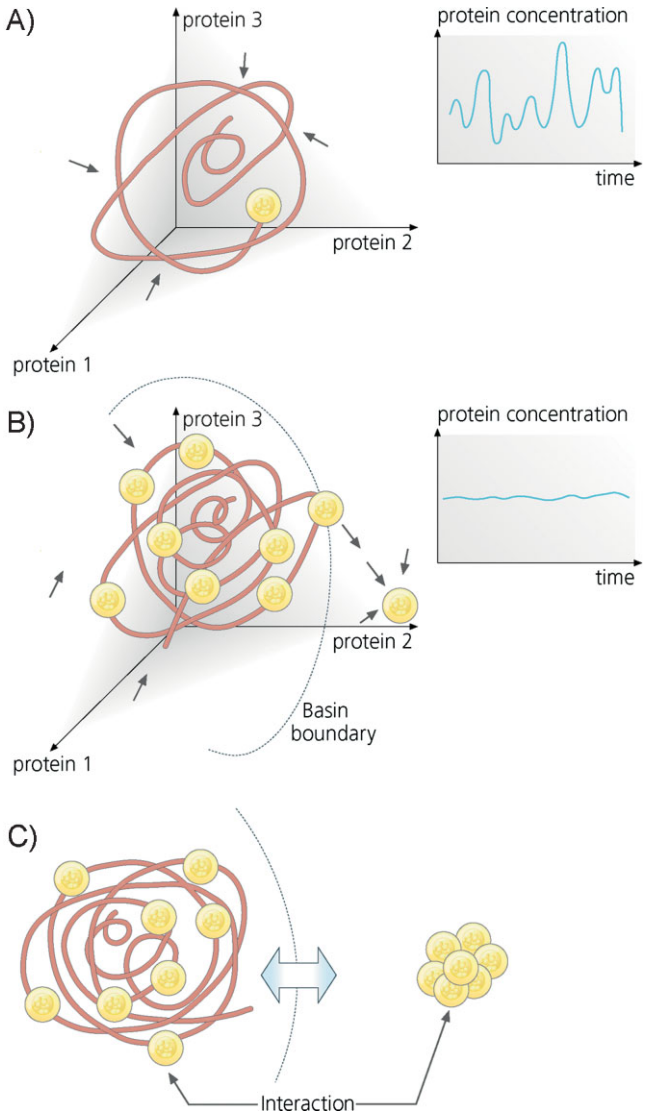

Figure 4. Schematic representation of the cellular differentiation process from multipotent stem cells, drawn by gene expression dynamics in the state space of protein concentrations. **A:** Oscillatory dynamics of a single stem cell, and illustration of the protein concentration of such a cell. Arrows represent the flow in the state space. **B:** With the increase in cell number, the orbit, the temporal course of protein concentrations, touches the boundary of its basin of attraction, so that some cells go out of the original attractor. Note that there are many directions in the state space. The original attractor remains attracted from most directions and the orbit goes out only through a limited direction at a specific timing. After escape from the original attractor, such cells are attracted to a different state in which the protein concentration is no longer oscillatory. **C:** Whether the attractor of the original cell is contacted with its basin boundary changes depending on the number ratio between the two cell types.

(2) With the interaction with the original cell type, the trajectory of such deviated cells went beyond the basin of attraction to the original attractor and was attracted to a new stationary state. Differentiation into a new cell type occurred, in which the concentration oscillation was lost (Fig. 4B).
(3) Under the presence of (a sufficient number of) differentiated cells, the flow of the cell state was shifted towards the original attractor, due to the interaction with them. The original state recovered stability (Fig. 4C), leading to its proliferation. In this way, this cell type, the *stem cell* in the model, differentiated if its number ratio was large, and proliferated if it was small. The two cell states remained stable as attractors only if the ratios of two cell types fell within a limited range. This gives a possible explanation of the macroscopic robustness of multicellular systems.

The model explains decreasing variation as differentiation proceeds

For complex GRNs, hierarchical differentiation progressed as in Fig. 5. Here, the initial cell type had potentiality to produce any other type including itself, while each type at a lower hierarchy produced only itself and the types downward. Thus, multipotency decreased. This decrease in multipotency was characterized by:

(i) Oscillations of protein concentrations decreasing as cells become more differentiated: the multipotent cells in the model presented here demonstrated temporal oscillation in the concentration of some proteins. This oscillation was not regular, and the cellular state itinerated over a few quasi-stable states. As differentiation progressed, this temporal variation decreased, so that the range of states that the cell could visit decreased with the developmental course (Fig. 5).
(ii) Cell-cell variation decreasing from stem to differentiated cell: this temporal variation was rather slow,

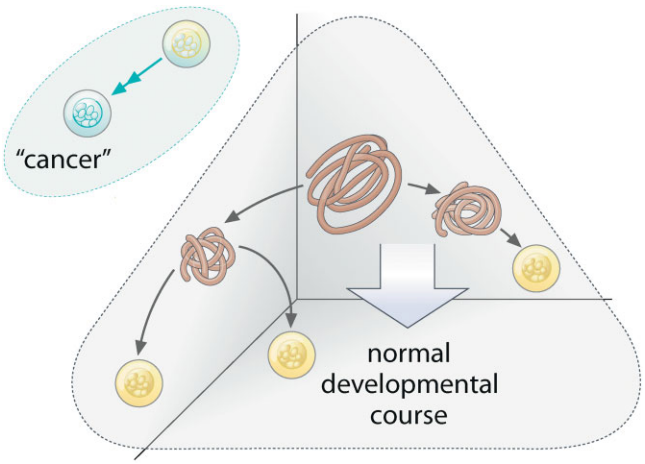

Figure 5. Differentiation in the normal developmental process and the existence of other attractors corresponding to cancer state. The normal developmental course starts from the stem-cell attractor, and progresses with the increase in the cell number and the following cell-cell interaction. Besides these normal attractors, a cancer attractor(s) exists that is reached when a large perturbation is applied to deviate from the normal developmental course. The stability of a cancer attractor could be increased by the genetic change. Schematic drawing in the state space.

so that at each snapshot in time, concentrations of some proteins differed distinctively across cells. By comparing cells at a given time, the cell-cell variation was large for multipotent stem cells and decreased as differentiation progressed.

Point (i) refers to the temporal variation of a single cell. Indeed, recent single-cell measurements of Hes1 protein concentrations using an imaging technique demonstrated the existence of oscillatory gene expression in embryonic stem cells within a period of a few hours [47]. This oscillation disappeared in differentiated cells [48]. Also, measurement of stem cell marker Sca-1 revealed the existence of slow transitory dynamics over metastable states for hematopoietic progenitor cells [49]. Point (ii), on the other hand, is concerned with the cell-to-cell variation. Heterogeneity in expressions of Stella, Nanog, and Hes1 expressions was recently observed in embryonic stem cells [50, 51]. To further examine this point, measurement of the variance of gene expressions over cells is important (as has been extensively carried out in bacteria and yeast [52, 53]). Decrease in cell-cell variations from embryonic stem cells to committed cells must be quantitatively confirmed.

Why differentiated cells can recover multipotency by changing expression of a few genes

In the simulations, the range of expression levels that the cellular state varied was larger for multipotent cells than for committed cells (see Fig. 3). In the stem cells in the model presented here, expressions of many genes mutually regulated each other. This complex regulation led to oscillatory dynamics with transitions over metastable states. In committed cell types, expressions were biased to fewer genes, which gave rise to stationary states. Thus, it is naturally expected that differentiated cells could recover the multipotency by perturbing expressions of a few genes, say by activating some genes whose expression is suppressed.

In fact, successful regaining of multipotency in animal cells was first achieved by nuclear transplant [54]. Furthermore, induced pluripotent stem cells recently demonstrated that pluripotency is restored by activating a few genes from differentiated skin cells, in agreement with the above discussion [55]. (There could be other ways to perturb gene expressions. Even by removing some gene, such reprogramming might be possible, as reported in [56].)

Unfortunately, from the above theoretical argument, we cannot predict which genes should be activated to restore pluripotency. Recalling, however, that oscillatory gene expression, generated as a result of feedbacks in gene regulation, is required for multipotency in our theoretical framework, it is expected that genes responsible for such oscillation should be activated to restore plasticity.

Evolutionary robustness of a multicellular system

So far I have reviewed the studies on the evolution of robustness and cell differentiation. Let us now discuss robustness of the multicellular system through evolution. As the gene expression in a cell is noisy, the discussion concerning robustness can also be applied here. First, the initial stem cell type will be stable at the single-cell level and remain so under a certain degree of cell-cell interaction introduced by the increase in cell number. The differentiated cell type must also be stable against perturbations, to maintain proliferation. These cell states must have achieved robustness against noise in gene expression dynamics, if they are necessary for survival of the multicellular organism, and thus under selection pressure. Following the discussion in the earlier section, this robustness is also expected to imply robustness to mutation. Thus, each cell type that appears over normal development will be robust to mutation.

Here recall that in the simulations described in the last section, we found each cellular state, as well as the number distribution of each cell type, was fairly stable against noise. Since the differentiation process here was based on cell-cell interaction, each cell type and the distribution would stabilize each other: robustness to noise at the single-cell and ensemble levels will consolidate each other. Recalling the relationship between robustness to noise and to mutation, the number distribution of cells of each type is also expected to be robust to genetic changes.

Cancer: An attractor that is not reached from the normal developmental path and lacks mutational robustness

Finally, I propose a hypothesis for cancer cells from the study on robust dynamical systems of development. Generally, GRNs are complex, involving a huge number of proteins that mutually influence the expression of others. Many attractors generally exist for such high-dimensional dynamical systems (as confirmed by simulations of a random GRN). The number of attractors might generally decrease as robustness increased through evolution [29], but considering the huge number of involved protein species, some other attractors in gene expression dynamics, besides those visited during normal development, may remain (Fig. 5). Indeed, in the earlier simulations by Furusawa and the author, such an attractor, which did not appear over normal development, was also identified [43].

Such states, as they are attractors, are stable to small perturbations. However, they are not generated as a result of normal cell-cell interaction, and not stabilized in the presence of other cell types, nor do they help the state of others to reach stability. These additional attractors would be *selfish* in that they can grow without forming stable relationships with others. This is in strong contrast to *normal* cells in our model, which were stabilized by cell-cell interaction and form mutually stabilizing relationships.

Far from a stable attractor: Why cancer cells are particularly sensitive to mutations

Recall that most attractors in a randomly chosen GRN were not robust to mutations as long as it was sufficiently complex, while robustness to noise and to mutation discussed previously was a result of evolution to produce the functional phenotype. Since the *aberrant* cell types here are not functionally necessary, and thus not under a selection pressure to preserve their state, they need not achieve high robustness against noise or mutation, through evolution. Accordingly, through divisions of such cells, their state would be more changeable by somatic mutation. Since the state is not yet optimized for the growth speed or robustness, they can be increased by such mutation. Hence, some mutations would increase the population of such cells. Accordingly, such cellular states would easily accumulate mutations. This is in contrast with the normal cell types that are expected to have already achieved robustness to noise and mutation through evolution, where somatic mutation can increase neither robustness nor growth speed.

To summarize, the cell types discussed here will be characterized as follows:

(i) They do not appear over the course of normal development, but are generated as a single-cell attractor when somehow perturbed sufficiently.
(ii) They are *selfish* and do not form stabilizing relationships with cells of other types.
(iii) Their phenotypes are vulnerable to noise or to changes in cell-cell interaction.
(iv) They are not robust to mutation.
(v) They can easily accumulate mutations, with which robustness can be increased.
(vi) They are in differentiated states compared with pluripotent cells, but are not terminally committed cell types.

Cells with these properties may fit with observations of cancer cells (or cancer stem cells). Indeed, the hypothesis of cancer cells as attractors was proposed by Kauffman [57] and recently put forward by Huang et al. [58, 59]. Here, my proposal concerns intra-intercellular dynamics rather than single-cell gene expression dynamics, and distinguishes *cancer* cells from *normal* cell types with regard to robustness. This distinction leads to the above six characteristic points of cancer-type cells.

According to point (i), a sufficiently large external perturbation may trigger the production of the cancer cell. Point (ii) might explain how cancer cells are not useful for other normal cell types. In addition, because the normal developmental course is a result of cell-cell interaction in the theory presented here, the appearance of *cancer-type* cells is also expected to depend on cell-cell interaction [14, 43].

The relatively large variation in cancer cell phenotypes observed [60–62] may reflect points (iii) and (iv). Their phenotype, even though they might be differentiated, could be changed more easily than the normal committed cells. Furthermore, their phenotype will change depending on interaction with other cells and mutations. The most plastic type will correspond to cancer stem cells [63].

Genetic instability in cancer: A result, rather than cause, of phenotypic instability?

According to point (v), genetic instability in cancer cells would not be a cause but a result of phenotypic instability in dynamics – a trait of the weak attractor. In this sense, genetic instability in cancer cells may be regarded as a genetic response to increase reproducibility of the phenotype under the environment in concern, a kind of *genetic assimilation* [1] in Waddington's sense. Phenotypic change as a result of gene expression dynamics would then induce changes in DNA methylation pattern [63], and later be fixed to genetic changes.

The question remains as to how cancer cells are quantitatively characterized. In the previous section, I proposed possible measures for multipotency. In the examples of earlier numerical studies [14, 43], chemical concentrations in such *cancer-type* cells did not show oscillatory change in time, but demonstrated larger variability across cells, compared with committed cells. This finding is consistent with the point (iii), weakness in phenotypic robustness. It was also found numerically that the chemical composition was more biased than that of the original stem cell. In summary, cancer-type cells are expected to have smaller temporal variation than embryonic stem cells, with cell-cell variation much larger than that of normally differentiated cell types. The chemical diversity of a cancer-type cell is expected to be smaller than that of embryonic stem cells and probably at the level of normally differentiated cell types.

Consequences of the hypothesis

The hypothesis presented here will lead to several consequences. According to the present picture, mutation would not be a direct cause of cancer cells, and generation of cancer cells would be strongly dependent on cell-cell interactions and environmental variation. In fact, density dependence of the frequency of tumor cells was reported by Rubin [64, 65]. Also, several experimental reports have shown that transplantation of cancer cells into some other tissue, which modifies their interaction with other cells, makes the cancer cells normalize [66–69].

From the view here, it is expected that cancer cells do not accumulate mutations when they are first generated. At this stage, they could revert to original multipotent cells by appropriate operations. In fact, such reprogramming has already been observed, by nuclear transplantation and nuclear cloning [70, 71]. If the change in cellular states can be observed by single-cell imaging, the present hypothesis could be confirmed by combining with the microarray analysis.

Conclusions

Cellular state is generated by gene expression pattern. Cells functioning in a given environment are robust to external perturbations. I proposed that robustness of the gene expression pattern to noise also leads to robustness to mutation, through evolution of such cells. Cells under selection pressure for some function are expected to achieve these two types of robustness.

In multicellular organisms, differentiation from stem cells to committed cells is also robust to perturbations over development. Here, stem cells have potentiality both to proliferate and differentiate. The former requires stability to keep the cellular state over cell divisions, and the latter requires plasticity to allow for change in the cellular state. Numerical simulations so far demonstrated that oscillatory gene expression dynamics switching between on and off make the robustness compatible with the plasticity. Cell-cell interactions, then, caused different cell types to stabilize each other, achieving robustness to external perturbations.

Since both the stem and differentiated cell states are necessary for a multicellular organism, robustness of each cell type to noise has to be achieved, which, in turn, would confer robustness to mutation.

In complex gene expression dynamics with many genes, however, there can be some other cellular states that are attractors but do not appear over the normal developmental course. In contrast to normal cell types, these cells do not necessarily form stabilizing relationships with other cell types. Since these cell types are not under selection pressure through evolution, their phenotype would not necessarily be robust to mutation, and the robustness of phenotypes to perturbations could increase by mutations. Hence, somatic mutations will easily be accumulated through cell divisions. I hypothesize that these cell types have something in common with cancer cells. This dynamical systems viewpoint provides a novel perspective on cancer cells, which can be verified experimentally.

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