Title: Phenotype switching through epigenetic conversion

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Abstract

Different cell types have been suggested as candidates for regenerative medicine. Embryonic pluripotent cells can give rise to all cells of the body and possess unlimited self-renewal. However they are unstable, difficult to control and show a risk of neoplastic transformation. Adult stem cells are safe but show limited proliferation and differentiation abilities, and, usually are not in easy access. In the last years, induced pluripotent cells became a new promising tool. However, the use of transgene vectors, commonly required for their creation, seriously limits their use in therapy. The same problem accompanies cells obtained through trans-differentiation. The developing knowledge of the mechanisms controlling epigenetic regulation of cell fate has boosted the use of epigenetic modifiers that drive the cells into a "highly permissive" state. We recently set up a new strategy for the conversion of an adult mature cell into another. We increased cell plasticity using 5-aza-cytidine and took advantage of a brief window of epigenetic instability to re-address cells to a different lineage. This approach is designated "epigenetic conversion". It is a simple, direct and safe way to obtain cells for therapy avoiding gene transfection and a stable pluripotent state.

Keywords: Epigenetic, cell conversion, cell plasticity, regenerative medicine

Gene expression and epigenetic mechanisms

All cells within a complex multicellular organism contain the same genome. However the body is composed of many different types of cells obtained through the adoption of a specific fate and specialization in several tissues. For instance, starting from a single cell with a half-genome from each parent, it is possible to obtain a wide variety of cell types different from each other, without any permanent loss of genetic material or alteration in the sequence of DNA. This is the result of cell differentiation processes that are regulated by the expression of different sets of genes, responsible for a distinct phenotype. More in detail, gene expression is regulated by factors both extrinsic and intrinsic to the cell (Swain, P. S., *et al.* 2002). Cell-extrinsic factors include environmental cues that can originate from other cells within the organism (such as small molecules, secreted proteins) or from the organism's environment (temperature and oxygen). By contrast cell-intrinsic regulation takes place through the cell's own machinery that chemically modifies the DNA. These changes are described as epigenetic modifications because they do not alter the primary DNA sequence, but instead affect gene expression by changing the accessibility of genes to transcription factors, in either a positive or a negative manner. They are responsible for heritable changes that stably maintain the genomic region activity state.

Two major mechanisms are involved in these regulatory processes: DNA methylation and histone modifications (Goldberg, A. D., *et al.* 2007). The first consists of a biochemical process where a methyl (CH3) group is added to the cytosine or adenine DNA nucleotides. The covalent addition of a CH3 group at the 5-carbon of the cytosine ring is controlled at several different levels and is carried out by a family of enzymes know as DNA methyltransferases (DNMTs). In particular, DNMT3a and DNMT3b are required for the establishment of new or de novo DNA methylation patterns (Okano, M., *et al.* 1999), while DNMT1 appears to be responsible for their maintenance (Takeshita, K., *et al.* 2011). DNA methylation rate differs strongly among species and it is

between 60% and 90% of all CpGs in mammals (Bird, A. P. 1986). These modalities of DNA modifications may affect the transcription of genes in two different ways. It may physically impede the binding of transcriptional proteins to the gene (Choy, M. K., *et al.* 2010) or it may be bound by proteins, known as methyl-CpG-binding domain proteins (MBDs), that recruit additional proteins to the locus, remodeling histones and forming compact, inactive chromatin, termed heterochromatin.

A second mechanism involved in the transcriptional regulation processes is the histone modification. Histones are subject to a complex and dynamic set of covalent modifications, including acetylation, phosphorylation, methylation, SUMOylation, citrullination, ADP ribosylation, and ubiquitination (Spivakov, M. and Fisher, A. G. 2007). The attachment of the different molecules on the histone tail allows or prevents transcription factors and other proteins to access the DNA.

All these modifications cause differences in gene expression that drive embryo development and cell differentiation. The acquisition of epigenetic marks culminates with the fixation of a specific lineage fate that has been considered stable and potentially irreversible for many years.

Reversion of cell fate

Mature cells of an adult organism acquire the differentiated state through a specification process that takes place during the embryo/fetus developmental phases. This process is characterized by differential gene expression and epigenetic restrictions that gradually limit cell differentiative potency (Hemberger, M., *et al.* 2009) to a more limited phenotype-related expression pattern, resulting in a progressive restriction in cell options (Zhou, Q. and Melton, D. A. 2008) and finally producing highly specialized committed populations.

Conrad Hal Waddington proposed a nice metaphor to describe epigenetics (Waddington, C. H. 1942) as "the branch of biology which studies the causal interactions between genes and their cellular product and puts the phenotype into being" . Based on these observations it is easy to see that, while the genotype is static and stable, the phenotype is dynamic and plastic. "Genetics proposes and epigenetics disposes", observed the Medawar in their philosophical dissertation on biology "From Aristotle to Zoos," in 1983, referring to the possibility of obtaining different phenotypes from the same DNA sequence (Medawar, P. B. and Medawar, J. S. 1983).

In Waddington's allegory the embryonic cell is represented by a ball that rolls from a non-committed state (pluripotent state) down a hill marked by slopes and valleys, that symbolize the many different and complex mechanisms involved in cell differentiation process. Rolling down, the ball is addressed, by slopes and valleys, towards a progressively more restricted potency pathway, down to a tissue specific differentiated state (unipotent state) (Waddington, C. H. 1957).

This final state is achieved and maintained through the epigenetic mechanisms that regulate gene expression (Li, M., *et al.* 2012). Furthermore, since this serie of events is extremely stable, a complete reversal of cell fate requires a wide reprogramming process that makes it inefficient and prone to errors (Plath, K. and Lowry, W. E. 2011). Indeed, although cellular differentiation is usually unidirectional *in vivo*, it can be reverted *in vitro* (De Carvalho, D. D., *et al.* 2010).

Over 30 years ago preliminary experiments demonstrated that human fibroblasts retain some plasticity and, in the presence of specific factors, they are able to express genes specific of another lineage (Chiu, C. P. and Blau, H. M. 1984). This study was the first evidence that differentiation is a bi-directional process and, more recent reports have expanded the knowledge about the reversibility of the process, showing that it is possible to force mature cells back to an increased potency state and stably maintain it (Chiu, C. P. and Blau, H. M. 1985). Using the

Waddington's landscape metaphor, it is possible to describe this event as the possibility to push the ball from the bottom of the valley up to the top of the hill in a counter-current direction.

To this end terminally differentiated somatic cells can be reprogrammed using defined factors to generate induced pluripotent stem (iPS) cells. Cell reprogramming requires higher levels of gene expression than the levels needed once the pluripotent state is reached, and equivalent to what is defined as “activation energy” (namely the energy necessary to initiate reprogramming even and required to surmount the cascades in an upstream, counter-current direction to create states of increased potency) (Hemberger, M., *et al.* 2009). This reflects the necessity to initiate epigenetic reprogramming events through the use of retroviral vectors carrying the transcription factors needed to reach the pluripotent state, namely OCT4, kruppel-like factor 4 (KLF4), SOX2 and MYC (Meissner, A., *et al.* 2007;Okita, K., *et al.* 2008;Takahashi, K., *et al.* 2007;Takahashi, K. and Yamanaka, S. 2006). Retroviral vectors integrate in the host genome and force ectopic over-expression of the reprogramming factors, resulting in the reactivation of endogenous genes and regaining a developmental potency akin to that of embryonic stem cells (ESCs).

Unfortunately this approach suffers from a number of severe limitations that prevent its possible use in regenerative medicine (Yamanaka, S. 2009). In particular, the need of potentially harmful genome integrating viruses to deliver reprogramming factor transgenes (Okita, K., *et al.* 2007;Takahashi, K. and Yamanaka, S. 2006) is associated with the risk of tumor formation and unsafe to human health. To by-pass this problem alternative gene factor delivery systems have been proposed, including non-integrating adenoviruses (Stadtfeld, M., *et al.* 2008), plasmid transfection (Okita, K., *et al.* 2008), doxycycline-inducible excisable piggyBac (PB) transposon system (Woltjen, K., *et al.* 2009), and non-integrating episomal vectors (Kaji, K., *et al.* 2009). Nonetheless other concerns associated with the risk to cause a tumor remain unsolved. Indeed, iPSs show low differentiation efficiency, that rarely exceeds 30%, leaving mature cells mixed

with undifferentiated ones (Cohen, D. E. and Melton, D. 2011). Caution is also suggested by the fact that the four factors used to induce reprogramming are strictly speaking oncogenes, increasing the risk of transformation to a cancer phenotype (Ibarretxe, G., *et al.* 2012). Furthermore the acquisition of a stable pluripotent state is not physiological and appears to be difficult to control.

To circumvent the latter problem a new technique, designated "trans-differentiation", has been introduced. This method consists in the direct inter-conversion of one fully differentiated adult cell type into another without an intermediate pluripotent state or progenitor cell type, but through a simultaneous down-regulation of one genetic programme and a concomitant up-regulation of the new one (Jopling, C., *et al.* 2011).

Several studies demonstrated the ability of the myogenic factor MyoD to reprogram different cell types into myogenic cells (Weintraub, H., *et al.* 1989), as well as the possibility to convert pancreatic cells into hepatocytes (Shen, C. N., *et al.* 2000;Zhou, Q., *et al.* 2008) or macrophages (Xie, H., *et al.* 2004), and fibroblasts into neurons (Vierbuchen, T., *et al.* 2010) or cardiomyocytes (Ieda, M., *et al.* 2010). All these promising approaches involve however the use of retrovirus for the overexpression of one or more specific transcription factors (Cohen, D. E. and Melton, D. 2011), leading to several limitations and making cells unsuitable for cell therapy and regenerative medicine.

To overcome all the problems described above, virus-free protocols for iPS derivation (Okita, K., *et al.* 2011;Stadtfeld, M., *et al.* 2008) or cell trans-differentiation were proposed, but, at present, they are technically demanding, less efficient (Lengner, C. J. 2010) and do not solve the problems arising from the use of transgenes.

Epigenetic direct conversion

During the last years several protocols that avoid the use of virally/non-virally introduced exogenous factors have been developed. A first study demonstrated that germ line cells could acquire pluripotency without the addition of exogenous transcription factors, using specific cell culture conditions (Sterneckert, J., *et al.* 2012). A following report showed that epiblast stem cells (EpiSCs) could be reverted to ESC using a chemical approach (Zhou, H., *et al.* 2010). More in detail Zhou et al. found that the TGFβ signaling pathway or histone demethylase LSD1 inhibition with small molecule was able to induce morphological changes in EpiSCs toward ESC phenotype, with simultaneous activation of inner cell mass-specific gene expression (Zhou, H., *et al.* 2010). Verma's lab also reported a novel technique for isolating ESCs from mammalian pre-implantation embryos by altering the epigenotype of embryonic explants with 5-aza-cytidine (5-aza-CR) (Lim, M. L., *et al.* 2011). The protocol was shown to work both in mouse and bovine and led to the generation of pluripotent cell lines. Further studies demonstrated that the inhibitor of histone deacetylases (HDACi) Trichostatin A (TSA) was very effective for the derivation of EG cells and accelerated the process of their dedifferentiation (Surani, M. A., *et al.* 2008) (see Table 1).

These findings have opened the way to new approaches in which small molecules and, more recently, epigenetic modifiers (Table 1) are used in order to directly convert cells from one type into another.

In 2007, Moschidou et al. demonstrated that cells isolated from amniotic fluid, which are not pluripotent (De Coppi, P., *et al.* 2007), can be reverted to a pluripotent state, without the use of ectopic reprogramming factors, but simply culturing them on matrigel in low growth factor medium (ESC medium) supplemented with the HDACi valproic acid (VPA) (De Coppi, P. 2013;Moschidou, D., *et al.* 2012). With this transgene-free technique, 82% of treated cells shared transcriptome identity with ESCs after long-term expansion, and were able to form embryoid

bodies (EBs) and teratomas, as well as to differentiate into lineages deriving from the three germ layers. Similar results were also obtained by treating adult human dermal fibroblasts with VPA (Rim, J. S., *et al.* 2012) (see Table 1). In this work the authors demonstrated the up-regulation of endogenous pluripotency transcription factor genes in the absence of any transgenes, while less encouraging results were obtained after incubation with zebularine, a de-methylating agent recently tested as a prototype of epigenetic therapy for cancer chemoprevention (Yoo, C. B., *et al.* 2008) (see Table 1).

It is however important to remember that the achievement of a stable and persistent pluripotent state is not physiological, does not occur during embryonic development, where pluripotency is limited to a short window of time (Wu, S. C. and Zhang, Y. 2010), and may lead to cell instability.

In this perspective alternative protocols have been recently developed in order to directly convert an adult mature cell into another differentiated cell type, avoiding a stable pluripotent state and the related limitations (Brevini, T. A., *et al.* 2014;Harris, D. M., *et al.* 2011;Pennarossa, G., *et al.* 2013;Pennarossa, G., *et al.* 2014) . Based on the concept that DNA methylation plays a fundamental role both during early embryonic development and cell lineage specification, we investigated and demonstrated that a brief exposure to a demethylating agent can push cells to a less committed state, increasing their plasticity. To this end we selected 5-aza-CR, a well-characterized DNA methyltransferase inhibitor, previously used to "boost" progenitor cell differentiation (Galvez, B. G., *et al.* 2008;Lefebvre, B., *et al.* 2010;Naeem, N., *et al.* 2013). This drug, mainly used in the treatment of myelodysplastic syndrome, is a chemical analogue of the cytosine and it is known to be a direct inhibitor of methyltransferase activity at low doses, as well as of methylation in newly synthesized DNA. These features give 5-aza-CR the ability to induce DNA hypomethylation, since the molecule substitutes for cytosine into DNA blocking DNA

methyltransferase function (Stresemann, C. and Lyko, F. 2008). This capability has been previously shown in mouse fibroblasts (Jones, P. A. 1985), where the drug has been demonstrated to remove the epigenetic "blocks" that are responsible for tissue specification. Furthermore, earlier studies also used this compound in order to modify gene expression and reactivate the transcription of silent genes (Jones, P. A. 1985), triggering phenotype changes in eukaryotic cells (Glover, T. W., *et al.* 1986;Taylor, S. M. and Jones, P. A. 1979). In line with these observations, Harris et al., successfully used 5-aza-CR to transform mesenchymal stromal cells and fibroblasts into hematopoietic cells (Harris, D. M., *et al.* 2011).

We recently demonstrated that adult skin fibroblasts and granulosa cells exposed to 5-aza-CR for 18 hours dramatically changed their phenotype, exhibiting reduced dimensions, increased nuclear volume (Figure 1) and displaying highly decondensed chromatin (Brevini, T. A., *et al.* 2014). These morphological features are distinctive of a high permissive state with cells containing more loosely packed chromatin than their differentiated counterparts, in order to maintain genes in a potentially open state and prepare them for future expression (Tamada, H., *et al.* 2006). These morphological modifications were accompanied by a specific and consistent gene regulatory response, that highlighted the acquisition of an increased plasticity. All these observations indicate that, in response to an epigenetic modifier, such as 5-aza-CR or valpoic acid, cells are pushed into a brief and transient "highly permissive state". Once entered into this higher plasticity window, they can easily be addressed toward a different phenotype if they are exposed to specific differentiation stimuli. Notably, if cells are returned to their standard culture medium, they revert to their original phenotype, suggesting the involvement of a reversible and non-toxic process.

With recent experiments we were able to convert fibroblasts exposed to 5-aza-CR toward the endodermic lineage commitment using a three step induction protocol that allows cells to transit

from early pancreatic differentiation stage to mature endocrine cells expressing the main hormones specific of the pancreatic tissue (Pennarossa, G., *et al.* 2013). This resulted in cell's rearrangement with the formation of large 3D spherical structures that tended to detach and float freely in the culture medium, reminiscent of in vitro cultured pancreatic islets (Figure 1). Most importantly epigenetically converted cells also expressed hormone (Figure 1) and glucose sensor genes distinctive of mature endocrine cells. Furthermore 35 ± 8.9% of cells were able to trigger active release of C-Peptide and Insulin after exposure to 20mM glucose, showing a dynamic response similar to pancreatic beta-cells, in which changes in ambient glucose represent the primary and physiological stimulus for insulin secretion. Converted cell functionality was also demonstrated in vivo after injection in immunodeficient SCID mice, whose β-cells had been selectively destroyed with streptozotocin. Cell transplantation was indeed able to restore normal glycemic levels in these diabetic animals and to stably maintain them (Pennarossa, G., et al. 2013).

A very recent report describes the possibility to transdifferentiate skin fibroblasts into insulin-expressing clusters only based on drug induction, avoiding both the necessity of using transgenic strategies and epigenetic modifications (Pereyra-Bonnet, F., *et al.* 2014). This approach is undoubtedly a promising one. However it should be considered with caution since no evidence about the involved mechanism is understood and further characterization of the transdifferentiated cells is still needed.

The possibility to apply epigenetic conversion to different cell types has been recently proved with the conversion of human granulosa cells (GCs) into muscle cells, through the use of 5-aza-CR followed by a 15 day culture with human recombinant VEGF (Brevini, T. A., *et al.* 2014). Over 80% cells changed their original phenotype, acquired elongated shape and became multinucleated. This was accompanied by molecular changes that demonstrated a switch from the

GC specific expression pattern (CYTOKERATIN17, HAS2, GREM1 and PTX3) to the one distinctive of muscle (DESMIN, MHC and MYOD).

The results obtained indicate that the conversion protocol may find wide application to different cell types suggesting a very promising potential for this approach as a safe option for the cure of several and diverse degenerative diseases (Figure 2).

Interestingly, the possibility to interact and modify cell phenotype through epigenetic remodeling has been shown in species other that the human, such as the porcine (Pennarossa, G., *et al.* 2014), the mouse, the dog and the cat (manuscript in preparation), with very encouraging and reproducible results.

In our understanding all these evidences further support the impact of epigenetic cell conversion and widen its application in human as well as veterinary regenerative medicine.

Conclusions

The increasing knowledge in epigenetic mechanisms has provided important insights for the understanding of cell commitment and, more in general, stem cell biology. In particular it is more and more evident that epigenetic events such as chromatin remodeling, histone modification and DNA methylation may impose flexible but precise control over the expression of important regulatory genes, allowing a dynamic interfacing between genotype and phenotype. The growing understanding of these aspects has greatly boosted the efficiency in cell reprogramming and transdifferentiation and has paved the way to the use of epigenetic modifiers for cell direct conversion.

These new developments in stem cell research promise to have important implications for better understanding of epigenetic cell fate control as well as for regenerative medicine advances, opening new avenues to cell therapy for human and animal diseases.

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FIGURE LEGENDS

Figure 1. Epigenetic converted cells are generated by exposing fibroblasts to 5-aza-CR and culturing them in pancreatic induction medium for 36 days. The brief exposure to the epigenetic modifier induces phenotype changes with cells exhibiting reduced dimensions and increased nuclear volume. After 36 days of pancreatic differentiation large 3D spherical structures, reminiscent of in vitro cultured pancreatic islets, become clearly visible. Cell immune-staining confirm the morphological changes with positivity for Vimetin (fibroblast-specific marker), Oct4 (pluripotency-related marker) and C-Peptide (mature pancreatic marker) at different time points of the conversion protocol.

436

437 **Figure 2. Epigenetic modifiers can be used to generate different cell types. An adult mature**
438 **cell can be pushed into a "highly permissive" state and then re-addressed towards different**
439 **cell lineages and phenotypes.**