



Mountain high and valley deep: epigenetic controls of pluripotency and cell fate

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Abstract

All the somatic cells composing a mammalian organism are genetically identical and contain the same DNA sequence. Nevertheless, they are able to adopt a distinct commitment, differentiate in a tissue specific way and respond to developmental cues, acquiring a terminal phenotype. At the end of the differentiation process, each cell is highly specialized and committed to a distinct determined fate. This is possible thanks to tissue-specific gene expression, timely regulated by epigenetic modifications, that gradually limit cell potency to a more restricted phenotype-related expression pattern. Complex chemical modifications of DNA, RNA and associated proteins, that determine activation or silencing of certain genes are responsible for the 'epigenetic control' that triggers the restriction of cell pluripotency, with the acquisition of the phenotypic definition and the preservation of its stability during subsequent cell divisions. The process is however reversible and may be modified by biochemical and biological manipulation, leading to the reactivation of hypermethylated pluripotency genes and inducing cells to transit from a terminally committed state to a higher plasticity one.

These epigenetic regulatory mechanisms play a key role in embryonic development since they drive phenotype definition and tissue differentiation. At the same time, they are crucial for a better understanding of pluripotency regulation and restriction, stem cell biology and tissue repair process.

Keywords: cell plasticity, differentiation, epigenetics.

Introduction

The temporal order of gene expression plays a fundamental rule to ensure lineage commitment and cell fate determination Brevini *et al.*, 2007. In 1942, Waddington coined the term 'epigenetics', which was defined as "changes in phenotype without changes in genotype". In recent years several studies have characterized the different mode of epigenetic regulation, such as DNA methylation, post-translational histone tail modifications, non-coding RNA control of chromatin structure, and nucleosome remodeling. All of them regulate the activation or repression of genes. Technological advances in epigenome analysis and

pluripotent stem cell technologies have been driver for elucidating the epigenetic control of cellular identity during development and reprogramming.

Here, we provide a brief overview of the main epigenetic mechanisms, such as DNA methylation and histone modifications. We then review the available knowledge on the possibility to erase the epigenetic memory through the use of cell reprogramming technologies. We also give prospective views of the epigenetic direct conversion of one cell type into another, in a safe and robust way, for regenerative medicine.

Cell commitment and Waddington model of epigenetic restriction

Over 230 different cell types are present in an adult multicellular organism. Although they all derive from one single cell and are genetically identical (containing the same DNA sequence), they are able to differentiate in a tissue specific way and to respond to specific developmental cues. Indeed, at the end of the differentiation process, each cells is highly specialized and committed to a distinct determined fate. This is possible thanks to tissue-specific gene expression, timely regulated by epigenetic restrictions, that gradually limit cell potency (Hemberger *et al.*, 2009) to a more limited phenotype-related expression pattern (Zhou and Melton, 2008).

More than 60 years ago, these concepts have been nicely depicted by Conrad Waddington, who first used the term "epigenetics" in his very famous landscape to describe the idea that a phenotype arises by a program, defined by the genome, under the influence of the organism's environment. In Waddington's metaphor, a ball represents the cell of an embryo, rolling from a non-committed, pluripotent condition down the hill, to a specific cell fate. The hill is marked by slopes and valleys representing the many different and complex process that characterize the events leading to cell differentiation. The ball is addressed along a progressively more restricted potency pathway, towards a favored position at the bottom of the hill, where the cell is unipotent and is characterized by a tissue specific differentiated state (Fig. 1).

Currently, epigenetics is at the center of modern biology, since it is considered a fundamental tool to understand stem cell biology as well as cell differentiation and de-differentiation processes.

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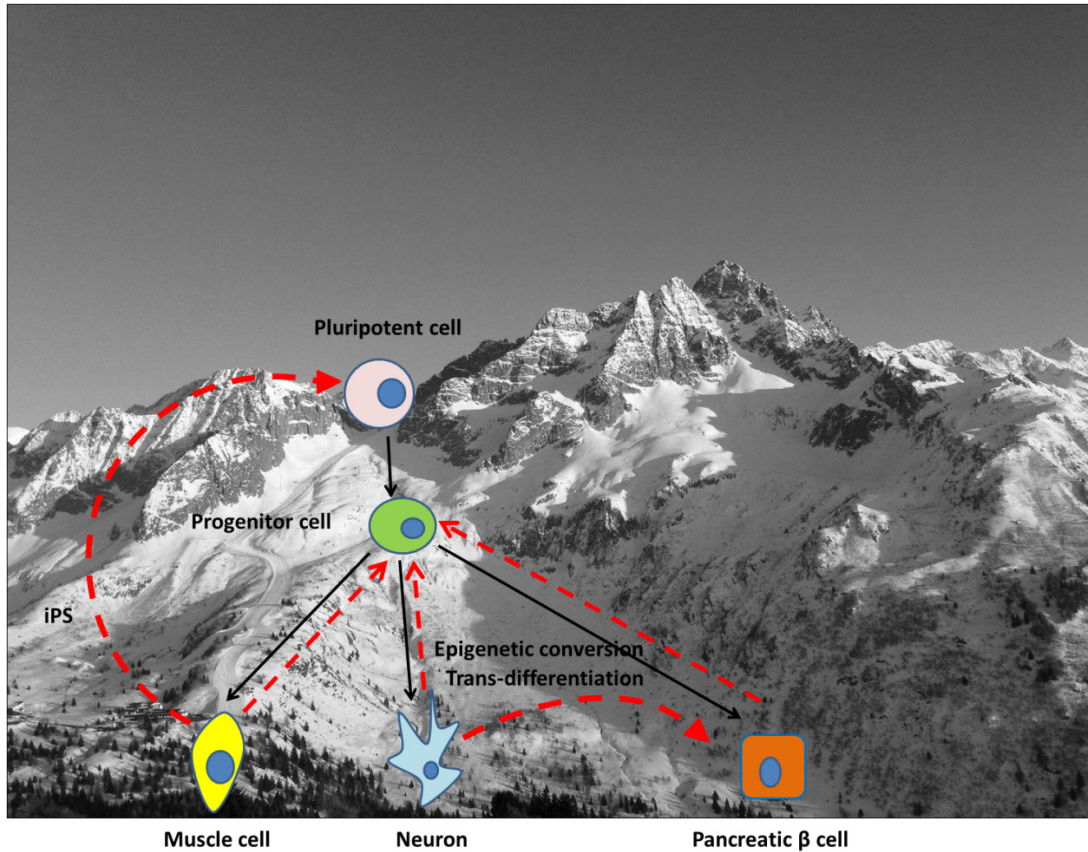


Figure 1. Representation of Waddington model. A pluripotent cell rolls from a non-committed condition down to the bottom of the hill, where the cell is unipotent and is characterized by a tissue specific differentiated state. The hill is marked by slopes and valleys, representing the many different and complex process that characterize the events leading to cell differentiation. Recent studies have shown that differentiated cells of an adult organism can be forced in an upstream, counter-current direction up the differentiation hill, transiting along different states of increased potency.

Epigenetic control mechanisms and “epigenetic memory”

The term “epigenetics” refers to complex chemical modifications of DNA, RNA and associated proteins, that determine activation or silencing of certain genes, without any permanent loss or alteration of genetic material (Goldberg *et al.*, 2007; Xie *et al.*, 2013). These mechanisms are responsible for the ‘epigenetic memory’ that triggers the phenotypic stability of the differentiated cell during subsequent cell divisions (Zhu *et al.*, 2013; Jost, 2014; Shipony *et al.*, 2014; Brevini *et al.*, 2015).

There are several types of epigenetic modifications (Table 1) that play a fundamental role in the regulation of chromatin structure and gene expression, namely histone post-translational modifications, covalent modifications of DNA, small (21- to 26-nt) non-coding RNAs, and recombinations of non-genic DNA (Goldberg *et al.*, 2007).

More in detail, histones can be subjected to acetylation, phosphorylation, methylation, SUMOylation, citrullination, ADP ribosylation, and ubiquitination (Spivakov and Fisher, 2007), that allow or prevent transcription factor and other protein access to DNA.

In parallel, the DNA can be methylated through a covalent addition of a methyl (CH₃) group at the 5-carbon of the cytosine, that physically impedes the binding of transcription factor proteins to the gene (Choy *et al.*, 2010) or recruits the methyl-CpG-binding domain proteins (MBDs). This modification also induces histone remodeling and the formation of compact, inactive chromatin, known as heterochromatin.

All these processes drive pluripotent cell differentiation and the acquisition of tissue specific epigenetic marks that are stable through the life span of a single individual and have been considered irreversible until not long ago.



Table 1. Mechanisms involved in epigenetic control and related epigenetic enzymes.

Mechanism	Epigenetic enzymes
DNA Methylation	DNA Methyltransferases DNA Demethylation Enzymes Methyl-CpG Binding Domains
Histone Acetylation	Histone Acetyltransferases Histone Deacetylases Bromodomains; Tandem PHD Fingers; Pleckstrin Homology Domains Protein Arginine Methyltransferases
Histone Arginine Methylation	Histone Demethylases Tudor Domains (recognize symmetrically dimethylated arginines); WD40 Domains Histone Lysine Methyltransferases
Histone Lysine Methylation	Histone Lysine Demethylases Chromodomains; Tudor Domains; PHD Fingers; MBT Domains; ZF-CW Proteins; WD40 Domains; PWWP Kinases (JAK2, ATM/ATR, PKC, PKA, Haspin, Aurora B Kinase, RSK2, AMPK, MSK, MEK)
Histone Phosphorylation	Protein Serine/Threonine Phosphatases; Protein Tyrosine Phosphatases Chromoshadow Domains (phosphoTyrosine); 14.3.3 Proteins (phosphoSerine); BIR Domains; BRCT Proteins
Histone Ubiquitination	Ubiquitin E2 Conjugases; Ubiquitin E3 Ligases Deubiquitinating Enzymes

Erasing of “epigenetic memory”

During the last years, many studies demonstrated that, although generally stable *in vivo*, the differentiated state of an adult cell can be reversed and forced in an upstream, counter-current direction up the Waddington’s differentiation hill, along different states of increased potency (De Carvalho *et al.*, 2010).

In 2006, the generation of induced pluripotent cells (iPSCs; Takahashi and Yamanaka, 2006) paved the way for a search of the mechanisms involved in the erasure of “epigenetic memory” and the re-establishment of pluripotency. Takahashi *et al.* demonstrated that an adult somatic cell can be brought back to an increased potency state, through the ectopic expression of four transcription factors (TFs; Takahashi and Yamanaka, 2006). Currently, various methodologies have been established for iPSC derivation, from virus-free (Okita *et al.*, 2008, 2010; Kaji *et al.*, 2009), to removable PiggyBac transposons (Woltjen *et al.*, 2009), minicircle systems (Jia *et al.*, 2010), episomal systems (Yu *et al.*, 2009), synthetic mRNAs (Kim *et al.*, 2009; Zhou *et al.*, 2009; Warren *et al.*, 2010), and microRNAs (Anokye-Danso *et al.*, 2011; Miyoshi *et al.*, 2011).

Nevertheless, cell reprogramming suffers from a number of severe limitations (Okita *et al.*, 2007). In particular, its efficiency remains low (Mikkelsen *et al.*, 2008; Pasque *et al.*, 2012; Gaspar-Maia *et al.*, 2013; Sridharan *et al.*, 2013; Nashun *et al.*, 2015), the extreme stability of adult somatic cell epigenetic signature makes iPSCs prone to errors (Plath and Lowry, 2011), and the use of DNA constructs and the subsequent possibility of exogenous sequence integration preclude their clinical use for safety concerns (Stadtfield *et al.*, 2008; Kim *et al.*, 2009; Zhou and Freed, 2009; Seki *et al.*, 2010). In order to circumvent these limits, small-

molecule compounds have been used to modulate the epigenetic state by inhibiting and/or activating, in a reversible way, specific signaling pathways (Huangfu *et al.*, 2008; Ichida *et al.*, 2009; Li *et al.*, 2011; Hou *et al.*, 2013). A recent study revealed that the endogenous pluripotency program can be re-activated through the use of a combination of seven small-molecule compounds, namely valproic acid (VPA), CHIR99021, 616452, TCP, Forskolin (FSK), 2-methyl-5-hydroxytryptamine (2-Me- 5HT), and D4476 (Hou *et al.*, 2013). Similarly, it was also demonstrated that the use of VPA, in combination with an embryonic stem cell (ESC) medium, is able to revert somatic cells into pluripotent ones, in the absence of any transgenes (Moschidou *et al.*, 2012; Rim *et al.*, 2012).

These results represent a significant progress in cell reprogramming technology, demonstrating the possibility to use new approaches that avoid the presence of retroviral and/or lentiviral vectors, and the insertion of transgenes. However, the major concern related to the acquisition of a stable and persistent pluripotent state, remains unsolved. Indeed, the achievement of a stable pluripotency is an unphysiological condition, since, physiologically is transient and limited to a short time window, during the first phases of embryonic development. Furthermore, iPSCs display cell instability (Wu and Zhang, 2010), are difficult to differentiate with an efficiency that rarely exceeds 30%, leaving mature cells mixed with undifferentiated and proliferating ones (Cohen and Melton, 2011).

Currently, all these aspects, severely limit the use of these cells in regenerative medicine, although useful information may be drawn when using iPSCs, as a model for a detailed understanding of cell plasticity and differentiation.



Epigenetic conversion: an alternative erasing of “epigenetic memory”

During the last years new approaches allowing the direct conversion of an adult mature cell into another differentiated cell type have been developed. These methods are based on the use of small molecules and epigenetic modifiers (Table 2), and avoid the use of transgenes, stably integrated into the genome. In 2004 we can list the first paper reporting the ability of the small molecule reversine to increase cell plasticity, inducing lineage committed myoblasts to become multipotent mesenchymal progenitor cells (Chen *et al.*, 2004). The activity of this molecule was subsequently tested in several type of cells, including 3T3E1 osteoblasts (Chen *et al.*, 2007), human primary skeletal myoblasts (Chen *et al.*, 2007), murine and human dermal fibroblasts (Anastasia *et al.*, 2006), and confirming in all treated cells the induction of an increased plasticity.

Since that time, several protocols that involve the use of epigenetic modifiers have been developed. They confirmed that specific chemical compounds can push cells to a transient less committed state, increasing cell plasticity for a relative short time-window, but sufficient to re-address an adult mature cell into another differentiated cell type (Harris *et al.*, 2011; Pennarossa *et al.*, 2013, 2014; Brevini *et al.*, 2014; Mirakhori *et al.*, 2015; Chandrakanthan *et al.*, 2016).

A very general concept at the base of these experiments is that, among several mechanisms that drive cell differentiation, DNA methylation plays a fundamental role during both early embryonic development and cell lineage specification. To this purpose, de-methylating agents, which are well-characterized DNA methyltransferase (DNMT) inhibitor, were selected and used to erase DNA epigenetic restrictions. An example comes from 5-azacytidine (5-aza-CR), that, when used at low doses, substitutes for cytosine and incorporate into DNA and RNA during replication (Stresemann and Lyko, 2008; Aimiwu *et al.*, 2012), forming covalent adducts with DNMT1. Thanks to its powerful effects, 5-aza-CR is able to induce global DNA hypo-methylation (Christman, 2002; Stresemann and Lyko, 2008), gene reactivation (Jones, 1985), and can facilitate adult somatic cell switch from one phenotype to a different one (Taylor and Jones, 1979; Glover *et al.*, 1986; Harris *et al.*, 2011).

In accordance with these findings, our laboratory demonstrated that an adult somatic cells can be converted into a new cell type after an 18 h exposure to 5-aza-CR (Pennarossa *et al.*, 2013, 2014; Brevini *et al.*, 2014, 2016). Cells acquired a ‘highly permissive state’ with significant changes in their phenotype and a specific gene regulatory response, that were paralleled by decrease in global DNA methylation. More in detail, following exposure to the demethylating agent, cells

exhibited reduced dimensions with large nuclei, displayed a global chromatin decondensation and expressed pluripotency-related genes such as OCT4, NANOG, REX1 and SOX2. These are common features of ESC, iPSC and, more in general, of pluripotent cells (Tamada *et al.*, 2006). It is interesting to consider that this condition was transient and reversible, and, if returned to their standard culture medium, cells reverted to their original phenotype. Expression of pluripotency related genes decreased gradually within a few days (Pennarossa *et al.*, 2013, 2014; Brevini *et al.*, 2014).

Once entered into the higher plasticity window, cells could easily be directed towards a different phenotype through the use of specific differentiation stimuli. In particular, adult skin fibroblasts, derived from different species namely human (Pennarossa *et al.*, 2013; Brevini *et al.*, 2014), porcine (Pennarossa *et al.*, 2014), and dog (Brevini *et al.*, 2016), were converted into pancreatic beta-cells through a three step pancreatic induction protocol. At the end of the epigenetic conversion, cells exhibited mature endocrine phenotype, expressing the main hormone and glucose sensor genes specific of the pancreatic tissue (Pennarossa *et al.*, 2013, 2014; Brevini *et al.*, 2016). The converted cell ability to restore normo-glycaemia and stably maintain glucose levels was also confirmed in vivo using diabetic mice (Pennarossa *et al.*, 2013, 2014). Notably, we also demonstrated that epigenetic conversion can be applied to different cell types, such as granulosa cells that were converted into muscle cells through the use of 5-aza-CR followed by a 15 day culture with human recombinant vascular endothelial growth factor (VEGF; Brevini *et al.*, 2014).

Furthermore, recent works carried out in other laboratories demonstrated the possibility to convert human skin fibroblasts into neural progenitor-like cells (Mirakhori *et al.*, 2015) and mature bone and fat cells into tissue-regenerative multipotent stem (iMS) cells (Chandrakanthan *et al.*, 2016) through the use of the demethylating agent 5-aza-CR, proving to be in agreement with our results. In addition, Cheng *et al.* reported that, using a cocktail containing inhibitors of histone deacetylation, glycogen synthase kinase and TGF- β pathway, it is possible to convert human and murine fibroblasts into proliferating chemical-induced neural progenitor cells (ciNPC), under physiological hypoxic conditions (5% O₂; Cheng *et al.*, 2015).

Furthermore, recent experiments described the possibility to epigenetically convert human skin fibroblasts into mature Schwann cells through the use of the histone deacetylase (HDAC) inhibitor VPA (Thoma *et al.*, 2014). In that work, cells were stimulated with a two-step neural induction protocol, in order to obtain a transient population of proliferating neural precursors and, subsequently, terminally differentiated Schwann cells (iSCs), that showed neuro-supportive and myelination capacity, and expressed proteins specific of the peripheral nervous system.

Table 2. List of epigenetic modifiers, their targets and applications.

Epigenetic Modifier (name and structure)	Target	Applications
5-aza-cytidine 	DNA methyltransferase inhibitor	Phenotype changes in eukaryotic cells; Transformation of mesenchymal stromal cells and fibroblasts into hematopoietic cells; Epigenetic cell conversion
5-aza-2'-deoxycytidine 	DNA methyltransferase inhibitor	Alteration of primitive HSC/HPC fate; Increasing of cell plasticity for somatic cell nuclear transfer; Transcriptional reactivation of tumor suppressor genes
Hydralazine 	DNA methyltransferase inhibitor	Epigenetic modification to revert multidrug-resistant phenotype
Zebularine 	DNA methyltransferase inhibitor	Epigenetic reprogramming of yak fibroblasts for cloning; Epigenetic modification of bovine adipose stem cells
Valproic acid 	HDAC inhibitor	Dedifferentiation of amniotic fluid cells and of human dermal fibroblasts
Vorinostat 	HDAC inhibitor	Epigenetic reprogramming to restore chemosensitivity
Trichostatin A 	HDAC inhibitor	Dedifferentiation of EG; Increasing of cell plasticity for somatic cell nuclear transfer; Increase iPS formation efficiency; Alteration of primitive HSC/HPC fate
Sodium butyrate 	HDAC inhibitor	In combination with A-83-01, PD0325901, and PS48, enables reprogramming of human somatic cells transduced with Oct4 only



Conclusions

The growing understanding of the epigenetic regulatory mechanisms controlling cell differentiation provides new perspective for both the embryology and the cell biology field. Accumulating evidence point to epigenetic modifications, such as histone modification and DNA methylation, as key cellular events that exert a precise control over gene expression and allow a dynamic crosstalk between genotype and phenotype, leading to cell fate commitment and tissue specification. This has, in turn, further widened our understanding of the regulatory pathways involved in cell reprogramming, transdifferentiation and conversion, and has boosted the use of epigenetic modifiers and small molecules to revert cells to high plasticity states and encourage the acquisition of terminal phenotype.

Altogether the results accumulating have important implications for a better understanding of epigenetic cell fate control but is also advantageous for stem cell therapy and for regenerative medicine of human and animals.

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