TITLE: All roads lead to Rome: the many ways to pluripotency

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ABSTRACT

Cell pluripotency, spatial restriction and development are spatially and temporally controlled by epigenetic regulatory mechanisms that occur without any permanent loss or alteration of genetic material, but rather through modifications "on top of it". These changes modulate the accessibility to transcription factors, either allowing or repressing their activity, thus shaping cell phenotype. Several studies have demonstrated the possibility to interact with these processes, reactivating silenced genes and inducing a high plasticity state, via an active demethylating effect, driven by ten-eleven translocation (TET) enzymes and an overall decrease of global methylation. In agreement with this, TET activities have been shown to be indispensable for mesenchymal to epithelial transition of somatic cells into iPSCs and for small molecule-driven epigenetic erasure. Beside the epigenetic mechanisms, growing evidences highlight the importance of mechanical forces in supporting cell pluripotency, which is strongly influenced by 3D rearrangement and mechanical properties of the surrounding microenvironment, through the activation of specific mechanosensing-related pathways.

In this review we discuss and provide an overview of small molecule ability to modulate cell plasticity and define cell fate, through the activation of direct demethylating effects. In addition, we describe the contribution of the Hippo signalling mechano-transduction pathway as one of the mechanisms involved in the maintenance of pluripotency during embryo development and its induction in somatic cells.

Keywords: cell plasticity, epigenetics, mechano-sensing, reprogramming, TET activities

Pluripotency, cell commitment and epigenetic restriction

Adult somatic cells are highly specialized and have a specific molecular pattern that regulates their functions and physiology. Although genetically identical, since derived from one-single cell with half-genome from each parent, they can display any of the over the 230 different cell types that are present in a complete multicellular organism. During the early phases of mammalian embryonic development, three germ layers, namely endoderm, mesoderm and ectoderm, are formed. Subsequently, each one of that responds to specific developmental cues giving rise to different set of tissue types and contributing to organ formation. These processes are driven by several factors both extrinsic and intrinsic to the cell [1], that induce tissue-specific gene expression, timely regulated by epigenetic restrictions. Cell commitment and differentiation are indeed fortified by the cell's own machinery that chemically modify the DNA, without any permanent loss or alteration of genetic material [2,3], also referred to as "epigenetic modifications". These latter gradually limit cell potency [4] to a more specific phenotype-related transcription pattern, resulting in a progressive restriction in cell options [5].

More than 60 years ago, Conrad Waddington used for the first time 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 [6]. In his metaphor, Waddington represents the cell of an embryo as a ball rolling from a non-committed, pluripotent condition down the hill, to a specific cell fate. The hill possesses slopes and valleys that represent the many different and complex processes characterizing the events leading to cell differentiation. The ball is addressed towards a favored position, along a progressively more restricted potency pathway, down to the bottom of the hill, where the cell is unipotent and characterized by a tissue specific differentiated state.

To date, it is widely known that embryo development, pluripotent cell differentiation and the acquisition of tissue specific marks are driven by epigenetic modifications that regulate the accessibility to transcription factors, in either a positive or negative manner. The two major mechanisms involved are DNA methylation and histone modifications [7]. The first is a biochemical process characterized by a covalent addition of a methyl (CH3) group at the 5-carbon of the cytosine. The added CH3 group physically impedes the binding of transcription factors to the gene [8] or recruits the methyl-CpG-binding domain proteins (MBDs), forming compact, inactive chromatin, known as heterochromatin. Histone modifications, that involve acetylation, phosphorylation, methylation, SUMOylation, citrullination, ADP ribosylation, or ubiquitination [9], encourage or prevent transcription factor and other protein access to DNA. All these processes are responsible for the so called 'epigenetic memory' and underly the stable maintenance of cell differentiated phenotype, that has been considered irreversible until not long ago [7,10–13].

In this review we focus on the recent knowledge of epigenetic mechanisms that modulate cell plasticity and define cell fate. We also discuss and provide an overview of the well- established methods to interact with the epigenetic signature of a cell, inducing and maintaining pluripotency. Interestingly, a surprising overlap among the molecular mechanisms that control cell reprogramming and the regulatory pathways acting in the early embryonic development phases, was found. The understanding of these processes is therefore not only an intriguing topic of study, but also sheds light on the genetic and environmental factors that affect the oocyte epigenetic landscape and determine the phenotypes of individuals and their offspring [14,15].

Erasing cell "epigenetic memory"

During the last years, many studies demonstrated that, although generally stable in vivo, a terminally differentiated cell can be reversed and forced in an upstream, counter-current direction up the Waddington's hill, along different states of increased potency [16].

The first attempt to modify the somatic cell identity dates back to the 1960s, when a somatic nucleus was "reprogrammed" by exposure to the oocyte's environment [17,18]. This early result paved the way for studying the mechanisms involved in the erasure of "epigenetic memory" and the re-establishment of pluri- or totipotency. It was therefore demonstrated that an adult somatic cell can be brought back a to a high plasticity state, using cell fusion techniques [19] or overexpressing master regulator transcription factors (TF), such as in the creation of induced pluripotent cells (iPSCs) [20]. However, one of the fundamental aspects for iPSC generation is the need for high levels of gene expression to reach an increased potency state for the requirement of elevated "activation energy" that allows initiation of cell reprogramming in an upstream, counter-current direction [4]. This demands the use of TF, such as OCT4, kruppel-like factor 4 (KLF4), SOX2 and MYC, that are carried into the cell by retroviral vectors, which, in turn integrate in the host genome, resulting in the reactivation of endogenous genes [21–24]. Currently, various methodologies avoiding the viral stable integration have been established for iPSC derivation, from virus-free [22,25], to removable PiggyBac transposons [26], minicircle systems [27], episomal systems [28], synthetic mRNAs [29–31], and microRNAs [32,33]. Although these advances have reduced safety concerns, iPSC induced pluripotent state remains stable and unphysiological, making them prone to acquire genomic alterations that increase the risk of mutagenesis [34].

Adult mature cells can also be pushed into a 'less committed state' through the use of small molecules and epigenetic modifiers. Following the pioneering work of Taylor and Jones [35], many groups have indeed reported the possibility to directly convert an adult cell type into another [36–45]. These methods are based on the use of chemical compounds able to interact and modify the cell epigenetic signature, avoiding the use of transgenes stably integrated into the genome, but

increasing plasticity for a short transient time-window, which is however sufficient to redirect cells towards a different lineage. The first paper reporting the possibility to use the small molecule “reversine” to induce myoblasts into multipotent mesenchymal progenitor cells, was published in 2004 [46]. Since then, several approaches that involve the use of epigenetic modifiers have been described. The general concept at the base of these new protocols is that, among the several mechanisms that drive cell differentiation, DNA methylation plays a fundamental role during both early embryonic development and cell lineage specification. For this reason, the well-characterized DNA methyltransferase (DNMT) inhibitor 5-azacytidine (5-aza-CR) has often been used to remove the epigenetic ‘blocks’ that are responsible for tissue differentiation [47]. Thanks to its powerful effects, 5-aza-CR is able to induce global DNA hypo-methylation [48,49] and silenced gene reactivation [50], promoting a high plasticity state that facilitates somatic cell switch from one phenotype to another [35,37,51]. In agreement with these findings, we demonstrated that an adult somatic cell can be reverted into a fully permissive state after an 18 h exposure to 5-aza-CR [40,41,43,45,52]. Erased cells underwent considerable changes in their phenotype and gene expression patterns, which were accompanied by a significant decrease in global DNA methylation. More in detail, following exposure to the demethylating agent, cells exhibited the most common features of ESCs, iPSCs and, more in general, of pluripotent cells [53], namely reduced dimensions, large nuclei, global chromatin decondensation, and the expression of pluripotency-related genes, such as OCT4, NANOG, REX1 and SOX2. It is interesting to highlight that this condition was transient and reversible, and, when cells were returned to their standard culture medium, they reverted to their original phenotype, gradually turning down the expression of pluripotency related genes within a few days [40,41,45,54]. On the other hand, this short high plasticity window was sufficient to allow cell transition towards a different phenotype (such as functional pancreatic beta [40–42,44,45,55], muscle [43], neural progenitor-like [38,39,56] or mature Schwann cells [57]), in response to specific differentiation stimuli, demonstrating the acquisition of fully functional high plasticity state.

TET demethylation waves and cell plasticity

At the beginning of development, DNA demethylation plays a key role in shaping the identity of mammalian embryos. In particular, demethylation waves allow the acquisition of the distinctive totipotent state in the zygote as well as the confinement of pluripotency to the inner cell mass (ICM). The specific epigenetic profiles of paternal and maternal gametes are erased shortly after fertilisation and syngamy through active ten–eleven translocation (TET)-mediated and passive DNMT-related demethylation processes. These allow embryos to activate transcriptional functionality and, together with polyadenylation regulatory mechanisms, to modulate the expression of specific genes [58], re-establishing pluripotency (Fig. 1). In agreement with this, it has been demonstrated that decreased global 5-mC methylation in ESCs is crucial for maintaining their naive state

and for antagonising differentiation signals [59]. Conversely, cell fate definition and differentiation are accompanied by a progressive increase in DNA methylation that silences pluripotency genes and establishes a phenotype-specific epigenetic pattern [60,61]. This hypermethylated state is then stably maintained in terminally differentiated somatic cells by copying the specific pattern onto daughter DNA strands during cell replication and division [62–64]. However, as described above, it has been recently demonstrated that cell phenotype can be reverted, either through the use of specific reprogramming factors or by exposure to chemical compounds, generating iPSCs or "epigenetically erased" cells, respectively. Since cell differentiated state is strictly controlled and stably maintained by specific methylation profiles, it is necessary to remove the epigenetic "blocks" and restrictions to allow the transition to a permissive state. Recent studies have shown that reprogramming events require active demethylation processes driven by the TET family of enzymes (Fig.1), which catalyse the oxidation of cytosine-5 methylation into 5hydroxymethyl-cytosine [65–67], leading to the transcription of previously silenced pluripotency genes. Similarly, oocyte TET enzymes showed reprogramming ability by reactivating pluripotency genes during early embryonic development, after both nuclear transfer and natural fertilisation [68]. All these findings point to the possibility that TET enzymes play a key role in somatic cell reprogramming to iPSCs, specifically in mesenchymal to epithelial transition (MET). This hypothesis was confirmed in experiments carried out using TET-deficient murine fibroblasts that failed to undergo MET, blocking their reprogramming potential [69]. In addition, recent studies demonstrated that the epigenetic eraser 5-aza-CR interferes with DNA methylation through a direct TET2-mediated mechanism that accompanies the well-known indirect DNMT-related effect (Fig.1). This indicates the possibility that 5-aza-CR action on cell plasticity may occur through alternative mechanisms that require the involvement of novel cellular targets [54]. Even more intriguing, these data suggest that TET proteins play a pivotal role in epigenetic tuning cell potency and that from the acquisition of the totipotent state of the zygote to somatic cell reprogramming and chemical erasing, they are the common actors controlling methylation levels that facilitate the acquisition of a high plasticity state.

Bio-mechanical effectors and cell plasticity

During the last years, increasing evidences have highlighted the importance of signal transduction pathways correlated to mechano-sensing, which play a fundamental role in cell behavior, both in physiological conditions as well as in many pathological situations. All cells respond to stimuli exerted by the surrounding microenvironment, such as traction or compression forces from neighboring cells or elasticity and stiffness of the extracellular matrix (ECM). These signals are constantly communicated across cell–ECM and cell–cell adhesion sites, influencing cell own stiffness and cytoskeleton

organization [70–73]. However, to date, in vitro and in vivo mechanical cues that control cell fate, and the molecular pathways that perceive and transduce this information, still remain poorly understood.

Currently, the term "mechano-transduction" is used to describe cell ability to recognize the surrounding microenvironment and appropriately respond to external physical stimuli. The main actors that regulate these processes belong to the Hippo signaling pathway and are the Yes-associated protein (YAP) and the WW domain-containing transcription regulator protein 1 (WWTR1 or TAZ) [22]. Recent studies have demonstrated that these two molecules respond to cytoskeletal-mediated mechano-sensing cues that control survival, proliferation and differentiation in somatic cells (Fig. 2), while no specific role has been fully identified neither in oogenesis nor in spermatogenesis [34]. However, they are present in both human and mouse gametes as well as in early stages of embryonic development [27,28, 35,36]. In particular, it was shown that YAP/TAZ are maternally accumulated and highly expressed in early zygotic stage, when maternal RNAs and proteins are exhausted [34], suggesting a developmental stage-specific function for these molecules. In agreement with this, it was demonstrated that distinct changes in TAZ/YAP localization are essential for determining early differentiation, where their nuclear/cytoplasmic compartmentalization defines the first cell fate choice. Indeed, at the blastocyst stage, YAP/TAZ distribution is strictly compartmentalized to the nucleus in the inner cell mass (ICM), while it appears more diffused in the outer trophoblast cells. This localization allows the two molecules to elicit their transcriptional co-activator functions. More in detail, once sorted to the nucleus, YAP and TAZ directly interact with SMAD2/3 [39]. This newly formed YAP/TAZ-SMAD2/3 complex binds to TEAD1/3/4 transcription factors as well as to OCT4, sustaining pluripotency-related gene transcription, buffering pluripotency and repressing differentiation processes [37,38] (Fig. 3). Conversely, nuclear exclusion of YAP/TAZ is directly related to the specification of the trophectoderm (TE) with the induction of CDX2 expression [40,41]. These observations are in line with recent study, demonstrating YAP/TAZ up-regulation and specific nuclear compartmentalization in parthenogenetic ESCs [42]. This distinctive behavior of the two proteins results in higher ability to form outgrowths [43–45], generate 3D spheroid colonies and increase high plasticity [46–48] in mono-parental cells, when compared to their bi-parental counterparts, suggesting that the exclusive maternal origin of these cells may be the main possible cause.

Bio-mechanical effectors and epigenetic erasing

The data currently present in the literature point to the possibility to combine cell reprogramming techniques with mechano-sensing, in order to achieve a stable high plasticity state. A relationship between cell fate commitment and 3D rearrangement was recently reported by Harrison et al. [74], who co-cultured murine ESCs and extra-embryonic trophoblast stem cells (TSCs) onto 3D scaffolds, generating aggregates whose morphogenesis was remarkably comparable to in vivo embryos.

Similarly, other studies described the possibility to obtain structures referred to as "organoids" [75] or “blastoids” [76,77] through the use of 3D in vitro cultures that mimic the natural conditions and the bio-mechanical effectors at work during in vivo embryogenic processes and differentiation [78].

Presently, organoids have been generated from different cell types using encapsulation systems that boost the formation of functional cell aggregation and provide optimal gas exchange between the interior liquid and the surrounding environment [52,79–83]. A successful strategy, in particular, involved the use of the super hydrophobic synthetic compound polytetrafluoroethylene (PTFE) to produce micro-bioreactors that allow scale down experiments and work in smaller volumes, amenable for higher throughput applications [52,83]. Previous studies reported that PTFE efficiently encouraged cell aggregation, facilitating the formation of embryoid bodies (EBs) from murine ESCs [84] and the establishment of olfactory ensheathing cell (OEC) spheroid structures [85]. Consistent with these observations, micro-bioreactor culture systems were demonstrated to induce epigenetically erased cells to self-assemble and form multicellular spheroids, displaying a uniform size geometry. This was paralleled by a global DNA demethylation and elevated transcription of pluripotency markers, suggesting that the use of PTFE microbioreactors may encourage cell aggregation and may boost the acquisition and maintenance of a long-term high plasticity state. Interestingly, these molecular and morphological modifications were accompanied by the activation of the Hippo-signalling pathway, with distinctive changes in YAP/TAZ localization. In particular, 3D cell confinement in PTFE encouraged their nuclear retention, that was mirrored by a parallel nuclear accumulation of SMAD2/3 and the activation of the OCT4 dependent pluripotency pathways [52].

Altogether, this evidence suggests that bio-mechanical effectors can be used to support cell plasticity, eliciting YAP/TAZ nuclear compartmentalization, as it happens in the blastocyst, and encouraging YAP/TAZ interaction with SMAD2/3, buffering pluripotency and repressing differentiation processes.

Conclusions

Thanks to the abundant data accumulated in the literature our understanding of pluripotency has significantly expanded in the past decade. Pluripotency, as we presently see it, is no longer a singular property but rather a flexible and dynamic state along the cell developmental program.

In vivo it is a unique feature of the inner cell mass/epiblast cells that can give rise to all cell lineages of the developing and adult organism. Embryonic pluripotency is short lived, and lacks self-renewal, since it is limited to cells transiting through early stages of development.

However, it can be captured and stabilized indefinitely in vitro, under artificial culture conditions, as it happens in ESC. Similarly, in vitro pluripotency can also be reactivated in differentiated somatic cells, using several alternative approaches (Fig.4), some of which were discussed in the present review. All these strategies, that may involve direct reprogramming with exogenous factors, or the induction of an hypomethylated state with an epigenetic modifier or the key contribution of bio-mechanical effectors, have the common ability to activate the network of transcription factors that prevents cell differentiation and maintains self-renewal, encouraging cells to return to a pluripotent state.

All this suggests that the finely tuned spatio-temporal signaling system, driving cell differentiation, can be covered in opposite directions, either specifying terminal cell differentiation or restoring its pluripotency-related functions. Similarly to what happened in ancient Rome, pluripotency is the Golden Milestone (the Milliarium Aureum) of the network, it is the point of origin but also the specific point towards which all roads lead back to, after covering distances and territories within the Empire.

FIGURE LEGENDS

Figure 1. TET demethylation waves and cell plasticity. Transitions where TET proteins play a key role in loss or gain of methylation are indicated as waves.

Figure 2. Mechano-sensing cues and cell plasticity. YAP/TAZ respond to cytoskeletal-mediated mechano-sensing cues, controlling cell survival, proliferation and pluripotency vs. differentiation.

Figure 3. Bio-mechanical effectors and cell plasticity. YAP/TAZ nuclear compartmentalization leads to direct interaction with SMAD2/3, sustaining pluripotency-related gene transcription, buffering pluripotency and repressing differentiation processes.

Figure 4. All roads lead to Rome: the many ways to pluripotency.

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