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Molecular and cellular mechanisms regulating epigenetic cell conversion

Doctoral Thesis

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"Some people want it to happen,

some wish it would happen,

others make it happen."

- Michael J. Jordan

"Insanity is doing the same thing over and over again

and expecting different results."

-Albert Einstein

INDEX

ENGLISH ABSTRACT

Phenotype definition is controlled by epigenetic regulations that allow cells to acquire their differentiated state. The process is reversible and cells can be driven back to a higher plasticity state with several different approaches, which include the interaction with the epigenetic set up through the use of epigenetic erasers, readers or writers. Among the many epigenetic modifiers presently available, in our previous experiments, we selected 5-azacytidine (5-aza-CR), which is a well-known DNA methyltransferase inhibitor, and has been previously shown to increase cell plasticity and facilitate phenotype changes in different cell types.

Beside the epigenetic mechanisms driving cell conversion processes, growing evidences highlight the importance of mechanical forces that directly influence cell plasticity and differentiation. Aims of my PhD were: a) characterization of the molecular and cellular mechanisms regulating cell phenotype b) analysis of the possible relation between mechano-sensing and epigenetic control of cell plasticity and differentiation. The experiments carried out confirmed the global demethylating effect of 5-aza-CR, with a transient upregulation of pluripotency markers. At the same time increased transcription of TET2 and several histones was detected. This was accompanied by changes in enzymes controlling histone acetylation and cell morphological rearrangement. Interestingly, the study of DNA methylation profile and its regulatory genes, revealed that the use of a 3D micro-bioreactor promotes and stabilizes the maintenance of the acquired plasticity for a long period of culture. In particular, the use of an adequate soft substrate

increased pancreatic conversion efficiency and induced the acquisition of a mono-hormonal phenotype, which is distinctive of terminally matured cell. Altogether these findings indicate that 5-aza-CR induced somatic cell transition to a higher plasticity state may be the result of multiple regulatory mechanisms that accompany the demethylating effect exerted by the modifier. The results described in my thesis also revealed that mechano-transduction-related responses modulate and maintain 5-aza-CR induced cell plasticity and significantly improve cell differentiation toward the pancreatic lineage.

ITALIAN ABSTRACT

La definizione del fenotipo è controllata da meccanismi epigenetici che consentono alle cellule di acquisire il loro stato differenziato. Questo processo è reversibile e le cellule possono essere ricondotte ad uno livello di plasticità più elevato grazie a diversi approcci, tra i quali l'interazione con l'assetto epigenetico attraverso l'utilizzo di modificatori epigenetici. A questo scopo, nei nostri esperimenti precedenti, abbiamo selezionato la 5-azacitidina (5-aza-CR), un noto inibitore della DNA metiltransferasi, capace di aumentare la plasticità delle cellule e facilitare i cambiamenti del fenotipo in diversi tipi cellulari.

Accanto ai meccanismi epigenetici che guidano i processi di conversione cellulare, numerosi studi hanno evidenziano l'importanza e la capacità delle forze meccaniche di influenzare direttamente la plasticità ed il differenziamento cellulare. Gli obiettivi del mio Dottorato di ricerca sono stati: a) la caratterizzazione dei meccanismi molecolari e cellulari che regolano il fenotipo della cellula, b) l'analisi della possibile relazione tra meccano-sensing e controllo epigenetico della plasticità e del differenziamento. Gli esperimenti svolti durante questi anni hanno confermato l'effetto demetilante della 5-aza-CR, che è accompagnato da una sovra-espressione transitoria dei marcatori di pluripotenza. Allo stesso tempo sono stati descritti alti livelli di trascrizione per TET2 e diversi istoni. Parallelamente, sono stati identificati cambiamenti negli enzimi che controllano l'acetilazione degli istoni ed un ri-arrangiamento morfologico delle cellule. È interessante notare come lo studio del profilo di metilazione del DNA, e dei suoi geni regolatori, abbia dimostrato che l'utilizzo di un micro-bioreattore 3D promuove e stabilizza il

mantenimento della plasticità per un lungo periodo di coltura. In particolare, selezionando un adeguato substrato morbido è possibile aumentare l'efficienza della conversione in cellule pancreatiche e l'acquisizione di un fenotipo mono-ormonale, distintivo di una cellula terminalmente differenziata. Complessivamente, i risultati ottenuti dimostrano che lo stato altamente plastico indotto dalla 5-aza-CR in cellule somatiche è regolato da molteplici meccanismi, che accompagnano l'effetto demetilante esercitato dal modificatore epigenetico. I dati descritti nella presente tesi hanno inoltre rivelato che le risposte correlate alla meccano-trasduzione modulano e mantengono la plasticità cellulare indotta dalla 5-aza-CR e migliorano significativamente il differenziamento cellulare verso un fenotipo pancreatico.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Epigenetics

The history of epigenetics is linked with the study of development and evolution. However, in parallel to our increased knowledge of the molecular mechanisms underlying regulation of gene expression in animals and human, the meaning of "epigenetics" has evolved. The term epigenetics, coined in 1942 by Waddington [1], was derived from the Greek word “epigenesis” which originally described the influence of genetic processes on development. During the years, there was a long debate about the nature and location of the components responsible for the organisms developmental plan. In particular two schools of thought opposed: those who thought that each cell contained preformed elements that enlarged during development (preformationism), and those who thought the process involved chemical reactions that executed a complex developmental plan (epigenesis). Although the definition for epigenetics has changed in accordance to our increasing knowledge, it is important to remember that the original question was: how can a fertilized egg give rise to a complex organism composed by cells with different phenotypes? Experiments carried out by Fleming and Morgan [2] provided strong evidence that the developmental program resided in the chromosomes. From that moment, rapid progresses were made in order to create linear chromosome maps in which individual genes were assigned to specific sites on them [3]. Over time, other questions of classic epigenesis found answers. It was demonstrated that both nucleic acid and proteins were present in chromosomes, but their relative contributions were not easily evident. Furthermore, data from Drosophila genetics identified the DNA

as the primary carrier of genetic information and suggested that heritable changes in phenotype could occur without changes in the genes. Ultimately, it was necessary to redefine epigenetics in order to distinguish heritable changes that arise from changes in DNA sequences from those that do not.

The present definition of epigenetics reflect our understanding that, although the DNA is essentially the same in all somatic cells of an organism, patterns of gene expression differ greatly among different cell types, and these patterns can be inherited. This led to definition of epigenetics that still today is generally accepted as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" [4].

1.1.1 DNA Methylation

DNA methylation is the most studied and well-characterized epigenetic modifications dating back to experiments done by Griffith in 1969.

Changes in DNA methylation allow mature cells of adult organisms to acquire their differentiated state through a gradual loss of potency and a progressive restriction in their options [5]. DNA of mammalian cells can be covalently modified through a biochemical process in which methyl group (CH3) is added to the 5-carbon of the cytosine residues. This modification is controlled at different levels and is performed by a family of enzymes know as DNA methyltransferases (DNMTs). In particular, DNMT3a and DNMT3b, named de novo DNA methyltransferases, are required for de novo cytosine methylation which consist in the addition of methyl-groups onto unmethylated DNA, establishing a specific

methylation patterns. On the other hand, after cell division, the existing methylation patterns are maintained by the Dnmt1, which prefers hemimethylated substrates. Despite enzymes that catalyse the addition of the methyl group have been well characterized over the years, those that are involved in DNA demethylation have remained unclear, until recently. Indeed, although DNA methylation is relatively stable compared with other epigenetic modifications, variations in methylation levels are primarily involved in different biological processes such as establishing parental-specific imprinting during gametogenesis as well as gene silencing on the inactivated X-chromosome. Interestingly, it has been demonstrated that these methylation changes can occur both by a passive or active way. Passive DNA demethylation refers to loss of 5mC during subsequent replications in the absence of functional DNA methylation maintenance machinery. By contrast, active DNA demethylation refers to an enzymatic process that removes or modifies the methyl group from 5mC. In line with this, it was recently demonstrated that Ten-eleven Translocation (TET) family of enzymes are the main actors of active DNA demethylation process accompanying the well-documented indirect DNMT-related action. In detail, several studies described TET proteins ability to catalyse the successive oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) [6, 7]. These observations provided the first evidence of a pathway for active DNA demethylation, showing that DNA methylation patterns are not as static as previously assumed [8].

1.1.2 Histone Modifications

Post-translational modifications of histones are among the most studied epigenetic modifications. They are characterized by the attachment of different molecules on the histone tail. that allow or prevent transcription factors and other proteins to access the DNA by modifying chromatin structure. Histone modifications are involved in several biological processes such as DNA damage/repair, transcriptional activation/inactivation and chromosome packaging. These modifications, that mainly include methylation and acetylation of conserved lysine residues on the amino terminal tail, are dynamically controlled by chromatin modifying enzymes with opposing activities. In particular, histone acetyltransferases (HATs) mediate lysine acetylation, marking transcriptionally competent regions, while lysine deacetylation catalysed by histone deacetylases (HDACs), tags transcriptionally inactive chromatin structures. Moreover, in order to guarantee additional regulation, histone methyltransferases (HMT), such as ESET, G9a, SUV39h, modulate lysine residues methylation that can be mono-, di-, or tri-methylated. Methylation of lysine 9 or lysine 27 on histone H3 (H3-K9/H3-K27) is a hallmark of heterochromatin, the tightly packed form of DNA, and is associated to a low or null transcriptional activity. In parallel, a recently discovered demethylase, LSD1, has been shown to remove a methyl group from di- or mono-methylated lysine 4 on histone H3 (H3-K4) [9], further demonstrating the dynamic nature of this epigenetic regulation. Another important post-translational modification is histones phosphorylation. In particular, histone tails contain specific sites that can be phosphorylated by a number of protein kinases and

dephosphorylated by phosphatases. Moreover, phosphorylation can occur on serine, threonine and tyrosine residues and takes place mainly during cellular response to DNA damage. Collectively, the presence of chromatin modifying enzymes that act in opposite way and the cross-talk between different epigenetic systems demonstrate the complexity of the gene regulation by epigenetic modifications (Table 1).

MODIFICATION	RESIDUES MODIFIED	FUNCTION
Acetylation	H3(K9, K14, K18, K56) H4 (K5, K8, K12, K16) H2B(K6, K7,K16, K17)	Transcription activation
Methylation	H3 (K4me2, K4me3, K36me3, K79me2)	Transcription activation
Methylation	H3 (K9me3, K27me3) H4 (K20me3)	Transcription repression
Phosphorilation	H3(S10)	Transcription activation, DNA repair

Table 1: Different histone modifications and its regulated biological functions

1.1.3 Small non coding RNAs

Pre- and post-transcriptional gene regulation by small non-coding RNAs is a new emerging epigenetic mechanism that add another level of control over the already complex systems described above. These mechanisms are the result of small noncoding pieces of RNA called small inhibitory RNA (siRNA) and microRNA (miRNA). siRNAs are generated by the cleavage of dsRNA by a member of the RNase III family called Dicer and have two nucleotide overhangs at each 3′ end. On the other hand, miRNAs are transcribed as parts of longer RNA molecules, processed in the nucleus into small hairpin-shaped molecules by ribonuclease named Drosha and cut to size in the cytoplasm by a Dicer enzyme. siRNA and miRNA operate in association with a protein, forming a

ribonucleoprotein complex called RNA-induced silencing complex (RISC), and inhibit translation by two different mechanisms. The proteins in RISC unwind siRNA and remain bound to a single antisense strand, which then binds to mRNA in a sequence-specific manner, allowing to a Slicer protein (a component of RISC) to cut the mRNA in the middle of the binding region. The cut mRNA is recognized as abnormal and subsequently destroyed. In the case of miRNA, a microRNA-induced silencing complex (miRISC) is associated with the mature miRNA, and the complex itself physically blocks translation binding to mRNA. In addition, several works demonstrated small non-coding RNAs ability not only to regulate the stability and translation of mRNAs but also to induce the formation of inactive chromatin structures of specific genomic sequences [10, 11].

1.2 Epigenetic Modifications in Embryonic Stem Cells (ESCs)

From the very beginning of development, DNA demethylation plays a key role in shaping the identity of the developing mammalian embryo. The first demethylating process occurs shortly after fertilization and allows the zygote to acquire the totipotent state and confinement of pluripotency to cells belonging to the inner cell mass (ICM). These events are followed by extensive de novo methylation that leads to the specification of the embryonic lineages. However, they are quickly followed by a second wave of reprogramming that takes place to allow the specification of Primordial Germ Cells (PGCs), relocating to and colonizing the genital ridges (Fig. 1). Loss of CpG methylation in the paternal genome is active, quick and is carried out by TET3-mediated

oxidation of 5-hmC to 5-fC and 5-caC [12-14]. Conversely, the maternal pronucleus remains more protected against TET3 activity [15] undergoing a more gradual methylation loss [16].

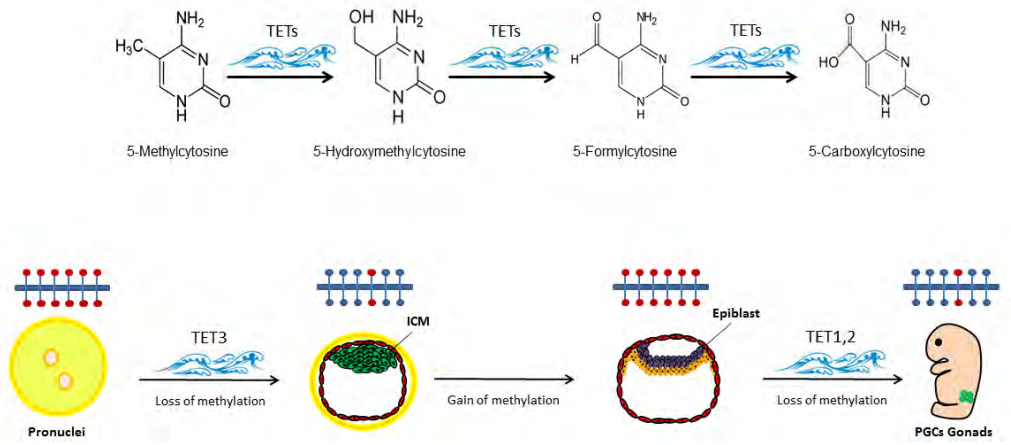


Fig. 1: TET-mediated oxidation of 5-methylcytosine generates 5-hydroxymethylcytosine, which is converted to 5-formylcytosine and 5-carboxylcytosine (upper panel). Changes in DNA methylation drive early embryo development and cell fate commitment (lower panel) (Brevini et al.; 2018).

In parallel, both genomes are targets for demethylation through DNMTs activity, that is passive and replication dependent. Therefore, epigenetic erasure, coordinating the progression of early development, involves both active and passive demethylation processes and is both replication dependent and independent. The very small number of cells available in the ICM or epiblast, makes the study of cell commitment and reprogramming *in vivo* very demanding [17, 18]. For this reason several studies are performed using embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) as models. These experiments have

demonstrated that DNA hypomethylation is crucial to promote self-renewal, maintain the naïve state and antagonize the self-activating differentiation signals. These observations point to the fact that demethylation process is essential for epigenome resetting and the re-establishment of pluripotency network [19]. In contrast, it has been demonstrated that cell differentiation is accompanied by a progressive increase of DNA methylation that silences pluripotency genes, such as Oct4 and Nanog, and establishes a phenotype-specific methylation pattern [20, 21]. Interestingly, studies performed using media supplemented with the two small molecule kinase inhibitor 2i [22, 23] reported the derivation of ESC lines that exhibit a higher level of hypomethylation than those derived using conventional media. The authors suggested that the addition of 2i increases TET-mediated conversion of 5-mC to 5-hmC, erasing cells into a naïve state in which the genome becomes hypomethylated and reminiscent of early blastomeres *in vivo*. In parallel, several studies have demonstrated that histone H3K4me3 and histone H3K27me3 play a particularly important role in regulating pluripotency of ESCs [24-26]. Indeed, while DNA methylation is mainly involved in the silencing of genes during differentiation, histone modifications are primarily implicated in the maintenance of the self-renewal property in ESCs [27]. In particular, H3K4me3 is catalysed by trithorax group (TrxG) enzymes and is associated with open chromatin state and active transcription [28], while H3K27me3 is catalysed by Polycomb group (PcG) proteins and is related to compact chromatin and gene repression [29]. However, in 2006 Azuara and Bernstein first described that several thousands of the H3K4me3-enriched

promoters in pluripotent cells also contain the repressive histone mark H3K27me3 [30, 31]. Since that moment, these regions were termed 'bivalent domains' and were proposed to maintain the pluripotent state of embryonic stem cells through the repression of developmental transcription factors acted by H3K27me3, while these genes are promptly activated during differentiation through a continued presence of H3K4me3. In addition, also the crosstalk between DNA methylation and histone modification plays an important role in the epigenetic regulation of ESCs gene expression. Different studies have identified an inverse relationship between H3K4 methylation and DNA methylation [32, 33]. Bird et al. observed that methylation at CpG-dense regions inhibit Trithorex activity, one of the protein involved in the modification of H3K4 methylation [34]. Since H3K4me3 is usually associated with active transcription, these mutually exclusive effects may be part of the coordinative mechanism underlying gene activation and silencing. Other experiments revealed an interaction at the protein level between specific methylcytosine binding proteins and histone deacetylases/methyltransferases enzymes [35, 36]. There are also works indicating that G9a and ESET, two methyltransferases for H3K9, can interact with DNMT3A/3B to facilitate local methylation of DNA [37, 38]. EZH2, an enzymatic component of the PRC2 complex involved in H3K27 methylation, can also interact with DNMTs to influence the DNA methylation status [39]. Altogether these observations demonstrate, once again, the complexity and dynamism of the mechanisms regulating transcription in eukaryotes.

1.3 Epigenetic Modifications in induced Pluripotent Stem Cells (iPSCs)

Differentiation has been considered stable and potentially irreversible for a long time. However, following the pioneering work of Taylor and Jones, many groups have reported the possibility to directly interact with cell fate definition and modify terminal commitment [40]. With regards to Waddington's metaphor, reprogramming refer to the process whereby cells travel back up their differentiation path, through the epigenetic landscape, to become more immature and finally convert into the pluripotent state [41] (Fig. 2).

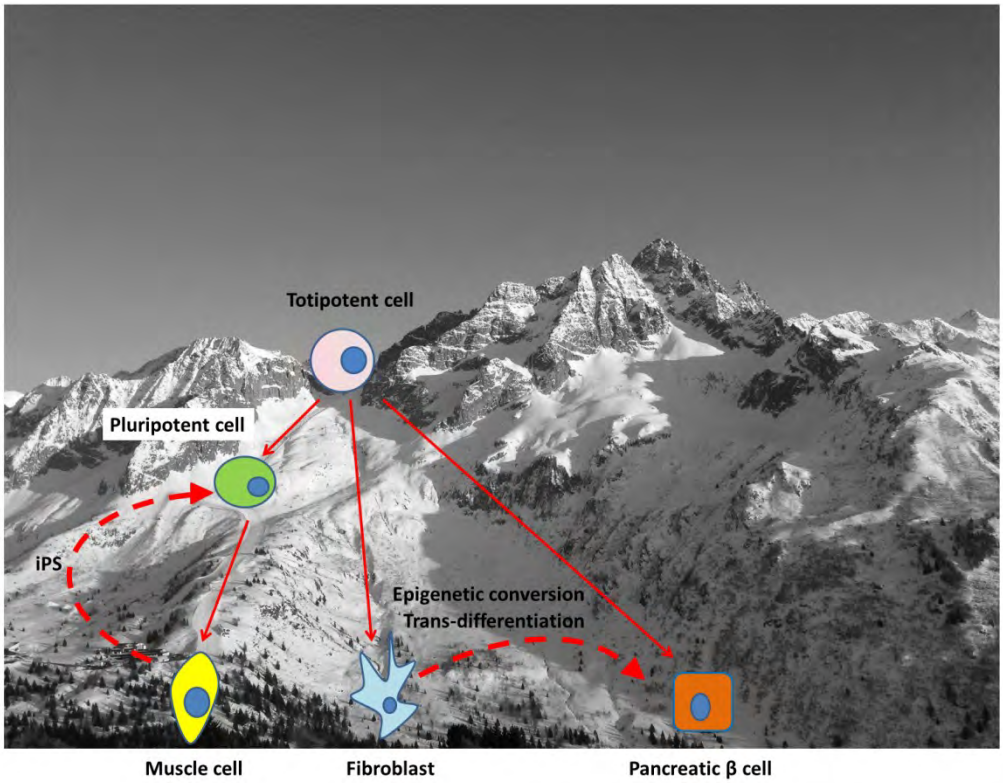


Fig. 2: Representation of Waddington model. (Brevini et al. 2017)

The concept of switching cell fate was first established by John Gurdon's experiments in Xenopus laevis, in the middle of the last century, at

approximately the same time as Waddington's doctrine emerged [42]. Later, the birth of a cloned sheep Dolly in the late 20th century, showed that erasing somatic cell fate was possible, even in mammals [43]. In 2006, Yamanaka demonstrated that overexpression of four transcription factors, Oct3/4, Sox2, Klf4 and c-Myc (OSKM factors), in differentiated somatic cells allowed the reach of a high plasticity state, as obtained in induced pluripotent stem cells (iPS) [44]. Since then, the induction of latent pluripotency in differentiated cells has been termed 'reprogramming'. Nevertheless, it is important to remember that the identity of a mature cell is ensured by a specific and unique epigenetic profile that maintains its lineage definition and prevents free transition among different phenotypes. Therefore, it is necessary to remove the epigenetic blocks in order to allow a switch in cell fate. Recent deep analysis of iPSCs revealed that the overall histone modification and DNA methylation landscapes are correctly reprogrammed in most authentic iPSC lines, whereas they are incompletely restored in partially reprogrammed iPSCs [45, 46]. Surprisingly, in a recent work it has been obtained iPSC colonies using deficient Dnmt3a and Dnmt3b cells, indicating that de novo methylation is dispensable for cellular reprogramming [47]. In contrast, reducing global genomic methylation levels, by treating cells with the demethylating drug 5-azacytidine (5-aza-CR), boosts cellular reprogramming [46], enhances colony formation and facilitates the conversion of partially reprogrammed into fully reprogrammed iPSCs. These observations suggest that, while maintenance of the somatic methylome is a barrier that must be overcome, de novo deposition of methylation is not a requirement for

successful iPSC reprogramming. In addition, several studies showed that experimental reprogramming requires not only passive but also active demethylation by the TET enzymes, which leads to activation of epigenetically silenced pluripotency genes [48, 49]. This finding point to the possibility that TET enzymes play a key role in reprogramming and mesenchymal to epithelial transition (MET). This hypothesis finds further support in experiments performed in mouse fibroblasts, in which TET genes were inactivated, resulting in a complete block of the reprogramming process [50]. These observations indicate that TET enzymes are indispensable for factor-driven reprogramming of somatic cells to iPSCs. In parallel, initial phase of reprogramming is marked by rapid changes of H3K4me2/3 distribution [51]. H3K4me2/3 peaks exhibit dramatic changes at promoter and enhancer regions of many pluripotency-related genes. In line with this, it has been recently demonstrated that WD repeat domain 5 (Wdr5), the key component of histone methyltransferase complex responsible for H3K4 methylation, directly interacts with Oct4 [52] and that promoters that exhibit H3K4me2/3 are significantly associated with overexpression of Oct4 and Sox2 [51]. In contrast to H3K4me2/3-containing regions, chromatin domains enriched for repressive H3K9me3 are refractory to initial OSKM binding [53]. Reduction of H3K9me3 levels through down-regulation of methyltransferases Suv39h enhances Oct4 and Sox2 binding at these regions and increases reprogramming efficiency. Moreover, bivalent histone modifications were also identified in human iPSCs [24, 45], marking developmentally regulated genes, similar to the situation found in hESCs. In addition, unlike to developmental progression, successful

iPS reprogramming requires the replacement of the somatic repressive chromatin with a highly dynamic pluripotent chromatin state that is largely devoid of heterochromatin [54].

1.4 Epigenetic Modification in Direct Converted Cells (EpiCCs)

In parallel to iPSC development, several protocols, that avoid the use of introduced exogenous factors, have been developed. These new approaches involve the use of small molecules and epigenetic modifiers (classified as erasers, writers and readers) in order to directly convert an adult mature cell into an alternative differentiated cell type [55-57]. The first paper reporting the ability of the small molecule Reversine to increase cell plasticity, inducing committed myoblasts to become multipotent mesenchymal progenitor cells, was published in 2004 [58]. In subsequent years, different works, that involved the use of epigenetic modifiers, have been described [59-62]. The Laboratory of Biomedical Embryology UNISTEM, where I carried out my PhD project, recently developed a new protocol based on the concept that DNA methylation plays a fundamental role both during early embryonic development and cell lineage specification. In particular, we hypothesized that a brief exposure to a de-methylating agent can increase cell plasticity, driving cells into a transient less committed state that is sufficient to readdress them to a differentiated cell type. To this purpose, among the many erasers presently available, we selected 5-aza-CR, a safe and approved drug used in medicine for myelodysplastic syndrome (MDS) therapy and chronic myelomonocytic leukemia (CMML). This molecule is known to induce reversible cell cycle arrest [63, 64] and acts as a direct inhibitor of

methyltransferase activity, decreasing methylation in newly synthesised DNA. The molecule substitutes for cytosine, incorporates into DNA and RNA, during replication [65], forms covalent adducts with DNA methyltransferase (DNMT) 1, thereby depleting the cells from enzyme activity, causing demethylation of genomic DNA [66, 67] as well as gene reactivation [68]. However, despite this molecule has long been known, the mechanisms underlying 5-aza-CR effects on cell plasticity and differentiation, as well as the epigenetic profile of direct converted cells, are still poorly understood and need to be better elucidated. Based on this, during my PhD, I dedicated part of the effort to better characterize the molecular and cellular mechanisms that regulate epigenetic conversion in EpiCCs.

1.5 Cell fate controlling cues

1.5.1 Mechanical cues

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 that are constantly transmitted across cell–ECM and cell–cell adhesion sites [69-71]. Cells balance these external forces thanks to the cytoskeleton that rapidly senses and adapts to changes in the microenvironment [72, 73]. 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 evidences demonstrate that cell shape, ECM elasticity and cytoskeletal tension play important roles in cell

behaviour, physiology and fate decision. 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 [74] 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 [75]. Moreover, Watt et al. reported that cell shape strongly influences cell fate decision, demonstrating its ability to affect the balance between keratinocyte self-renewal and differentiation [76]. Similarly human Mesenchymal Stem Cells (MSCs) differentiate into osteoblasts when allowed to spread, whereas they differentiate into adipocytes when they are confined to a round shape [77]. In addition, *in vitro* experiments showed MSCs phenotype changes in response to ECM substrates with different elasticity. In particular, MSCs differentiate into osteoblasts when seeded on a synthetic matrix with bone-like stiffness, acquire a specific myoblast phenotype when grown on ECMs with intermediate stiffness or differentiate into neurons and adipocytes when cultured on a soft ECM [78]. In the same way, skeletal muscle stem cells require a substrate with adult muscle-like stiffness in order to preserve high regenerative capacity when cells are engrafted back into mice [79, 80]. Several studies have also reported the formation of structures referred to as organoids, obtained from different cell types using culture media that mimic the conditions of embryogenetic processes and differentiation [81, 82]. These models, mostly obtained by cell encapsulation in micro-bioreactors, have been developed as model to study organ development and pathologies 'in a

dish'. All these evidences point to the fact that the *in vitro* microenvironment, provided by the traditional polystyrene culture systems, fails to imitate the physiological and biochemical features of cells and causes deviations in cell response. This is mainly related to the significant differences between the stiffness of the original tissue and that of several gigapascal (GPa) of the plastic supports traditionally used (Fig. 3). Given these characteristics, plastic provides a static environment, does not allow a detailed comprehension of the natural tissue architecture, and leads to the development of a limited model. For this reason, the use of 3D matrices, micro-bioreactors and scaffolds, that display an architecture closer to the *in vivo* one and allow a more physiological cell organization, has become widespread.

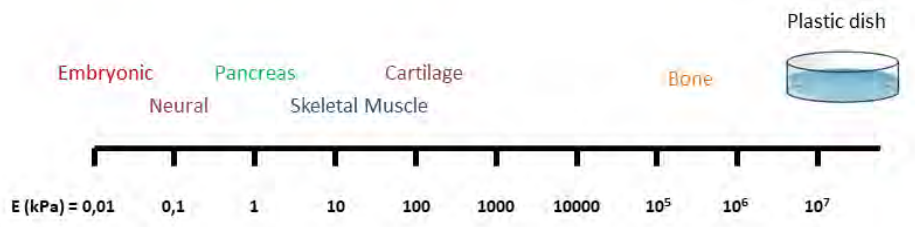


Fig. 3: the biomechanical properties of the tissues, expressed as stiffness (E; elastic modulus) and measured in kilopascals (kPa), vary specifically among organs and are strictly related to its function.

Despite these increasing evidences, the way and the molecular components that perceive and transduce mechanical signals into the cells remain poorly characterized. Recent studies identified yes-associated protein/WW domain containing transcription regulator 1 (YAP/TAZ), the main transcriptional effectors of the Hippo signalling pathway, as key mechano-transducers acting by nuclear relays of mechanical stimuli. In mammals, the Hippo pathway is constituted of a cascade of kinases, such as MST 1/2 and LATS 1/2, which lastly phosphorylate YAP/TAZ leading

to its inactivation and exclusion for nuclear accumulation, while its dephosphorylation cause YAP/TAZ activation and translocation from the cytoplasm to the nucleus [83]. Several works revealed the role of YAP/TAZ as main sensor for mechanical stimuli including cell density, matrix stretch and stiffness. Experiments from the Piccolo's laboratory, as well as others, have examined YAP/TAZ activation after altering cellular or extracellular mechanical properties [84, 85]. However, the impact of other mechanical factors on YAP/TAZ activity require further investigation, as well as it is yet unclear if matrix elasticity is able to influence cell epigenetic profiles, inducing changes in methylation levels and/or in gene expression [86].

1.5.2 Chemical cues

In parallel to mechanical cues, chemical cues have been shown to play key roles in a wide range of biological processes, including development, inflammation and cancer metastasis, determining the function and the fate of cells *in vivo* [87]. One of the more important chemical property of cellular microenvironment is the chemical gradient. In recent years, it grew more and more the need to develop *in vitro* methods for exposing cells to chemical gradients that are quantifiable, controllable, and mimic those present *in vivo*. Gradient signalling is a highly-regulated process, in which the ultimate cellular response is determined by the unique concentration, complement, and spatio-temporal characteristics of the gradients to which cells are exposed. While initial approaches were focused on creating several forms of gradients in 2D standard condition, it is obvious that a gradient across 3D systems more closely mimics the *in*

vivo environment. The ability to create complex chemical environments, composed of precise molecule concentrations, greatly advanced biological research and helped to elucidate how biological processes are affected by gradients of biomolecules.

An alternative approach to modulate the *in vitro* microenvironment is based on the principles of macromolecular crowding (MMC), a biophysical phenomenon that directs the intra- and extra-cellular milieu in multicellular organisms and increases thermodynamic activities and biological processes [88]. MMC uses the principles of volume occupancy, in which large macromolecules occupy volumes larger than their 'real' volume owing to their high hydrodynamic radius, thereby hampering the space for other macromolecules in the system [89]. It is known that biological macromolecules, such as enzymes and proteins, function in a highly dense environment. However, standard culture media are very diluted in this respect and contain macromolecules at levels below 20 g/l which fails to imitate even the most dilute of the body fluids. Moreover it has been demonstrated that the addition of neutral or negatively charged molecules to the culture media accelerated collagen type I deposition in *in vitro* culture of human fibroblasts. This can be explained by the fact that *in vivo* cells are entrapped in highly crowded extracellular space, where the conversion of the de novo synthesised procollagen to collagen takes place rapidly, whereas in the diluted culture environment the conversion of procollagen to collagen is very slow [90]. In line with this, recent studies demonstrated that MMC enhanced collagen I deposition and alignment in human Mesenchymal Stem Cells (MSC), which in turn

increased alignment of actin cytoskeleton and proliferation but decreased cell motility [91].

More recently, it has been demonstrated that also microvesicles play an important role in cell behaviour and fate decision. 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 [92, 93] and to act as transcription modulators, affecting cell phenotypes [94] The concept that extracellular vesicles (EVs) derived from cells are capable of transferring information not only in a paracrine manner, but also in an endocrine manner, has revolutionised our understanding of cell–cell communication. The pivotal study of Ratajczak et al. showed that Evs derived from stem cells exert profound effects on the microenvironment by transferring stem cell-specific proteins and mRNAs [95]. In that study it was demonstrated that microvesicles derived from ESCs contained Wnt-3and mRNAs implicated as pluripotent transcription factors. These molecular components were transferred and translated into proteins by neighbouring cells, thus reprogramming haematopoietic progenitors. This experiment was the first to describe epigenetic changes in recipient cells following transfer of mRNA. 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 [96, 97]. Following these experiments, the exchange of genetic information mediated by Evs has been suggested as a fundamental component of stem cell biology, where the environmental stimuli are critical for the differentiation decision of stem cells [98]. In the context of tissue injury, EV-mediated exchange of

information could be bidirectional between stem and injured cells. For example, Bruno et al. demonstrated that injured tissue cells may induce gene expression and differentiation decisions in stem cells. Conversely, stem cell-derived vesicles may reprogram injured cells by activating regenerative mechanisms [99]. In particular, the transfer of transcriptional factors, such as non-coding RNAs, may induce epigenetic modifications in recipient cells, which could be exploited in regenerative medicine. Based on these observations, 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.

CHAPTER 2

AIM

2. AIM

Recent findings have demonstrated that adult cells may modify their phenotype in response to compounds that interact with the epigenetic signature. In particular, over the years, many studies have described the demethylating effect of the epigenetic eraser 5-azacytidine. However, despite this molecule has long been known, the mechanisms underlying 5-aza-CR effects on cell plasticity and differentiation are still poorly understood. In addition, several evidences have shown that changes in cell fate are directly influenced by cell microenvironment and that biophysical signals, conveyed by the extracellular matrix, can regulate cell pluripotency and cell differentiation state. In my PhD, I dedicated part of the effort to better characterize the molecular and cellular mechanisms that regulate epigenetic conversion. In particular, my research activity was addressed to:

- Further elucidate the data collected during my Master thesis, obtained through WTA analysis
- Investigate the mechanisms driving 5-aza-CR epigenetic erasing and modulating cell plasticity and cell commitment
- Determine the relation between mechanosensing and epigenetic control of cell plasticity using a micro-bioreactor culture systems that promote 3D cell rearrangement
- Evaluate the effect of an adequate 3D systems, that display *in vivo* tissue-like stiffness, on cell pancreatic differentiation

The elucidation of these aspects may be useful to expand our knowledge on 5-aza-CR mechanisms of action as well as to better characterize the

general process that controls cell phenotype. Moreover, the experiments described in the present thesis provide a novel approach to *in vitro* culture that may find useful application in stem cell organoid technology and long-term cell culture. At the same time, they may be useful to further improve the pancreatic conversion protocol, accelerating and supporting a promising therapeutic approach for the cure of the diabetes.

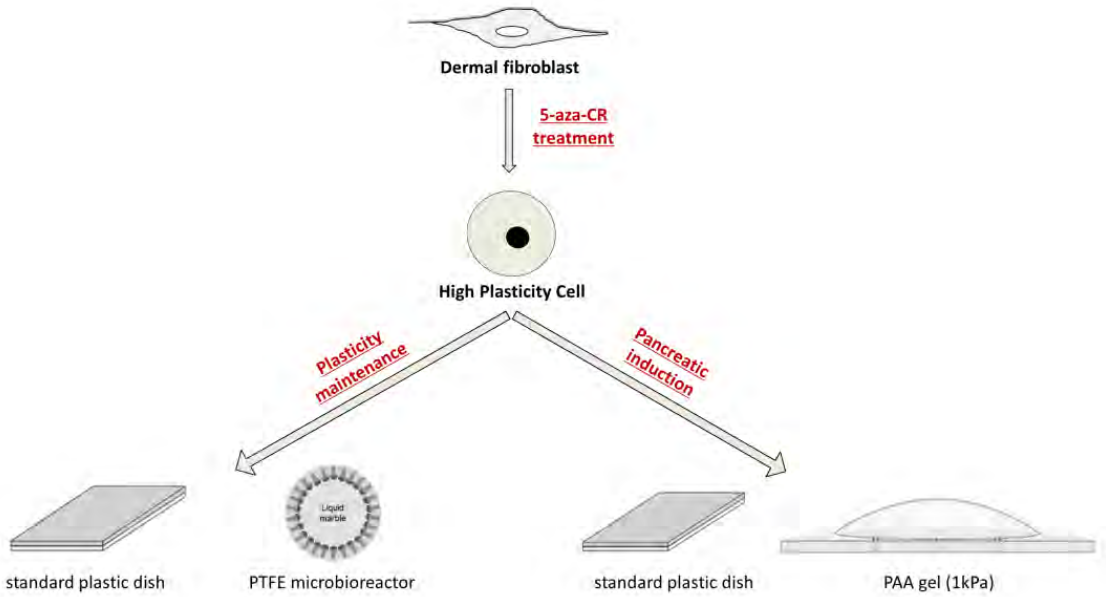
CHAPTER 3

MATERIALS AND METHODS

3. MATERIAL AND METHODS

3.1 Experimental Design

The following imagine represents the general experimental design of the present thesis, regardless of the starting cell types used:



3.2 Isolation and Culture of Dermal Fibroblasts

In the experiments described in the present thesis different cell types were used:

- Murine dermal fibroblasts isolated from 7-week-old C57BL/6N male mice and obtained from Charles River.
- Human dermal fibroblasts established from skin biopsies of eight healthy patients.

Fragments of murine and human dermal tissue of approximately 2 mm^3 were transferred onto 0.1% gelatin (Sigma) pre-coated Petri dish (Sarstedt) and cultured in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 20% Fetal Bovine Serum (FBS), 2 mM glutamine (Sigma) and antibiotics. After 6 days of culture, fibroblasts started to grow out of the tissue fragments and the latter were carefully removed. Murine and human cells were maintained in the medium described above. Fibroblasts were grown in 5% CO2 at 37°C, and passaged twice a week in a 1:3 ratio. All experiments were performed in triplicate on at least three different lines.

3.3 Treatment of Dermal Fibroblasts with 5-aza-CR

Murine and human fibroblasts were epigenetically erased with 1 μM 5-aza-CR (Sigma) for 18 hours. Concentration and time of exposure were selected according to previous works [55, 100].

3.4 Cell Seeding and Encapsulation

3.4.1 Seeding of Dermal Fibroblasts on Plastic

Dermal fibroblasts were plated in 4-well multidish (Nunc) previously treated with 0.1% gelatin (Sigma) at concentration of 7.8 X 10^4 cells/cm^2. 24 hours after plating, cells were erased with 5-aza-CR as described above.

3.4.2 Seeding of Dermal Fibroblasts on Gel

Based on data from the literature showing that *in vivo* beta-cells are surrounded by a rich network of soft tissue (0.1– 1 kPa) [101, 102] and that islet encapsulation in 1 kPa silk hydrogel increased insulin secretion and expression of functional genes [103], I selected thin polyacrylamide (PAA) gels with elastic modulus of 1 kPa. Dermal fibroblasts were plated

PAA gels at concentration of 7.8 X 10^4 cells/cm^2. 24 hours after plating, cells were erased with 5-aza-CR as described above.

3.4.3 Encapsulation of Dermal Fibroblasts in PTFE

The polytetrafluoroethylene (PTFE) micro-bioreactor was created inside a Petri dish by preparing PTFE powder bed with particle size of 1 μm (Sigma 430935). Spatula was used to gently make a curved gully at the center of the powder bed. A micropipette was used to dispense 4 X 10^4 cells resuspended in 30 μl of 1 μM 5-aza-CR, on the PTFE powder bed. The Petri dish was then gently shaken in a circular motion to ensure that the powder particles completely covered the surface of the liquid drop. PTFE drops were incubated in 35 mm Petri dishes at 37 °C in 5 % CO2 in air. To increase humidity and avoid dehydration, the Petri dish was placed in a larger Petri dish containing sterile water.

3.5 Cell culture for Pluripotency Maintenance

At the end of the 18-hour exposure, 5-aza-CR treated fibroblast were cultured in ESC medium (Table 2). More in details, medium was removed and replaced on cells grown in adhesion. While for cells encapsulated in PTFE, liquid marbles were broken by puncturing with a needle. Formed organoids were recovered using a 200 μl pipette tip cut at the edge, washed in ESC medium and re-encapsulated in PTFE micro-bioreactors. Culture medium was refreshed daily until day 28, when culture was arrested.

PRODUCT	COMPANY	CATALOG N°	COMPOSITION (%)
Ham's F-10 Nutrient Mix	Life Technologies	31550-023	40%
DMEM Low Glucose + Pyruvate	Life Technologies	31885-023	40%
KnockOut™ Serum Replacement	Life Technologies	10828-028	10%
Fetal Bovine Serum	Life Technologies	10108-165	5%l
Antibiotic Antimycotic Solution (100×)	Sigma	A5955	1%l
L-Glutamine solution	Sigma	G7513	1%
NUCLEOSIDE MIX ***(Table 3)***	---	---	1%
MEM Non-Essential Amino Acids Solution	Life Technologies	11140-035	1%
2-Mercaptoethanol	Sigma	M7522	1%
ESGRO® (LIF)	Millipore	ESG1106	0,1%
Recombinant Human FGF basic (bFGF)	R&D System	233-FB-025	0,1%

Table 2: ESC medium formulation.

PRODUCT	COMPANY	CATALOG N°	COMPOSITION (in 50 ml water)
Guanosine	Sigma	G6264	0.042 g
Adenosine	Sigma	A4036	0.040 g
Cytidine	Sigma	C4654	0.036 g
Uridine	Sigma	U3003	0.036 g
Thymidine	Sigma	T1895	0.012 g

Table 3: Nucleoside Mix composition.

3.6 Cell culture for Pancreatic Induction

At the end of the 18-hour exposure, 5-aza-CR treated fibroblast were incubated with ESC medium (Table 2 above) for 3 hours. After the incubation period in ESC medium, cells were grown in specific culture media in order to induce the pancreatic differentiation. In details, cells were cultured in pancreatic medium basal supplemented with specific reagents (Table 4).

PRODUCT	COMPANY	CATALOG N°	COMPOSITION (%)
DMEM/F12	Life Technologies	11320-074	92%
B-27® Supplement Minus Vitamin A (50X)	Life Technologies	12587-010	2%
N-2 Supplement (100X)	Life Technologies	17502-048	1%
MEM Non-Essential Amino Acids Solution, 100X	Life Technologies	11140-035	1%
Antibiotic Antimycotic Solution (100×)	Sigma	A5955	1%
L-Glutamine solution	Sigma	G7513	1%
2-Mercaptoethanol	Sigma	M7522	1%
Albumin from bovine serum (BSA)	Sigma	A3311	1%

Table 4: Pancreatic Medium Basal formulation.

In the murine model, the conversion process requires 10 days of culture and pancreatic differentiation was induced using a three-step protocol:

- DAY 1: pancreatic medium basal + ACTIVIN A (1 μL/mL)

- DAY 2: pancreatic medium basal + ACTIVIN A (1 μL/mL) + RETINOIC ACID (1 μL/mL)
- DAYS 3-10: pancreatic medium basal + Insulin-Transferrin-Selenium (ITS; 10 μL/mL) + B27 supplement (20 μL/mL) + bFGF (1 μL/mL) (FINAL MEDIUM)

3.7 DNA Analysis

3.7.1 Global Methylation Analysis

Genomic DNA was extracted using PureLink® Genomic DNA Kits following the manufacturer's instructions. DNA was converted to single-stranded DNA by incubation at 95°C for 5 min, followed by rapid chilling on ice. Samples were then digested to nucleosides by incubating the denatured DNA with nuclease P1 for 2 h at 37°C in 20 mM sodium acetate (pH 5.2). Alkaline phosphatase was added and incubated for 1 h at 37°C in 100 mM Tris (pH 7.5). After centrifugation, the supernatant was used for ELISA assay using Global DNA Methylation ELISA Kit (5'-methyl-2'-deoxycytidine Quantitation; CELL BIOLABS) according to the manufacturer's protocol. Highest expression set to 1 and all other times relative to this.

3.7.2 DNA Dot Blot

Genomic DNA was extracted with PureLink® Genomic DNA Kits as previously described. DNA concentration was assessed with NanoDrop 8000 (Thermoscientific). Aliquots of 200 ng total DNA were prepared in a total volume of 2 μL per sample and spotted onto nylon membranes (Hybond-N+, Amersham). The DNA spots were air dried for 15 min, UV-

crosslinked for 1 min and probed with primary antibodies against 5-Carboxylcytosine (Active Motif, 1:1500) and 5-Formylcytosine (Active Motif, 1:1500). Dots were visualized with a WesternBreeze chemiluminescent kit. Signal intensity was quantified by densitometric analysis, using the Image J analysis software (National Institutes of Health).

3.8 Ultrastructural Analysis

Cells were collected for transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

TEM analysis samples were fixed for 2 hours in 0.1 M cacodylate buffer pH 7.2, containing 2% glutaraldehyde. Specimens were then washed in the same buffer and post-fixed for 2 hours with 1% osmic acid in cacodylate buffer. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica). Semi-thin sections were stained by conventional methods (crystal violet and basic fuchsin) and observed under a light microscope (Olympus). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol).

For SEM, cells were fixed and dehydrated as described above, then treated with hexamethildisilazane and mounted on polylysinated slides, air dried and subsequently covered with a 9 nm gold film by flash evaporation of carbon in an Emitech K 250 sputter coater (Emitech). Specimens were examined with a SEM-FEG Philips XL-30 microscope (Philips).

3.9 Gene Expression Analysis

RNA was extracted using the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. Quantitative PCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) using predesigned gene-specific primers and probe sets from TaqManGene Expression Assays, listed in Table 5. GAPDH, ACTB, and RPS 18 were used as internal reference genes. CFX Manager software (Bio-Rad Laboratories) was used for target gene quantification. Gene expression levels are reported with the highest expression set to 1 and all other times relative to this.

GENE	DESCRIPTION	SPECIES	CATALOG N°
Actb	Actin beta	Mouse	Mm02619580_g1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mouse	Mm99999915_g1
Gcg	Glucagon	Mouse	Mm00801714_m1
Hat1	Histone aminotransferase 1	Mouse	Mm00509140_m1
Hdac1	Histone deacetylase 1	Mouse	Mm02745760_g1
Ins	Insulin	Mouse	Mm01950294_s1
Nanog	Nanog homeobox	Mouse	Mm02019550_s1
Oct4	POU class 5 homeobox 1	Mouse	Mm03053917_g1
Rex1	ZFP42 zinc finger protein	Mouse	Mm03053975_g1
Rps18	Ribosomal protein S18	Mouse	Mm02601777_g1
Sox2	Sex determining region Y-box 2	Mouse	Mm03053810_s1
Sst	Somatostatin	Mouse	Mm00436671_m1
Tet2	tet methylcytosine dioxygenase2	Mouse	Mm00524395_m1
ACTB	Actin beta	Human	Hs01060665_g1
CCNG1	Cyclin G1	Human	Hs00171112_m1
CDH1	Cadherin 1	Human	Hs01023895_m1
COL6A3	collagen, type VI, alpha 3	Human	Hs00915125_m1
EPCAM	Epithelial cell adhesion molecule	Human	Hs00901885_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Human	Hs02786624_g1

H2AFX	H2A histone family, member X	Human	Hs00266783_s1
H2AFZ	H2A histone family, member Z	Human	Hs01888362_g1
HAT1	histone acetyltransferase 1	Human	Hs00186320_m1
HDAC1	histone deacetylase 1	Human	Hs02621185_s1
HIST1H1D	histone cluster 1, H1d	Human	Hs00271187_s1
HIST1H2AB	histone cluster 1, H2ab	Human	Hs01001083_s1
HIST1H2AC	histone cluster 1, H2ac	Human	Hs00374312_s1
HIST1H2AH	histone cluster 1, H2ah	Human	Hs00544732_s1
HIST1H2AJ	histone cluster 1, H2aj	Human	Hs04191486_s1
HIST1H2AM	histone cluster 1, H2am	Human	Hs01920904_s1
HIST1H2BB	histone cluster 1, H2bb	Human	Hs00606684_s1
HIST1H2BG	histone cluster 1, H2bg	Human	Hs00374317_s1
HIST1H2BH	histone cluster 1, H2bh	Human	Hs00374322_s1
HIST1H3B	histone cluster 1, H3b	Human	Hs00605810_s1
HIST1H3D	histone cluster 1, H3d	Human	Hs00371415_s1
HIST1H4C	histone cluster 1, H4c	Human	Hs00543883_s1
HIST1H4E	histone cluster 1, H4e	Human	Hs00374346_s1
HIST1H4L	histone cluster 1, H4l	Human	Hs00361930_s1
HIST2H2AB	histone cluster 2, H2ab	Human	Hs00602439_s1
HIST2H2AC	histone cluster 2, H2ac	Human	Hs00543838_s1
NANOG	Nanog homeobox	Human	Hs02387400_g1
OCT4	POU class 5 homeobox 1	Human	Hs00999632_g1
REX1	ZFP42 zinc finger protein	Human	Hs00399279_m1
SOX2	Sex determining region Y-box 2	Human	Hs01053049_s1
TET1	tet methylcytosine dioxygenase1	Human	Hs00286756_m1
TET2	tet methylcytosine dioxygenase2	Human	Hs00325999_m1
TET3	tet methylcytosine dioxygenase3	Human	Hs00379125_m1
THY1	Thy-1 cell surface antigen	Human	Hs00174816_m1

Table 5: List of primers used for quantitative PCR analysis.

3.10 Protein Analysis

3.10.1 ELISA

Cells were detached from the culture dish with trypsin and collected by centrifugation. They were then washed three times with cold PBS (1x) and subjected to ultrasonication. After centrifugation and dilution, samples were used for ELISA assay using the commercially available

Enzyme-linked Immunoassorbent Assay Kit (MyBioSource) for the specific histone (Table 6), according to the manufacturer's protocol. Optical density (O.D.) measurements were done using a Multiskan™ FC Microplate Photometer (Thermo Fisher) at the wavelength of 450 nm. Protein concentration in the samples was determined by comparing the O.D. of the samples to the standard curve.

PROTEIN	DESCRIPTION	COMPANY	CATALOG N°
H2AX	H2A histone family, member X(H2AFX), ELISA Kit	MyBioSource	MBS9335115
H2A.Z	H2A histone family, member Z(H2AFZ), ELISA Kit	MyBioSource	MBS2025145
H1.3	Histone cluster 1, H1d (HIST1H1D), ELISA Kit	MyBioSource	MBS914357
H2A type 1-B/E	Histone Cluster 1, H2ab (HIST1H2AB), ELISA Kit	MyBioSource	MBS9321468
H2A type 1-C	Histone cluster 1, H2ac (HIST1H2AC), ELISA Kit	MyBioSource	MBS9333153
H2A type 1-H	Histone cluster 1, H2ah (HIST1H2AH), ELISA Kit	MyBioSource	MBS9320153
H2A type 1-J	Histone cluster 1, H2aj (HIST1H2AJ), ELISA Kit	MyBioSource	MBS9343145
H2B type 1-B	Histone cluster 1, H2bb (HIST1H2BB), ELISA Kit	MyBioSource	MBS9317197
H2B type 1-C/E/F/G/I	Histone cluster 1, H2bc (HIST1H2BG), ELISA Kit	MyBioSource	MBS9324014
H2B type 1-H	Histone cluster 1, H2bh (HIST1H2BH), ELISA Kit	MyBioSource	MBS9319723
H3.1	Histone cluster H3.1 (HIST1H3A), ELISA Kit	MyBioSource	MBS937951
H4	Histone H4 (HIST1H4A), ELISA Kit	MyBioSource	MBS2884038
H2A type 2-B	Histone cluster 2, H2ab (HIST2H2AB), ELISA Kit	MyBioSource	MBS9338734
H2A type 2-C	histone cluster 2, H2ac (HIST2H2AC), ELISA Kit	MyBioSource	MBS900872

Table 6: List of ELISA kits used for quantification of histone protein levels

3.10.2 Immunocytochemistry

Cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma), washed three times in PBS and permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma) in PBS. Samples were treated with blocking solution containing 5% (vol/vol) BSA and 5% (vol/vol) not immune serum in PBS. Primary antibodies were incubated over-night at +4°C and their working dilutions are listed in Table 7. Cells were incubated with suitable secondary antibodies (Alexa Fluor) for 45 min. Nuclei were stained with 4',6-diamidino-2- phenylindole (DAPI, Sigma). Samples were observed under a Nikon Eclipse TE200 and Zeiss Apotome. Cells were fixed, permeabilized and treated with blocking solution as described above. When cells formed spherical structures, these were dissociated with 0.25% trypsin-EDTA and Accutase (Innovative Cell Technologies) at 37°C for 10–15 min, and attached to slides, using a cytocentrifuge (Cytospin 4, Thermo Shandon).

The number of immune-positive cells was counted in 10 randomly selected fields at 200× total magnification. A minimum of 500 cells were counted in three independent replicates. The number of positively stained cells was expressed as a percentage of the total cell counted.

Antibody	Host species	Company	Cat. N°	Application	Dilution
C-PEPTIDE	Rabbit	Abcam	ab14181	ICC	1:100
GLUCAGON	Mouse	Abcam	ab10988	ICC	1:100
PHALLOIDIN	---	Sigma	P1951	ICC	32µg/ml
SOMATOSTATIN	Rabbit	Abcam	ab103790	ICC	1:100
VIMENTIN	Mouse	Abcam	ab8978	ICC	5µg/ml
YAP	Rabbit	Cell Signaling	14074	ICC	1:100

Table 7: List of antibodies and working dilutions used for immunocytochemical (ICC) analysis.

3.10.3 Western Blotting

Cells were lysed and constitutive proteins were extracted using a ReadyPrep Protein Extraction Kit (Bio-Rad). Nuclear extracts from the cells were isolated using the NXtract CelLytic NuCLEAR Extraction Kit (Sigma). Protein concentration was assessed by Coomassie Blue-G Dye-binding method. 100 µg of proteins were resuspended in sample buffer (1:1) consisting of 4% (wt/vol) SDS, 10% 2-mercaptoethanol, 20% (wt/vol) glycerol, 0.004% bromophenol blue, and 0.125 M Tris–HCl at pH 6.8. Equal amounts of total protein were loaded and electrophoresed on a SDS-polyacrylamide gels. Proteins were then transferred onto 0.45 µm pore size nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences) and probed with primary antibodies listed in Table 8. Protein bands were visualized by the WesternBreeze chemiluminescent kit. Densitometric analysis was performed with Quantity One 1-D analysis software (Bio-Rad).

Antibody	Host species	Company	Cat. N°	Application	Dilution
B-ACTIN	Rabbit	Abcam	AB8227	WB	1:1000
SMAD2	Rabbit	Novus Biologicals	NB100-56462	WB	1:500
TAZ/WWTR1	Rabbit	Novus Biologicals	NB110-58359	WB	1:5000
GAPDH	Mouse	Abcam	ab8245	WB	1:1000
LATS1	Rabbit	Cell Signaling	9153	WB	1:1000
MOB1	Rabbit	Cell Signaling	3863	WB	1:1000
pLATS1	Rabbit	Cell Signaling	9157	WB	1:1000
pMOB	Rabbit	Cell Signaling	8699	WB	1:1000
pYAP	Rabbit	Cell Signaling	13008	WB	1:1000

Table 8: List of antibodies and working dilutions used for western blotting (WB) analysis.

3.11 siRNA Transfection

siRNA transfection was performed using Lipofectamine RNAi-MAX in antibiotics-free Opti-MEM® medium according to manufacturer's instructions. Validated Stealth RNAi siRNA used in the present study were specific for TAZ/WWTR1 (Thermo Fisher) and TET2 (Santa Cruz).

3.12 In Vitro Functional Analysis

EpiCC functional activity was evaluated measuring insulin release in supernatant. Cells were stimulated for 1 hour with 20mM and 5 mM D-glucose (final concentration) in basal medium without ITS. Glucose-dependent insulin release was assessed with Insulin ELISA (Mercodia) following the manufacturer's instruction. Values were normalized against DNA content of the stimulated cells. DNA was extracted with PureLink® Genomic DNA Kits (Thermo Fisher Scientific) and its concentration was assessed using NanoDrop8000 (Theromoscientific).

3.13 Statistical Analysis

Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant.

CHAPTER 4

RESULTS

4. RESULTS

4.1 Epigenetic erasing: characterization of the early stages

4.1.1 5-aza-CR induces global DNA methylation changes

Exposure to 5-aza-CR induced a significant decrease of global DNA methylation (Fig. 4). In particular, human fibroblasts (T0) treated for 18 hours with the epigenetic modifier (Post 5-aza-CR), showed a significant decrease in DNA methylation. Cells maintained comparable methylation levels when they were cultured in ESC medium for 24 and 48 hours respectively (24h ESC; 48h ESC). No methylation changes were observed in cells that were cultured in the ESC medium, without a previous exposure to 5-aza-CR (MEDIUM ESC), indicating that the medium alone is not able to affect DNA methylation levels in differentiated cells.

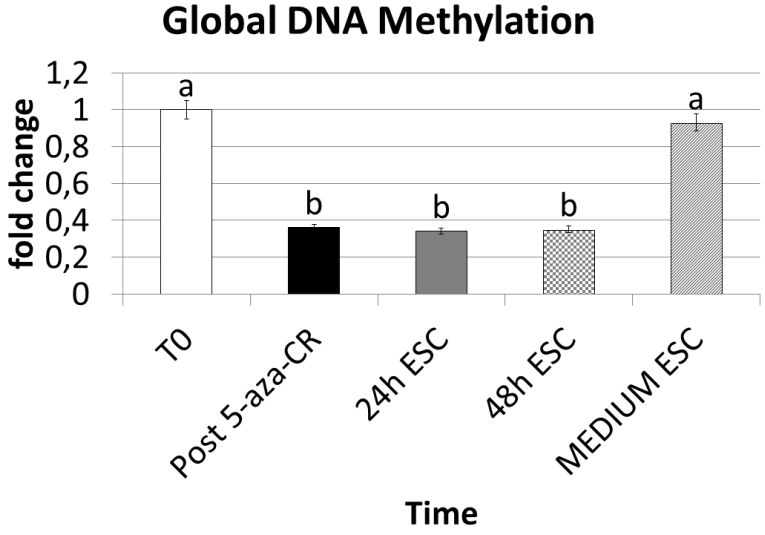


Fig. 4: Global DNA methylation of cells exposed to 5-aza-CR and to ESC medium for 24 and 48 hours. Different superscripts denote significant differences between groups ($P<0.05$).

4.1.2 Human fibroblasts acquire a high plasticity state after epigenetic erasing with 5-aza-CR

5-aza-CR induced the onset of pluripotency gene expression [POU class 5 homeobox 1 (OCT4), sex determining region Y-box 2 (SOX2), Nanog homeobox (NANOG), and ZFP42 zinc finger protein (REX1)] (Fig. 5a, Post 5-aza-CR). ESC medium maintained high expression levels of all these genes for the first 2 days of culture. Methylation decrease was also accompanied by the up-regulation of the ten-eleven translocation 2 (TET2) gene (Fig. 5b, Post 5-aza-CR). However, the modifier did not affect all the TET family members, but rather it exerted a distinct effect on TET2 transcription, while no significant effect was observed for TET1 and TET3.

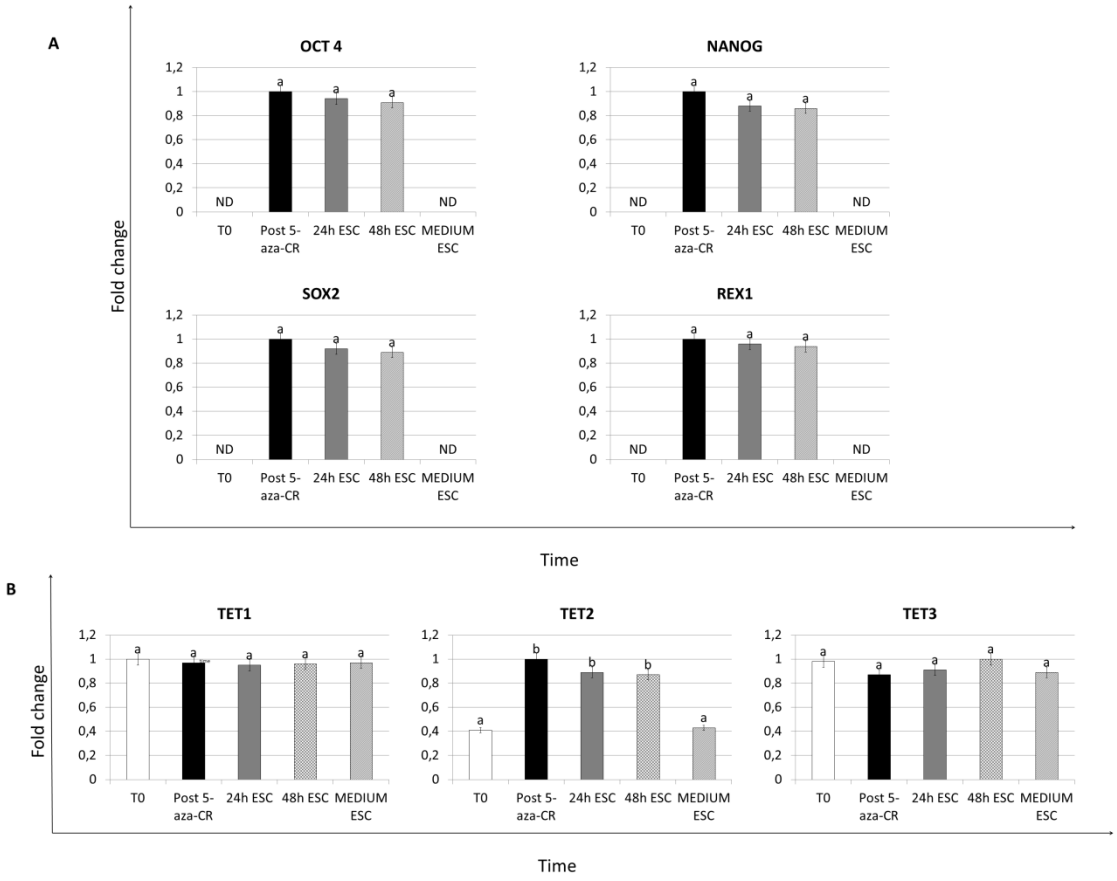


Fig. 5: (**A**) 5-aza-CR treatment induced the onset of pluripotency gene expression, increasing the transcription of OCT4, SOX2, NANOG and REX1 (Post 5-aza-CR). Culture in ESC medium for two days (48h ESC) maintained upregulated expression of all the genes. (**B**) Effect of 5-aza-CR on TET gene family transcription. The modifier upregulated TET2, while leaving the other TET members unchanged. Different superscripts denote significant differences between groups ($P<0.05$).

4.1.3 TET2 upregulation results in 5-Formylcytosine and 5-Carboxylcytosine increase

5-aza-CR induced TET2 upregulation resulted in significant increase of 5fC and 5caC (Fig. 6). These increased levels were also present in cells cultured in ESC medium for 24 h and 48 h (24h ESC; 48h ESC). No changes were observed in cells that were cultured in the EESC medium, without a previous exposure to 5-aza-CR (MEDIUM ESC).

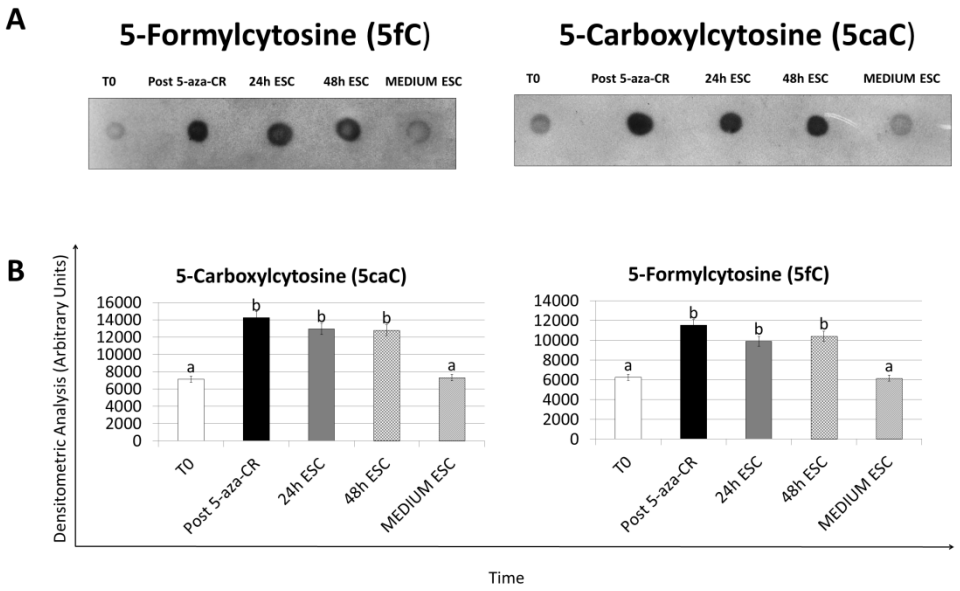


Fig. 6: (**A**) DNA dot blot analysis of 5-Formylcytosine (5fC) and 5-Carboxylcytosine (5caC) in cells exposed to 5-aza-CR and to ESC medium for 24 and 48 hours. (**B**) Histogram represents dot-blot signal intensity quantified by densitometric analysis. Different superscripts denote significant differences between groups ($P<0.05$).

4.1.4 TET2 downregulation affects 5-aza-CR induced global DNA demethylation

Global DNA demethylation induced by 5-aza-CR was decreased in response to TET2 RNA silencing (Fig. 7). Significant differences in methylation levels were also detected in cells cultured in ESC medium (24h ESC; 48h ESC).

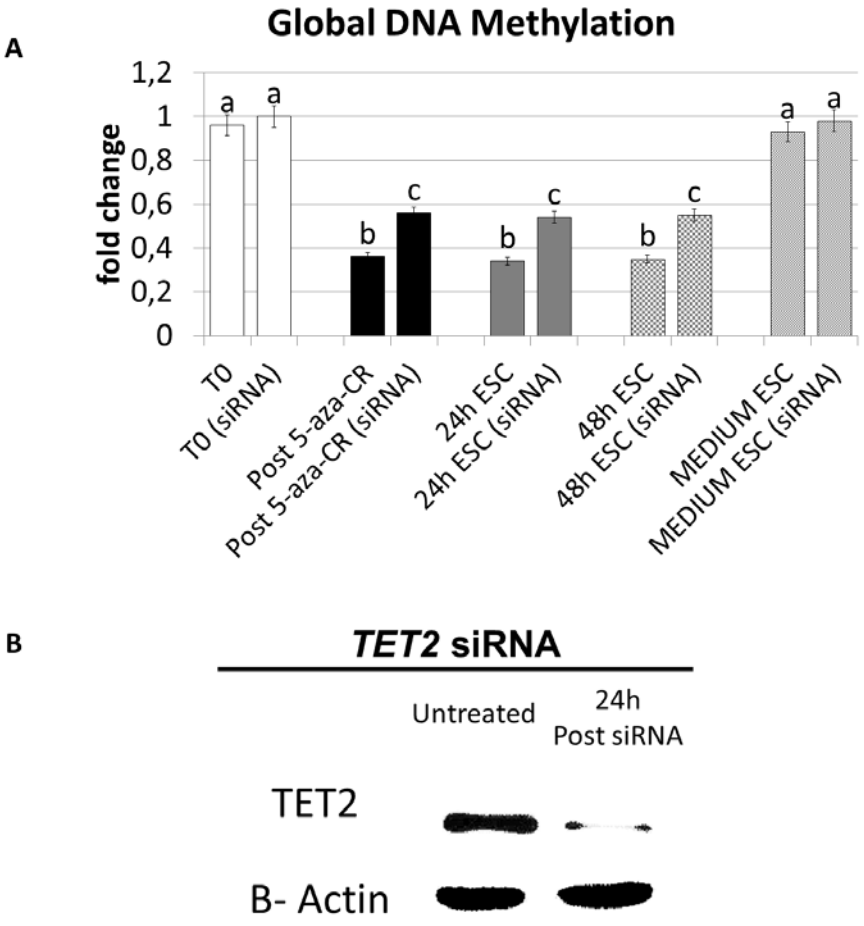


Fig. 7: (**A**) Global DNA methylation of cells, before and after siRNA transfection, exposed to 5-aza-CR and to ESC medium for 24 and 48 hours. Different superscripts denote significant differences between groups ($P<0.05$). (**B**) TET2 silencing was demonstrated by the decrease of protein level after siRNA treatment

4.1.5 5-aza-CR modulates expression of histones and histone acetylating enzymes in human fibroblasts

Expression analysis demonstrated 5-aza-CR ability to significantly upregulate the transcription of histones within the 1, 2, 3 and 4 families, and their encoded protein (Fig. 8). Histones showed a significant increase post 5-aza-CR and persisted at high levels in cells cultured in ESC medium for 24 h and 48 h (24h ESC; 48h ESC). No significant change was detected in cells cultured in ESC medium, without prior exposure to 5-aza-CR.

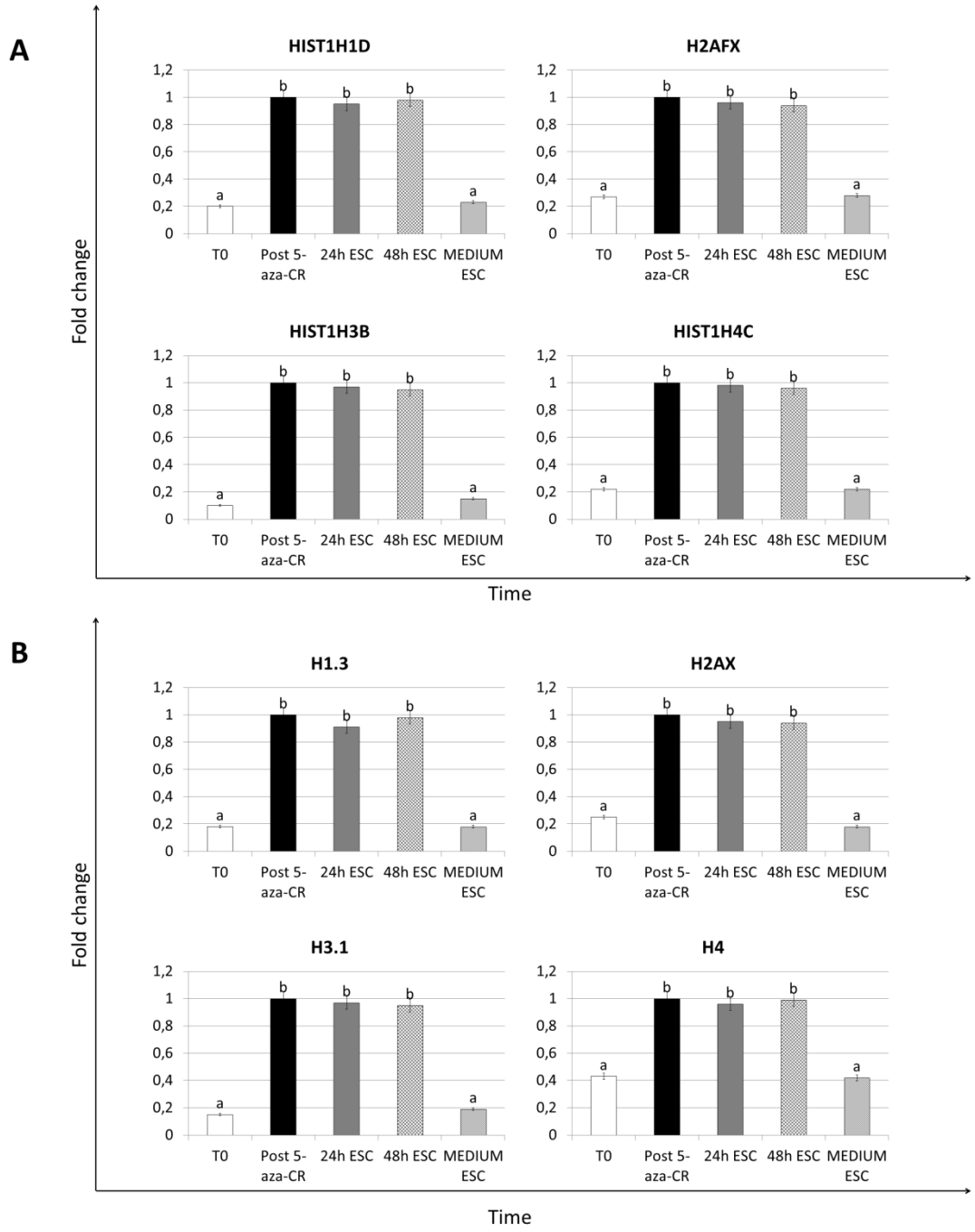


Fig. 8: (**A**) Histone transcription changes in adult human skin fibroblasts exposed to 5-aza-CR and cultured in ESC medium for 24 and 48 hours. (**B**) Histone protein levels in adult human skin fibroblasts exposed to 5-aza-CR and cultured in ESC medium for 24 and 48 hours. Different superscripts denote significant differences between groups ($P<0.05$).

Moreover, a parallel downregulation of a fibroblast specific gene (COL6A3) was detected. Furthermore, increased expression of histones was supported by the upregulation of the acetylating enzyme Histone acetyltransferase 1 (HAT1) and by the decreased expression of the Histone deacetylase enzyme 1 (HDAC1) (Fig. 9).

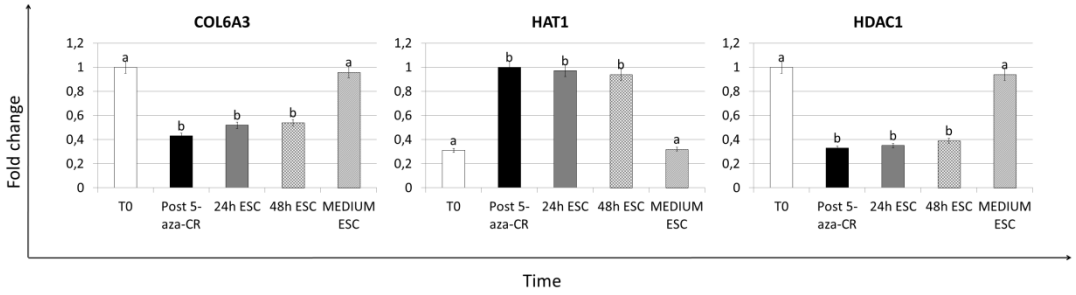


Fig. 9: Exposure to the 5-aza-CR resulted in downregulation of COL6A3. Furthermore, a sharp upregulation of HAT1, and a decrease of HDAC1 was detected after exposure to the epigenetic modifier. Different superscripts denote significant differences between groups ($P<0.05$).

4.1.6 5-aza-CR induces a high plasticity-related cell morphology and chromatin organization

T0 cells displayed a typical fibroblast morphology and were characterized by nuclei with condensed areas close to the nuclear membrane and large vacuole. The perinuclear compartment contained rough endoplasmic reticulum cisternae (RER), widely scattered mitochondria, vesicles and electron dense granules (Fig. 10 panels a-c).

Count in semi-thin sections demonstrated that 86,31 ± 4,13 % cells remarkably changed their morphology after exposure to 5-aza-CR, showing a reduced cell size and either few short microvilli on the plasma membrane or a smooth cell surface. At higher magnification, in the electronlucent cytoplasm, I could detect large roundish or lobed nuclei that displayed a global chromatin decondensation, mitochondria, RER, empty or filled vacuoles (Fig. 10 panel d), as well as numerous lysosomes and autophagosomes (Fig. 10 panel e). Simultaneously with the autophagic phenomena, a cytoplasmic peripheral ring, less dense and organule-free, became visible (Fig. 10 panel f), which is likely to be responsible for a final reduction in cell size. These morphological features, that are typically related to a high plasticity phenotype, were maintained by 84,24 ± 5,05 % and 83,74 ± 5,52 % of cells cultured in ESC medium for 24 h and 48 h respectively, as confirmed at higher magnification (Fig. 10 panels g-i), possibly suggesting that, once acquired, the increased plasticity state can be sustained by signals derived from medium.

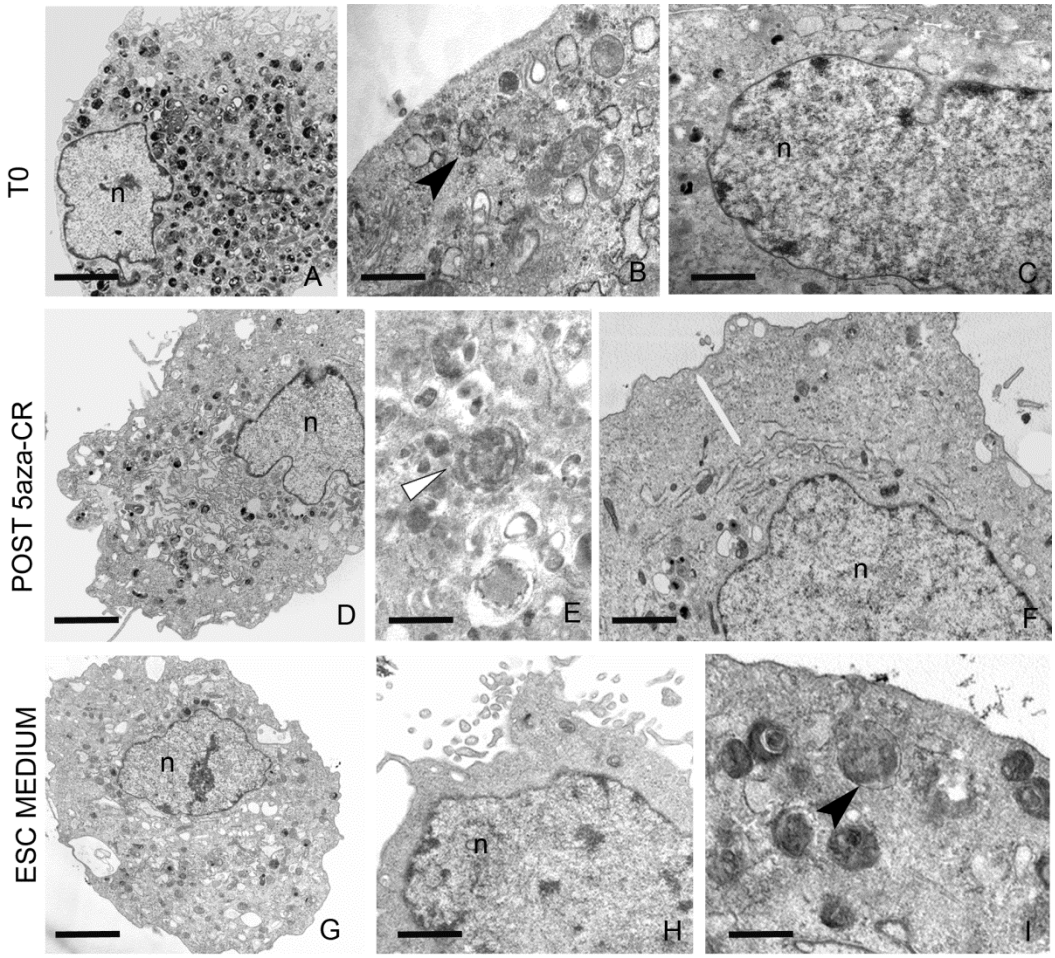


Fig. 10: 5-aza-CR induced cell plasticity is unravelled by morphological changes. The distinct fibroblastoid morphology (panels a-c) change after exposure to 5-aza-CR (panels d-f). Post 5-aza-CR cells cultured in ESC medium (panels g-i) maintain the undifferentiated phenotype (n: nucleus; autophagosomes: arrowhead). Scale bars: a, 2.5μm; b,1μm; c,1μm; d, 2μm; e,0.8μm; f, 2μm; g, 4μm; h, 1.4μm; i, 0.6μm.

4.2 Effect of 3D microenvironment in the induction and maintenance of high plasticity

In the experiments here described I used PTFE micro-bioreactor to promote 3D cell rearrangement and maintain high plasticity in epigenetically erased fibroblasts.

4.2.1 The PTFE micro-bioreactor promotes 3D cell rearrangement in epigenetically erased human fibroblasts

Human dermal fibroblasts displayed a dramatic change in their morphology, when exposed to 5-aza-CR, regardless of the culture system used. In particular, fibroblasts plated on standard plastic dishes as well as those encapsulated in PTFE became rounded, with large and granulated nuclei. However, while plastic cells retained a monolayer distribution for the entire length of the experiments, cells encapsulated in PTFE formed 3D spherical structures (Fig. 11), that were stably maintained for the entire length of the experiments.

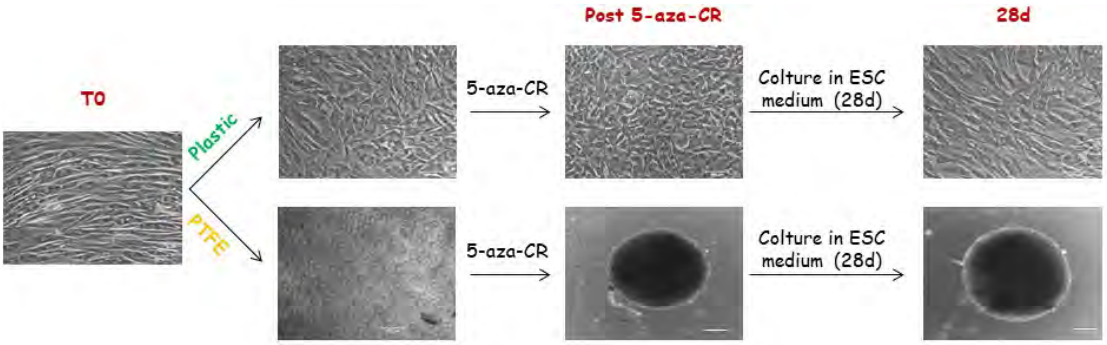


Fig. 11: The PTFE micro-bioreactor promotes 3D cell rearrangement and induces ultrastructural modifications in epigenetically erased human fibroblasts. (Scale bar, 100 μm).

4.2.2 The PTFE micro-bioreactor induces ultrastructural modifications in epigenetically erased human fibroblasts

The fine structure of cells encapsulated in PTFE was analyzed by SEM and TEM starting from T0, after 5-aza-CR exposure and up to 28 days. 5-aza-CR treatment induced changes in morphology. Cells lost their characteristic spindle shape (Fig. 12a, b) and became ovoidal (Fig. 12c,d), showing a cytoplasm filled with autophagic vacuoles (Fig. 12d).

Starting from 24 hours after 5-aza-CR treatment and for the entire length of the experiments, cells within 3D structures maintained a roundish shape with a high nucleus to cytoplasm ratio, few organelles and large intercellular spaces (Fig. 12e, f). Their cytoplasm contained free ribosomes, polyribosomes, elongated tubular mitochondria, well-developed rough endoplasmic reticulum (RER), Golgi complexes, few reticulum cisternae and lipid droplets. Furthermore, cells showed nuclei with euchromatin and large reticulated nucleoli that are typical of ESC 21. Occasionally, scattered cells with cytoplasm occupied by large autophagic vacuoles were visible (Fig. 12f).

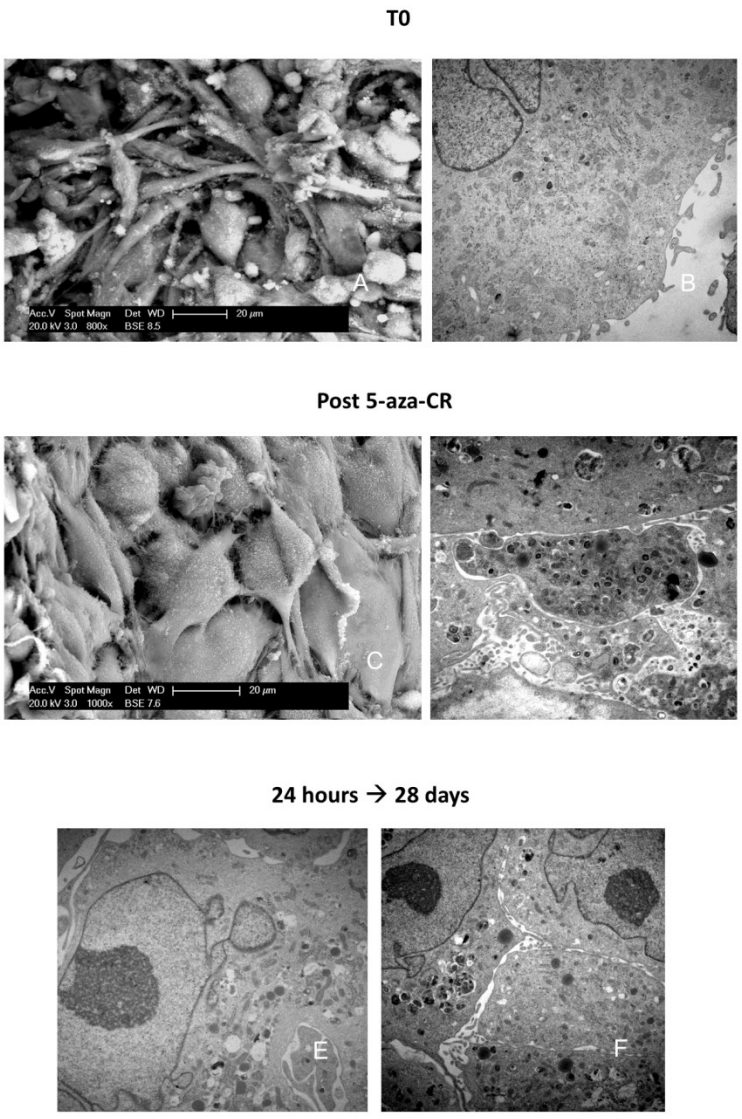


Fig. 12: The PTFE micro-bioreactor induces ultrastructural modifications in epigenetically erased human fibroblasts.

4.2.3 The PTFE micro-bioreactor enhances 5-aza-CR demethylating effect in epigenetically erased human fibroblasts

5-aza-CR caused a significant decrease in global DNA methylation in fibroblasts belonging to the two experimental Groups (Fig. 13). However, PTFE cells exhibited significantly lower DNA methylation levels compared to those on plastic, at all time points analyzed, and remained significantly hypomethylated for the entire length of the experiments. In contrast, cells plated on plastic dishes, although displayed a decreased methylation for 72 hours, slowly increased and returned comparable to untreated fibroblasts (T0) by day 7 of culture.

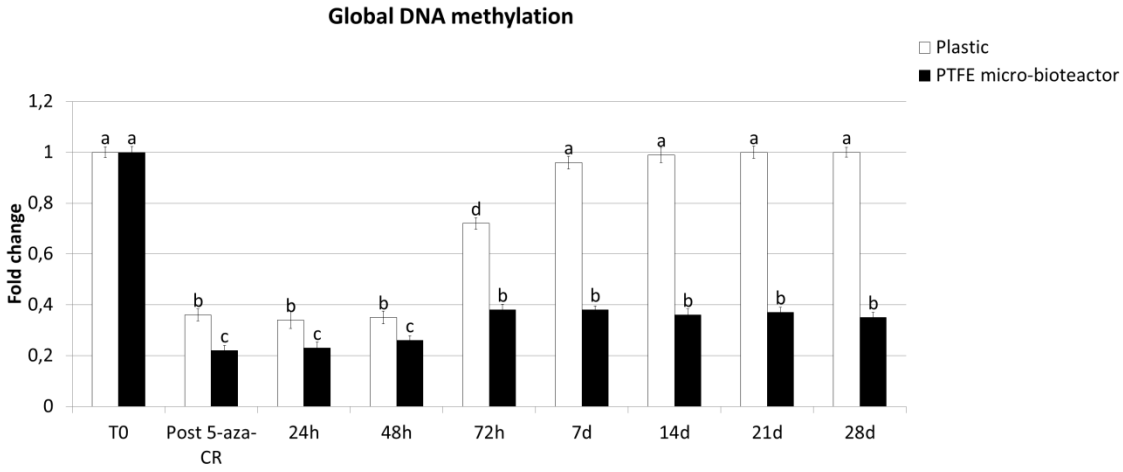


Fig. 13: The PTFE micro-bioreactor enhances the demethylating effect of 5-aza-CR. Different superscripts denote significant differences between groups ($P<0.05$).

4.2.4 The PTFE micro-bioreactor boosts pluripotency gene transcription and maintains long-term high plasticity in epigenetically erased human fibroblasts

Morphological changes and methylation decrease were accompanied by the onset of the expression of pluripotency-related genes, namely POU class 5 homeobox 1 (OCT4), Nanog homeobox (NANOG), ZFP42 zinc finger protein (REX1), and sex determining region Y-box 2 (SOX2), which were undetectable in untreated fibroblasts (T0; Fig. 14). Cells in PTFE showed significantly higher expression levels of these genes, when compared to cell on plastic. Furthermore, encapsulation in the micro-bioreactor maintained high expression levels for the entire length of the experiments. In contrast, cells on plastic transcribed for pluripotency-related markers until 72 hours and turned down their expression by day 7 of culture.

5-aza-CR treatment also induced the up-regulation of TET2, epithelial cell adhesion molecule (EPCAM), and cadherin 1 (CDH1) genes in both groups (Fig. 14). More in detail, PTFE cells showed significantly higher expression levels of these genes, compared to those on plastic. Furthermore, TET2, EPCAM, and CDH1, expression profile paralleled that described above for pluripotency- related genes.

In agreement with these observations, I detected a significant downregulation of a fibroblast specific gene (Thy-1 cell surface antigen, THY1) in micro-bioreactor cells for the entire length of the experiments. Plastic group cells showed, in contrast, decreased levels for the first 72 hours of culture, and returned to values comparable to those of untreated fibroblasts by day 7 (Fig. 14).

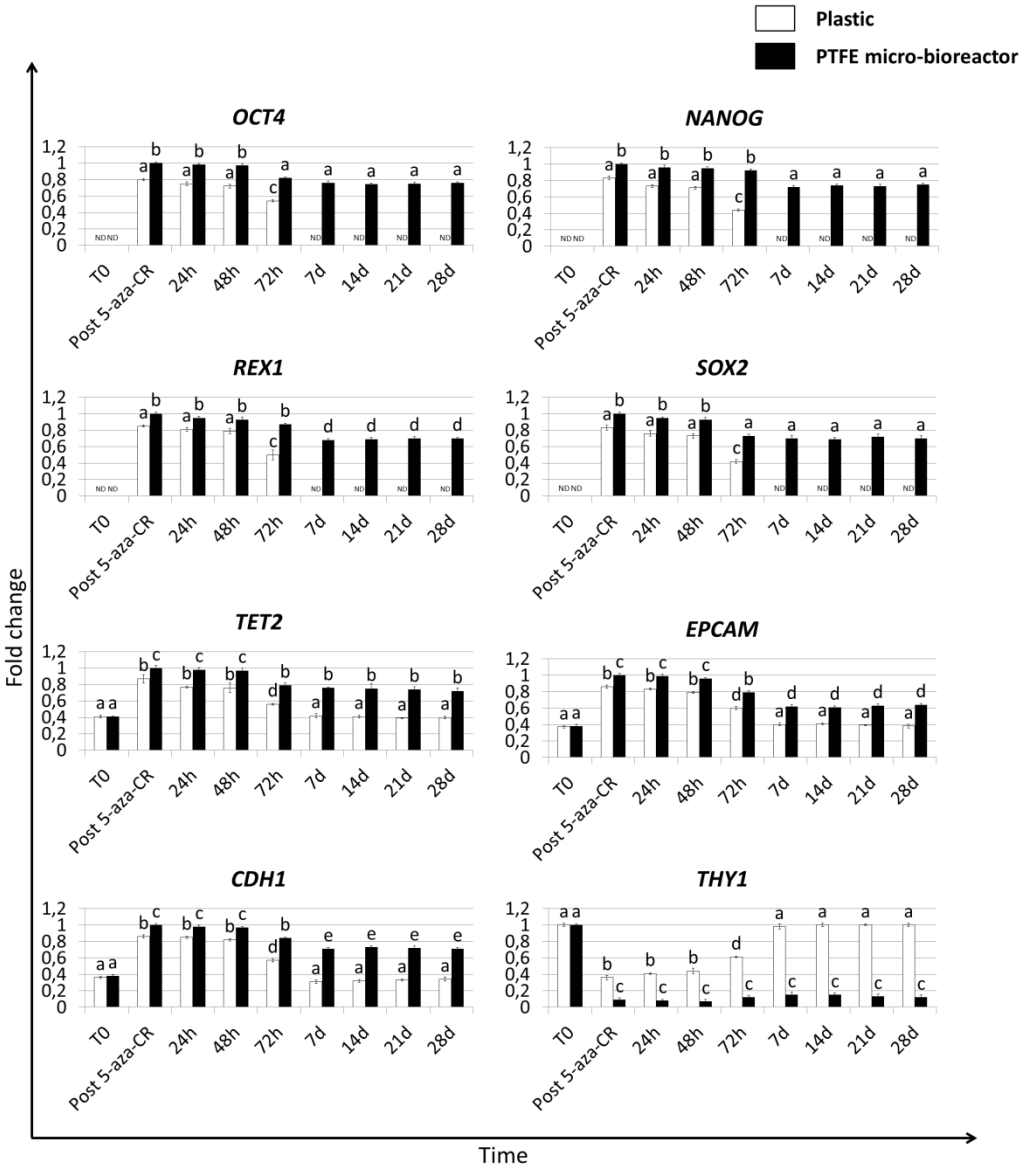


Fig. 14: The PTFE micro-bioreactor boosts pluripotency gene transcription and maintains long-term high plasticity in epigenetically erased human fibroblasts. Different superscripts denote significant differences between groups ($P<0.05$).

4.2.5 The PTFE micro-bioreactor activates the Hippo signalling pathway to maintain high plasticity in epigenetically erased human fibroblasts

Fibroblasts exposed to 5-aza-CR (Post 5-aza-CR) showed TAZ nuclear accumulation both in cells plated onto plastic plates (Fig. 15a) and in those encapsulated in PTFE (Fig. 15b). TAZ nuclear confinement was however lost by cells on plastic, with the molecule relocating to the cytoplasm, by day 7 of culture (Fig. 15a). In contrast, 3D cell confinement of PTFE cells encouraged TAZ nuclear retention, that was stably maintained for the entire length of the experiments (Fig. 15b). Interestingly, TAZ localization was mirrored by a parallel compartmentalization of SMAD2. More in detail, cells displaying nuclear localized TAZ showed SMAD2 nuclear accumulation, while cells with cytoplasmic TAZ exhibited SMAD2 cytoplasmic distribution (Fig. 15 a,b).

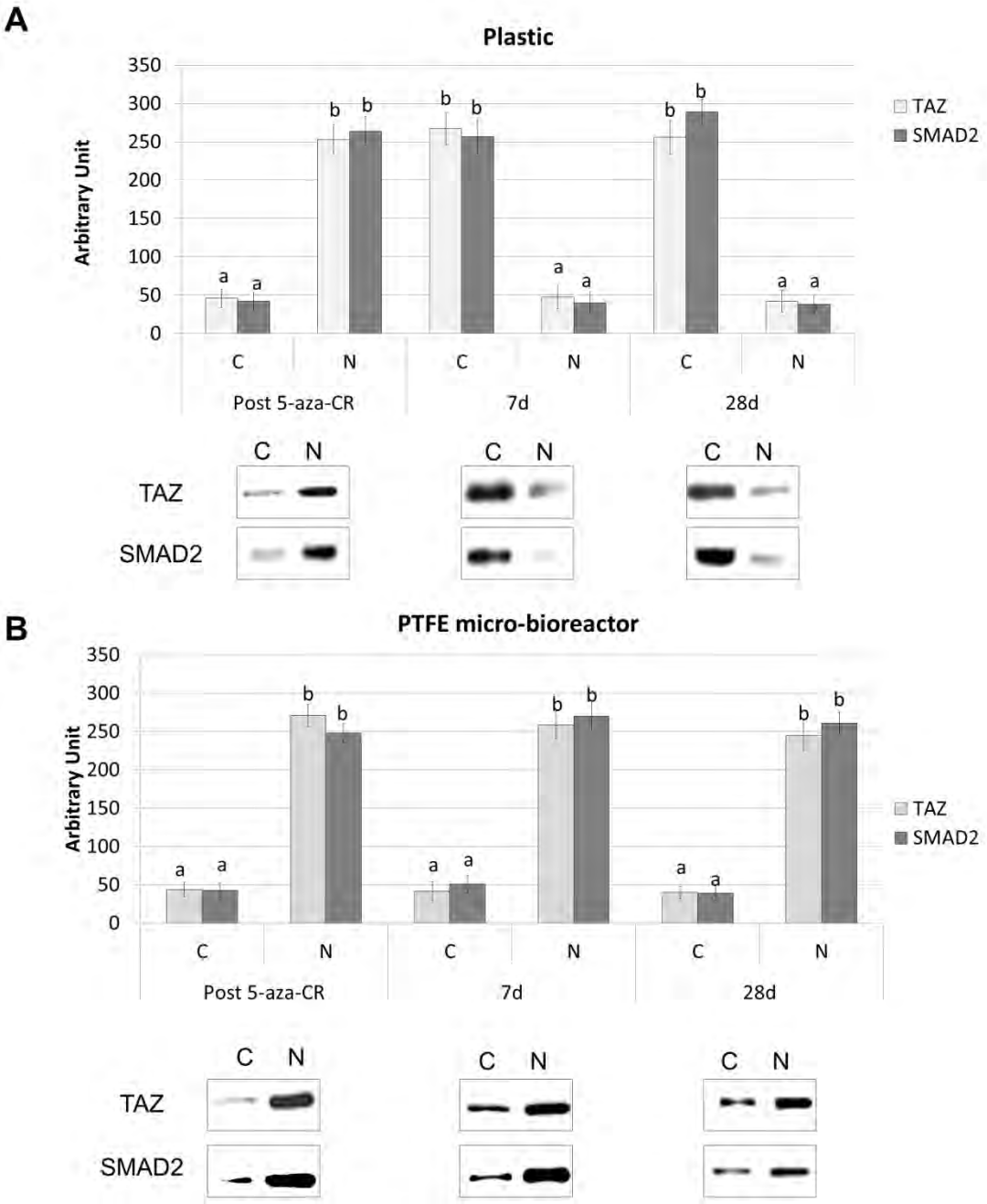


Fig. 15: Activation of the Hippo signaling pathway in PTFE encapsulated cells. Western blots for TAZ and SMAD2 proteins in epigenetically erased fibroblasts, plated on standard plastic dishes (**A**) or encapsulated in PTFE (**B**). Different superscripts denote significant differences between groups ($P<0.05$).

In addition, siRNA knockdown of TAZ caused inhibition of SMAD2 nuclear accumulation (Fig. 16a) as well as the loss of pluripotency marker transcription (Fig. 16b), suggesting the direct involvement of TAZ in SMAD2 shuttling and high plasticity maintenance, boosted by the PTFE 3D microenvironment.

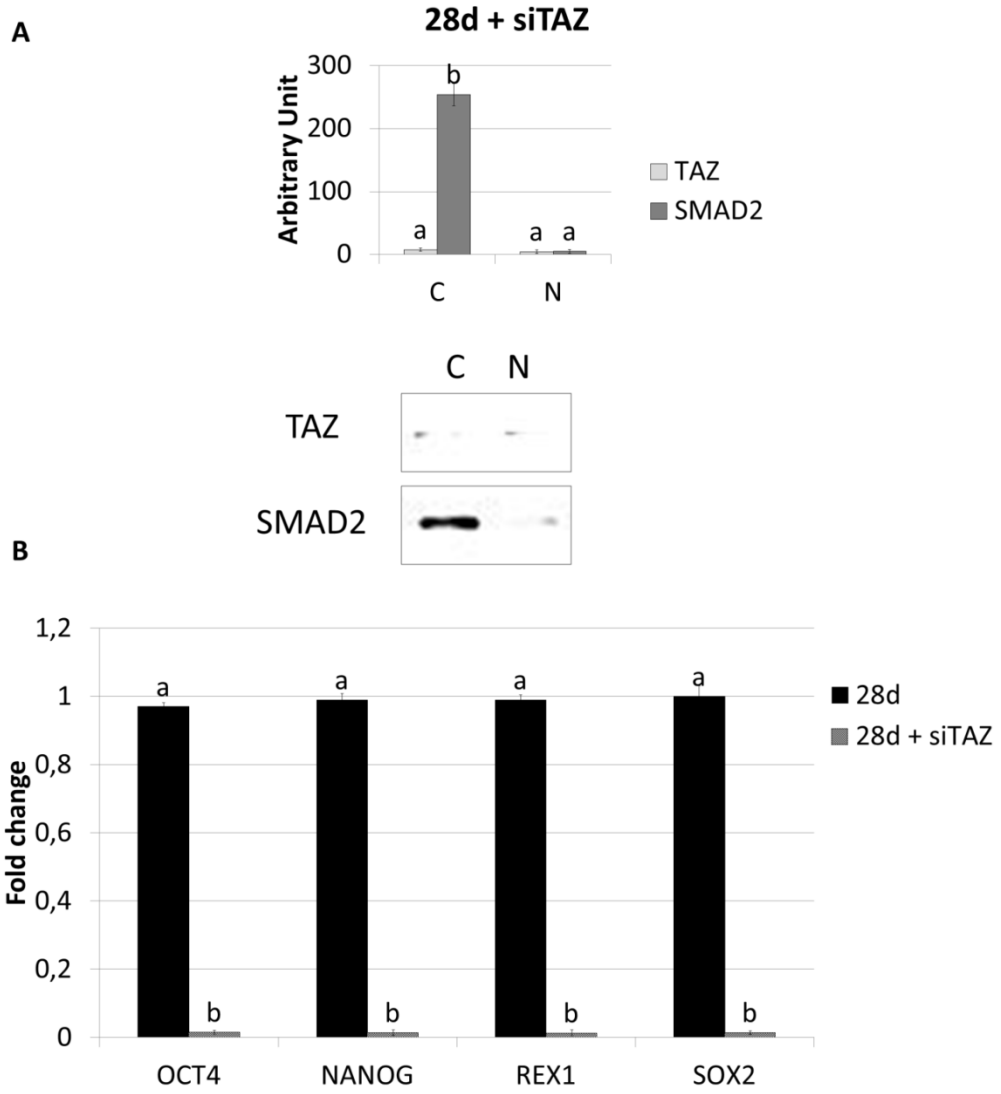


Fig. 16: **(A)** Western blots for TAZ and SMAD2 proteins in PTFE encapsulated cells at day 28 of culture, after TAZ siRNA transfection (28d + siTAZ). Different superscripts denote significant differences between groups ($P<0.05$). Representative Western blots for each protein are also shown. **(B)** Pluripotency-related gene (OCT4, NANOG, REX1, and SOX2) expression levels in PTFE cells at day 28 of culture (28d) and after TAZ siRNA transfection (28d + siTAZ). Different superscripts denote significant differences between groups ($P<0.05$).

4.3 Effect of low-stiffness substrate in the induction of epigenetic erasing and pancreatic differentiation

In the experiments here described 1kPa PAA gel was used to boost epigenetic erasing and pancreatic differentiation .

4.3.1 Isolation and characterization of murine dermal fibroblasts

Fibroblasts obtained from dorsal skin biopsies grew out of the original explants forming a monolayer (Fig. 17). They displayed a standard elongated morphology with a uniform immune-positivity for the fibroblast specific marker vimentin (VIM) and a complete absence of the pancreatic markers (C-PEP, GLUC and SOM) (Fig. 17).

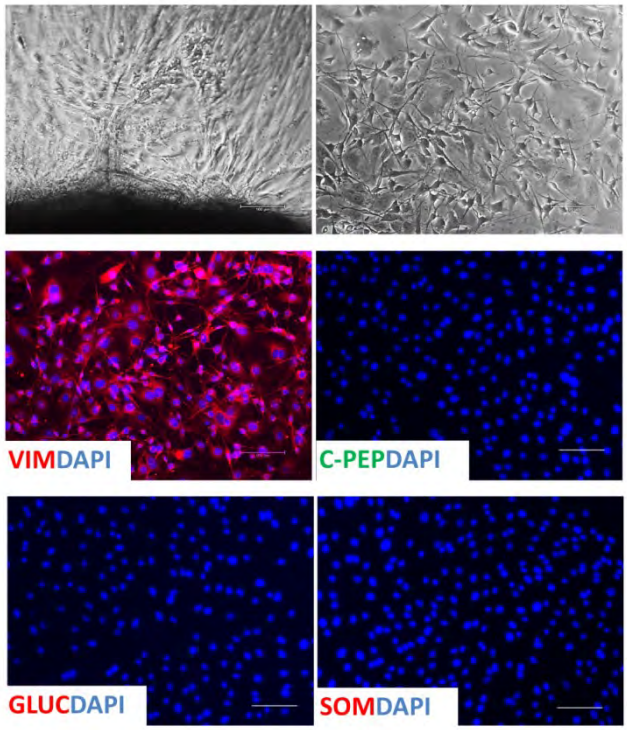


Fig. 17:. Characterization of murine skin fibroblasts. Scale bars: 100 μm.

4.3.2 Effect of matrix elasticity on DNA methylation changes after 5-aza-CR exposure

After exposure to 5-aza-CR, both cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg) showed a significant decrease in global DNA methylation compared to untreated fibroblasts seeded on plastic plates (T0p) and PAA gels (T0g) (Fig. 18). However, Post 5-aza-CRg cells exhibited a significantly lower methylation level compared to that of Post 5-aza-CRp. No methylation changes were observed in cells maintained in medium without 5-aza-CR (w/o 5-aza-CRp; w/o 5-aza-CRg), indicating that the substrate alone is not able to affect DNA methylation levels in 18 hours.

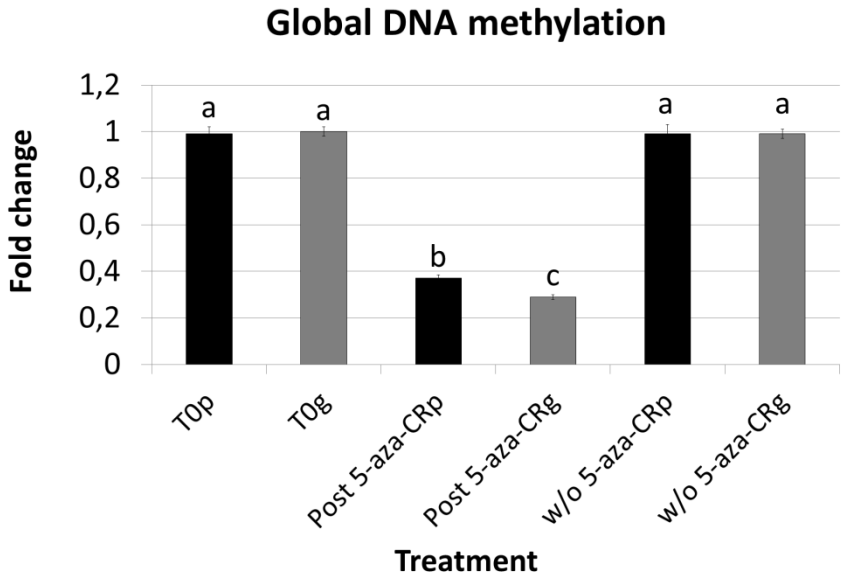


Fig. 18: Global DNA methylation changes in cells plated on plastic dishes and PAA gels and exposed to 5-aza-CR. Different superscripts denote significant differences between groups ($P<0.05$).

4.3.3 Effect of matrix elasticity on cell plasticity after 5-aza-CR exposure

5-aza-CR induced methylation changes were accompanied by the up-regulation of the ten-eleven translocation 2 (*Tet2*) and histone aminotransferase 1 (*Hat1*) genes. Moreover, a parallel decreased in the histone deacetylase enzyme 1 (*Hdac1*) transcription levels was detected (Fig. 19a). Increase of cell plasticity was also shown by the onset of pluripotency genes, namely POU class 5 homeobox 1 (*Oct4*), nanog homeobox (*Nanog*), ZFP42 zinc finger protein (*Rex1*), and sex determining region Y-box 2 (*Sox2*), which were undetectable in untreated fibroblasts (T0p, T0g; Fig. 19b) as well as in cells not exposed to 5-aza-CR (w/o 5-aza-CRp, w/o 5-aza-CRg; Fig. 19b). Interestingly, Post 5-aza-CRg cells showed higher expression levels of all the genes analyzed, when compared to those of Post 5-aza-CRp. No expression changes were observed between cells plated onto plastic and PAA gels not subjected to 5-aza-CR exposure (w/o 5-aza-CRp, w/o 5-aza-CRg), indicating that the substrate alone is not able to affect gene expression levels in 18 hours.

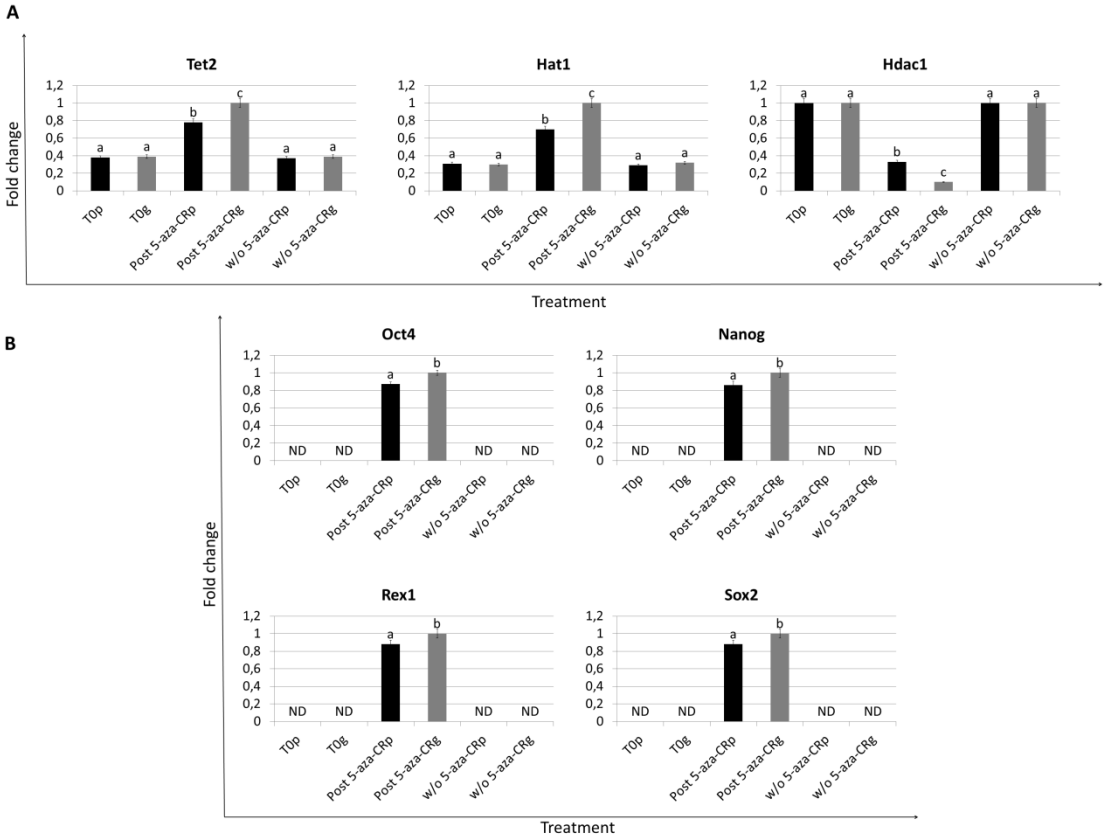


Fig. 19: (**A**) 5-aza-CR treatment induce the up-regulation of Tet2 and Hat1 genes, and a decrease of Hdac1 transcription. (**B**) After 5-aza-CR treatment, cells also show the onset of pluripotency genes, namely Oct4, Nanog, Rex1 and Sox2. Different superscripts denote significant differences between groups ($P<0.05$).

4.3.4 Effect of matrix elasticity on EpiCC morphological changes

No differences in cell morphology were observed between untreated fibroblasts plated onto plastic (T0p; Fig. 20a) and PAA gels (T0g; Fig. 20b). Cells appeared large, flat and elongated, regardless of the support used. By contrast, morphological changes become evident at the end of the pancreatic induction, in cells grown on plastic (EpiCCp, Fig. 20a) as well as in those differentiated on gel (EpiCCg, Fig. 20b). Both cell populations lost the typical elongated shape of untreated fibroblasts and acquired an epithelioid morphology. However, EpiCCp mainly kept a reticular organization and formed only small aggregates (Fig. 20a), while EpiCCg were able to organize in distinct large three-dimensional spherical structures (Fig. 20b).

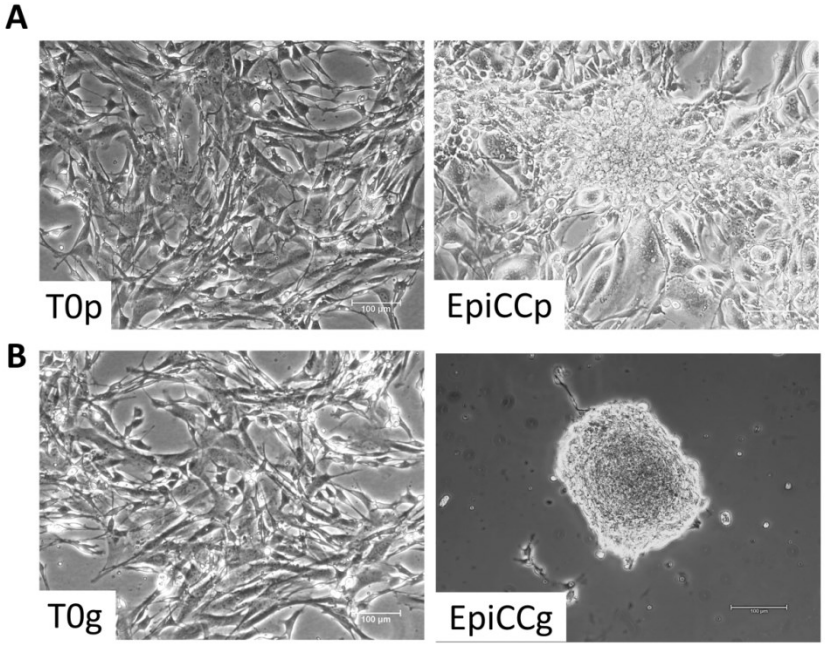


Fig. 20: Effect of matrix elasticity on cell morphology. (**A**) Untreated fibroblasts (T0p) and differentiated cells (EpiCCp) on plastic dish. (**B**) Untreated fibroblasts (T0g) and converted cells (EpiCCg) on substrate with low mechanical compliance. Scale bars: 100 μm.

4.3.5 Effect of matrix elasticity on mechano-responses and biochemical signals

Immunocytochemical studies revealed that yes-associated protein (YAP) was evenly distributed between cytoplasm and nucleus in untreated fibroblasts (T0p, T0g; Fig. 21a). No effect on its nuclear accumulation was detected after 18-hour exposure to 5-aza-CR either in cells plated onto plastic plates (Post 5-aza-CRp; Fig. 21a) or in those grown on PAA gels (Post 5-aza-CRg; Fig. 21a). In contrast, at the end of pancreatic induction, YAP localization markedly shifted into the cytoplasm (EpiCCp, EpiCCg; Fig. 21a), with a significantly higher number of cells showing nuclear immune-positivity exclusion in EpiCCg (Fig. 21b).

Consistent with these observations, significant changes in YAP phosphorylation were detected in EpiCCp and EpiCCg. More in detail, EpiCC showed significantly higher levels of pYAP compared to untreated fibroblasts (T0p, T0g) and 5-aza-CR treated cells (Post 5-aza-CRp, Post 5-aza-CRg; Fig. 21c). Interestingly, Hippo-pathway regulating kinases, namely Large Tumor Suppressor 1 (LATS1) and MOB kinase activator 1 (MOB1), changed their phosphorylation levels in parallel to those of YAP protein (Fig. 21 c,d).

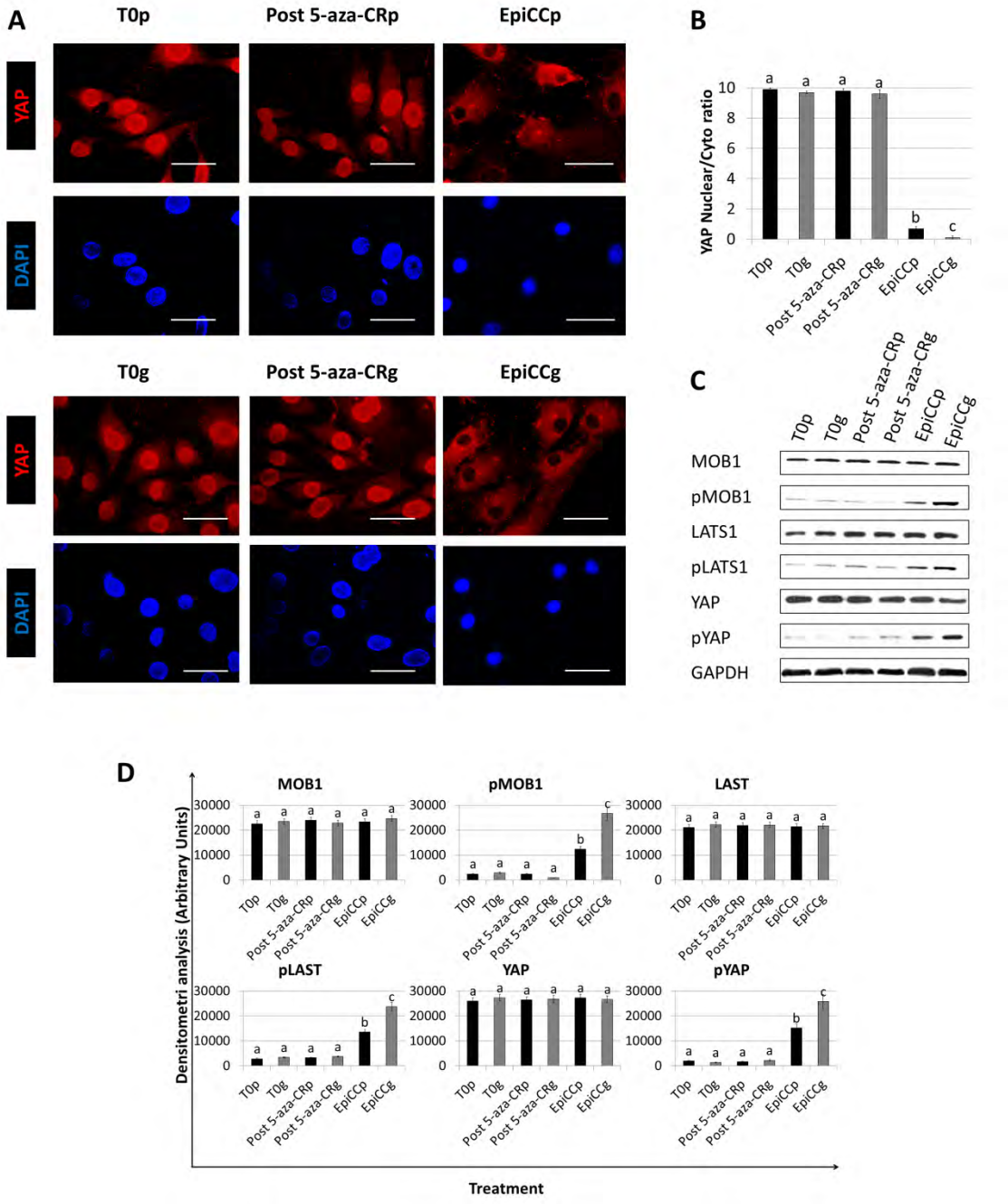


Fig. 21: (**A**) YAP (red) cellular localization determined by immunofluorescence staining. Nuclei are stained with DAPI. Scale bars: 50 μm. (**B**) Quantification of the nuclear/cytoplasmic ratio of YAP. (**C**) Representative western blot immune-detection of Hippo signaling pathway-related molecules and GAPDH during epigenetic conversion. (**D**) Densitometric analysis of the Western blots. The values are reported as relative optical density of the bands normalized to GAPDH. Different superscripts denote significant differences between groups ($P<0.05$).

4.3.6 Matrix elasticity boosts differentiation efficiency and EpiCC mono-hormonal phenotype acquisition

At the end of pancreatic induction, EpiCC obtained both on plastic plates (EpiCCp) and on PAA gels (EpiCCg) displayed immune-positivity for endocrine pancreatic hormones, namely C-PEP, GLUC and SOM. In particular, in all immune-reactive cells I detected a co-localization of the three pancreatic hormones within each single cell (Fig. 22a). Gene expression analysis confirmed these results, showing the onset of active transcription for mature pancreatic specific genes, namely Ins, Gcg and Sst (Fig. 22d). Significant differences in conversion efficiency were observed, when comparing cells differentiated onto standard plastic plates vs. PAA gels. In particular, the percentage of hormone immune-positive cells significantly increased from 26.86±5.8% in EpiCCp to 82.83±6.8% in cells differentiated onto the soft substrate (EpiCCg; Fig. 22b). Low stiffness PAA gels significantly promoted the acquisition of a mature pancreatic phenotype with 65.33±2.5% of EpiCCg showing a mono-hormonal staining distribution, while only 17.5±0.98% remained poly-hormonal (Fig. 22b). Furthermore, in depth analysis of these cells demonstrated that 13.57±1.1% were positive for SOM, 23.76±2.4% for GLUC, and 45.5±3.1% C-PEP (Fig. 22c).

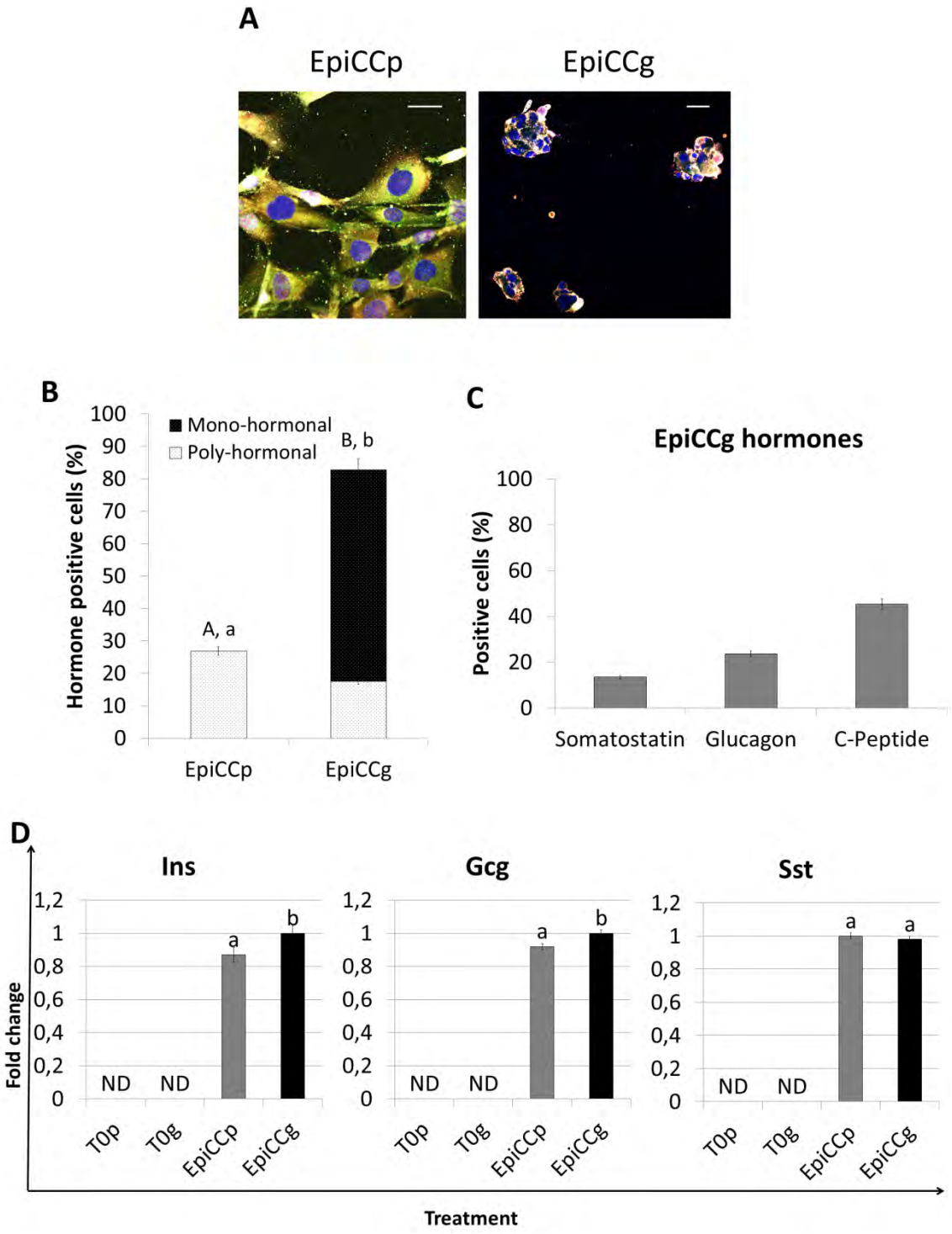


Fig. 22: (**A**) EpiCC show immune-positivity for C-peptide (white), glucagon (green) and somatostatin (red). Nuclei are stained with DAPI. (**B**) Hormone immune-positive cell rate and poly/mono-hormonal cell percentages in EpiCCp and EpiCCg. (**C**) Somatostatin, glucagon and C-peptide positive cell rates in EpiCCg. (**D**) Ins, Gcg and Sst gene expression in EpiCCp and EpiCCg. Different superscripts denote significant differences between groups ($P<0.05$).

4.3.7 Matrix elasticity enhances EpiCC insulin release

EpiCC grown on plastic plates and on low stiffness PAA gels were able to respond to 1-hour exposure to 20mM glucose and actively released insulin in cell supernatants. However, significantly higher concentrations of insulin were released by EpiCCg (4.15 ± 0.07 μg /μg DNA) compared to EpiCCp (1.91 ± 0.09 μg /μg DNA) (Fig. 23).

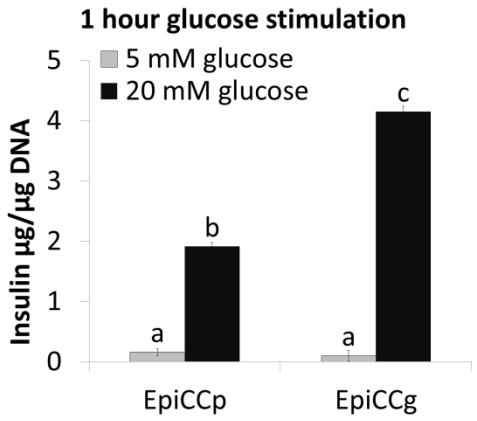


Fig. 23: Quantification of insulin release in cell supernatant in response to 5 mM and 20 mM D-glucose exposure for 1 h. Different superscripts denote significant differences between groups ($P<0.05$).

CHAPTER 5

DISCUSSION

5. DISCUSSION

The epigenetic modifier 5-aza-CR has been shown to incorporate into DNA during cell replication, leading to a global demethylation and gene reactivation [104]. Thanks to this powerful effect, the compound has been used to increase chromatin plasticity and facilitate phenotype changes in different models [40, 55, 56, 100, 105, 106]. Although the DNA demethylation capabilities of 5-aza-CR have been well characterized, both *in vitro* and *in vivo*, there is also reason to believe that this drug exhibits alternative mechanisms of action [107, 108]. The experiments carried out during my PhD confirmed the global effect of 5-aza-CR on methylation. As shown in Fig. 4, decreased methylation in fibroblasts exposed to 5-aza-CR was detected and these levels were maintained in cells cultured in ESC medium, suggesting that DNA methylation levels may be modulated by the milieu of the cells, including signals deriving from the medium. These findings are in agreement with Blaschke et al. [109] and Yin et al. [110] that demonstrated changes in DNA methylation in response to specific media formulation and the contained nutritional factors. Interestingly, the changes found in my experiments were accompanied by an increased expression of several genes. Following preliminary Whole Transcriptome Analysis (WTA) results, described in my Master thesis, and showing that more than one thousand genes are differentially expressed during 5-aza-CR epigenetic erasing and differentiation, I took into consideration and investigated in details some of them.

In particular, upregulated transcription of the TET genes, which was recently shown to support 5-aza-CR dependent demethylation process in

hepatocarcinoma cell lines [111], was confirmed in my study (Fig. 5b). Interestingly, 5-aza-CR did not affect all the members of the TET family, but rather it exerted a distinct effect on TET2, leaving the other members unchanged. In detail, upregulation of TET2 resulted in an increase of both 5fC and 5caC (which are both products of TET2 enzyme-mediated oxidation of 5-methylcytosine, Fig. 6), indicating 5-aza-CR ability to activate a direct and active demethylating effect, mediated via TET2 protein. Furthermore, the possibility of a synergistic action between direct and indirect demethylation processes was also demonstrated thank to TET2 siRNA experiments, where downregulation of TET2 protein resulted in an overall decrease of global DNA demethylation (Fig. 7), that however still persisted in response to 5-aza-CR, possibly due to the unaffected action of 5-aza-CR on DNMTs.

TET2 upregulation was also in agreement with the acquisition of the higher plasticity state detected in post 5-aza-CR cells, since TET2 has been reported to have a role in pluripotency [112]. Indeed, it has been demonstrated that TET enzymes play an essential role in ESC pluripotency maintenance [6, 7] and are upregulated during mesenchymal to epithelial transition (MET), as well as during somatic cell reprogramming, affecting the establishment of an open chromatin state, and contributing to the re-activation of endogenous pluripotency genes [113]. The fundamental functions exerted by TET factors in iPS generation were previously confirmed by experiments demonstrating inability of TET-deficient fibroblasts to complete reprogramming processes [50]. Exposure to 5-aza-CR also caused the onset of pluripotency gene expression, such as OCT4, NANOG, REX1 and SOX2 (Fig. 5a). This result

is also consistent with the molecule ability to reactivate previously silent genes and to alter the differentiation state of eukaryotic cells, as well as with recent data showing that 5-aza-CR demethylation of one or more loci facilitates a rapid and stable transition of partially reprogrammed iPSCs to a fully reprogrammed state [46]. Interestingly, culture in ESC medium maintained upregulated expression of all the genes described, suggesting the acquisition of a high plasticity state.

Expression of histones belonging to 1, 2, 3 and 4 families was also affected by the exposure to the epigenetic eraser. The genes and their encoded proteins showed increases, in cells treated with 5-aza-CR, and persisted at high levels in cells cultured in ESC medium for 24 and 48 hours (Fig. 8).

The hypothesis was that 5-aza-CR effect on histone gene transcription was unlikely to be accounted for a general demethylating effect, since exposure to the modifier, resulted in parallel downregulation of fibroblast specific genes (COL6A3) and did not affect the expression of several other histone genes (see appendix II). In agreement with this hypothesis, Komashko et al. demonstrated that several changes in gene expression after 5-aza-CR treatment were related to an alternative DNA-hypomethylating independent effect [114]. Interestingly, Takebayashi et al. demonstrated a DNA methylation independent mechanism, directly related to an increase in histone hyperacetylation [107].

This was also in agreement with a significantly higher transcription level of HAT1 detected in cells exposed to 5-aza-CR, suggesting the possible correlation among the exposure to the modifier, the overexpression of HAT1, the consequent histone hyperacetylation and increased transcription. This possibility was further supported by the parallel

decreased expression of HDAC1 (Fig. 9) and was also coherent with previous works that showed how modulation of histone acetylation was an integral component of gene expression control [115, 116].

Morphological analysis further demonstrated the impact of 5-aza-CR on cell plasticity and differentiation. In particular, it was interesting to underline that adult skin fibroblasts changed after 5-aza-CR exposure and reshaped into an undifferentiated progenitor-like phenotype. In this condition, cells showed a nuclei/cytoplasm ratio similar to what previously described in hESCs [117] and in hiPSCs obtained from human dermal fibroblasts [118]. 5-aza-CR increased plasticity was also accompanied by autophagic phenomena, that have been reported to play a critical role in the maintenance of adult stem cells [119], possibly reducing senescence via the active removal of immune reactive species [120]. Interestingly the presence of autophagic vacuoles was previously reported in ultrastructural analysis of hiPSC [121]. Furthermore, in my experiments, post 5-aza-CR cell nuclei were characterized by sparse chromatin and appeared devoid of compact formations. This was consistent with the observation that an open chromatin structure is required for a high plasticity state [122] and is one of the hallmarks of stemness (Fig. 10) [123]. Interestingly, this condition was maintained by ESC medium, although for a short period, possibly suggesting that, once acquired, the decreased methylation state can be sustained by factors contained in the medium and modulated by adequate microenvironment properties. Starting from these observations and from the notion that 3D culture systems may have a profound influence on cell phenotype and plasticity, I investigated whether the use of PTFE micro-bioreactor could

promote 3D cell rearrangement and aggregation, stabilizing the transient high plasticity window in epigenetically erased cells for a prolonged time.

As previously described, after treatment with the epigenetic eraser, both cells plated onto plastic plates and those encapsulated in PTFE displayed significant morphological changes, compared to untreated fibroblasts (T0). However, while cells on plastic retained a monolayer distribution for the entire length of the experiments, the use of the micro-bioreactor allowed cells to self-assemble and form multicellular spheroids, displaying a uniform size geometry (Fig. 11). This was consistent with previous studies indicating that the use of PTFE is able to efficiently encourage cell aggregation, facilitating the formation of embryoid bodies (Ebs) from murine ESC [124] or the establishment of olfactory ensheathing cell (OEC) spheroid structures [125].

Ultrastructural analysis demonstrated that cells in the 3D spherical structures showed significant intercellular spaces, high nucleus to cytoplasm ratio, nuclei containing euchromatin and large reticulated nucleoli. Cytoplasm was characterized by the presence of free ribosomes, polyribosomes, elongated tubular mitochondria, well-developed rough endoplasmic reticulum (RER), Golgi complexes, few reticulum cisternae and lipid droplets. As previously described, these features resemble those described for human ESC [126] and induced pluripotent cells (iPS) (Fig. 12) [121]. These observations suggest that the use of the PTFE micro-bioreactor encourages not only cell aggregation, but also boosts the formation and stable maintenance of morphological properties previously described in pluripotent cells. Similar to what described

above, after 5-aza-CR treatment, I detected DNA demethylation. However, while cell methylation on plastic returned comparable to untreated fibroblasts (T0) by day 7 of culture, PTFE encapsulated cells remained significantly hypomethylated for the entire length of the experiments (Fig. 13). Even in this case, hypomethylated state is due to the effect of a direct and active demethylation mechanism controlled by TET proteins, as confirmed by gene expression results. Increased expression of TET2 was accompanied by the onset of the pluripotency-related genes, OCT4, NANOG, REX1, and SOX2, as well as by the up-regulation of EPCAM, and CDH1 genes, confirming and expanding previous results. These changes in gene expression were detected in both experimental Groups. However, as described in methylation study, cells on plastic returned to values comparable to those of untreated fibroblasts by day 7 of culture, demonstrating that high plasticity state was transient and reversible. In contrast cells encapsulated in PTFE displayed high expression levels for the entire length of the experiments. These results were also well in line with the morphological observations and suggested that the acquisition of a high plasticity phenotype was paralleled by the concomitant decrease of fibroblast specific marker, THY1, the onset of pluripotency-related genes, and the upregulation of key MET markers (EPCAM, CDH1). Interestingly, these changes were promoted and stably maintained by the use of the PTFE micro-bioreactor, suggesting that 3D cell confinement boosts pluripotency gene transcription and maintains long-term cell plasticity (Fig. 14). To better understand the mechanisms associated to the use of the PTFE to generate a 3D cell confinement that favors the induction and maintenance of cell plasticity, I investigated the

involvement of mechanotransduction-related signaling pathways (Fig. 15). Results demonstrated that the morphological and molecular changes described, were accompanied by the activation of the Hippo-signaling pathway with distinctive modifications in TAZ localization. In particular, after 5-aza-CR treatment, TAZ protein displayed a nuclear accumulation in both experimental Groups. This localization was however lost by cells on plastic, with TAZ molecule relocating to the cytoplasm, by day 7 of culture. In contrast, 3D cell confinement of PTFE encapsulated cells encouraged TAZ nuclear retention, that was stably maintained for the entire length of the experiments. This is consistent with a recent study showing a clear TAZ nuclear localization in large spheroids and organoid-like structures [127]. In the experiments described, TAZ localization was mirrored by a parallel compartmentalization of SMAD2. In particular, cells displaying nuclear localized TAZ showed concomitant SMAD2 nuclear accumulation, while cells with cytoplasmic TAZ, also exhibited SMAD2 cytoplasmic distribution. This observation is in line with previous reports that indicate a direct interaction between the transcriptional cofactor TAZ and SMAD proteins, where TAZ defines a hierarchical system, regulating SMAD complexes shuttling and coupling to the transcriptional machinery [128, 129]. Interestingly, Panciera et al. showed that transient expression of exogenous TAZ in terminally differentiated cells can induced conversion to a progenitor cell state [130] and that the loss of TAZ expression induces failure of SMAD nuclear accumulation, disappearance of OCT4 and subsequent differentiation [128]. Consistent with these findings, the results described also showed that siRNA knockdown of TAZ, with the concomitant failure of SMAD

nuclear accumulation, caused loss of pluripotency marker transcription in PTFE encapsulated cells (Fig. 16). Base on this, I speculate that the activation of the Hippo-signaling pathway induced by the PTFE micro-bioreactor and the related TAZ-dependent SMAD shuttling represent the base of the maintenance of high plasticity in encapsulated cells. These results showed that the use of PTFE micro-bioreactor, that promotes 3D cell rearrangement mimicking embryo environment, maintains high plasticity in erased cells for a long period of culture. This notion highlighted the importance of mechanotrasduction-related stimuli in cell fate decisions. Starting from this, the next step was to evaluate whether the use of a substrate able to recapitulate the *in vivo* pancreas stiffness may influence the epigenetic conversion and differentiation of dermal fibroblasts into insulin-producing cells. To this end I selected mouse fibroblasts because cells isolated from this species showed low conversion efficacy and a more limited tendency to form 3D aggregates compared to other such as human, pig and dog. The obtained results revealed that , as previously discussed in my thesis, after 5-aza-CR treatment, both cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg) showed a significant decrease in global DNA methylation. However, Post 5-aza-CRg cells exhibited a significantly lower methylation level compared to that of Post 5-aza-CRp. Since no DNA methylation changes were observed in cells plated on PAA gels, without exposure to 5-aza-CR (w/o 5-aza-CRp and w/o 5-aza-CRg, Fig. 18), the methylation decrease observed is unlikely to be accounted for the substrate and its elasticity “per sè”. This is in line with Schellenberg et al.

that previously demonstrated no influence by Young's moduli on mesenchymal cell methylation profiles [86].

I also confirmed whether the use of low-stiffness substrate may influence regulatory genes that are involved in DNA acetylation and methylation changes. The results obtained showed a significant up-regulation of Hat1 gene transcription in cells exposed to 5-aza-CR, which was increased by the use of soft substrate (Fig. 19a). This was accompanied by a significant decreased expression of Hdac1 gene, that was lower in cells plated on PAA gels compared to that seeded on plastic dishes (Fig. 19a). These observations validate the results described in the previous paragraphs, demonstrating the key role played by these two genes in transcriptional regulation changes after 5-aza-CR treatment. Once again, I correlated DNA demethylation with Tet2, which expression was increased by the soft substrate (Post 5-aza-CRg, Fig. 19a) and paralleled by the onset of pluripotency genes, (Fig. 19b), confirming the results described above also in the mouse species. Pluripotency-related genes expression was detected in cells erased on plastic, but was boosted by the presence of the soft matrix, which induced a 19%, 14%, 23% and 25% increment in Oct4, Nanog, Rex1, and Sox2 gene expression respectively. These observations are in agreement with previous data, reporting that iPSC cultured on soft PAA gels showed higher expression of pluripotency genes, than the same cells plated on rigid plastic dishes [131] and are in line with the possibility to maintain and promote self-renewal of murine ESC (mESC), in the absence of exogenous LIF, through the use of soft substrates that match the intrinsic stiffness of the mESC [132].

Interestingly, cell transition to pancreatic phenotype induced the activation of the Hippo signaling pathway and modifications in cellular localization of the main transcriptional effector YAP (Fig. 21 a,b). Indeed, while fibroblasts showed an even distribution of this protein between cytoplasm and nucleus, regardless of the substrate utilized (T0p and T0g), EpiCC displayed a restricted YAP localization to the cytoplasmic compartment with a significantly higher number of cells showing nuclear immune-positivity exclusion in EpiCCg compared to EpiCCp (Fig. 21b). These data are altogether in agreement with recent studies, demonstrating a correlation between cytoplasmic retention and the subsequent complete nuclear exclusion of YAP [133, 134]. Notably, cytoplasmic confinement of YAP has been reported to be distinctive of differentiating cells, while ESC showed the presence of the protein in the nucleus as well as in the cytoplasm [5, 131, 132]. Similarly, I detected YAP equal localization in both compartments of cells exposed to 5-aza-CR (Fig. 21 a,b), regardless of the matrix elasticity and, most likely related to the newly acquired high plasticity state. These results are well in line with recent work, demonstrating nuclear YAP essential role in ESC self-renewal and in the control of the levels of the pluripotency genes Oct4, Nanog and Sox2 [135-137]. Nuclear/cytoplasmic translocation of YAP has also been described to be controlled by the phosphorylation levels of the protein, which are higher in differentiating cells, compared to undifferentiated ones [135]. In particular, phosphorylation via MOB and LATS kinases, that are core components of the Hippo signaling pathway, resulted in YAP phosphorylation and subsequent exclusion from the nuclear compartment [134]. Consistent with this, the data reported in this

thesis, demonstrate the phosphorylation of the Hippo-pathway regulating kinases, that paralleled changes in the YAP protein phosphorylation levels (Fig. 21c) and in its nuclear/cytoplasmic distribution. The results obtained supports the robustness of the epigenetic conversion method, confirming and extending to the mouse our previous experiments carried out in human, pig, and dog [55, 100, 106].

However, some species-specific differences became evident. In contrast to what observed in the other species, murine converting cells on plastic showed a more limited tendency to form aggregates and organized in smaller and scattered clusters (Fig. 20a). Similarly, conversion efficiency was 26.86 ± 5.8% (Fig. 21b), while a significantly higher percentage of C-peptide immune-positive cells was obtained in the human (35 ± 8.9%), pig (38.1±9.2 %), and dog (38±6.1%).

Interestingly, the use of a substrate with lower than plastic elastic modulus, allowed mechanotransduction-related stimuli, that guided cells to rearrange into distinct large three-dimensional spherical structures (Fig. 20b) and induced the morphological changes described, that were paralleled by a significantly higher pancreatic differentiation efficiency (82.83 ± 6.8% vs. 26.86 ± 5.8%) (Fig. 21b). These results suggest that the formation of tridimensional clusters is a crucial aspect of pancreatic differentiation *in vitro* and a key point in order to encourage and boost the acquisition of a mature pancreatic phenotype. This is consistent with recent studies that demonstrated soft gel encapsulation system ability to enhance cell differentiation towards the endodermal lineage [138-140].

In the experiment described in this thesis, epigenetic conversion of murine skin fibroblasts led to the acquisition of a pancreatic phenotype. Differentiation process was supported both by active transcriptional activity for pancreatic genes as well as immune-positivity for C-PEP, SOM and GLUC. However, while EpiCCp displayed a co-localization of the three pancreatic hormones in all immune-reactive cells, EpiCCg showed a mono-hormonal staining distribution in over 65% of the population. Interestingly, it has been observed that primitive endocrine cells, typical of early fetal stages, co-express insulin and glucagon, while they mature into a mono-hormonal phenotype later in development [141, 142]. Hormone compartmentalized localization of EpiCCg may therefore be suggestive of a more mature phenotype and to be distinctive of terminally differentiated cells.

Altogether, these results indicate that the use of a soft substrate has a general effect on the differentiation of epigenetically erased fibroblasts and indicate a positive impact both on efficiency and on the promotion of a mono hormonal mode. *In vitro* functional experiments, showed that, when challenged with 20mM D-glucose, EpiCC actively released insulin (Fig. 23), demonstrating their ability to respond to the primary and physiological stimulus for insulin secretion, and supporting the achievement of a functional phenotype. However, it is interesting to note that a significantly higher amount of insulin was released by EpiCCg (Fig. 23). This is likely to be related to the acquisition of a more mature/mono-hormonal phenotype which was obtained thanks to the use of a soft substrate.

CHAPTER 6

CONCLUSIONS

6. CONCLUSIONS

Altogether, the experiments performed during my PhD further elucidate the mechanisms underlying 5-aza-CR effects on cell plasticity and differentiation. In particular, data presented in this thesis, indicate 5-aza-CR ability to increase TET2 gene expression, modulate transcription of histones and histone acetylating enzymes, which result in specific morphological changes. Moreover, the results obtained, also suggest the molecule ability to interfere with DNA methylation through a direct TET2-mediated mechanism, that accompanies the well-known indirect DNMT related one. In addition, our findings give evidence that the use of the PTFE micro-bioreactor boosts the induction and maintenance of high plasticity state in epigenetically erased cells. This model system provides a novel in vitro culture technique that induces distinctive 3D cell rearrangement and specific cell-to-cell interactions. We are also convinced that the PTFE micro-bioreactor may represent a notable innovation in stem cell organoid technology and may constitute an advantageous micro-environment for long-term culture of different cell types, from epigenetically erased cells, to ESC and iPSC, as well as MSC.

Moreover, in the present thesis, I describe the epigenetic conversion of dermal fibroblasts in the murine species and demonstrate that cell mechano-sensing and biomechanical properties of the surrounding matrix, may influence the acquisition of cell plasticity and enhance tissue differentiation, increase conversion efficiency and encourage the acquisition of a mature pancreatic phenotype. The results confirm and strengthen previous data obtained in other species, and expands our knowledge on the mechanisms underlying the epigenetic erasing and

conversion processes. Furthermore, the data here reported may have interesting technological impacts in order to increase reliability and increment efficiency of the conversion process. In my opinion, this aspect is crucial for clinical translation of the results, since it allows swift scale-up culture procedure that reduces the time required from skin biopsy to therapeutic cell engraftment.

CHAPTER 7

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7. BIBLIOGRAPHY

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CHAPTER 8

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Erase and Rewind: Epigenetic Conversion of Cell Fate

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Abstract The potential of cell therapy in regenerative medicine has greatly expanded thanks to the availability of sources of pluripotent cells. In particular, induced pluripotent stem cells (iPS) have dominated the scenario in the last years for their ability to proliferate and differentiate into specific cell types. Nevertheless, the concerns inherent to the cell reprogramming process, limit iPS use in therapy and pose questions on the long-term behavior of these cells. In particular, despite the development of virus-free methods for their obtainment, a major and persisting drawback, is related to the acquisition of a stable pluripotent state, that is unphysiological and may lead to cell instability. The increased understanding of epigenetic mechanisms has paved the way to the use of "small molecules" and "epigenetic modifiers" that allow the fine tuning of cell genotype and phenotype. In particular, it was demonstrated that an adult mature cell could be directly converted into a different cell type with the use of these chemicals, obtaining a new patient-specific cell, suitable for cell therapy. This approach is simple and direct and may represent a very promising tool for the regenerative medicine of several and diverse degenerative diseases.

Keywords Cell conversion · Epigenetic memory · Regenerative medicine · Reprogramming · Trans-differentiation

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Cell Fate and Establishment of "Epigenetic Memory"

Cells in adult organisms are highly specialized and have a specific molecular pattern that regulates their functions and physiology. Despite the fact that these cells arise from a single cell with half-genome from each parent, they are able to give rise to a wide variety of cell types, different from each other, that allow the formation of a complete multicellular organism.

During embryo/fetus development, the differentiation ability progressively decreases. The process is driven by several factors both extrinsic and intrinsic to the cell [1], that induce differential gene expression and epigenetic restrictions. These gradually limit cell potency [2] to a more limited phenotype-related expression pattern, resulting in a progressive restriction in cell options [3].

In particular, cell commitment and differentiation are fortified by the cell's own machinery that chemically modify the DNA, without any permanent loss or alteration of genetic material [4, 5].

These changes are referred to as epigenetic modifications and regulate the accessibility to transcription factors, in either a positive or a negative manner. They are responsible for the 'epigenetic memory' that underlies the phenotypic stability of the differentiated cell state, during subsequent cell divisions [6–9].

The two major mechanisms involved in the regulation of cell commitment and differentiation are the DNA methylation and the histone modifications [4]. 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 factor proteins to the gene [10] or recruits the methyl-CpG-binding domain proteins (MBDs), remodeling histones and forming compact, inactive chromatin, known as heterochromatin.

In the second process, histones are subjected to several covalent modifications, such as acetylation, phosphorylation, methylation, SUMOylation, citrullination, ADP ribosylation, and ubiquitination [11], allowing or preventing transcription factor and other protein access to DNA.

These processes drive embryo development 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.

Cell Reprogramming and "Epigenetic Memory" Erasure

During the last years, several works demonstrated that, although generally stable in vivo, the differentiated state of a cell can be reversed [12].

The first evidences date back to the 1960s, when it was shown that a somatic cell identity can be reversed by exposing the somatic nucleus to the oocyte environment [13] [14]. This paved the way for a search of the mechanisms involved in the erasure of "epigenetic memory" and the re-establishment of pluri- or totipotency (Fig. 1). Indeed, it has subsequently been demonstrated that terminally differentiated cells can be brought back a to an increased potency state, using cell fusion techniques [15] or over-expressing master regulator transcription factors (TF) [16].

More recently, somatic cell reprogramming was achieved through the ectopic expression of four TF, with the production of the so called "induced pluripotent stem cells" (iPS) [17].

The reprogramming process requires complete erasure of the existing somatic epigenetic memory, followed by the establishment of a new cell type specific epigenetic signature [18]. Due to the extreme stability of adult somatic cell epigenetic signature, this process makes cells prone to errors [19]. Furthermore, although iPS derivation is technically less demanding than cell fusion or somatic cell nuclear transfer (SCNT), the efficiency of cell reprogramming remains low [18, 20–23]. This may reflects the lack of a complete functional machinery that makes the other two techniques more efficient [24].

Cell reprogramming also suffers from a number of severe limitations that prevent its possible use in regenerative medicine [25]. In particular, the use of potentially harmful genome-integrating viruses to deliver the TFs needed to reach the pluripotent state [17, 26–28] is associated with the risk of tumor formation.

During the last years, several studies focused on the improvement of iPS methodology, increasing efficiency, accelerating kinetics, and reducing safety concerns related to the use of virus-mediated gene, and the expression/reactivation of oncogenes. In particular, several groups demonstrated that treatment with cytokines or small molecules significantly increases efficiency and speed of iPS derivation [29–31]. Furthermore, virus-free [28, 32, 33], removable PiggyBac transposons [34], minicircle systems [35], and episomal systems [36] methods have been developed. However, the use of DNA constructs and the subsequent possibility of exogenous sequence integration cannot preclude the safety concerns [37–39]. More recently, the use of recombinant proteins, synthetic mRNAs [40, 41] [42], or microRNAs [43, 44] was also proposed to induce cell reprogramming, but the robustness of these approaches remains uncertain.

A major concern, regardless of the use of viruses or alternative virus-free protocols, is related to the acquisition of a stable and persistent pluripotent state, a fundamental aspect shared by all types of iPS. This is an un-physiological condition because,

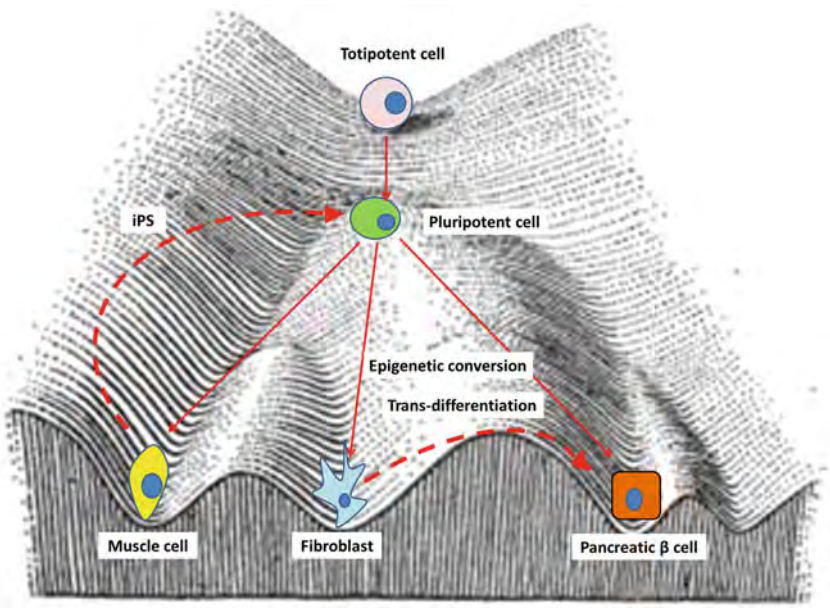


Fig. 1 From totipotent to terminally differentiated cells and back: a multi-directional process

physiologically, pluripotency is transient and limited to a short time window during the first phases of embryonic development. The establishment of a persistent pluripotent state creates safety issues since it may lead to cell instability [45]. Furthermore, iPS proliferation and differentiation are difficult to control. When induced to differentiate, iPS show a low efficiency, that rarely exceeds 30 %, leaving mature cells mixed with undifferentiated and proliferating ones [46]. All these aspects are matter of caution because they are related to a high risk of malignant transformation and subsequent tumor formation that severely question the use of these cells in regenerative medicine.

Transdifferentiation and "Epigenetic Memory" Modification

In an attempt to circumvent the problems described above, a new strategy, designated 'transdifferentiation' or 'direct reprogramming', has been proposed. This approach is based on the direct inter-lineage conversion of a terminally differentiated adult somatic cell into another (Fig. 1), through the simultaneous down-regulation of one genetic program and the concomitant up-regulation of new one [47] (see Table 1). This method has several advantages: it reduces the risk of tumorigenesis associated with the induced pluripotent state and makes the process faster and more efficient [24]. Ectopic expression of the myogenic factor *MYOD* was able to induce the conversion of different cell types into myogenic cells [48]. Similarly, it was shown that *C/EBPα* or *C/EBPβ* genes promoted the transition of pancreatic cells into macrophages [50] or hepatocytes [51, 52], while *GATA1* or *PU1* genes encouraged the switch between myeloid cells and megakaryocyte/erythroid cells [49].

However, this approach, once again, involved the use of retroviral vector to transfer one or more specific transcription factors [46], making transdifferentiated cells unsuitable for cell therapy in regenerative medicine.

Virus-free protocols were recently proposed, but they are technically demanding, less efficient [59] and do not eliminate the problems deriving from the use of transgenes.

"Epigenetic Memory" and Epigenetic Modifiers

During the last years several studies reported that it is possible to directly convert one somatic cell type into another, without the ectopic expression of transgenes that are often stably integrated into the genome [60].

Indeed, it has been firstly demonstrated that epiblast stem cells (EpiSCs) could be reverted to ESC, inhibiting the TGFβ signaling pathway or the histone demethylase LSD1 with small molecules [61]. Similarly, germ line cells could be brought back to a pluripotent state, through the use of specific cell culture conditions [62]. De Coppi et al. demonstrated that cells isolated from amniotic fluid, which are not pluripotent [63], can be induced to pluripotency, simply culturing them on matrigel in ESC medium, supplemented with the HDAC inhibitor valproic acid (VPA) [64, 65].

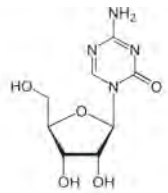
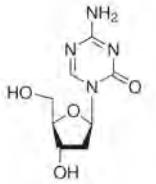
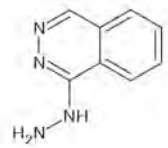
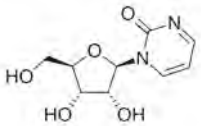
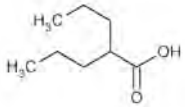
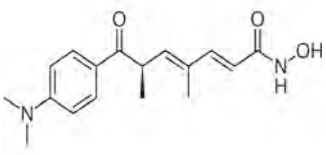
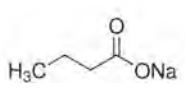
These findings have opened the way to the idea that small molecules and epigenetic modifiers, not only increase the efficiency of cell reprogramming, but may also provide a powerful tools to induce conversion of one cell type into another (Table 2), functionally replacing ectopic expression of TF [24].

Rim et al. recently demonstrated that human dermal fibroblasts up-regulated endogenous pluripotency transcription factor genes after exposure to VPA, in the absence of any

Table 1 Recent studies reporting trans-differentiation by ectopic expression of key developmental genes

Original cell type	Transdifferentiated cell type	Ectopic expressed gene	Reference
Melanoma, neuroblastoma, liver cells and adipocytes	Myogenic cells	*MYOD*	Weintraub et al., 1989 [48]
Myeloid cells	Megakaryocytes and erythroid cells	*GATA1* *PU1*	Graf et al., 2002 [49]
Pancreatic cells	Macrophages	*C/EBPα* *C/EBPβ*	Xie et al., 2004 [50]
Pancreatic cells	Hepatocytes	*C/EBPα* *C/EBPβ*	Shen et al., 2000 [51] Zhou et al., 2008 [52]
Postnatal or fetal fibroblasts	Neurons	*BRN2*, *ASCL1*, *MYT1L*, *NEUROD*	Pang et al., 2011 [53]
Postnatal or adult fibroblasts	Neurons	*miR124*, *BRN2*, *MYT1L*	Ambasudhan et al., 2011 [54]
Foreskin, dermal or adult cardiac fibroblasts	Cardiomyocytes	*GATA4*, *HAND2*, *TBX5*, *MYOD*, *miR1*, *miR133*	Nam et al., 2013 [55]
Dermal and cardiac fibroblasts	Cardiomyocytes	*GATA4*, *MEF2C*, *TBX5*, *ESRRG*, *MESP1*, *MYOCD*, *ZFPM2*	Fu et al., 2013 [56]
Foreskin fibroblasts	Retinal pigment epithelial-like cells	*CMYC*, *MITF*, *OTC2*, *RAX*, *CRX*	Zhang et al., 2014 [57]
Neonatal fibroblasts	Chondrogenic cells	*CMYC*, *KLF4*, *SOX9*	Outani et al., 2013 [58]

Table 2 Epigenetic modifiers used for cell fate control

Epigenetic Modifier (name and structure)	Target	Applications	Reference
5-aza-cytidine 	DNA methyltransferase inhibitor	Phenotype changes in eukaryotic cells; Transformation of mesenchymal stromal cells and fibroblasts into hematopoietic cells; Epigenetic cell conversion	Glover et al., 1986[66]; Harris et al., 2011[67]; Pennarossa G et al., 2013[68]; Brevini T.A.L. et al, 2014[69].
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	Enright BP et al., 2003[70]; Wozniak R.J. et al, 2007[71]; Ding X et al., 2008[72]. .
Hydralazine 	DNA methyltransferase inhibitor	Epigenetic modification to revert multidrug-resistant phenotype	Segura-Pacheco et al., 2007 [73].
Zebularine 	DNA methyltransferase inhibitor	Epigenetic reprogramming of yak fibroblasts for cloning; Epigenetic modification of bovine adipose stem cells	Xiong X. et al., 2013[74].
Valproic acid 	HDAC inhibitor	Dedifferentiation of amniotic fluid cells and of human dermal fibroblasts	De Coppi 2007[63]; Moschidou 2012[64]; Rim 2012[75].
Vorinostat 	HDAC inhibitor	Epigenetic reprogramming to restore chemosensitivity	T Bhatla 2012[76].
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	Milhem M et al., 2004[77]; Kishigami S. et al., 2007[78]; Shi L:H. et al., 2008[79]; Surani A. et al., 2008[80]; Huangfu D. et al., 2008[81]. .
Sodium butyrate 	HDAC inhibitor	In combination with A-83-01, PD0325901, and PS48, enables reprogramming of human somatic cells transduced with Oct4 only	Zhu S. et al., 2010[82].

transgenes [75]. Chemically induced pluripotent stem cells (CiPSC) were also obtained in the mouse through the use of a combination of small molecules, namely Forskolin (FSK), 2-methyl-5-hydroxytryptamine (2-Me-5HT), and D4476 [83].

In this contest, it is however important to highlight that pluripotency is transient and limited to a short time window during embryonic development. The induction of a stable and persistent pluripotent state is therefore not physiological and may lead to cell instability [45].

Based on these observations, alternative protocols were identified in order to directly convert an adult mature cell into another differentiated cell type, avoiding an un-physiological stable pluripotent state that make cells prone to error. [60, 67–69, 85, 90].

We recently developed a new protocol based on the concept that DNA methylation plays a fundamental role both during early embryonic development and cell lineage specification. In particular, we hypothesized that a brief exposure to a de-methylating agent can increase cell plasticity, driving cells into a transient less committed state that is sufficient to re-address them to a differentiated cell type. To this purpose, we used the de-methylating drug 5-aza-cytidine (5-aza-CR), which is a chemical analogue of the cytosine, directly inhibiting methyltransferase activity. When used at low doses, 5-aza-CR substitutes for cytosine into DNA and blocks DNA methyltransferase function, preventing methylation [85]. These features give 5-aza-CR the ability to induce DNA hypo-methylation and to remove the epigenetic "blocks" that are responsible for tissue specification, as previously shown by Jones in the 1985 [86]. Furthermore, this molecule was also demonstrated to trigger gene expression modifications, reactivation of silent gene transcription [87], and phenotype changes in eukaryotic cells [66, 67, 88].

In agreement with this observation, we recently demonstrated that an adult somatic cells can be converted into a new cell type after an 18 h exposure to 5-aza-CR [68, 69, 90]. More in details, human skin fibroblasts were converted into pancreatic beta-cells and at the end of the three step pancreatic induction protocol, 35±8.9 % of epigenetically converted cells exhibited mature endocrine phenotype, expressing the main hormone and glucose sensor genes specific of the pancreatic tissue [89]. These modifications in gene expression were accompanied by cell morphological changes and 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 (Fig. 2). The cells obtained were also able to actively release C-Peptide and Insulin, when exposed to 20 mM glucose, showing a dynamic response similar to that of pancreatic beta-cells, where changes in ambient glucose represent the primary and physiological stimulus for insulin secretion. In addition epigenetically converted cells were shown to restore normal glycemic levels and stably maintain them, when injected into diabetic animals [89].

Comparable results were obtained also in species other that the human, such as the porcine [68], the mouse, and the dog (manuscript submitted), demonstrating that it is possible to interact and modify cell phenotype through epigenetic remodeling with high reproducibility and in a wide range of species and cell types.

To strengthen this concept, 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 VEGF [69]. In these experiments over 80 % cells changed their original phenotype, acquired an elongated shape and became multinucleated. This was accompanied by molecular changes that demonstrated a switch from the granulosa cell specific expression pattern (*CYTOKERATIN17*, *HAS2*, *GREM1* and *PTX3*) to the one distinctive of muscle (*DESMIN*, *MHC* and *MYOD*).

In agreement with our results, Cheng et al. developed a protocol to convert human and murine fibroblasts into proliferating chemical-induced neural progenitor cells (ciNPC), through the use of a cocktail containing inhibitors of histone deacetylation, glycogen synthase kinase, and TGF-β pathway under physiological hypoxic conditions (5 % O_2) [90]. Successful epigenetic conversion was also demonstrated in a recent study showing that human skin fibroblasts exposed to the HDAC inhibitor VPA, can be re-addressed to mature

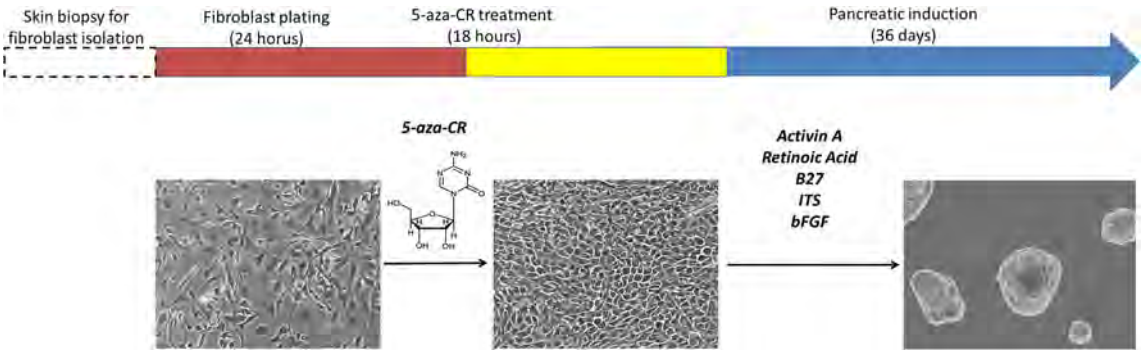


Fig. 2 Epigenetic conversion of adult skin fibroblasts into insulin-secreting cells using 5-aza-CR and pancreatic induction protocol

Schwann cells using a two-step protocol. More in details, during the first step, the Authors obtained a transient population of proliferating neural precursors. In the second one, these precursors were differentiated into functional induced Schwann cells (iSCs). These cells showed neuro-supportive and myelination capacity, and expressed proteins specific of the peripheral nervous system [60]. In addition, Mirakhori et al. also demonstrated that it is possible to obtain neural progenitor-like cells from human fibroblasts, avoiding the use of any genetic manipulation, but simply changing culture conditions from monolayer into cell suspension, in the presence of 5-aza-CR [84].

Conclusions

Altogether, the results accumulated during the last years have paved the way to the use of epigenetic modifiers for direct cell conversion. The increased understanding of epigenetic mechanisms, that regulate cell commitment and differentiation, have demonstrated that it is possible to dynamically interact with cell genotype and phenotype and change cell fate using specific and controlled conditions.

This new approach may represent a very promising tool for the regenerative medicine of several and diverse degenerative diseases both in human as well as in animal patients.

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Compliance with Ethical Standards

Disclosures The authors indicate no potential conflicts of interest.

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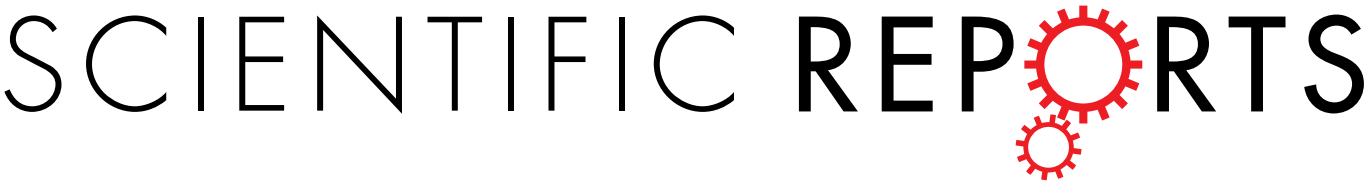

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5-azacytidine affects TET2 and histone transcription and reshapes morphology of human skin fibroblasts

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Phenotype definition is controlled by epigenetic regulations that allow cells to acquire their differentiated state. The process is reversible and attractive for therapeutic intervention and for the reactivation of hypermethylated pluripotency genes that facilitate transition to a higher plasticity state. We report the results obtained in human fibroblasts exposed to the epigenetic modifier 5-azacytidine (5-aza-CR), which increases adult cell plasticity and facilitates phenotype change. Although many aspects controlling its demethylating action have been widely investigated, the mechanisms underlying 5-aza-CR effects on cell plasticity are still poorly understood. Our experiments confirm decreased global methylation, but also demonstrate an increase of both Formylcytosine (5fC) and 5-Carboxylcytosine (5caC), indicating 5-aza-CR ability to activate a direct and active demethylating effect, possibly mediated via TET2 protein increased transcription. This was accompanied by transient upregulation of pluripotency markers and incremented histone expression, paralleled by changes in histone acetylating enzymes. Furthermore, adult fibroblasts reshaped into undifferentiated progenitor-like phenotype, with a sparse and open chromatin structure. Our findings indicate that 5-aza-CR induced somatic cell transition to a higher plasticity state is activated by multiple regulations that accompany the demethylating effect exerted by the modifier.

DNA methylation is essential for mammalian development, gene regulation, genomic imprinting, and chromatin structure[1]. Changes in methylation allow mature cells of adult organisms to acquire their differentiated state through a gradual loss of potency[2] and a progressive restriction in their options[3].

The process is reversible and may be altered by biochemical and biological manipulation, making it an attractive target to reactivate hypermethylated pluripotency genes[4] and facilitate cell transition to a higher plasticity state[5].

The epigenetic modifier 5-azacytidine (5-aza-CR) is a chemical analogue of cytosine. It is known to induce reversible cell cycle arrest[6,7] and acts as a direct inhibitor of methyltransferase activity, decreasing methylation in newly synthesised DNA. The molecule substitutes for cytosine, incorporates into DNA and RNA, during replication[8,9], forms covalent adducts with DNA methyltransferase (DNMT) 1, thereby depleting the cells from enzyme activity, causing demethylation of genomic DNA[8,10], as well as gene reactivation[11]. Thanks to its powerful effects, this compound may be used to increase chromatin plasticity and facilitate phenotype changes[12,13]. Several studies reported 5-aza-CR ability to facilitate adult somatic cell switch from one phenotype to a different one[14–16]. In particular, we demonstrated that a short exposure to 5-aza-CR allows a transient passage through a plastic chromatin state. This is sufficient to allow a complete directed differentiation of an adult mature cell into a different cell type[17–20].

All these studies are very promising because they allow to obtain safe and viral vector free cells that may be used for regenerative medicine. However, the mechanisms underlying 5-aza-CR effects on cell plasticity and differentiation are still poorly understood and need to be better elucidated. In this manuscript we expose 5-aza-CR treated cells either to: 1) embryonic stem cell (ESC) medium (to promote and maintain cell plasticity);

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2) pancreatic differentiation (PANCR) medium (to encourage and boost differentiation); 3) standard fibroblast (FB) culture medium (to allow cells to revert to their original phenotype).

We analyze the global methylation changes taking place in cells exposed to the epigenetic modifier and in the different experimental conditions tested. Based on preliminary Whole Transcriptome Analysis, obtained with an Applied Biosystems SOLiD 5500xl Sequencer, we investigate in details changes in transcriptions of the TET family genes, that affect methylation[21,22] and play an essential role in pluripotency regulation of ESC[21,22] and in the very early stage of somatic cell reprogramming toward induced Pluripotent Stem Cells (iPSCs)[23]. We study whether upregulation of TET2 results in increased levels of 5-Formylcytosine (5fC) and 5-Carboxylcytosine (5caC), which are both products of TET2 enzyme-mediated oxidation of 5-methylcytosine. This would suggest the possibility of a direct demethylating mechanism, accompanying the well documented indirect DNMT related action. To further support this hypothesis, we then investigate the effect of siRNA TET2 on the global DNA demethylation caused by 5-aza-CR. We also analyze the expression of histones belonging to the 1, 2 A, 2B, 3 and 4 families. Furthermore, we characterize the ultrastructural phenotypic modifications related to the exposure to 5-aza-CR and to the different culture conditions.

The data presented confirm the well-known effect on methylation but also highlight new cellular targets accompanying 5-aza-CR effects on epigenetic regulation of cell plasticity and differentiation.

Results

Global methylation changes in response to 5-aza-CR. Exposure to 5-aza-CR induced a significant decrease of global DNA methylation (Fig. 1a). In particular, human fibroblasts (T0) treated for 18 hours with the epigenetic modifier (Post 5-aza-CR), showed a significant decrease in DNA methylation. Cells maintained comparable methylation levels when they were cultured in ESC medium for 24 and 48 hours respectively (24 h ESC; 48 h ESC). In contrast, methylation significantly increased in cells exposed to PANCR medium (24 h PANCR; 48 h PANCR) as well as in those returned to FB medium (24 h FB; 48 h FB). No methylation changes were observed in cells that were cultured in the different media, without a previous exposure to 5-aza-CR (MEDIUM ESC; MEDIUM PANCR; MEDIUM FB), indicating that the medium alone is not able to affect DNA methylation levels in differentiated cells.

Human fibroblasts acquire a transient high plasticity state after epigenetic erasing with 5-aza-CR. Methylation decrease was accompanied by the up-regulation of the ten-eleven translocation 2 (TET2) gene (Fig. 1c, Post 5-aza-CR). However, the modifier did not affect all the TET family members, but rather it exerted a distinct effect on TET2 transcription, while no significant effect was observed for TET1 and TET3.

5-aza-CR also induced the onset of pluripotency gene expression [POU class 5 homeobox 1 (OCT4), sex determining region Y-box 2 (SOX2), Nanog homeobox (NANOG), and ZFP42 zinc finger protein (REX1)] (Fig. 1b, Post 5-aza-CR). ESC medium maintained high expression levels of all these genes for the first 2 days of culture. By contrast, pluripotency gene expression significantly decreased by day 2, when post 5-aza-CR cells were cultured in differentiation media (Fig. 1b; 2d, 4d, 6d PANCR; 2d, 4d, 6d FB). Furthermore, even when maintained in ESC medium over a longer period of time, cells turned down the transcription of these genes by day 6 of culture (Fig. 1b; 6d ESC).

TET2 upregulation resulted in 5-Formylcytosine and 5-Carboxylcytosine increase. 5-aza-CR induced TET2 upregulation resulted in significant increase of 5fC and 5caC (Fig. 1d and Suppl. Figure 1). These increased levels were also present in cells cultured in ESC medium for 24 h and 48 h (24 h ESC; 48 h ESC), while decreased when cells were transferred to PANCR (24 h PANCR; 48 h PANCR) and FB (24 h FB; 48 h FB) media. No changes were observed in cells that were cultured in the different media, without a previous exposure to 5-aza-CR (MEDIUM ESC; MEDIUM PANCR; MEDIUM FB).

TET2 downregulation affects 5-aza-CR induced global DNA demethylation. Global DNA demethylation induced by 5-aza-CR was decreased in response to TET2 RNA silencing (Fig. 1a). Significant differences in methylation levels were also detected in cells cultured in ESC medium (24 h ESC; 48 h ESC). No differences were detected in the other groups (T0; 24 h PANCR; 48 h PANCR; 24 h FB; 48 h FB; MEDIUM ESC; MEDIUM PANCR; MEDIUM FB).

5-aza-CR modulates expression of histones and histone acetylating enzymes in human fibroblasts. Expression analysis demonstrated 5-aza-CR ability to significantly upregulate the transcription of histones within the 1, 2, 3 and 4 families, and their encoded protein (Figs 2 and 3). Histones showed a significant increase post 5-aza-CR and persisted at high levels in cells cultured in ESC medium for 24 h and 48 h (24 h ESC; 48 h ESC). In contrast, their expression levels were significantly down-regulated when cells were transferred to PANCR (24 h PANCR; 48 h PANCR) and returned to values comparable to T0 after 48 h in FB medium (48 h FB). No significant change was detected in cells cultured in the different media, without prior exposure to 5-aza-CR.

5-aza-CR did not affect the expression of HIST1H2AC (H2A type 1-c) and HIST2H2AC (H2A type 2-C, Figs 3 and 4). Moreover, we detected a parallel downregulation of a fibroblast specific gene (COL6A3) as well as a decreased expression of a transcript involved in cell cycle control (CCNG1). Furthermore, increased expression of histones was supported by the upregulation of the acetylating enzyme Histone acetyltransferase 1 (HAT1) and by the decreased expression of the Histone deacetylase enzyme 1 (HDAC1).

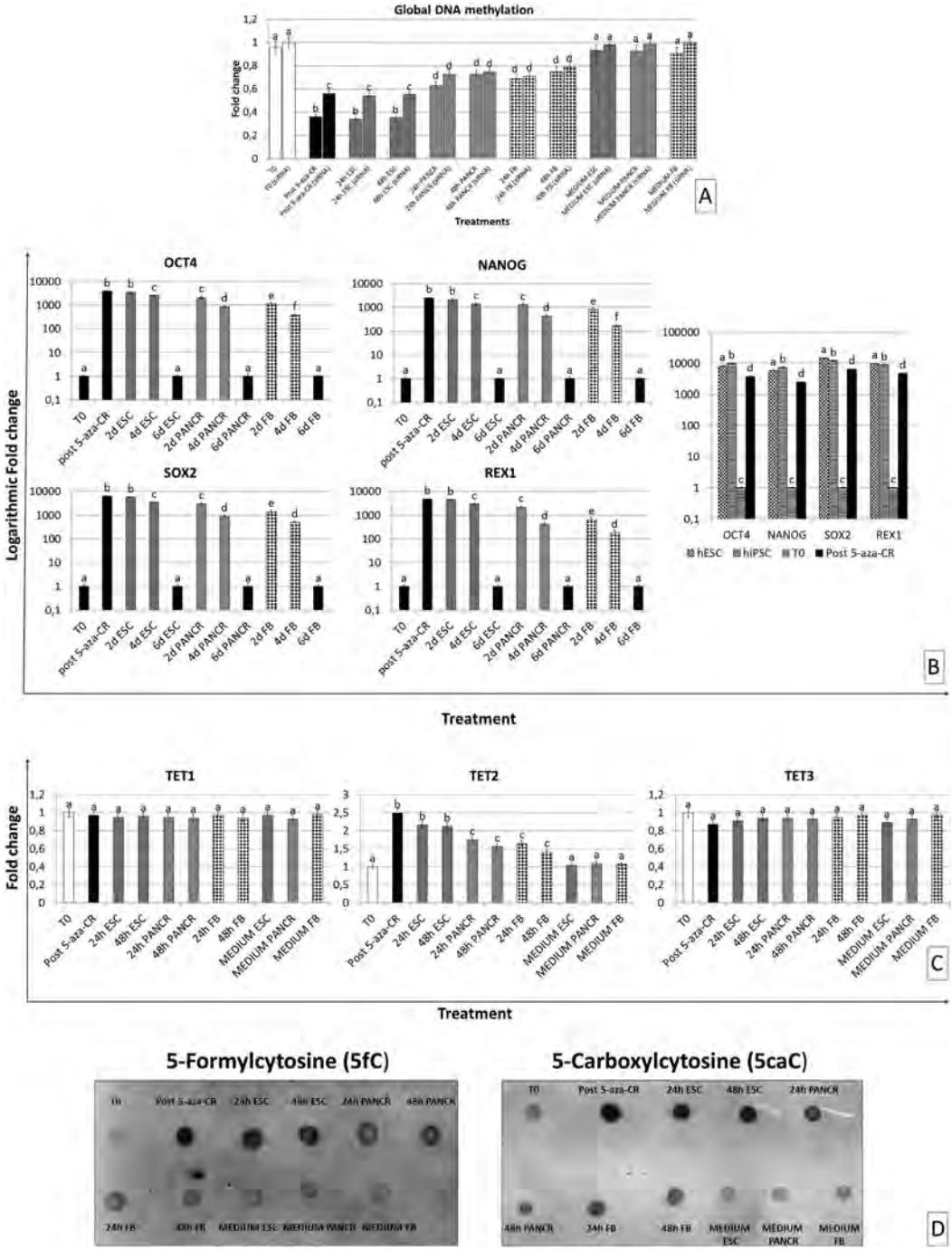

Figure 1. (**a**) Global DNA methylation of cells before and after siRNA transfection, exposed to 5-aza-CR and to different treatments for 24 and 48 hours. Highest expression set to 1 and all other times relative to this. Bars represent the mean ± SD of two independent experiments with three independent replicates, obtained from three different fibroblast primary lines. Different superscripts denote significant differences between groups ($P < 0.05$). (**b**) 5-aza-CR treatment induced the onset of pluripotency gene expression, increasing the transcription of OCT4, SOX2, NANOG and REX1 (Post 5-aza-CR). Culture in ESC medium for two days (2d ESC) maintained upregulated expression of all the genes, that are turned down by day 6 of culture (6d ESC). 5-aza-CR treated cells (Post 5-aza-CR) display pluripotency gene transcription levels in the range of 45% of those detected in human ESC (hESC) and iPSC (hiPSC). Gene expression levels are reported with the T0 expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P < 0.05$). (**c**) Effect of 5-aza-CR on TET gene family transcription. The modifier upregulated TET2, while leaving the other TET members unchanged. Gene expression levels are reported with the T0 expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P < 0.05$). (**d**) DNA dot blot analysis of 5-Formylcytosine (5fC) and 5-Carboxylcytosine (5caC) in cells exposed to 5-aza-CR and to different treatments for 24 and 48 hours.

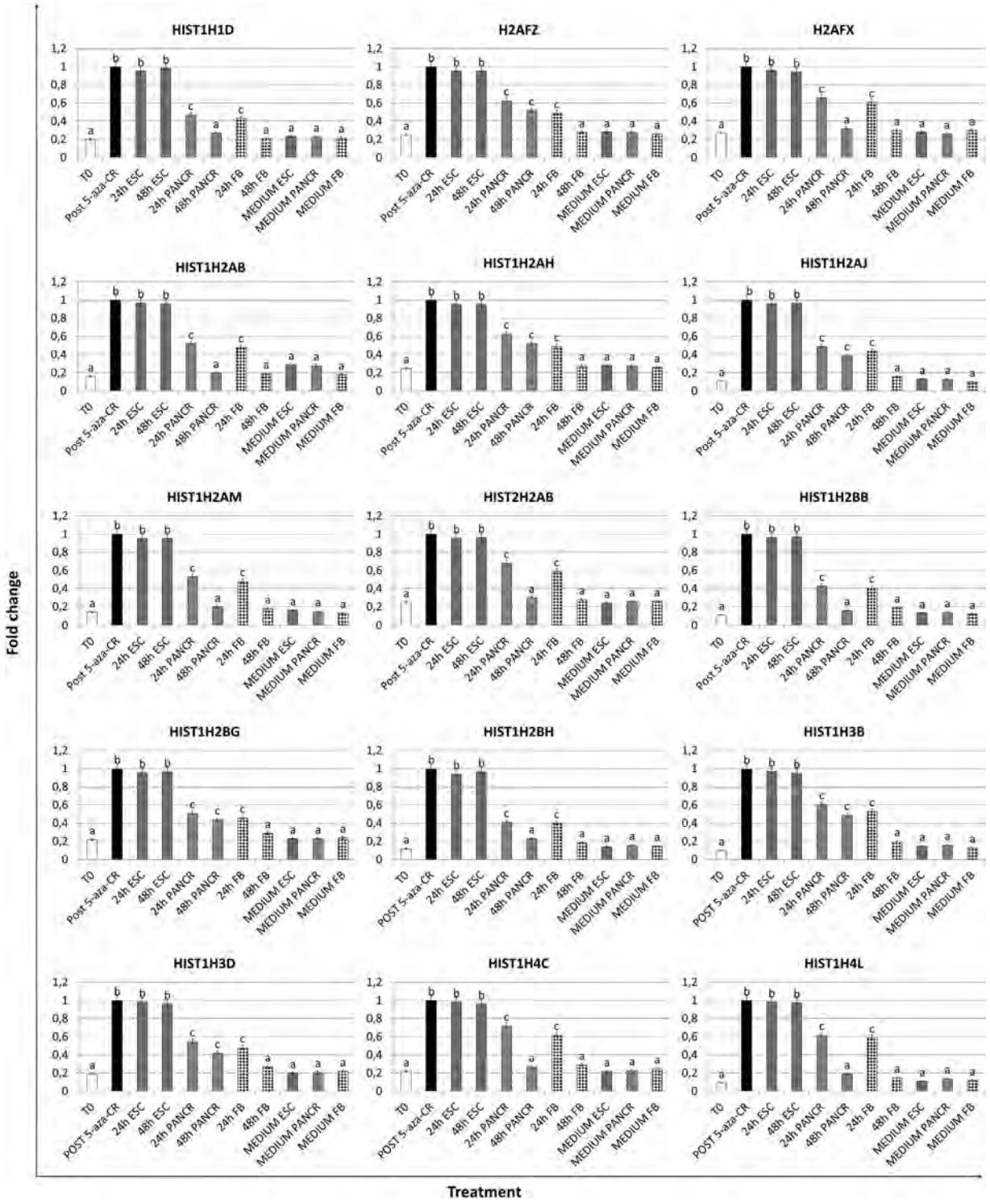

Figure 2. Histone transcription changes in adult human skin fibroblasts exposed to 5-aza-CR and subjected to three different culture conditions for 24 and 48 hours. Transcription was affected by the epigenetic eraser and persisted at high levels in cells cultured in ESC medium (24 h and 48 h ESC). It decreased when post 5-aza-CR cells where exposed to differentiation media. (24 h and 48 h PANCR; 24 h and 48 h FB). Gene expression levels are reported with the highest expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P < 0.05$).

5-aza-CR affects cell morphology and chromatin organization. T0 cells displayed a typical fibroblast morphology and were characterized by nuclei with condensed areas close to the nuclear membrane and

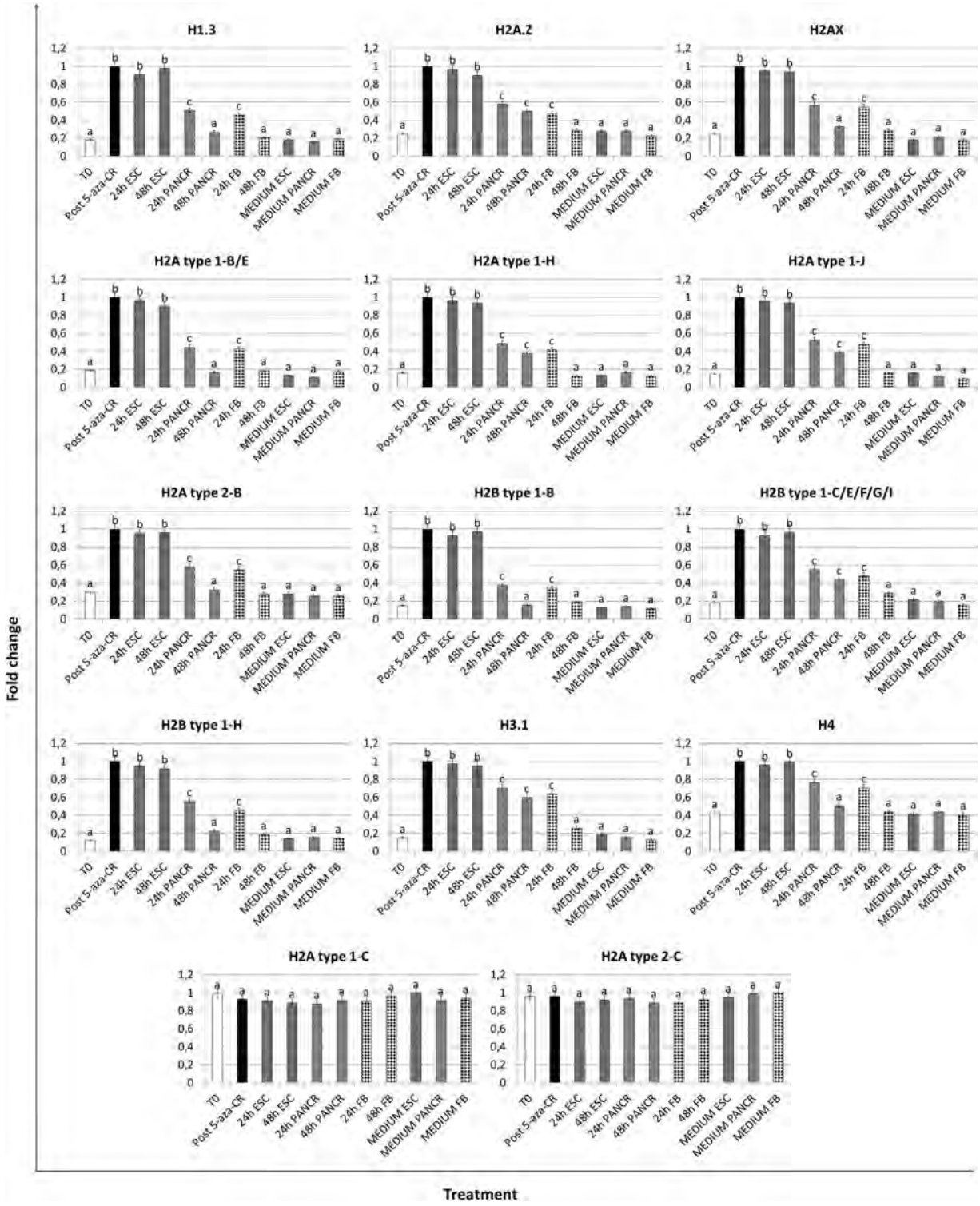

Figure 3. Histone protein levels in adult human skin fibroblasts exposed to 5-aza-CR and subjected to three different culture conditions for 24 and 48 hours. 5-aza-CR significantly increased histone protein concentrations that persisted at high levels in ESC medium (24 h and 48 h ESC). Protein levels decreased when post 5-aza-CR cells were cultured in differentiation media. (24 h and 48 h PANCR; 24 h and 48 h FB). Notably no changes were detected for H2A type 1-C and H2A type 2-C. Different superscripts denote significant differences between groups ($P < 0.05$).

large vacuoli. The perinuclear compartment contained rough endoplasmic reticulum cisternae (RER), widely scattered mitochondria, vesicles and electron dense granules (Fig. 5 panels a–c).

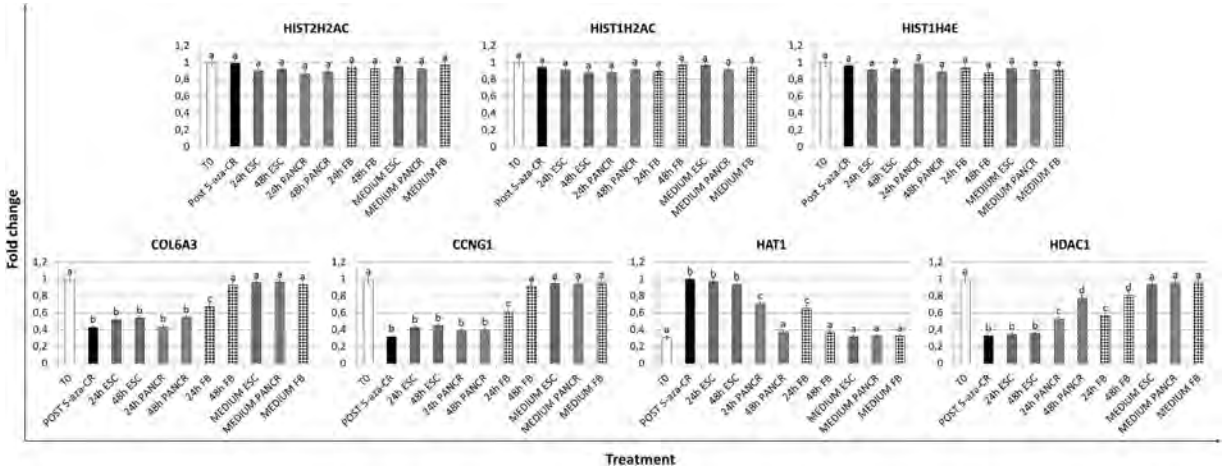

Figure 4. A gene specific effect accompanies 5-aza-CR demethylating action. Exposure to the modifier didn't affect the transcription of several histones (HIST1H2AC, HIST2H2AC and HIST1H4E), or resulted in downregulation of COL6A3 and CCNG1. Furthermore, a sharp upregulation of HAT1, and a decrease of HDAC1 was detected after exposure to 5-aza-CR. Different superscripts denote significant differences between groups ($P < 0.05$).

Count in semi-thin sections demonstrated that $86{,}31 \pm 4{,}13\%$ cells remarkably changed their morphology after exposure to 5-aza-CR, showing a reduced cell size and either few short microvilli on the plasma membrane or a smoth cell surface. At higher magnification, in the electron-lucent cytoplasm, we could detect large roundish or lobed nuclei that displayed a global chromatin decondensation, mitochondria, RER, empty or filled vacuoles (Fig. 5 panel d), as well as numerous lysosomes and autophagosomes (Fig. 5 panel e). Simultaneously with the autophagic phenomena, a cytoplasmic peripheral ring, less dense and organule-free, became visible (Fig. 5 panel f), which is likely to be responsible for a final reduction in cell size. These morphological features, that are typically related to a high plasticity phenotype[24], were maintained by $84{,}24 \pm 5{,}05\%$ and $83{,}74 \pm 5{,}52\%$ of cells cultured in ESC medium for 24 h and 48 h respectively, as confirmed at higher magnification (Fig. 5 panels g–i), possibly suggesting that, once acquired, the increased plasticity state can be sustained by signals derived from medium.

Interestingly, cells returned to FB medium after exposure to 5-aza-CR re-established the fibroblast phenotype (Fig. 5 panels j–l), indicating that the effect of the modifier is reversible and allows the re-acquisition of the original cell phenotype.

When post 5-aza-CR cells were transferred to PANCR medium, which has been previously shown to address them towards the pancreatic endoderm differentiation[17], they adopted a completely different spatial organization. After 24 hours in this medium, $80{,}12 \pm 4{,}41\%$ cells were variously shaped and showed numerous and long cytoplasmic projections. At higher magnification, nuclei were characterized by indented profiles, clustered dense chromatin close to the nuclear envelope and prominent nucleoli (Fig. 6 panels a, b, d, e). Cytoplasm contained mitochondria, lysosomes, granules and numerous vacuoles which enclosed materials of different electron density (Fig. 6 panels c, d, f). Numerous RER dilated cisternae, wrapping fine reticular network could be appreciated close to the cell surface (Fig. 6 panels a, c). At 48 hours, the same rate of cells had spotted contacts (Fig. 6 panels g, i) or became tightly attached due to the presence of gap-like junctions (Fig. 6 panel h). Cytoplasmic bridges among adjacent cells were visible (Fig. 6 panel j). Vacuoles with heterogeneous material, granules and autophagosomes were present in the inner region of cytoplasm, while bundles of filaments were localized at the periphery of the cells (Fig. 6 panel k).

Discussion

The epigenetic modifier 5-aza-CR has been shown to incorporate into DNA during cell replication, leading to a global demethylation and gene reactivation[11]. Thanks to this powerful effects, the compound has been used to increase chromatin plasticity and facilitate phenotype changes in different models[12,13,17–20]. Although the DNA demethylation capabilities of 5-aza-CR have been well characterized, both *in vitro* and *in vivo*, there is also reason to believe that this drug exhibits alternative mechanisms of action[25,26]. Here we confirm the global effect on methylation and, as shown in Fig. 1a, we detect a decreased methylation in fibroblasts exposed to 5-aza-CR. These levels are maintained in cells cultured in ESC medium, suggesting that DNA methylation levels may be modulated by the milieu of the cells, including signals deriving from the medium. These findings are in agreement with Blaschke *et al.*[27] and Yin *et al.*[28] that demonstrated changes in DNA methylation in response to specific media formulation and the contained nutritional factors. This possibility is also confirmed by the observation that cells, treated with the modifier, but exposed to FB medium and or PANCR medium, restored their methylation levels. Interestingly, the changes described were accompanied by an increased expression of several genes. Following preliminary NGS analysis of untreated fibroblasts vs. 5-aza-CR exposed cells, we here confirm upregulated transcription of the TET2 gene, which has been recently shown to support 5-aza-CR dependent demethylation

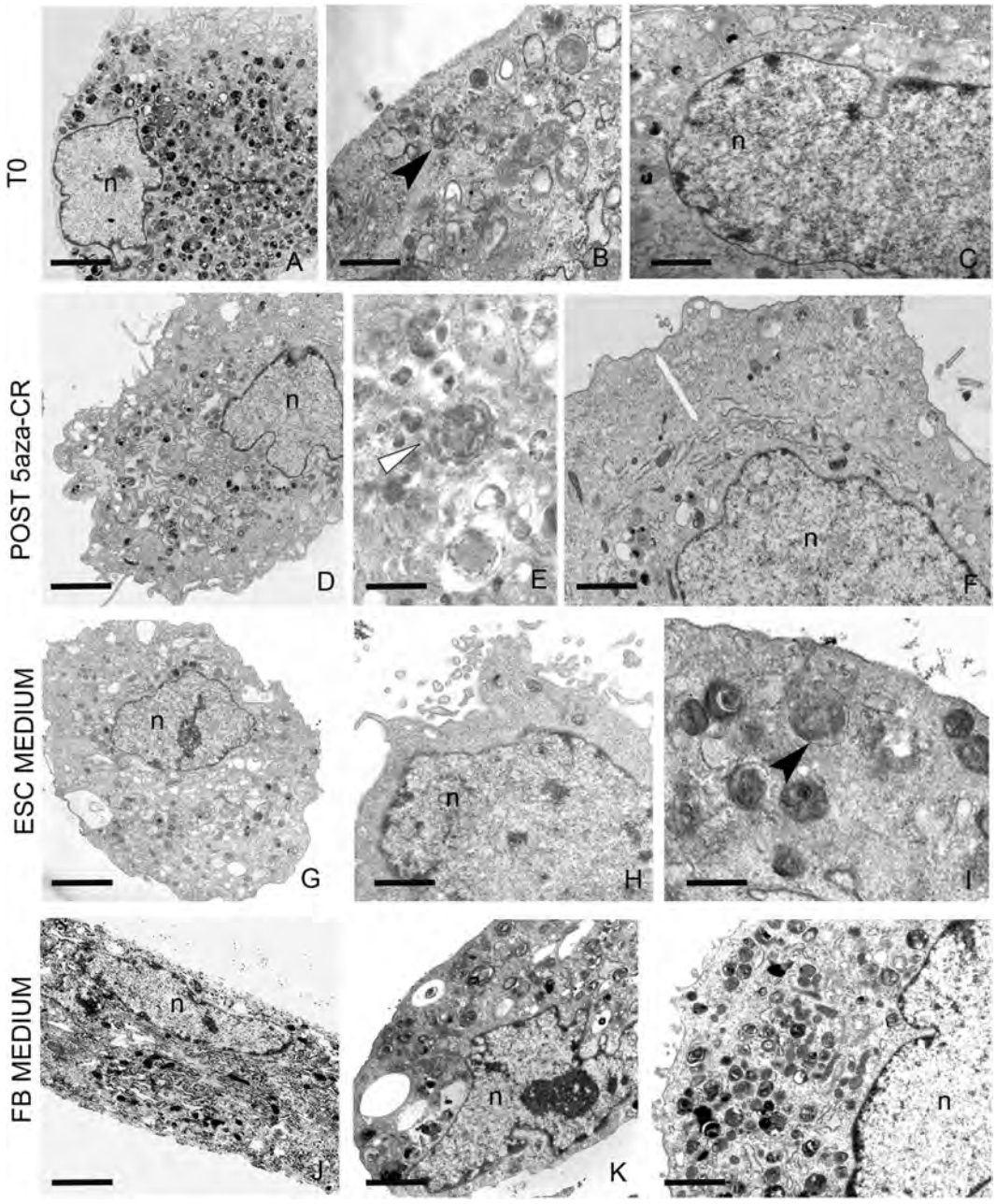

Figure 5. 5-aza-CR induced cell plasticity is unravelled by morphological changes. The distinct fibroblastoid morphology (panels a–c) change after exposure to 5-aza-CR (panels d–f) with the acquisition of an undifferentiated progenitor-like phenotype characterized by nuclei (n) showing chromatin decondensation (panels d, f) and autophagic phenomena (panel e, arrowhead). Cells can be characterized by cytoplasmic peripheral ring without organules that are present in the perinuclear area (panel f, white arrow). Post 5-aza-CR cells cultured in ESC medium (panels g–i) maintain the undifferentiated phenotype (n: nucleus; autophagosomes: arrowhead). Post 5-aza-CR cells cultured in FB medium (panels j–l) show re-established features typical of fibroblasts. Scale bars: a, 2.5 μm; b,1 μm; c,1 μm; d, 2 μm; e,0.8 μm; f, 2 μm; g, 4 μm; h, 1.4 μm; i, 0.6 μm; j, 2 μm; k, 2 μm; l, 1.7 μm.

process in hepatocarcinoma cell lines[29]. In particular, we show that upregulation of TET2 results in an increase of both 5fC and 5caC (Fig. 1d), indicating 5-aza-CR ability to activate a direct and active demethylating effect, possibly mediated via TET2 protein. Furthermore, the possibility of a synergistic action between direct and indirect demethylation processes is also demonstrated thank to TET2 siRNA experiments, where we demonstrate that downregulation of TET2 protein results in an overall decrease of global DNA demethylation (Fig. 1a), that however still persist in response to 5-aza-CR, possibly due to the unaffected action of 5-aza-CR on DNMTs.

TET2 upregulation is also in agreement with the acquisition of the higher plasticity state detected in post 5-aza-CR cells, since TET2 has been reported to have a role in pluripotency induction and maintenance[30].

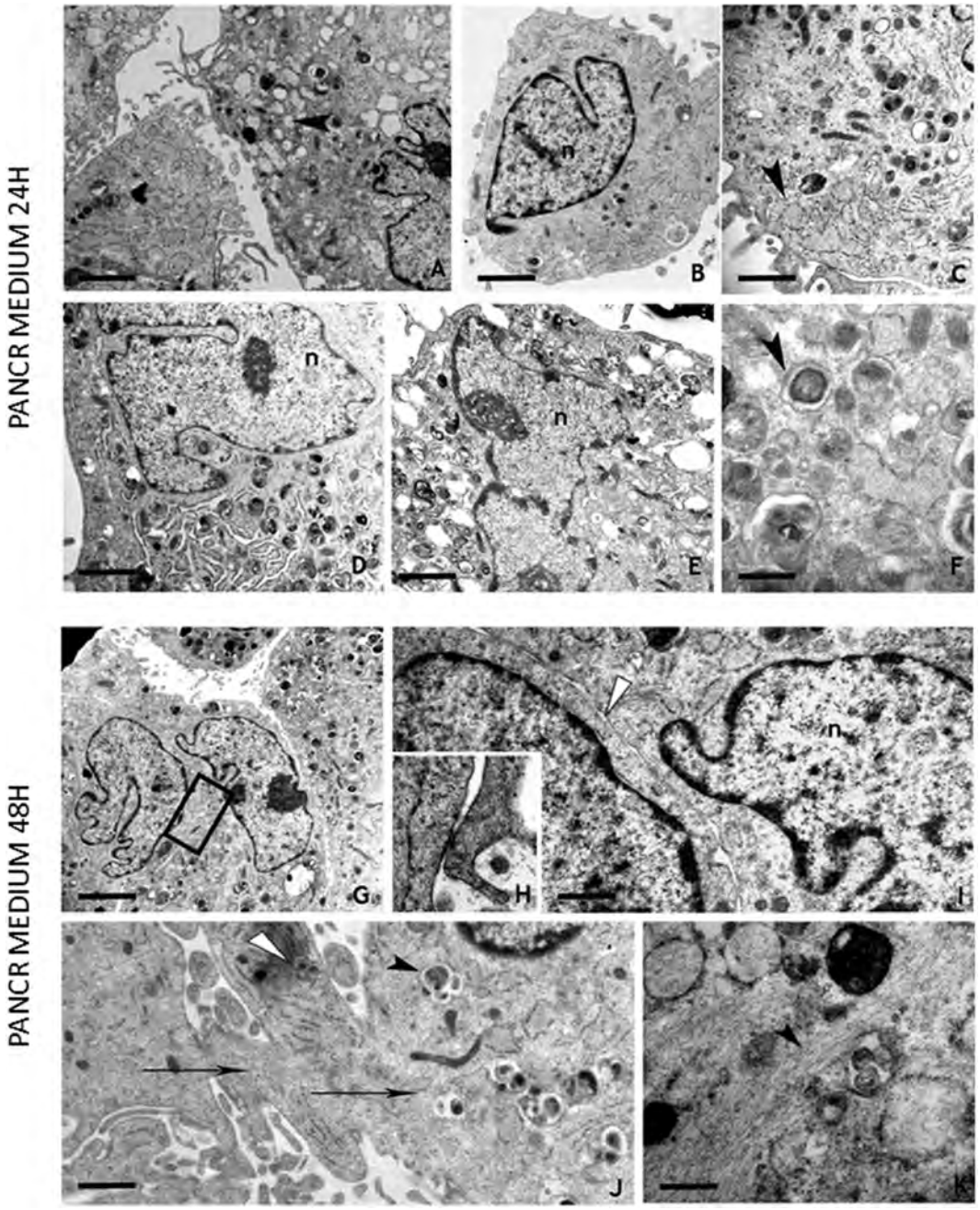

Figure 6. Post 5-aza-CR cells cultured in PANCR medium (panels a–k) show peculiar morphological changes. After 24 hours, cells are characterized by cytoplasmic projections (panel a), indented nuclei (n) (panels a, b, d, e) and large nucleoli. The cytoplasm is occupied by numerous organules, large dilated cisternae of endoplasmic reticulum (panels a, c, arrowheads) and autophagosomes (panel f, arrowhead). After 48 hours, cells can be in close contact (panels g, detail; i, white arrowehead) or tightly joined by gap-like junction (see enlargement in panel h) or can show cytoplasmic bridges (panel j, arrows). In the cytoplasm, phagolysosomes (panel j, arrowhead) and bundles of filaments (panel k, arrowhead) are visible. Scale bars: a, 2 μm; b, 2 μm; c, 3.3 μm; d, 1.7 μm; e, 1.4 μm; f, 0.5 μm; g, 2 μm; i, 1 μm; j, 1.3 μm; k, 0.5 μm.

Interestingly, 5-aza-CR did not affect all the members of the TET family, but rather it exerted a distinct effect on TET2, leaving the other members unchanged. Based on previous observations, showing that TET2 does not affect methylation levels at gene promoters and transcription start sites, but, in contrast, mainly regulates gene bodies and exon boundaries, our results suggest the possibility of a specific effect of the modifier on the latter.

Exposure to 5-aza-CR also caused the onset of pluripotency gene expression, such as OCT4, NANOG, REX1 and SOX2 (Fig. 1b). As shown in Fig. 1b, these genes were induced by 5-aza-CR, displaying transcription levels in the range of 45% of those detected in human ESCs and iPSCs. This result is also consistent with the molecule ability to reactivate previously silent genes and to alter the differentiation state of eukaryotic cells, as well as with

recent data showing that 5-aza-CR demethylation of one or more loci facilitates a rapid and stable transition of partially reprogrammed iPSCs to a fully reprogrammed state[31].

Interestingly, culture in ESC medium maintained upregulated expression of all the genes described, suggesting the acquisition of a high plasticity state. However, this condition was transient and reversible, since expression of pluripotency related genes decreased gradually when post 5-aza-CR cells where exposed to differentiation media (24 h and 48 h PANCR; 24 h and 48 h FB). Furthermore, even when maintained in ESC medium over a longer period of time, cells turned down the transcription of these genes by day 6 of culture (Fig. 1b).

The expression of histones belonging to 1, 2, 3 and 4 families was also affected by the exposure to the epigenetic eraser. The genes and their encoded proteins showed increases (between 3 to 11 fold), in cells treated with 5-aza-CR, and persisted at high levels in cells cultured in ESC medium for 24 and 48 hours (Figs 2 and 3). The effect on transcription was evident in replication-dependent as well as in replication-independent histones, indicating that it may not only be a secondary indirect consequence of 5-aza-CR on the cell cycle. Furthermore, histones belonging to the same cluster was either upregulated or unchanged by 5-aza-CR, suggesting a histone specific effect rather than a cluster specific one. We have no ready explanation for this, although it is known that separate sets of histone genes are transcribed in a precise temporal sequence during development and differentiation of lower species[32]. On the other hand, the contribution of histone transcription to the maintenance of high plasticity versus differentiation in mammalian is presently still to be fully understood and further studies are required.

We postulate that the 5-aza-CR effect on histone gene transcription is unlikely to be accounted for a general demethylating effect, since exposure to the modifier, resulted in parallel downregulation of fibroblast specific genes (COL6A3) as well as of transcripts involved in the control of cell cycle (CCNG1) and did not affect the expression of several histones genes (HIST1H2AC, HIST2H2AC and HIST1H4E, Fig. 4). In agreement with this hypothesis, Komashko *et al.* demonstrated that several changes in gene expression after 5-aza-CR treatment were related to an alternative DNA-hypomethylating independent effect[33]. Furthermore, consistent with the idea that this epigenetic modifier has additional modes of action, several works indicated that 5-aza-CR can induce expression of genes lacking CpG methylation[34,35]. Interestingly, Takebayashi *et al.* demonstrated a DNA methylation independent mechanism, directly related to an increase in histone hyperacetylation[25]. This is also in agreement with our observation that HAT1 transcription was significantly higher in cells exposed to 5-aza-CR, suggesting the possible correlation among the exposure to the modifier, the overexpression of HAT1, the consequent histone hyperacetylation and increased transcription. This possibility is further supported by the parallel decreased expression of HDAC1 (Fig. 4), reported in our manuscript. It is also coherent with previous works that show how modulation of histone acetylation is an integral component of gene expression control[36] and demonstrate a key role of the specific enzymatic activity of HDACs in transcriptional regulation[37].

Morphological analysis further unravelled the impact of 5-aza-CR on cell plasticity and differentiation. In particular, it is interesting to underline that adult skin fibroblasts changed after 5-aza-CR exposure, became smaller in size and reshaped into an undifferentiated progenitor-like phenotype. In this condition, cells showed a nuclei/cytoplasm ratio similar to what previously described in hESCs[38] and in hiPSCs obtained from human dermal fibroblasts[39]. 5-aza-CR increased plasticity was also accompanied by autophagic phenomena, that have been reported to play a critical role in the maintenance of adult stem cells[40], possibly reducing senescence via the active removal of oxigen reactive species[41]. Interestingly the presence of autophagic vacuoles have been previously reported in ultrastructural analysis of hiPSC[42]. Furthermore, post 5-aza-CR cell nuclei were characterized by sparse chromatin and appeared devoid of compact formations. This is consistent with the observation that an open chromatin structure is required for a high plasticity state[43] and is a common feature of iPSC and, more in general, one of the hallmark of stem cells[43,44]. Interestingly, this condition was maintained by ESC medium, possibly suggesting that, once acquired, the decreased methylation state can be sustained by factors contained in the medium such as LIF, which has been recently demonstrated to induce murine cell hypomethylation[45]. The acquisition of a transient high plasticity state is also consistent with post 5-aza-CR cell ability to respond to differentiation protocols and acquire a differentiated phenotype[17–20]. Indeed, when 5-aza-CR treated cells were transferred to PANCR medium, which address them towards the pancreatic endoderm differentiation[17], they adopted a different phenotype. Nuclei were characterized by indented profiles, prominent nucleoli and clustered dense chromatin, mostly locating close to the nuclear envelope. These morphological changes, involving a packed chromatin conformation, and its clustering near the nuclear envelope, have been previously described and considered to be distinctive of a differentiated state[24] and suggest that post 5-aza-CR cells may reverse their high plasticity state and respond to differentiation stimuli. This is consistent with the methylation and transcriptional changes described above and indicates that the mechanisms exerted by the modifier have also deep effects at the phenotype level. Such hypothesis is further supported by the observation that the majority of cells showed gap-like junctions and cytoplasmic bridges, all features required to guarantee a correct differentiation processes[46,47].

The reversibility of the effects exerted by 5-aza-CR, was confirmed by the re-acquisition of the original phenotype when cells were returned to FB medium, indicating that a short exposure to the modifier allows a transient high plasticity chromatin state, sufficient to allow cell reshaping.

Altogether, the data presented in this manuscript, indicate 5-aza-CR ability to increase TET2 gene expression, modulate transcription of histones and histone acetylating enzymes, which result in specific morphological changes. Moreover, the results obtained, also suggest the molecule ability to interfere with DNA methylation through a direct TET2-mediated mechanism, that accompanies the well-known indirect DNMT related one.

GENE	DESCRIPTION	CATALOG NO.
ACTB	Actin, beta	Hs01060665_g1
CCNG1	Cyclin G1	Hs00171112_m1
COL6A3	collagen, type VI, alpha 3	Hs00915125_m1
H2AFX	H2A histone family, member X	Hs00266783_s1
H2AFZ	H2A histone family, member Z	Hs01888362_g1
HAT1	histone acetyltransferase 1	Hs00186320_m1
HDAC1	histone deacetylase 1	Hs02621185_s1
HIST1H1D	histone cluster 1, H1d	Hs00271187_s1
HIST1H2AB	histone cluster 1, H2ab	Hs01001083_s1
HIST1H2AC	histone cluster 1, H2ac	Hs00374312_s1
HIST1H2AH	histone cluster 1, H2ah	Hs00544732_s1
HIST1H2AJ	histone cluster 1, H2aj	Hs04191486_s1
HIST1H2AM	histone cluster 1, H2am	Hs01920904_s1
HIST1H2BB	histone cluster 1, H2bb	Hs00606684_s1
HIST1H2BG	histone cluster 1, H2bg	Hs00374317_s1
HIST1H2BH	histone cluster 1, H2bh	Hs00374322_s1
HIST1H3B	histone cluster 1, H3b	Hs00605810_s1
HIST1H3D	histone cluster 1, H3d	Hs00371415_s1
HIST1H4C	histone cluster 1, H4c	Hs00543883_s1
HIST1H4E	histone cluster 1, H4e	Hs00374346_s1
HIST1H4L	histone cluster 1, H4l	Hs00361930_s1
HIST2H2AB	histone cluster 2, H2ab	Hs00602439_s1
HIST2H2AC	histone cluster 2, H2ac	Hs00543838_s1
NANOG	Nanog homeobox	Hs02387400_g1
OCT4	POU class 5 homeobox 1	Hs00999632_g1
REX1	ZFP42 zinc finger protein	Hs00399279_m1
SOX2	Sex determining region Y-box 2	Hs01053049_s1
TET1	tet methylcytosine dioxygenase 1	Hs00286756_m1
TET2	tet methylcytosine dioxygenase 2	Hs00325999_m1
TET3	tet methylcytosine dioxygenase 3	Hs00379125_m1

Table 1. List of primers used for quantitative PCR analysis of human cells.

Experimental Procedures

All chemicals were purchased from Life Technologies (Italy) unless otherwise indicated.

Ethics statement. Adult human skin fibroblast primary lines were kindly donated by Dr. Gianpaolo Zerbini (Scientific Institute San Raffaele). Cells were isolated from adult patients aged between 35 and 49 years, after written informed consent and approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa. All the methods in our study were carried out in accordance with the approved guidelines.

Skin fibroblast culture (FB). Three adult skin fibroblast primary lines were isolated from human healthy patients and grown in standard culture medium (FB), consisting of DMEM with 20% (vol/vol) Fetal Bovine Serum (FBS), 2 mM glutamine (Sigma), and antibiotics (Sigma). Cells were passaged twice a week in a 1:3 ratio. All experiments were performed in triplicate.

5-aza-CR treatment. Fibroblasts were plated in 4-well multidish (Nunc) previously treated with 0.1% gelatin (Sigma) at concentration of 7.8×10^4 cell/cm^2. They were then incubated with 1 μM 5-aza-CR (Sigma) for 18 hours. Concentration and time of exposure were selected according to our previous work[17].

Experimental design. At the end of 5-aza-CR treatment cells were divided in three experimental groups and cultured for 24 and 48 hours, 4 and 6 days. In the first group, cells were returned in FB medium. The second group of cells were cultured in medium specific for pluripotency maintenance (ESC). Cells of the last group we differentiated toward pancreatic lineage (PANCR).

Medium for pluripotency maintenance (ESC). Cells were cultured in DMEM low glucose/ Ham's F-10 Nutrient Mix (1:1) supplemented with 10% KnockOut Serum Replacement, 5% FBS, 1% antibiotics (Sigma), 0.1 mM β- mercaptoetanol (Sigma), 2 mM glutamine (Sigma), 1 mM MEM Non-Essential Amino Acids, 1% Nucleoside mix, 20 ng/ml Recombinant Human FGF basic (bFGF) and 10^3 units/ml ESGRO (LIF)[48].

Medium for pancreatic induction (PANCR). Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with 1% B27, 1% N2, 0.1 mM β- mercaptoetanol (Sigma), 2 mM glutamine

PROTEIN	DESCRIPTION	COMPANY	CATALOG NO.
H2AX	H2A histone family, member X(H2AFX), ELISA Kit	MyBioSource	MBS9335115
H2A.Z	H2A histone family, member Z(H2AFZ), ELISA Kit	MyBioSource	MBS2025145
H1.3	Histone cluster 1, H1d (HIST1H1D), ELISA Kit	MyBioSource	MBS914357
H2A type 1-B/E	Histone Cluster 1, H2ab (HIST1H2AB), ELISA Kit	MyBioSource	MBS9321468
H2A type 1-C	Histone cluster 1, H2ac (HIST1H2AC), ELISA Kit	MyBioSource	MBS9333153
H2A type 1-H	Histone cluster 1, H2ah (HIST1H2AH), ELISA Kit	MyBioSource	MBS9320153
H2A type 1-J	Histone cluster 1, H2aj (HIST1H2AJ), ELISA Kit	MyBioSource	MBS9343145
H2A type 1	Not commercially available		
H2B type 1-B	Histone cluster 1, H2bb (HIST1H2BB), ELISA Kit	MyBioSource	MBS9317197
H2B type 1-C/E/F/G/I	Histone cluster 1, H2bc (HIST1H2BG), ELISA Kit	MyBioSource	MBS9324014
H2B type 1-H	Histone cluster 1, H2bh (HIST1H2BH), ELISA Kit	MyBioSource	MBS9319723
H3.1	Histone cluster H3.1 (HIST1H3A), ELISA Kit	MyBioSource	MBS937951
H4	Histone H4 (HIST1H4A), ELISA Kit	MyBioSource	MBS2884038
H2A type 2-B	Histone cluster 2, H2ab (HIST2H2AB), ELISA Kit	MyBioSource	MBS9338734
H2A type 2-C	histone cluster 2, H2ac (HIST2H2AC), ELISA Kit	MyBioSource	MBS900872

Table 2. List of ELISA kits used for quantification of histone protein levels.

(Sigma), 1 mM MEM Non-Essential Amino Acids, 0.05% bovine serum albumin (BSA, Sigma), and with 30 ng/mL activin A.

siRNA transfection. The targeting siRNA used in the present study was TET2 siRNA (Santa Cruz) and siRNA induced protein knock-down was carried out as previously described[49]. More in detail, cells were transfected 24 hours before exposure to the modifier and during 5-aza-CR treatment, with 90 pM of siRNA, using Lipofectamine RNAi MAX Reagents diluted in Opti-MEM® Medium, following the manufacturer's instructions. For negative controls, cells were incubated with Lipofectamine alone or with Lipofectamine plus 90 pM non-silencing siRNAs. The success of the silencing occurrence was assessed by monitoring the decrease of TET2 protein. After transfection global DNA methylation was assessed as described below. All experiments were performed three times in triplicate samples.

Global methylation analysis. Genomic DNA was extracted from untreated fibroblasts and after their siRNA transfection at different time points: T0, after 5-aza-CR treatment (Post 5-aza-CR), 24 and 48 hours after culture in fibroblast (FB), ESC or pancreatic induction (PANCR) media. PureLink® Genomic DNA Kits was used according to the manufacturer's instructions. DNA was converted to single-stranded DNA by incubation at 95 °C for 5 min, followed by rapid chilling on ice. Samples were then digested to nucleosides by incubating the denatured DNA with nuclease P1 for 2 h at 37 °C in 20 mM sodium acetate (pH 5.2). Alkaline phosphatase was added and incubated for 1 h at 37 °C in 100 mM Tris (pH 7.5). After centrifugation, the supernatant was used for ELISA assay using Global DNA Methylation ELISA Kit (5′-methyl-2′-deoxycytidine Quantitation; CELL BIOLABS) according to the manufacturer's protocol.

DNA dot blot. Genomic DNA was extracted with PureLink® Genomic DNA Kits as previously described. DNA concentration was assessed with NanoDrop 8000 (Thermoscientific). Aliquots of 200 ng total DNA were prepared in a total volume of 2 μL per sample and spotted onto nylon membranes (Hybond-N+, Amersham). The DNA spots were air dried for 15 min, UV-crosslinked for 1 min and probed with primary antibodies against 5-Carboxylcytosine (Active Motif, 1:1500) and 5-Formylcytosine (Active Motif, 1:1500). Dots were visualized with a WesternBreeze chemiluminescent kit. Signal intensity was quantified by densitometric analysis, using the Image J analysis software (National Institutes of Health).

Gene expression analysis. RNA was extracted with the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. Predesigned gene-specific primer and probe sets from TaqManGene Expression Assays (Applied Biosystems) were used for gene study (Table 1). PCR runs and fluorescence detection were carried out in a 7500 Real-Time PCR System (Applied Biosystems). β-actin was used as internal standard. For each individual gene, the

number of amplification cycles for the fluorescent reporter signal to reach a common threshold value (Ct) were estimated and then normalized against the Ct value obtained for β-actin of the same sample to give the ΔCt value.

Quantification of histone protein levels. Cells were detached from the culture dish with trypsin and collected by centrifugation. They were then washed three times with cold PBS (1x) and subjected to ultrasonication. After centrifugation and dilution, samples were used for ELISA assay using the commercially available Enzyme-linked Immunoassorbent Assay Kit (MyBioSource) for the specific histone (Table 2), according to the manufacturer's protocol. Optical density (O.D.) measurements were done using a Multiskan™ FC Microplate Photometer (Thermo Fisher) at the wavelength of 450 nm. Protein concentration in the samples was determined by comparing the O.D. of the samples to the standard curve.

Morphological analysis. Cells were analyzed at different time points: immediately before exposure to 5-aza-CR (T0), after 5-aza-CR treatment (Post 5-aza-CR), 24 and 48 hours after culture in standard fibroblast (FB), in ESC or in pancreatic induction (PANCR) media.

Electron microscopy. Samples were fixed for 2 hours in 0.1 M cacodylate buffer pH 7.2, containing 2% glutaraldehyde. Specimens were then washed in the same buffer and post-fixed for 2 hours with 1% osmic acid in cacodylate buffer. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica, Austria). Semi-thin sections were stained by conventional methods (crystal violet and basic fuchsin) and observed under a light microscope (Olympus, Japan) to score cells for different morphological changes. Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol, Japan).

Statistical analysis. Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

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Author Contributions

T.A.L.B and F.G. designed the study and wrote the manuscript. E.F.M.M., G.P., and G.T. performed the experiments and analyzed data. M.D.E. provided expertise for electron microscopy and imaging.

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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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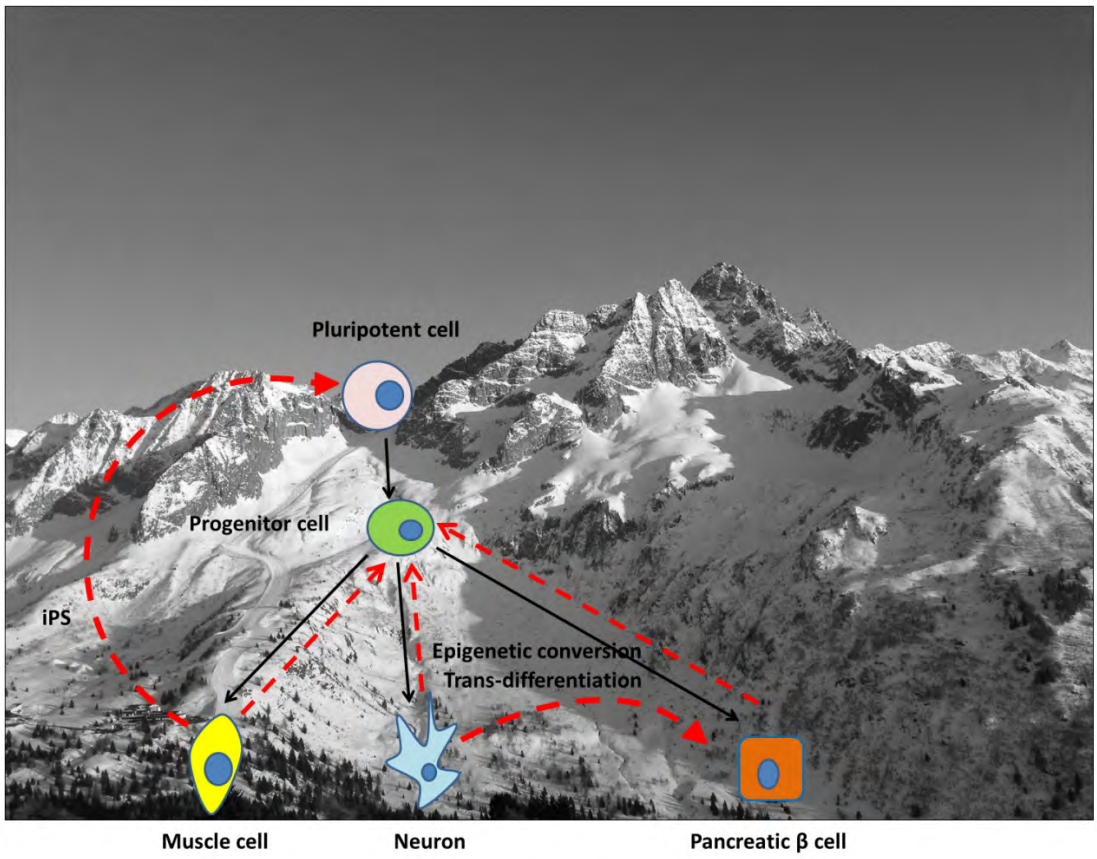


Figure 1. Representation of Waddington model. A pluripotent cell rolls from a non-committed condition down to the bottom of the hill, where the cell is unipotent and is characterized by a tissue specific differentiated state. The hill is marked by slopes and valleys, representing the many different and 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 trough 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
Histone Arginine Methylation	Protein Arginine Methyltransferases Histone Demethylases Tudor Domains (recognize symmetrically dimethylated arginines); WD40 Domains
Histone Lysine Methylation	Histone Lysine Methyltransferases Histone Lysine Demethylases Chromodomains; Tudor Domains; PHD Fingers; MBT Domains; ZF-CW Proteins; WD40 Domains; PWWP
Histone Phosphorylation	Kinases (JAK2, ATM/ATR, PKC, PKA, Haspin, Aurora B Kinase, RSK2, AMPK, MSK, MEK) 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 (Stadtfeld *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 un-physiological 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 draw 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; Aimiuwu *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).

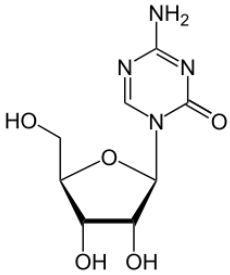
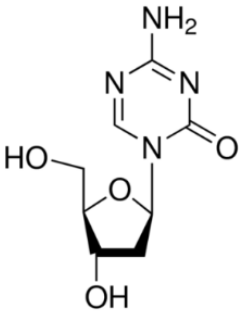
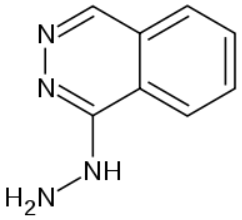
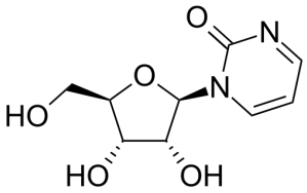
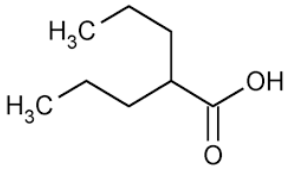
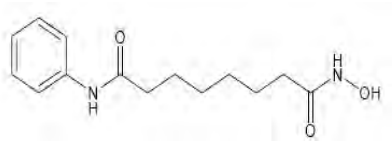
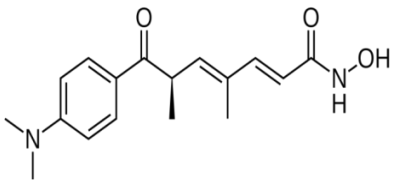
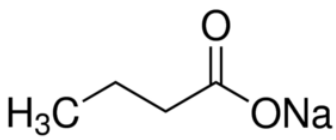
In accordance with these findings, our laboratory demonstrated that an adult somatic cells can be converted into a new cell type after an 18 h exposure to 5-aza-CR (Pennarossa *et al*., 2013, 2014; Brevini *et al*., 2014, 2016). Cells acquired a 'highly permissive state' with significant changes in their 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 de-methylating 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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Clinical Epigenetics

REVIEW Open Access

The quest for an effective and safe personalized cell therapy using epigenetic tools

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Abstract

In the presence of different environmental cues that are able to trigger specific responses, a given genotype has the ability to originate a variety of different phenotypes. This property is defined as plasticity and allows cell fate definition and tissue specialization. Fundamental epigenetic mechanisms drive these modifications in gene expression and include DNA methylation, histone modifications, chromatin remodeling, and microRNAs. Understanding these mechanisms can provide powerful tools to switch cell phenotype and implement cell therapy. Environmentally influenced epigenetic changes have also been associated to many diseases such as cancer and neurodegenerative disorders, with patients that do not respond, or only poorly respond, to conventional therapy. It is clear that disorders based on an individual's personal genomic/epigenomic profile can rarely be successfully treated with standard therapies due to genetic heterogeneity and epigenetic alterations and a personalized medicine approach is far more appropriate to manage these patients.

We here discuss the recent advances in small molecule approaches for personalized medicine, drug targeting, and generation of new cells for medical application. We also provide prospective views of the possibility to directly convert one cell type into another, in a safe and robust way, for cell-based clinical trials and regenerative medicine.

Keywords: Epigenetic conversion, Epigenetics, iPSCs, Molecular medicine, Personalized medicine, Regenerative medicine, Small molecules

Background

Epigenetics is at the center of modern biology and medicine, since it is currently considered a fundamental tool to understand embryo development and stem cell biology, as well as to explain the relationship among an individual's genetic background, the environmental influences, aging, and disease susceptibility.

The most exciting idea is that epigenetics may provide new clues to intervene at the junction between the genome and the environment, modifying the effects of deleterious genes [1]. It would be also useful to develop new strategies for disease prevention and therapy and to master tissue reprogramming in regenerative medicine.

In particular, during the last years, great attention was given to epigenetics in order to prevent, diagnose, and treat different diseases. Indeed, it has been demonstrated that malignant transformations as well as several disorders, such as autism, bipolar disorder, familial hypertrophic cardiomyopathy, schizophrenia, and syndromes, namely Prader-Willi, Angelman, Beckwith-Wiedemann, and Silver-Russell, are directly or indirectly caused by epigenetic alterations in form of mutation of DNA methylation or incorrect histone modifications [2–5]. In particular, DNA methyltransferase (DNMT) inhibiting nucleoside analogs, non-nucleoside analogs, and histone deacetylase (HDAC) inhibitors have been proposed as potential anti-cancer drugs. In parallel, several researches are focusing on the development of direct disease treatments with small molecules, based on individual personal genomic profile and epigenetic characteristics of each patient, in order to improve outcomes.

Presently, a growing problem is also represented by degenerative diseases that, despite decades of research, still lack effective cures. Regenerative medicine has earned increased attention and represents an attractive option as a potentially novel approach for the treatment

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of neurodegenerative, cardiovascular and liver diseases, diabetes, spinal cord injury, and corneal degeneration. In this field, the use of small molecules in cell reprogramming technology has allowed for the development of protocols that avoid the use of retroviral and/or lentiviral vectors, and the insertion of transgenes for the generation of induced pluripotent cells (iPSC). However, although these cells may represent a promising stem cell source, the induction of a stable pluripotent state and the deriving cell instability severely limits their use in cell therapy.

In order to circumvent these limits, a new small-molecule-based method able to directly convert a terminally differentiated cell into a different cell type has been recently proposed. This new approach demonstrated that it is possible to dynamically interact with cell genotype and phenotype through the use of epigenetic modifiers [2–7].

We here discuss the recent advances in small molecule approaches for drug targeting, personalized medicine, and generation of new cells for medical application. We also provide prospective views on the possibility to directly convert one cell type into another, in a safe and reproducible way, in order to obtain cells that may find application in clinical trials and regenerative medicine.

Review

Molecular basis of epigenetics

The molecular basis of epigenetics is a complex phenomenon that determines activation or silencing of certain genes, without changing the DNA sequence.

There are several types of epigenetic mechanisms that play an essential role in the regulation of chromatin structure and gene expression, namely histone post-translational modifications, covalent modification of DNA, small (21- to 26-nt) non-coding RNAs (ncRNAs), and recombination of non-genic DNA.

These processes are driven by different proteins that are usually categorized based on their molecular nature. In particular, the enzymes involved in epigenetic control are classified as epigenetic writers, epigenetic erasers, and epigenetic readers (see Table 1).

The firsts catalyze modifications either on DNA, RNA, or histone proteins by adding of chemical groups on top of them. This group includes the following:

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

Mechanism	Writer	Eraser	Reader
DNA methylation	DNA methyltransferases (DNMT1, DNMT3) [67, 68]	DNA demethylation enzymes (TET) [69]	Methyl-CpG binding domains (MECP2, MBD1, MBD2, and MBD4) [70]
Histone lysine acetylation	Histone acetyltransferases (GCN5/PCAF, MYST, P300/CBP, SRC/p160) [71]	Histone deacetylases (HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, HDAC10, HDAC11); Sir2-like proteins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7) [72]	Bromodomain, tandem PHD [73, 74]
Histone arginine methylation	Histone arginine methyltransferases (PRMT1, PRMT2, PRMT3, PRMT5, PRMT6, PRMT7, CARM1) [75]	Histone arginine demethylase (bifunctional arginine demethylase and lysyl-hydroxylase JMJD6) [76]	Tudor, ADD, WD40 [73, 74]
Histone lysine methylation	Histone lysine methyltransferases (EZH, SET1, SET2, SMYD, SUV39, SUV4-20, RIZ, SET8/PR-SET7, SET7/9, PRDM) [77]	Histone lysine demethylases Lysine-specific demethylase (LSD1, LSD2); Jumonji histone demethylases (JHDM1, JHDM2, JHDM3/JMJD2, JARID, JMJC, PHF2/PHF8, UTX/UTY) [77]	Chromodomain, ADD, ankyrin, BAH, chromobarrel Tudor, PHD fingers, MBT, ZF-CW, WD40, PWWP [73, 74]
Histone phosphorylation	Histone kinases (AGC, CaMK, CMGC, protein-tyrosine kinase, MEK) [78]	Histone phosphatases Serine/threonine phosphatases (PPP, PPM); tyrosine phosphatases (PTP, VH1-like dual-specificity phosphatase, cdc25) [79, 80]	Chromoshadow, 14.3.3 proteins, BIR, BRCT [73, 74]
Histone lysine ubiquitination	Histone lysine ubiquitinases (E1 enzyme, E2 ubiquitin conjugases); E3 ubiquitin-protein ligases (HECT domain, RING finger domain) [81]	Histone lysine deubiquitinases (UCH, USP, MJD, OTU, JAMM) [82]	Unknown
Histone arginine citrullination	Histone arginine deiminases (PAD1, PAD2, PAD3, PAD4, PAD6) [83]	Unknown	Unknown
Histone lysine biotinylation	Histone lysine biotinases (HLCS) [84]	Unknown	Unknown
Histone lysine ribosylation	Histone lysine ribosylases (PARP1) [85]	Histone lysine deribosylase (PARG) [85]	Unknown

- Histone methyltransferases (HMTs), which are further subdivided into lysine methyltransferases (PKMTs) and arginine methyltransferases (PRMTs) according to their target residue
- Histone acetyltransferases (HATs)
- Enzymes that catalyze the phosphorylation of histone tails
- Ubiquitin-conjugating enzymes
- DNA methyltransferases (DNMTs)

In contrast, epigenetic erasers remove the structural modifications introduced by the writers. They comprise the following:

- Histone deacetylases (HDACs)
- Histone serine/threonine/tyrosine phosphatases
- Histone deubiquitinases (DUBs)
- Histone lysine/arginine demethylases
- DNA demethylation enzymes

Lastly, epigenetic readers are effector proteins that recognize specific structural units in nucleic acids and proteins and are recruited to specific marks on histones or nucleotides. Their structure is characterized by a cavity in which to accommodate a specific epigenetic mark. The interaction between the reader domain and the modified amino acid allows to distinguish similar epigenetic marks. Furthermore, they can be also contained in writer or eraser enzymes and are classified into four groups:

- Chromatin architectural proteins
- Chromatin remodeling enzymes
- Chromatin modifiers
- Adaptor proteins

Epigenetic in medicine

During the last years, the understanding of genetic and epigenetic is becoming increasingly important for the prevention, diagnosis, and treatment of several diseases, and much attention has been given to molecular medicine. In this contest, it has been demonstrated that several disorders were directly or indirectly caused by epigenetic modifications in form of impaired DNA methylations or incorrect histone modifications [8]. Human diseases, such as autism, bipolar disorder, diabetes, familial hypertrophic cardiomyopathy, schizophrenia, and syndromes, namely Prader-Willi, Angelman, Beckwith-Wiedemann, and Silver-Russell, have been related to alteration of DNA methylation and modifications of normal imprinting patterns [9–11]. In particular, these human rare syndromes appear to be directly linked to aberrant expression of long ncRNAs [12]. They are involved in the epigenetic controls of coding genes, through the up- or down-regulation of messenger RNAs (mRNAs), methylation, and transcription of specific gene polymorphisms [13], thus exerting a powerful effect on a number of physiological processes. Their aberrant levels are likely to cause disorders associated with protein dysregulations [14]. Despite the present advances, regulatory mechanisms and functions of long non-coding RNA (lncRNA), and their association with the majority of the diseases, need to be further elucidated in order to improve patient management, as well as the prevention and treatment of the related genetic diseases.

It is well known that genetic aberrations can also promote malignant transformations. Many studies demonstrated that initiation and progression of several form of cancer are related to epigenetic aberrations that alter the complex functional interaction and balance between oncogenes and tumor suppressor genes [15, 16]. One of the main actors is hypermethylation of many tumor suppressor genes, such as those involved in DNA repair (BRCA1, MGMT and MLH1), signal transduction (RASSF1A), cell cycle regulation (p16INK4a), apoptosis (DAPK and TMS1), and angiogenesis (THBS1) [17–19]. Indeed, epigenetic disruption was one of the main abnormality identified in cancer cells [20] and might lead to gene activation, promoting overexpression of oncogenes, and might represents a fundamental mechanism of cancer development [17].

Alteration of normal patterns of covalent histone modifications is yet another hallmark of cancer. The most characteristic examples are, in this respect, related to the overexpression, mutations, and/or chromosomal translocations of histone acetylation/deacetylation (HAT/HDAC) and methylation/demethylation (HMT/HDM or sirtuins) enzymes [21].

In this context, the development of molecular medicine, the fast progress of the new epigenetic approaches, and the reversible nature of the epigenome offer great advances in the fields of drug targeting and personalized medicine.

Based on these observations, DNMT-inhibiting cytosine nucleoside analogs and non-nucleoside analogs (see Table 2)

Table 2 List of nucleoside and non-nucleoside analog DNMT inhibitors

Nucleoside analogs	Non-nucleoside analogs
5-6-Dihydro-azacytidine	(−)-Epigallocatechin-3-galate
5-Fluoro-2-deoxycytidine	Curcumin
Azacytidine	Mithramycin A
CP-4200	Nanomycin A
Decitabine	Natural compounds: flavonoids
NPEOC-DAC	NSC-106084
SGI-110	NSC-14778
Zebularine	PRIMA-1
	Psammaplin A
	RG-108
	SGI-1027
	Synthetic compounds: procaine

have been proposed as potential anti-cancer drugs. The most characterized nucleoside analogs, 5-azacytidine (Vidaza®) and 5-aza-2′-deoxycytidine or decitabine (Dacogen®), have been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML). Several clinical studies have also shown promising results in patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) [22]. Preliminary experiments also demonstrated that dihydro-5-azacytidine (DHAC) and zebularine are less cytotoxic than the 5-aza-nucleosides in cultured cells and that are able to inhibit tumorigenesis in various cancer cell lines [23–27]. However, further studies are needed in order to demonstrate safety and efficacy and, eventually, enter into the clinical phase.

Non-nucleoside analogs are also being studied. For instance, procainamide and its analog procaine have shown DNMT inhibitory effects in various cancer types, as well as other synthetic compounds, such as RG108, MG98, PRIMA-1, and SGI-1027, and natural compounds, namely flavonoids, psammaplin A, and curcumin. However, none of them have entered clinical development yet, since there is still a long way to go before we may obtain the identification of novel, selective, non-nucleoside DNMT inhibitors.

As described above, cancer cells can also be characterized by alterations of histone methyltransferases/demethylases and overexpression of histone deacetylases (HDACs). Several reports indicate that HDAC inhibitors are able to induce a cell cycle arrest at G1 or G2-M stage, cancer cell differentiation and apoptosis. Furthermore, these molecules can inhibit angiogenesis and metastasis and enhance cell sensitivity to chemotherapy [28]. Several HDACi are being tested in phase II–III trials as reported in Table 3 and include both natural and synthetic compounds [29]. Vorinostat and romidepsin are the first agents approved by the FDA and EMA for the treatment of progressive or recurrent cutaneous T cell lymphoma (CTCL) [30], while several other molecules, listed in Table 2, are in the early phases of clinical development [31].

Currently, several clinical trials are testing the use of a different combination of DNMT and HDAC inhibitors, together with cyclin-dependent kinase inhibitors (CDKi) or proteasome inhibitors or engineered transcriptional factors [32–36].

Another new concept derives from the observation that patients with the same disease may have different symptoms and may not or only poorly respond to conventional therapy. This brings about the concept of "personalized medicine," also known as "precision medicine." This new branch of medicine, basically, encompasses the tailoring of medical treatment on the basis of individual characteristics, needs, and preferences of each patient, in order to improve outcomes [37]. As very prematurely stated by Hippocrates "It's far more important to know what person the disease has than what disease the person has," introducing for the first time the idea of the "individuality" of disease and the importance of prescription of "different" medicines to "different" patients.

Table 3 List of HDACs and their current status in clinical trials

Group	Example	Current status
Short-chain fatty acid	Valproic acid	Phase II CT
	Phenyl butyrate	Phase II CT
	Pivanex	Phase II CT
Hydroxamic acids	Vorinostat	FDA approved
	Panobinostat	Phase III CT
	Belinostat	Phase II CT
	Abexinostat	Phase II CT
	Resminostat	Phase II CT
	Givinostat	Phase II CT
	Dacinostat	Phase II CT
	Pracinostat	Phase II CT
Cyclic tetrapeptide	Romidepsin	FDA approved
	Apicidin	Phase II CT
	Trapoxin A	No data
Benzamide	Mocetinostat	Phase II CT
	Entinostat	Phase II CT
	Rocilinostat	Phase II CT

In this contest, gene-expression profiling and genomic studies represent potential tools for improving patient management through their classification into clinically relevant subtypes for prevision therapy [38]. Although FDA has already approved some expression profiling platforms for clinical use, strong claims cannot yet be made about the clinical value of these signatures. Other important technological platforms are being developed to analyze epigenetic changes in DNA, microRNAs, and proteins. These allow to identify biomarkers for individual's classification into subpopulations that differ in their susceptibility to a particular disease or in their response to a specific treatment. Furthermore, it is important to highlight that, although a few drugs used for personalized medicine have been approved by FDA, various challenges still exist, given the observation that each patient is unique and, similarly, displays a unique epigenomic signature.

Epigenetic in stem cell research

Access to unlimited numbers of specific cell types represents the major goal in regenerative medicine. Recent advances in the stem cell field led to the production of iPSCs that were generated in 2006 through genetic reprogramming of adult somatic cells. Following these studies, several researchers succeeded in producing iPSCs. However, although various methodologies have been established for

their derivation, the efficiency of iPSC induction remains low. Furthermore, the integration of transgenes severely limits their use in clinical studies [39]. Therefore, several reprogramming technologies that increase efficiency, accelerate kinetics, and eliminate the use of virus-mediated gene have been developed. Different approaches have been tested, from virus-free [40–42] to removable PiggyBac transposons [43], minicircle systems [44], and episomal systems [45]. Nevertheless, evidence persists demonstrating the problems related to residual exogenous DNA and chromosomal disruptions that result in harmful genetic alterations [46].

In order to circumvent these limits related to low efficiency and the introduction of exogenous transcription factors, small-molecule compounds have been used to modulate the epigenetic state increasing reprogramming efficiency, by inhibiting and activating, in reversible way, specific signaling pathways [47–50].

Huangfu et al. reported that the HDAC inhibitor, valproic acid (VPA), not only improves reprogramming efficiency by more than 100-fold but also enables efficient induction of human and murine iPSCs, without introduction of the myelocytomatosis oncogene (c-Myc) [47].

Subsequently, it was demonstrated that murine embryonic and adult fibroblasts could be reprogrammed by stimulating cells with a specific chemical combination of VPA, CHIR99021, 616452, and tranylcypromine (TCP), in the presence of a single transcription factor, POU domain, class 5, transcription factor 1 (*Oct-4*), without the use of transgenes for SRY (sex-determining region Y)-box 2 (*Sox2*), Kruppel-like factor 4 (gut) (*Klf4*), and *c-Myc* [48].

A recent study also reveals that endogenous pluripotency program can be re-activated through the use of small molecules that modulate molecular pathways nonspecifically related to pluripotency, without the introduction of exogenous genes. In that report, Hou et al. generated iPSCs from murine somatic cells at a frequency up to 0.2%, using a combination of seven small-molecule compounds, namely VPA, CHIR99021, 616452, TCP, forskolin (FSK), 2-methyl-5-hydroxytryptamine (2-Me-5HT), and D4476 [50]. In line with these findings, Moschidou et al. demonstrated that the use of VPA in combination with a low growth factor medium (embryonic stem cell (ESC) medium) is able to revert 82% of amniotic fluid cells into a pluripotent state that shares transcriptome identity with ESC and ability to form embryoid bodies (EB) and teratomas, as well as to differentiate into cell lineages deriving from all the three germ layers [51]. Similarly, endogenous pluripotency transcription factor genes were re-activated in adult human dermal fibroblasts using VPA, in the absence of any transgenes [52].

Although the exact mechanisms underlying iPSC generation still remain to be elucidated, these results suggest that epigenetic modifiers improve cell reprogramming altering chromatin structure and directly modulating the epigenetic enzymes. These events possibly drive cells to a more permissive state that allow changes in the epigenome, activating specific signaling pathways that influence cell fate during reprogramming processes.

Altogether, the data obtained represent a significant progress in cell reprogramming technology, with new approaches that avoid the use of retroviral and/or lentiviral vectors and the insertion of transgenes.

Epigenetic and direct cell conversion: a new alternative

In recent years, several protocols that avoid the use of virally or non-virally introduced exogenous factors as well as the establishment of a stable 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 another differentiated cell type (Fig. 1).

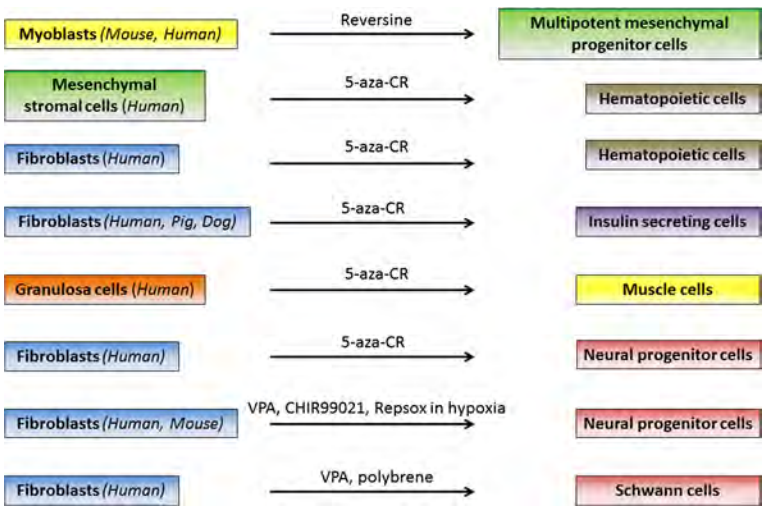

Fig. 1 Schematic representation of epigenetic conversion experiments

The first paper reporting the ability of a small molecule to induce a de-differentiation in murine C2C12 myoblasts was published in 2004 [53]. In these experiments, cells were initially treated with a library of 50,000 small molecules for 4 days, with the final goal of identifying target compounds that can induce de-differentiation. The results obtained demonstrated that reversine, a 2,6-disubstituted purine, was able to increase cell plasticity, inducing lineage-committed myoblasts to become multipotent mesenchymal progenitor cells. The activity of this molecule was subsequently tested in several type of cells, including 3T3E1 osteoblasts [54], human primary skeletal myoblasts [54], and murine and human dermal fibroblasts [55], confirming the induction of an increased plasticity in treated cells.

More recent experiments demonstrated that a brief exposure to a demethylating agent can push cells to a less committed state, increasing their plasticity for a short window of time sufficient to re-address cells towards a different cell type [2–7]. The starting hypothesis was that the processes associated with differentiation are driven by several mechanisms. Among these, DNA methylation plays a fundamental role during both early embryonic development and cell lineage specification, causing silencing of large fraction of the genome and subsequent expression of gene essential for the maintenance of the differentiated and tissue-specific phenotype. Based on this, 5-azacytidine (5-aza-CR), a well-characterized DNMT inhibitor, was selected in order to remove the epigenetic "blocks" that are responsible for tissue specification [3–5, 7]. This drug is a chemical analog of cytosine, it can be incorporated into DNA and RNA, causing an increased effect in resting as well as in dividing cells, and it is known to be a direct inhibitor of methylation in newly synthesized DNA by blocking DNMT function [56]. These features give 5-aza-CR the ability to induce DNA hypomethylation, modify gene expression, and reactivate the transcription of silent genes in eukaryotic cells [57–62].

In agreement with these findings, human mesenchymal stromal cells (MSC) and skin fibroblasts were transformed into hematopoietic cells after an incubation with 5-aza-CR, granulocyte-macrophage colony-stimulating factor (GM-CSF), and stem cell factor (SCF) [2].

Moreover, our laboratory demonstrated that adult skin fibroblasts and granulosa cells, derived from different species, namely human [3, 5], porcine [4], and dog [63], can be converted into a different cell type, belonging to the same embryonic layer or even to a different one.

The "highly permissive state" enriched by cells, after 5-aza-CR exposure, was paralleled by decrease in global DNA methylation and was accompanied by significant changes in cell phenotype and a specific and consistent gene regulatory response. Indeed, after demethylating agent treatment, both cell types used in the experiments, adult skin fibroblasts and granulosa cells, exhibited reduced dimensions, increased nuclear volume, and highly decondensed chromatin [3–5]. These observations are in agreement with the morphological features distinctive of highly plastic cells that contain more loosely packed chromatin than their differentiated counterparts, in order to maintain genes in a potentially open state and prepare them for future expression [64]. Preliminary data obtained in our laboratory with next-generation sequencing analysis of 5-aza-CR exposed cells indicate changes of several pathways, mostly related to histone transcription and cell adhesion. This suggests the possibility that, beside the well-known effect on DNMTs and cell methylation, 5-aza-CR action on cell plasticity and differentiation may take place through alternative mechanisms that require the involvement of novel cellular targets (manuscript under revision). Notably, this process is completely reversible and does not show toxic effects, since cells returned to their standard culture medium, reverted to their original phenotype within a few days. The absence of genotoxic effects is further supported by cytogenetic analysis showing that 5-aza-CR-treated cells maintained a normal karyotype throughout the entire length of the experiments [3–5]. We also demonstrated that, once cells entered into the higher plasticity window, they could easily be directed towards a different phenotype if they were exposed to specific differentiation stimuli.

In particular, skin fibroblasts of human, porcine, and canine origin were converted towards the pancreatic lineage, using a three-step induction protocol. This allowed cells to transit from the early endodermic and pancreatic differentiation stage to mature endocrine cells. At the end of the epigenetic conversion, cells formed large three-dimensional spherical structures, reminiscent of in vitro-cultured pancreatic islets. They expressed the main hormones and glucose sensor genes specific of pancreatic tissue and were able to actively release of C-peptide and insulin after exposure to 20 mM glucose, showing a dynamic response similar to pancreatic β cells, in which changes in ambient glucose represent the primary and physiological stimulus for insulin secretion. Furthermore, cell functionality was also demonstrated in vivo using immunodeficient severe combined immunodeficiency (SCID) mice whose β cells had been selectively destroyed with streptozotocin, demonstrating converted cell ability to restore normo-glycaemia and stably maintain mice glucose levels [3, 4].

The possibility to apply epigenetic conversion to different cell types has been demonstrated using granulosa cells as starting cell population and converting them into muscle cells through the use of 5-aza-CR followed by a 15-day culture with human recombinant vascular endothelial growth factor (VEGF) [5]. At the end of the conversion, over 80% of granulosa cells change the original phenotype and become elongated and multinucleated. These morphological changes were paralleled by the up-regulation of

muscle-specific genes, such as desmin (DES), myosin heavy chain (MHC), and myogenic differentiation (MYOD). In contrast, markers distinctive of granulosa cells (cytokeratin 17 (KRT17), hyaluronan synthase 2 (HAS2), gremlin 1 (GREM1), and pentraxin 3 (PTX3)) were turned down.

In agreement with our results, the demethylating agent 5-aza-CR was also demonstrated to convert human foreskin fibroblasts into neural progenitor-like cells [6]. At the end of the 14-day neural conversion, cells down-regulated fibroblast specific protein 1 (FSP1) and expressed high levels of neural progenitor markers, namely SOX2, NESTIN, PAX6, EN1, LMX1A, and WNT1. Molecular switch was accompanied by morphological changes, with cells becoming smaller, acquiring radial arrangement, and producing neurosphere-like aggregates.

Cheng et al. reported that it is possible to convert human and murine fibroblasts into proliferating chemical-induced neural progenitor cells (ciNPC), using a cocktail containing inhibitors of histone deacetylation, glycogen synthase kinase, and TGF-β pathway under physiological hypoxic conditions (5% O_2) [65].

Furthermore, recent experiments described the possibility to epigenetically convert human skin fibroblasts into mature Schwann cells through the use of the HDAC inhibitor VPA [66]. 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.

Conclusions

Altogether, the results accumulated during the last years have paved the way to the use of small molecules for personalized medicine, drug targeting, and the induction of changes in cell fate. Some of these molecules have been already approved for patient's treatment and are currently used for the cure of disease caused be epigenetic aberrations, while other chemical compounds are tested in several clinical trials. In this context, various challenges still exist given the observation that each patient is unique and displays a unique epigenomic signature, and more studies are indeed in order to develop epigenetic biomarkers, technologies, and tools to classify individuals into subpopulations that differ in their susceptibility to a particular disease or in their response to a specific treatment.

Epigenetic modifiers are also been used to replace TFs for iPSC generation. Indeed, mouse and human iPSCs have been generated using a small-molecule-based reprogramming protocol, without the use of genetic material. However, although these cells may represent a promising stem cell source, it is important to highlight that the induction of a stable pluripotent state, and the deriving cell instability, severely limits their use in regenerative medicine.

The new proposed method of epigenetic cell conversion demonstrated that it is possible to dynamically interact with cell genotype and phenotype through the use of epigenetic modifiers. This approach allows to directly convert a terminally differentiated cells into a different cell type, without the use of transgenes, and increase cell plasticity only for a short and transient period, and avoid the induction of a stable pluripotent state. This makes epigenetic conversion a very promising tool for regenerative medicine. Furthermore, the results obtained indicate that this protocol is robust since it was successfully applied to different cell types as well as in several species [3–5].

All this evidences support for the importance of epigenetic related approaches widen their application both to human as well as to veterinary regenerative medicine for the cure of several and diverse degenerative diseases.

Abbreviations

5-aza-CR: 5-Azacytidine; ciNPC: Chemical-induced neural progenitor cells; DNMTs: DNA methyltransferases; DUBs: Deubiquitinases; EB: Embryoid bodies; ESC: Embryonic stem cell; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HATs: Histone acetyltransferases; HDACs: Histone deacetylases; iPSCs: Induced pluripotent cell; MSC: Mesenchymal stromal cells; PKMTs: Lysine methyltransferases; PRMTs: Arginine methyltransferases; SCF: Stem cell factor; SCID: Immunodeficient severe combined immunodeficiency; TCP: Tranylcypromine; VEGF: Vascular endothelial growth factor; VPA: Valproic acid

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Authors' contributions

TALB and FG wrote the manuscript. GP, EFMM, CEG and AZ collected the information and draw figure and tables. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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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 phenotype. 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 plasticity, 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 remodelling 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 during the early stage of development, whereas other methyltransferases, such as DNMT1, primarily maintain the established patterns by copying them onto daughter DNA strands during cell replication and division. DNMT-controlled changes in methylation 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

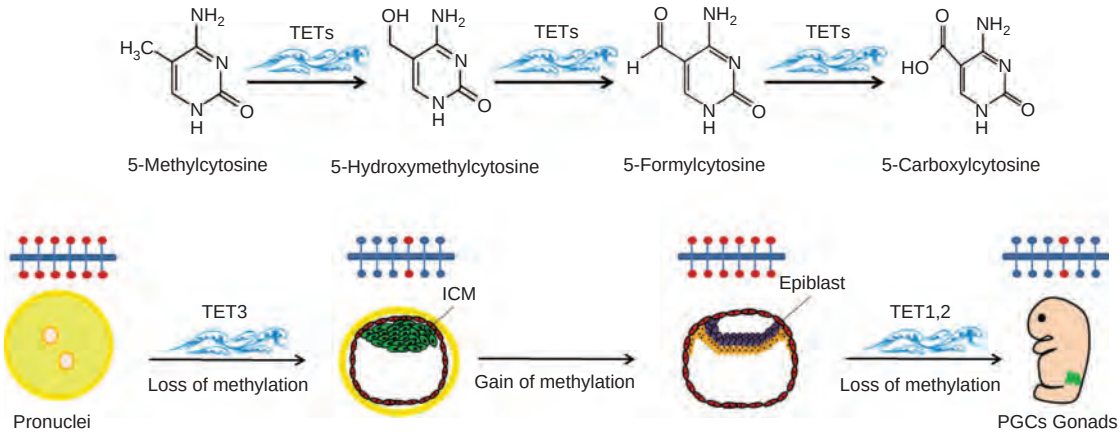

Fig. 1. Ten–eleven translocation (TET)-mediated oxidation of 5-methylcytosine generates 5-hydroxymethylcytosine, which is subsequently converted to 5-formylcytosine and 5-carboxylcytosine (upper panel). Changes in DNA methylation drive early embryo development and cell fate commitment (lower panel). Transitions where TET proteins are critical are highlighted, as are waves, where loss or gain of methylation take place.

converts and oxidises 5-methylcytosine (5-mC) to 5-formylcytosine (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 possibility 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 mammalian 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 demethylation 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 modulate 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 maintaining 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 commitment, 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 temporal activation and spatial restriction, allowing cells to acquire distinct differentiation traits, stabilising the cell terminal phenotype (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 pluripotency genes. In agreement with these observations, it has been reported that oocyte TET enzymes exhibited reprogramming activity for pluripotency gene reactivation during early embryonic development, after nuclear transfer and natural fertilisation (Gu *et al.* 2011). Together, these findings point to the possibility that TET enzymes may play a key role in reprogramming 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. Interestingly, 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 undergoing 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 commitment (Brevini *et al.* 2015).

Several protocols that avoid using virally or non-virally introduced exogenous factors, as well as the establishment of a stable 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 compounds 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 methylation 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 inhibitor, 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 transformed 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 significant changes in their phenotype and a specific gene regulatory response that was paralleled by a decrease in global DNA

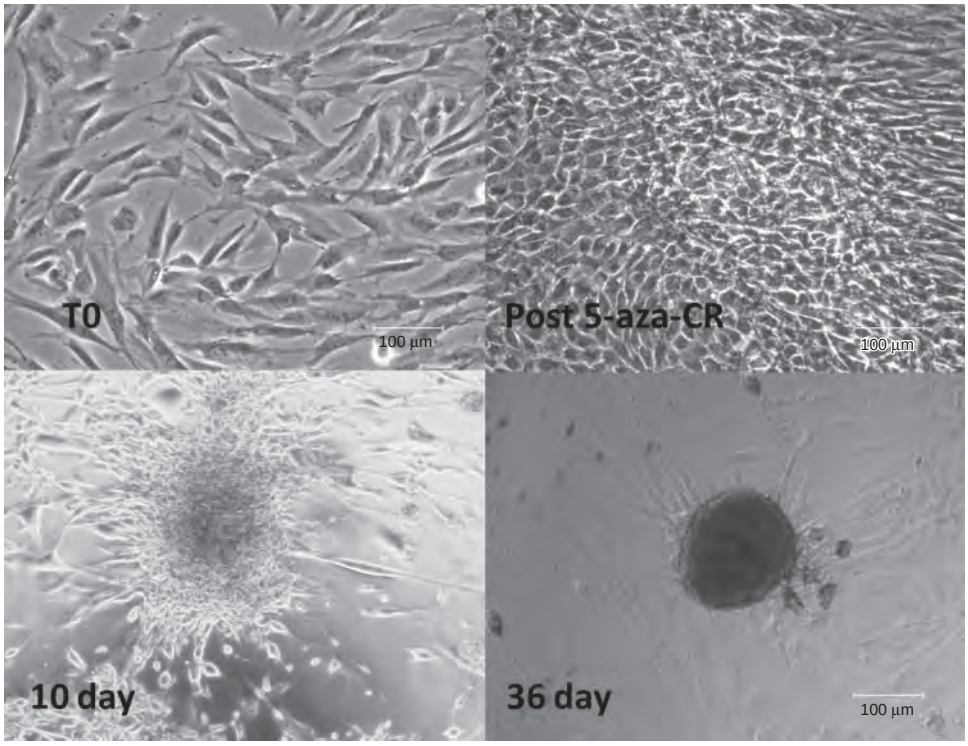

Fig. 2. Small molecules interact with cell fate definition. Cat skin fibroblasts undergoing epigenetic conversion to insulin-secreting cells exhibit marked morphological changes. Untreated cells (T0) lost their typical elongated shape and adopted a round epithelioid aspect in response to 18-h exposure to 5-azacytidine (5-aza-CR; 'post 5-aza-CR'). After 10 days pancreatic differentiation, cells are rearranged in a reticular pattern and clustered in distinguishable aggregates. These formations progressed with time, aggregating in large three-dimensional spherical structures, reminiscent of typical pancreatic islets *in vitro* (Day 36).

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 pluripotent 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 into proliferating chemical-induced neural progenitor cells using a cocktail containing inhibitors of histone deacetylation, glycogen synthase kinase and transforming growth factor β signalling under physiologically hypoxic conditions (5% O_2). Furthermore, recent experiments described the possibility of epigenetically 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, pluripotent cells are highly sensitive to extracellular signals that play a critical role in the maintenance of stemness, differentiation 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, reprogramming of somatic cells involves a complex interaction between intracellular and extracellular signals, leading to epigenetic 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 extracellular vesicles (EVs) derived from cells are capable of transferring 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 microenvironment 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 haematopoietic 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 preservation 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 fundamental 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 expression 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 be exploited in regenerative medicine. Based on these observations, 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 constantly 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 cytoskeleton 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 evidence 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 balance 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 behaviour. In addition, it has also been shown that ECM elasticity plays a crucial role in cell fate control. Studies of MSC differentiation 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

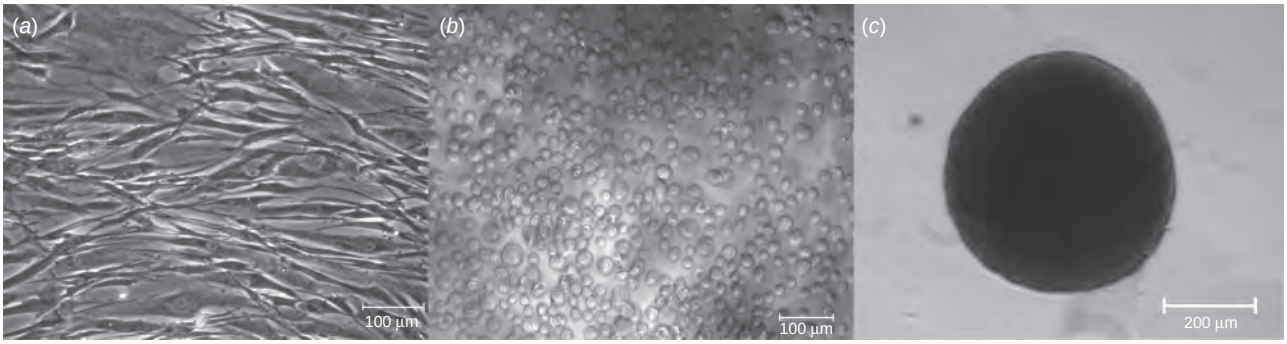

Fig. 3. Cell plasticity and differentiation is controlled by mechanical cues. (*a*) Fibroblasts were encapsulated in a three-dimensional (3D) microbioreactor and subjected to epigenetic erasure. (*b*) A non-adhesive 3D coating allowed cells to freely interact with each other and form 3D spherical structures, with distinct spatial organisation and the stable maintenance of the desired commitment state (*c*).

intermediate stiffness or differentiate into neurons and adipocytes when cultured on a soft ECM (Engler *et al.* 2006). Similarly, 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 elasticity 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 pathologies '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 microenvironment 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 phenotype. 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 cytoskeletal remodelling and with the involvement of specific mechanosensing-related pathways.

Conflicts of interest

The authors declare no conflicts of interest.

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Epigenetic Erasing and Pancreatic Differentiation of Dermal Fibroblasts into Insulin-Producing Cells are Boosted by the Use of Low-Stiffness Substrate

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Abstract
Several studies have demonstrated the possibility to revert differentiation process, reactivating hypermethylated genes and facilitating cell transition to a different lineage. Beside the epigenetic mechanisms driving cell conversion processes, growing evidences highlight the importance of mechanical forces in supporting cell plasticity and boosting differentiation. Here, we describe epigenetic erasing and conversion of dermal fibroblasts into insulin-producing cells (EpiCC), and demonstrate that the use of a low-stiffness substrate positively influences these processes. Our results show a higher expression of pluripotency genes and a significant bigger decrease of DNA methylation levels in 5-azacytidine (5-aza-CR) treated cells plated on soft matrix, compared to those cultured on plastic dishes. Furthermore, the use of low-stiffness also induces a significant increased up-regulation of ten-eleven translocation 2 (*Tet2)* and histone acetyltransferase 1 (*Hat1)* genes, and more decreased histone deacetylase enzyme1 (*Hdac1)* transcription levels. The soft substrate also encourages morphological changes, actin cytoskeleton re-organization, and the activation of the Hippo signaling pathway, leading to yes-associated protein (YAP) phosphorylation and its cytoplasmic translocation. Altogether, this results in increased epigenetic conversion efficiency and in EpiCC acquisition of a mono-hormonal phenotype. Our findings indicate that mechano-transduction related responsed influence cell plasticity induced by 5-aza-CR and improve fibroblast differentiation toward the pancreatic lineage.

Keywords Cell plasticity · Epigenetic conversion · Hippo signaling pathway · Matrix elasticity · 5-Azacytidine · Insulin-producing cells

Introduction

Development and phenotype definition are regulated by complex epigenetic mechanisms that control genomic imprinting, specific gene transcription programs and chromatin structure [1]. However, the differentiation process is reversible and may be altered by biochemical and biological manipulations, making it an attractive target to reactivate hypermethylated genes [2] and facilitate cell phenotype changes [3]. During the last years, the possibility to interact with the epigenetic signature of terminally differentiated cells and with a preexisting quiescent sub-population of pluripotent stem cells detected in different adult tissue, has been described as a possible tool for regenerative medicine [4–14]. In particular, we demonstrated that a short exposure to the epigenetic eraser 5-azacytidine (5-aza-CR) allows the acquisition of a transient high plasticity state [4–6, 9, 11], which is achieved through the well-known 5-aza-CR ability to deplete DNA methyltransferase (DNMT) 1 enzymatic activity [15, 16]. Furthermore, we recently demonstrated that these events are related to a direct and active ten-eleven translocation 2 (TET2)-mediated demethylating effect [11]. The increased high plasticity, induced by 5-aza-CR treatment, is transient but sufficient to allow a complete and direct differentiation into a new mature and functional cell type [4–6, 9].

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Parallel studies addressed their attention to tissue architecture and mechanical forces and showed their involvement (together with chemical signals) in the control of cell plasticity and differentiation. In particular, it was demonstrated that the microenvironment, provided by the traditional polystyrene culture systems, fails to imitate the physiological and biochemical features of cells and can cause deviations in cell response. This is related to the significant differences between the stiffness of the original tissue and that of several GPa of the support used. In contrast, the use of a surface that matches the stiffness of native tissues, exerts a direct effect on lineage commitment, positively influences cell differentiation [17–22] and might be crucial for specific cellular functions [23]. However, it is yet unclear if matrix elasticity is able to impact cell epigenetic profiles, inducing changes in methylation levels and/or in gene expression [23].

In the present work, we investigate whether matrix elasticity may affect the epigenetic conversion process. In particular, we focus our attention on the two main steps of the protocol, namely cell erasing and cell differentiation. In order to investigate whether a soft substrate may influence epigenetic erasing induced by 5-aza-CR treatment, we monitor DNA methylation changes and the expression levels of its regulatory genes, namely ten-eleven translocation 2 (*Tet2*), histone acetyltransferase 1 (*Hat1*), and histone deacetylase enzyme 1 (*Hdac1*). We also analyze the expression of pluripotency-related genes and the morphological changes that take place in response to the exposure to the epigenetic eraser. We then examine the effect of the selected low-stiffness substrates on the differentiation efficiency of fibroblasts into insulin-producing cells (EpiCC) and monitor the impact of matrix elasticity on the acquisition of a mono-hormonal phenotype, which is distinctive of terminally differentiated pancreatic cells.

Finally, in order to better understand the mechanisms involved, we analyze the activation of the Hippo signaling mechano-transduction pathway along the processes.

Materials and Methods

All reagents were purchased from Thermo Fisher Scientific unless otherwise indicated.

Ethics Statement

Murine dermal fibroblasts were isolated from 7-week-old C57BL/6N male mice obtained from Charles River. All studies were reviewed and approved by the Ethical Committee of the University of Milan. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH).

Isolation and Culture of Murine Skin fibroblasts

Primary dermal fibroblast cultures were established using skin fresh biopsies from 7-week-old C57BL/6N mice. Fragments of skin tissue of approximately 2 mm^3 were transferred onto 0.1% gelatin (Sigma) pre-coated Petri dish (Sarstedt) and cultured in DMEM supplemented with 20% Fetal Bovine Serum (FBS), 2 mM glutamine (Sigma) and antibiotics. After 6 days of culture, fibroblasts started to grow out of the tissue fragments and the latter were carefully removed. Cells were maintained in the medium described above, grown in 5% CO_2 at 37 °C, and passaged twice a week in a 1:4 ratio. All experiments were performed in triplicate on at least three different lines.

Substrate Preparation

Based on data from the literature showing that *in vivo* beta-cells are surrounded by a rich network of soft tissue (0.1– 1 kPa) [24, 25] and that islet encapsulation in 1 kPa silk hydrogel increased insulin secretion and expression of functional genes [26], here we selected thin polyacrylamide (PAA) gels with elastic modulus of 1 kPa.

PAA gels of controlled stiffness (1 kPa) were produced according to a protocol previously described by Pelpham and Wang [27].

Briefly, a thin layer (approximately 100 μm thickness) of polyacrylamide was deposited onto a glass slide. The stiffness of the final gel was tuned by varying the percentage of acrylamide and the ratio acrylamide/bisacrylammide and measured by Atomic Force Microscope (AFM). Aiming to allow cell attachment and proliferation, the polyacrylamide surface was activated by treatment with sulfosuccinimidyl 6 (49-azido-29-nitrophenyl-amino) hexanoate (Sulfo-SANPAH; Pierce) and coated by overnight incubation at 4 °C with 0.2 mg/ml type I collagen (Worthington).

Treatment of Murine Dermal Fibroblasts with 5-aza-CR

Murine dermal fibroblasts were plated either onto standard plastic plates or on PAA gels, at a concentration of 7.8×10^4 cells/cm^2. Twenty-four hours after plating, cells were exposed to 1 μM 5-aza-CR (Sigma) for 18 h. Concentration and time of exposure were selected according to our previous works [4–6, 9]. At the end of the 18-hour exposure, cells were rinsed three times with PBS and incubated for 3 h with ESC culture medium [28].

Pancreatic Induction

Pancreatic differentiation was induced using the three-step protocol modified by Shi et al. [29]. 5-aza-CR treated cells were cultured in basal medium composed by DMEM/F12 supplemented with 1% N2, 1% B27, 0.1 mM β-mercaptoethanol (Sigma), 2 mM glutamine (Sigma), MEM Non-Essential Amino Acids and 0.05 mg/ml bovine serum albumin (BSA, Sigma). During the first 24 h of pancreatic induction, the basal medium was supplemented with 30 ng/ml activin A. On the second day, 10 µM retinoic acid (Sigma) was added. From the day after onward, cells were cultured in basal medium supplemented with 1% B27, 5 ng/ml basic fibroblast growth factor (bFGF) and 1% insulin–transferrin–selenium (ITS) to further encourage differentiation. Medium was refreshed daily. Cells were differentiated for a total of 10 days.

Global Methylation Analysis

Genomic DNA was extracted with PureLink® Genomic DNA Kits according to the manufacturer's instructions. DNA was converted to single-stranded DNA by incubation at 95 °C for 5 min, followed by rapid chilling on ice. Samples were then digested to nucleosides by incubating the denatured DNA with nuclease P1 for 2 h at 37 °C in 20 mM sodium acetate (pH 5.2). Alkaline phosphatase was added and incubated for 1 h at 37 °C in 100 mM Tris (pH 7.5). After centrifugation, the supernatant was used for ELISA assay using Global DNA Methylation ELISA Kit (5′-methyl-2′-deoxycytidine Quantitation; CELL BIOLABS) according to the manufacturer's protocol.

Gene Expression Analysis

RNA was extracted using the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. Quantitative PCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) using predesigned gene-specific primers and probe sets from TaqManGene Expression Assays (Thermo Fisher Scientific), listed in Table 1. *Gapdh* and *Rps18* were used as internal reference genes. CFX Manager software (Bio-Rad Laboratories) was used for target gene quantification.

Western Blotting

Cells were lysed and constitutive proteins were extracted using a ReadyPrep Protein Extraction Kit (Bio-Rad). Protein concentration was assessed by Coomassie Blue-G Dye-binding method. 100 µg of proteins were resuspended in sample buffer (1:1) consisting of 4% (wt/vol) SDS, 10% 2-mercaptoethanol, 20% (wt/vol) glycerol, 0.004% bromophenol blue, and 0.125 M Tris–HCl at pH 6.8. Equal amounts of total protein were loaded and electrophoresed on a SDS-polyacrylamide gels. Proteins were then transferred onto 0.45 µm pore size nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences) and probed with primary antibodies listed in Table 2. Protein bands were visualized by the WesternBreeze chemiluminescent kit. Densitometric analysis was performed with Image-J Software. The protein expression was normalized to GAPDH protein expression by calculating the optical density ratio.

Table 1 List of primers used for quantitative PCR analysis

GENE	DESCRIPTION	Catalog number ID
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Gcg	Glucagon	Mm00801714_m1
Hat1	Histone acetyltransferase 1	Mm00509140_m1
Hdac1	Histone deacetylase 1	Mm02745760_g1
Ins	Insulin	Mm01950294_s1
Nanog	Nanog homeobox	Mm02019550_s1
Oct4	POU domain, class 5, transcription factor 1	Mm03053917_g1
Rex1	Zinc finger protein 42	Mm03053975_g1
Rps18	Ribosomal protein S18	Mm02601777_g1
Sox2	SRY (sex determining region Y)-box 2	Mm03053810_s1
Sst	Somatostatin	Mm00436671_m1
Tet2	Tet methylcytosine dioxygenase 2	Mm00524395_m1

Immunocitochemistry

Cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma), washed three times in PBS and permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma) in PBS. Samples were treated with blocking solution containing 5% (vol/vol) BSA and 5% (vol/vol) not immuno serum in PBS. Primary antibodies were incubated over-night at +4 °C and their working dilutions are listed in Table 2. Cells were incubated with suitable secondary antibodies (Alexa Fluor) for 45 min. Nuclei were stained with 4′,6-diamidino-2- phenylindole (DAPI, Sigma). Samples were observed under a Nikon Eclipse TE200 and Zeiss Apotome.

Quantification of the Nuclear/Cytoplasmic Ratio of YAP

The number of YAP immuno-positive cells was counted in 5 randomly selected fields at 200 × total magnification. A minimum of 100 cells were counted in three independent replicates. YAP fluorescent signal was calculated using

Table 2 List of antibodies and working dilutions used for western blotting (WB) and immunocytochemical (ICC) analysis

ANTIBODY	Host species	Company	Cat. Number	Working dilution WB	Working dilution ICC
C-PEPTIDE	Rabbit	Abcam	ab14181	---	1:100
GAPDH	Mouse	Abcam	ab8245	1:1000	---
GLUCAGON	Mouse	Abcam	ab10988	---	1:100
LATS1	Rabbit	Cell Signaling	9153	1:1000	---
MOB1	Rabbit	Cell Signaling	3863	1:1000	---
PHALLOIDIN	---	Sigma	P1951	---	32 µg/ml
pLATS1	Rabbit	Cell Signaling	9157	1:1000	---
pMOB	Rabbit	Cell Signaling	8699	1:1000	---
pYAP	Rabbit	Cell Signaling	13,008	1:1000	---
SOMATOSTATIN	Rabbit	Abcam	ab103790	---	1:100
VIMENTIN	Mouse	Abcam	ab8978	---	5 µg/ml
YAP	Rabbit	Cell Signaling	14,074	1:1000	1:100

ImageJ software. YAP nucleus/cytoplasm ratio was obtained dividing the sum of the intensity values for the pixels in the nuclear/nuclear corresponding area per the sum of the intensity values for the pixels in the cytoplasm/ cytoplasm corresponding area, as previously described [30].

Cell Counting

Cells were fixed, permeabilized and treated with blocking solution as described above.

Sample were then incubated overnight at + 4 °C with primary antibodies specific for glucagone (GLUC), somatostatin (SOM) and C-peptide (C-PEP; see Table 2 for working dilutions) and, subsequently, with secondary antibodies (Alexa Fluor – 488; Alexa Fluor – 549; Alexa Fluor – 633). Nuclei were stained with 4′,6-diamidino-2- phenylindole (DAPI, Sigma). Samples were observed Zeiss Apotome.

When cells formed spherical structures, these were dissociated with 0.25% trypsin-EDTA and Accutase (Innovative Cell Technologies) at 37 °C for 10–15 min, and attached to slides, using a cytocentrifuge (Cytospin 4, Thermo Shandon).

The number of immuno-positive cells was counted in 10 randomly selected fields at 200 × total magnification. A minimum of 500 cells were counted in three independent replicates. The number of positively stained cells was expressed as a percentage of the total cell counted.

Flow Cytometry

EpiCCg were dissociated with 0.25% trypsin-EDTA and Accutase (Innovative Cell Technologies) at 37 °C for 10–15 min. Cells were washed and fixed with 2% (wt/vol) paraformaldehyde in PBS at room temperature for 45 min and permeabilized with 0.2% TRITON X- 100 in PBS for 15 min. Before incubation with primary antibodies, pellets were resuspended in blocking solution containing 10% (vol/vol) BSA in PBS and incubated for 30 min. Cells were incubated with primary antibodies specific for GLUC, SOM and C-PEP (see Table 2 for working dilutions) and, subsequently, with appropriate secondary antibodies (Alexa Fluor – 488; Alexa Fluor – 549; Alexa Fluor – 633). Cells were then washed and resuspended in PBS. Samples incubated with primary isotypic antibodies were used as a control. Flow cytometry was carried out with a FACS Canto II (BD Bioscience) and analyzed with BD FACSDiva v6.1.3 software.

Confocal Analysis

Confocal analysis was carried out under a LSM-710 Zeiss Confocal microscope. Images were acquired with a Z-stacking protocol. In order to ensure the quantification of each fluorescence channel and to determine the fluorescence intensity in the inner vs. the outer part of EpiCC aggregates, PMT voltages and digital image enhancement settings were set to register identical intensities in reference isolated cells showing equal staining levels for the three considered antigens. Z-stack images were then acquired using identical numbers of optical sections along Z-axis and using the same pinhole opening value. In order to obtain aggregate-specific fluorescence distribution patterns, the maximum intensity projection of each of the Z-Stacks was derived using ZEN software (Zeiss). The measures of the fluorescence intensity for the three antigen channels (Alexa Fluor – 488; Alexa Fluor – 549; Alexa Fluor – 633) were obtained by profiling the fluorescence along an arbitrarily set diameter-like axis on each of the images and then transformed into data tables where fluorescence intensity was plotted against the axis length by Graph Pad (Prism). Fluorescence density plots representing in a 2D dimension the intensity of the antigens

fluorescence intensity were obtained by maximum intensity projection image post processing using Image-J software.

In vitro Functional Analysis

EpiCC functional activity was evaluated measuring insulin release in supernatant. Cells were stimulated for 1 h with 20 mM and 5 mM D-glucose (final concentration) in basal medium without ITS. Glucose-dependent insulin release was assessed with Mouse Insulin ELISA (Mercodia) following the manufacturer's instruction. Values were normalized against DNA content of the stimulated cells. DNA was extracted with PureLink® Genomic DNA Kits (Thermo Fisher Scientific) and its concentration was assessed using NanoDrop8000 (Theromoscientific).

Statistical Analysis

Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

Results

Isolation and Characterization of Murine Dermal Fibroblasts

Fibroblasts obtained from dorsal skin biopsies grew out of the original explants forming a monolayer (Fig. 1). They displayed a standard elongated morphology with a uniform immuno-positivity for the fibroblast specific marker vimentin (VIM) and a complete absence of the pancreatic markers (C-PEP, GLUC and SOM) (Fig. 1).

Effect of Matrix Elasticity on DNA Methylation Changes after 5-aza-CR Exposure

After exposure to 5-aza-CR, both cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg) showed a significant decrease in global DNA methylation compared to untreated fibroblasts seeded on plastic plates (T0p) and PAA gels (T0g) (Fig. 2a). However, Post 5-aza-CRg cells exhibited a significantly lower methylation level compared to that of Post 5-aza-CRp. No methylation changes were observed in cells maintained in medium without 5-aza-CR (w/o 5-aza-CRp; w/o 5-aza-CRg), indicating that the substrate alone is not able to affect DNA methylation levels in 18 h.

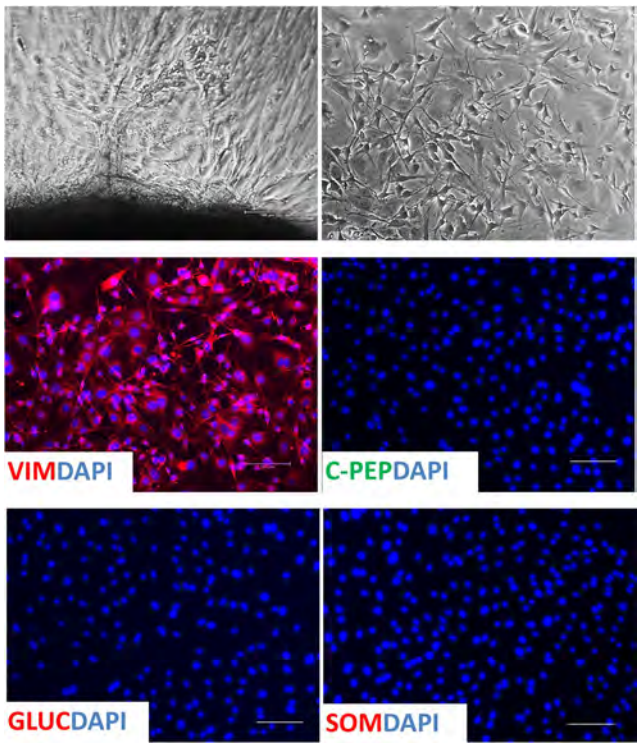

Fig. 1 Characterization of murine skin fibroblasts. Fibroblasts obtained from murine skin biopsies form a monolayer and display a standard elongated morphology and uniform immuno-positivity for vimentin (VIM). No signals are detected for C-peptide (C-PEP), glucagon (GLUC) and somatostatin (SOM). Nuclei are stained with DAPI. Scale bars: 100 μm

Effect of Matrix Elasticity on Cell Plasticity after 5-aza-CR Exposure

5-aza-CR induced methylation changes were accompanied by the up-regulation of the ten-eleven translocation 2 (*Tet2*) and histone acetyltransferase 1 (*Hat1*) genes. Moreover, we detected a parallel decreased in the histone deacetylase enzyme 1 (*Hdac1*) transcription levels (Fig. 2c). Increase of cell plasticity was also shown by the onset of pluripotency genes, namely POU class 5 homeobox 1 (*Oct4*), nanog homeobox (*Nanog*), ZFP42 zinc finger protein (*Rex1*), and sex determining region Y-box 2 (*Sox2*), which were undetectable in untreated fibroblasts (T0p, T0g; Fig. 2b) as well as in cells not exposed to 5-aza-CR (w/o 5-aza-CRp, w/o 5-aza-CRg; Fig. 2b). Interestingly, Post 5-aza-CRg cells showed higher expression levels of all the genes analyzed, when compared to those of Post 5-aza-CRp. No expression changes were observed between cells plated onto plastic and PAA gels not subjected to 5-aza-CR exposure (w/o 5-aza-CRp, w/o 5-aza-CRg), indicating that the substrate alone is not able to affect gene expression levels in 18 h.

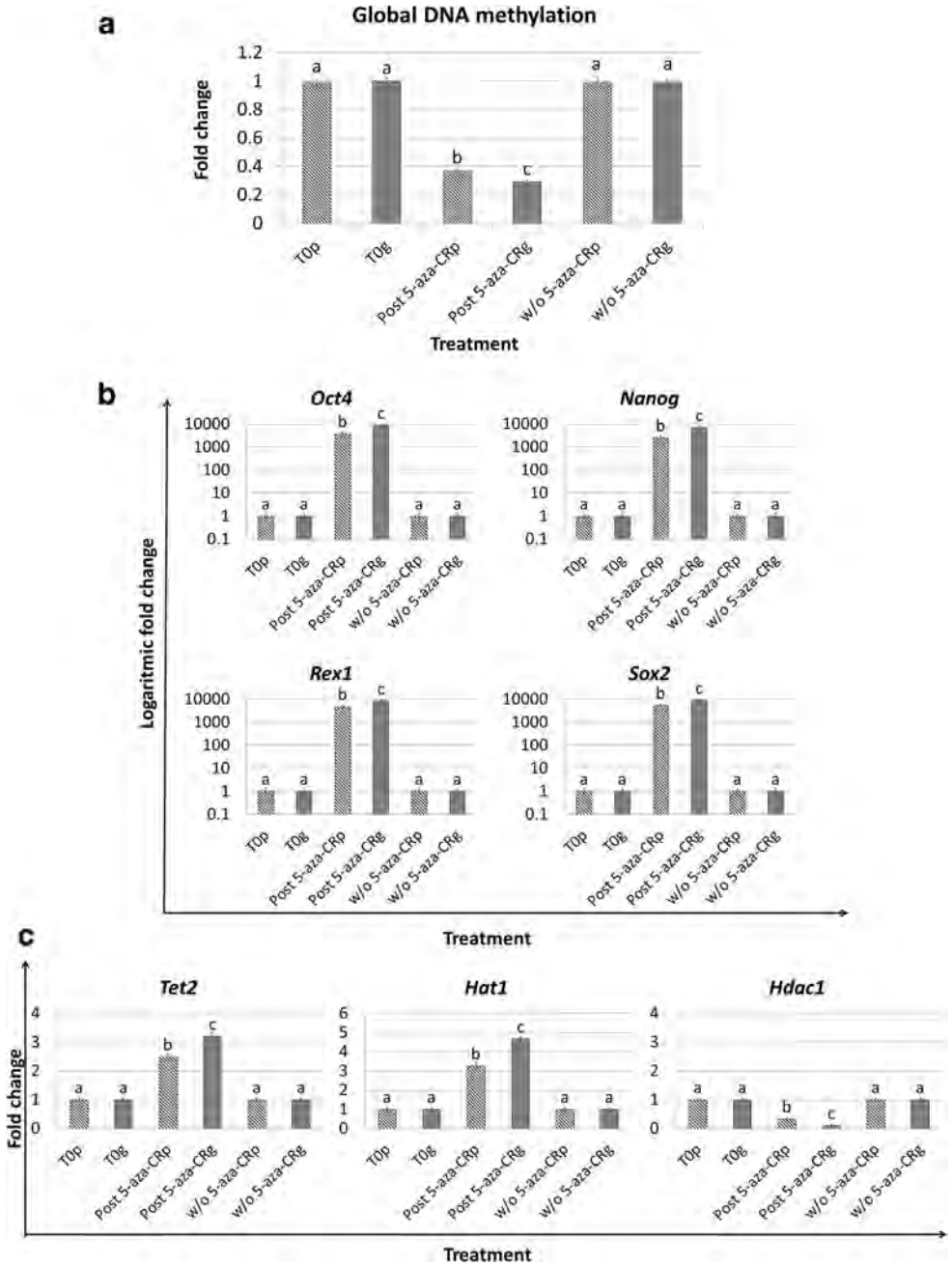

Fig. 2 Effect of matrix elasticity on methylation and cell plasticity. **a** Global DNA methylation changes in cells plated on plastic dishes and PAA gels and exposed to 5-aza-CR. Highest level set to 1 and all other relative to this. Bars represent the mean ± SD of three independent replicates. Different superscripts denote significant differences between groups ($P < 0.05$). **b** After 5-aza-CR treatment, cells show the onset of pluripotency genes, namely *Oct4, Nanog, Rex1* and *Sox2*. Gene expression levels are reported with the T0 expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P < 0.05$). **c** 5-aza-CR treatment also induce the up-regulation of *Tet2* and *Hat1* genes, and a decrease of *Hdac1* transcription. Gene expression levels are reported with the T0 expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P < 0.05$)

Effect of Matrix Elasticity on EpiCC Morphological Changes

No differences in cell morphology were observed between untreated fibroblasts plated onto plastic (T0p; Fig. 3a) and PAA gels (T0g; Fig. 3b). Cells appeared large, flat and elongated, regardless of the support used. Furthermore, no variations in filamentous actin distribution and organization were evident between them (T0p F-ACTIN DAPI; T0g F-ACTIN DAPI, Fig. 3a, b), with rhodamine-phalloidin

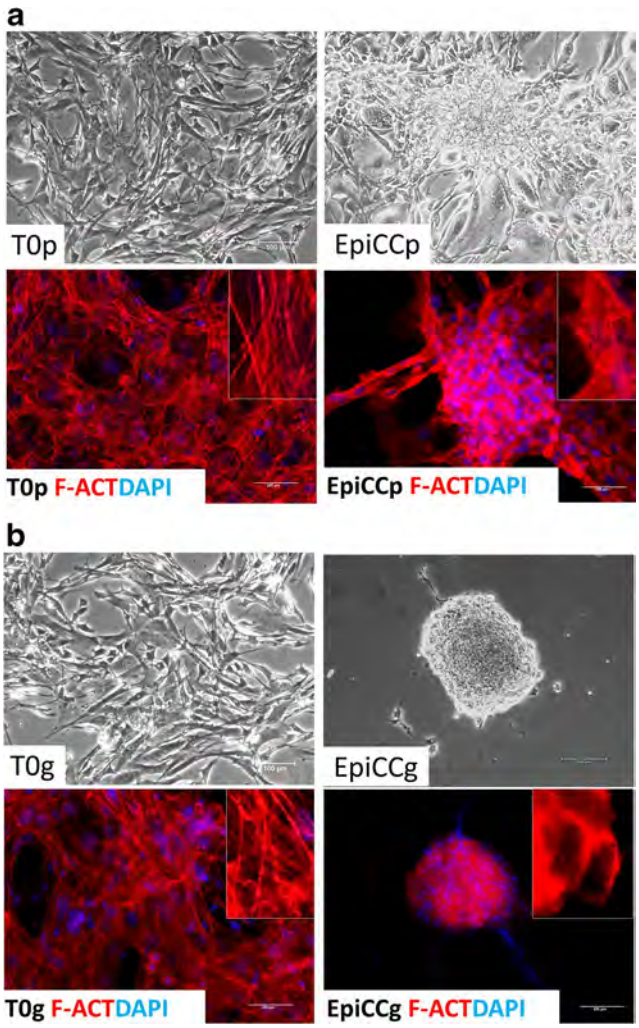

Fig. 3 Effect of matrix elasticity on cell morphology. a Untreated fibroblasts plated on plastic dish (T0p) appear large, flat and elongated, showing well-spread F-actin bundles and fine filaments (T0p F-ACTIN DAPI). At the end of pancreatic induction, cells differentiated on plastic (EpiCCp) acquire an epithelioid morphology and mainly kept a reticular organization, or aggregated in small and scattered clusters, displaying F-actin de-polymerized fibers (EpiCCp F-ACTIN DAPI). Scale bars: 100 µm. **b** Untreated fibroblasts plated on substrate with low mechanical compliance (T0g) appear large, flat and elongated, with well-spread F-actin bundles and fine filaments (T0g F-ACTIN DAPI). At the end of the conversion protocol, EpiCCg form large three-dimensional spherical structures and exhibit de-polymerized fibers, mainly localized at the cortical area (EpiCCg F-ACTIN DAPI). Scale bars: 100 µm

stainings, showing well-spread F-actin bundles and fine filaments.

By contrast, morphological changes become evident at the end of the pancreatic induction, in cells grown on plastic (EpiCCp, Fig. 3a) as well as in those differentiated on gel (EpiCCg, Fig. 3b). Both cell populations lost the typical elongated shape of untreated fibroblasts and acquired an epithelioid morphology. However, EpiCCp mainly kept a reticular organization and formed only small aggregates (Fig. 3a), while EpiCCg were able to organize in distinct large three-dimensional spherical structures (Fig. 3b). Interestingly, EpiCC showed a striking re-organization of the actin cytoskeleton, with depolarized F-actin filaments. These changes were more evident in EpiCCg, where we detected an intense rhodamine-phalloidin staining at the cortical area, immediately adjacent to the plasma membrane (EpiCCg F-ACTIN DAPI, Fig. 3b).

Effect of Matrix Elasticity on Mechano-responses and Biochemical Signals

Immunocytochemical studies revealed that yes-associated protein (YAP) was evenly distributed between cytoplasm and nucleus in untreated fibroblasts (T0p, T0g; Fig. 4a). No effect on its nuclear accumulation was detected after 18-hour exposure to 5-aza-CR either in cells plated onto plastic plates (Post 5-aza-CRp; Fig. 4a) or in those grown on PAA gels (Post 5-aza-CRg; Fig. 4a). In contrast, at the end of pancreatic induction, YAP localization markedly shifted into the cytoplasm (EpiCCp, EpiCCg; Fig. 4a), with a significantly higher number of cells showing nuclear immuno-positivity exclusion in EpiCCg (Fig. 4b).

Consistent with these observations, significant changes in YAP phosphorylation were detected in EpiCCp and EpiCCg. More in detail, EpiCC showed significantly higher levels of pYAP compared to untreated fibroblasts (T0p, T0g) and 5-aza-CR treated cells (Post 5-aza-CRp, Post 5-aza-CRg; Fig. 4b). Interestingly, Hippo-pathway regulating kinases, namely Large Tumor Suppressor 1 (LATS1) and MOB kinase activator 1 (MOB1), changed their phosphorylation levels in parallel to those of YAP protein (Fig. 4c, d).

Effect of Matrix Elasticity on Conversion Efficiency

At the end of pancreatic induction, EpiCC obtained both on plastic plates (EpiCCp) and on PAA gels (EpiCCg) displayed immuno-positivity for endocrine pancreatic hormones, namely C-PEP, GLUC and SOM (Fig. 5a). Gene expression analysis confirmed these results, showing the onset of active transcription for mature pancreatic specific genes, namely *Ins, Gcg* and *Sst* (Fig. 5e).

Significant differences in conversion efficiency were observed, when comparing cells differentiated onto standard plastic plates vs. PAA gels. In particular, the percentage of hormone immuno-positive cells significantly increased from $26.86 \pm 5.8\%$ in EpiCCp to $82.83 \pm 6.8\%$ in cells differentiated onto the soft substrate (EpiCCg; Fig. 5b).

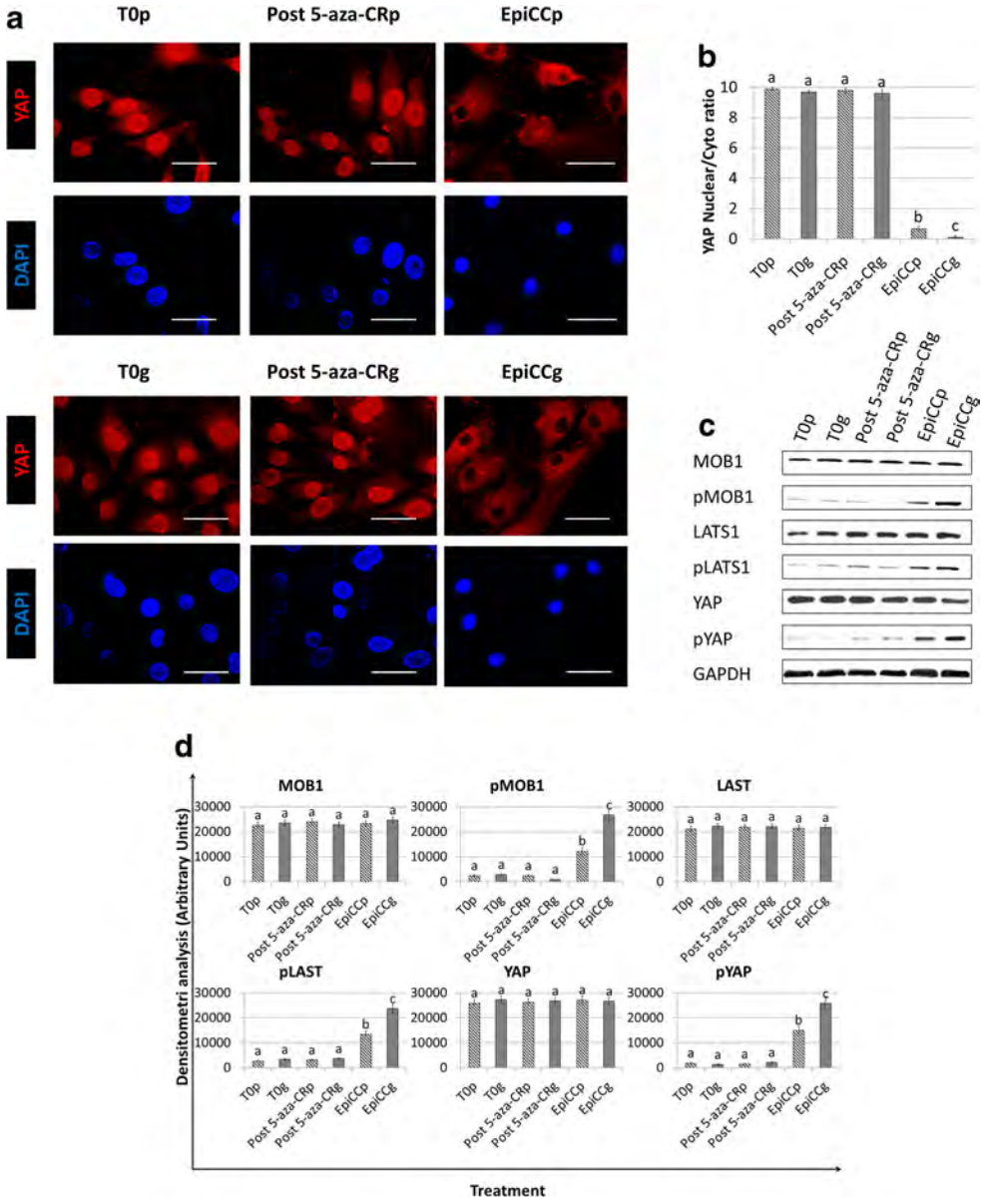

Fig. 4 Effect of matrix elasticity on mechanoresponses and biochemical signals. a YAP protein is evenly distributed between the cytoplasm and nucleus in untreated fibroblasts (T0p, T0g) and after 18-hour exposure to 5-aza-CR both in cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg). At the end of pancreatic induction YAP staining is excluded from the nucleus and shifts to the cytoplasm (EpiCCp, EpiCCg). Nuclei are stained with DAPI. Scale bars: 50 µm. **b** Quantification of the nuclear/cytoplasmic ratio of YAP. Bars represent mean ± SD of three independent replicates. Different superscripts (a, b, and c) denote significant differences between groups ($P < 0.05$). **c** Representative western blot immuno-detection of MOB1, pMOB1, LATS1, pLATS1, YAP, pYAP and GAPDH during epigenetic conversion. Full-length blots are included in the supplementary information. **d** Densitometric analysis of the Western blots. The values are reported as relative optical density of the bands normalized to GAPDH. Different superscripts denote significant differences between groups ($P < 0.05$)

Effect of Matrix Elasticity on EpiCC Acquisition of a Mono-hormonal Phenotype

EpiCCp displayed positivity for C-PEP, GLUC and SOM. In particular, in all immuno-reactive cells we detected a co-localization of the three pancreatic hormones within each single cell (EpiCCp, Fig. 5a, b).

Low stiffness PAA gels significantly promoted the acquisition of a mature pancreatic phenotype with 65.33 ± 2.5% of EpiCCg showing a mono-hormonal staining distribution, while only 17.5 ± 0.98% remained poly-hormonal (Fig. 5a, b). Furthermore, in depth analysis of these cells demonstrated that 13.57 ± 1.1% were positive for SOM, 23.76 ± 2.4% for GLUC, and 45.5 ± 3.1% C-PEP (Fig. 5c, d).

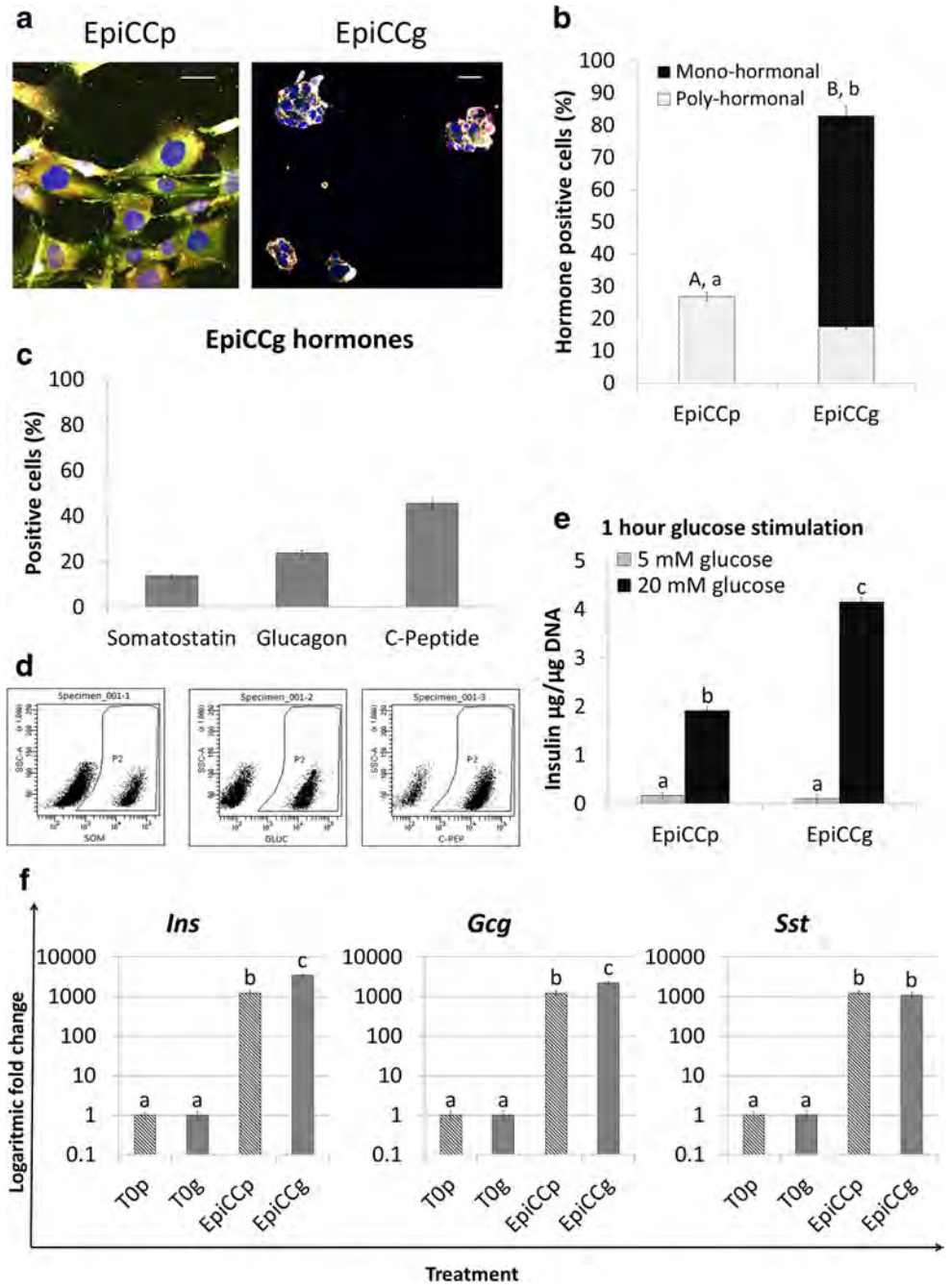

Fig. 5 Effect of matrix elasticity on epigenetic conversion. a EpiCC show immuno-positivity for C-peptide (white), glucagon (green) and somatostatin (red). EpiCCp display a co-localization of the three pancreatic hormones within each single immuno-reactive cell, while EpiCCg exhibit a mono-hormonal staining distribution. Nuclei are stained with DAPI. **b** Hormone immuno-positive cell rate and poly/mono-hormonal cell percentages in EpiCCp and EpiCCg. Different superscripts denote significant differences between groups ($P < 0.05$). **c** Somatostatin, glucagon and C-peptide positive cell rates in EpiCCg. **d** Representative output of flow cytometer analysis of EpiCCg showing somatostatin (SOM), glucagon (GLUC) and C-peptide (C-PEP) labeled cells. **e** Quantification of insulin release in cell supernatant in response to 5 mM and 20 mM D-glucose exposure for 1 h. Bars represent the mean ± SD of three independent replicates. Different superscripts denote significant differences between groups ($P < 0.05$). **f** At the end of pancreatic induction cells show the up-regulation of *Ins, Gcg* and *Sst* genes. Gene expression levels are reported with the T0 expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P < 0.05$)

The 3D spherical structures formed by EpiCCg were then analyzed using confocal microscopy. The results obtained showed poly-hormonal cells confined to the surface of the spheres, with mono-hormonal ones localized to the core (Fig. 6a). Moreover, fluorescence intensity measurement along the sphere diameter indicated SOM and C-PEP signal peaking on the external shell of the sphere, and glucagon expression homogeneously distributed along the diameter (Fig. 6b, c).

Effect of Matrix Elasticity on EpiCC Insulin Release

EpiCC grown on plastic plates and on low stiffness PAA gels were able to respond to 1-hour exposure to 20 mM glucose and actively released insulin in cell supernatants. However, significantly higher concentrations of insulin were released by EpiCCg (4.15 ± 0.07 µg /µg DNA) compared to EpiCCp (1.91 ± 0.09 µg /µg DNA) (Fig. 5d).

Discussion

The results obtained in the present study indicate that matrix elasticity may have a profound influence on the epigenetic conversion and differentiation of murine dermal fibroblasts into insulin-producing cells. In particular, the data obtained suggest that the presence of a soft surface is able to affect the different steps involved in the epigenetic conversion protocol, influencing both the transient acquisition of cell plasticity and the efficiency of differentiation.

After treatment with 5-aza-CR, both cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg) showed a significant decrease in global DNA methylation, compared to untreated fibroblasts, seeded on plastic plates (T0p) and PAA gels (T0g) (Fig. 2a). However, Post 5-aza-CRg cells exhibited a significantly lower methylation level compared to that of Post 5-aza-CRp.

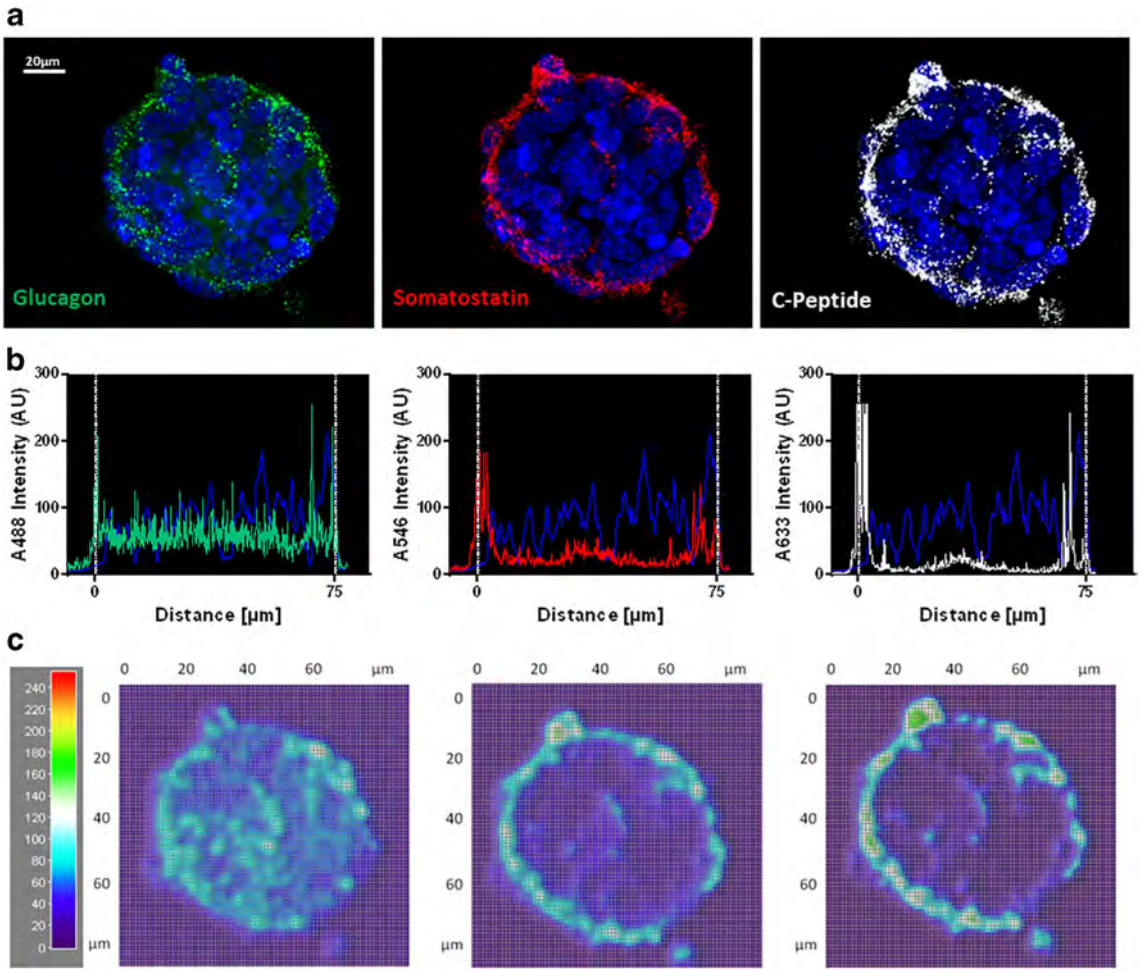

Fig. 6 Confocal analysis of 3D spherical structures formed by EpiCCg. a EpiCCg spheres display immuno-positivity for glucagon, somatostatin and C-peptide. **b** Fluorescence intensity analysis, along an arbitrarily set diameter-like axis, shows poly-hormonal cells confined to the surface of the spheres and mono-hormonal ones localized to the core. **c** Density plots representing glucagon, somatostatin and C-Peptide fluorescence intensity

Since no DNA methylation changes were observed in cells plated on PAA gels, without exposure to 5-aza-CR (w/o 5-aza-CRp and w/o 5-aza-CRg, Fig. 2a), the methylation decrease observed is unlikely to be accounted for the substrate and its elasticity "per sè". This is in line with Schellenberg et al. that previously demonstrated no influence by Young's moduli on mesenchymal cell methylation profiles [23]. In our understanding, the effect on methylation is more probably due to the combined actions of the matrix and the compound. On the other hand, it cannot be ruled out that the substrate alone may exert a direct effect, but it may require a longer time of exposure than the one described in these experiments.

Based on the results previously obtained in our laboratory [11], we also investigated whether the use of low-stiffness substrate may influence regulatory genes that are involved in DNA acetylation and methylation changes. The results here obtained showed a significant up-regulation of *Hat1* gene transcription in cells exposed to 5-aza-CR, which was increased by the use of soft substrate (Fig. 2c). This was paralleled by a significant decreased expression of *Hdac1* gene, that was lower in cells plated on PAA gels compared to that seeded on plastic dishes (Fig. 2c). These results are in agreement with our previous work demonstrating the key role played by these two genes in transcriptional regulation changes after 5-aza-CR treatment [11].

It is also tempting to speculate that the decrease in methylation may be the result of the upregulation of *Tet2*, which we have shown to play a direct and active demethylating action [11], and the expression of which was increased by the soft substrate (Post 5-aza-CRg, Fig. 2b). TET family genes also have an essential role in pluripotency regulation of ESC [31, 32] and in the very early stage of somatic cell reprogramming toward iPSC [33]. Interestingly, our results show that epigenetic erasing through 5-aza-CR, caused an increase in *Tet2* (Fig. 2c), paralleled by the onset of other pluripotency-related genes (*Oct4, Nanog, Rex1*, and *Sox2*, Fig. 2b), confirming previous studies by our laboratory, that demonstrated induction of OCT4, NANOG, REX1, SOX2 and TET2 in species other than the mouse [4–6, 11]. The mechanisms driving epigenetic erasing have been previously investigated and a relation with 5-aza-CR demethylating ability, either by inhibiting DNMT activity and/or modulating TET protein transcription was demonstrated [11]. The resulting global demethylation is likely to lead to a decrease of energy gradients required for the transition of mature cells to a higher plasticity state [34]. On the other hand, multiple and synergistically acting mechanisms may be hypothesized: the involvement and recruitment of a preexisting quiescent sub-population of pluripotent stem cells, recently detected in different adult tissues [12, 13], can be a distinct possibility. Their existence in very few number [14] can explain the lack of positivity for pluripotency related genes that might be below detection levels in T0 cell population.

Oct4, Nanog, Rex1, and *Sox2* expression was detected in cells erased on plastic, but was boosted by the presence of the soft matrix, which induced a 19%, 14%, 23% and 25% increment in *Oct4, Nanog, Rex1*, and *Sox2* gene expression respectively. These observations are in agreement with previous data, reporting that iPSC cultured on soft PAA gels showed higher expression of pluripotency genes, than the same cells plated on rigid plastic dishes [35] and are in line with the possibility to maintain and promote self-renewal of murine ESC (mESC), in the absence of exogenous LIF, through the use of soft substrates that match the intrinsic stiffness of the mESC [36].

Cell transition to pancreatic phenotype induced evident morphological rearrangements of the cytoskeletal organization, that shifted from F-actin with fine filaments in untreated fibroblasts (T0) to strikingly re-organized and depolarized actin in EpiCC. These changes were, however, more evident in EpiCCg, where F-actin destabilization led to a clear modification in its localization, that concentrated under the cortical area, immediately adjacent to the plasma membrane. This is consistent with recent studies demonstrating the presence of many stress fibers oriented along the line of applied force in cells cultured on stiff surfaces, while showing, in contrast, random and depolarized actin networks in cells grown on soft matrixes [37, 38].

Interestingly, the morphological changes described in the present manuscript, were also accompanied by activation of the Hippo signaling pathway (Fig. 4c) and by modifications in YAP cellular localization (Fig. 4a, b). Indeed, while fibroblasts showed an even distribution of this protein between cytoplasm and nucleus, regardless of the substrate utilized (T0p and T0g), EpiCC displayed a restricted YAP localization to the cytoplasmic compartment (Fig. 4b) with a significantly higher number of cells showing nuclear immuno-positivity exclusion in EpiCCg compared to EpiCCp (Fig. 4b). These data are altogether in agreement with recent studies, demonstrating a correlation between cytoplasmic retention, the subsequent complete nuclear exclusion of YAP and F-ACTIN destabilization/disruption [39–41]. Notably, cytoplasmic confinement of YAP has been reported to be distinctive of differentiating cells, while ESC showed the presence of the protein in the nucleus as well as in the cytoplasm [34–37]. Similarly, in the experiments here reported, we detected YAP equal localization in both compartments of cells exposed to 5-aza-CR (Fig. 4a, b), regardless of the matrix elasticity and, most likely related to the newly acquired high plasticity state. These results are well in line with recent work, demonstrating nuclear YAP essential role in ESC self-renewal and in the control of the levels of the pluripotency genes *Oct4, Nanog* and *Sox2* [42–45]. Nuclear/cytoplasmic translocation of YAP has also been described

to be controlled by the phosphorylation levels of the protein, which are higher in differentiating cells, compared to undifferentiated ones [42]. In particular, phosphorylation via MOB and LATS kinases, that are core components of the Hippo signaling pathway, resulted in YAP phosphorylation and subsequent exclusion from the nuclear compartment [41]. Consistent with this, the data here presented, demonstrate the phosphorylation of the Hippo-pathway regulating kinases, that paralleled changes in the YAP protein phosphorylation levels (Fig. 4c) and in its nuclear/cytoplasmic distribution (Fig. 4a, b).

In this manuscript, we report for the first time the epigenetic conversion of dermal fibroblasts in the murine species. This result supports the robustness of the method, confirming and extending to the mouse our previous experiments carried out in human, pig, and dog [4, 5, 9]. On the other hand, some species-specific differences became evident. In contrast to what observed in the other species, murine converting cells showed a more limited tendency to form aggregates and organized in smaller and scattered clusters (Fig. 3a). Similarly, conversion efficiency was 26.86 ± 5.8% (Fig. 5b), while a significantly higher percentage of C-peptide immuno-positive cells was obtained in the human (35 ± 8.9%) [4], pig (38.1 ± 9.2%) [5], and dog (38 ± 6.1%) [9]. Interestingly, the use of a substrate with lower than plastic elastic modulus, allowed mechanotransduction-related stimuli, that guided cells to rearrange into distinct large three-dimensional spherical structures (Fig. 3b) and induced the morphological changes described, that were paralleled by a significantly higher pancreatic differentiation efficiency (82.83 ± 6.8% vs. 26.86 ± 5.8%) (Fig. 5b). This is consistent with recent studies that demonstrated soft gel encapsulation system ability to enhance cell differentiation towards the endodermal lineage [46–48].

In the experiment here described, epigenetic conversion of murine skin fibroblasts led to the acquisition of a pancreatic phenotype. Genuine differentiation was supported both by active transcriptional activity for pancreatic genes as well as immuno-positivity for C-PEP, SOM and GLUC. However, while EpiCCp displayed a co-localization of the three pancreatic hormones in all immuno-reactive cells, EpiCCg showed a mono-hormonal staining distribution in over 65% of the population (Fig. 5a, b). Interestingly, it has been observed that primitive endocrine cells, typical of early fetal stages, co-express insulin and glucagon, while they mature into a mono-hormonal phenotype later in development [49, 50]. Hormone compartmentalized localization of EpiCCg may therefore be suggestive of a more mature phenotype and to be distinctive of terminally differentiated cells.

Altogether, these results indicate that the use of a soft substrate has a general effect on the differentiation of epigenetically erased fibroblasts and indicate a positive impact both on efficiency and on the promotion of a mono hormonal mode. Furthermore, the low compliance substrate used in the present experiments appear to support a spatial-related maturation process, with a clear compartmentalization of poly-hormonal cells to the surface of the spheres, and of mono-hormonal ones in the core of the structures, as shown by the confocal analysis (Fig. 6). Although further studies are needed in order to better understand this aspect, a substrate-dependent maturation gradient could be hypothesized.

In vitro functional experiments, showed that, when challenged with 20 mM D-glucose, EpiCC actively released insulin (Fig. 5d), demonstrating their ability to respond to the primary and physiological stimulus for insulin secretion, and supporting the achievement of a functional phenotype. However, it is interesting to note that a significantly higher amount of insulin was released by EpiCCg (Fig. 5d). This is likely to be related to the acquisition of a more mature/mono-hormonal phenotype which was obtained thanks to the use of a soft substrate. Needless to say that, although these results are very promising, more tests assessing the efficacy of EpiCCg in vivo are needed in order to further characterize these cells and the impact of the soft substrate on their functional activity.

In conclusion, in the present manuscript, we describe the epigenetic conversion of dermal fibroblasts in the murine species and demonstrate that cell mechano-sensing and biomechanical properties of the surrounding matrix, may influence the acquisition of cell plasticity and enhance tissue differentiation, increase conversion efficiency and encourage the acquisition of a mature pancreatic phenotype. The results confirm and strengthen previous data obtained in other species [4, 5, 9, 51], and expand our knowledge on the mechanisms underlying the epigenetic erasing and conversion processes. Furthermore, the data here obtained may have interesting technological impacts in order to increase reliability and increment efficiency of the conversion process. In our opinion, this aspect is crucial for clinical translation of the results, since it allows swift scale-up culture procedures that are essential for cell therapy and tissue engineering applied to human regenerative medicine.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Safety and Efficacy of Epigenetically Converted Human Fibroblasts Into Insulin-Secreting Cells: A Preclinical Study

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Abstract

Type 1 Diabetes Mellitus (T1DM) is a chronic disease that leads to loss of insulin secreting β-cells, causing high levels of blood glucose. Exogenous insulin administration is not sufficient to mimic the normal function of β-cells and, consequently, diabetes mellitus often progresses and can lead to major chronic complications and morbidity. The physiological control of glucose levels can only be restored by replacing the β-cell mass.

We recently developed a new strategy that allows for epigenetic conversion of dermal fibroblasts into insulin-secreting cells (EpiCC), using a brief exposure to the demethylating agent 5-aza-cytidine (5-aza-CR), followed by a pancreatic induction protocol. This method has notable advantages compared to the alternative available procedures and may represent a promising tool for clinical translation as a therapy for T1DM. However, a thought evaluation of its therapeutic safety and efficacy is mandatory to support preclinical studies based on EpiCC treatment.

We here report the data obtained using human fibroblasts isolated from diabetic and healthy individuals, belonging the two genders. EpiCC were injected into 650 diabetic severe combined immunodeficiency (SCID) mice and demonstrated to be able to restore and maintain glycemic levels within the physiological range. Cells had the ability to self-regulate and not to cause hypoglycemia, when transplanted in healthy animals. Efficacy tests showed that EpiCC successfully re-established normoglycemia in diabetic mice, using a dose range that appeared clinically relevant to the concentration 0.6×10^6 EpiCC. Necropsy and histopathological investigations demonstrated the absence of malignant transformation and cell migration to organs and lymph nodes.

The present preclinical study demonstrates safety and efficacy of human EpiCC in diabetic mice and supports the use of epigenetic converted cells for regenerative medicine of diabetes mellitus.

Keywords

Diabetes · Efficacy · Epigenetically converted cells · Glycaemia · Preclinical study · Safety

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1 Introduction

In Type 1 Diabetes Mellitus (T1DM) patients, the onset of overt disease is assumed to occur when the β-cell mass falls below 20% of the normal range (Gepts 1965; Pipeleers and Ling 1992; Butler et al. 2007). Exogenous insulin administration is not sufficient to mimic the normal function of β-cells. Consequently, diabetes mellitus often progresses and can lead to major chronic complications and morbidity (Nathan et al. 1993). The physiological control of blood glucose levels can only be restored by replacing the β-cell mass. In patients with T1DM, transplantation of pancreatic islet cells has proven successful for functional replenishment of damaged islets (Keymeulen et al. 1998; Shapiro et al. 2000; Ryan et al. 2001). However, to achieve sustained metabolic control for 1 year, at least 2 million β-cells per kg body weight need to be transplanted (Keymeulen et al. 2006), which usually requires 2–3 donor pancreata. Indeed, many β-cells are lost during the isolation procedures, during the early period of post-engraftment neo vascularization and possibly also as a result of inflammation at the transplantation site. Furthermore, the functional β-cell mass of the graft seems to decline after 1 year, and after 5 years only 20% of transplanted patients retain a functioning graft (Ryan et al. 2005). This, together with the limited availability of donor organs, severely limit the widespread application of transplantation therapy in patients with T1DM.

In line with this, during the last years several studies have been carried out in order to identify an alternative source of viable insulin-producing cells for regenerative medicine.

We recently described a protocol for the epigenetic conversion of human dermal fibroblasts into insulin-secreting cells (EpiCC) (Pennarossa et al. 2013; Brevini et al. 2016). This method is based on a brief exposure (18 h) to the demethylating drug 5-aza-cytidine (5-aza-CR), followed by a three-step induction protocol. This allows cells to transit from the early endodermic and pancreatic differentiation stage to mature endocrine cells. At the end of the epigenetic conversion process, fibroblasts acquire an epithelial morphology and form large three-dimensional spherical structures that tend to detach and float freely in the culture medium, reminiscent of in vitro-cultured pancreatic islets. Most importantly, EpiCC express the main pancreatic hormones and glucose sensor genes, distinctive of mature endocrine cells. Furthermore, 35 ± 8.9% of starting cell population is able to actively release of C-peptide and insulin after exposure to 20 mM glucose, showing a dynamic response similar to pancreatic β-cells, in which changes in ambient glucose represent the primary and physiological stimulus for insulin secretion. Preliminary experiments also demonstrate *in vivo* functionality of EpiCC, after their injection in streptozotocin (STZ)-induced diabetic mice. Indeed, cell transplantation lead to a restoring of physiological glycemic levels, that are stably maintained for a long period in diabetic animals.

The epigenetic conversion method has notable advantages compared to the alternative available procedures: it is highly efficient, it does not require any transgenic modification, and it does not induce a stable pluripotent stage that may be cause malignant transformations. Based on these observations, EpiCC look very promising candidates for their clinical translation as a therapy for diabetes including type I diabetes mellitus (T1DM).

In this perspective, preclinical studies in animal models are necessary in order to ensure the safety of cell treatment and to establish the adequate dose that enables patient adherence to treatment.

2 Approaches

2.1 Ethical Statement

Cells were isolated from adult patients, after written informed consent and approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa and by the Ethical Committee of the University of Milan. All the methods in our study were carried out in accordance with the approved guidelines. Animals were handled and treated in agreement with the

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

2.2 Epigenetic Cell Conversion into Insulin-Secreting Cells

In this study, four adult human dermal fibroblast primary lines were used. Two lines were isolated from diabetic patients and kindly donated by Gianpaolo Zerbini (Scientific Institute San Raffaele, University of Milan, Milan, Italy). One line was obtained from male healthy individual, and one from female healthy individual.

All dermal fibroblasts were treated as previously described (Pennarossa et al. 2013; Brevini et al. 2016). Briefly, cells were plated on 0.1% gelatin pre-coated dishes (Sarstedt) at concentration of 7.8×10^4 fibroblasts/cm^2. After 24 h, they were epigenetically erased by exposure to 1 μM 5-aza-CR (Sigma) for 18 h, and, subsequently, incubated for 3 h with ESC culture medium (14) (Brevini et al. 2009). Pancreatic differentiation was induced culturing cell in in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 1% B27, 1% N2, 0.1 mM β-mercaptoetanol (Sigma), 2 mM glutamine (Sigma), 1 mM MEM Non-essential amino acids and 0.05% bovine serum albumin (BSA, Sigma). During the first 6 days, medium was enriched with 30 ng/ml activin A. The following 2 days, 10 μM retinoic acid (Sigma) was added. From day 9 onward, a medium containing 1% B27, 20 ng/ml basic fibroblast growth factor and 1% insulin–transferrin–selenium was used to further encourage differentiation. Medium was refreshed daily. Cells were in vitro differentiated for a total of 36 days.

2.3 Experimental Design

EpiCC Safety Assessment To exclude the possible risk of EpiCC tumorigenicity, 300 eight-week-old male SCID mice (Harlan) were xenograted with 5×10^6 EpiCC. Experimental diabetes was induced in 200 mice, by a single intraperitoneal injection of streptozotocin (STZ; Sigma, 150 mg/kg of body weight) freshly dissolved in 0.1 M of citrate buffer, pH 4.6 (Lumelsky et al. 2001). STZ-induced diabetic animals were randomly divided in 4 groups of 50 individuals. Group 1 received diabetic female EpiCC (DF), group 2 was injected with EpiCC obtained from diabetic male (DM), group 3 was xenografted with healthy female cells (HF), and group 4 was transplanted with healthy male EpiCC (HM). Furthermore, in order to rule out tumorigenic potential also in the presence of an autologous source of insulin, the scheme described above was applied on 100 SCID mice that were not treated with STZ and animals received 5×10^6 EpiCC obtained to the four different cell lines: diabetic female (DF, $n = 25$), diabetic male (DM, $n = 25$), healthy female (HF, $n = 25$), and healthy male (HM, $n = 25$).

Six days after STZ treatment, all animals were anesthetized by isoflurane (3.5% gas in oxygen, Vet-Merial). EpiCC were injected subcutaneously in the shoulder area through a 19-gauge hypodermic needle. Mice were macroscopically monitored daily for signs of tumor formation/growth or visible morbidity. Blood glucose levels were measured using Accu-Chek glucometer (Roche) at 1 week intervals. Mice were euthanized 50 weeks after transplantation. At the end of the experiment, randomly selected mice from each group were subjected to a complete necropsy and histopathological evaluation for any sign of tumorigenic transformation and/or cell migration from the injection site.

EpiCC Efficacy Assessment 210 STZ-induced diabetic SCID mice were xenografted, as described above, with different doses of EpiCC obtained from the four different cell lines: diabetic female (DF, $n = 50$), diabetic male (DM, $n = 50$), healthy female (HF, $n = 50$), healthy male (HM, $n = 50$). The following doses were tested: 10×10^6 ($n = 40$), 5×10^6 ($n = 40$),

2.5×10^6 (n = 40), 1.25×10^6 (n = 40), 0.6×10^6 (n = 40), and 0 (n = 10) cells.

40 additional animals received STZ treatment and 5×10^6 untreated control fibroblasts belonging to the four cell lines: DF, n = 10; DM, n = 10; HF, n = 10; and HM, n = 10. Mice were carefully monitored as described above and euthanized 4 and 30 weeks after fibroblasts and EpiCC transplantation, respectively. At the end of the experiment, randomly selected mice from each group were subjected to a complete necropsy and histopathological evaluation.

Statistical Analysis Statistical analysis was performed using ANOVA test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

3 Observations

3.1 EpiCC Safety Assessment

EpiCC obtained from the four different human cell lines described above were able to quickly restore and maintain blood glucose physiological levels in all STZ-induced diabetic mice (Table 1 and Fig. 1A). Interestingly enough, EpiCC ability to release insulin was comparable and completely independent from the health state of the patient of origin. Furthermore, when cells were xenografted in healthy animals (no STZ treatment), glycemic values remained normal and were not affected by the injection, demonstrating EpiCC ability to self-regulate and to secrete insulin in a controlled physiological manner.

Necropsy and histopathological evaluations, carried out 50 weeks after transplantation, demonstrated the complete absence of malignant transformation and no traces of cell migration to organs and lymph nodes of all subjects analyzed. Furthermore, no major advice effects were observed.

3.2 EpiCC Efficacy Assessment

EpiCC efficacy experiments demonstrated re-establishment of normoglycemia in STZ-induced diabetic SCID mice with all the different doses tested (Table 2 and Fig. 1B). More in detail, the normal and physiological glucose concentrations were restored 2 weeks after transplantation in a cell concentration ranging from 10×10^6 to the lower dose of 0.6×10^6. Furthermore, all dose range were able to steady maintain glycemic levels for the entire length of the experiments (30 weeks).

In contrast, injection of vehicle only (no EpiCC) or untreated control fibroblasts obtained from DF, DM, HF and HM patients, resulted in not reduction of hyperglycemic values in diabetic animals.

Necropsy and histopathological evaluations, carried out 30 weeks after injection, demonstrated the complete absence of malignant transformation and/or no traces of cell migration to organs and lymph nodes in all experimental groups. Furthermore, no major advice effects were observed.

4 Discussion

According to the World Health Organization (WHO), more than 400 million individuals suffer from diabetes and it is estimated that this disease will represents the seventh leading cause of death in 2030 (Mathers and Loncar 2006). Similar to other autoimmune diseases, caused by a combination of genetic and environmental factors, the incidence of T1DM is on the increase at an alarming rate in industrialized countries, leading, in the first place, to high levels of blood glucose, and, subsequently, acute and late complications, such as ketoacidosis, and atherosclerosis, retinopathy, kidney failure, neuropathy, and infection, respectively.

Exogenous insulin administration is a life-saving treatment rather than a cure, and individuals suffering from T1DM and receiving

Table 1 EpiCC safety assessment

Weeks	−1	−0.5	0	1	2	3	4
STZ + DF	123.1 ± 6.4	423.2 ± 26.1*	459.5 ± 7.6*	235.4 ± 31.3**	162.7 ± 11.0	153.8 ± 16.1	147.3 ± 13.4
STZ + DM	122.2 ± 10.1	411.0 ± 31.2*	443.8 ± 9.2*	236.3 ± 25.5**	148.7 ± 14.2	156.5 ± 8.9	147.0 ± 17.1
STZ + HF	130.6 ± 9.2	407.5 ± 21.8*	443.8 ± 11.4*	237.6 ± 22.6**	151.8 ± 16.1	158.4 ± 12.3	157.2 ± 16.2
STZ + HM	123.1 ± 12.3	400.0 ± 21.9*	441.0 ± 10.4*	228.1 ± 23.2**	176.1 ± 9.7	166.8 ± 10.8	144.2 ± 11.2
No-STZ + DF	129.6 ± 8.5	146.9 ± 11.3	122.7 ± 8.4	134.8 ± 13.4	156.5 ± 11.6	128.4 ± 8.7	138.3 ± 9.9
No-STZ + DM	126.7 ± 7.6	132.5 ± 12.1	131.2 ± 12.3	127.6 ± 8.7	138.3 ± 12.3	131.4 ± 9.1	141.1 ± 10.4
No-STZ + HF	131.1 ± 10.2	101.0 ± 9.5	125.6 ± 11.7	133.8 ± 8.1	140.8 ± 9.8	127.4 ± 12.1	144.6 ± 15.5
No-STZ + HM	123.0 ± 9.1	104.4 ± 9.7	146.4 ± 10.9	133.2 ± 9.8	138.6 ± 8.8	131.6 ± 10.7	145.2 ± 9.1

Weeks	8	12	16	20	30	40	50
STZ + DF	136.7 ± 14.5	153.0 ± 14.7	136.8 ± 9.8	136.1 ± 15.4	134.1 ± 13.1	124.6 ± 12.1	127.5 ± 16.4
STZ + DM	145.1 ± 14.6	128.4 ± 8.8	132.2 ± 15.0	111.3 ± 13.2	126.8 ± 15.6	122.1 ± 9.9	134.0 ± 13.8
STZ + HF	141.6 ± 8.9	135.0 ± 11.0	134.2 ± 8.7	134.6 ± 8.8	136.8 ± 17.2	143.2 ± 11.3	138.6 ± 10.7
STZ + HM	141.8 ± 9.2	140.2 ± 9.8	128.3 ± 14.2	129.6 ± 9.7	140.0 ± 7.9	144.6 ± 12.3	135.0 ± 9.2
No-STZ + DF	142.3 ± 11.9	128.5 ± 12.7	134.3 ± 10.7	140.2 ± 14.7	127.1 ± 12.1	132.8 ± 9.9	129.0 ± 12.7
No-STZ + DM	135.4 ± 9.3	137.6 ± 10.8	127.8 ± 11.4	145.0 ± 8.5	124.2 ± 9.9	137.6 ± 8.9	136.3 ± 8.6
No-STZ + HF	135.2 ± 11.1	133.4 ± 7.8	128.4 ± 13.6	171.0 ± 10.0	137.2 ± 8.9	105.2 ± 11.1	166.0 ± 9.9
No-STZ + HM	147.6 ± 10.8	147.2 ± 9.2	133.8 ± 7.9	111.8 ± 9.3	167.4 ± 11.2	194.4 ± 13.4	129.8 ± 11.1

Blood glucose values (means ± SD) in STZ-treated (STZ) and healthy (no-STZ) mice transplanted with 5×10^6 EpiCC obtained from the four different cell lines (*DF* diabetic female, *DM* diabetic male, *HF* healthy female, and *HM* healthy male). Different superscripts denote significant differences between groups ($P < 0.05$)

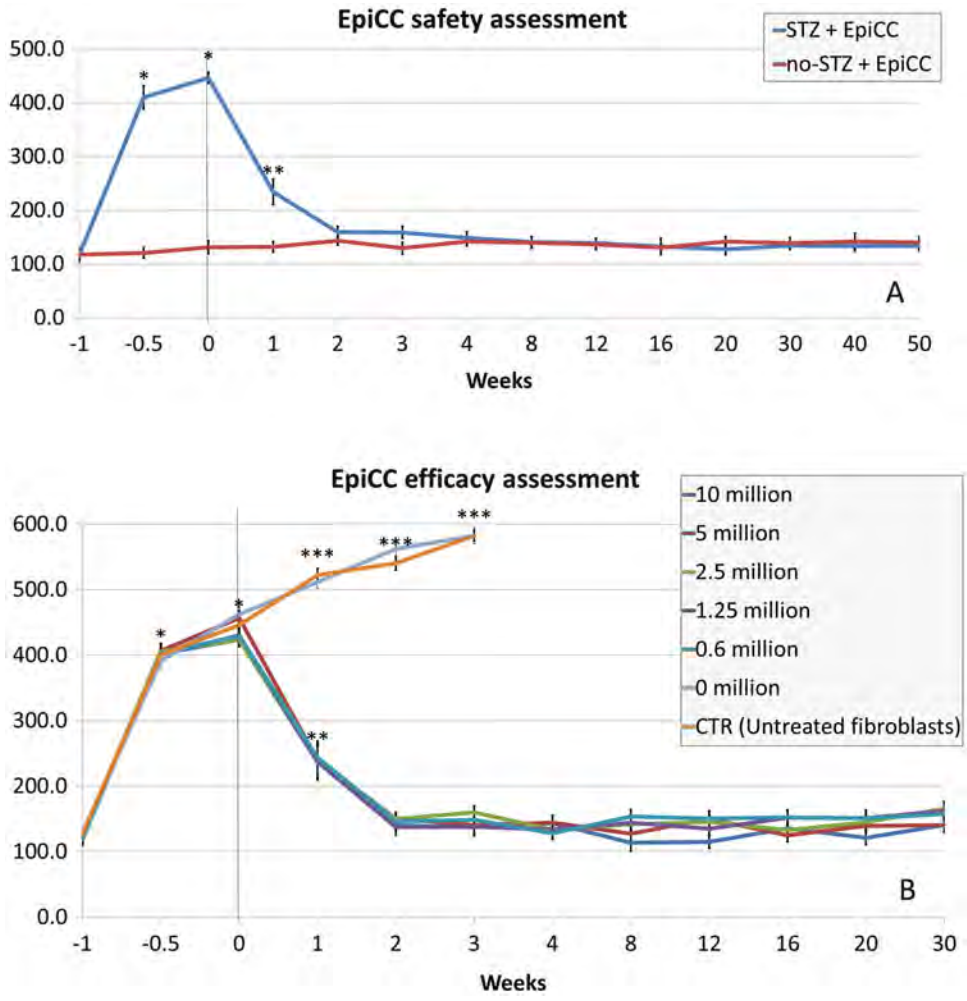

Fig. 1 (**a**) Blood glucose levels (means±SD) in STZ-treated (STZ) and healthy (no-STZ) mice transplanted with 5×10^6 human EpiCC. (**b**) Blood glucose values (means±SD) in STZ-treated (STZ) mice transplanted with different doses of human EpiCC. Different superscripts denote significant differences between groups ($P < 0.05$)

insulin still show an unacceptable 15 year reduction in life expectancy and complications of the disease, according to the Juvenile Diabetes Research Foundation (JDRF, http://www.jdrf.org.uk/page.asp?section=163&sectionTitle=FAQs+about+type+1+diabetes). Indeed, this treatment is not sufficient to mimic the normal function of β-cells and the physiological control of blood glucose levels can only be maintained or restored by preventing the evolution of the disease or by the replacement/regeneration of β-cells mass. In line with this, during the last years, several studies have been carried out to find novel treatments to cure T1DM. Much investigation has been directed at interrupting its process both during the stage of evolution or at the time of disease onset, using immune-therapeutic approaches. The goal of this intervention is to arrest the immune destruction and thus delay or prevent clinical disease. However, to effectively accomplish this requires identification of individuals at risk of T1DM (Ziegler and Nepom 2010). This represents a relevant limitation, since the major symptoms of the disease

Table 2 EpiCC efficacy assessment

Weeks	−1	−0.5	0	1	2	3
DF (10×10^6)	123.2 ± 8.9	399.3 ± 13.0*	411.5 ± 9.7*	225.1 ± 25.1**	116.8 ± 13.2	122.2 ± 12.5
DM (10×10^6)	131.6 ± 7.6	410.7 ± 9.9*	423.0 ± 11.3*	245.5 ± 24.2**	131.1 ± 11.2	126.1 ± 16.5
HF (10×10^6)	142.4 ± 8.2	401.9 ± 11.9*	408.6 ± 12.0*	238.2 ± 26.2**	144.4 ± 10.4	149.4 ± 9.0
HM (10×10^6)	109.3 ± 7.1	399.8 ± 13.8*	451.3 ± 10.7*	238.2 ± 24.1**	168.5 ± 9.1	151.5 ± 14.1
DF (5×10^6)	126.7 ± 9.9	421.9 ± 11.0*	432.6 ± 11.1*	244.2 ± 28.7**	169.2 ± 9.4	145.6 ± 13.2
DM (5×10^6)	131.1 ± 7.6	387.5 ± 12.1*	461.4 ± 9.8*	240.2 ± 21.6**	156.1 ± 8.7	141.9 ± 9.6
HF (5×10^6)	112.7 ± 6.6	417.4 ± 13.2*	481.1 ± 12.5*	243.4 ± 22.4**	125.3 ± 9.9	127.8 ± 11.2
HM (5×10^6)	108.4 ± 5.9	401.1 ± 8.9*	452.4 ± 11.9*	231.8 ± 24.6**	151.2 ± 13.5	147.6 ± 10.2
DF (2.5×10^6)	118.3 ± 11.9	409.0 ± 11.1*	461.7 ± 14.0*	234.6 ± 27.0**	157.5 ± 11.7	177.5 ± 11.7
DM (2.5×10^6)	127.0 ± 8.5	387.6 ± 10.1*	416.8 ± 8.2*	247.4 ± 26.1**	148.1 ± 12.2	172.7 ± 8.3
HF (2.5×10^6)	131.1 ± 6.7	420.1 ± 9.8*	407.2 ± 10.8*	242.3 ± 24.2**	140.4 ± 9.8	128.1 ± 9.6
HM (2.5×10^6)	125.2 ± 7.4	406.7 ± 8.8*	409.2 ± 9.1*	237.4 ± 23.4**	153.7 ± 7.8	161.8 ± 11.2
DF (1.25×10^6)	116.8 ± 10.1	403.4 ± 9.6*	413.3 ± 14.2*	231.1 ± 23.1**	122.5 ± 11.2	121.9 ± 8.6
DM (1.25×10^6)	124.4 ± 6.8	379.9 ± 11.2*	437.1 ± 9.8*	220.7 ± 29.9**	149.4 ± 8.5	151.0 ± 10.9
HF (1.25×10^6)	131.5 ± 7.3	419.3 ± 12.1*	448.8 ± 9.6*	241.6 ± 28.9**	137.5 ± 15.5	133.5 ± 9.4
HM (1.25×10^6)	119.8 ± 8.8	402.1 ± 8.9*	422.2 ± 11.1*	255.8 ± 29.4**	140.4 ± 13.5	147.6 ± 14.2
DF (0.6×10^6)	131.9 ± 9.5	412.3 ± 12.0*	417.3 ± 12.0*	229.0 ± 22.3**	135.3 ± 11.3	130.3 ± 15.1
DM (0.6×10^6)	124.7 ± 9.8	399.4 ± 11.6*	412.4 ± 13.3*	253.1 ± 28.9**	169.8 ± 10.8	172.1 ± 8.1
HF (0.6×10^6)	117.3 ± 7.6	388.6 ± 8.9*	435.6 ± 8.4*	243.4 ± 27.4**	129.0 ± 9.6	147.5 ± 8.6
HM (0.6×10^6)	104.0 ± 11.0	411.7 ± 9.9*	452.4 ± 11.3*	249.4 ± 23.1**	152.2 ± 9.5	146.6 ± 12.4
VEHICLE	127.8 ± 8.1	391.2 ± 11.5*	462.3 ± 9.6*	511.3 ± 9.7***	562.1 ± 12.3***	582.1 ± 12.5***
DF (CTR)	139.1 ± 8.5	386.3 ± 13.1*	477.5 ± 10.8*	502.3 ± 12.8***	506.1 ± 11.1***	577.7 ± 8.5***
DM (CTR)	129.6 ± 8.3	415.3 ± 7.5*	454.4 ± 7.9*	502.2 ± 7.9***	507.8 ± 13.2***	584.6 ± 10.0***
HF (CTR)	127.6 ± 7.5	389.8 ± 10.1*	436.8 ± 10.2*	524.1 ± 8.6***	553.6 ± 9.7***	569.7 ± 7.4***
HM (CTR)	113.5 ± 9.8	410.1 ± 9.8*	416.9 ± 11.2*	561.4 ± 11.3***	594.9 ± 7.8***	598.8 ± 7.9***

Weeks	4	8	12	16	20	30
DF (10×10^6)	142.4 ± 13.2	121.4 ± 15.9	108.9 ± 9.8	131.3 ± 8.7	112.0 ± 9.9	114.2 ± 12.1
DM (10×10^6)	145.0 ± 9.3	110.4 ± 9.1	123.8 ± 10.5	142.3 ± 11.1	134.0 ± 10.4	138.6 ± 14.1
HF (10×10^6)	148.3 ± 7.4	103.8 ± 10.9	100.4 ± 11.2	128.6 ± 7.9	108.6 ± 11.1	155.6 ± 9.7
HM (10×10^6)	139.1 ± 9.9	119.7 ± 15.3	129.5 ± 9.5	139.5 ± 9.8	130.2 ± 12.1	154.5 ± 7.8
DF (5×10^6)	143.4 ± 15.1	120.1 ± 11.4	141.7 ± 11.1	109.6 ± 12.2	121.6 ± 9.8	136.5 ± 7.9

(continued)

Table 2 (continued)

Weeks	4	8	12	16	20	30
DM (5×10^6)	173.5 ± 11.6	118.4 ± 9.7	181.2 ± 14.3	122.1 ± 9.7	154.1 ± 6.9	146.3 ± 13.1
HF (5×10^6)	137.0 ± 9.7	124.2 ± 10.7	131.2 ± 15.9	125.4 ± 9.9	137.8 ± 12.4	130.7 ± 9.8
HM (5×10^6)	123.1 ± 8.7	146.3 ± 12.1	146.2 ± 9.8	142.6 ± 8.9	144.8 ± 11.0	149.0 ± 9.9
DF (2.5×10^6)	134.5 ± 14.4	156.7 ± 12.3	139.5 ± 12.3	129.4 ± 16.1	122.7 ± 11.5	168.1 ± 8.9
DM (2.5×10^6)	137.6 ± 11.3	135.5 ± 9.7	157.0 ± 9.8	135.6 ± 9.4	162.5 ± 12.1	172.0 ± 9.9
HF (2.5×10^6)	115.3 ± 16.4	146.4 ± 8.6	160.1 ± 7.9	151.6 ± 11.3	171.6 ± 9.8	138.4 ± 12.5
HM (2.5×10^6)	148.6 ± 9.8	129.2 ± 9.4	125.2 ± 14.5	117.4 ± 9.1	123.6 ± 11.4	182.4 ± 11.9
DF (1.25×10^6)	131.6 ± 15.3	149.1 ± 11.0	145.7 ± 8.9	159.8 ± 10.9	149.4 ± 11.2	188.3 ± 11.2
DM (1.25×10^6)	128.7 ± 8.2	151.3 ± 13.2	130.5 ± 12.1	137.1 ± 14.5	170.2 ± 15.1	134.8 ± 12.1
HF (1.25×10^6)	119.1 ± 10.9	157.9 ± 7.9	121.5 ± 9.2	177.3 ± 12.3	145.9 ± 13.2	140.4 ± 9.8
HM (1.25×10^6)	155.3 ± 12.3	119.8 ± 8.9	144.6 ± 14.2	134.5 ± 9.8	141.6 ± 9.9	186.8 ± 13.2
DF (0.6×10^6)	126.2 ± 12.5	134.3 ± 9.7	131.7 ± 12.3	146.1 ± 12.1	184.1 ± 8.7	157.7 ± 11.2
DM (0.6×10^6)	124.0 ± 9.9	189.1 ± 8.4	174.2 ± 9.9	142.0 ± 10.7	136.7 ± 6.9	124.2 ± 13.4
HF (0.6×10^6)	134.1 ± 8.6	143.3 ± 10.9	142.1 ± 9.1	172.3 ± 9.1	139.4 ± 7.9	165.1 ± 9.4
HM (0.6×10^6)	128.6 ± 7.9	148.4 ± 12.4	154.3 ± 15.1	148.6 ± 11.1	144.8 ± 11.6	184.1 ± 7.9
VEHICLE	–	–	–	–	–	–
DF (CTR)	–	–	–	–	–	–
DM (CTR)	–	–	–	–	–	–
HF (CTR)	–	–	–	–	–	–
HM (CTR)	–	–	–	–	–	–

Blood glucose values (means ± SD) in STZ-treated mice transplanted with different doses of EpiCC, obtained from the four different cell lines (DF: diabetic female, DM: diabetic male, HF: healthy female, and HM: healthy male), vehicle only (VEHICLE) or untreated control fibroblasts (CTR) isolated from the four patients (*DF* diabetic female, *DM* diabetic male, *HF* healthy female, and *HM* healthy male). Different superscripts denote significant differences between groups ($P < 0.05$)

usually become manifest only once the number of β-cells mass has become inadequate (Weir and Bonner-Weir 2013). Transplantation of pancreas or pancreatic islets, with the obligatory immunosuppression, has been the most commonly used alternative, applied for over two decades in patients suffering from hypoglycemia (Alejandro et al. 2008). Even this procedure does not represent a definitive cure, since the pre-existing autoimmunity cause ~85% of islet transplants to fail 10 years post-transplantation (Ryan et al. 2005). Furthermore, the limited availability of an adequate source of transplantable β-cells and the low number of donors still represent a critical and primary obstacle for this therapy (Halban et al. 2010).

Accordingly, a series of potentially innovative solutions have been proposed during the last years. In particular, stem-cell research had a progressive improvement in this field, exploring a variety of cells, namely, embryonic, mesenchymal, bone marrow, cord blood, adipocyte-derived, and others, for the derivation of insulin-producing cells. To date, no standard protocol has been established to obtain terminally differentiated and functional β-cells from embryonic stem cells (ESCs) and transplantation of pancreatic endocrine progenitors, which can further differentiate and mature *in vivo*, has been proposed. However, a severe limit may derive from the risk that undifferentiated cells could be potentially included among them, with a tendency to form teratomas (Kroon et al. 2008; Sui et al. 2013), severely limiting their use in human patients. The same issue can be hypothesized for induced pluripotent stem cells (iPSC), where the use of DNA-based reprogramming techniques and the introduction of oncogenic reprogramming factors could lead to insertional mutagenesis and increase risk of tumoural transformation. Multipotent stem cells, obtained from the adult pancreas and capable of β-cell neogenesis are also suggested as an alternative source of cells for pancreatic regeneration. Currently, their identification is under investigation, but the preliminary results are heavily controversial. In contrast, mesenchymal stem cells (MSCs) outside the pancreas are well-characterized and easily accessible. They can be obtained from umbilical cord blood, bone marrow or adipose tissue, highly expandable *in vitro* and can give rise to multiple cell lineages (Nombela-Arrieta et al. 2011). However, it must be noted that the involvement of MSCs in epithelial tissue regeneration and their ability to differentiate in functional pancreatic cells remains uncertain (Mathews et al. 2004).

Transdifferentiation or 'direct reprogramming' (Nicholas and Kriegstein 2010) has also been proposed as a possible option. This method is based on the conversion of differentiated cells into another phenotype without inducing a stable multipotent or pluripotent state and introducing key transcription factors, usually with the help of viral vectors. In the case of the pancreas, the transcription factors successfully used are known to regulate the major transitions during embryonic morphogenesis, and are PDX-1 (pancreatic and duodenal homeobox 1; associated with the primary transition to multipotent pancreatic progenitors) (Jonsson et al. 1994), NGN-3 (neurogenin 3; involved in the secondary transition to endocrine precursors) (Gradwohl et al. 2000) and MafA (MAF bZIP transcription factor A; implicated in the transition from immature to functional β cells) (Matsuoka et al. 2004). This approach is very promising, however, similar to some of the techniques described above, bears the limit related to the risks of insertional mutagenesis and oncogenesis, associated with the insertion of exogenous DNA.

More recently, different studies demonstrated that acinar cells can dedifferentiate in vitro to a state resembling embryonic multipotent progenitors, through the induction of endogenous gene expression, via stimulation with soluble signaling factors (Pinho et al. 2011). Several different lineages can then be derived, including β cells (Lardon et al. 2004). Yet, the general scarcity of donor organs still poses a problem for this approach, as acinar cells (unlike stem cells) have limited expansion potential.

Interestingly, although the approaches discussed above have some limits that hinder their potential use in patients, they paved the way for other possible easier and safer alternatives to translate, based on epigenetic

tools, to the clinical setting. A small-molecule-based method, able to directly convert a terminally differentiated cell into a different cell type, has been recently proposed. This new approach demonstrated that it is possible to dynamically interact with cell genotype and phenotype through the use of epigenetic modifiers, with no toxic effect and without the induction of a stable pluripotent state, that makes cells prone to transformation (Harris et al. 2011; Pennarossa et al. 2013; Brevini et al. 2014; Pennarossa et al. 2014; Mirakhori et al. 2015; Brevini et al. 2016; Chandrakanthan et al. 2016). In particular, a brief exposure to a demethylating agent, able to remove the epigenetic 'blocks' that are responsible for tissue specification (Jones and Taylor 1981; Taylor and Jones 1982; Jones et al. 1983; Jones 1985a, b; Glover et al. 1986), can push cells to a less committed state, increasing their plasticity for a short window of time, though sufficient to re-address cells towards a different phenotype (Harris et al. 2011; Pennarossa et al. 2013, 2014; Brevini et al. 2014, 2016; Mirakhori et al. 2015; Chandrakanthan et al. 2016). In agreement with these findings, we demonstrated that adult skin fibroblasts, derived from different species, namely human (Pennarossa et al. 2013), porcine (Pennarossa et al. 2014), dog (Brevini et al. 2016), cat (Brevini et al. 2018), and mouse (Pennarossa et al. 2018), can be converted towards the pancreatic lineage. This is achieved using a three-step induction protocol that allow cells to transit from the early endodermic and pancreatic differentiation stage to mature endocrine cells. At the end of the epigenetic conversion, over 30% of cells expressed the main pancreatic hormones and were able to actively release C-peptide and insulin. Similar to β-cells, they dynamically responded to 20 mM glucose stimulation as a primary and physiological stimulus for insulin secretion. These results demonstrated that the use of an epigenetic modifier provides notable advantages, compared to the alternative available procedures, and could represent a very promising tool for cell therapy of diabetes. In order to translate these very encouraging experimental findings, it was however mandatory to progress through preclinical in vivo animal studies, in order to evaluate efficacy and toxicity.

The data presented in this manuscript confirm in vivo cell functionality and demonstrate converted cell ability to restore normo-glycaemia, and stably maintain it within the physiological range, in diabetic STZ-induced SCID mice. A very interesting point is related to the results obtained when treatment was carried out in healthy non-diabetic animals. In particular, EpiCC engrafted into non-STZ treated SCID mice did not cause hypoglycemia, but, rather sensed the glucose concentration ambient, and responded in an adequate manner, suggesting cell acquisition of self-regulatory property.

Autoptic investigations and histopathological analysis showed the complete absence of malignant transformation. This finding clearly indicates that epigenetic conversion confers a steady pancreatic phenotype to human fibroblasts, which is independent of the health state and gender of the patient of origin and is stably maintained even after a 50-week engraftment period. Interestingly, no migrated cells were detected in any organ and lymph node, suggesting that cells colonize the area of injection and do not spread elsewhere, making the adoption of a containing safety device unnecessary.

In our understanding, a key aspect was represented by the establishment of the dose range that may restore normal glycemic values in diabetic individuals. Our findings point out that it is possible to gain and steadily maintain physiological levels in a cell concentration ranging from 10×10^6 to the lower dose of 0.6×10^6. The possibility to obtain a therapeutic effect with a low concentration as the latter, represents a great advantage for the overall procedure, since it requires a shorter fibroblast expansion time from patient biopsy.

In conclusion, this preclinical study demonstrates that epigenetic conversion of

dermal fibroblasts into insulin secreting cells may represent a powerful, safe, and promising approach for personalized regenerative medicine of diabetic patients.

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Conflict of Interest The authors declare no conflict of interest in relation to this article.

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Use of a PTFE Micro-Bioreactor to Promote 3D Cell Rearrangement and Maintain High Plasticity in Epigenetically Erased Fibroblasts

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Abstract
Phenotype definition is driven by epigenetic mechanisms as well as directly influenced by the cell microenvironment and by biophysical signals deriving from the extracellular matrix. The possibility to interact with the epigenetic signature of an adult mature cell, reversing its differentiated state and inducing a short transient high plasticity window, was previously demonstrated. In parallel, in vitro studies have shown that 3D culture systems, mimicking cell native tissue, exert significant effects on cell behavior and functions. Here we report the production of "PTFE micro-bioreactors" for long-term culture of epigenetically derived high plasticity cells. The system promotes 3D cell rearrangement, global DNA demethylation and elevated transcription of pluripotency markers, that is dependent on WW domain containing transcription regulator 1 (TAZ) nuclear accumulation and SMAD family member 2 (SMAD2) co-shuttling. Our findings demonstrate that the use of 3D culture strategies greatly improves the induction and maintenance of a high plasticity state.

Keywords Epigenetic erasing · High plasticity · Micro-bioreactor · PTFE · SMAD2 · TAZ

Introduction

Embryo development, gene regulation and cell specification are driven by complex epigenetic mechanisms, that lead to a gradual loss of potency [1] and a progressive restriction in cell options [2]. However, the differentiation process is reversible and it has been recently demonstrated that it is possible to interact with the epigenetic signature of an adult mature cell, switching its original phenotype into a different one [3–10]. This is achievable through biochemical and biological manipulations that are able to reactivate hypermethylated genes [11–15]. In previous studies, we used the epigenetic modifier 5-azacytidine (5-aza-CR) to interfere with DNA methylation and induce a transient high plasticity state, sufficient to allow a complete, directed differentiation of an adult mature cell into a different functional cell type [3–5, 8]. We also demonstrated that 5-aza-CR is able to decrease methylation levels through a direct ten-eleven translocation 2 (TET2)-mediated action [10], beside the well-known indirect DNA methyltransferase (DNMT) inhibition mechanism [16, 17]. The transient high plasticity state induces the transcription of pluripotency-related genes, that, however gradually decreased, even when epigenetically erased cells were maintained in embryonic stem cell (ESC) medium, and disappeared by day 6 of culture [10].

Parallel studies addressed their attention to tissue architecture and mechanical forces that are involved in the control of cell plasticity and/or differentiation. Indeed, it is known that changes in cell fate are directly influenced by cell microenvironment and biophysical signals conveyed by the extracellular matrix (ECM) [18, 19]. In vitro studies have shown that 3D culture systems, mimicking cell native tissue, exert significant effects on cell behavior, influencing cellular molecular mechanisms and related functions. Furthermore, a recent report highlighted the possibility and advantage to use a fiber

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bioreactor to increase efficiency of mesenchymal stem cells (MSC) expansion, allowing a GMP-compliant process and leading to cost effective manufacturing procedure [20].

In the present work, we describe a new method to promote 3D cell rearrangement and specifically extend and maintain the transient high plasticity window in epigenetically erased cells. To this end we use polytetrafluoroethylene (PTFE), a non-reactive hydrophobic synthetic compound, to produce an easy to generate and efficient micro-bioreactor, that permits the production of cellular microenvironment unachievable through the use of traditional 2D culture systems.

We assess whether biophysical effectors linked to 3D cell confinement may positively impact on cell plasticity, induced by epigenetic erasing. In particular, we examine if the use of the micro-bioreactor may influence global DNA methylation, boost pluripotency gene transcription and maintain long-term high plasticity. In order to better understand the mechanisms linking 3D cell rearrangement and the maintenance of high plasticity, we investigate the involvement of the Hippo signaling mechanotransduction pathway and demonstrate that 3D cell confinement induces the activation of WW domain containing transcription regulator 1 (TAZ, also known as WWTR1), which is one of the key molecule in the pathway. We then evaluate TAZ interaction with the crucial pluripotency regulator SMAD family member 2 (SMAD2) and demonstrate co-accumulation of the two molecules in the cell nuclear compartment. Furthermore, we show that TAZ knockdown causes loss of SMAD2 nuclear retention and loss of pluripotency marker transcription. Altogether, our findings indicate that the use of a micro-bioreactor and 3D cell confinement activates the Hippo signaling machinery, which, in turn, is required to maintain high plasticity.

Materials and Methods

All reagents were purchased from Thermo Fisher Scientific unless otherwise indicated.

Ethics Statement

Murine cells were isolated from 7-week-old male mice carrying Oct4–GFP (B6;129S4-Pou5f1^{tm2Jae}/J, stock #008214) obtained from Jackson Laboratory. All studies were reviewed and approved by the Ethical Committee of the University of Milan. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH).

Human cells were isolated from adult patients, after written informed consent and approved by the Ethical Committee of the Ospedale Maggiore Policlinico, Milano. All the methods in our study were carried out in accordance with the approved guidelines.

Isolation, Culture and Transfection of Dermal Fibroblasts

Murine dermal fibroblasts were isolated from skin biopsies of four Oct4-GFP transgenic mice (B6;129S4-Pou5f1^{tm2Jae}/J; Jackson Laboratory, stock #008214) [21].

Human dermal fibroblasts were established from skin biopsies of five patients.

Fragments of murine and human dermal tissue of approximately 2 mm^3 were transferred onto 0.1% gelatin (Sigma) pre-coated Petri dish (Sarstedt) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS), 2 mM glutamine (Sigma) and antibiotics. After 6 days of culture, fibroblasts started to grow out of the tissue fragments and the latter were carefully removed. Cells were maintained in the medium described above, grown in 5% CO2 at 37 °C, and passaged twice a week in a 1:3 ratio.

siRNA transfection was performed using Lipofectamine RNAi-MAX in antibiotics-free Opti-MEM® medium according to manufacturer's instructions. Validated Stealth RNAi siRNA used in the present study were specific for TAZ/WWTR1 (Human: HSS119545).

All experiments were independently repeated at least three times for each cell line derived from mouse ($n = 4$) and human patients ($n = 5$).

Treatment of Dermal Fibroblasts with 5-Aza-CR

Murine and human fibroblasts were randomly allocated in two groups: A) standard plastic dish group, and B) polytetrafluoroethylene (PTFE) group. Cells belonging to the two groups were epigenetically erased with 1 μM 5-aza-CR (Sigma) for 18 h. Concentration and time of exposure were selected according previous works [3, 22].

Group A- Standard Plastic Dish

Cells were plated in 4-well multidish (Nunc) previously treated with 0.1% gelatin (Sigma) at concentration of 7.8 X 10^4 cell/cm^2. 24 h after plating, cells were erased with 5-aza-CR as described above.

Group B- PTFE

The PTFE micro-bioreactor was created inside a Petri dish by preparing PTFE powder bed with particle size of 1 μm (Sigma 430,935). Spatula was used to gently make a curved gully at the centre of the powder bed. A micropipette was used to dispense 4 X 10^4 cells resuspended in 30 μl of 1 μM 5-aza-CR, on the PTFE powder bed. The Petri dish was then gently shaken in a circular motion to ensure that the powder particles completely covered the surface of the liquid drop. PTFE drops

were incubated in 35- mm Petri dishes at 37 °C in 5% CO_2 in air. To increase humidity and avoid dehydration, the Petri dish was placed in a larger Petri dish containing sterile water.

Culture of Epigenetically Erased Fibroblasts

At the end of the 18-h exposure, 5-aza-CR treated cells belonging to the two experimental groups (A and B) were cultured in ESC culture medium [22, 23]. More in details, group B liquid marbles were broken by puncturing with a needle. Formed organoids were recovered using a 200 μl pipette tip cut at the edge, washed in ESC medium and re-encapsulated in PTFE micro-bioreactors. Culture medium was refreshed daily until day 28, when culture was arrested.

Ultrastructural Analysis

Cells encapsulated in PTFE were collected for transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

TEM analysis samples were fixed for 2 h in 0.1 M cacodylate buffer pH 7.2, containing 2% glutaraldehyde. Specimens were then washed in the same buffer and post-fixed for 2 h with 1% osmic acid in cacodylate buffer. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica). Semi-thin sections were stained by conventional methods (crystal violet and basic fuchsin) and observed under a light microscope (Olympus). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol).

For SEM, cells were fixed and dehydrated as described above, then treated with hexamethildisilazane and mounted on polylysinated slides, air dried and subsequently covered with a 9 nm gold film by flash evaporation of carbon in an Emitech K 250 sputter coater (Emitech). Specimens were examined with a SEM-FEG Philips XL-30 microscope (Philips).

Karyotyping

Cells were cultured in medium containing Colcemid (0.1 μg/ml) for 3 h, incubated for 30 min in hypotonic solution, fixed, and stained using Giemsa (Kario MAX Giemsa). Metaphases were examined under a Leica HC microscope equipped with a digital camera Leica DC250. Images were analyzed using Leica CW4000 Karyo software.

Global Methylation Analysis

Genomic DNA was extracted using PureLink® Genomic DNA Kits following the manufacturer's instructions. DNA was converted to single-stranded DNA by incubation at 95 °C for 5 min, followed by rapid chilling on ice. Samples were then digested to nucleosides by incubating the denatured DNA with nuclease P1 for 2 h at 37 °C in 20 mM sodium acetate (pH 5.2). Alkaline phosphatase was added and incubated for 1 h at 37 °C in 100 mM Tris (pH 7.5). After centrifugation, the supernatant was used for ELISA assay using Global DNA Methylation ELISA Kit (5′-methyl-2′-deoxycytidine Quantitation; CELL BIOLABS) according to the manufacturer's protocol.

Gene Expression Analysis

RNA was extracted using the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. Quantitative PCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) using predesigned gene-specific primers and probe sets from TaqManGene Expression Assays, listed in Table 1. *GAPDH* and *ACTB* were used as internal reference genes. CFX Manager software (Bio-Rad Laboratories) was used for target gene quantification.

Western Blot Analysis

Cells were lysed and constitutive proteins were extracted using a ReadyPrep Protein Extraction Kit (Bio-Rad). Nuclear extracts from the cells were isolated using the NXtract CelLytic NuCLEAR Extraction Kit (Sigma). Protein concentration was assessed by Coomassie Blue-G Dye-binding method. 100 μg of proteins were resuspended in sample buffer (1:1) consisting of 4% (wt/vol) SDS, 10% 2-mercaptoethanol, 20% (wt/vol) glycerol, 0.004% bromophenol blue, and 0.125 M Tris–HCl at pH 6.8. Equal amounts of total protein were loaded and electrophoresed on a SDS-polyacrylamide gels. Proteins were then transferred onto 0.45 μm pore size nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences) and probed with primary antibodies listed in Table 2. Protein bands were visualized by the WesternBreeze chemiluminescent kit. Densitometric analysis was performed with Quantity One 1-D analysis software (Bio-Rad).

Statistical Analysis

Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant.

Table 1 List of primers used for quantitative PCR analysis

Gene	Description	Species	Catalog No.
ACTB	Actin beta	Human	Hs01060665_g1
Actb	Actin beta	Mouse	Mm02619580_g1
CDH1	Cadherin 1	Human	Hs01023895_m1
EPCAM	Epithelial cell adhesion molecule	Human	Hs00901885_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Human	Hs02786624_g1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mouse	Mm99999915_g1
NANOG	Nanog homeobox	Human	Hs02387400_g1
Nanog	Nanog homeobox	Mouse	Mm02019550_s1
OCT4	POU class 5 homeobox 1	Human	Hs00999632_g1
Oct4	POU class 5 homeobox 1	Mouse	Mm03053917_g1
REX1	ZFP42 zinc finger protein	Human	Hs00399279_m1
Rex1	ZFP42 zinc finger protein	Mouse	Mm03053975_g1
SOX2	Sex determining region Y-box 2	Human	Hs01053049_s1
Sox2	Sex determining region Y-box 2	Mouse	Mm03053810_s1
TET2	Tet methylcytosine dioxygenase 2	Human	Hs00325999_m1
THY1	Thy-1 cell surface antigen	Human	Hs00174816_m1

Results

The PTFE Micro-Bioreactor Promotes 3D Cell Rearrangement and Maintain High Plasticity in Epigenetically Erased Murine Fibroblasts: Preliminary Studies

Preliminary experiments were carried out using fibroblast primary cell lines, isolated from skin biopsies of four Oct4-GFP transgenic mice (B6;129S4-Pou5f1tm2Jae/J; Jackson Laboratory, stock #008214) [21]. After encapsulation in PTFE and 5-aza-CR exposure, cells formed 3 dimensional (3D) spherical structures (Fig. 1a) and became GFP positive (Fig. 1b), indicating the onset of *Oct4* gene expression. High plasticity state achievement was also confirmed by gene expression analysis. Indeed, the morphological changes were accompanied by the expression of pluripotency-related genes, namely POU class 5 homeobox 1 (*Oct4*), Nanog homeobox (*Nanog*), Zfp42 zinc finger protein (*Rex1*), and sex determining region Y-box 2 (*Sox2*), which were undetectable in untreated fibroblasts (T0, Fig. 1c). Interestingly, GFP positivity as well as pluripotency gene transcription were high for the entire length of the experiments.

The PTFE Micro-Bioreactor Promotes 3D Cell Rearrangement in Epigenetically Erased Human Fibroblasts

Human dermal fibroblasts obtained from five patients displayed a dramatic change in their morphology, when exposed to 5-aza-CR, regardless of the culture system used. In particular, fibroblasts plated on standard plastic dishes (Group A) as well as those encapsulated in PTFE (Group B) became rounded, with large and granulated nuclei (Fig. 2a). However, while Group A cells retained a monolayer distribution for the entire length of the experiments, cells encapsulated in PTFE (Group B) formed 3D spherical structures (Fig. 2b), that were stably maintained for the entire length of the experiments.

The PTFE Micro-Bioreactor Induces Ultrastructural Modifications in Epigenetically Erased Human Fibroblasts

The fine structure of cells encapsulated in PTFE was analyzed by SEM and TEM starting from T0, after 5-aza-CR exposure and up to 28 days. 5-aza-CR treatment induced changes in

Table 2 List of antibodies used for immunoblotting studies

Antibody	Description	Company	Cat. No.	Application	Dilution
Anti-β-Actin	Rabbit polyclonal Immunogen: Synthetic human peptide	Abcam	AB8227	WB	1:1000
Smad2 Antibody	Rabbit polyclonal Immunogen: Human protein	Novus Biologicals	NB100–56462	WB	1:500
TAZ/WWTR1 Antibody	Rabbit polyclonal Immunogen: Synthetic human peptide	Novus Biologicals	NB110–58359	WB	1:5000

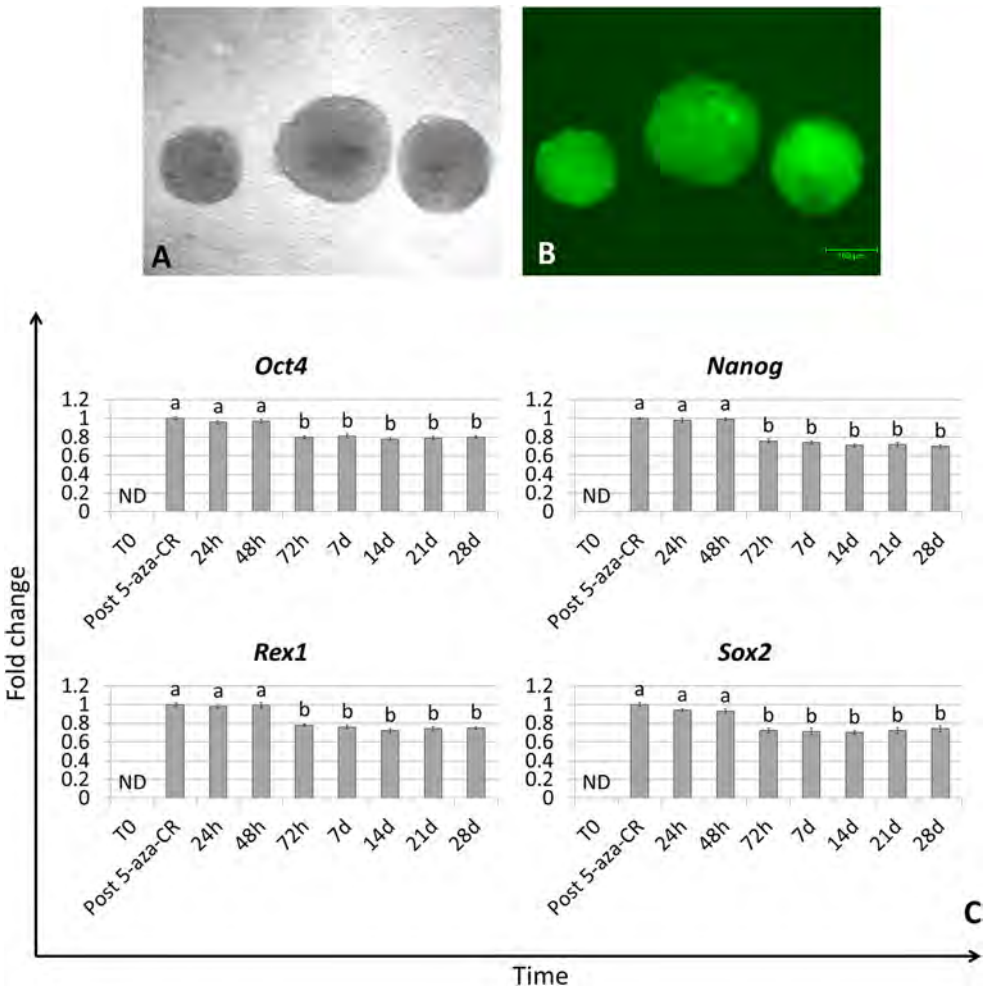

Fig. 1 The PTFE micro-bioreactor promotes 3D cell rearrangement and maintains high plasticity in epigenetically erased Oct4-GFP murine fibroblasts. (a) Cells encapsulated in PTFE and exposed to 5-aza-CR for 18 h formed 3D spherical structures, that were stably maintained for the entire length of the experiments (Scale bar, 100 μm). **(b)** Fibroblasts became GFP positive, indicating the onset of *Oct4* gene expression (Scale bar, 100 μm). **(c)** Gene expression profiling of pluripotency-related genes (*Oct4*, *Nanog*, *Rex1*, and *Sox2*) in untreated fibroblasts (T0), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR) and at different time points of culture. Gene expression levels are reported with highest expression set to 1 and all other times relative to this. Different superscripts denote significant differences ($P < 0.05$)

morphology. Cells lost their characteristic spindle shape (Fig. 2d, e) and became ovoidal (Fig. 2f, g), showing a cytoplasm filled with autophagic vacuoles (Fig. 2g).

Starting from 24 h after 5-aza-CR treatment and for the entire length of the experiments, cells within 3D structures maintained a roundish shape with a high nucleus to cytoplasm ratio, few organelles and large intercellular spaces (Fig. 2h, i). Their cytoplasm contained free ribosomes, polyribosomes, elongated tubular mitochondria, well-developed rough endoplasmic reticulum (RER), Golgi complexes, few reticulum cisternae and lipid droplets. Furthermore, cells showed nuclei with euchromatin and large reticulated nucleoli that are typical of ESC [24]. Occasionally, scattered cells with cytoplasm occupied by large autophagic vacuoles were visible (Fig. 2i).

The PTFE Micro-Bioreactor Enhances the Demethylating Effect of 5-Aza-CR in Epigenetically Erased Human Fibroblasts

5-aza-CR caused a significant decrease in global DNA methylation in fibroblasts belonging to the two experimental Groups (Fig. 3a). However, Group B cells exhibited significantly lower DNA methylation levels compared to those of Group A, at all time points analyzed, and remained significantly hypomethylated for the entire length of the experiments. In contrast, cells plated on plastic dishes (Group A), although displayed a decreased methylation for 72 h, slowly increased and returned comparable to untreated fibroblasts (T0) by day 7 of culture.

Interestingly, cells maintained a normal karyotype throughout the entire length of the experiments (Fig. 2c), indicating that methylation changes did not cause chromosome copy number variations or cytotoxic effects.

The PTFE Micro-Bioreactor Boosts Pluripotency Gene Transcription and Maintains Long-Term High Plasticity in Epigenetically Erased Human Fibroblasts

Morphological changes and methylation decrease were accompanied by the onset of the expression of pluripotency-related genes, namely POU class 5 homeobox 1 (*OCT4*), Nanog homeobox (*NANOG*), ZFP42 zinc finger protein (*REX1*), and sex determining region Y-box 2 (*SOX2*), which were undetectable in untreated

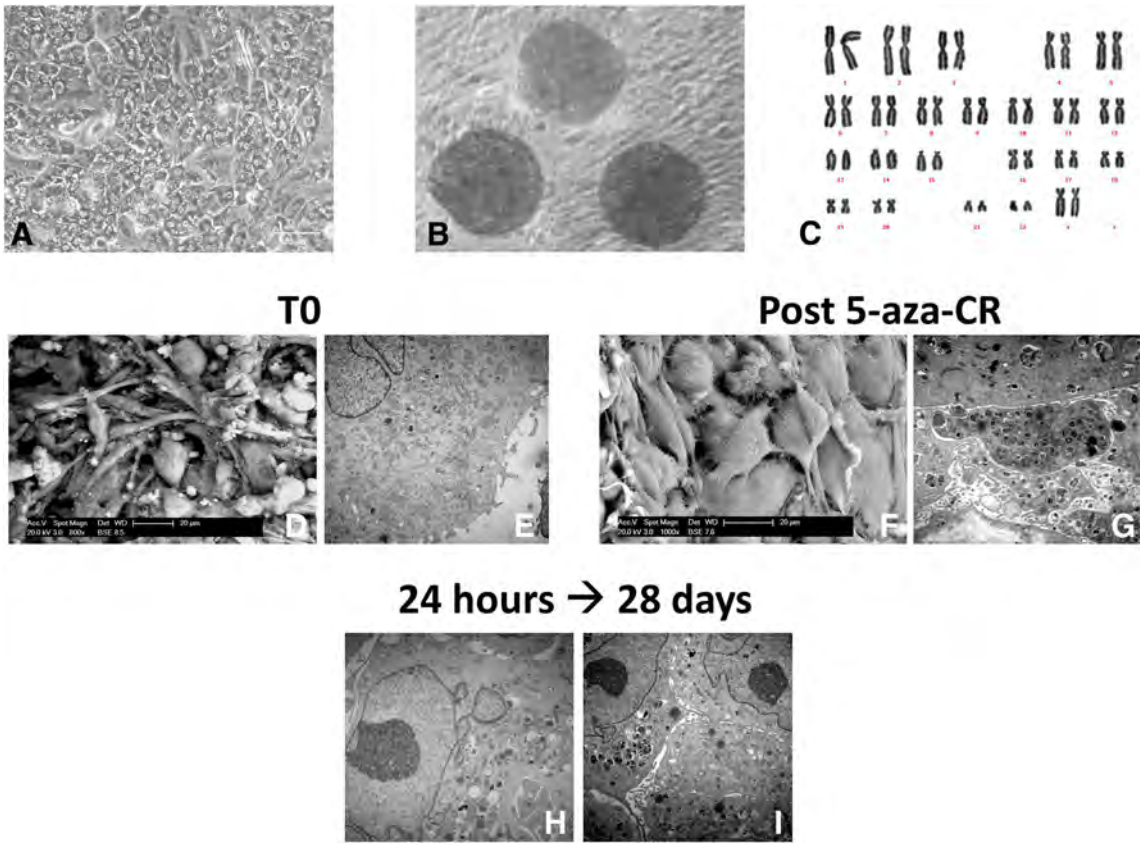

Fig. 2 The PTFE micro-bioreactor promotes 3D cell rearrangement and induces ultrastructural modifications in epigenetically erased human fibroblasts. (a) After 5-aza-CR incubation, fibroblasts plated on plastic dishes (Group A) changed their typical elongated shape into a round epithelioid aspect and retained a monolayer distribution. Cell size was smaller, and nuclei became larger and granular. (Scale bar, 100 μm). **(b)** Cells encapsulated in PTFE (Group B) and treated with the demethylating agent formed 3D spherical structures, that were stably maintained for the entire length of the experiments (Scale bar, 200 μm). **(C)** Cells maintained a normal karyotype. **(d-e)** Untreated fibroblasts displayed a spindle shaped morphology. **(f-g)** Cells encapsulated in PTFE and subjected to 5-aza-CR treatment became ovoidal with autophagic phenomena. **(h-i)** They showed a roundish shape, high nucleus to cytoplasm ratio, nuclei with euchromatin and large reticulated nucleoli, few organelles, and large intercellular spaces for the entire length of the experiments

fibroblasts (T0; Fig. 3b). Group B cells showed significantly higher expression levels of these genes, when compared to Group A. Furthermore, encapsulation in PTFE (Group B) maintained high expression levels for the entire length of the experiments. In contrast, Group A cells transcribed for pluripotency-related markers until 72 h and turned down their expression by day 7 of culture.

5-aza-CR treatment also induced the up-regulation of *TET2*, epithelial cell adhesion molecule (*EPCAM*), and cadherin 1 (*CDH1*) genes in both groups (Fig. 3b). More in detail, Group B cells showed significantly higher expression levels of these genes, compared to those of Group A. Furthermore, *TET2*, *EPCAM*, and *CDH1*, expression profile paralleled that described above for pluripotency- related genes.

In agreement with these observations, we detected a significant downregulation of a fibroblast specific gene (Thy-1 cell surface antigen, *THY1*) in Group B cells for the entire length of the experiments. Group A cells showed, in contrast, decreased levels for the first 72 h of culture, and returned to values comparable to those of untreated fibroblasts by day 7 (Fig. 3b).

The PTFE Micro-Bioreactor Activates the Hippo Signaling Pathway to Maintain High Plasticity in Epigenetically Erased Human Fibroblasts

Fibroblasts exposed to 5-aza-CR (Post 5-aza-CR) showed TAZ nuclear accumulation both in cells plated onto plastic plates (Group A; Fig. 4 A) and in those encapsulated in PTFE (Group B; Fig. 4b). TAZ nuclear confinement was however lost by Group A cells, with the molecule relocating to the cytoplasm, by day 7 of culture (Fig. 4a). In contrast, 3D cell confinement of Group B cells encouraged TAZ nuclear retention, that was stably maintained for the entire length of the experiments (Fig. 4b).

Interestingly, TAZ localization was mirrored by a parallel compartmentalization of SMAD2. More in detail, cells displaying nuclear localized TAZ showed SMAD2 nuclear

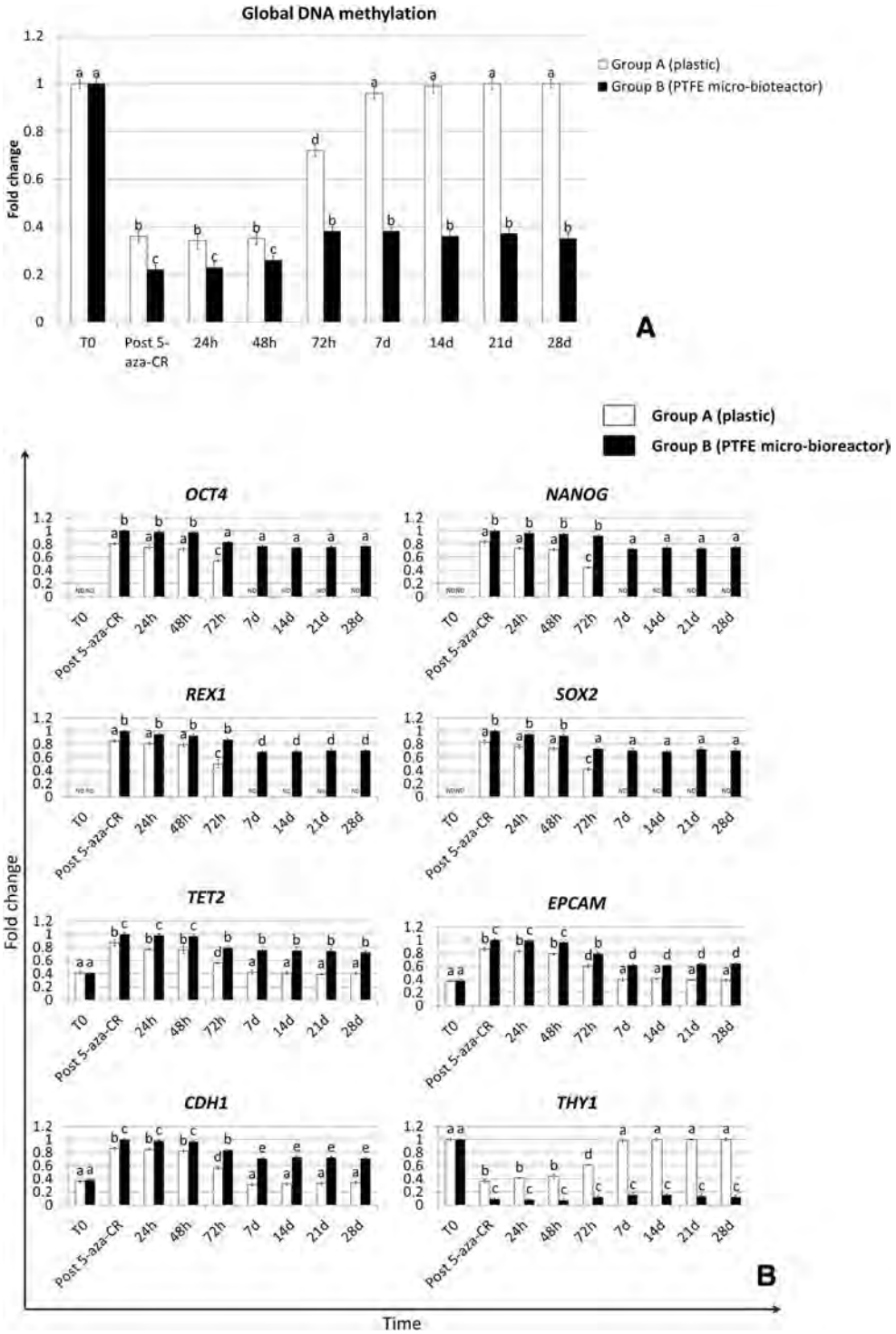

Fig. 3 The PTFE micro-bioreactor enhances the demethylating effect of 5-aza-CR, boosts pluripotency gene transcription and maintains long-term high plasticity in epigenetically erased human fibroblasts. (a) Global DNA methylation levels of cells plated on standard plastic dishes (Group A) or encapsulated in PTFE (Group B), exposed to 5-aza-CR (Post 5-aza-CR) and cultured in ESC medium. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P < 0.05$). **(b)** Gene expression changes in epigenetically erased fibroblasts plated on standard plastic dishes (Group A) or encapsulated in PTFE (Group B). Expression pattern of pluripotency-related genes (*OCT4*, *NANOG*, *REX1*, and *SOX2*), ten-eleven translocation family member *TET2*, MET markers (*EPCAM*, *CDH1*) and fibroblast specific marker (*THY1*). Gene expression levels are shown for untreated fibroblasts (T0), fibroblasts exposed to 5-aza-CR (Post 5-aza-CR), and at different time points of culture. Values are reported with highest expression set to 1 and all other times relative to this. Different superscripts denote significant differences (P < 0.05)

accumulation, while cells with cytoplasmic TAZ exhibited SMAD2 cytoplasmic distribution (Fig. 4a, b).

In addition, siRNA knockdown of TAZ caused inhibition of SMAD2 nuclear accumulation (Fig. 4c) as well as the loss of pluripotency marker transcription (Fig. 4d), suggesting the direct involvement of TAZ in SMAD2 shuttling and high plasticity maintenance, boosted by the PTFE 3D microenvironment.

Discussion

The results obtained in the present study demonstrate that the use of the PTFE micro-bioreactor is able to induce significant morphological changes, 3D cell rearrangements and to enhance the acquisition and maintenance of high plasticity in 5-aza-CR exposed cells. These observations extend previous

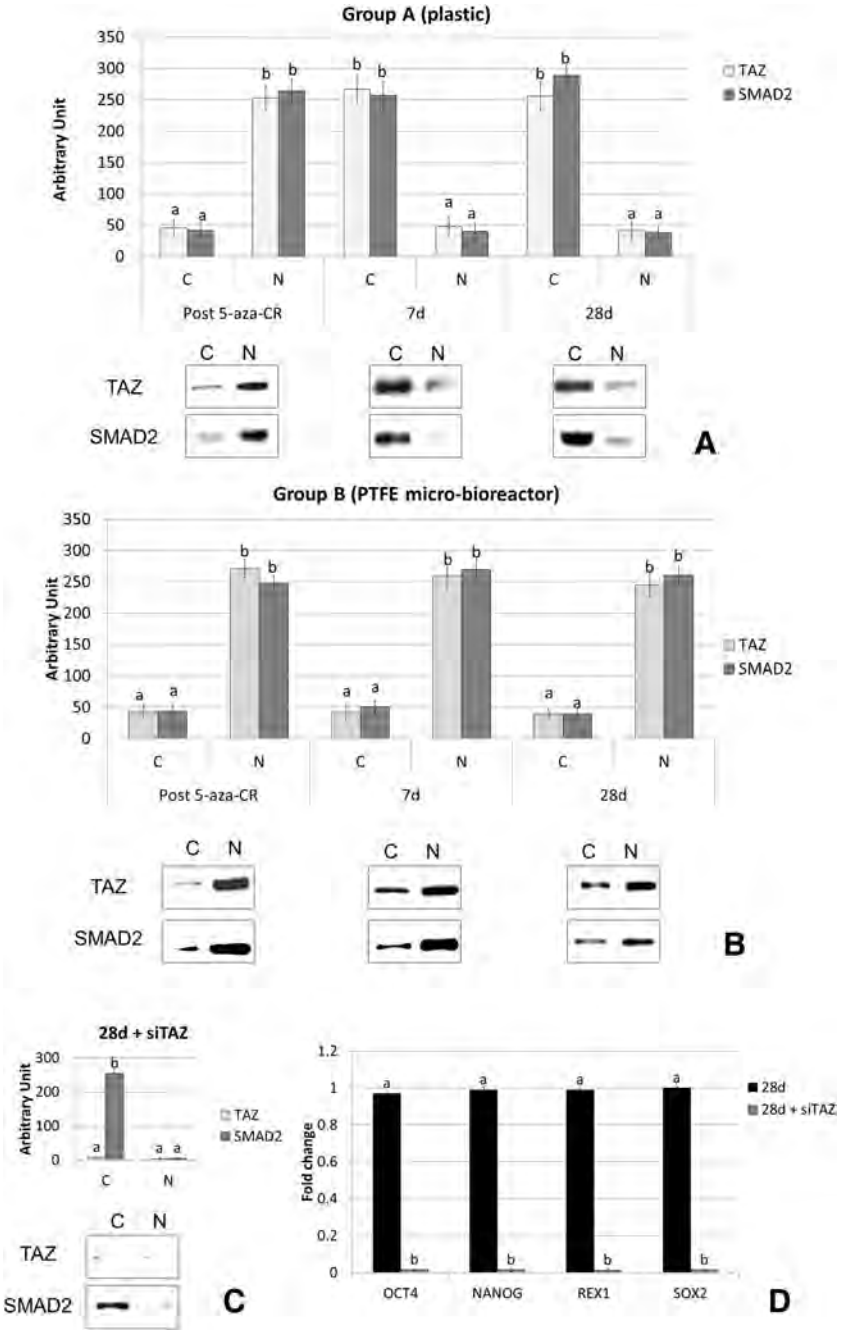

Fig. 4 Activation of the Hippo signaling pathway in PTFE encapsulated cells. (a) Western blots for TAZ and SMAD2 proteins in epigenetically erased fibroblasts, plated on standard plastic dishes (Group A). The densitometry results (arbitrary units) of the Western blots are shown as a bar graph. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P < 0.05$). Representative Western blots for each protein are also shown. **(b)** Western blots for TAZ and SMAD2 proteins in epigenetically erased fibroblasts, encapsulated in PTFE (Group B). The densitometry results (arbitrary units) of the Western blots are shown as a bar graph. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P < 0.05$). Representative Western blots for each protein are also shown. **(c)** Western blots for TAZ and SMAD2 proteins in PTFE encapsulated cells at day 28 of culture, after TAZ siRNA transfection (28d + siTAZ). The densitometry results (arbitrary units) of the Western blots are shown as a bar graph. Bars represent the mean ± SD of three independent experiments with five independent biological replicates. Different superscripts denote significant differences ($P < 0.05$). Representative Western blots for each protein are also shown. **(d)** Pluripotency-related gene (*OCT4*, *NANOG*, *REX1*, and *SOX2*) expression levels in Group B cells at day 28 of culture (28d) and after TAZ siRNA transfection (28d + siTAZ). Values are reported with highest expression set to 1 and all other times relative to this. Different superscripts denote significant differences ($P < 0.05$)

evidences indicating that 3D microenvironment may have a profound influence on cell phenotype and plasticity [25–28].

After treatment with the epigenetic eraser, both cells plated onto plastic plates (Group A) and those encapsulated in PTFE (Group B) displayed significant changes, compared to untreated fibroblasts (T0). In particular, the typical fibroblast elongated morphology, was replaced by a round or oval shape. Cells belonging to the two Groups became considerably smaller in size, with larger and granulated nuclei, acquiring the typical morphological features described for pluripotent cells [29, 30]. However, while Group A cells retained a monolayer distribution for the entire length of the experiments, the use of the micro-bioreactor allowed cells to self-assemble and form multicellular spheroids, displaying a uniform size geometry. This is consistent with previous studies indicating that the use of PTFE is able to efficiently encourage cell aggregation, facilitating the formation of embryoid bodies (EBs) from murine ESC [31] or the establishment of olfactory ensheathing cell (OEC) spheroid structures [32].

Ultrastructural analysis demonstrated that cells in the 3D spherical structures showed significant intercellular spaces, high nucleus to cytoplasm ratio, nuclei containing euchromatin and large reticulated nucleoli. Cytoplasm was characterized by the presence of free ribosomes, polyribosomes, elongated tubular mitochondria, well-developed rough endoplasmic reticulum (RER), Golgi complexes, few reticulum cisternae and lipid droplets. These features resemble those described for human ESC [24] and induced pluripotent cells (iPS) [30], that have been shown to display large intercellular spaces, accessible euchromatin and morphology typical of undifferentiated cells, reminding of inner cell mass (ICM) cells of blastocysts [24, 29, 30, 33–36]. These observations suggest that the use of the PTFE micro-bioreactor encourages not only cell aggregation, but also boosts the formation and stable maintenance of morphological properties previously described in pluripotent cells.

Incubation with the epigenetic eraser significantly modified global DNA methylation. Indeed, cells belonging to both the two experimental Groups showed a significant decrease in methylation levels, compared to the starting cell population (T0). However, while Group A cell methylation returned comparable to untreated fibroblasts (T0) by day 7 of culture, PTFE encapsulated cells (Group B) remained significantly hypomethylated for the entire length of the experiments. Methylation changes have been previously described during fibroblast reprogramming [36–38], where the hypomethylated state is due to the effect of a direct and active demethylation mechanism controlled by TET proteins [38]. Indeed, it has been demonstrated that TET enzymes play an essential role in ESC pluripotency maintenance [39, 40] and are upregulated during mesenchymal to epithelial transition (MET) as well as during somatic cell reprogramming, affecting the establishment of the open chromatin and contributing to the reactivation of endogenous pluripotency genes [41]. The fundamental functions exerted by TET factors in iPS generation were also confirm by experiments demonstrating inability of *TET*-deficient fibroblasts to complete reprogramming processes [42]. In line with these observations, our results show that epigenetic erasing lead to an increased expression of the ten-eleven translocation family member *TET2*. This was accompanied by the onset of the pluripotency-related genes, *OCT4*, *NANOG*, *REX1*, and *SOX2*, as well as the up-regulation of *EPCAM*, and *CDH1* genes, confirming and expanding previous studies carried out in our laboratory [3–5, 10]. These changes in gene expression were detected in both experimental Groups. However, as described in methylation study, Group A cells returned to values comparable to those of untreated fibroblasts by day 7 of culture, while those encapsulated in PTFE (Group B) displayed high expression levels for the entire length of the experiments. These results are also well in line with the morphological observations and suggest that the acquisition of a high plasticity phenotype is paralleled by the concomitant decrease of fibroblast specific marker, *THY1*, the onset of pluripotency-related genes (*OCT4*, *NANOG*, *REX1*, and *SOX2*), and the upregulation of key MET markers (*EPCAM*, *CDH1*). Interestingly, these changes were promoted and stably maintained by the use of the PTFE micro-bioreactor, suggesting that 3D cell confinement boosts pluripotency gene transcription and maintains long-term cell plasticity.

A common denominator of all the experiments here described is the use the PTFE micro-bioreactor to generate a 3D cell confinement that favors the induction and maintenance of cell plasticity. In order to better understand the mechanisms associated, we investigated the possible involvement of mechanotransduction-related signaling pathways. Our results demonstrated that the morphological and molecular changes described in the previous paragraphs, were accompanied by the activation of the Hippo-signaling pathway with distinctive modifications in TAZ localization. In particular, after 5-aza-CR treatment, TAZ protein displayed a nuclear accumulation both in Group A and B. This localization was however lost by Group A cells, with TAZ molecule relocating to the cytoplasm, by day 7 of culture. In contrast, 3D cell confinement of Group B cells encouraged TAZ nuclear retention, that was stably maintained for the entire length of the experiments. This is consistent with a recent study showing a clear TAZ nuclear localization in large spheroids and organoid-like structures [43]. In our experiments, TAZ localization was mirrored by a parallel compartmentalization of SMAD2. In particular, we observed that cells displaying nuclear localized TAZ showed concomitant SMAD2 nuclear accumulation, while cells with cytoplasmic TAZ, also exhibited SMAD2 cytoplasmic distribution. This evidence is in line with previous reports that indicate a direct interaction between the transcriptional cofactor TAZ and SMAD proteins, where TAZ defines a hierarchical system, regulating SMAD

complexes shuttling and coupling to the transcriptional machinery [44, 45]. Interestingly, Panciera et al. showed that transient expression of exogenous TAZ in terminally differentiated cells can induced conversion to a progenitor cell state [46] and that the loss of TAZ expression induces failure of SMAD nuclear accumulation, disappearance of *OCT4* and subsequent differentiation [44]. Consistent with this findings, our results showed that siRNA knockdown of TAZ, with the concomitant failure of SMAD nuclear accumulation, caused loss of pluripotency marker transcription in PTFE encapsulated cells. Base on this, we speculate that the activation of the Hippo-signaling pathway induced by the PTFE micro-bioreactor and the related TAZ-dependent SMAD shuttling may represent the base of the maintenance of high plasticity in encapsulated cells.

In conclusion, our findings demonstrated that the use of the PTFE micro-bioreactor boosts the induction and maintenance of high plasticity state in epigenetically erased cells. This model system could provide a novel in vitro culture technique that induces distinctive 3D cell rearrangement and specific cell-to-cell interactions. We are convinced that the PTFE micro-bioreactor may represent a notable advance in stem cell organoid technology and may constitute an advantageous micro-environment for long-term culture of different cell types, from epigenetically erased cells, to ESC and iPSC, as well as MSC.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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