

Genomic-Epigenomic Signaling Pathways Changes in Cellular Differentiation Process

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Abstract Cellular differentiation is a highly complex process and we need a deeper understanding of their mechanisms. Reprogramming somatic cells follows the inverse order to the physiologic differentiation process. Reprogramming somatic cells may be used as a simplistic model to understand the cellular differentiation process. The generation of induced pluripotent stem cells (iPSCs) requires going along through a complex network of genetic and epigenetic pathways. Dedifferentiation from somatic cells to iPSCs involves multiple genetic-epigenetic signaling pathways to obtain high levels of plasticity, self-renewal, motility and loss of specialized cellular functions. Eleven main signaling pathways have been involved in cell fate control and embryonic patterning. Extensive crosstalk among epigenetic pathways modifies DNA, histones and nucleosomes which make up the epigenetic mechanisms of gene regulation in differentiation and reprogramming processes.

Keywords: cellular differentiation process, reprogramming, genetic and epigenetic mechanisms

1. Introduction

New knowledge has been unveiled about differentiation cellular programs, particularly in eukaryotic gene expression regulation.

Intracellular genetic expression dynamics and their regulatory mechanisms in different enhancement of transcription conditions have been better known recently. Changes in cellular expression signatures correspond to specific cellular phenotypes. 2600 transcription factors and additional proteins such as coactivators, chromatin remodelers, histone acetylases, deacetylases kinases and methylases are playing crucial roles in gene regulation. These complex biomolecules group bind to either enhancer or promoter regions of DNA adjacent to the genes that they regulate. They bind to the DNA and help to initiate a program of increased or decreased gene transcription. In eukaryotes, DNA must be accessible to transcriptional factors and the nucleosome should be actively removed by molecular motors such as chromatin remodelers. Different transcription factors and additional proteins drive the basal transcriptional regulation and the differential enhancement expression. This last group is critical to ensure that genes are expressed in the right cell at the right time and in the right amount.

Many transcription factors and cofactors in multicellular organisms are involved in development, these turn on/off the transcription of appropriate genes, which, in turn, allow changes in cell growth/apoptosis and cellular differentiation [1].

Different cellular differentiation models contain scarce information of epigenetic mechanism control of enhanced expression. Embryonic stem cells and progenitor cells have been the classic model to understand the mechanisms regulating cell differentiation [2]. Transdifferentiation of somatic cells as the four types of dendritic cells and the multiple types of effector and memory CD8+ T cells shows relevant advances in signaling pathways and transcriptional programmes comprehension [3,4]. Recently, the human induced pluripotent stem cells model has obtained a great deal of genetic/epigenetic clues of the cellular differentiation process. In this model, a differentiated cell is converted directly into a pluripotent stem cell. Induced pluripotent stem cells can be driven through steps similar to those that occur during embryonic development [5].

The cellular differentiation process involves genetic and epigenetic signaling pathways related with the intracellular expression dynamics. Important epigenetic cell signaling pathways have been unveiled in the last years. The human induced pluripotent stem cell model identifies genetic and epigenetic instability to understand phenotype changes related with self-renewal, proliferation and motility [6,7]. Although this model is the reverse of the differentiation process, enough biological information has been accumulated to increase our understanding of this crucial cellular process.

Human pluripotent stem cells have the capacity to give rise to all somatic cell types. Alternatively, human induced pluripotent stem cells (iPSCs) have been reprogrammed from somatic cells. Different genomic, epigenomic and signaling pathways in reprogramming somatic cells correspond to gain cellular functions for pluripotency and

to lose specialized cellular functions [5,6,7]. In cellular reprogramming, somatic cells acquire stable spatio-temporal genomic, epigenomic and signaling pathways features of pluripotent stem cells. Here, reprogramming process is used as a simplistic model to understand the main genetic and epigenetic changes in cellular differentiation process.

2. Genetic and Epigenetic Changes in the Reprogramming Cell Process

iPSCs show high level of plasticity, renewal, motility; low level of apoptosis and loss of specialized cellular functions. iPSCs also present asymmetric division (Table 1). Asymmetric division is regulated by maintaining the stem cell orientation, and this is regulated by its spatial relationship with cells of the niche. In stem cells, loss of polarity leads to impairment of asymmetric cell division, altering cell fates, rendering daughter cells unable to respond to the mechanisms that control proliferation. Specific genetic and epigenetic changes are related with these iPSCs phenotype modifications. We point out the similarities and differences that have been observed between embryonic stem cells (ESC)/iPSCs and somatic cell types.

The most frequent changes that accumulate in ESC/iPSCs in low-resolution karyotypic analyses were gain (amplifications) of chromosomes 12, 17 and, to a lesser extent, X. These alterations are also common in germ cell tumors. Furthermore, less-frequent recurrent amplifications that were observed in both cells types are gains of chromosomes 8, 12p, i(20)q10 and X. Trisomy of chromosome 12 is the predominant abnormality. Nevertheless, most of the pluripotent stem cell lines maintain the normal karyotype. Most focal alterations in high-resolution studies are copy number variants (CNVs) and loss-of-heterozygosity changes. The most recurrent CNV hotspot, amplification of gene-rich locus at 20q11.21 (observed in around 15% of iPSC lines) contains the antiapoptotic gene BCL2-like 1, the pluripotency associated gene inhibitor of DNA binding I, dominant negative helix-loop-helix protein, and the DNA methyltransferase 3B. Other recurrent duplications CNVs spanning genes associated with pluripotency are NANOG homeobox pseudogene1 locus, the pluripotency associated transcription factor SRY box 2 (SOX2), transforming growth factor- β signaling regulators and MYC. The number of CNVs and mutations has been reported to be higher in iPSCs than in the corresponding fibroblasts from which the iPSCs were derived [8]. Also, reprogramming was associated with deletions in tumour-suppressor genes (Table 2).

Specific transcription factors interface with the genome and induce changes in cellular identity in the context of reprogramming [7]. Some transcriptional factors work as master regulators in the cellular dedifferentiation process. Human genes related to the cellular differentiation process can be classified in pluripotency genes, differentiation genes and housekeeping genes (e.g. GAPDH, 18SrRNA). Genes used to induce reprogramming, dedifferentiation or transdifferentiation correspond to transcription factors (such as OSMK, *Gata 4*, *Lin28*, *Mafa*, *Mef2c*, *Myc*, *Nanog*, *Ngn3*, *Rb1*, *Tbx5*), chromatin modulators (*Baf60c*, *Rb1*),

protein kinase (*p38 mapk*), protein kinase inhibitor (*Arf*), and growth factor (*Fgf1*) [9]. The key transcription factors of pluripotency form positive reciprocal and autoregulatory loops that maintain the expression of OCT4, SOX2 and NANOG. The key factors also synergistically co-occupy numerous downstream target genes that promote self-renewal and maintenance pluripotency, while repressing developmentally regulated genes that drive differentiation. Examples of pluripotency genes are OCT4, SOX2, NANOG, Gdf3, Esrrb, Zic3, Salt4, Rift, Jarid2, Stat3, cell surface antigens markers (Tra-1-60, Ssea-4). Examples of differentiation genes are Cdx2, Dkk1, Hand1, Otx1, Gata6, HoxB1, Pax6, Afp, Sox17, ectodermal lineage markers [10,11,12]. The transcription factors OCT4, SOX2, NANOG and MYC are among the pluripotency master regulators.

Table 1. Main phenotypes changes during the somatic cells reprogramming to iPSCs

Phenotypes changes	Somatic cells	iPSCs
Somatic programme regulation (SPR) ¹	↑	↓
Epigenetics changes	↓	↑
DNA damage response	↓	↑
Global genome reorganization	↓	↑
Metabolism shift (glycolysis)	↓	↑
Cell stress and senescence	↑	↓
Genetic changes	↓	↑
G1 cell cycle phase	Extended	Short
Cell morphology	Specialized	Non specialized
Mesenchymal-to-epithelial transition	↓	↑
Lineage conversion	↓	↑
Pluripotent programme regulation (PPR) ¹	↓	↑
Stem cell self renewal	↓	↑
Asymmetric cell division	↓	↑
Rejuvenation	↓	↑

Each one of main phenotypes are related to specific subphenotype changes such as overproduction of specific patterns of gene expression, signaling pathways, suborganelle and organelle changes; i.e preferential-transit signaling pathways and production of biomolecules-specialized functions in SPR1 and PPR1

Reprogramming cells to a pluripotent state entails global epigenetic remodeling and introduces epigenetic changes, some of which are necessary for reprogramming to occur. A failure to demethylate pluripotency genes is associated with reprogramming in iPSCs. Other epigenetic gene expression regulations modify histones, nucleosome packaging and rearrangement, higher-order chromatin structures and the dynamic interplay between chromatin and the nuclear lamina. Increasing evidence suggest the existence of extensive crosstalk epigenetic pathways that modify DNA, histones and nucleosomes. Gaining insights into the spatiotemporal regulation of epigenetic pathways in pluripotent stem cells will not only further our understanding of human development and diseases, but also guide our effort to better manipulate these cells in the clinical setting [13].

Table 2. Main genomic and epigenomic changes during somatic cell reprogramming to iPSCs

Genetic reprogramming
Repression of differentiation genes by specific- TF
Activation of developmental genes by specific-TF
Occasional amplifications of chromosomes 12,8, X
Focal alterations as copy number variants (i.e. 20q11.21) and loss-of-heterozygosity
Deletions in tumor-suppressor genes (i.e. p53, cyclin-dependent kinase inhibitors p16 ^{INK4} and p21 ^{CIP1})
Epigenetic reprogramming (somatic genes silencing and pluripotency genes expressing)
Chromatin modifications: post-transcriptional histone changes, Polycomb Repressive Complex
Chromatin modifications: post-transcriptional regulation through non-coding RNAs (microRNAs)
Chromatin remodeling around the transcriptional start site: H3K27me3, H3K4me3 (repressive marks)
Chromatin plasticity: converting to an open hyperdynamic chromatin
DNA methylation at pluripotency promoters-genes

3. Extrinsic and Intrinsic Signaling in Cellular Differentiation Process

In cellular differentiation process, cells that produce the signals are “inducing cells”, and the receiving cells are “responders”. Plasticity or competence is the ability of cells to respond to the inducers, usually reflects the presence of a receptor at the top of a pathway that regulates the expression of specific transcription factors in the responding cells. The responding cells, in turn, may become inductive and change the fate of their neighbors by producing new signals. Eleven signaling pathways have been involved in development, they are identifiable by the ligand or signal transducers involved: Notch, FGF, EGF, Wnt/Wingless, Hedgehog, transforming growth factor β /BMPs, cytokine pathway (nonreceptor tyrosine kinase JAK-STAT), Hippo, Jun kinase, NF-kb, and retinoic acid receptor. These pathways involve either juxtacrine signaling or cell-to-cell contact via surface proteins (Notch and Hippo) and paracrine signaling or secreted diffusible growth and differentiation factors (other nine pathways) [2]. The response to signaling-pathways activation is usually complex and involves the regulation of many processes such as control of cell fate, apoptosis, cell proliferation, cytoskeletal reorganization, cell polarity, adhesion and cell migration. Many pathways activate feedback loops that modulate or terminate the incoming signal.

Changes in gene expression rely, in part, on the activation of signaling pathways by cell-cell communication. Juxtacrine signaling such as Notch regulates a broad range of cellular processes in organisms. Notch signaling is a simple linear pathway with no amplification step, but Notch has different modes of action, depending on the cell context: lateral inhibition, lineage decisions and inductive signaling. Notch signaling operates in lateral inhibition roles for a single cell within a group of equivalent cells, and as a control of lineage decisions and inductive signaling among non-equivalent cells (cells asymmetrically express regulators of the Notch pathway). The distribution of ligands (mode of transport, endocytosis, interaction with heparin-sulfate proteoglycans) in paracrine signaling such as EGF and FGF has diverse distribution and can generate a graded

signaling profile. These morphogen gradients provide spatial information and generate different cell types in a distinct spatial order.

Many of the mechanisms underlying cell-type specification and formation of distinct tissues rely on interactions between signaling pathways. Often the activation of one pathway leads to activation of a second, the two pathways acting in a sequential or relay mode, may act in parallel, modulates the activity of a canonical component of another and converge to regulate the activity of the same target. Such integration of signaling pathways at the promoter/enhancer level allows each gene to define its rules of regulation. Two given pathways can act synergistically in one setting and antagonistically in another.

During reprogramming, signaling pathways specify cell fates by activating transcriptional programs in response to extracellular signals. The cell reprogramming process from the differentiated to the pluripotent state, suggested by Takahashi and Yamanaka [14,15], leads to an accessible study model of extrinsic and intrinsic signaling in the process of cellular differentiation. Cell reprogramming reverts cells to pluripotent/multipotent from programmed states, by re-establishing epigenetic markers modification. During reprogramming to pluripotency using OSKM factors, it takes at least 1 to 2 weeks for the first reprogrammed cells to emerge in the culture. Importantly, only a few of the somatic cells that initially express the reprogramming factors eventually convert to the pluripotent state within this timeframe. The main steps of fibroblast reprogramming to induced pluripotent stem cells are induction of proliferation and downregulation of fibroblast specific transcription, followed by acquisition of epithelial characteristics and activation of some ESC markers.

The first step in reprogramming cellular fate is the silencing of the somatic programme and the change in the cell division rate with a decrease in cell size, this occurs as early as 24 hours after induction of the reprogramming factors. These changes are accompanied molecularly by the induction of proliferation genes and downregulation of the somatic expression programme. Next step is gaining epithelial cell character; fibroblasts after suppression of the somatic transcriptional programme undergo coordinated changes in cell-cell and cell-matrix interactions, which correspond with a loss of mesenchymal features and acquisition of epithelial cell characteristics (mesenchymal-to-epithelial transition). After epithelial cell character has been established (E-cadherin-positive cells) and larger colonies are formed, other ESC genes are upregulated, particularly those that are involved in anabolic housekeeping functions, and the expression of pluripotency networks is activated (transcriptional or developmental regulators endogenously encoded such as OCT4, SOX2, NANOG and many other pluripotency-related genes [16]. The upregulation of this core pluripotency network is considered to be the final step of reprogramming [17].

OCT4 is a pluripotency-associated factor that maintains ESCs phenotype. The OCT4-centric module includes 14 transcriptional factors and other proteins, which are the downstream effectors for signaling pathways controlled by MAPK, TGF- β and Wnt signaling pathways. The *OCT4* genes are themselves bound with multiple transcription

factors, including OCT4 itself. OCT4 is found to target genes involved in diverse cellular functions such as transcriptional regulation (e.g. TFIID-associated factor), chromatin modifications (e.g. demethylases, histone modifier components of Polycomb Repressive Complex 2, PRC2, chromatin remodeling complex), and post-transcriptional regulation through non-coding RNAs and microRNAs (*mir302* and *mir290* clusters). OCT4

positively regulates the expression of histone modifiers [18]. The reprogramming process requires a wide variety of molecular changes until cells reach a bona-fide pluripotent state. Perhaps the only way to truly understand the reprogramming process will be to extend the recent studies that combined single cell analysis with fine temporal resolution [19] (Figure 1).

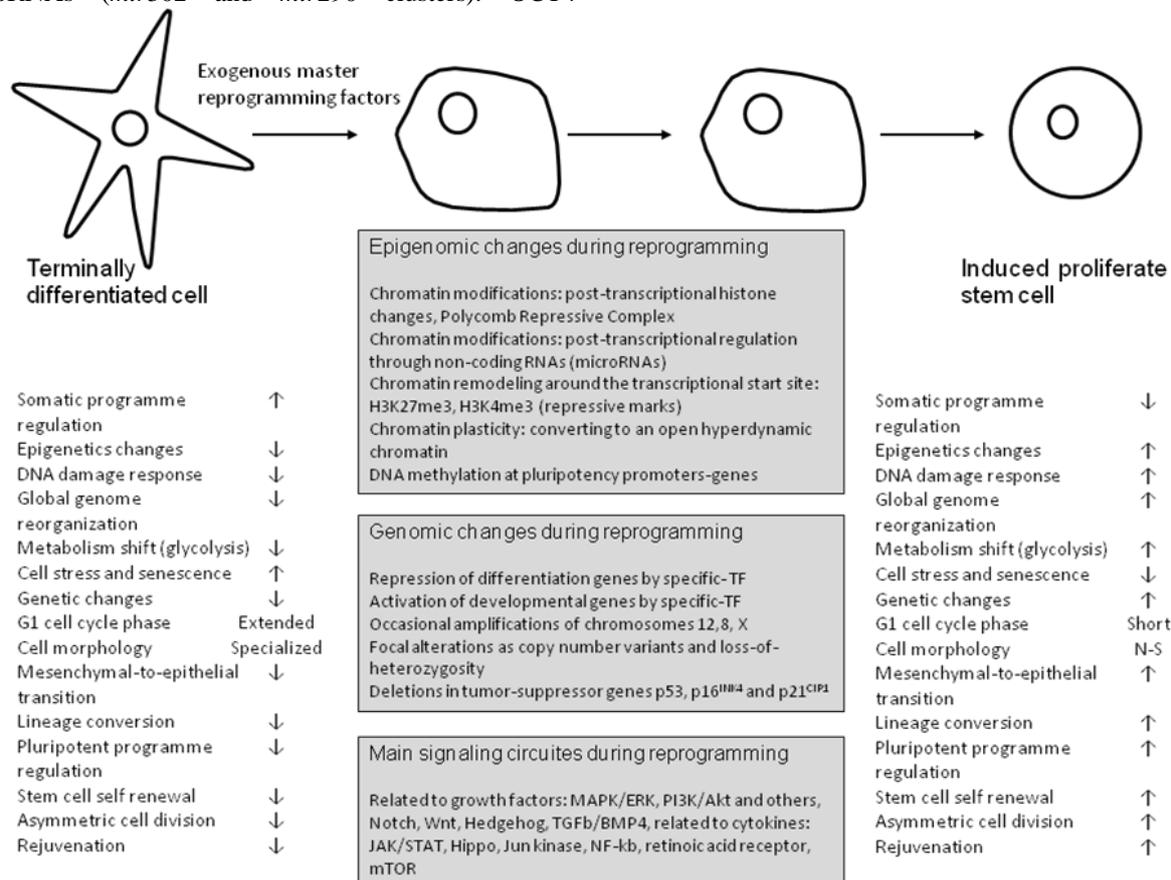


Figure 1. Genomic-epigenomic-signalling pathways changes in somatic cells reprogramming to iPSCs

4. Regulatory Signaling Networks in Reprogramming Cell Fate to Pluripotency

The integration of extrinsic and intrinsic signals is required to preserve the self-renewal and tissue regenerative capacity of adult stem cells. Pluripotency process during development is progressively and irreversibly lost through a mechanism that requires strict coordination of the signaling pathways involved in cell proliferation, migration and differentiation. However, recent breakthroughs have highlighted evidence pointing out that terminally differentiated cells can be reprogrammed into pluripotent stem cells, prompting a re-evaluation of the reversibility of cell differentiation. Generations of pluripotent cells can arise from somatic cells following ectopic expression of specific transcription factors; however these factors might not be the unique essential reprogramming factors. Furthermore, they might be the end-point target of signaling pathways. Indeed, recent evidence shows that modulation of the Wnt/β-catenin, MAPK/ERK, TGF-β or PI3K/Akt signaling

pathways strikingly enhances somatic cell reprogramming [20].

The transcriptional network that regulates cell reprogramming shows mimic like-events in the differentiation processes. Starting from the original discovery of Takahashi and Yamanaka, who identified the four OCT4, SOX2, KLF4 and c-MYC (OSKM) factors that are able to restore pluripotency, additional studies have demonstrated that OSKM are not strictly required and are not the only factors that can induce reprogramming [21]. One of the main barriers that have to be overcome to obtain fully reprogrammed clones is an irreversible arrest during G1 transition of the cell cycle. This arrest is implemented primarily through activation of p53 and up-regulation of the cyclin-dependent kinase inhibitors p16^{INK4} and p21^{CIP1}. It was shown that down-regulation of the p53 gene or reductions in down-stream factors such as p21 significantly increase the reprogramming efficiency of human somatic cells. Down-regulation of p53 appears to allow OCT4 and SOX2 to remodel chromatin to a threshold required for expression of sufficient NANOG to drive the subsequent event involved in iPSC generation, resulting from an enhancement of the proliferation rate of reprogrammed clones. But in some cases p53 inactivation promotes genome instability and tumorigenesis in iPSCs [20].

4.1. Wnt/ β -catenin Signaling Pathway

Activation of the Wnt/ β -catenin signaling pathway enhances somatic-cell reprogramming. Conditional medium for ESC *in vitro* cultures has been supplied (historically) with the cytokine leukaemia inhibitory factor (LIF), which activates the Janus tyrosine kinase (JAK), and in turn STAT3, that acts as a transcriptional factor in the sustaining of pluripotency in mouse ESCs. In human ESCs, LIF cannot promote self-renewal, but fibroblast growth factor (FGF) does it. Human ESCs can be sustained in an undifferentiated state by the activation of Wnt signaling, which maintains the expression of OCT4, REX1 and NANOG through the action of β -catenin, an intracellular signaling molecule that is part of the canonical Wnt signaling pathway. In absence of Wnt activation, β -catenin is phosphorylated by a complex (APC, axin and GSK3 β) and degraded. Upon Wnt-ligand binding with receptors Frizzled and LRP5/6, β -catenin accumulates in the nucleus where it binds with LEF/TCF and targeted self-renewal genes. Wnt3a-conditioned medium showed a 20-fold promotion of reprogramming of OSK-murine fibroblasts. Wnt signaling is involved in epithelial-to mesenchymal transition (EMT) and stemness, many β -catenin target genes are linked to the acquisition of stem cell competence [20,22]. For instance survivin, a stem cell marker participates in proliferation and apoptosis regulation being a direct β -catenin target.

4.2. MEK and GSK3 Signaling Pathways

The inhibition of MEK and GSK3 pathways enhances somatic cell reprogramming. Glycogen synthase kinase 3 (GSK3) is an important component of Wnt signaling, and together with MEK-MAPK/ERK pathway, is considered a fundamental player in the intracellular signaling that controls ESC pluripotency [20]. ERK signaling triggers differentiation of pluripotent ESCs to lineage commitment; and inhibition of GSK3 activity might also maintain ESC self-renewal.

4.3. TGF β Signaling Pathway

The TGF β signaling modulates the stem cell polarity in cell reprogramming. A TGF β superfamily member bone morphogenic protein 4 (BMP4) cooperates with LIF in the maintenance of pluripotency in mouse ESCs. BMP induces mesenchymal-to-epithelial transition (MET) [23]. MET is characterized by the upregulation of epithelial junctional components and morphological transformation into epithelial-like colonies, and seems to be a crucial step in the reprogramming process. Inhibition of signaling by TGF β improves reprogramming, because TGF activity prevents MET by inhibiting the upregulation of epithelial markers and the downregulation of the mesenchymal transcriptional repressor Snail zinc-finger protein. It was shown that OCT4 and SOX2 suppress the promesenchymal regulator Snail, whereas KLF induces the epithelial programme by direct binding towards the activation of epithelial genes, including E-cadherin [23].

4.4. PI3K/Akt Signaling Pathway

Phosphoinositide 3-kinases (PIKs) are lipid kinases that promote the generation of signaling lipid phosphatidylinositol 3,4,5-triphosphate upon activation by many different growth factor receptor tyrosine kinases, such as FGF, EGF and PDGF. This in turn regulates a complex signaling cascade. One of the main players in this pathway is Akt1, a serine/threonine kinase that modulates the functions of numerous substrates, such as Mdm2 and IKK, and elicits various cellular responses, including cell proliferation, adhesion, growth and death. Activation of Akt maintains the pluripotency of ESCs, on the contrary, treatment of ESCs with PI3K inhibitor, results in a loss of ESC features [20] (Table 3).

Table 3. Signaling circuits during somatic cell reprogramming to iPSCs

Main signaling pathways during reprogramming
Related to growth factors: MAPK/ERK, PI3K/Akt, and others
Notch
Wnt
Hedgehog
TGF β /BMP4
Related with cytokines: JAK/STAT
Hippo
Jun kinase
NF-kb
Retinoic acid receptor
mTOR

5. Epigenomic Changes in Somatic Cell Reprogramming Process

Despite the development of numerous methods to introduce the reprogramming factors into somatic cells, only a small percentage of cells expressing the endogenous factors complete the journey to the pluripotent state. It is now believed that the inefficiency of reprogramming is attributable to epigenetic hurdles that are only infrequently defeated. Recent data also demonstrate that repressive chromatin states comprise a major mechanistic barrier to the induction of pluripotency [13,24,25].

Although much progress in iPSC-induction technology has been made since Yamanaka and Takahashi landmark study, many of the molecular mechanisms that underlie reprogramming still remain elusive. One of the up-and-coming ideas is that ectopic expression of the Yamanaka cocktail of OSKM transcription factors triggers a sequence of epigenetic events. The epigenetic aspects in ESCs, iPSCs and reprogramming will contribute to the understanding of the cellular differentiation process.

The chromatin condensation is subject to regulation by several factors. Covalent modifications in N-terminal tails of histones and methylation of cytosine residues of DNA can influence the degree of condensation of chromatin *per se* or/and facilitate the recruitment of structural proteins (heterochromatin protein 1, HP1) or activator proteins, such as remodeling complexes. Certain regions of the genome are enriched with genes that are silenced but that can be active in certain situations or in different cell types.

Although the mechanisms of gene silencing may be heterogeneous and gene-specific, these areas are overall occupied by the Polycomb complex and marked with H3K27me3. Genes encoding many developmental regulators are located in such regions, and thus, are subject to intense regulation. While some silent genes can be activated by certain signals, others remain permanently silent and refractory to stimulation. This property is displayed in cell specific ways and defines both cell identity and plasticity. Certain cell types, such as stem cells, have highly plastic chromatin that makes them extremely sensitive to environmental signals. Dynamic chromatin is essential for pluripotency. ESC chromatin is hyperdynamic due to a combination of a loose association of histones and chromatin binding proteins with DNA, and rapid turnover of chromatin-binding proteins and histones [26]. Heterochromatin binding proteins such as HP1 and histones associate less tightly with chromatin in ESCs than in cells undergoing differentiation. As cell differentiate, particular genes become silent with a consequent loss of regulatory potential.

The ESCs contain specific epigenetic landmarks. In ECSCs, the core regulatory network phenotype is governed by the transcription factors OCT4, SOX2 and NANOG. These three factors are able to stimulate the expression of each other and also to control self-renewal and pluripotency through different mechanisms [21]. First, they bind to the regulatory regions of genes involved in self-renewal and stimulate their transcriptional activity. Second, they can also occupy the regulatory regions of critical genes involved in development and differentiation and presumably contribute to maintain these genes in a silenced but poised state for activation during differentiation, which constitutes the basis of pluripotency.

Embryonic stem cells can be maintained *in vitro* in the presence of signaling molecules such as LIF, FGF or TGF- β . All these complexes systems converge into two main responses: maintenance of very high rates of transcription of genes that belong to the pluripotency network, and “poising” of developmental genes. Globally, ESCs display high rates of transcriptional activity compared to differentiated cells, they express high levels of general transcription factors and of certain complex involved in transcriptional activation such as the ATP-remodeling BAF complex, remodeling factors Chd1 or Brg1, the chromatin remodeling complex INO80, the Mediator complex and TBP-associated factors (TAFs). Some of these factors have been found to co-occupy the regulatory regions of self-renewal genes with OCT4, SOX2 or NANOG [20].

ESC chromatin presents different structural characteristics compared to differentiated cells, their heterochromatin appears more relaxed. ESCs also display unique modification patterns, referred to as bivalent domains, at the regulatory regions of developmental genes. These are formed by large regions of H3K27me3 that harbor smaller regions of H3K4me3 around the transcriptional starting site. The coexistence of these two antagonistic marks has been suggested to play a role in silencing developmental genes in ECSCs, while keeping them poised for activation upon initiation of specific developmental pathways. Bivalent domains seem to be tightly regulated by the activating and repressing activities of chromatin and transcription related complexes such as

Polycomb complex. Specific transcription factors that bind the regulatory regions of bivalent genes such OCT4, SOX2 and NANOG contribute to Polycomb complex recruitment or stabilization [13,19]. Bivalent genes are further enriched in CpG islands that in ESCs are non-methylated. Despite being transcribed at very low levels, bivalent genes have considerable levels of transcriptionally engaged RNA Polymerase II near their transcription start sites, but greatly reduced level of productive elongating Pol II [22].

During cell differentiation, complex epigenetic changes take place. The *in vitro* differentiation of ESCs is achieved through the removal of molecules that promote self-renewal, such as LIF or FGF, and the addition of factors that induce differentiation. These changes in culture conditions lead to down-regulation of the pluripotency network and to the activation or repression of developmental genes in germ layer specific fashion. The physiological function of bivalent domains might be used to maintain important regulatory sequences, accessible to the binding of relevant transcription factors that are activated by the differentiation signals. The regulatory areas which were accessible at the undifferentiated stage, and which are not targeted by transcription factors, “close up” during differentiation, becoming further inaccessible. Therefore, differentiation to one particular lineage implies the permanent and irreversible silencing of genes involved in alternative lineages. Bivalent domains repress differentiation-genes, and repression might be further reinforced by the incorporation of other repressive marks such as H3K9me3 or DNA methylation [21], ensuring the permanent silencing of developmental genes. The resolution of bivalent domains requires the coordinated action of histone lysine methyltransferases and demethylases. Also, changes in subunit composition of chromatin-related complexes, and in the expression of histone variants might also contribute to establish the new epigenetic landscapes of differentiated cells, such is the case of the CBX subunits of the Polycomb complex, and of histone linker variant H1.0.

The silencing of genes that belong to the pluripotency network is a critical event for proper differentiation. These genes become passively downregulated due to the absence of LIF or FGF signaling and more actively due to the action of transcriptional repressors that are induced during differentiation. The fact that in differentiated cells the regulatory regions of different genes of the pluripotency network are marked with different combinations of repressive modifications, suggests that these mechanisms are likely to be gene-specific.

Epigenetic road map during cell reprogramming suffers hardships. The low efficiency in the iPSCs generation with any variants in reprogramming methods, suggests that somatic cells present barriers that prevent switches in cell identity. The fact that the efficiency of reprogramming can be increased by using inhibitors of DNA methyltransferases, histone methyltransferases and deacetylases [16], points to a critical role of chromatin: as a barrier that prevents reprogramming. Reprogramming is a progressive process, in early stages cells acquire the ability to self-renew and downregulate cell specific programs. At this stage, cells can be trapped in a partially reprogrammed state in which they self-renew and continue to depend on the expression of the transgenes. A second

critical phase consists of the activation of the endogenous pluripotency network, including the genes *OCT4*, *SOX2* and *NANOG*. This event allows the maintenance of pluripotency in an autonomous way and independently of the transgenes. However, this stage is reached at a low frequency likely due to the inability of the transcriptional factors to bind and activate the regulatory regions of the endogenous pluripotency genes [19,20]. Although the first stage of reprogramming lead to downregulation of the expression of specific genes, the complete erasure of this transcriptional memory takes place gradually after the activation of the pluripotency network [13,21]. The silencing of tissue specific genes appears to be more important than previously thought for the process of reprogramming. Importantly, epigenetic aberrations in reprogrammed cells have been correlated with defects in differentiation.

The regulatory regions of some pluripotency related genes, such as *OCT4*, *NANOG* and others, are hypermethylated at the DNA level in somatic cells and pre-iPSCs, and lack the activating mark histone H3 lysine 4 trimethylation (H3K4me3) [27]. Many pluripotency genes are enriched for repressive H3K27 and/or H3K9 methylation in somatic cells. DNA demethylation and loss of repressive histone methylation marks in pluripotency genes probably occur at the end of the reprogramming process. These findings support the idea that repressive chromatin at promoters and enhancers of pluripotency related genes may initially block engagement of the reprogramming factors. During reprogramming, silencing of somatic genes is also associated with chromatin structure change, at their enhancers and promoters, and in particular with a rapid loss of histone H3K4me3 [17].

Reprogramming of somatic cells to pluripotency is accompanied by dynamic changes in DNA methylation patterns. These changes include DNA demethylation of key pluripotency genes such as *OCT4* and *NANOG*. In somatic cells, the promoters of *OCT4* and *NANOG* are highly methylated, reflecting their transcriptionally repressed state. The formation of iPSCs involves activation of these genes, and their demethylation is widely used to monitor successful reprogramming. Demethylation can occur by a passive mechanism (inhibition of DNA methyltransferase 1) or by an active mechanism in which the methylated base is removed from nonreplicating DNA [28] (Table 2, Figure 1).

An example of differentiation cell process is the generation of fatty and bone cells from mesenchymal stem cells. Somatic or adult stem cells have a finite self-renewal capacity and are lineage-restricted. For example, mesenchymal stem cells (MSCs) are adult stem cells that can differentiate into osteogenic, adipogenic, chondrogenic, or myogenic lineages. MSCs isolated from various tissues have similar phenotypic, transcriptomic, functional and epigenetic characteristics. Particularly, the epigenetic state of ESCs changes as they differentiate into MSC. Osteogenic differentiation of MSCs is a complex process that is tightly controlled by numerous signaling pathways and transcription factors [29]. But runt-related transcription factor 2 (*runx2*) is considered a master regulator of osteogenic differentiation and is expressed at many stages of bone development and maturation. Epigenetic regulation results in structural changes in chromatin that alter the ability of *runx2* and other

transcription factors to osteogenic promoter regions (e.g. osteocalcin and osteopontin promoters). Such as osteogenic differentiation, the adipogenic differentiation of MSC is a highly coordinated process that involves numerous transcription factors performing specific function at various time points. The nuclear hormone receptor peroxisome proliferator-activated receptor- γ (PPAR- γ) serves as a master regulator. Many coregulators and transcription factors central to adipogenesis have chromatin-modifying activities, supporting the role of epigenetic regulation during the differentiation of MSCs to adipocytes (e.g. PPAR- γ 2, leptin, fatty acid-binding protein 4, lipoprotein lipase, promoters) [30]. Like this, MSC are subject of different levels of control, and numerous epigenetic modifications occur concomitantly during both osteogenic and adipogenic differentiation of MSCs.

6. Conclusions

In summary, iPSCs generation from somatic cells requires going along a complex network of genetic and epigenetic pathways to maintain a delicate balance between self-renewal and potential multilineage differentiation. Somatic reprogramming shows that a few pluripotency-associated transcription factors are sufficient to instigate the pluripotency genetic/epigenetic networks, but multiple epigenetic/genetic pathways are needed to obtain the pluripotent cell phenotype.

Eleven main signaling pathways have been involved in the control of cell fate and embryonic patterning. Extensive crosstalk among epigenetic pathways modifies DNA, histones and nucleosomes which make up the epigenetic mechanisms of gene regulation in differentiation and reprogramming processes.

A deeper insight/knowledge into the mechanisms of epigenetic regulation is necessary to improve the understanding of the cellular differentiation and reprogramming processes.

Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

iPSCs:	induced pluripotent stem cells
ESC:	embryonic stem cells
CNVs:	copy number variants
SOX2:	pluripotency associated transcription factor SRY box 2
PRC2:	Polycomb Repressive Complex 2
OSKM:	four <i>OCT4</i> , <i>SOX2</i> , <i>KLF4</i> and <i>c-MYC</i> factors that are able to restore pluripotency
LIF:	cytokine leukaemia inhibitory factor
JAK:	Janus tyrosine kinase
FGF:	fibroblast growth factor
EMT:	epithelial-to mesenchymal transition
GSK3:	glycogen synthase kinase 3
BMP4:	bone morphogenic protein 4

MET: mesenchymal-to-epithelial transition
PIKs: phosphoinositide 3-kinases
HP1: heterochromatin protein 1
TAFs: TBP-associated factors
H3K4me3: histone H3 lysine 4 trimethylation
MSCs: mesenchymal stem cells
runx2: runt-related transcription factor 2
PPAR-γ: nuclear hormone receptor peroxisome proliferator-activated receptor-gamma

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