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Dynamical systems theory of cellular reprogramming

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In cellular reprogramming, almost all epigenetic memories of differentiated cells are erased by the overexpression of a few genes, resulting in regaining pluripotency, the potential for differentiation. Considering the interplay between oscillatory gene expression and slower epigenetic modifications, such reprogramming is perceived as an unintuitive, global attraction to the unstable manifold of a saddle, which represents pluripotency. The universality of this scheme is confirmed by the repressilator model and by gene regulatory networks that are randomly generated and extracted from embryonic stem cells.

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In the development of multicellular organisms, cells with identical genomes differentiate into distinct cell types. This cellular differentiation process has often been picturized as balls falling down the epigenetic landscape, as originally proposed by Waddington [1]: Balls start from the top of the landscape and as development progresses, they fall into distinct valleys corresponding to differentiated cell types. In modern biology, such landscapes are believed to be formed by epigenetic regulation, including DNA and chromatin modifications [2–5]. For pluripotent cells, these modifications are small, whereas each differentiated cell type has a different epigenetic modification pattern [6–9]. Cells with pluripotency, such as embryonic stem (ES) cells, are located in the vicinity of the first branching point into the valleys, because they can easily differentiate into different cell types with only slight stimuli [10].

In 2006, the seminal study by Takahashi and Yamanaka demonstrated that differentiated cells can regain pluripotency only by overexpressing a few genes (the so-called four Yamanaka factors). This was termed as reprogramming to form induced pluripotent stem (iPS) cells [11]. Reprogramming is often described as “climbing” the epigenetic landscape [12–14]. However, this hypothesis has a basic issue that needs to be resolved. In the cellular reprogramming reported by Takahashi and Yamanaka and others, only a few genes are overexpressed, without direct manipulation of the epigenetic state, even though the erasure of epigenetic memories is a key factor for cellular reprogramming [12]. In other words, epigenetic memories are erased only by the perturbation of gene expression. Moreover, only a few genes (e.g., four genes) are overexpressed, which are notably fewer than the total number of genes involved. By such simple manipulation, the cellular state successfully returns to the top of the landscape, which is an unstable point, as it is followed by differentiation. Thus, the following problem in cellular reprogramming needs to be solved: How can reprogramming robustly make cells head toward an “unstable” pluripotent state via the overexpression of only a few genes?

Theoretically, these issues should be resolved based on the dynamical systems theory. The interplay between fast gene regulation and slow epigenetic dynamics shapes the epigenetic landscape, and differentiated cells are represented by different attractors [15–17]. Therefore, upon reprogramming, cellular states starting from different attractors first converge into a unique pluripotent state, which is unstable, followed by their progression toward various attractors. At first glance, these requirements seem incompatible; an unstable state (e.g., repeller) is not attracted from different initial conditions. Therefore, to satisfy these requirements, the pluripotent cell is expected to be represented at least by a saddle that is attracted from many directions and departs only along unstable directions (manifolds), which represent the cell differentiation process, leading to attractors of different destinations. To regain pluripotency by reprogramming, cellular states must be placed on the stable manifold of the saddle by common manipulations from different attractors. However, such manipulation would require fine-tuned control. In contrast, reprogramming is mediated by the overexpression of a few common genes across various differentiated cell types. Therefore, some dynamical systems concept beyond just a saddle is needed.

Recent advances in experiments provide some clues on this subject. Temporal oscillations in gene expression have been reported during cell differentiation and in embryonic stem cells. The relevance of oscillatory expression or dynamic heterogeneity to pluripotency has been noted experimentally [18–25] and theoretically [26–32]. Cell differentiation processes accompanied by loss of pluripotency have been studied in relation with the loss of oscillation [19,26]. The possible relationship between oscillatory cellular dynamics and pluripotency is also observed in cell cycle changes through cellular differentiation [33,34]. Notably, oscillatory
epigenetic factors for cellular differentiation [42–44]. Specifically, Polycomb (PcG) group proteins, which are two essential systems that modeling the entire epigenetic modification process is quite difficult. For instance, DNA methylation, one of the popular epigenetic processes, modifies DNA segments, whereas histone modification changes the efficiency of the DNA compaction process [3–5]. Although diverse molecular mechanisms are involved in epigenetic modification, they generally change the feasibility of gene expression. Hence, we adopted a simple “coarse-grained” model that captures this modification in gene expression feasibility to elucidate how the interplay between gene expression dynamics and epigenetic modification leads to cellular differentiation and reprogramming. We adopted reinforcement dynamics, which are inevitably required for a stable heritage of differentiated cell type, wherein the gene expression state is imprinted on the epigenetic state.

We consider a cell model in which the cellular state is represented by the expression $x_i$ and epigenetic modification level $\theta_i$ for each gene $i$, with $i = 1, 2, \ldots, N$. As mentioned above, this set of $\theta_i$ values represents the “coarse-grained” epigenetic state. It can correspond to various epigenetic modification levels, such as the DNA methylation level, histone modification level, and chromatin density. Gene expression dynamics, with faster timescales, are governed by GRN with mutual activation or inhibition by transcription factors [37–41], whereas slower epigenetic dynamics change the feasibility of gene expression, which follows gene expression patterns. We assumed the epigenetic feedback reinforcement, meaning that as a gene is expressed more or silenced, the more feasible or harder it is to express, respectively. This hypothesis was based on experimental observations on the Trithorax (TrxG) and Polycomb (PcG) group proteins, which are two essential epigenetic factors for cellular differentiation [42–44]. Specifically, we adopted

$$\frac{dx_i}{dt} = F \left( \sum_j J_{ij} x_j + \theta_i + I_i(t) \right) - x_i, \quad (1a)$$

$$\frac{d\theta_i}{dt} = \frac{1}{\tau} (x_i - \theta_i). \quad (1b)$$

In Eq. (1a), gene expression shows an on-off response to the input by adopting the function $F(z) = \tanh(\beta z)$, whereas $\beta = 40$ [45]. If $J_{ij}$ is positive or negative, gene $j$ activates or inhibits gene $i$, respectively, whereas $I_i(t)$ is set to 0 if no regulation exists. External input $I_i(t)$ is applied only during reprogramming manipulation to flip the expression of the gene $i$. For simplicity, $I_i(t)$ takes a constant nonzero value when gene $i$ is overexpressed for reprogramming manipulation and is zero otherwise.

In Eq. (1a), $-\theta_i$ functions as a threshold of the expression of gene $i$, which represents the epigenetic modification status (when there is no epigenetic modification, it takes the value zero). Equation (1b) represents epigenetic feedback regulation. Following the experimental observation of positive epigenetic feedback [46–52], we adopted this simple form as its specific form, which is yet to be confirmed [32,53–55]. Here, $\tau$ denotes the characteristic timescale for epigenetic modifications, which is assumed to be sufficiently larger than 1; the change in epigenetic modification is much slower than that of gene regulatory dynamics [56–58].

Recalling the relevance of oscillatory dynamics, we chose a GRN in which oscillatory dynamics were generated for appropriate $\theta_i$ values (specifically at $\theta_i \sim 0$). First, we adopted a repressor model as a minimal model [see Fig. S4(a) [59]], consisting of three genes that repress the expression of the next gene in a cyclic manner [60]. Specifically, we chose $J_{12} = J_{23} = J_{13} = -g = -0.4$ in Eq. (1a).

The expression of $x_i$ in this model showed a limit-cycle oscillation when $\theta_i$ was close to zero. Thus, for epigenetic modification to change $\theta_i$ following Eq. (1b), the states were differentiated into three fixed-point attractors $\{\theta_1, \theta_2, \theta_3\} = \{-1, 1, 1\}$, $\{1, -1, 1\}$, $\{1, 1, -1\}$ [61], after first approaching a straight line $\theta_1 = \theta_2 = \theta_3$, as shown in Fig. 1(a) [62] [see also Fig. 5(a) [59]]. In these fixed points, $dx_i/dt = 0$ and $d\theta_i/dt = 0$ were satisfied, i.e., the differentiation of expression $x_i$ was embedded into the epigenetic modification $\theta_i$.

Next, we considered “reprogramming.” Starting from one of the differentiated fixed points, we added external input $I_i(t)$ to again invoke transient oscillation [black dotted line in Fig. 1(b)]. Later, $I_i(t)$ was set to zero. After reprogramming manipulation, they approached a line with $\theta_1 = \theta_2 = \theta_3$ around the origin and then deviated from the line to one of the three fixed points [Fig. 1(b)], in the same manner as the differentiation process. During this reprogramming process, the memory of the differentiated states was erased. Once the oscillation in $x$ was recovered, the approach to the straight line and deviation from it always followed [Fig. 1(c)].

Next, we studied how attraction to the straight line occurs, followed by the progression of differentiation. For this, we considered the adiabatic limit of $\tau \rightarrow \infty$. For a fixed $\theta_i$, we first obtained the attractor $x_i$. Then, the evolution of $\theta_i$ was obtained by replacing $x_i$ in Eq. (1b) by its time average $\bar{x}_i$ for a given $\theta_i$, as follows:

$$\frac{d\theta_i}{dt} = \bar{x}_i(\theta_i) - \theta_i(\equiv \Theta_i). \quad (2)$$

In the three-variable Eq. (2), $\{\theta_1, \theta_2, \theta_3\} = \{0, 0, 0\}$ is a fixed-point solution because $x_i(t)$ shows a symmetric limit-cycle oscillation, such that $\bar{x}_i = 0$ for all $i$ therein for $\{\theta_1, \theta_2, \theta_3\} = \{0, 0, 0\}$. By slightly perturbing $\theta_i$ as a parameter, $\bar{x}_i$ changed.
all trajectories converged agreed with the unstable manifold (c) Eigenvector represents the saddle point at origin { \hat{v}_k } \{ \hat{\theta}_u, \hat{\theta}_s \} without it (\hat{u}_s, reprogramming manipulation (black), and the subsequent process (blue) without it (\hat{u}_t = 0). We plotted the time development of \( x_i, \theta_i \) [see Figs. S5(a) and S5(b) [59] for the time series of all variables]. Left: Three trajectories were sampled from slightly different initial conditions near \( \hat{\theta}_t = 0 \). Right: From the fixed point \{−1, 1, 1\}, we tested three slightly different time spans to add external input (520, 530, 540). After reprogramming manipulation, the cellular state first approached \( \hat{\theta}_1 = \hat{\theta}_2 = \hat{\theta}_s \) and then it differentiated to the three fixed points again. (b) Trajectories through reprogramming and differentiation were plotted in the \( (\hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_s) \) space. Ten attempts were overlaid by considering the initial conditions in \( x_i \in [-1, 1], \hat{\theta}_i \in [-0.25, 0.25], \) which allowed oscillation. Attraction toward the straight line \( \hat{\theta}_1 = \hat{\theta}_2 = \hat{\theta}_s \) and departure from it was discernible. (c) Eigenvector \( \{ \hat{v}_k \} \) and variables \{ \hat{\theta}_i \} (see text). The black X mark represents the saddle point at origin \{0, 0, 0\}, whereas the three colored points represent the fixed points \{−1, 1, 1\} (blue), \{1, −1, 1\} (orange), and \{1, 1, −1\} (green).

Accordingly, from \( \partial x_i / \partial \theta_j \), we obtained the Jacobi matrix \( \partial \theta_i / \partial \theta_j \) with eigenvalues \{ \lambda_k \} and eigenvectors \( \{ \hat{v}_k \} \). As shown in Fig. 1(d), the \{ \hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_s \} = \{0, 0, 0\} fixed point was a saddle, with the eigenvector \( \hat{v}_3 \) = \{1, 1, 1\}/\sqrt{3} corresponding to \( \lambda_u > 0 \) (unstable axis), and \( \hat{v}_2 \) = \{2, −1, −1\}/\sqrt{6}, \( \hat{v}_1 \) = \{0, −1, 1\}/\sqrt{2} for \( \text{Re}(\lambda_s) = \text{Re}(\lambda_{s2}) < 0 \) [see Supplemental Material (SM) Sec. 1A [59]]. To investigate \( \theta \) dynamics along each of the eigenvectors \( \hat{v}_k \) (\( k = u, s_1, s_2 \)), we introduced the variable \( \hat{\theta}_k \), a projection of \( \theta \) on \( \hat{v}_k \) (i.e., \( \hat{\theta}_k = \theta \cdot \hat{v}_k \), with \( \hat{v}_k \) normalized). Notably, owing to the symmetry of the repressilator, the unstable manifold was in line with the eigenvector \( \hat{v}_u \) (see SM Sec. 1A [59]).

As shown in Figs. 1(c) and 1(d), the straight line to which all trajectories converged agree with the unstable manifold \( \hat{v}_u \) [Fig. 2(a)]. Of course, attraction to the \( \hat{v}_u \) axis was natural if the initial conditions were restricted onto the stable manifold for \{\( \hat{\theta}_1, \hat{\theta}_2, \hat{\theta}_s \} = \{0, 0, 0\}. However, we observed attraction toward the unstable axis over a wide range of initial conditions for \{\( \hat{\theta}_s \)\}, supporting the oscillation of \( x_s \). Furthermore, the magnitudes of eigenvalues for the stable and unstable eigenvectors were of the same order \( |\text{Re}(\lambda_u)| = 0.31, |\text{Re}(\lambda_s)| = |\text{Re}(\lambda_{s2})| = −0.66, \) see Fig. S5(c) [59]. Thus, the reprogramming dynamics shown in Fig. 1(b) could not be explained only by linear stability.

To elucidate whether the nonlinear effect suppresses instability along the \( \hat{v}_u \) axis, we computed \( \partial \hat{\theta}_u / \partial t \). As shown in Fig. 2(c), \( \partial \hat{\theta}_u / \partial t \) was drastically reduced from that in the linear case. We also computed \( \partial \hat{\theta}_u / \partial t, \partial \hat{\theta}_s / \partial t \) for a certain \( \hat{\theta}_u \) value [i.e., the flow structure in the \( (\hat{\theta}_s, \hat{\theta}_u) \) plane, sliced along the \( \hat{\theta}_u \) axis], which showed that \( \hat{\theta}_u = 0 \) changed from stable to unstable at \( \hat{\theta}_u = \hat{\theta}_u^{\text{th}} \approx 0.4 \) [see Figs. 2(d) and S6 [59]]. Up to \( \hat{\theta}_u < \hat{\theta}_u^{\text{th}} \approx 0.4 \), \( \hat{\theta}_u \) in the \( (\hat{\theta}_s, \hat{\theta}_u) \) plane was attracted to the \( \hat{\theta}_u \) axis. By further increasing \( \hat{\theta}_u \) beyond \( \hat{\theta}_u^{\text{th}} \), \( \hat{\theta}_u \) departed from the \( \hat{\theta}_u \) axis rotating in the \( (\hat{\theta}_s, \hat{\theta}_u) \) plane, leading to differentiation toward three distinct fixed points.

To understand how slow motion along \( \hat{\theta}_u \) and attraction to \( \hat{\theta}_u \) occurred, we first fixed \( \hat{\theta}_u \) and studied the change in the \( \hat{\theta}_u \) attractor, as shown in Fig. 2(b). In the green and blue region, the \( x \) attractor was a limit cycle and fixed point, respectively, for \( (\hat{\theta}_u, \hat{\theta}_s) \). At the line \( \hat{\theta}_u = −\hat{\theta}_s/\sqrt{2} + \sqrt{6}/5 \) (as discussed in SM Secs. 1B and 1C [59]), \( x \) dynamics exhibited bifurcation from the limit cycle to a fixed point \{−1, 1, 1\} (see Fig. S7 [59] for more details). Considering the symmetry of the repressilator, bifurcations to three
fixed points \([-1,1,1], [1,-1,1], [1,1,-1]\) coexisted in the \((\theta_1, \theta_σ)\) plane. With the increase in \(\theta_u\), the limit cycle approached the three fixed points.

Next, we discuss the mechanism of slow motion along \(\theta_u\). From Eq. (2), movement along \(v_u\) followed \(d \dot{\theta}_u / dt = \bar{x}_u(\theta_u) - \theta_u\) (we defined \(x_u\) as a projection on \(v_u\)). As shown in Fig. 3(a), the limit cycle approached the plane spanned by the three fixed points \([-1,1,1], [1,-1,1], [1,1,-1]\) as \(\theta_u\) increased. In the plane, \(\bar{x}_u\) comprised \(1/\sqrt{3}\), and \(\theta_i\) increased following Eq. (2) (see SM Sec. 1D \([59]\) for more details). Then, as \(\theta_u\) approaches \(1/\sqrt{3}\), \(d \dot{\theta}_u / dt\) was minimized, as shown in Fig. 2(c).

Next, we considered how attraction to the \(v_u\) from the \((\theta_1, \theta_σ)\) plane was lost at the \(\theta_u = \bar{x}_u\) plane. By considering \(\theta_u\) as a parameter, the direction of flow in the \((\theta_1, \theta_σ)\) plane toward the \(v_u\) was determined by the sign of \(\partial \bar{x}_u / \partial \theta_1\). As shown in Fig. 2(b), with the increase in \(\theta_u\), \(\bar{x}_u\) approached \(1/\sqrt{3}\), \(\theta_u\) reached fixed points. Accordingly, \(\partial \bar{x}_u / \partial \theta_1\) increased beyond one, so that \(\partial \bar{x}_u / \partial \theta_1\) became positive at \(\theta_u\), approaching \(\theta_u = 0.4\), as shown in Figs. 2(d) and 2(b).

Thus, we unveiled how attraction to the unstable manifold is achieved by slow epigenetic fixation of the oscillation of fast gene expression in the repressor model. Following this picture, reprogramming is possible by forcing the cells to return to the oscillatory state. Then, the cell is attracted to a pluripotent state with low epigenetic modification \(\theta_i = 0\), followed by differentiation to distinct cell types with specific \(\theta\) values. Notably, the differentiation process \([63]\), as well as the present reprogramming process, is robust against internal or external noise because the initial pluripotent state is globally attracted (see Fig. S8 \([59]\) \([64]\)).

To verify the generality of this reprogramming scheme, we examined several GRN models with more degrees of freedom. As discussed in Ref. \([63]\), differentiation from oscillatory states is often observed in GRNs (e.g., 20% of randomly generated GRNs show oscillatory dynamics for \(N = 10\)). An example is shown in Fig. S9(a) \([59]\). From a differentiated state, we overexpressed three genes to regain oscillatory expression [black line in Fig. S9(a) \([59]\)]. Later, global attraction to the unstable manifold also occurred as discussed above. The cell states then branched again to distinct fixed point states [blue line in Fig. S9(a) \([59]\)]. In these cases, the original pluripotent state with \(\theta = 0\) was an unstable fixed point, with one positive eigenvalue for the Jacobi matrix of \(\theta\) dynamics [Fig. S9(d) \([59]\)], as in the repressor model. Even though the degrees of freedom increased, the unstable manifold was one dimensional, and attraction to the manifold occurred from a higher-dimensional state space. This implies that reprogramming manipulation requires only partial degrees of freedom compared with the total number of genes. In fact, overexpression of three genes is sufficient for reprogramming in GRN models with \(N = 10\), as far as we have investigated.

The present mechanism also works in a model extracted from the GRN of an embryonic stem cell \([65]\), as a core network with five genes \((\text{Nanog, Oct4, Gata6, Gata4, and Klf4})\) \([32]\) [see Fig. S4(b)] \([66]\). Oct4, Sox2, and Klf4 are known as factors to induce reprogramming. The model involves a negative feedback loop, as in the repressor, in addition to positive feedback regulation. In this five-gene model, \(x_i\) and \(\theta_i\) oscillate in the region near the origin, and then differentiation to three fixed points progresses as in the case of the repressor [three lines in Fig. 4(a)], whereas \(\theta_i = 0\) for all \(i\) represents a saddle point with one unstable manifold and four stable manifolds, as shown in Fig. S10(b). After overexpression of Oct4, Nanog, and Klf4 from one of the differentiated cell types for a certain time span [black dotted line in Fig. 4(b)] \([67]\), the epigenetic state \(\theta_i\) approaches the unstable manifold for the unstable fixed point \(\theta_i = 0\), leading to the recovery of pluripotency [blue line in Fig. 4(b)].

In this Letter, we have shown that oscillatory gene expression dynamics with slow epigenetic modifications lead to cellular reprogramming by the overexpression of only a few genes. Global attraction to the unstable manifold of the saddle point explains the reprogramming process. Now, the return to the top of the landscape by reprogramming, which is seemingly unstable, is explained by the strong attraction toward the unstable manifold of the saddle, and its suppressed instability...
along with the unstable manifold, owing to the approach of the limit cycle of bifurcation to the fixed points. The memory of the cellular state before reprogramming manipulation was erased through this reprogramming process.

According to the present study, regaining oscillation is the main requirement for reprogramming and elaborate manipulation to induce cells into a specific state is not necessary. This explains the role of oscillations in the gene expression in pluripotent cells [19] and epigenetic modification through the differentiation process [35]. It also explains how reprogramming is possible by overexpressing only a few genes among thousands [11,14]. Timescale separation between the fast expression dynamics and slow epigenetic modification feedback required is also consistent with observations of previous studies [56,57].

If we treat slowly varying $\theta_i$ values as parameters, our model could be represented by a gene expression dynamical system with external bifurcation parameters $\theta_i$, as also discussed, for instance, in Ref. [68]. However, in developmental processes, the epigenetic state changes slowly and autonomously depending on the gene expression state, as elucidated in the current study. It will thus be important to make a general formulation for such slow-fast dynamical systems [69,70].

Although there are some experimental reports on oscillation in gene expression [23] and epigenetic modification during cellular differentiation, such as reports on DNA methylation levels [35] and chromatin compaction states [71], it is further important to elucidate the role of such oscillation in epigenetic modifications to differentiation and reprogramming, by measuring its time course under controlled conditions. Our theory suggests that through cellular reprogramming, the epigenetic state will first converge to a common state from differentiated cell types, with transient oscillation. The genes to be overexpressed for reprogramming (e.g., the Yamanaka factor) will then be those needed to recover oscillation in gene expression dynamics.

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