Effects of the *in vitro* manipulation of stem cells: epigenetic mechanisms as mediators of induced metabolic fluctuations

The increasing popularity of stem cells in life science research has at least two major causes. On one hand, the study of stem cells may provide insights into one of the major secrets of biology: the mechanisms of cell differentiation. On the other hand, stem cells are potentially promising tools for regenerative therapy. The understanding of how environmental stimuli are translated into phenotypic differentiation through gene expression changes and how the same stimuli at the same time may perturb the normal process of cellular differentiation, growth and maintenance is a central issue for fundamental research but is also essential for the development of efficient and safe procedures for therapeutic use. This article assembles the known facts, as pieces of a puzzle, into a coherent picture around the idea of why stem cells are so sensitive to their culture environment and what practical consequences this implies.

KEYWORDS: cell differentiation ■ epigenetic mechanism ■ metabolism ■ pluripotency ■ stem cell ■ stochastic model

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Stem cells are commonly defined as cells that have the capacity for unlimited self-renewal and at the same time conserve the potential to differentiate. Embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells are the emblematic representatives of this category; they can differentiate *in vitro* into any cell type. When mouse ESCs or iPS cells are placed in an *in vivo* environment by injection into the cavity of a blastocyst they are able to contribute to all tissues of the developing chimeric animal. Adult stem cells can be isolated from adult tissues; their differentiation potential is limited to lineage-specific cell phenotypes. ESCs, iPS and tissue-derived stem cells are usually considered as a distinct category of cells that share some common features of 'stemness': unlimited self-renewal capacity and pluripotency. These fundamental characteristics rely on an implicit assumption: the stem cell phenotype is a cell-autonomous, intrinsic property that is highly resistant to environmental stimuli [1]. However, it is less recognized that the concept of stem cells together with the above-mentioned seductively simple definition emerged from *a priori* theoretical speculations rather than resulting from observations and experimental studies of ESCs or tissue stem cells. This is well illustrated by the fact that the existence of a common progenitor in the hematopoietic system, for example, was proposed a long time ago, but almost 100 years were necessary for the discovery of the first cell type that could fit the theoretical concept [2]. The same hierarchical logic has also more recently been applied to solid tissues and allowed the identification of tissue-specific cell types also considered as stem cells. The experimental derivation of ESC lines and, more recently, the creation of induced pluripotent cells that have no *in vivo* equivalent at all are also founded on the concept of intrinsic stability and unlimited self-renewal capacity.

Nevertheless, the reality turned out to be more complex than the speculative definition. Accumulating experimental evidence suggests that what actually unifies the stem cell phenotypes of various origin is, in fact, their highly dynamic nature and responsiveness to environmental stimuli. Without the ambition of exhaustively reviewing the tremendous amount of literature published on stem cell biology, this article will try to assemble the known facts, as pieces of a puzzle, into a coherent picture around the idea of why stem cells are so sensitive to their culture environment and what the practical consequences of this are. I will argue that this unstable behavior is due to the high chromatin plasticity of the stem cells that itself is the result of dynamic interactions between epigenetic mechanisms and the basic metabolic network. This plasticity makes stem cells particularly reactive to environmental influences during *in vitro* manipulations.

Pieces of the puzzle

The present review will focus essentially on *in vitro* findings on how the environment impacts stem cells. There are several reasons

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for this. First, ESCs and iPS cells have no exact *in vivo* equivalents, and even in the case of adult tissue-derived stem cells it is sometimes difficult to design their exact equivalent. *In vivo* cells with tissue regenerative capacity are located in a specific anatomic environment – the stem cell niche. The interactions between these stem cells and their niche are likely to be very complex, and can be better viewed as a mutual dependence rather than a unidirectional regulatory effect. The discussion of these interactions is beyond the scope of this paper. The second reason why *in vitro* observations are discussed here resides in the fact that, when isolated *in vitro*, tissue-derived stem cells still reproduce many characteristics of *in vivo* stem cells providing the basis for their practical use. *In vitro* observations, therefore, have strong relevance for the understanding of stem cell biology and are absolutely necessary for the development of suitable therapy-oriented procedures.

Fluctuating phenotypes

One of the earliest observations made on ESCs was their propensity to differentiate spontaneously into various cell types in standard culture medium, and that specific culture conditions (conditioned or factor-supplemented media) are required to maintain their undifferentiated state [3–5]. Different kinds of specific culture conditions using defined factors allow the canalization of the spontaneous differentiation toward defined pathways. Induced pluripotent cells also display similar behavior [6]. Perhaps one of the most intriguing features of ESCs or iPS cells is that, even under conditions favoring the undifferentiated state, they remain phenotypically heterogeneous, unstable with distinct functional and epigenetic states. Single cell comparison of the transcription profiles of 42 genes involved in the pluripotent state between individual human ESCs and iPS cells revealed that the transcriptional profiles were markedly heterogeneous; no two cells with an identical profile were found [7]. The individual ESCs were found to continuously fluctuate between phenotypically different states and generate a dynamic heterogeneity at the population level [8,9]. Although the phenotype of individual cells fluctuates, under the same culture conditions the distribution of the different phenotypic forms in the whole population remains stable over time. Due to the dynamic nature of the individual cellular phenotype, a single cell can reconstitute the original distribution of phenotypes in the population after a period of time [8,10]. The phenotypic fluctuations are likely to be the manifestation of so-called 'gene expression noise'. In pluripotent cells this noise generates oscillations in the transcriptional regulatory network organized around *Sox2*, *Oct4* and *Nanog*. Although these three genes are considered markers of pluripotency, they are not expressed simultaneously in the same cell. Based on the known interactions between these three genes it was possible to describe the dynamic behavior of the gene network as a noise-driven excitable system. In addition, the differentiation capacity of an individual cell was dependent on the actual phase of the network dynamics: a cell with low levels of Nanog is more prone to differentiate than during the high Nanog expressing phase [10]. Since the transition between the two phases is driven by stochastic transcription noise of the core gene network, the sensitivity of the cells to external stimuli that can increase or decrease the transcriptional noise is likely to be an important factor determining the overall phenotypic dynamics of the cells in the niche.

The phenomenon of phenotypic fluctuations is not a distinguishing feature of ESCs. Such fluctuations generating heterogeneity within a cell population and allowing reversible transitions between different differentiation stages have also been observed in proliferating tissue-derived stem cells. For example, in muscle-derived cultures stem cell-like 'side-population' cells are continuously generated from more differentiated cells [11]. The back and forth transition between the two phenotypes occurred spontaneously with a frequency that was dependent on the local cell density. A similar dynamic population structure has been described in human primary muscle cell cultures [12]. The cells oscillated between myoblast and fibroblast phenotypes with slow noise-driven bistable kinetics. Dynamic distribution of phenotype states with very similar noise-driven bistable kinetics has also been reported for hematopoietic progenitors [13]. Cells with a distinct phenotype had different developmental potential but because of the rapid phenotypic interconversion they tended to restore the population to the initial composition [13]. These observations show that in addition to pluripotent cells, dynamic phenotypic fluctuations are also a distinguishing feature of tissue stem cells.

The observation of continuous phenotypic fluctuations fundamentally modifies our ideas on the existence of a pluripotent state associated with a fixed pattern of gene expression and suggests that pluripotency is best represented

by a dynamic heterogeneity of a cell population driven by transcriptional fluctuations within individual cells. Phenotypic fluctuations both underlie the generation of heterogeneity within a cell population and control a stable population composition in a given environment. In other terms, instead of being a strictly intrinsic cellular state, pluripotency can be seen as an emergent property of the whole cell population.

In vitro culture & metabolism

Should we consider pluripotency as a cell-autonomous or population-level characteristic? Accumulating data clearly show that this is not a self-sustaining property; maintaining it requires special culture conditions such as growing the stem cells on feeder cells or complementation of the media with LIF or other soluble factors that inhibit spontaneous differentiation. Deciphering the mechanisms of differentiation inhibition is of major interest both for understanding pluripotency and the possibility of manipulating it experimentally. Perhaps the most interesting recent observation in this respect is the use of pharmacologic inhibitors of the GSK3 and FGF–MAPK signaling cascade (CHIRON99021 and PD0325901, respectively). These inhibitors facilitate the derivation and maintenance of pluripotent cells [14]. Although these molecules are specific for their target enzymes, it is difficult to deduce their exact mechanism of action: both Fgf and Gsk-3 signaling are promiscuous and have a plethora of targets involved in multiple basic intracellular processes such as energy metabolism and stress response [15–17]. In line with this idea, it has been shown that 'reprogramming' of somatic cells into an induced pluripotent state is facilitated by the manipulation of some basic metabolic pathways [18]. Stimulation of glycolytic metabolism by drugs [19] or by culturing the cells under hypoxia [20] enhances the transformation of somatic differentiated cells into a pluripotent state. Inhibition of glycolysis or stimulation of oxidative phosphorylation has an opposite effect [19,21]. Remarkably, the exact opposite happens during the process of differentiation. For example, when ESCs differentiate their metabolism gradually shifts from glycolytic to oxidative [22]. This observation is not really unexpected, because ESCs follow the rule of the glycolytic-to-oxidative metabolic shift, which is already known to occur in somatic cell differentiation [23]. The true novelty resides in the fact that manipulation of cellular metabolism using small pharmacomolecules, or by varying the nutrient and oxygen concentration provides a promising approach to increase the efficiency of the derivation and culture of pluripotent cells for therapy-oriented applications where the use of feeder cells or serum is not recommended. In addition to their practical importance, these observations also deliver a fundamental message: pluripotency is dynamic. It is the manifestation of the cell's general physiology and cannot be simply reduced to a particular molecular regulatory pathway or the expression of a small set of stemness genes. As a corollary, it is becoming increasingly clear that characterization of stem cells on the basis of a small number of biomarkers is biased, and hence unreliable, and global systemic detection methods of multiple features followed by a multiparametric statistical analysis of the data are needed to provide reliable results [24]. Similar conclusions showing that the use of a small number of biomarkers can be misleading were reached in other domains of molecular diagnosis [25].

Another manifestation of the dynamic nature of stem cells is the fact that ESCs and iPS cells, as well as tissue-derived stem cells, undergo gradual alterations in culture and loose their capacity to differentiate. These changes are reminiscent of cell senescence, and include epigenetic and genetic instability [26]. Genetic instability of human ESC and iPS cell lines appeared to be essentially independent of culture methods [27]. The case of epigenetic alterations is different. Environmental stress associated with *in vitro* manipulation may generate such changes, particularly if it is associated with metabolic changes. One of the earliest observations was the high epigenetic instability of ESCs in culture that led to developmental defects in fetuses and newborn mice derived from ESCs [28,29]. In a recent example, culture in 5% O_2 helped in maintaining pluripotency and suppressing spontaneous differentiation of human ESCs, while also preventing precocious X chromosome inactivation in female ESCs [30]. Culture in 20% O_2 accelerated the accumulation of the repressive H3K27-3M epigenetic mark on the inactivated X chromosomes [30]. Another recent study demonstrated that human ESCs respond rapidly to altered culture conditions by changing the expression profile and methylation pattern of a large number of genes [31]. Strikingly, upon reversion to the original culture conditions some of the methylation changes persisted indicating that the cells keep – at least partially – the 'memory' of their previous history [31]. This phenomenon suggests that cells

of the same pluripotent line but with different culture history may have different differentiation potential. Indeed, recently, low-passage iPS cells derived from adult murine tissues were found to harbor residual DNA methylation signatures characteristic of their somatic tissue of origin. The epigenetic 'memory' of the donor phenotype favored the differentiation of the cells along lineages related to the donor cell and restricted alternative cell fates [32]. A recent study has shown that a large number of errors are generated in reprogramming CpG methylation and many of them are transmitted at a high frequency during cell division, so an iPS cell-reprogramming signature was maintained even after differentiation [33].

Similar to ESCs and iPS cells, adult tissue-derived stem cells are also dependent on the environment for both self-renewal and differentiation. *In vitro* studies have demonstrated that the environmental stimuli influencing cell fate choice can be of any kind: chemical (various growth factors), mechanical or metabolic. For example, mesenchymal stem cells are capable of neuronal, myogenic or osteogenic differentiation depending on the surface rigidity on which they grow. Soft matrices were found to promote neurogenic differentiation of the mesenchymal stem cells, stiffer matrices stimulated myogenic transformation, while more rigid matrices that mimic collagenous bone were demonstrated to be osteogenic [34]. In addition to the physical environment, oxygen concentration and nutrient availability also seem to be among the determining factors. For example, *in vitro* studies have shown severe impairment of adipogenesis and osteogenesis at low oxygen tensions [35]. Muscle satellite cell proliferation and survival of mature fibers increased in 6% O_2 versus nonphysiologic 20% O_2 [36]. In general, one can consider that low oxygen tension supports the undifferentiated state [37]. In all cases, prolonged *in vitro* culture has a tendency to diminish the differentiation potential of the stem cells [38] and can lead to accumulation of signs of senescence [39].

All these observations point to the extreme sensitivity of pluripotent ESCs or iPS and tissue-derived stem cells to *in vitro* culture conditions. This sensitivity is probably due to multiple complex mechanisms related, among others, to the oxidative bioenergetics metabolism, and is manifested by the continuously fluctuating gene expression. Chromatin-related epigenetic mechanisms are likely to be major players in the generation of these variations as mediators of metabolic changes (see below). However, the role of chromatin is more complex. It also deserves particular attention for its role in the 'cellular memory'. Epigenetic mechanisms allow the chromatin to 'keep the record' of previous gene expression patterns even after multiple cell divisions; this is the basis for stable cell differentiation. The corollary of this property is that the establishment of the pluripotent state is not a simple erasure of some epigenetic marks but a new stage added to the previous life history of the cell.

The above list of observations demonstrates that highly dynamic and complex behavior, and high environmental responsiveness/dependence are the truly distinguishing features of stem cells. They underlie the capacity of these cells for rapid phenotypic conversion in response to environmental changes. The repertoire of accessible phenotypes usually called 'differentiation potential' can be smaller or larger depending on the previous life history of the cells. From this point of view, the potential for differentiation or self-renewal appear as two complementary faces of the same feature and can be described by the concepts of robustness and plasticity. For differentiated cells the balance is on the side of robustness, so they have a stable phenotype that resists change. In stem cells, plasticity prevails, so they can rapidly provide an adaptive response to environmental changes. The concept of plasticity poses a number of challenging questions: how do the same epigenetic mechanisms confer the cells the capacity to respond to any new environmental stimulus they have never encountered before and simultaneously make them robust? How are dynamic fluctuations, metabolism and chromatin-related mechanisms related? How can we compile these very different characteristics into a single logical scheme?

Chance in the life of the cell

The prevailing view is that cells can sense environmental changes using sophisticated signal transduction machinery that transmit the signal to the gene regulatory network and specifically induce lineage-specific gene expression. This deterministic theory is based on the assumption that the cells have an appropriate response to all possible environmental stimuli. How do we explain then that pluripotent cells spontaneously differentiate into various cell types in the absence of specific instructive signals or in response to nonspecific stress? This issue is much older than the discovery of pluripotent cells; it is as old as the concept of cell

differentiation. The hypothesis that cell fate commitment is a stochastic process was first proposed for hematopoietic stem cells by Till and colleagues in the early 1960s on the basis of experimental observations [40]. Observations on lineage commitment in the absence of instructive signaling by lineage-restricted cytokine receptors [41] or *in vivo* observations on the differentiation of hematopoietic stem cells support the model [42]. The idea that cell differentiation relies on stochastic events was generalized by Kupiec who proposed that robust cell differentiation and development requires a Darwinian mechanism: ordered expression patterns characteristic for differentiated genes emerge from the stochastic expression of many genes under the effect of selective pressure of cell–environment and cell–cell interactions [43–45]. The issue of stochasticity in cell differentiation is now widely debated [46]. The Darwinian model became generally accepted to explain the capacity of the immune system to generate antibody and T-cell receptor diversity. *In vivo* observations on the stem cell fate choice in adult tissues, across a range of tissue types and organisms cells can also be interpreted on the basis of the stochastic model [47], as well as the above cited observations on the phenotypic fluctuations in various *in vitro* stem cell model systems [8,10,12,13]. Strong experimental support in favor of the stochastic model came from the study of the early events of transformation of somatic into induced pluripotent cells. In order to explain why the reprogramming process is inefficient and frequently incomplete, and why only a small fraction of somatic cells are finally transformed into iPS cells, Yamanaka compared two alternatives: the deterministic 'elite' and stochastic models [48]. He concluded that the stochastic model fitted better to the available data. Briefly, this model predicts that although all cells have the potential to gain pluripotency, the rarity of the event can only be explained by the rare coincidence of stochastic factors followed by a selection required to retrieve these cells in the culture. In fact, the stochastic model is a reformulation of Kupiec's Darwinian model. Recently, the Yamanaka–Kupiec prediction has been submitted to a rigorous experimental test using a single-cell approach in monoclonal cell populations [49,50]. These studies demonstrated that the forced expression of the reprogramming factors induce a stochastic transcriptional response. Instead of activating specific genes in all or most of the cells, the expression pattern in each cell is unique. The process becomes ordered only at later stages, and only in a small fraction of the cells where the activation of the pluripotency genes is correct and the cells can successfully proliferate in the selective culture environment. In addition, it has been shown that the activation of pluripotency genes is possible by various combinations of factors and even in the absence of generic transcription factors [49]. These results show that the reprogramming is a continuous stochastic process and the data clearly disqualify the elite model.

Differentiation and 'dedifferentiation' can be considered as similar processes; both require changing the previous gene expression pattern into a new one – a condition for robust phenotype changes – and both can be explained by the same stochastic mechanism.

Putting the pieces of the puzzle together

Biologists trained on deterministic reasoning are usually skepticical about stochastic models. The traditional view of gene expression regulation is deterministic: transcriptional factors specifically activate the transcription of the target gene through binding of the gene's regulatory sequences. However, this qualitative description depicts the average situation for a large number of cells and over a period of time, exceeding the time scale of molecular events by several orders of magnitude. In a single cell each step of the gene activation and transcription process is subject to stochastic fluctuations. The cause of these fluctuations is the low copy number of the various molecules participating in the biochemical reaction of gene transcription. For example, most of the genes are present in only two copies in the cell, but the number of transcription factors sand other protein molecules is also usually very low. The resulting stochasticity of gene expression is now well known; it is readily measurable, providing the observation is performed on single cells [51]. Many different sources contribute to the variation of gene expression, including gene transcription, translation and protein degradation, but also protein–protein interactions [52]. It is now widely accepted that gene expression variation is an active player in the process of cell fate decision-making [53].

One of the major sources of gene expression noise in mammalian cells is the chromatin [54]. DNA is inaccessible for the transcription factors most of the time, because it is packaged into

chromatin. Chromatin proteins – like any other protein in the nucleus – are highly mobile. Their dissociation from DNA provides a window of opportunity for the initiation of transcription. Weak interactions between the chromatin proteins lead to dynamic turnover of the chromatin and provide more opportunities for transcription, while strong interactions slow down the turnover and stabilize the existing chromatin structure [55]. Therefore, cells with higher chromatin dynamics can induce the expression of new genes more rapidly than those with low dynamics. The dynamics of chromatin protein assembly and dissociation is essentially determined by the covalent post-translational modification of histones, other chromatin-associated proteins and the DNA molecule itself. It is well known how the epigenetic state of the chromatin determines its dynamics. Some modifications increase the stability of the interactions between the chromatin components; consequently decreasing chromatin dynamics. Other modifications do the opposite: by decreasing the stability of the interactions they accelerate the exchange of components and increase the chances for transcription. Therefore, cells with dynamic chromatin are expected to be able to express new genes rapidly. It is not surprising that pluripotent cells fall in this category. The epigenetic state of the ESC genome was found to be hyperdynamic [56]. These cells possess high levels of histone acetylation and relatively low levels of H3K9 methylation, which contribute to the high plasticity of their genome [57]. The data indicate that the genome's epigenetic state in pluripotent cells modulates chromatin plasticity and determines, in this way, the cell's differentiation potential. Epigenetic modifications are themselves reversible with a high turnover rate due to the simultaneous presence of enzymes catalyzing the on and off reactions. Even DNA methylation, thought to be the most stable modification, has been shown to have an unexpectedly high turnover rate [58,59]. Therefore, the level of each modification in the genome is the result of a dynamic equilibrium.

How can the chromatin of pluripotent cells achieve the equilibrium required for the hyperdynamic state? It was proposed some time ago that the major regulator of epigenetic modifications is the central carbon metabolism itself, because it produces substrates for the modifications [60]. This has since been firmly demonstrated [61,62]. The link between epigenetic modifications and metabolism is first of all mechanistic: all substrates used by the epigenetic enzymes to modify chromatin are key metabolic intermediates that play a major role in the regulation of the redox metabolism of the cell (e.g., acetyl coenzyme A [acetyl-CoA], NAD^+ and 2-oxoglutarate) or are directly synthetized from them (e.g., *S*-adenosyl methionine and UDP *N*-acetylglucosamine) [63]. It is essential that the metabolic intermediates are not cofactors, but true substrates that are consumed by the enzymes for the modification of the chromatin. Therefore, they are not available for energy production or biosynthesis. For example, one molecule of acetyl-CoA is required for the monoacetylation of an $-NH_3$ lysine residue. The full oxidation of an acetyl-CoA molecule in the Krebs cycle and terminal oxidation produces 17 ATP molecules. Therefore, the energy cost of chromatin acetylation is high. The intracellular concentration of the metabolic intermediates varies depending on the actual carbohydrate substrate availability and oxygen tension that determines metabolic flux. These fluctuations can directly influence the epigenetic status of the genome, giving chromatin the capacity to sense the metabolic flux and the energetic status of the cell. Compared with the ubiquitous conservation of the small metabolic intermediates and their role in epigenetic modifications, regulatory mechanisms known for their role to adapt the cells to hypoxia or other metabolic stresses are likely to be secondary evolutionary acquisitions. These mechanisms reinforce the means by which metabolic events impact on the epigenetic state of the genome.

On the basis of these data it is possible to assemble into a coherent picture the puzzle of the apparently unrelated pieces of observations on the gene expression fluctuations, epigenetic plasticity and metabolic particularities of the pluripotent cells. These features are tightly linked and form a unique system that conveys the environmental effects to the genome and translates it into phenotypic change (Figure 1). Initially, the environmental stress, such as specific action of signaling molecules through their receptors or nonspecific environmental change of the concentration of metabolic substrates, oxygen, pH or mechanical environment, produce perturbations in the cell's energy and metabolic balance. The resulting fluctuations of the key intracellular metabolite levels are transmitted to the chromatin through epigenetic mechanisms. These mechanisms use specific substrates and modify specific chromatin proteins, but are not specific for individual genes. Nevertheless, their action may be different in different chromatin regions depending

on the pre-existing epigenetic profile of each region and the actual concentration of the metabolic substrate used. The final outcome can be a substantial alteration in the local chromatin dynamics that allows the transcriptional activation of previously silent genes in regions with increased chromatin turnover or decreased transcriptional activity in regions where the chromatin turnover is reduced. In this way, environmental changes act as a gene expression noise generator and create a favorable ground for the rapid emergence of a coordinated expression response under the selective constraints of the new environment. Stabilization of the new expression pattern is likely to occur if the new phenotype resulting from the random activation of genes allows the cell a better adaptation to the new environmental conditions by recovering a metabolic flux compatible with the cell's energy and biosynthetic demands. In addition to the stabilizing action of the emerging new gene expression regulatory network [64], the tight connection between metabolism and epigenetic regulation is also likely to play a stabilizing role by reducing the chromatin dynamics. The stochastic variation-generating phase of this scenario has already found experimental support [50]; however, the exact mechanism of the second stabilizing phase remains an unresolved issue.

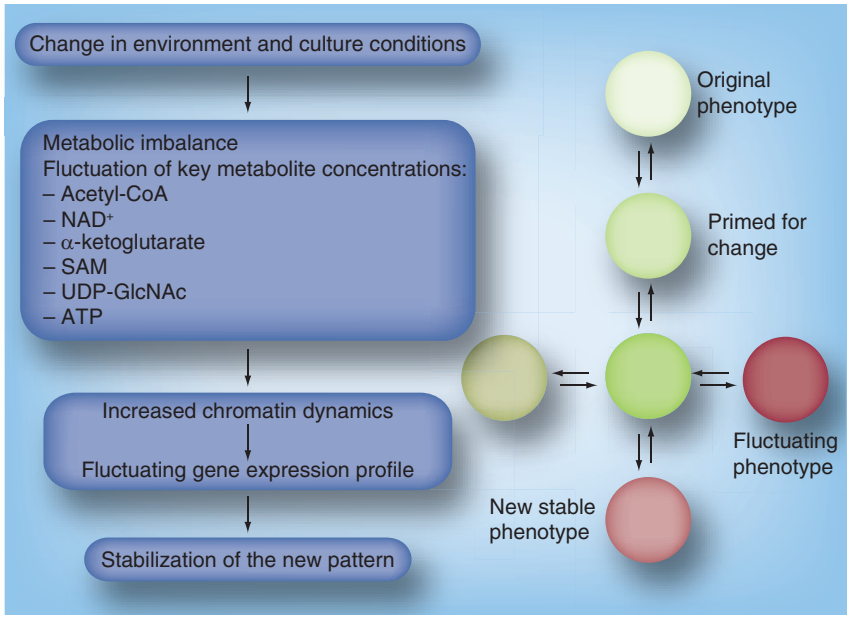

Figure 1. Stochastic model for differentiation. Environmental changes impact on the energy and metabolic balance of the cell, and induce fluctuations in the concentration of key metabolites. These fluctuations are transmitted to the chromatin and increase the rate of the dynamic turnover of its components through epigenetic mechanisms that use the metabolites as substrates. The increased chromatin turnover allows the expression of many genes in a noncoordinated stochastic way. Fluctuating gene expression patterns provide the cells with the opportunity to enter new phenotypic states. A new phenotype is stabilized if it allows the cell to recover the metabolic equilibrium.
Acetyl-CoA: Acetyl coenzyme A; GlcNAc: *N*-acetylglucosamine; SAM: *S*-adenosyl methionine.

Future perspective

Manipulation of pluripotent cells has shown that the major way the environment impacts on cellular differentiation makes use of epigenetic mechanisms. The model described here provides a scenario of how changes in the cell environment that cause metabolic perturbations are first translated into stochastic gene expression fluctuations by epigenetic mechanisms, then select and stabilize the phenotype best adapted to the new conditions, with the contribution of epigenetic mechanisms. If definitively confirmed, this model will help better the understanding of the fundamental issue of cell differentiation by placing on the central stage the connection between metabolic perturbations induced by environmental changes and epigenetic mechanisms. In addition to the fundamental significance, the model has important practical implications for the *in vitro* culture and manipulation of stem cells. On the one hand, environmental stress associated with cell manipulation is essential for the induction of the desired cell phenotype; on the other hand the very same manipulations inevitably induce epigenetic changes that may be 'memorized' by the cells and produce undesired consequences at later stages. Accumulating data show that the risk may not just be hypothetical [65,66]. Therefore, the developers of new *in vitro* culture methods aiming to safely manipulate pluripotent cells for therapeutic use have to learn how to manipulate this double-edged sword. However, the stochastic mechanism of phenotypic change also provides an opportunity to directly reprogram cells to any phenotype. The initial phase of stochastic gene expression is likely to be common to any differentiation/dedifferentiation process. It is the selection process that determines the phenotype the cells will converge to. It is, therefore, theoretically possible to develop new culture methods that will allow direct reprogramming of somatic cells into another somatic phenotype without passing through a pluripotent stage. This would considerably simplify the use of cell reprogramming for practical purposes and reduce the associated risks of potential long-term epigenetic effects.

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Executive summary

Dynamic stem cell phenotype

- Embryonic, induced pluripotent- and tissue-derived stem cells display a highly unstable phenotype; they continuously fluctuate between different states. An increasing body of evidence suggests that this dynamic instability is the result of randomly fluctuating gene expression patterns.

High environmental sensitivity

- Alterations of the culture environment may induce changes in the metabolic and energy balance of cells. This results in fluctuations in the intracellular concentration of key metabolites. This is the first step in the process of adaptation to the new environment. The fluctuations cease when the metabolic and energy flux is re-established. If expression of new genes also occurs, the process is considered to be cell differentiation (or dedifferentiation).

Epigenetic mechanisms: a dynamic interface between the cellular metabolism & gene expression

- Environmental effects are conveyed to the genome by epigenetic mechanisms. Key metabolites are used by the epigenetic mechanisms as substrates to modify chromatin dynamics. As a result, metabolic fluctuations may act as a generator of stochastic gene expression patterns and open the way toward new cellular states.

Conclusion

- The stochastic mechanism of cell phenotype change provides theoretical and practical opportunities. From a fundamental point of view, the stochastic model can reconcile the apparent contradiction between how stochastic fluctuations can lead to a stable and well-defined phenotype. From a practical perspective, the stochastic model may inspire strategies to directly reprogram one somatic phenotype to another.

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