Methylation mechanisms and biomechanical effectors controlling cell fate

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Abstract

Mammalian development and cell fate specification are controlled by multiple regulatory mechanisms that interact in a coordinated way to ensure proper regulation of gene expression and spatial restriction, allowing cells to adopt distinct differentiation traits and a terminal phenotype. For example, cell potency is modulated by changes in methylation that are under the control of methyltransferases and ten–eleven translocation (TET) enzymes, which establish or erase a phenotype-specific methylation pattern during embryo development and mesenchymal to epithelial transition (MET). Cell plasticity is also responsive to extracellular factors, such as small molecules that interact with cell fate definition and induce a transient pluripotent state that allows the direct conversion of an adult mature cell into another differentiated cell type. In addition, cell-secreted vesicles emerge as powerful effectors, capable of modifying cell function and phenotype and delivering different signals, such as octamer-binding transcription factor-4 (*Oct4*) and SRY (sex determining region Y)-box 2 (*Sox2*) mRNAs (implicated in the preservation of pluripotency), thus triggering epigenetic changes in the recipient cells. In parallel, mechanical properties of the cellular microenvironment and three-dimensional rearrangement can affect both cell potency and differentiation through marked effects on cytoskeletal remodelling and with the involvement of specific mechanosensing-related pathways.

Additional keywords: epigenetic modifier, extracellular vesicles, mechanosensing, methyltransferase, ten–eleven translocation (TET) enzyme.

Introduction

Cell fate specification and tissue differentiation are controlled by tight regulatory mechanisms that interact to properly direct gene expression and spatial restriction, thus driving the correct progression of the developmental process and allowing cells to adopt distinct differentiation traits, related to a terminal phe- notype. Epigenetic restriction plays a key role in the changes driving cell fate definition; in particular, changes in methylation finely tune cell potency and add or erase a phenotype-specific methylation pattern, during both embryo development and cell reprogramming. At the same time, extracellular factors, such as small molecules, and cell-secreted vesicles act as powerful effectors, both interacting with the epigenetic regulatory loops and exerting direct effects, and capable of affecting cell plas- ticity, modifying cell function and having a considerable effect on phenotype. Further aspects that need to be considered are the three-dimensional (3D) rearrangement and mechanical properties of the cellular microenvironment. These can interact with the signals described above and regulate cell potency and differentiation through marked effects on cytoskeletal remo- delling and with the involvement of specific mechanosensing- related pathways.

Active and passive demethylation in mammalian cells

Epigenetic regulatory mechanisms are essential for mammalian development, gene regulation, genomic imprinting and chromatin structure (Bird 2002). In particular, changes in methylation allow mature cells of adult organisms to acquire their differentiated state through a gradual loss of potency (Hemberger *et al.* 2009) and a progressive restriction in their options (Zhou and Melton 2008). Cells from mammalian organisms modify the methylation of cytosine predominantly in cytosine–phosphorous–guanine (CpG0 dinucleotide islands. This process is controlled by two classes of *de novo* DNA methyltransferases (DNMTs). Specifically, DNMT3a and DNMT3b arrange DNA

methylation patterns dur- ing the early stage of development, whereas other methyl- transferases, such as DNMT1, primarily maintain the established patterns by copying them onto daughter DNA strands during cell replication and division. DNMT-controlled changes in methyla- tion take place during cell replication and are considered a passive process, whereby modification or erasure are possible primarily when a cell divides (Wu and Zhang 2010). Conversely, ten–eleven translocation (TET) enzymes can affect cytosine methylation through an active mechanism that converts and oxidises 5-methylcytosine (5-mC) to 5-formylcy- tosine (5-fC) and 5-carboxylcytosine (5-caC), with an overall decrease in global methylation (Manzoni *et al.* 2016). The process is replication independent and demonstrates the possi- bility of a direct demethylating mechanism accompanying the well-documented indirect DNMT-related action in regulating the loss of methylcytosine from the genome.

Changes in methylation control cell fate and cell potency

From the very beginning of development, DNA demethylation plays a key role in shaping the identity of the developing mam- malian embryo. Demethylation waves allow the acquisition of the distinctive totipotent state of the zygote, confinement of pluripotency to cells belonging to the inner cell mass (ICM) and orchestrate the specification of primordial germ cells (PGCs) relocating to and colonising the genital ridges (Fig. 1). Loss of CpG methylation in the paternal genome is brisk and is achieved by TET3-mediated oxidation of 5-hydroxymethylcytosine (5-hmC) to 5-fC and 5-caC (Gu *et al.* 2011; Iqbal *et al.* 2011; Wossidlo *et al.* 2011). Conversely, active and quick demethyla- tion of the paternal DNA is accompanied by a more gradual methylation loss in the maternal pronucleus (Santos *et al.* 2002) that remains more protected against TET3 activity (Nakamura *et al.* 2012). Both genomes, in parallel, are targets for passive DNMT-related demethylation that is replication dependent. Therefore, epigenetic erasure coordinating the progression of early development is both replication dependent and independent

and involves both active and passive demethylation processes. Interestingly, the acquired epigenetic traits distinctive of the gametes are erased shortly after fertilisation and syngamy. This process allows the embryo to activate transcriptional activity and, together with polyadenylation regulatory mechanisms, to mod- ulate the expression of specific genes (Brevini *et al.* 2004). The overall result leads to re-establishment of pluripotency and to the development of the haploid gametes that are required to establish the next generation.The very small number of cells available in the mammalian ICM or epiblast and their constant transition state makes the study of cell commitment and reprogramming *in vivo* very demanding (Smith *et al.* 2012; Hackett *et al.* 2013). Therefore, many studies have been performed using immortal surrogates such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs; Nichols and Smith 2009). Several studies have reported that ESCs show characteristics of decreased global 5-mC in these models (Leitch *et al.* 2013) and have demonstrated that DNA hypomethylation is crucial for main- taining the naïve state of ESCs and antagonising the self- activating differentiation signal, resetting the epigenome and re-establishing the pluripotency network (Grabole *et al.* 2013). Furthermore, downregulation of DNMTs is correlated with boosting symmetry in cell division (Jasnos *et al.* 2013), further supporting the possibility that demethylation may play a major role in promoting self-renewal to maintain cells in their most naïve state. In agreement with these observations, cell fate restriction and subsequent differentiation is accompanied by a progressive build-up of DNA methylation. Indeed, it has been demonstrated that lineage specification is supported by dynamic epigenetic changes and genome-wide redistribution of DNA methylation that silence pluripotency genes and establish a phenotype-specific methylation pattern (Berdasco and Esteller 2011; Oda *et al.* 2013). In particular, during cell fate commit- ment, pluripotency genes such as octamer-binding transcription factor 4 (*Oct4*) and *Nanog* undergo silencing and *de novo* DNA methylation in their promoter and enhancer regions. This hypermethylated state is then maintained in differentiated somatic cells (Li *et al.* 2007; Epsztejn-Litman *et al.* 2008).

DNA methylation has also been shown to promote the adequate and proper regulation of gene expression, ensuring both tempo- ral activation and spatial restriction, allowing cells to acquire distinct differentiation traits, stabilising the cell terminal pheno- type (Oda *et al.* 2013) and maintaining the established patterns by copying them onto daughter DNA strands during cell replication and division. Interestingly, studies performed recently using media supplemented with the two small molecule kinase inhibitor 2i (inhibitors PD0325901 and CHIR99021) (Habibi *et al.* 2013; Leitch *et al.* 2013) report the derivation of ESC lines that exhibit a higher level of hypomethylation than those derived using conventional media. The authors suggest that the addition of 2i increases TET activity, boosting TET-mediated conversion of 5-mC to 5-hmC, which accompanies the simultaneous DNMT- related passive effect, easing cells into a 'naïve state' in which the genome becomes hypomethylated (and reminiscent of early blastomeres seen *in vivo*; Hu *et al.* 2014).

Mesenchymal to epithelial transition requires TET-mediated DNA demethylation

Cell phenotype can be reversed by transferring a somatic nucleus into a previously enucleated oocyte. Similarly, somatic cells can be exposed *in vitro* to specific reprogramming factors and can be converted to iPSCs. However, it is fundamental to keep in mind that the identity of a differentiated cell is guaranteed by a unique methylation profile that maintains its lineage definition and prevents free transition among different cell types. Therefore, methylation blocks and restriction must be removed in order to allow a switch in phenotype. For example, demethylation of pluripotency genes is a hallmark of somatic cell reprogramming into a pluripotent state (Takahashi and Yamanaka 2006; Gurdon and Melton 2008). Recently, studies have shown that experimental reprogramming requires active demethylation by the TET family of enzymes, which catalyse oxidation of 5-mC (Tahiliani *et al.* 2009; He *et al.* 2011; Ito *et al.* 2011), leading to activation of epigenetically silenced plur- ipotency genes. In agreement with

these observations, it has been reported that oocyte TET enzymes exhibited reprogram- ming activity for pluripotency gene reactivation during early embryonic development, after nuclear transfer and natural fer- tilisation (Gu *et al.* 2011). Together, these findings point to the possibility that TET enzymes may play a key role in repro- gramming and mesenchymal to epithelial transition. This hypothesis finds further support in experiments performed in mouse fibroblasts, in which TET genes were inactivated, resulting in cell failure to undergo mesenchymal to epithelial transition and a complete block of the reprogramming potential of mouse embryonic fibroblasts (MEFs; Hu *et al.* 2014). These observations indicate that TET enzymes are indispensable for factor-driven reprogramming of somatic cells to iPSCs. Inter- estingly, the same authors showed that TET-deficient MEFs failed to reactivate microRNAs, such as miR-200 s, miR-200a and miR-200b, which play a critical role in mesenchymal to epithelial transition (MET) and are upregulated in cells under- going reprogramming. Indeed, Hu *et al.* (2014) showed that the expression of the miR-200 family diminished in TET-deficient MEFs, and this was accompanied by the reprogramming block. However, ectopic expression of miR-200s was able to restore the MET process and rescue o up to 80% of the reprogramming efficiency of wild-type fibroblasts (Hu *et al.* 2014).

Ability of small molecules to define cell fate

The achievement of a specific cell fate has been considered stable and potentially irreversible for a long time. However, following on from the pioneering work of Taylor and Jones (1979), many groups have reported that it is possible to directly interact with cell fate definition and modify terminal commit- ment (Brevini *et al.* 2015).

Several protocols that avoid using virally or non-virally introduced exogenous factors, as well as the establishment of astable pluripotent state, have been developed. These new approaches involve the use of small molecules and epigenetic modifiers in order to directly convert an adult

mature cell into an alternative differentiated cell type (Pennarossa *et al.* 2013; Brevini *et al.* 2014; Chandrakanthan *et al.* 2016; Manzoni *et al.* 2016). 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, was published in 2004 (Chen *et al.* 2004). In subsequent years, this molecule was confirmed to induce increased plasticity in treated 3T3E1 osteoblasts (Chen *et al.* 2007), human primary skeletal myoblasts (Chen *et al.* 2007) and murine and human dermal fibroblasts (Anastasia *et al.* 2006). In response, several protocols that involve the use of epigenetic modifiers have been developed. The resulting chemical com- pounds can push cells to a transient 'less committed state', increasing cell plasticity for a short time, sufficient to redirect them towards a different cell type (Harris *et al.* 2011; Pennarossa *et al.* 2013, 2014; Brevini *et al.* 2014; Mirakhori *et al.* 2015; Chandrakanthan *et al.* 2016). The general concept forming the basis of these experiments is that cell differentiation is regulated by the expression of different sets of genes, responsible for a distinct phenotype, under the control of complex regulatory mechanisms. Of these regulatory mechanisms, DNA methyla- tion plays a fundamental role during both early embryonic development and cell lineage specification. For this reason, 5-azacytidine (5-aza-CR), a well-characterised DNMT inhibi- tor, was selected and used to remove the epigenetic 'blocks' that are responsible for tissue specification (Pennarossa *et al.* 2013; Brevini *et al.* 2014; Chandrakanthan *et al.* 2016). This chemical compound is an analogue of cytosine and can be incorporated into DNA and RNA during replication (Stresemann and Lyko 2008; Aimiuwu *et al.* 2012), forming covalent adducts with DNMT1. Because of its powerful effects, 5-aza-CR is able to induce global DNA hypomethylation (Christman 2002) and gene reactivation (Jones 1985), and can facilitate adult somatic cell switching from one phenotype to another (Taylor and Jones 1979; Glover *et al.* 1986; Harris *et al.* 2011). Indeed, human mesenchymal stromal cells and skin fibroblasts were trans- formed into haematopoietic cells after coincubation with 5-aza-CR, granulocyte–macrophage colony-stimulating factor (GM-

CSF) and stem cell factor (SCF; Harris *et al.* 2011). Moreover, we have demonstrated that adult skin fibroblasts and granulosa cells can be converted into different cell types, in the human as well as in the pig, dog and mouse (Pennarossa *et al.* 2013, 2014; Brevini *et al.* 2014, 2016). The fate switch was proved to be possible in cells belonging to the same embryonic layer or changing between different embryonic layers (Fig. 2). This process is possible due to the fact that after 18 h exposure to 5-aza-CR, cells acquired a 'highly permissive state' with signif- icant changes in their phenotype and a specific gene regulatory response that was paralleled by a decrease in global DNA methylation. Moreover, following exposure to the demethylating agent, cells exhibited reduced dimensions with large nuclei and global chromatin decondensation, as well as expression of pluripotency-related genes such as *OCT4*, *NANOG*, ZFP42 zinc finger protein (*REX1*) and SRY (sex determining region Y)-box 2 (*SOX2*). Our observations are in agreement with the common morphological features distinctive of ESCs, iPSCs and pluripo- tent cells in general (Tamada *et al.* 2006). More recently, it has been demonstrated that 5-aza-CR interferes with DNA methylation through a direct TET2-mediated mechanism that accompanies the well-known indirect DNMT-related effect, indicating the possibility that 5-aza-CR actions on cell plasticity and differentiation may occur through alternative mechanisms that require the involvement of novel cellular targets (Manzoni *et al.* 2016). It is important to note that this process is completely reversible and does not exhibit toxic effects, because cells returned to their standard culture medium reverted to their original phenotype within a few days (Pennarossa *et al.* 2013; Manzoni *et al.* 2016). In agreement with our findings (Pennarossa *et al.* 2013, 2014; Brevini *et al.* 2014, 2016), 5-aza-CR has been used to convert human skin fibroblasts into neural progenitor- like cells (Mirakhori *et al.* 2015) and mature bone and fat cells into tissue-regenerative induced multipotent stem cells (Chandrakanthan *et al.* 2016). In addition, Cheng *et al.* (2015) reported the conversion of human and murine fibroblasts intoproliferating chemical-induced neural progenitor cells using a cocktail containing inhibitors of histone deacetylation, glycogen synthase

kinase and transforming growth factor b signalling under physiologically hypoxic conditions (5% O_2). Furthermore, recent experiments described the possibility of epigeneti- cally converting human skin fibroblasts into terminally differentiated induced Schwann cells by using the histone deacetylase (HDAC) inhibitor Valproic acid (VPA) (Thoma *et al.* 2014). These studies are particularly intriguing because they allow a deeper understanding of epigenetic restriction, while at the same time better clarify the mechanisms leading to the acquisition of a mature somatic phenotype. Needless to say, these studies may contribute key information that may find advantageous applications in stem cell therapy and regenerative medicine in human and animal species.

Microvesicle-mediated genetic transfer modifies cell function and phenotype

Cell fate is determined by complex interactions between several factors within a defined microenvironment. In particular, plu- ripotent cells are highly sensitive to extracellular signals that play a critical role in the maintenance of stemness, differentia- tion and interplay with somatic cells. Several environmental factors, including growth factors, oxygen concentration and mechanical, metabolic and biochemical conditions, have been shown to be relevant in cell differentiation and have been reviewed extensively (Discher *et al.* 2009). Similarly, repro- gramming of somatic cells involves a complex interaction between intracellular and extracellular signals, leading to epi- genetic remodelling (Buganim *et al.* 2013). Therefore, changes in cell phenotype are determined by multiple signals in a defined microenvironment. The observation that cell-secreted vesicles are an integral component of the intercellular exchange of information is based on their ability to transfer different types of signals between cells (Ratajczak *et al.* 2006*b*; Cocucci *et al.* 2009) and to act as transcription modulators, affecting cell phenotypes (Bhat and Bissell 2014). The concept that extra- cellular vesicles (EVs) derived from cells are capable of trans- ferring information not only in a paracrine manner (i.e. between cells of the same origin),

but also in an endocrine manner (i.e. to distant target cells) has revolutionised our understanding of cell–cell communication. Several studies indicate that EVs, because of their complex composition, may modify cell function and phenotype, delivering different signals to recipient cells. The pivotal study of Ratajczak *et al.* (2006*a*) showed that EVs derived from stem cells exert profound effects on the microen- vironment by transferring stem cell-specific proteins and mRNAs. In their study, Ratajczak *et al.* (2006*a*) demonstrated that microvesicles derived from ESCs contained Wnt-3 and mRNAs implicated as pluripotent transcription factors. These molecular components were transferred and translated into proteins by neighbouring cells, thus reprogramming hae- matopoietic progenitors. This experiment was the first to describe epigenetic changes in recipient cells following transfer of mRNA. In addition, other experiments have demonstrated that EVs released by ESCs may also transfer embryonic stem cell mRNAs, such as *Oct4* and *Sox2*, implicated in the preser- vation of pluripotency, to retinal progenitor Müller cells, along with mRNAs related to embryonic and early retinal genes (Katsman *et al.* 2012).

Others have further demonstrated the functional role of EVs, focusing on the ability of EVs to modify the phenotype of bone marrow cells by transferring nucleic acids and proteins (Badiavas *et al.* 2003; Abedi *et al.* 2004; Dooner *et al.* 2004). In addition, Castellana *et al.* (2009) showed that EVs are able to tRNA from injured lung cells to bone marrow cells, inducing lung-specific gene expression.

These experiments shed new light on stem cell plasticity, indicating that vesicles derived from various organs may induce phenotypic changes in cells. Therefore, the exchange of genetic information mediated by EVs has been suggested as a funda- mental component of stem cell biology, where the environmental stimuli are critical for the differentiation decision of stem cells (Quesenberry *et al.* 2010). In the context of tissue injury, EV-mediated exchange of information could be bidirectional between stem and injured cells. For example, Bruno *et al.* (2009) demonstrated that injured tissue cells may induce gene expres- sion and differentiation decisions

in stem cells. Conversely, stem cell-derived vesicles may reprogram injured cells by activating regenerative mechanisms. In particular, the transfer of transcriptional factors, such as non-coding RNAs, may induce epigenetic modifications in recipient cells, which could beexploited in regenerative medicine. Based on these observa- tions, it is important to fully understand the mechanisms involved in the biogenesis and composition of EVs, and how they depend on environmental stimuli, in order to design possible new therapeutic interventions.

Mechanical signals control cell fate

The study of signal transduction pathways has been a focus of intense research in recent years. Every cell responds to the mechanical properties of its environment, such as the elasticity and stiffness of the extracellular matrix (ECM) and traction or compression forces exerted by neighbouring cells that are con- stantly transmitted across cell–ECM and cell–cell adhesion sites (Discher *et al.* 2009; Jaalouk and Lammerding 2009; Mammoto and Ingber 2009; Wozniak and Chen 2009). Cells balance these external forces by adjusting the stiffness of their cytoskeleton (Vogel and Sheetz 2006; Parsons *et al.* 2010). Reciprocally, forces generated inside the cell by contraction of the cytoskel- eton are transmitted across adhesion sites to surrounding structures. Thus, the cytoskeleton rapidly senses and adapts to changes in the mechanical properties of the microenvironment. The way mechanical cues control cell fate *in vitro* and *in vivo* and the molecular components that perceive and transduce such signals remain poorly understood. However, increasing evi- dence is accumulating, and demonstrates that cell shape, ECM elasticity and cytoskeletal tension play important roles in cell behaviour, physiology and many diseases. This has led to the development of a new discipline termed ‘mechanobiology’. The first observation that cellular shape is an important regulator of cell behaviour dates back to 1978, when Folkman and Moscona (1978) showed that gradual changes in substrate adhesiveness regulate cell proliferation and differentiation. This was followed by evidence that the degree of

cell shape distortion is itself a fundamental and dose-dependent signal for proliferation control (Singhvi *et al.* 1994; Chen *et al.* 1997). In these latter two studies, microprinted ECM islands of different sizes were engineered to control the extent of cell spreading of a single endothelial cell. Strikingly, it was observed that well-spread cells proliferate, whereas cells confined to small adhesive areas do not proliferate and instead undergo apoptosis. Similarly, cell shape has been reported to strongly affect cell fate. In particular, Watt *et al.* (1988) demonstrated that cell shape affects the bal- ance between keratinocyte self-renewal and differentiation and the differentiation of human mesenchymal stem cells (MSCs). Indeed, human MSCs differentiate into osteoblasts when allowed to spread, whereas they differentiate into adipocytes when they are confined to a round shape (McBeath *et al.* 2004), showing that cell shape is a key determinant of cellular behav- iour. In addition, it has also been shown that ECM elasticity plays a crucial role in cell fate control. Studies of MSC differ- entiation provided a powerful example of how cells respond to the stiffness of their surroundings. Furthermore, several studies reported that, *in vitro*, MSCs change their phenotype in response to ECM substrates with different elasticity. For example, MSCs differentiate into osteoblasts when seeded on a synthetic matrix engineered to have a bone-like stiffness, whereas they acquire a specific myoblast phenotype when grown on ECMs with intermediate stiffness or differentiate into neurons and adipo- cytes when cultured on a soft ECM (Engler *et al.* 2006). Simi- larly, skeletal muscle stem cells require an ECM substrate that mimics the stiffness of the adult muscle in order to preserve high regenerative capacity when cells are engrafted back into mice (Engler *et al.* 2004; Gilbert *et al.* 2010). Moreover, ECM elas- ticity exerts effects to control cell proliferation (Klein *et al.* 2009), whereas the various elastic properties of the different tissues seem to affect tissue regeneration.

The use of 3D culture systems and scaffolding that are able to rearrange cells in a specific cytoarchitectural pattern has also been suggested as a way to preserve the physiological features distinctive of the original tissue. In particular, a relationship between fate commitment and 3D

rearrangement was recently reported by Harrison *et al.* (2017), who demonstrated the possibility of combining mouse ESCs and extra-embryonic trophoblast stem cells (TSCs) using a 3D scaffold to generate aggregates whose morphogenesis was remarkably similar to natural embryos and involved Nodal signalling. Similarly, several studies have reported the formation of structures referred to as organoids (Simian and Bissell 2017), obtained through 3D *in vitro* culture of cells. Presently, organoids have been generated from different types of cells using culture media that mimic the conditions of embryogenetic processes and differentiation (Clevers 2016), mostly to model organ development and patholo- gies 'in a dish'. Encapsulation of cells in microbioreactor material with hydrophobic properties may further boost the formation of functional organoids that are not only protected by the supporting surface, but are also provided with optimal gas exchange between the interior liquid and the surrounding environment (Fig. 3; Arbatan *et al.* 2012; Sarvi *et al.* 2013, 2015; Tian *et al.* 2013;

Serrano *et al.* 2015; Brevini *et al.* 2017). Together, accumulating evidence indicates that, similar to soluble growth factors, cell morphology and the mechanical properties of the cellular micro-environment can affect both cell growth and cell differentiation.

Conclusions

The development of a mammalian organism and its cell fate specification are under the tight control of multiple regulatory mechanisms that interact, in a coordinated way, to orchestrate proper regulation of gene expression and spatial restriction. This ensures correct progression of the events and allows cells to adopt distinct differentiation traits, related to a terminal phe- notype. Changes in methylation finely tune cell potency and establish or erase a phenotype-specific methylation pattern, during both embryo development and MET. At the same time, cell plasticity is responsive to extracellular factors, such as small molecules, and cell-secreted vesicles that act as powerful effectors, able to modify cell function and phenotype. The 3D rearrangement and

mechanical properties of the cellular microenvironment interact with these signals and regulate cell potency and differentiation through marked effects on cyto- skeletal remodelling and with the involvement of specific mechanosensing-related pathways.

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