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A Population Shift View of Cellular Reprogramming

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ABSTRACT

Cellular reprogramming can offer valuable insight into disease mechanism and has the potential to provide novel tools for regenerative medicine. Yet it remains an inefficient and often incomplete process. However, experiments show that almost all somatic cells eventually give rise to the pluripotent state, albeit at different latencies, as long as expression of reprogramming transcription factors is maintained. Furthermore, it appears that specific subpopulations of cells can be identified that show enhanced propensities to be reprogrammed to the pluripotent state. It has been proposed that an initial stochastic process is responsible for this initial priming that is followed by a deterministic process that directs the primed cells into the pluripotent state. Here, we propose a population shift view of cellular reprogramming, which explains these observations and reconciles the stochastic and deterministic nature of this process. According to this view, a small population of cells, whose states are closer to the pluripotent state and reside in pre-existing energetically favorable trajectories, will be initially selected for reprogramming. Moreover, by maintaining ectopic expression of reprogramming factors, other cells enter these pathways as a result of transcriptional and epigenetic stochastic variations. Consequently, increasing numbers of cells reach the pluripotent state, and the cell population distribution shifts toward this state. Importantly, additional perturbations can change the epigenetic landscape, allowing cells more access to the reprogramming trajectories, thereby increasing reprogramming efficiency. Knowledge of the initial cellular subpopulations and pathways of states that lead to the final cellular state should allow us to design alternative perturbation strategies to improve reprogramming efficiency and fidelity.

INTRODUCTION

Cells in tissues and in culture exist as populations in a dynamic landscape characterized by gene expression states occupied by cells with different probabilities. Furthermore, this gene expression landscape can be viewed as an energy landscape, where the energy of each cellular state is determined by its underlying transcriptional and epigenetic regulatory interactions. Although, there are an enormous number of possible configurations in this landscape, cell fates (distinct functional phenotypic states of cells) correspond to discrete steady stable states (attractors) determined by gene regulatory networks and epigenetic regulation [1, 2]. Despite these regulatory constraints, individual cells are exposed to multiple simultaneous input cues from the

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environment, including cell-cell interactions, and gene/protein expression noise [1]. Therefore, they can follow specific trajectories to explore different states with different probabilities depending on the energetic barrier separating these states. The probability distribution of different states has been experimentally supported; for instance disturbance of the population equilibrium with respect to their constituent sub-population proportions by removing a specific sub-population, induces cellular transitions that recover the initial equilibrium distribution. This equilibrium recovery has been experimentally observed respect to different sub-populations of embryonic stem cells [3-6] and among different phenotypic states within cancer cells [7].

The gene expression landscape is generally irregular, containing a hierarchy of valleys with sub-valleys (attractors with sub-attractors), which explain different levels of heterogeneity observed in cultures of various lines of genetically identical cells even in controlled environments ([3]). Attractors roughly correspond to different cell types, whereas each sub-attractor, within a main attractor, represents a possible sub-state of a cell-type specifying attractor. The intra-attractor heterogeneity has been experimentally observed in different cellular systems; this can be seen in the emergence of different sub-populations of embryonic stem cells characterized by the expression of different combinations of genes [8], and specific pluripotent markers, like Nanog [4, 9], or Stella [6]. Furthermore, it is expected that distinct cell-type specifying attractors have different numbers of sub-attractors corresponding to different sub-populations. For example, pluripotent stem cells have a permissive and dynamic chromatin structure [9] that allows them to exist in a larger number of sub-attractor states compared to fully differentiated cells that have a more condensed chromatin structure [10]. These observations show that cells remain in a dynamic equilibrium transitioning back and forth between different sub-populations. Hence, different cells can transiently reside in different states each of which is more or less primed to respond to different reprogramming-inducing perturbations. Indeed, these perturbations change the energy landscape in such a way that cells seek more energetically favorable states. In this process, cells follow different trajectories depending on their initial states, and not all trajectories reach the final phenotypic state (that state with the lowest energy) due to roadblocks that include transcriptional and epigenetic regulations. These reprogramming trajectories can be defined as a time ordered sequence of cellular states leading cells from the differentiated state to the pluripotent state (or directly to alternative differentiated states). Among all possible sequences of cellular states potentially leading cells from the differentiated state to the pluripotent state, only a subset is characterized by high transition probabilities between consecutive states these regulatory transitions are dictated by transcriptional and epigenetic regulatory mechanisms. We refer to these pathways as energetically favorable pathways and are analogous to the example of a ball rolling down a Waddington landscape to reach the lowest energy stable state following energetically favorable pathways that avoid high energy barriers. This picture is reminiscent of the protein folding process, through which the non-native protein conformation, which possess a higher energy, transitions toward the lower energy native state. This transition is accomplished via energetically favorable pathways, which are characterized by high transition probabilities between sequential intermediate states, dictated by the free energies of these states [11-14]. Further, it is reasonable to assume that the more similar the transcriptional and epigenetic profiles between the starting and product cell types, the fewer roadblocks encountered, rendering reprogramming more efficient. Experimental evidence supports this notion. For example, human keratinocytes from skin biopsies can be reprogrammed to pluripotency at much higher frequency and faster speed than fibroblasts. This difference is attributed to the finding that

keratinocytes express much higher levels of endogenous c-Myc and Klf4 than fibroblasts, which may accelerate the conversion of keratinocytes to iPS cells [15]. In addition, given that mesenchymal-to-epithelial transition (MET) is a crucial early phase during the reprogramming of fibroblasts into iPS cells [16], fibroblasts unlike keratinocytes, which are an epithelial cell type, may need to undergo an initial MET during the reprogramming process. This additional transition step may result in reduced efficiency and a prolonged duration to reprogram fibroblasts to reach pluripotency [15]. Another example relates to neural progenitors, which express Sox2 and low levels of Klf4, and can be reprogrammed more efficiently than fibroblasts and minimally require only ectopic Oct4 [17]. Furthermore, it has been shown that trans-differentiation events between cells sharing a common direct progenitor can be accomplished more efficiently (frequently involving a change in state of a bistable toggle switch) than between cells from different lineages [18].

Population shift model in cellular reprogramming

Despite the initial cellular state heterogeneity and the fact that some cellular states are more primed to initially respond to reprogramming-inducing stimuli, most cell types can potentially be reprogrammed. Recent experimental results have shown that in fact almost all mouse donor fibroblasts eventually give rise to iPSCs, given sufficient time i.e. up to 18 weeks [19]. How can this happen? As we discussed above, in cell cultures and tissues, cell types exist in an ensemble of sub-attractor states occupying the gene expression landscape around their steady stable attractor states [12]. Perturbation of this system with the ectopic expression of the OKSM factors changes the gene expression landscape; and those cells residing in these ‘primed’ pre-existing states respond more efficiently to the reprogramming-inducing perturbation. These pre-existing states, which usually share some common features with the pluripotent state, occupy energetically favorable trajectories that lead to the pluripotent state. Transitions between states in these pathways occur with high probabilities, dictated by transcriptional and epigenetic regulatory mechanisms. In other words, these small populations of cells, whose states are close to the pluripotent state and reside in these pre-existing trajectories, will be initially selected for reprogramming. Further, if ectopic expression of the reprogramming factors is maintained over time, other cells can enter these pathways as a result of stochastic events in gene expression and epigenetic modifications, and thus more and more cells should be able to reach the pluripotent state. Consequently, there will be a shift in the cell population distribution toward the pluripotent state. Hence, the traditional Waddington landscape can be used to visualize reprogramming as a population of balls rolling down in the landscape along the valleys, seeking their lowest energy point. Those balls, which are closer to the lowest energy point and follow energetically favorable pathways, reach this point first (pluripotent state). On the other hand, others take different pathways that are not able to direct them to the lowest energy point due to roadblocks impeding their progression. Further, stochastic events can eventually enable these latter ones to enter reprogramming trajectories to finally reach the lowest energy point, and in doing so, result in a population shift towards the lowest energy state (Fig. 1). This perspective builds on previous models [20] [19], including the elite and stochastic models for iPSC generation proposed by Yamanaka [20]. According to the elite model, only a small number of cells, either determined before or after the reprogramming-inducing perturbation, can be reprogrammed either partially or completely. In the stochastic model, most differentiated cells can potentially become iPSCs, providing that stochastically the four factors are expressed in the right amount and epigenetic stochastic events lock them into the pluripotent state. Our model also acknowledges the existence of specific primed sub-populations of cells that are able to progress to the pluripotent state via pre-existing trajectories, because of their initial transcriptional and epigenetic states. However, in addition, we propose that other cells outside of

this primed subpopulation can also potentially enter these trajectories as a result of stochastic events, and thus over time, reach the pluripotent state. As a result, a cell population shift occurs from the differentiated state to the pluripotent state. This two stage process is consistent with a study that used a tamoxifen inducible cre inserted into the 3'UTR of the Oct4 gene to allow lineage tracing of Oct4+ reprogrammed cells [21]. Reprogramming using the OKSM cocktail led to induction of endogenous Oct4 starting as early as five days post-tamoxifen treatment however only a fraction of these cells subsequently activated Nanog expression and adopted an ESC phenotype, indicating that other stochastic (or unknown deterministic) factors are required for subsequent transition to a fully reprogrammed pluripotent state and additional markers need to be considered to fully characterize states in the reprogramming pathways. This model is corroborated by a recent single-cell study on the reprogramming of mouse embryonic fibroblasts into iPSCs [22]. In this study, ectopic expression of the four Yamanaka factors (OKSM) in a population of mouse embryonic fibroblasts induce stochastic gene expression changes measured in a subset of 48 genes, including pluripotency genes. However early endogenous expression of specific 'mark' genes (Esrrb, Utf1, Lin28, and Dppa2) was shown to indicate those few cells that were destined to become iPSCs via direct or indirect activation of Sox2 locus. Further, at a later stage, when cells start to express Sox2, then a more deterministic phase ensues leading to activation of the pluripotency circuitry. In this case, early expression of mark genes that leads to Sox2 activation, and subsequent activation of other pluripotent genes, characterizes pre-existing states that reside in energetically favorable trajectories that lead to the pluripotent state. Moreover, the sequential order of states in these trajectories is dictated by the regulatory relationships between these genes. In fact, the early mark gene Esrrb activates Sox2, which in turn constitutes a master regulator of other pluripotent genes and contributes to the stabilization of the pluripotency circuitry by participating in a positive feedback circuit [22].

So is there any evidence to support the population shift model *in vivo*? In a seminal study from the Yamanaka laboratory, it was shown that only a subpopulation of reprogrammed cells showed full functional pluripotency, as assessed by their ability to generate chimeras using blastocyst injection [23]. In a later study, use of the OKSM Yamanaka cocktail to reprogram neural stem cells led to rapid induction of an ESC type morphology, that was accompanied by elevated levels of endogenous Oct4 and Fgf4, but only minimal levels of Nanog were induced and the resultant 'reprogrammed' cells failed to generate chimaeras upon blastocyst injection. Only after "2i" inhibition of Mek/Erk and GSK3 was full functional pluripotency established [24]. It should be noted that routinely, generation of chimeras requires multiple cells to be injected into multiple blastocysts, followed by multiple blastocyst transplantation. Consequently, it is not possible to say whether all reprogrammed cells contribute to the progeny, i.e. generation of chimeras tests the pluripotentiality of the cell population not the individual cell.

We have presented this population shift perspective from the vantage point of reprogramming cells to a pluripotent ground state, the iPSC. However, this perspective can be applied in equal measure to direct reprogramming (or transdifferentiation) by positing pre-existing states, characterized by the expression of specific genes, which belong to energetically favorable pathways leading to the final reprogrammed states. At present there are few studies that have tackled the potential existence of multiple states during direct reprogramming. However, one study that has addressed this issue used C.Elegans to study the *in vivo* natural direct reprogramming of a rectal cells into a motorneuron. Importantly, it was shown that this transdifferentiation occurred in two stages; first the rectal cell loses epithelial identity and second, takes on neural characteristics. Importantly, the stages can be

clearly dissected by the UNC-3 dependence of the latter but not the former. In the absence of UNC-3, the reprogramming is stalled in an intermediate stage that lacks both rectal epithelial and neural markers and phenotypes [25].

It is important to recognise that the behaviour of cells in culture may not reflect all aspects of their *in vivo* counterparts. Intrinsic gene regulatory networks are subject to regulation by extracellular signals, which induce signalling cascades culminating in regulation of both transcription factor activity and the epigenetic landscape. Indeed, such signals arising from stem cell niches are required for balancing quiescence, expansion and differentiation of stem cells *in vivo* [26]. As such, cellular states not only depend on intrinsic transcriptional and epigenetic regulation, but also on extrinsic cell-cell interactions. Thus, differences between the epigenetic landscape *in vitro* and *in vivo* are reflected in the stability properties of cellular states, the epigenetic barriers that separate the cell states, and consequently the cellular dynamics. For example, pluripotency is a metastable state, which can only be maintained under very specific culture conditions. *In vivo*, pluripotency is by nature transient and unstable, serving as transit between totipotency and germ layer differentiation [27]. The importance of considering epigenetic barriers separating cell states can be gleaned from the observed modifications to the epigenetic landscape during reprogramming. Numerous studies have used small molecule inhibition of epigenetic writers to increase efficiency of reprogramming in mouse embryonic fibroblasts, including inhibition of DNA methyltransferase using 5'azacytidine and inhibition of histone deacetylase activity using valproic acid [28], and inhibition of G9a histone methyltransferase activity with BIX-01294 [29] ,while inhibition of the NAD-dependent deacetylase/ ADP ribosyltransferase, sirtuin-3 [30], leads to increased expression of pluripotency markers in bovine fibroblasts. The resultant epigenetic changes can allow cells to have more access to the pre-existing trajectories, and therefore to increase reprogramming efficiency; stated otherwise, this could be thought of as placing most cells in the population into primed attractor states, such that subsequent reprogramming to the pluripotent state follows a purely deterministic pathway. In fact, a recent study showed that depletion of the methyl-binding protein 3, (Mbd3) together with the OKSM transduction yields reprogramming efficiencies of up to 100% [31, 32]. These results suggest that depletion of Mbd3 eliminates or lowers epigenetic barriers in such a way that cells can enter these pre-exsiting reprogramming trajectories and thus initiate a more deterministic transition towards pluripotency. From a Waddington perspective, barriers between attractor states are lowered by epigenetic modifications, while transcriptional manipulation directs the population flow into the primed attractor state(s); both increase the fraction of cells in the primed state i.e. increase reprogramming efficiency.

In summary, according to the population shift model of cellular reprogramming, after the reprogramming-inducing perturbation, those cells whose transcriptional and epigenetic states are closer to the pluripotent state and can follow energetically favorable trajectories are initially selected for reprogramming. Given sufficient time, stochasticity in the transcriptional and epigenetic regulatory mechanism will allow other cells to access these trajectories and eventually reach the final state (Fig.1). This model can be generalized to any kind of cellular transition, where initial sub-population of cells with similar transcriptional and epigenetic profiles to the final cellular state responds more efficiently to the reprogramming-inducing perturbation by following energetically favorable trajectories towards the final state. Interestingly, this view is reminiscent of an established concept used to understand protein allostery, where the allosteric ligand selects for binding the pre-existing most complementary conformer within the population. The conformer selected may not have initially the lowest energy; however, binding and subsequent induced fit

minor conformational changes will stabilize it leading to 'population shift' toward this conformer [33].

What will this perspective offer? Understanding the cell state space through which reprogrammed cells must traverse will ultimately lead to greater understanding of mechanism but may also lead to identification of key genes and pathways that underwrite therapeutic reprogramming events. For instance, a cocktail of Ngn3, Pdx1 and MafA can reprogram pancreatic exocrine cells [34] or liver cells [35] into insulin+ ß-like cells *in vivo*. In the latter case the insulin cells were seen to arise exclusively from Sox9+ cells arising from the interlobular ductules and bile ducts, indicating susceptibility of a specific subpopulation of cells to reprogramming. Knowing the pathways that determine the reprogrammable cell state and those that direct the reprogramming will be essential if we are to exploit the potential of in vivo reprogramming.

Future Perspectives

The model presented here explains why only a few cells, which initially display specific expression patterns, are more likely to reach the final state, and why most cells can potentially be reprogrammed. Knowledge of the initial cellular subpopulations and trajectories of states that lead to the final cellular state should allow us to design alternative perturbation strategies to improve reprogramming efficiency and fidelity. Furthermore, prior knowledge of initial cellular sub-populations that are more primed to be reprogrammed should facilitate the design of more efficient reprogramming strategies for therapeutic applications. Acknowledgement that cell populations exist in a dynamic flux between unprimed and primed states and that this transition can be controlled by transcriptional and epigenetic manipulation opens up opportunities for increasing reprogramming efficiency. There is no fundamental difference between addressing transcriptional and epigenetic modifications; the current predilection for interpreting chromatin modifcations as proxies for epigenetic status simply poses transcription factors as recruiters of epigenetic readers and writers. Nevertheless, targetting epigenetic modifiers may produce more druggable targets than targetting transcription factors, a thinking that lies behind such consortia as the Structural Genomics Consortium (http://www.thesgc.org). There is abundant evidence pointing to reprogramming and transdifferentiation being multi-stage processes, characterised by stochastic and deterministic phases. These observations are consistent with a population shift model that posits multiple pre-existing attractor states among which cells can transition. Ultimately, experimental validation of the population shift model requires single cell studies that allow fate adoption in marked individual cells to be monitored during reprogramming. The increasing use of single cell transcriptomic platforms, such as Fluidigm, combined with lineage tagged progenitor cells will fuel these approaches.

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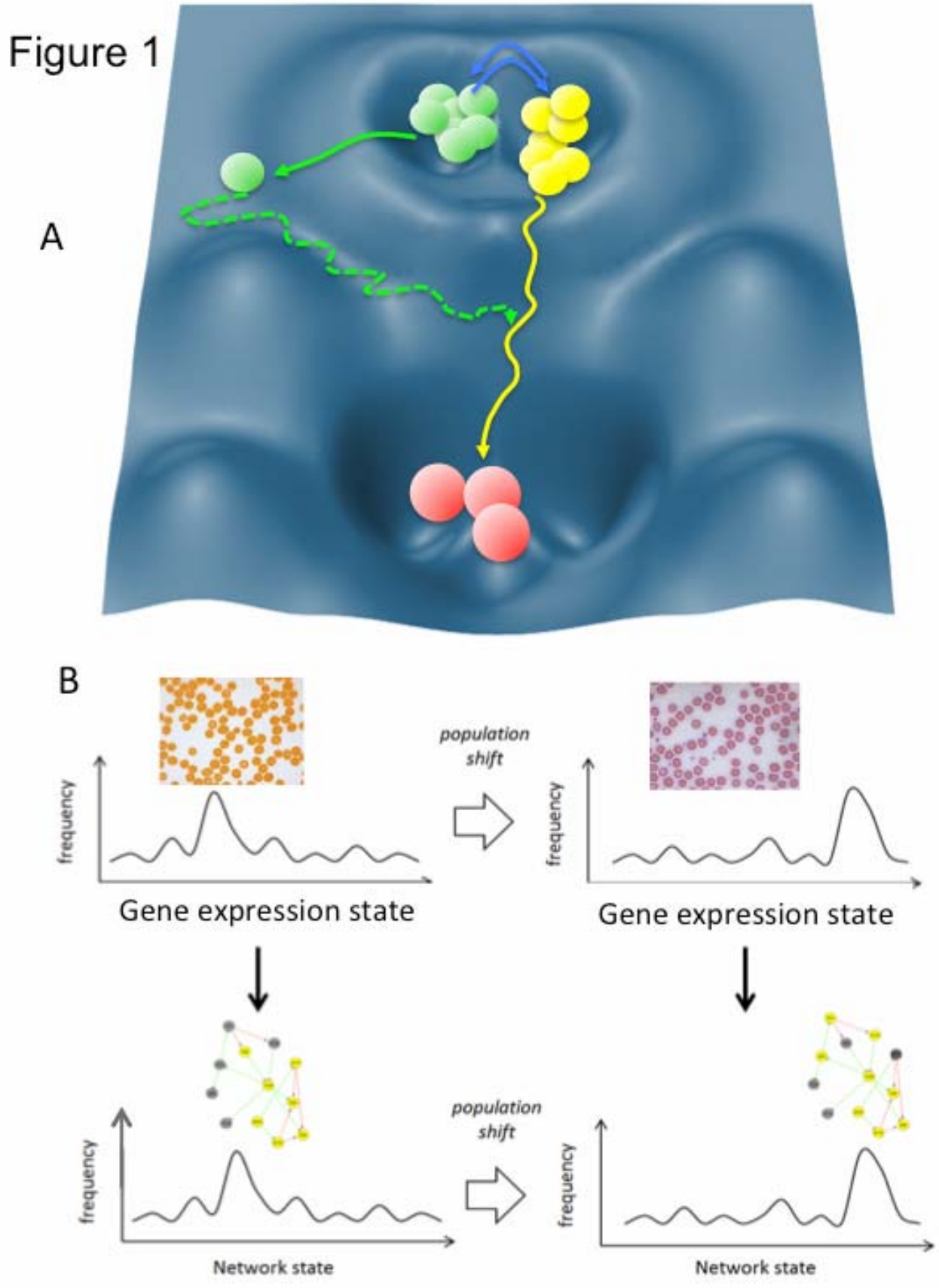
Figure 1. Population shift in cellular reprogramming. (a) Gene expression landscape comprising two attractors- the initial differentiated state (cells represented with green and yellow balls) and the final pluripotent state (cell represented with pink balls). Yellow balls represent the primed sub-population that is in a specific initial sub-attractor state capable of responding to the reprogramming-inducing perturbation. After the perturbation, the landscape changes and cells seek minimum energy states. As a result, some cells undergo transitions to other sub-attractors within the initial attractor, whereas other (primed) cells leave the initial attractor following the reprogramming trajectories (yellow lines) to reach the pluripotent state. Other cells may follow alternative trajectories (solid green lines), which cannot guide them to the pluripotent state due to transcriptional or epigenetic roadblocks. However, if the maintenance signal is retained then stochastic events allow the later cells to enter the reprogramming trajectories (dashed green lines), which lead them to the pluripotent state. In addition, removal of cells from the primed sub-attractor will thus favour more cells to enter this sub-attractor (blue arrows) to re-establish the steady state. Consequently, more cells can progressively reach the pluripotent state, inducing a cellular population shift towards this state. (b) The initial cellular population probability distribution shifts toward a new distribution as result of the reprogramming-inducing perturbation. The probability distributions are determined by transcriptional and epigenetic regulatory interactions.

Figure 1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