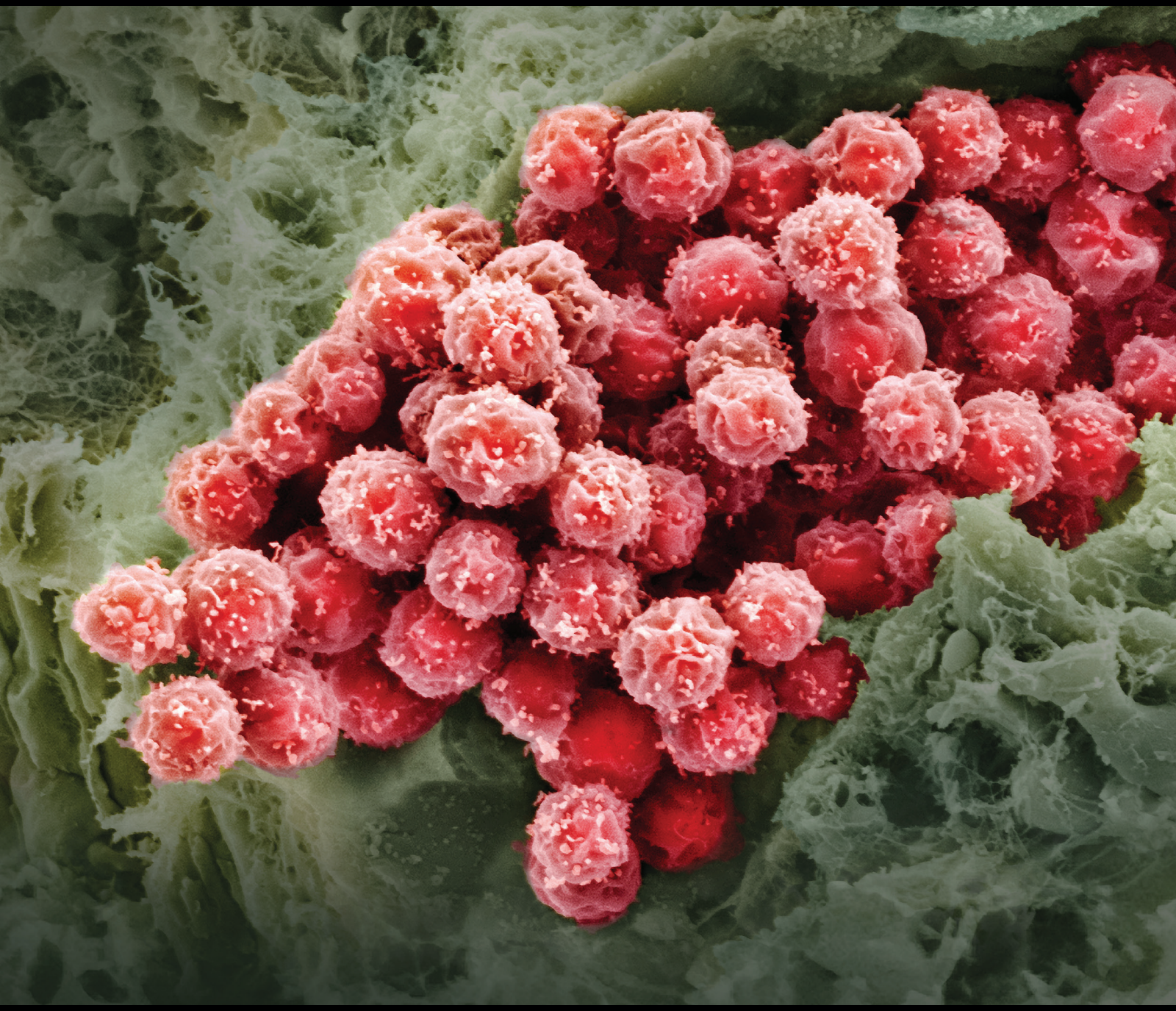
Stem Cells International

Epigenetic Regulation Shapes the Stem Cells State

Guest Editors: Giuseppina Caretti, Libera Berghella, Aster Juan, Lucia Latella, and James Ryall



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Editorial Board

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Editorial

Epigenetic Regulation Shapes the Stem Cells State

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Pluripotent stem cells are endowed with the dual capacity to self-renew and to differentiate towards all lineages. Genetic and genome-wide studies in different model organisms have provided compelling evidence for the importance of epigenetic factors both in the maintenance of pluripotency and in the establishment of cell lineage commitment, during embryonic differentiation and in regenerative events occurring in postnatal life.

In this special issue, we have collected reviews and reports highlighting the plasticity of the epigenome in embryonic, induced pluripotent and adult stem cells, providing readers with an overview of different molecular mechanisms, spanning from DNA methylation, histone modifications and variants, and regulatory RNAs.

In response to signals from the external niche and/or to intracellular signaling pathways, embryonic and adult stem cells engage epigenetic factors in the transition process towards differentiation. L. Fagnocchi et al. have summarized the current understanding of the cross-talk between extrinsic/intrinsic signaling pathways and epigenetic factors and how they cooperatively regulate the fate of different stem cell lineages.

Together with signaling molecules from the niche, metabolites and cofactors derived from the environment modulate intracellular pathways and the epigenetic response. A. J. Harvey et al. review several examples of metabolites and cofactors, which interface metabolic pathways and epigenetic targets, affecting histone marks and transcription.

DNA methylation, once believed to be an irreversible signature restricted to germ cells and embryo development, is now recognized as a dynamic modification, occurring in all cell types. R. C. Laker and J. G. Ryall present recent advances in our knowledge of the role of DNA methylation and hydroxymethylation in skeletal muscle stem cells, with an emphasis on recent whole genome sequencing results that show genomic enrichment for these modifications outside promoter regions and underscore their plastic role in sensing environmental cues.

Recently, the novel function of long noncoding RNAs (lncRNAs) in maintaining pluripotency of ESCs has been explored. A. Rosa and M. Ballarino present an overview of the underlying molecular mechanisms of lncRNAs in regulating ESC pluripotency and differentiation. Another class of noncoding RNAs are presented in the review by A. D. Haase, in which PIWI-interacting RNAs (piRNAs) are described. piRNAs developed transcription and posttranscription strategies to limit the spread of transposon elements, which are mobile genetic elements threatening genomic integrity. The author describes piRNAs as an RNA-based immune system guarding the genome integrity through non-self-memory and adaptive protection against transposons.

Adult stem cells hold great promise for their clinical relevance in regenerative medicine.

In the article by S. Consalvi et al., the authors describe many of the epigenetic regulators involved in the differentiation of skeletal muscle stem cells. The authors focus

predominantly on the processes of histone acetylation and deacetylation but also describe a potentially novel role for noncoding RNAs in the epigenetic regulation of differentiation and the potential for epigenetic modulation of skeletal muscle stem cells for the treatment of Duchenne muscular dystrophy (DMD).

In the review by F. A. Choudry and M. Frontini, the authors give an overview on the changes of the epigenetic landscape within the haematopoietic stem cell (HSC) compartment occurring in the elderly, which may be linked to increased occurrence of myeloproliferative disorders, myeloid malignancy, and thrombosis observed in the elderly. Epigenetic changes in the HSC compartment affect HSC activity, survival, and function and they might lead to the selection and expansion of particular HSC clones generating myeloid and platelet skewing of the haematopoietic system distinctive of the elderly population.

The review by L. Rouhana and J. Tasaki focuses on the process of tissue regeneration in lower order organisms. The authors discuss the careful integration of DNA methylation, histone modifications, and noncoding RNAs in the regulation of regeneration, as well as the important role of programmed cell death.

In contrast to changes to the DNA sequence, epigenetic modifications are reversible and are therefore considered promising therapeutic targets for the use of stem cells in the treatment of human diseases. In their review, R. Fernández-Santiago and M. Ezquerra describe how induced pluripotent stem cells are becoming a valuable model for neurodegenerative disorders, recapitulating key disease-associated molecular events. Furthermore, these authors highlight the potential of epigenetic regulation of patient-specific iPSC-derived neural models to develop novel therapeutic approaches for human disorders.

During the cellular reprogramming of somatic cells, distinctive chromatin status coupled with gene expression changes is an important determinant for the reprogramming efficiency towards pluripotency. In the research paper contributed by F. Dong et al., the authors showed that redistribution of histone variants H2A.Z during the reprogramming process alters nucleosome stability to increase expression of genes that promote reprogramming.

Together with kinase inhibitors, cocktails of epigenetic modulators can be used to promote reprogramming and to probe stem cells functions. In their report, Y.-C. Han et al. describe a novel method to induce neuronal stem cells from mouse embryonic fibroblasts, with the use of small molecules, and suggest that the reprogramming is enhanced by histone demethylation and histone acetylation and decreased DNA methylation.

Transdifferentiation is an alternative approach to somatic reprogramming of induced pluripotent stem cells, which allows the direct conversion of one cell type into another, bypassing safety concerns related to the pluripotent cell state. G. Palazzolo and colleagues present an original research paper documenting a transdifferentiation process used to convert fibroblasts from golden retriever dogs with muscular dystrophy (GRMD) directly to cardiac-like myocytes. While the induced cells do not exhibit spontaneous contraction *in vitro*, when transplanted into the hearts of neonatal mice, the induced cells were found to participate in cardiac myogenesis.

Overall, this special issue highlights recent advances in our understanding of epigenetic regulation of stem cells and describes several new approaches to investigate stem cell biology to model human disorders and develop novel therapies for disease states.

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Review Article

DNA Methylation in Skeletal Muscle Stem Cell Specification, Proliferation, and Differentiation

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An unresolved and critically important question in skeletal muscle biology is how muscle stem cells initiate and regulate the genetic program during muscle development. Epigenetic dynamics are essential for cellular development and organogenesis in early life and it is becoming increasingly clear that epigenetic remodeling may also be responsible for the cellular adaptations that occur in later life. DNA methylation of cytosine bases within CpG dinucleotide pairs is an important epigenetic modification that reduces gene expression when located within a promoter or enhancer region. Recent advances in the field suggest that epigenetic regulation is essential for skeletal muscle stem cell identity and subsequent cell development. This review summarizes what is currently known about how skeletal muscle stem cells regulate the myogenic program through DNA methylation, discusses a novel role for metabolism in this process, and addresses DNA methylation dynamics in adult skeletal muscle in response to physical activity.

1. Introduction

The term "epigenetics" literally means "above genetics" and is defined by the NIH Roadmap Epigenomics project as "both heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable." Epigenetics underlies the ability of embryonic stem cells (with an identical DNA code) to commit to the three germ layers (mesoderm, endoderm, and ectoderm) during the early stages of development and eventually commit to specific cell fates to generate all the different cell types in an organism, including skeletal muscle. These biological trait variations are not a result of changes in the DNA code, but rather structural modifications to the DNA and/or histones, or posttranscriptional gene silencing via small RNAs (including miRNA, siRNA, and piRNA) [1].

Considering the interest surrounding epigenetics and in particular DNA methylation, in the regulation of stem cell identity, this review aims to discuss some of the recent findings regarding methylation, with a particular focus on skeletal muscle stem cells (MuSCs, also referred to as satellite cells). While not discussed in this review, it is worth mentioning that, in addition to direct DNA modifications, structural epigenetic control is conferred at the level of histones. The core histone proteins H2A, H2B, H3, and H4 all contain long N-terminal tails which are highly susceptible to posttranslational modifications including methylation (me), acetylation (ac), phosphorylation (p), SUMOylation (sumo), ubiquitination (ub), ADP-ribosylation (ADP), and citrullination (cit) (reviewed in [2]). Each of these modifications influences the structure of the chromatin and directly regulates transcription. The complexity of many of these histone modifications has recently been documented in a series of publications arising from the Roadmap Epigenomics project (selected publications [3–5]).

2. DNA Methylation

Before discussing the role of DNA methylation in MuSC biology, it is essential to first define the process of methylation.

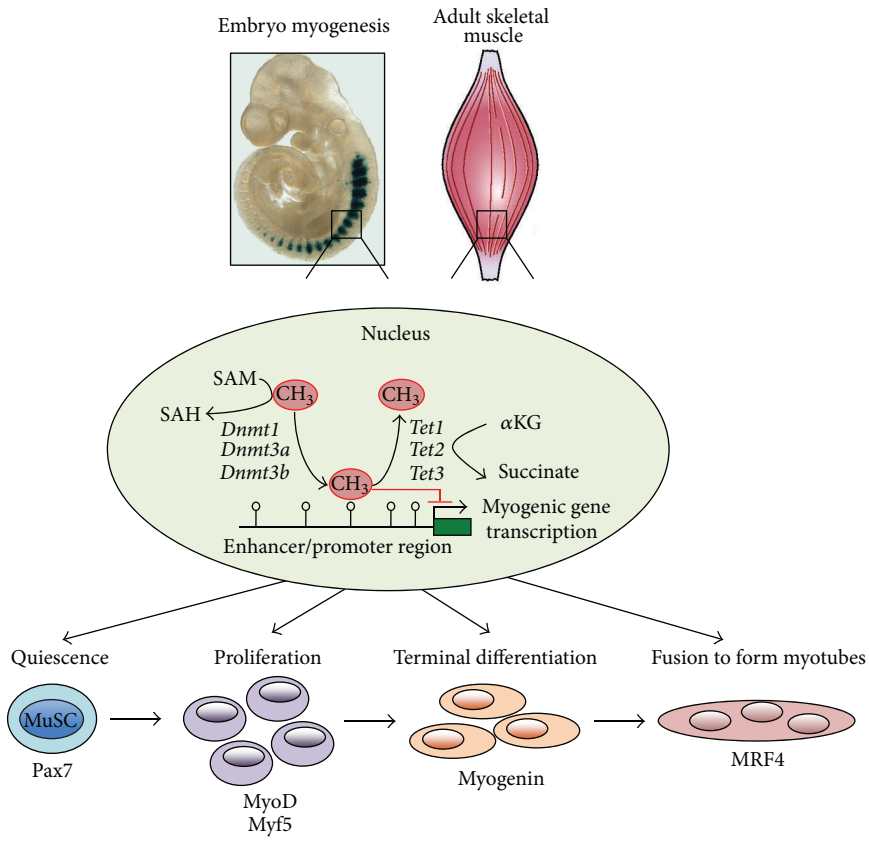

FIGURE 1: Transient DNA methylation and demethylation via specific *Dnmt* and *Tet* isoforms, respectively, regulate the expression of myogenic genes during embryonic MuSC specification, proliferation, and differentiation and in adult MuSC following an environmental stimulus to induce stem cell activation and muscle regeneration. Furthermore, the regulation of methylation and demethylation may be dependent on cellular metabolism since availability of the methyl group (CH_3) is derived from S-adenosyl methionine (SAM), which is converted to S-adenosyl homocysteine (SAH), while Tet dependent demethylation relies heavily on the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (αKG), which is converted to succinate.

Methylation of DNA is a well-described phenomenon and primarily occurs on the 5′ position of cytosine bases within CpG dinucleotide pairs and leads to the formation of 5-methylcytosine (5mC) and a context specific effect on transcription. DNA methylation within the promoter region of genes is typically linked to transcriptional repression due to recruitment of methyl CpG binding domain (MBD) proteins, which block transcription factor and RNA polymerase access [6]. In contrast, intragenic DNA methylation has been observed to have variable effects on gene transcription and can regulate the process of alternative splicing [7–9]. Finally, like promoter methylation, intergenic DNA methylation has been linked to gene repression likely as a result of inhibiting the actions of long range gene enhancers [10, 11]. Although research to date has focused on the role of promoter region methylation, the emergence of whole genome sequencing techniques has highlighted the potential for alterations to intragenic and intergenic methylated regions in response to environmental stimuli. Their involvement in the regulation of gene expression programs will greatly enhance our understanding of tissue specific transcriptional programs.

The processes of DNA methylation and demethylation are carefully regulated by a family of DNA methyltransferases (DNMTs) and demethylases (the ten-eleven translocation (TET) enzymes) (Figure 1). The methyltransferases DNMT3a and DNMT3b are primarily responsible for the generation of *de novo* DNA methylation [12], while DNMT1 has been found to maintain the methylation patterns following mitosis [13]. Interestingly, while the vast majority of DNA methylation is limited to CpG pairs, several recent studies have identified a significant proportion of CpH (H = A/C/T) methylation sites in a range of cells and tissues, including skeletal muscle and neurons [14, 15]. In neurons, CpH methylation was found to be DNMT3a dependent and was observed to lead to gene repression [15]. In contrast to DNMT enzymes, the TET1, TET2, and TET3 isoforms convert the 5mC to 5-hydroxymethyl cytosine (5hmC, as well as 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)), which can then be removed through base excision repair mechanisms [16, 17].

DNA methylation was originally thought to occur exclusively during germ cell development and in preimplantation embryos [21, 22]. It is now clear that methylation events occur in response to a variety of environmental cues and may play a larger role in regulating adaptation in adult tissues throughout the lifespan [21, 22]. An overall increase in DNA methylation has been observed as embryonic stem cells

transition to late stage progenitor cells and fully differentiated somatic cells [23–25]. These methylation events likely mediate the silencing of gamete specific and pluripotency genes in the transition towards a specific cellular identity. While less common, the loss of methylation can occur in a loci-specific manner to further drive specification [26–28]. These observations provide evidence for a role of transient epigenetic patterning in cell fate decisions and lineage pathways. Whether these epigenetic patterns can be manipulated or even reversed to withdraw differentiated cells from commitment and back towards pluripotency will be of significant focus in the epigenetic and stem cell fields.

3. Transcriptional Regulation of Skeletal Muscle Stem Cells

Skeletal muscle is derived from a population of mesodermal progenitor cells that undergo proliferation, differentiation, fusion, and maturation to form skeletal muscle fibers, a process known as myogenesis. Importantly, a subpopulation of these cells exit the cell cycle early and enter a state of quiescence (G_0). These cells are located between the basal lamina and sarcolemma of adult muscle fibers and make up the adult muscle stem cell (MuSC) population (also known as satellite cells). It is this population of cells that confer the high regenerative capacity characteristic of adult skeletal muscle, which in response to injury or trauma become activated and enter the myogenic program to generate new muscle fibers.

The paired domain homeobox 3 (Pax3) transcription factor is critical for successful migration of myogenic progenitor cells to the developing limb bud and subsequent muscle formation [29], while the closely related Pax7 is absolutely critical for the maintenance of the adult MuSC population [30–32]. In addition to Pax3/7, developmental myogenesis is primarily controlled through the actions of the myogenic regulatory factor (MRF) family of transcription factors. The MRFs are basic helix-loop-helix (bHLH) proteins and include myogenic factor 5 (Myf5), myogenic differentiation 1 (MyoD), myogenin, and myogenic regulatory factor 4 (MRF4). The MRFs undergo a strict program of spatial and temporal expression during development to control an array of muscle-specific genes to drive cell identity. The earliest detection of MRF proteins occurs during mid-to-late gestation and is characterized by the appearance of Myf5, closely followed by MyoD [29, 33–35]. These two proteins drive proliferation of the myogenic progenitors and initiate myogenic specification. Myogenin expression soon follows, leading to cells exiting the cell cycle and undergoing terminal differentiation [36, 37]. Fusion and maturation of these cells are regulated (at least in part) by MRF4, which plays a major role in primary and secondary fiber formation [29, 33, 38–40]. From this brief description of the transcriptional regulation of MuSCs, it is clear that the activation of specific transcriptional pathways must be carefully regulated, both spatially and temporally, as cells shift from proliferation to differentiation to a mature muscle fiber.

Indeed, in skeletal muscle biology, one of the most intriguing and pressing questions relates to the processes of MuSC activation, specification to the myogenic lineage, and eventual differentiation. Several studies have provided important evidence linking methylation of the promoter and enhancer regions of myogenic regulators to the initiation of the myogenic transcriptional program in the somites [41, 42]. Although whole genome methylation patterns have been reported in adult skeletal muscle, this type of comprehensive analysis has not yet been applied to the early stages of skeletal muscle development or in purified populations of adult MuSCs. The following section will detail the research to date regarding DNA methylation and its role in mature skeletal muscle function as well as MuSC specification, proliferation, and differentiation.

4. Methylation and Skeletal Muscle Stem Cells

4.1. DNA Methylation and Demethylation in Quiescent, Proliferating, and Differentiating MuSCs. The differential regulation of DNMT and TET expression and activity during muscle development is critical for understanding the link between environmental cues, intracellular signaling, DNA methylation, and gene expression. Evidence suggests that these methyltransferases and demethylases may be regulated in an isoform-specific manner during myogenesis. Indeed, *Dnmt1* has been found to be downregulated during myogenic differentiation with alternative isoforms of *Dnmt1* and *Dnmt3b* detected specifically in mature skeletal muscle [43–45]. In addition, RNA microarray data has shown that *Tet1* and *Tet2* have increased expression in myoblasts and myotubes in culture when compared with 19 other cell types [46]. In support of elevated demethylase activity in muscle maturation, these authors also reported increased 5hmC levels in adult muscle compared with myoblasts or myotubes [47]. Interestingly, a recently published whole transcriptome dataset from quiescent and proliferating MuSCs showed the nonspecific downregulation of all *Tet* isoforms as well as *Dnmt3a* during MuSC activation, while the expression of *Dnmt1* was robustly increased [18]. These observations suggest that specific DNMT and TET isoforms may be critical for initiating the MRF transcriptional program and/or regulating cell cycle in the transition between quiescence and proliferation and from proliferation to differentiation.

Advances in fluorescent activated cell sorting (FACS) techniques, coupled with downstream gene arrays (Affymetrix microarrays) or whole transcriptome sequencing (RNAseq), have allowed for the generation of transcriptome signatures for pure stem cell populations, including MuSCs [18–20]. A careful analysis of the extensive datasets from these studies reveals a clear pattern of expression for *Dnmt* and *Tet* genes (Table 1). In one such dataset from Ryall and colleagues, the expression of *Dnmt1* was found to increase fourfold and *Dnmt3a* decreased threefold in MuSCs activated *ex vivo*. A similar change in *Dnmt1* and *Dnmt3a* gene expression was observed in two other studies using both *in vivo* and *ex vivo* activated MuSCs [19, 20]. In contrast, *Dnmt3b* expression was unchanged in response to MuSC activation. Interestingly, MuSC activation has been associated with a 2–10-fold decrease in the expression of *Tet1–3* genes (Table 1). Together, these results support the need for a direct

Table 1: A summary of differential gene expression in DNA methyltransferases and demethylases following MuSC activation (fold change compared to quiescent MuSCs).

	Ryall et al. 2015 [18] (RNAseq, *in vitro* MuSC activation)	Pallafacchina et al. 2010 [19] (microarray, *in vitro* MuSC activation)	Liu et al. 2013 [20] (microarray, *in vivo* MuSC activation)	Pallafacchina et al. 2010 [19] (microarray, MuSCs from one-week-old mice)	Pallafacchina et al. 2010 [19] (microarray, MuSCs from *mdx* dystrophic mice)
Dnmt1	↑ 4-fold	↑ 6-fold	↑ 6-fold	↑ 7-fold	↑ 3-fold
Dnmt3a	↓ 3-fold	↓ 3-fold	NA	↑ 3-fold	↔
Dnmt3b	↔	NA	NA	NA	NA
Tet1	↓ 10-fold	NA	↓ 5-fold	NA	NA
Tet2	↓ 2-fold	↓ 13-fold	↓ 3-fold	NA	NA
Tet3	↓ 2-fold	NA	NA	NA	NA

DNMT: DNA methyltransferase; Tet: ten-eleven translocase; MuSC: muscle stem cell; NA: not available.

measurement of the methylation status in quiescent versus actively proliferating MuSCs.

While the methylation status of quiescent MuSCs has not been investigated in detail, several authors have attempted to define a DNA methylation signature in proliferating versus differentiating MuSC cultures. Tsumagari et al. (2013) assessed DNA methylation in proliferating human myoblasts and differentiated myotubes but did not find significant differences between methylation patterns [47]. However, when the DNA methylation profiles of proliferating and differentiating myogenic cells were compared with adult skeletal muscle, they reported a loss of ~90% of the hypermethylated sites in mature fibers [47] with similar findings reported by Carrió et al. (2015) [48]. Interestingly, many of the demethylated genes were associated with homeobox and Tbox transcription factors. Tsumagari and colleagues also reported hypermethylation of the *Pax3* gene in both myogenic cells and mature skeletal muscle. Given the role of Pax3 in migration and early lineage commitment, it would perhaps be interesting, and more informative, to determine the methylation status of this gene during somitogenesis and early specification [47]. Two additional genes observed to be differentially methylated were *Obscn* (encoding a giant muscle associated protein) and *Myh7b* (the gene encoding the slow, cardiac myosin heavy chain) which were both demethylated [47]. In contrast, Miyata et al. (2015) found a small but significant increase in global DNA methylation as myogenesis progressed from myoblast to myotube stage. Gene ontology analysis showed hypermethylation of promoter regions was associated with genes involved in muscle contraction and other muscle processes. Furthermore, two binding motifs recognized by the transcription factors ID4 and ZNF238 were significantly enriched in hypermethylated promoter regions [49]. An important consideration, however, is that the methods used in this study did not distinguish between 5mC and 5hmC. This distinction will be critical for future studies when interpreting the functional impact of methylation changes and the role of 5hmC in gene regulation.

The specific enrichment of 5hmC in either gene bodies or enhancer regions is often associated with activation and has been identified in human embryonic stem cells [50]. In a recent study from Terragni and colleagues, the presence of 5mC and 5hmC within specific gene regions of the Notch signaling pathway was assessed in myoblasts, myotubes, and mature skeletal muscle [51]. Paracrine Notch signaling is critical for the regulation of several developmental pathways, including the proliferation of MuSCs [52]. Using genome-wide profiles of DNA methylation, Terragni et al. identified hypomethylated regions within or near Notch signaling genes including *Notch1* and its ligands *Dll1* and *Jag2* in all skeletal muscle lineages compared with other cell types [51]. Subsequent enzymatic assays revealed enrichment of 5hmC in or near these same genes in mature skeletal muscle, but not myoblasts or myotubes [51]. The 5hmC modification in this context may function as a fine tuning mechanism for rapid induction of gene expression and intercellular signaling to the MuSC niche when skeletal muscle regeneration and/or repair is required.

Brunk and colleagues were the first to perform studies that linked DNA methylation to muscle cell identity [41]. In this study, it was shown that the distal enhancer of *Myod1*, located 20 Kb upstream of the transcriptional start site, was completely unmethylated at all CpG sites examined in myogenic cells and a subpopulation of somite cells. Furthermore, nonmyogenic cells displayed methylation of the enhancer at an average level of >50% [41]. Importantly, the lack of methylation was found to be sufficient for activation of the gene during embryogenesis. More recently, Palacios et al. reported that demethylation of the myogenin promoter occurs in an anterior-posterior manner in cells during somitogenesis, which correlated with myogenin expression and subsequent muscle development [42]. Lucarelli et al. also reported that the myogenin promoter is unmethylated in differentiated muscle cells and correlates with its expression [53].

Since the seminal work by Brunk and colleagues in 1996, Carrió et al. investigated the methylation status of a 110 kb enhancer region of Myf5/Myf6 (known as a "super-enhancer" because it has a high density of enhancer elements) [54]. Of the five enhancer elements analyzed within this region, all were highly methylated in ESCs and almost totally

demethylated in myoblasts, myotubes, and skeletal muscle in concert with increased Myf5 gene expression [48]. Importantly, these were muscle-specific observations and localized to the enhancer regions [48]. Together these findings suggest that DNA methylation/demethylation plays a critical role in regulating gene expression to control muscle cell specification and highlights an important role in gene regulation for DNA methylation changes outside promoter regions.

To better characterize the role of DNA demethylation in myogenic development, several studies have utilized 5-azacytidine (5AC), a potent inhibitor of DNA methylation, which acts via the sequestration of DNMT1 and results in global loss of methylation. Mouse fibroblasts (C3H10T1/2) treated with 5AC for 10 days resulted in the emergence of several cell types including those of adipogenic and osteogenic lineages. However, the majority of cells underwent transformation towards the myogenic lineage [55]. Similar results have been observed in fibroblasts following DNMT1 inhibition via antisense RNA [56]. Together, these findings provide strong evidence that DNA methylation plays an important role in dictating cell fate.

In the immortalized C2C12 myogenic cell line, proliferating myoblasts treated with 5AC exhibited increased expression of muscle-specific genes (including myogenin), enhanced myotube maturation, spontaneous contraction, and Ca^{2+} transients [57–59]. These results suggest that DNA demethylation in cells already committed to the myogenic lineage likely induces a permissive chromatin configuration, allowing muscle-specific transcription factors to bind their target gene promoters to promote differentiation. In similar experiments with 5AC, increased protein expression of cyclin D (linked to differentiation) and p21 (associated with the maintenance of the postmitotic state), as well as the gene expression of the myogenic regulators *Myf5* and *Myod1*, has been observed [60]. These findings support a role for DNA demethylation during myogenic differentiation. However, as these studies were conducted in proliferating myoblasts that are already committed to the myogenic lineage, it is important that future studies investigate DNA methylation in each broad step of myogenesis (quiescence, proliferation, and differentiation). Furthermore, genome-wide methylation analysis such as reduced representation bisulfite sequencing will provide comprehensive and important information regarding the earliest stages of muscle development and regeneration.

4.2. Dnmt and Tet Enzymes in Embryonic Development. The generation of *Tet* and *Dnmt* isoform-specific knockout (KO) mice has greatly enhanced our understanding of DNA methylation in embryonic stem cells and development. Of particular interest, the deletion of TET1 in embryonic stem cells results in reduced 5hmC and dysregulation of 221 genes, including muscle development and contractile genes [61]. However, *Tet1* KO mice remain viable with only a slightly reduced body size. Similarly, loss of *Tet2* also results in viable mice; however, these animals display myeloid malignancies associated with the dysregulation of hematopoietic stem cells as a result of the loss of 5hmC and elevated levels of 5mC in bone marrow cells [62, 63]. In embryonic stem cells lacking both *Tet1* and *Tet2*, there is a greater loss of 5hmC than that observed in cells lacking only *Tet1* or *Tet2*, but these cells still remain pluripotent. The resulting double KO mice demonstrate partial perinatal lethality, with those surviving mice displaying reduced fertility [64]. Finally, loss of *Tet3* leads to neonatal lethality with abnormal hydroxylation and impaired demethylation of the paternal genome [65]. These findings suggest that the TET isoforms functions are not redundant and play specific roles in cell fate decisions and organ development. In contrast to the loss of either *Tet1* or *Tet2*, DNMTs appear to be far more critical for survival. *Dnmt1* KO embryos arrest at the 8th somite stage and display ~70% reduction in methylation levels [66] and *Dnmt3a* KO mice survive only to ~4 weeks of age, while *Dnmt3b* KO mice are not viable [67]. Altogether, it is clear that regulation of DNA methylation is critical for embryonic development and DNMT and TET enzymes play important and potentially tissue specific roles in cell fate. Future studies taking advantage of conditional and inducible KO models will be crucial to dissect the complex interaction of DNA methylation and demethylation in the regulation of transcriptional networks and tissue development.

4.3. A Novel Role for Metabolism in the Regulation of MuSC DNA Methylation. In addition to the differential regulation of *Dnmt* and *Tet* expression during myogenesis, the activity of these proteins can be regulated in a metabolic dependent manner (Figure 1) [68, 69]. The process of DNA methylation involves the attachment of a methyl group ($-CH_3$) to the 5′ position of a cytosine base. This methyl group is derived from S-adenosyl methionine (SAM), which is in turn produced via one-carbon metabolism (specifically the folate and methionine cycles). The precursors necessary to produce SAM for DNA methylation are derived from the diet (folate) or glycolysis (3-phosphoglycerate (3PG) → serine → glycine + 5,10-methylenetetrahydrofolate (metTHF)). In contrast, TET dependent DNA demethylation requires the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (αKG) to proceed. This dependency of both DNMT and TET proteins on metabolites suggests that significant changes in cellular metabolism may be associated with dramatic changes in the cellular DNA methylation patterns [70].

Recent work has identified a process of metabolic reprogramming in MuSCs as they move from quiescence to proliferation, with fatty-acid oxidation predominating during quiescence and glycolysis increasing during proliferation [18]. While this process of metabolic reprogramming was linked to altered transcription and gene expression as a result of increased histone acetylation, it seems likely that such a dramatic shift in metabolism (and cell state) would likely be associated with several epigenetic changes, including DNA methylation. Of particular interest to the current discussion are findings suggesting that the shift from quiescence to proliferation in MuSCs is associated with a significant increase in the expression of genes associated with the enzymatic conversion of 3PG to metTHF (phosphoglycerate dehydrogenase, *Phgdh*; phosphoserine aminotransferase 1, *Psat1*; phosphoserine phosphatase, *Psph*; and serine hydroxymethyltransferase, *Shmt*) and a decrease in the expression of isocitrate

dehydrogenase 1 (*Idh1*, responsible for the conversion of isocitrate to αKG). These changes in the expression level of key metabolic enzymes, coupled with the previously identified changes in *Dnmt* and *Tet* (Table 1), suggest a likely increase in DNA methylation during MuSC activation/proliferation [18, 20]. Future studies investigating the link between the metabolic switch during MuSC activation and changes in DNA methylation will be fundamental for our understanding of MuSC specification and the downstream transcriptional program.

5. Skeletal Muscle DNA Methylation and Physical Activity

Several studies have compared CpG methylation patterns in adult skeletal muscle to that in other tissue types in order to define the DNA methylation signature of skeletal muscle. One human study assessed seventeen thousand CpG islands of which 178 were specifically hypermethylated in skeletal muscle compared with other cell types including blood, sperm, brain, and spleen [71]. A similar study identified 183 differentially methylated CpG sites in 22 skeletal muscle samples, within a set of 1,628 human tissues [72]. Finally, Calvanese et al. identified 47 genes that were hypomethylated exclusively in skeletal muscle, with some of these encoding contractile proteins such as obscurins, myotilin, and the slow-twitch myosin heavy chain [27]. Together, these findings have clearly demonstrated that different tissue types display distinct DNA methylation patterns appropriate for genetic control of their function and structure, but they fail to provide information regarding the dynamic methylation processes that may occur in response to environmental stimuli or during tissue development.

It is now accepted that DNA methylation is a dynamic process, and as skeletal muscle is a highly plastic tissue able to rapidly respond to changes in demand, DNA methylation may be a particularly important mediator of these adaptations. Skeletal muscle responds to endurance and resistance training through adaptation of contractile apparatus and metabolic capacity. Barrès and colleagues have previously reported that acute exercise, in humans and mice, is linked to transient DNA demethylation in the promoter region of genes including peroxisome proliferator activated receptor γ (*Pparg*) coactivator-1α (*Ppargc1a*), pyruvate dehydrogenase kinase 4 (*Pdk4*), and *Ppard* in skeletal muscle, which corresponded to transient induction of gene expression in a time and intensity dependent manner [73]. This data suggests that at least part of skeletal muscle adaptation to exercise/contraction may be mediated through transient regulation of DNA methylation. Others have provided evidence that histone modifications also occur in human skeletal muscle following an acute bout of exercise [74], while long-term training may cause sustained impacts on DNA methylation patterns of muscle-specific genes [75].

There is also mounting evidence that during perinatal development skeletal muscle is susceptible to insults or stimuli that may alter the epigenetic program, which has consequences for gene transcription and functional outcomes later in life [76–80]. For example, in mice, obesity in the mother caused DNA hypermethylation at the promoter of the metabolic master regulator, *Ppargc1a*, in skeletal muscle of the offspring [76]. This was detected at birth and up to 12 months of age and led to functional consequences for *Ppargc1a* mRNA levels and downstream gene expression (*Glut4*, *Cox4*, and *CytC*) [76]. Furthermore, these epigenetic changes were associated with metabolic dysfunction later in life [76]. Interestingly, when the obese mother was allowed to exercise prior to and during pregnancy, the hypermethylation of the promoter region of *Ppargc1a* was abolished in the skeletal muscle of the offspring along with the associated functional consequences [76]. It is therefore possible, and highly likely, that other genes important for skeletal muscle development may be epigenetically regulated in early life and susceptible to environmental stimuli during critical periods of cell growth.

6. Conclusions

Additional studies are warranted to further characterize how DNA methylation and hydroxymethylation differ between MuSCs in different dynamic states and what specifically regulates these methylation events. In doing so, these studies will reveal novel mechanisms to regulate MuSC identity and growth. Furthermore, the identification of isoform-specific roles for *Dnmt* and *Tet* enzymes in regulating the MRF transcriptional program would provide new insight into DNA methylation dynamics and MuSC function and could be extended to investigate periods of muscle adaptation and plasticity. Finally, whole genome sequencing will allow us to take this research beyond the classical muscle-specific genes and also extend our reach to regions of intra- and intergenic DNA methylation in regulating transcriptional programs. These findings will be crucial for furthering our fundamental understanding of stem cell biology and epigenetic regulation and may lead to the development of novel techniques to induce pluripotency in committed cells and unveil new therapeutic targets.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Epigenetic Reprogramming of Muscle Progenitors: Inspiration for Clinical Therapies

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In the context of regenerative medicine, based on the potential of stem cells to restore diseased tissues, epigenetics is becoming a pivotal area of interest. Therapeutic interventions that promote tissue and organ regeneration have as primary objective the selective control of gene expression in adult stem cells. This requires a deep understanding of the epigenetic mechanisms controlling transcriptional programs in tissue progenitors. This review attempts to elucidate the principle epigenetic regulations responsible of stem cells differentiation. In particular we focus on the current understanding of the epigenetic networks that regulate differentiation of muscle progenitors by the concerted action of chromatin-modifying enzymes and noncoding RNAs. The novel exciting role of exosome-bound microRNA in mediating epigenetic information transfer is also discussed. Finally we show an overview of the epigenetic strategies and therapies that aim to potentiate muscle regeneration and counteract the progression of Duchenne Muscular Dystrophy (DMD).

1. Introduction

Epigenetic regulation of chromatin structure is fundamental to achieve the activation or repression of transcriptional programs governing cell development and differentiation. Changing cell phenotype without affecting genotype, epigenetics controls the spatial and temporal regulation of gene expression that ensures the quality, stability, and heritability of cell identity. At least three systems, including DNA methylation, posttranslational histone tail modifications, and noncoding RNA, are currently involved in epigenetic regulation [1]. Epigenetic changes occur naturally in normal development and health but can also be influenced by several factors including aging and diseases. Indeed aberrant epigenetic control can cause abnormal activation or silencing of genes. Importantly epigenetic modifications are reversible and sensitive to the environment, having therefore the potential to be therapeutically manipulated. Thus, epigenetics is currently a hot topic for research and the number of studies relating to various models of epigenetic regulation is tremendously increasing. Moreover, advances in genome-wide technologies trying to elucidate epigenetic profiling (i.e., ChIP-seq, ChIA-PET, and Hi-C) hold the promise to deeply clarify the epigenetic control of cellular identity in health and disease.

Adult stem cells are candidate targets of epigenetic therapies toward repairing injured or diseased tissues, so they represent a key issue in regenerative medicine. In this context, skeletal muscle regeneration provides an insightful model for the study of the epigenetic events supporting the synchronized activation and repression of gene expression during stem cells differentiation. Indeed adult muscle stem cells remain in an embryonic-like state during development with the long-term ability to self-renewal and differentiate in response to injury [2]. A global genome reorganization allows activation, proliferation, and subsequent differentiation of quiescent progenitor muscle cells into functional multinucleated myofibers. Satellite cells are the main source of muscle stem cells (MuSCs) that regenerate adult skeletal muscles during postnatal life [3]. Intriguingly during aging or muscular disorders in which there is a chronic loss of skeletal muscle structure, the satellite cells function is compromised [4] even if their endogenous capacity to regenerate is not affected [5]. In fact it was demonstrated that the muscle

environment is critical to permit effective muscle regeneration [6]. In particular the recently identified population of muscle interstitial cells, named fibroadipogenic progenitors (FAPs), plays a key role in supporting MuSCs activity and regeneration. However, in chronic muscle damage these cells lose their ability to support MuSCs mediated muscle regeneration and differentiate into fibroblasts and adipocytes [7–11]. An extensive analysis of the epigenome of these cells in healthy and diseased muscles is currently missing and would be crucial to better understand and pharmacologically manipulate changes that affect their regeneration activity.

The most severe neuromuscular disease is the Duchenne Muscular Dystrophy (DMD), a rare X-linked genetic disease caused by mutations in the dystrophin gene. DMD is characterized by a rapid progression of muscle degeneration that leads to the loss of ambulation and death within the second decade of life. In DMD the unbalanced regeneration of muscles exposed to continuous waves of degeneration leads to replacement of contractile myofibers with fibrotic and fatty tissue [12, 13]. Nowadays there is not available cure for dystrophic patients and treatment is restricted to strategies that counteract the progression of the disease. The only therapy is limited to using corticosteroids as drugs to improve muscle strength. A huge number of studies for the treatment of the muscular disease are arising and some of them are undergoing clinical investigation. Gene and cell-therapies, acting to repair the genetic defect, represent the most promising curative approach in the treatment of DMD but are still far from clinical translation [14].

Otherwise, pharmacological approaches that target the pathological consequences of the genetic defect are easy prompt to clinical practice translation. Actually, the pharmacological therapy for DMD includes nitric oxide (NO) administration, insulin-like growth factor 1 (IGF-1) stimulation, and myostatin inhibition in way to increase skeletal muscle mass; otherwise, therapies leading the inhibition of the transforming growth factor-beta (TGFβ) pathway, modulation of nuclear factor-κB (NF-κB), and tumor necrosis factor-α (TNF-α) signalling are used to reduce fibrosis and inflammation in muscle [15].

However, the major limitation of manipulating target pathways consists in the lack of selectivity resulting in undesired side effects. Thus, regenerative medicine is providing novel strategies developing several epigenetic drugs aimed to manipulate the chromatin targets of individual signalling pathways. In the context of DMD, Histone Deacetylase Inhibitors (HDACi) are emerging as promising treatment to increase the functional and morphological recovery of dystrophic muscles [16–18]. Most of the beneficial effects of HDACi arise from their ability to activate a microRNA-SWI/SNF based epigenetic network in FAPs that redirects their lineage commitment from a fibroadipogenic toward a myogenic fate [19].

MicroRNAs (miRs) belong to the small noncoding RNAs family and are known to control numerous biological processes representing the most prevalent regulatory mechanism of mRNA availability in cells [20]. Apart from their role in regulating cell-autologous epigenetic events, miRs are involved also in cell-to-cell communication being involved in epigenetic regulation of recipient cells. miR shuttle between cells appears to be preserved and mediated by extracellular vesicles (i.e., exosomes) that are emerging as potent genetic transfer agents [21]. Interestingly stem cell-derived extracellular vesicles appear to be naturally equipped to mediate tissue regeneration and recent evidence suggests their therapeutic potential for targeted delivery of exogenous miRs [22].

In this review, we will focus on the principal epigenetic regulatory mechanisms underpinning skeletal muscle regeneration and their potential manipulation to develop pharmacological therapies for the treatment of DMD.

2. Chromatin-Modifying Enzymes: Epigenetic Writers and Erasers Regulating Cell Epigenome

The temporally regulated gene expression that controls pluripotency and differentiation is achieved by highly coordinated epigenetic events that ensure lineage commitment and cell fate determination. Epigenetic regulation of chromatin structure is fundamental to the activation or repression of specific transcriptional programs and is mainly controlled by chromatin-modifying enzymes that induce DNA methylation, posttranslational histone tail modifications, and nucleosome remodelling.

DNA methylation is a heritable, yet reversible, epigenetic modification that plays a central role in transcriptional repression. DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from cofactor *S*-adenosylmethionine to carbon 5 of the cytosines (5mC) that typically reside within a CpG dinucleotide. Regions of high CpG density, known as CpG islands, are typically devoid of DNA methylation [23]. Conversely, genes regulated by methylation usually contain low CpG density promoters and are demethylated and expressed in a cell-type-specific manner during differentiation [24–26]. This process is well illustrated during skeletal muscle cell fate commitment and differentiation. During development, pluripotent cells show a progressive loss of methylation leading to muscle stem cells with a unique DNA methylation signature associated with its specialized functions. Specific-myogenic factors such as MyoD and Myogenin are activated in a demethylation-dependent manner driving the activation of the myogenic program (reviewed in [27]). Simultaneously, myogenesis is accompanied by DNA methylation of pluripotency and developmental genes (i.e., Hox genes) [28]. Seminal works demonstrated that treatment with 5-azacytidine, a potent inhibitor of DNA methylation, triggers myogenic differentiation in nonmuscle cells, linking for the first time *MyoD* tissue-specific demethylation and cell fate commitment [29–31]. DNA demethylation may also provide a transcriptionally poised state of muscle fiber genes that would be activated during differentiation, upon the acquisition of transcription factors and positive histone marks.

Indeed changes in DNA methylation and histone modifications strongly cooperate to achieve the global genome

reorganization of progenitor cells necessary to establish myogenic identity, proliferation, and subsequent differentiation.

Satellite cells represent the main source of stem cells for adult muscle regeneration. Following muscle injury, they are readily activated and induced to proliferate and differentiate in multinucleated myofibers [32, 33]. The myogenic lineage in satellite cells is determined by the expression of Pax3 and Pax7 genes, while the expression of basic helix-loop-helix Myogenic Regulatory Factors (MRFs; MyoD, Myf5, Myogenin, and MRF4) in cooperation with myocyte enhancer factor-2 (MEF2) family proteins confers their ability to form differentiated myofibers [34]. Satellite cells activation is reflected in drastic changes at specific chromatin regions via the action of chromatin-modifying enzymes [35].

There are several classes of posttranslational histone modifications (i.e., phosphorylation, acetylation, methylation, and ubiquitylation) that affect chromatin structure and accessibility [36].

Histone acetylation has generally been linked to transcriptional active chromatin and is dynamically regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). A large amount of work illustrated the fundamental role of HATs and HDACs in regulating muscle development and differentiation. HATs catalyse the transfer of acetyl groups to lysine residues of histones, resulting in the relaxation of chromosomal DNA permissive for transcription. The histone acetyltransferases p300/CBP and PCAF activate muscle gene expression by acetylation of MyoD and modulation of its recruitment at target loci [37]. Interestingly recent studies have highlighted the ability of MyoD to preset the chromatin landscape of myoblasts for the activation of muscle-specific genes. Indeed genome-wide binding of MyoD has been associated with HATs recruitment and regional histone acetylation [38], while MyoD-bound distal enhancers have been linked to the recruitment of additional transcription factors and the regional enrichment of H3K4 monomethylation (H3K4me1) and H3K27 acetylation (H3K27ac), two typical markers of active enhancers [39].

HDACs function to reverse histone acetylation, causing chromosomal DNA condensing and preventing the unscheduled transcriptional activation of muscle-specific genes in undifferentiated cells. There are currently 18 known human HDACs grouped into four classes [40]. Classes I, II, and IV HDACs are zinc-dependent proteins, while class III HDACs require NAD^+ [41].

Interestingly, class I HDACs (HDAC1 and HDAC2) show constitutive nuclear localization and preferentially associate with MyoD [42], while class II HDAC members (HDAC4 and 5) shuttle between the nucleus and the cytoplasm and are dedicated repressors of MEF2-dependent transcription [43, 44]. Upon differentiation, displacement of HDACs from the chromatin of target genes correlates with the hyperacetylation at muscle loci and activation of muscle gene transcription (i.e., Myogenin and Myosin heavy chain) [45].

Given the importance of the balance between acetylation and deacetylation in regulating muscle gene transcription, HDACi are emerging as promising drugs to manipulate the regenerative potential of stem cells in diseased muscle [15] (see below). Despite the general assumption that HDAC inhibition would indiscriminately cause a global hyperacetylation in all organs and tissues, several studies revealed a surprising selective effect of HDACi on embryonic and adult stem cells [46] and in particular on genes with bivalence or with preexisting activator marks [15, 47]. Accordingly genome-wide Chip-seq analysis in myoblasts showed that the large majority of HDACi induced genes were involved in the myogenic differentiation program (i.e., Myosin 7 (MyH7), Enolase 3 (ENO3), and Myomesin 1 (MYOM1)) and showed bivalent (42%) or active (57%) epigenetic marks [48]. This data indicates that, in myoblasts, HDACi enforce and anticipate the expression of genes that are normally induced during differentiation. A bivalent chromatin structure builds an epigenetic signature that identifies genes poised for transcription typically enriched in stem cells. Poised genes show bivalent promoters marked by the presence of both active and repressive histone methylation marks [49, 50].

Methylation is linked to both active and inactive chromatin regions depending on the specific histone and lysine residue that is targeted. In particular, tri-methylation of lysine 4 on histone H3 (H3K4me3) is associated with transcriptionally active promoters, while tri-methylation of lysine 27 (H3K27me3) leads to chromatin condensation [51].

Differentiated cells usually resolve bivalent promoters into an active or repressive state [52, 53] and become resistent to HDACi treatments. Indeed HDACi have been shown to potentiate myogenesis and the gene expression profile selectively in proliferating myoblasts and not in terminally differentiated myotubes. As differentiation precedes, muscle specific genes (i.e., Myogenin and MCK) gradually lose H3K27me3 and gain H3K4me3 [54]. Conversely, progenitor-specific transcription factors (i.e., Pax7) require H3K27me3-mediated epigenetic repression for myotube maturation [55]. This process is finely regulated by the activity of two classes of histone lysine methyltransferases: the Polycomb group proteins responsible of H3K27me3 for epigenetic silencing and the Trithorax group (TrxG) which activates gene transcription catalyzing H3K4me3.

Dynamic changes in the epigenetic landscape of muscle progenitors during differentiation are coordinated by extracellular signals that specifically target the activity and recruitment of chromatin modifier enzymes. For instance, the regeneration-activated p38 signalling targets multiple components of the myogenic transcriptosome that is assembled on the chromatin of muscle genes in response to locally released regeneration cues. p38α/β kinases phosphorylate MEF2D mediating the recruitment of the Trithorax enzymatic subunit Ash2L to the chromatin of muscle genes [56]. Concomitantly p38α kinase promotes the phosphorylation of EZH2, the enzymatic subunit of the Polycomb Repressive Complex 2 (PRC2), targeting Pax7 promoter for repression [55]. Finally p38 signalling promotes the recruitment of the chromatin remodelling SWI/SNF complex to the regulatory regions of MyoD-target muscle genes by the phosphorylation of BAF60c [57].

SWI/SNF complex comprises two mutually exclusive enzymatic sub-units (the ATPases Brg1 and Brm) and several

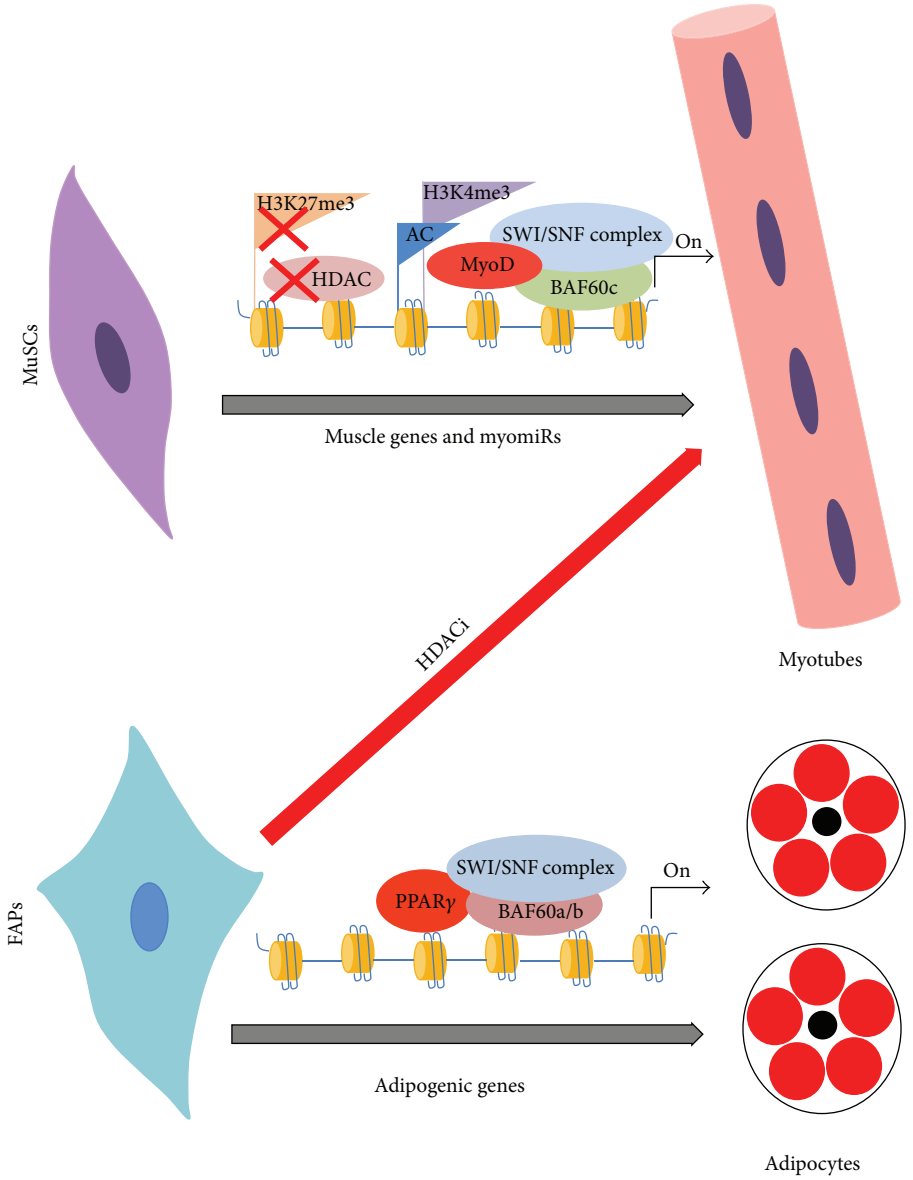

Figure 1: Epigenetic reprogramming of MuSCs and FAPs during differentiation. MuSCs adopt a chromatin permissive structure on muscle genes in which MyoD and BAF60c-based SWI/SNF complex promote transcription (on the top). FAPs differentiation into adipocytes is mediated by BAF60a/b-based SWI/SNF complex (on the bottom). HDACi treatment in dystrophic muscles activates a myomiR/MyoD/BAF60c network that, switching the BAF60 subunits assembled in the SWI/SNF complex, reprograms FAPs toward the acquisition of a myogenic phenotype.

Brg1/Brm associated factors (BAFs) [58]. In particular, three alternative variants of Baf60 sub-unit (BAF60a, BAF60b, and BAF60c) confer the affinity for tissue-specific transcription factors regulating lineage determination in many cell types [19, 59–61]. BAF60c is essential to activate both skeletal and cardiac muscle programs [57, 61], while BAF60a and BAF60b activate alternative lineages, including lipid metabolism [62]. During embryo myogenesis, the negative regulation of BAF60a and BAF60b leads in progenitor cells the activation of a BAF60c-mediated muscle differentiation program [63].

Intriguingly our recent study demonstrated that BAF60 selection can drive lineage determination in a population of fibro-adipogenic progenitors (FAPs) resident in skeletal muscles. Favouring BAF60c incorporation in SWI/SNF complex at expense of BAF60a/b directs the switch from the fibro-adipogenic to the myogenic lineage reducing fibrosis and fat deposition in dystrophic muscles (Figure 1) [19, 64]. These data suggest that therapeutic approaches aim to selectively target the combinatorial assembly of the SWI/SNF complex could be used to manipulate cell fate determination in several disorders.

3. Non-Coding RNAs as Epigenetic Regulators of Gene Expression

A novel emerging level of gene expression regulation is mediated by non-coding RNAs (ncRNAs): functional RNA molecules not translated into proteins, composite of structural and regulatory RNAs. ncRNAs are divided by their size into *long non-coding RNA* (lncRNAs) greater than 200 nucleotides to over 100 kb in length and *small non-coding RNA* (sncRNAs) with a non-coding transcript long less than 200 nucleotides [65].

LncRNAs localize both in the nucleus and cytoplasm and have roles in chromatin remodelling, transcription, intracellular trafficking and post-translational processes controlling cell identity and lineage commitment [66, 67]. LncRNAs located in the nucleus regulate transcription recruiting chromatin-modifying enzymes or interacting with RNA sequences to influence their splicing. Many nuclear lncRNAs associate with EzH2/PRC2 and control the formation of nuclear compartments (i.e., speckles, para-speckles, polycomb bodies) [68, 69]. lncRNAs identified in *cytoplasm* regulate protein localization, mRNA translation and stability. Intriguingly it was recently described their role as sponge for miRNAs: reducing miRNAs levels, they inhibit the miRNA-mRNA mediated target degradation [66]. lncRNAs strongly regulate MuSCs differentiation. The muscle-specific linc-MD1 manages the time of muscle differentiation acting as a sponge to sequester miR-133 and miR-135 that regulate the expression of MAML1 and MEF2C, pro-myogenic transcription factors [66]. lncRNAs transcribed from MyoD enhancer (Enhancer RNAs (eRNAs)) regulate also MyoD and Myogenin expression [70].

sncRNAs family include transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), as well as microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs) [65]. Recent studies suggest the existence of thousands of ncRNAs, many of them involved in the epigenetic regulation of development, physiology, tissue regeneration and disease [71, 72]. The most studied small non-coding RNAs are the miRNAs, molecules containing about 22 nucleotides, expressed in eukaryotes and found well conserved in plants and animals. miRNAs regulate numerous biological processes inhibiting translation of their target mRNA and also mediating their degradation through recognition of imperfect complementary sites, usually located in the 3′-untranslated regions [73, 74]. It seems that miRNAs regulate the expression of more than 50% of mammalian genes making them the most prevalent regulatory mechanism of mRNA availability [20, 75, 76].

miRNAs may fine tuning distinct processes targeting specific epigenetic regulators: DNA methylases, PRC components, Histone Deacetylases and chromatin remodelling complexes members [77]. Given their consistent epigenetic role, miRNAs are important regulators of embryonic and adult myogenesis [78] controlling MuSCs quiescence, proliferation and differentiation [79, 80]. For instance, Rando's group identified in a microarray expression study, about twenty quiescence-specific miRNAs that actively maintain the quiescent state of satellite cells (i.e., miR-489 that targets the oncogene Dek) and 351 miRNAs regulating satellite cells activation [81].

One crucial step for MuSCs activation is miR-31 downregulation that allows Myf5 translation in myoblasts [82]. Myf5, together with MyoD, leads the activation of miR-133a/b that inhibit the adipogenic regulator PRDM16 preventing muscle progenitors cells commitment to adipose cell fate [83, 84]. miR-133 controls also myoblasts proliferation acting as SRF regulator [85] During myogenesis, miR-1, miR-29 and miR-206 target HDAC4 promoting the activity of the myogenic transcriptional elements Mef-2 and MRFs [86]. MRFs in turn regulate the expression of miR-1, miR-133a/b and miR-206, muscle specific miRNA defined as "myomiRs". Finally miR-1 and miR-206 control Pax3/7 repression [87] while miR-26a targets the Ezh2 methyltransferase, to allow muscle differentiation [54, 88].

Different studies demonstrated that miRNAs could modulate the composition of SWI/SNF chromatin remodelling complexes in a way to epigenetically reprogram cell fate determination. Crabtree showed a microRNA mediated switching of chromatin-remodelling complexes in neural development: miR-9 and miR-124 target BAF53a sub-unit driving differentiation of progenitor cells into neurons [89]. Similarly our group identified in muscle interstitial FAPs an analogous miR-based mechanism that regulates the balance toward myogenic versus alternative fates (fibro-adipogenesis). In FAPs myomiRs (miR-1,2as, miR-133a and miR-206) favour the composition of the pro-myogenic BAF60c-SWI/SNF complex by targeting the alternative BAF60a and BAF60b variants [19]. Similarly in Embryonic muscle progenitors myomiRs negatively regulate BAF60a/b to promote the BAF60c-SWI/SNF complex [63].

Interestingly miRNAs derived from various tissues and organs, being stable and resistant to nuclease digestion, are easily detectable in both plasma and serum and may serve as diseases biomarker. Indeed circulating miRNAs profile dynamically change in many diseases such as cancer, myocardial infarction, heart failure, myotonic Distrophy type I and DMD [90–95]. MyomiRs for instance, have been identified in serum of muscular dystrophy animal models and patients where they are passively released as a consequence of myofibers degeneration and breakdown. Their putative active role is still unkown and currently they are proposed as novel diagnostic markers of disease progression. Indeed myomiRs detection in serum is inversely correlated to muscle health, representing a more sensible biomarker than the commonly used Creatine Kinase (CK) [94, 96].

4. Extracellular Vesicles for Genetic Information Transfer and Cell Phenotype Modulation

Extracellular vesicles are emerging as potent sources of genetic information transfer between cells and are involved in regulating stem cell plasticity via epigenetic reprogramming and their ability to alter gene regulatory networks [21]. Cell-derived vesicles such as exosomes and microvesicles possess the capability to mediate intercellular communication by

fusing with the plasma membrane of recipient cells and subsequently delivering their cargo, consisting of functional proteins, mRNAs and miRNAs able to modulate gene expression and cell phenotype [97]. Exosomes are homogenous small particles, usually 30 to 100 nm in size, of endosomal origin. Microvesicles, instead, constitute a larger and heterogeneous population of extracellular vesicles, 50 to 1000 nm in size, and are directly produced through the plasma membrane budding [22].

Multiple cell types have been described to release vesicles in extracellular medium, including mesenchymal cells, adipocytes, fibroblasts, immune cells and myoblasts. Little is known about vescicles regulation of MuSCs in health and diseased muscles. However several studies reporting muscle-exosomes are emerging [98–100]. Myoblasts and myotubes use exosome clustered miRNAs as "endocrine signals" to control important signaling pathways (i.e., Wnt signaling pathway) for muscle homeostasis and regeneration. MiRNAs secreted in exosome by myotubes are functionally able to silence the HDAC Sirt1 in myoblasts, controlling their commitment to differentiation [88]. Muscle behaviour is also influenced by vesicles released from different sources, like mesenchymal stem cells. Indeed it has been recently shown that miRNAs (i.e., miR-494 and myomiRs) released in exosomes from mesenchymal stem cells promote muscle regeneration following injury by enhancing myogenesis and angiogenesis [101]. Indeed exosomes appear to be naturally equipped to mediate tissue regeneration and their cargo constitute a rapid response, protected by the oxidative environment, to initiate tissue repair [102]. Vesicles from mesenchymal stem cells were found to confer therapeutic benefit in a range of different diseases: kidney [103–105] and hepatic injuries [106] myocardial ischaemia and infarction [107–109] and peripheral arterial disease [110]; this "regenerative" effect is mainly due to the ability of extracellular-vesicles to induce phenotypic changes in local stem cells through epigenetic reprogramming to stimulate tissue repair and regeneration [111]. Notably, the transfer of tissue-specific mRNAs, miRNAs and protein-based transcription factors through the extracellular microvesicles was shown to induce phenotype change in bone marrow cells when co-cultured with cells derived from various tissues (brain, heart, liver and lung) [112–114].

Extracellular vesicles mediate communication even between distally located cells and tissue and can be found in many biological fluids including blood, saliva, urine, and breast milk [22]. For instance tumor cells can induce apoptosis in distal skeletal muscles via exosome assembled miR-21, which signals through the Toll-like 7 receptor (TLR7) on myoblasts to promote cell death and cancer cachexia [115].

Given their ability to be readily isolated from most body fluids, circulating miRNAs packed into exosomes are emerging as useful biomarkers to determine the development and progression of various diseases. Moreover, their natural role in transferring genetic material both locally and systemically has inspired pharmacological strategies to exploit these vesicles as therapeutic agents via the introduction of exogenous genetic cargoes such as siRNA (see below).

5. Epigenetic Therapies and Future Perspectives for Muscle Regeneration in DMD

In last years, regenerative medicine focused on the study of plasticity of stem cells epigenome and the recent findings lead the researchers to concentrate on strategies aimed to reprogram the stem cell fate in numerous diseases.

Myogenesis is coordinated by a complex interplay between epigenetic events that are crucial to control lineage determination and differentiation of adult stem cells. Basic research and recent studies of next generation sequencing are clarifying the fine epigenetic regulation of myogenesis and which are the epigenetic players that create changes in the epigenome opening new therapeutic options in muscle diseases as DMD.

HDACi are considered as the first generation of epigenetic drugs with proven clinical efficacy in the treatment of some lymphoid malignancies [116] and are now in clinical trials for a number of other diseases including DMD. Indeed preclinical studies in dystrophic mice (mdx) showed their ability to alleviate both morphological and functional consequences of the primary genetic defect [16, 17]. The current availability of HDACi in clinical practice gave the opportunity for an immediate translation of these drugs into pharmacological treatments of DMD in human patients. The HDACi ITF2357 (Givinostat) represents the first epigenetic drug included into a study therapy for DMD.

Givinostat has already been tested in pediatric populations and received an Orphan Drug Designation by EMA for the treatment of systemic-onset juvenile idiopathic arthritis (SOJIA) [117, 118]. This knowledge encouraged its traslation into a phase I/II clinical trial with children affected by DMD (ClinicalTrials.gov identifier: NCT01761292). After one year of treatment Givinostat efficacy has been monitored showing very promising results on muscle histology and functionality without severe adverse effects on children health; thus the trial has been prolonged for a second year. Obviously this study requires defining the activity of Givinostat in long-term treatments to assess its persistent effect in dystrophic muscles and to monitor possible adverse events.

The functional characterization of the recently identified epigenetic network that determines the ability of HDACi to promote regeneration of dystrophic muscles, at expense of fibrosis and fat deposition, highlights the role of FAPs as key cellular mediators of HDACi activity and DMD progression [18, 19].

FAPs are multipotent mesenchymal cells located in muscle interstitium with the ability to proliferate and support satellite cells mediated muscle regeneration in response to local injury or disease. However, beyond their beneficial role, FAPs have been shown to be the major source of fibro-adipocytes in degenerating muscles [9, 10].

Importantly we have demonstrated that treatment with HDACi at early stages of DMD induces in FAPs a myogenic fate at expense of their fibro-adipogenic lineage. HDACi de-repress a latent myogenic program by activating a MyoD/BAF60c/myomiR network that leads muscle

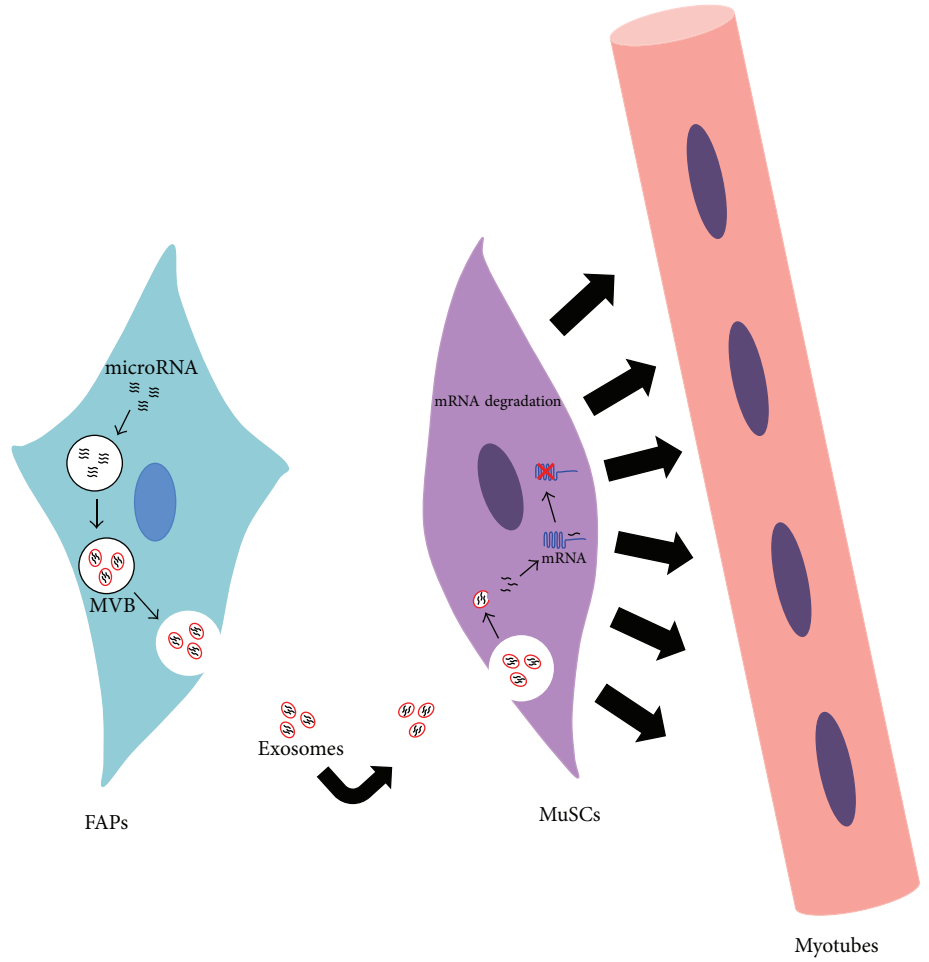

Figure 2: Exosomes as putative mediators of the functional interaction between FAPs and MuSCs. In this model, exosomes released by activated FAPs support myoblasts differentiation through a mechanism by which their cargo of miRNA can be transferred to MuSCs.

differentiation. Indeed HDACi induce MyoD and BAF60c expression, two core components of the myogenic transcriptional machinery, and up-regulate myomiRs (miR-1.2, -133 and -206), which target the alternative BAF60 variants A and B. Switching of the BAF60 sub-units assembled in the SWI/SNF complex reprograms FAPs toward the acquisition of a pro-myogenic phenotype. However the progressive impairment of the integrity of this network prevents HDACi efficacy at late stages of DMD. Indeed, with the progress of the disease FAPs becomes resistant to HDACi and acquire a constitutive fibro-adipogenic lineage replacing the muscle loss with fatty and fibrotic tissues [18]. Importantly, transplantation of "young" FAPs into muscles of "old" dystrophic mice, restored the ability of HDACi to promote regeneration at advanced stages of disease [18]. This suggests that a powerful future therapeutic strategy will be to epigenetically reprogram aged FAPs with selective delivery of Baf60c and myomRs. In this context the natural ability of exosomes to transfer material both locally and systemically encourage the possibility of exploiting these vesicles for therapeutic purposes.

While these data provide new insight into the molecular pathogenesis of DMD and therapeutic approaches to delay the disease, they also highlight the potential of miRs detection as clinical biomarkers of disease progression. The increase of circulating myomiRs in the peripheral blood of dystrophic patients correlates with the severity of the disease, suggesting that myomiR quantification in blood of DMD patients might represent a sensible diagnostic and prognostic marker [64, 94]. On the other hand our recent data, show a great increase of FAPs derived myomiRs in muscle interstitium of mdx mice after HDACi exposure suggesting an inverse correlation between local and circulating myomiRs [19]. This suggests that detection of muscular (local) versus circulating myomiRs could provide a novel more accurate biomarker for diagnosis of DMD progression and efficacy of therapeutic drugs [64].

MiR stability in extracellular environment seems to be preserved by vesicles budding and intriguingly myomiRs have been detected *in vitro* in exosomes released by mesenchymal cells to support myoblasts differentiation [101]. An analogous mechanism is probably involved *in vivo* between FAPs and MuSCs to promote muscle regeneration (Figure 2).

It would be important to investigate if this functional cross-talk mediated by exosome is somehow affected in muscle disorders. Furthermore these data strongly encourage the possibility to re-engineer naturally derived exosomes for DMD epigenetic therapy.

6. Conclusions

In the last years, great advances have been made in the comprehension of the epigenetic mechanisms regulating, via chromatin organization, different transcriptional programs. The functional characterization of the variety of epigenetic regulations in healthy and disease states has the prospect to identify novel targets for epigenetic-based therapies.

HDACi represent the first generation of epigenetic drugs. Their clinical efficacy is currently being tested in a phase I/II clinical trial on children affected by DMD. The pro-regenerative effects of HDACi are mediated by FAPs, a population of muscle-resident stem cells. However, dystrophic muscles at late stages of the disease are resistant to HDACi-induced beneficial effects. This unresponsiveness might be due to a decreased chromatin plasticity of FAPs caused by epigenetic silencing pathways. The identification of the epigenetic players preventing HDACi responsiveness at advanced stages of DMD will be crucial to devise new personalized and selective strategies to re-establish HDACi sensitivity. In this context, a comprehensive epigenetic mapping of the chromatin landscape of key populations involved in muscle regeneration is becoming urgent to identify in the near future both therapeutic effectiveness and inclusion criteria of DMD patients to epigenetic therapy.

Exosome-bound miRNAs are emerging as a crucial mechanism to transfer epigenetic information between cells. New evidence showing the therapeutic relevance of these vesicles in both unmodified and modified forms make them attractive therapeutic agents for further study. Moreover detection of specific miRNAs secreted in muscle interstitium and blood of dystrophic patients holds the promise to develop new painless methodologies, less invasive than classic biopsy, such as blood sampling or fine needle aspiration techniques, to diagnose DMD.

Disclosure

The authors declare that there is no conflict of interests regarding the publication of this paper.

Conflict of Interests

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Review Article

Metaboloepigenetic Regulation of Pluripotent Stem Cells

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The differentiation of pluripotent stem cells is associated with extensive changes in metabolism, as well as widespread remodeling of the epigenetic landscape. Epigenetic regulation is essential for the modulation of differentiation, being responsible for cell type specific gene expression patterns through the modification of DNA and histones, thereby establishing cell identity. Each cell type has its own idiosyncratic pattern regarding the use of specific metabolic pathways. Rather than simply being perceived as a means of generating ATP and building blocks for cell growth and division, cellular metabolism can directly influence cellular regulation and the epigenome. Consequently, the significance of nutrients and metabolites as regulators of differentiation is central to understanding how cells interact with their immediate environment. This review serves to integrate studies on pluripotent stem cell metabolism, and the regulation of DNA methylation and acetylation and identifies areas in which current knowledge is limited.

1. Introduction

Resurgence in metabolic research has revealed metabolism to be at the heart of cell-sensing mechanisms. Not only does metabolism provide ATP to maintain homeostasis and cell replication and intermediates that form the basic building blocks for cell proliferation, but also metabolic processes and products can modulate signalling pathways, transcription factor activity, and gene expression. Metabolites can induce long-term changes to the cell through the regulation of the epigenome, a phenomenon referred to as metaboloepigenetics. Every cell type has a unique metabolic phenotype and a unique epigenetic profile, reflecting their cellular niche and function. It is hypothesized that not only does the pattern of metabolism observed in different cell types serve to fulfil that cell's specific functions, but also metabolism is involved in establishing the epigenome of the cell during development. This implies that the intra- and extracellular metabolic environment, in which cells reside, either *in vivo* or *in vitro* can have a profound effect on cellular phenotype. Further, the ability of cells themselves to modify their own environment in order to facilitate their function warrants consideration.

The pluripotent epigenome must maintain transcription of pluripotency-related genes, while being poised for rapid, lineage-specific gene activation upon differentiation [1–3]. Concomitantly, cells constantly modulate their metabolic state in response to extracellular signals, including nutrient availability [4]. Significant changes in metabolism accompany the transition from the early embryo through differentiation [5, 6]. The availability and activity of metabolic cofactors and enzyme substrates, generated through cellular metabolism, can impact the regulation of transcription through modulation of epigenetic processes, including histone methylation and acetylation. Metabolism is consequently emerging as a central player in the regulation of epigenetics and gene expression.

Here we review recent advances in our understanding of the roles of metabolites and cofactors in modulating the pluripotent stem cell epigenome. We discuss how stem cell metabolism and chromatin modifications are interconnected, how their interactions can impact stem cell state and differentiation, how culture conditions have the potential to induce (erase/generate) epigenetic marks, how these processes could significantly impact the utility of cells, and the potential

for metabolic alterations to induce epigenetic deregulation. We refer the reader to existing reviews on mitochondrial characteristics of pluripotent stem cells [7–9].

2. Defining Pluripotent Stem Cell States

In the embryo and in culture, pluripotent cells have been shown to comprise a lineage of temporally distinct cell states (reviewed in [10]). Pluripotent stem cells, either embryonic (derived from the inner cell mass (ICM) of the blastocyst stage preimplantation embryo; ES cells) or reprogrammed from a somatic cell to an embryonic stem cell-like state (induced pluripotent stem cells; iPS cells) are defined by their ability to self-renew (to proliferate indefinitely) and by pluripotency, as shown by the ability to act as a founder cell population for all the cells of the embryo and adult. These properties underpin the potential use of these cells as a source of clinically relevant cells for therapeutics and drug discovery. Many studies have focused on defining the molecular properties of ES cells but only recently have we begun to investigate the physiology and metabolism of these cells.

Mouse and human ES cells differ in their growth factor requirements *in vitro*, a consequence of their origins from different developmental stages. Mouse ES cells isolated from the ICM are reliant on leukemia inhibitory factor (LIF) for ongoing propagation, while also requiring serum [11]. Alternatively, mouse ES cells can be isolated in medium supplemented with inhibitors of Mek/Erk and GSK3 activation [12]. Human ES cells are derived from a later stage pluripotent cell population, more similar to postimplantation epiblast [13], and are dependent on activin/nodal and fibroblast growth factor (FGF) signaling for self-renewal and pluripotency [14–16]. The tissue of origin and gene expression profile of human ES cells suggest that they are representative of a later stage pluripotent cell state. Pluripotent cells have been isolated from the postimplantation epiblast or primitive ectoderm of the mouse. Like human ES cells, epiblast stem cells (EpiSC) require FGF and activin A for self-renewal and pluripotency [17–19]. In culture these cells adopt the phenotype of the anterior primitive ectoderm from the late gastrula stage embryo [17].

Mouse ES cells can be cultured with inhibitors of Fgf, Mek/Erk, and Gsk3 to form a naïve ES cell state, representative of the early inner cell mass [20–22]. Inclusion of the GSK3 inhibitor minimizes the negative regulation of biosynthetic pathways [12], thereby modulating proliferative capacity. Alternatively, mouse ES cells can be cultured in medium supplemented with the amino acid L-proline to form early primitive ectoderm-like (EPL) cells that represent a stage of pluripotency intermediate to ES cells and EpiSC [23].

Each population, isolated or cultured, represents a stem cell state within the continuum of the pluripotent lineage.

3. Framework of Pluripotent Stem Cell Metabolism

Pluripotent stem cells and the pluripotent cells of the ICM from which they are derived exhibit a metabolism characterized by high levels of glucose consumption, combined with the production of lactate (reviewed by [9, 24]; Figure 1). This pattern of metabolism is maintained in conditions of oxygen sufficiency, distinguishing it from anaerobic glycolysis, and has therefore been termed aerobic glycolysis. Warburg [25] first described aerobic glycolysis in cancer cells in culture, which produce large amounts of lactate even in the presence of sufficient oxygen for the complete oxidation of glucose (the Warburg effect). Initially it was thought that aerobic glycolysis was specific to cancers, but it has since been shown that this metabolic trait occurs in other proliferating cells types such as lymphocytes [26]. Gardner [27, 28] identified similarities between cancers and blastocysts and the significance of the Warburg effect for the development of the late stage embryo.

What are the cellular advantages of aerobic glycolysis to pluripotent stem cells? Glucose is typically considered in its capacity as an energy source. Pluripotent cells are highly proliferative, with reports of cell cycle rates reducing to as little as 5–7 hours in the developing embryo [29]. To maintain this growth rate these cells will have to generate building blocks for proteins, nucleic acids, lipids, and carbohydrates. The glycolytic metabolism of glucose may not generate as much ATP as oxidative phosphorylation per mol of glucose, but it can readily create equal amounts of ATP by an increased flux of glucose. Further, enhanced glycolytic rate plays a significant role in the generation of metabolic intermediates for biosynthesis. The carbon in glucose is utilized in the synthesis of triacylglycerols and phospholipids. Glucose is precursor for complex sugars of mucopolysaccharides and glycoproteins [27, 28]. Metabolism of glucose through the pentose phosphate pathway (PPP) generates ribose moieties required for DNA and RNA synthesis and the NADPH required for the biosynthesis of lipids and other complex molecules [30, 31]. Aerobic glycolysis is therefore the mechanism that ensures sufficient carbon flux through biosynthetic pathways in rapidly dividing cells [27, 30, 32, 33].

The preferential metabolism of glucose through glycolysis reduces the use of oxidative metabolism in pluripotent cells, with a concomitant decrease in the formation of reactive oxygen species (ROS) associated with oxidative phosphorylation [34]. From a developmental perspective, this reduces oxidative stress within the cell, thereby reducing the risk of DNA damage. Given that pluripotent cells *in vivo* and *in vitro* act as founders for all cell types of the embryo and adult, a metabolism that promotes genetic stability would represent an evolutionary adaptation for successful and faithful propagation.

4. Key Metabolites Define the *In Vivo* Pluripotent Stem Cell Niche

Maintenance of pluripotency relies on a balance of complex cellular and acellular signals within the surrounding microenvironment. High levels of aerobic glycolysis in pluripotent cells form a localized area or niche, characterized by relatively high concentrations of lactate and low extracellular pH surrounding the blastocyst (and potentially around cell colonies in culture). The blastocyst uses this microenvironment to facilitate the implantation process [24].

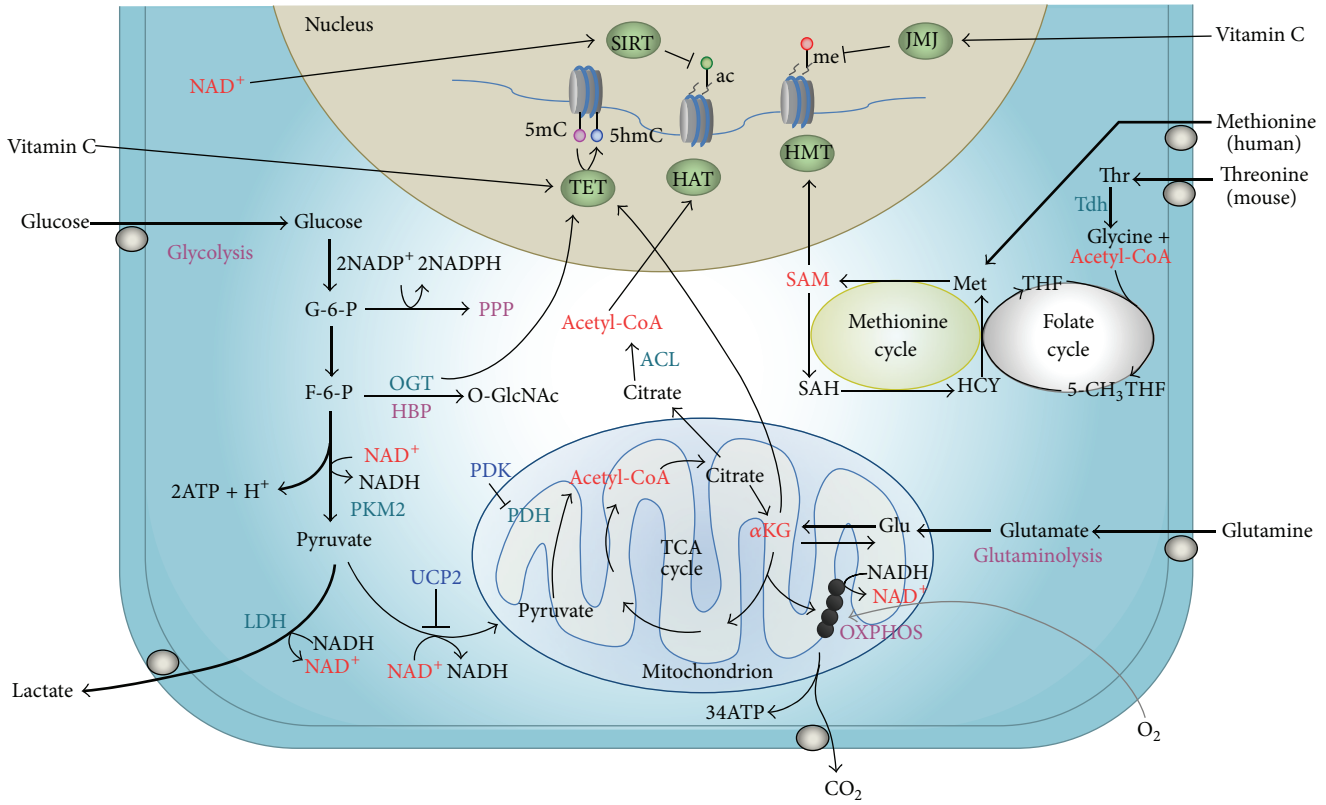

Figure 1: Metabolic regulation of the pluripotent epigenetic landscape. Pluripotent stem cells are characterized by spherical, electron-poor mitochondria, which contain few cristae. These cells rely heavily on glycolysis for ATP generation (thick black arrows), resulting in significant lactate production through the conversion of pyruvate to lactate by lactate dehydrogenase (LDH). Consequently, oxidative phosphorylation (OXPHOS) contributes minimally to total ATP. Glucose metabolized by the pentose phosphate pathway (PPP) generates the ribose moieties required for DNA and RNA synthesis and the NADPH required for the biosynthesis of lipids and other complex molecules. Intermediate metabolites generated via metabolic pathways act as cofactors for epigenetic modifying enzymes. Threonine and methionine metabolism is required for S-adenosyl methionine (SAM) generation via the folate and SAM cycles in mouse and human pluripotent stem cells, respectively [60, 61]. SAM acts as a methyl donor for histone methyltransferases (HMT) as well as DNA methyltransferases. Demethylation of SAM yields S-adenosylhomocysteine (SAH), which is, in turn, hydrolyzed to homocysteine. Transfer of a methyl group to homocysteine from the folate pathway regenerates methionine. Acetyl coenzyme A (Acetyl-CoA) acts as a cofactor for histone acetyltransferases (HAT). Acetyl-CoA, generated from glucose derived pyruvate, modulates human pluripotent stem cell histone acetylation [76], although pyruvate dehydrogenase kinase (PDK) activity may limit the conversion of pyruvate to acetyl-CoA [79]. Similarly, uncoupling protein 2 (UCP2) functions to shunt pyruvate away from the tricarboxylic acid (TCA) cycle, facilitating lactate production [79]. Acetyl-CoA can also be generated from threonine catabolism [62]. Alpha-ketoglutarate (αKG) [65] or supplementation with vitamin C in culture [122] reduces histone and DNA methylation in human pluripotent stem cells, respectively. These metabolites modulate histone and DNA demethylation reactions catalyzed by Jumonji (JMJ) and Ten-Eleven Translocation (TET) demethylases, respectively [101]. The hexosamine biosynthetic pathway (HBP) is an alternative route of glucose utilization that generates the coenzyme UDP-GlcNAc, which together with O-linked N-acetylglucosamine transferase (OGT) leads to histone O-GlcNAcylation [82]. Flux through glycolysis and oxidative phosphorylation determines the NAD^+ : NADH ratio, known to regulate the activity of the NAD^+-dependent histone deacetylases sirtuins (SIRT; [90]). In addition, a proline-dependent mechanism of epigenetic regulation has been reported in pluripotent stem cells [74]; however, it is unclear how the metabolism of proline interacts with these pathways. Metabolic regulators of chromatin-modifying enzymes are highlighted in red.

This environment assists in extracellular matrix degradation, angiogenesis, and immune-modulation of the mother at the implantation site. Lactate, as it would appear, is a very important signalling molecule that elicits numerous effects in the cell of origin and surrounding tissues. Some of these effects could be modulated through lactate-responsive transcription factors. Many cancers appear to recreate an embryonic-like phenotype and coopt embryonic pathways. Cancers, like blastocysts, generate a microenvironment characterized by high lactate and reduced external pH, created through aerobic glycolysis, to facilitate tissue invasion, angiogenesis, and immunomodulation. The role of such a microenvironment in *in vitro* stem cell culture has not been considered, though it is likely to have a profound effect on pluripotent stem cells and those cells surrounding them.

Low oxygen is a characteristic of the stem cell niche *in vivo*, where the oxygen concentration within the reproductive tract approximates 2–8% [35, 36]. Within the oviduct, early

embryo development takes place in an oxygen concentration from 5 to 8.5% (35–60 mm Hg) in the rabbit, hamster, and rhesus monkey [35]. Around the time of compaction, coinciding with the first lineage specification event, the embryo traverses into the uterus, which has a lower oxygen concentration of 1.5–2% oxygen in the rhesus monkey, 3.5% in the rabbit, 5% in the hamster [35], and 4% in the rat [36]. Decreased oxygen in the uterus is particularly evident at the time of implantation, when in rabbits and hamsters a decrease from 5.3% to 3.5% oxygen is seen [35]. Therefore, embryos appear to encounter a decreasing oxygen concentration gradient as they progress from the oviduct to the uterus. Furthermore, during the time of early implantation, hypoxia and even anoxic conditions confront the invading trophectoderm (reviewed by [37]). At concentrations of 7% oxygen and lower the activation of hypoxia-inducible factors (HIFs; [38], reviewed by [39]) occurs within cells. HIFs modulate cellular homeostasis through the regulation of glucose metabolism, pH, angiogenesis, and iron metabolism, supporting a high rate of glycolysis.

5. Regulation of Epigenetic Modifiers by Metabolic Cofactors

Cell state transitions are characterized by global changes in the epigenetic landscape [40, 41]. As differentiation proceeds, epigenetic modifications progressively restrict gene expression, silencing pluripotency genes and activating lineage-specific genes [42]. Underlying pluripotency, ES cells are characterized by an open and highly dynamic chromatin landscape (reviewed by [43]). Progression of the pluripotent lineage and early events in differentiation are accompanied by changes in the genomic architecture. This is evidenced in changes in mean replication timing (MRT) at loci across the genome. Changes in MRT provide evidence of changes in the genomic organization that underpin the establishment of cell identity [40]. Large changes in MRT accompany the global genome reorganization, also known as autosomal lyonization, and occur as EPL cells differentiate to cells representative of a later primitive ectoderm (EpiSC and EBM6; [44]) and the germ lineage progenitors. Coincident with autosomal lyonization are changes in the nuclear architecture and the formation and accumulation of late-replicating heterochromatin at the nuclear periphery [44].

Stable modifications to DNA are catalyzed by DNA methyltransferases (DNMTs). In general, DNA methylation can modify chromatin architecture and prevent transcription factor binding within gene promoters, restricting gene expression. Methylation of lysine and arginine residues within histones H3 and H4 is catalyzed by residue-specific methyltransferases (HMT) and can be associated with either transcriptional repression or activation. Establishment of methylation patterns such as H3K4 di- or trimethylation (H3K4me2/me3) and H3K27 trimethylation (H3K27me3) are generally associated with transcriptional activation, while H3K9me2/3 and H3K27me2/3 are associated with transcriptional repression. S-Adenosyl methionine (SAM) acts as the primary methyl donor for DNA and histone methylation, generated through one-carbon metabolism. This pathway integrates the folate and methionine cycles (Figure 1), the latter having metabolic inputs from methionine, serine, and glycine metabolism. ES cells are characterized by elevated global transcriptional activity [45]. Repressive marks, such as H3K9me3, are low in ES cells compared with differentiated cells [46]. Bivalent methylation, marked by a combination of active H3K4me3 and repressive H3K27me3 at a subset of developmental regulators, has been proposed to establish a primed epigenetic state, ready for activation prior to ES cell differentiation [2] and to safeguard differentiation [3].

DNA demethylation takes place either passively or actively. Passive demethylation occurs with DNA replication in the absence of maintenance methyltransferases. The process of active demethylation is catalyzed by Ten-Eleven Translocation (TET) dioxygenases, responsible for the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [47–49]. TET activity is dynamically regulated by alpha-ketoglutarate (αKG), a product of the TCA cycle, and succinate [50]. Tet1 and Tet2 are highly expressed in mouse ES cells [51], with Tet1 also enriched in the inner cell mass of mouse blastocysts [51]. High 5hmC levels are present in mouse ES cells and decrease significantly after differentiation [49, 52]. In a similar manner, Jumonji demethylases are regulated by αKG [53]. Jmjd1a and Jmjd2c knockdown leads to mouse ES cell differentiation, regulating pluripotent gene expression [54].

Histone acetylation participates in multiple chromatin-dependent processes, including gene regulation, DNA replication, and DNA damage repair. Acetylation is generally associated with a more open chromatin configuration, permissive to transcription, while deacetylation is associated with condensed, compact chromatin leading to transcriptional repression. Acetylation is catalyzed by histone acetyltransferases (HATs), which transfer an acetyl group from acetyl coenzyme A (acetyl-CoA) to lysine residues, with the concomitant production of CoA. Cellular acetyl-CoA levels fluctuate in response to various physiological cues, including nutrient availability and metabolic activity. A major source of acetyl-CoA in cells is the conversion of citrate via ATP citrate lyase (ACL). siRNA-mediated silencing of ACL significantly decreases histone H2B, H3, and H4 acetylation in HCT116 colorectal cancer cells [55]. Glycolysis plausibly has a significant role in modulating acetyl-CoA levels, and glucose availability can affect histone acetylation in an ACL-dependent manner [55].

Histone deacetylation is catalyzed by NAD^+-independent or NAD^+-dependent deacetylases (HDACs). Class I and II HDACs are dependent on zinc, while the activities of the sirtuin family of histone deacetylases (class III) are reliant on NAD^+ for their catalytic activity. Sirtuins (SIRTs) act as sensors of environmental stimuli and deacetylate histone and nonhistone substrates. In addition, they have roles in the regulation of a number of metabolite pathways, including glycolysis, the TCA cycle and fatty acid oxidation, telomere maintenance, tolerance to oxidative stress, and DNA repair. High rates of glycolysis establish a high $NADH/NAD^+$ ratio, which downregulates sirtuin activity. Studies of pluripotent stem cell histone acetylation have largely focused on

the regulation of deacetylation by class I and II HDACs and their inhibitors, in the context of differentiation. A genome-wide reduction of H3K9 acetylation (H3K9ac) is required for mouse and human ES cell differentiation [56, 57]. Partial inhibition of ES cell HDAC activity has been shown to promote ES cell self-renewal [58, 59]. Therefore, acetylation maintains a highly dynamic configuration permissive to transcriptional activation.

6. Linking Pluripotent Stem Cell Metabolism with Epigenetics and Cell State

6.1. The Role of Essential Amino Acids in Modulating Pluripotent Stem Cell Methylation. Pivotal studies in mouse ES cells highlight the role of specific amino acids in pluripotent cell regulation. Depletion of individual amino acids from ES cell culture identified threonine as a critical regulator of pluripotency. Threonine catabolism supports mouse ES cell self-renewal, while elimination of threonine from culture medium results in slowed proliferation and increased differentiation [60, 61]. Threonine catabolism contributes to cellular glycine and acetyl-CoA levels, the former being required for SAM synthesis through the SAM cycle (Figure 1). Depletion of threonine from the culture medium or knockdown of threonine dehydrogenase (Tdh) in mouse ES cells decreased SAM accumulation [60] and altered differentiation potential [62]. Analysis of ^{13}C-labelled threonine demonstrated that threonine contributes significantly to the acetyl-CoA pool in mouse ES cells and that glycine derived from ^{13}C-Thr contributed to SAM synthesis. Removal of threonine leads to the loss of methylated histone H3. Threonine reduction, not sufficient to induce cell death, was accompanied by a decrease in H3K4me3 [62], suggestive of a more repressive epigenetic landscape. Loss of H3K4me3 could be rescued by supplementation with threonine or with glycine and pyruvate, associated with an increase in the SAM/SAH ratio [62].

Human ES cells also require SAM but generated through an alternative metabolic pathway, a consequence of their lack of a functional TDH [63]. Systematic elimination of amino acids from culture medium identified methionine as a critical amino acid. Methionine deprivation resulted in a reduction in cell number within 5 hours, attributable to increased cell death, and was associated with a reduction in SAM levels and NANOG expression [64]. Knockdown of the methionine adenosyltransferases, MAT2A and MAT2B, that catalyze the conversion of methionine to SAM similarly decreased cell numbers after 48 h, suggesting that SAM, rather than methionine, was essential for cell survival. The early reduction in cell numbers on methionine depletion is reversible but later impacts on cell proliferation following prolonged methionine deprivation are not [64]. We interpret this to mean that maintenance of SAM levels is critical for stem cell survival and reductions in the metabolite interfaces with apoptosis machinery. Short (5 h) and long (24 h) term methionine deprivation led to a rapid decrease in H3K4me3, accompanied by a modest reduction in global DNA methylation; the effects of short term deprivation could be abrogated through supplementation with SAM [64].

These approaches identify SAM as a major methyl donor within pluripotent cells and show that reduction of SAM reduces histone methylation. The processes used to reduce SAM within the cells likely reduce levels below physiological ranges and impact on the pools of other important metabolites. Shyh-Chang et al. [62] detected reduced NADH/NAD$^+$ and glycine and increased ATP, glucose-6-phosphate, and fructose-6-phosphate, within the first 6 hours of threonine depletion, suggesting that culture media are rapidly depleted of other nutrients, essentially starving cells. This alone may account for the increased cell death observed in both studies, particularly as human ES cells were shown to replenish SAM within 24 hours through recycling of homocysteine [64]. These data suggest that threonine and potentially methionine are critical to maintain metabolic balance and therefore cell survival, within pluripotent stem cells in roles independent of SAM generation. The question remains whether the modulation of SAM concentrations within cells within a physiological range provides a mechanism for the cells to link their metabolome with their epigenome. These data suggest that homocysteine may be important in modulating cell survival. The methionine pathway is reliant on transfer of a methyl group to homocysteine from the folate pathway to regenerate methionine. The ability of glycine, in the presence of pyruvate, to restore cell survival after short term methionine withdrawal implicates the folate pathway in modulating pluripotent cell survival.

Shiraki et al. [64] noted that short term methionine deprivation potentiated subsequent cell differentiation, with more cells exhibiting lineage-specific marker expression on day 4 when induced to differentiate with known differentiation-inducing conditions. It will be important to understand whether methionine deprivation potentiates differentiation by poising cells in a more primed state relative to methionine supplemented conditions or whether methionine deprivation selects a population of cells more receptive to differentiation-inducing conditions.

6.2. Glutamine Regulates ES Cell Methylation. Glutamine has been shown to regulate pluripotency and histone methylation. Most proliferating mammalian cells rely on the catabolism of two molecules, glucose and glutamine, to fulfill their energy, carbon, and nitrogen requirements [4]. As expected, naïve and primed cells consume glucose and glutamine, although steady-state levels of TCA cycle intermediates were lower in naïve ES cells [65]. Neither naïve nor primed mouse ES cells were able to proliferate in the absence of glucose [65], demonstrating an absolute requirement for this metabolite. These cell states could, however, be distinguished by their ability to proliferate in the absence of glutamine, with naïve, but not primed, ES cells able to proliferate, albeit at a reduced rate, in glutamine deficient medium [65]. The proliferation of naïve ES cells in glutamine-deficient medium was supported by an increase in glutamate production from glucose, while the addition of precursors of glutamine synthesis to the medium of primed ES cells enabled proliferation in glutamine-depleted medium [65].

This suggests that the transition from naïve to primed ES cells establishes a reliance on glutamine and TCA cycle activity to support proliferation.

Naïve ES cells exhibited an increased αKG : succinate ratio (Carey et al. 2015), where elevated αKG could impact the epigenome through modulation of Jumonji and TET activity. Following glutamine deprivation, increased H3K9me3, H3K27me3, H3K26me3, and H4K20me3 levels were detected in naïve ES cells, which could be reversed through medium supplementation with cell permeable αKG [65]. These results suggest that the high levels of intracellular αKG found in naïve ES cells, sustained through glucose-dependent glutamate production, maintain an epigenetic landscape characterized by low levels of histone methylation. As cells progress to the primed state, their metabolic phenotype changes, glucose-dependent glutamate production and intracellular αKG levels decrease, and a concomitant increase of histone methylation accumulates, consistent with the higher levels of methylation seen in these ES cells.

6.3. L-Proline Metabolism Induces Changes in the Epigenome That Reflect Pluripotent Cell Identity. Pluripotent early primitive ectoderm-like (EPL) cells can be formed from primed ES cells in culture [66]. The loss of ICM- and ES cell-specific marker gene expression, coupled with increased expression of the primitive ectoderm markers [23, 66–68], increased proliferation rate [23], and a restricted ability to form cell populations characteristic of the primitive endoderm lineage [69, 70] show EPL cells to be distinct from ES cells and align them with the embryonic primitive ectoderm. The amino acid L-proline has been shown to induce the differentiation of ES cells to EPL cells [23, 71–73]. L-Proline activity is facilitated by uptake via the amino acid transporter, SNAT2; inhibition of L-proline uptake through SNAT2 prevents EPL cell formation [71]. L-Proline activity is reliant on intracellular L-proline concentration and L-proline metabolism. Inhibition of proline dehydrogenase prevents the formation of EPL cells ([72], Rathjen unpublished), and removal of L-proline from EPL cells leads to the reestablishment of the ES cell phenotype. Suppression of L-proline biosynthesis, and creation of a shortage within the cell, has been hypothesized to safeguard ES cell identity and prevent autoregulation of differentiation [73].

The addition of L-proline to ES cells induces changes to the epigenome and transcriptome. Analysis of the gross genomic organization of ES and EPL cells has shown these cells to be similar, but repeatable changes in MRT do occur with EPL cell formation and these can be used to distinguish the two cell types [40]. It follows that the metabolism of L-proline and the cell identity changes induced by this process are manifest in changes to genome architecture. Analysis of histone methylation patterns in proline-treated cells showed that epigenetic remodelling, in part, regulated changes to the transcriptome. The addition of L-proline increased the methylation of H3 at lysines 9 and 36 and induced a reprogramming of H3K9 and H3K36 methylation status across the genome [74]. Changes in methylation correlated with loci that were regulated by L-proline. The epigenetic changes induced by L-proline were suppressed when ascorbic acid was added to the cells; to date, the method of ascorbic acid action is not known. The availability of L-proline to the ES cell, which represents the balance of L-proline availability and synthesis in the cell and subsequent metabolism, regulates pluripotent cell identity, with low availability enforcing an ES cell state and increased levels of L-proline inducing EPL cell formation [23, 71, 73]. As part of this process L-proline induces changes to the epigenome characteristic of the EPL cell state.

6.4. Glucose Regulation of the Pluripotent Stem Cell Epigenetic Landscape. A high glycolytic rate drives citrate synthesis, leading to the production of cytosolic acetyl-CoA. In turn, acetyl-CoA can act as a cofactor for histone acetylation [75]. Moussaieff et al. [76] have shown that acetyl-CoA levels in human ES cells were twofold higher than those found in their differentiating counterparts. In ES cells, glucose flux through glycolysis was the primary contributor to the acetyl-CoA pool; as cells differentiated the ability to generate acetyl-CoA through this pathway was lost [76]. Acetyl-CoA levels reduce significantly upon mouse ES cell differentiation, although in these cells this was a result of reduced threonine catabolism [61]. The addition of acetate, a precursor of acetyl-CoA, to differentiating human ES cells delayed cell differentiation [76].

The initiation of human ES cell differentiation led to a loss of H3K9/K27 acetylation (H3K9/H3K27ac), marks that are associated with transcriptional repression, while the addition of acetate to differentiating cells blocked this loss. Inhibition of glycolysis by 2-deoxyglucose, previously associated with differentiation [77], similarly decreased H3K9/H3K27ac, an effect that could be reversed with the addition of acetate [76]. The study by Moussaieff et al. suggests that the generation of acetyl-CoA, from glucose-derived pyruvate, is required to support maintenance of a pluripotent epigenetic landscape. Others have questioned the use of pyruvate by pluripotent stem cell mitochondria [78, 79]. Direct quantification of acetyl-CoA flux from pyruvate is needed to clarify the activity of this pathway.

These data support a link between metabolites, metabolism, and epigenetic regulation (summarized in Table 1), but it is still difficult to define this relationship in pluripotent cells. There remains a need to understand more fully the regulation of pluripotent stem cell metabolism, the relative activity of metabolic pathways, and the impact of nutrient availability on pathway activity. The study by Moussaieff et al. (2015) has identified glucose as a source for acetyl-CoA, correlated acetate availability with the level of histone acetylation in pluripotent stem cells, and differentiated derivatives. Coincident with changes in metabolism with differentiation [76], lower levels of H3K9ac have been observed following the initiation of differentiation compared with ES cells [56, 80], along with other global changes in the epigenetic landscape [43, 44]. Conceivably, modulation of the metabolic intermediate pool may serve to facilitate these dynamics and the widespread nature of epigenetic change with the initiation of differentiation.

6.5. Other Potential Pathways Modifying Epigenetic Regulator Activity. Pluripotent cells in the embryo and in culture are poised to undergo the most extensive epigenetic regulation

Table 1: Summary of metabolites linked with epigenetic modifications in pluripotent stem cells.

Metabolite	Epigenetic intermediate	Metabolic pathway	Epigenetic target	References
Threonine	SAM (via glycine) Acetyl-CoA	Folate/SAM cycles	H3K4me3	[60–62]
Methionine	SAM	SAM cycle	H3K4me3	[64]
Glutamine	αKG	TCA	H3K9me3 H3K27me3 H3K26m3 H4K20me3	[65]
L-Proline	*TBD*	*TBD*	MRT H3K9me H3K36me	[74]
Glucose	Acetyl-CoA (from citrate)	(glycolysis-derived) Pyruvate oxidation	H3K9/K27ac	[76]
TBD	*TBD*	O-GlcNAc	me/TETs	[83]
TBD	NAD^+	*TBD*	ac/sirtuins	—

event that will occur within the organism's lifespan or during ES cell differentiation, respectively. Not surprisingly, within these cells a number of metabolic pathways appear to be poised to change activity, potentially to provide or limit the pools of modification donors, such as SAM and acetyl-CoA, and to respond to the processes with metabolic regulatory cues. Our knowledge of the metabolic positioning of a pluripotent cell is, however, remarkably sparse. It is highly likely that similar roles for other metabolic pathways in pluripotency, cell state transitions, and lineage specification will be shown. Pathways that are still to be examined include biosynthetic pathways, like the hexosamine biosynthesis pathway and many amino acid synthetic pathways, nutrient sensing mechanisms, and metabolic regulatory mechanisms, such as the sirtuin family of histone deacetylases.

The O-GlcNAc transferase (Ogt) is essential for ES cell viability and loss of the gene disrupts embryogenesis [81] and ES cell self-renewal [82]. More recently, Ogt has been shown to associate preferentially with transcriptional start sites of a number of genes in ES cells and regulate gene expression of genes involved in metabolic and signaling pathways. Ogt associates with Tet1 in a complex in these cells, and Tet1 promotes DNA binding of Ogt [83]. It is tempting to speculate that Ogt is affecting gene regulation in association with Tet1 and through localized epigenetic modifications in gene promoters. The activity of Ogt would appear to be pleiotropic within the cell and alternative mechanisms of gene activation and repression may account for Ogt activity. Further, the colocalization of Ogt, Tet1, and H3K4me3 at hypomethylated, CpG-rich gene promoters may act to maintain these areas free of methylation and modulate the epigenome to maintain the pluripotent state.

It is known that SIRT1, a member of the sirtuin histone deacetylase family, is expressed in the preimplantation embryo and in ES cells [84–86]. Inhibition of SIRTs in the embryo negatively impacts blastocyst development and increases ROS production [87, 88]. In pluripotent cells, SIRT1 levels are downregulated as the cells commence differentiation [84, 89]. These proteins have been shown to play key roles in linking the metabolome with the epigenome by acting as sensors of environmental stimuli, regulating a number of metabolic pathways, and modulating the acetylation of histone and nonhistone substrates (reviewed by [90]). It remains to be determined if any of the roles SIRT1 plays in the early embryo and pluripotent cells are attributable to SIRT1-mediated modulation of histone acetylation and the epigenome.

7. Replicating the *In Vivo* Environment and Cell States *In Vitro*

Within the niche the metabolism of pluripotent cells is finely balanced, configured to supply the metabolites required for growth and for maintenance of DNA, and yet poised to respond to the challenges that will be placed on the cell as it differentiates and specifically the requirements of the massive DNA remodeling that accompanies the early events of differentiation. Optimization of *in vitro* culture conditions for pluripotent cells has largely centered on propagating cells that are pluripotent, differentiation competent, and grossly karyotypically normal. It has been assumed that *in vitro* culture conditions, based on commonly used tissue culture media and developed with a focus on growth factor regulation, will sustain the metabolism of the cell. It is known that common culture media are not suitable for embryo culture and significant increases in embryo viability have been achieved by developing media that recapitulate the physiological environment [91]. Our greater understanding of how metabolites can impact not only cell physiology, but also epigenetics, raises questions about the impact of medium metabolite concentrations on the pluripotent epigenome and on cell state.

The composition of the culture medium can significantly influence metabolic pathway use in pluripotent cells, and variations in culture conditions could underlie much of the variability that exists in studies elucidating pluripotent metabolism. We have shown that serum supplementation and supplementation with serum replacer of human ES cell culture changed the production and consumption rates of amino acids and increased glucose uptake by the cells [92]. In contrast to others, however, we were unable to show alterations of metabolism on the induction of differentiation, a difference we ascribe to the maintenance of base medium composition throughout differentiation in our experiments [92]. In a similar way, metabolic differences between naïve and primed ES cells may merely reflect base medium variability. Zhou et al. (2012) attributed metabolic change with cell state without recognizing the potential influence of changing base media [93]. In contrast, Carey et al. (2015) showed that glutamine independence could be established in primed cells independent of base media. Clearly there are metabolic differences between cell states; however it is difficult to distinguish true metabolic differences when base medium composition is not maintained.

A number of protocols for establishing naïve pluripotency have recently been developed for human cells [94–97]. Each of these methodologies used distinct culture conditions that will establish inherent differences in underlying metabolite use. The consequence of these changes in metabolism may not impact pluripotency, differentiation capacity, or gross karyotype but may result in significant alterations to the epigenome. Epigenetic codes will likely be perpetuated during self-renewal and differentiation, potentially influencing future cell events. We have advocated the need to understand the interplay between metabolism and the culture medium to enable true optimization of development in the embryo [98, 99] and in pluripotent stem cells [9].

Abnormalities in cellular metabolism have been linked with alterations in the epigenetic landscape, contributing to numerous diseases including cancer [100, 101]. It will, therefore, be important to establish the metabolic mechanisms regulating pluripotent stem cell epigenetics that underlie pluripotency and differentiation and examine the impact of metabolic perturbations on epigenetic control to ensure these cells and their differentiated derivatives exhibit a normal physiology and are not predisposed to disease states.

7.1. Oxygen: The Forgotten Metabolite. The majority of all tissue culture, including the majority of pluripotent cell culture, is performed in the presence of atmospheric oxygen (~20%). The *in vivo* environment in which prevascularisation embryos develop constitutes a relatively low oxygen environment (2–8%). Departure from a physiological oxygen concentration in culture significantly impacts preimplantation embryo development. While embryos are capable of developing under 20% oxygen, this has been associated with increased DNA fragmentation [102–104], altered genomic [105, 106] and proteomic profiles [107], and perturbed metabolic activity [91, 108]. The changes induced by atmospheric oxygen are not consistent with the viability of the blastocyst [99]. Despite the embryonic requirement for physiological oxygen and the negative impact of atmospheric oxygen on embryo viability, cell derivatives of the embryo, including pluripotent cells, are routinely cultured and characterized in 20% oxygen.

We have documented oxygen-dependent changes in pluripotent cell metabolism that occur in the absence of overt changes in standard measures of self-renewal in human ES cells [109]. Others have described similar changes in metabolite use in response to oxygen [110–112], consistent with a conserved cellular response to oxygen availability [39]. Further, the availability of oxygen can significantly impact the pluripotent epigenome. Increased 5′ methylcytosine staining in response to high oxygen has been described in preimplantation embryos [113], with increased expression of SIRT1 and TET1 also reported [114]. Maintenance of blastocyst integrity under low oxygen is likely mediated by HIF2α [105]. HIF2α has been shown to be responsible for long-term adaptation to hypoxia in human ES cells [115], binding directly to OCT4, NANOG, and SOX2 proximal promoters in human ES cells cultured at low oxygen concentrations [116]. Methylation of the OCT4 hypoxia response element (HRE) in human ES cells is marked by a significant increase in H3K36me3, a marker of transcriptional activation, under 5% oxygen conditions compared with atmospheric oxygen. Within the NANOG and SOX2 HREs, human ES cells maintained under atmospheric conditions exhibited high H3K9me3 levels, representing a marker of transcriptional silencing, and significantly reduced H3K4me3 and H3K36me3 compared to cells cultured under hypoxic conditions [116], consistent with a more closed conformational chromatin with atmospheric oxygen culture.

The oxygen concentration used to isolate human ES cells has been shown to impact X inactivation status, with those cultured under physiological oxygen able to maintain both active X chromosomes [117]. In contrast, those cultured under atmospheric conditions readily inactivate an X chromosome. SIRT activity is responsive to alterations in cellular redox including oxidative stress (reviewed by [118]) and may be one mechanism by which oxygen-regulated changes to the epigenome are established. Alternatively, HIF activation has been shown to upregulate MAT2A transcription in hepatoma cells, reducing SAM levels and leading to DNA demethylation [119]. Whether a similar relationship exists in pluripotent stem cells remains to be determined.

7.2. Regulation of Demethylation by Vitamin C. Vitamin C is commonly added to culture as an antioxidant, yet it has been shown to regulate DNA methylation dynamics in human and mouse pluripotent stem cells, acting as a key regulator of TETs [120] and the Jumonji family of histone demethylases [121]. The culture of human ES cells without added vitamin C increased DNA methylation, while the presence of vitamin C promotes DNA demethylation [122]. In mouse ES cells, supplementation with vitamin C leads to a rapid increase in 5hmC, dependent on Tet activity [123]. Addition of vitamin C to culture establishes an epigenetic landscape more similar to the inner cell mass of the embryo [123], potentially revealing a role for vitamin C in culture independent of its antioxidant capacity.

8. The Significance of Metaboloepigenetics to Pluripotent Stem Cell Biology

It is clear that metabolism can drive cell state transitions through interacting with the signalling machinery and more subtly through modification of the epigenome. What is less clear is how perturbations in metabolism impact subsequent potency and cell function. A consequence of perturbing metabolism is that heritable changes to DNA are passed on to daughter cells.

In vitro embryo culture has been found to be associated with alterations in DNA methylation and the expression of imprinted loci [124–128]. Assisted reproductive technologies that involve the culture of the preimplantation embryo have been associated with the early onset of metabolic disease (reviewed by [129]) and an increased frequency of epigenetic disorders [130] in offspring. Imprinting abnormalities following *in vitro* culture have been described in embryos cultured with different media formulations [131]. A key difference in these media formulations is the provision of amino acids, providing a correlation between metabolite concentration in the environment of the preimplantation embryo and lifelong impacts on the resulting child. The importance of establishing correct metabolic regulation in embryos in culture cannot be underestimated. There is still much work to do in medium optimization. Studies on the impact of the various commercially available media formulations on human ES cell epigenetics are essentially lacking.

Independently derived human ES cells display relatively stable methylation patterns [132] and share equivalent genomic arrangements [40]. These analyses do not encompass the entirety of the epigenetic landscape and smaller differences may exist between cell lines and between what is considered the norm of cells in culture versus cells in the embryo. A heavy reliance on glycolysis may, by default, activate the major pathways that regulate the epigenetic landscape, providing sufficient intermediates to enable the maintenance of pluripotency. However, subtle differences in medium formulation may impact less well-characterized modifications. The process of ES cell isolation must, by its very nature, place selective pressure on cells that is likely to be resolved, in part, through heritable modifications to the epigenome that embed changes in gene expression. Significant differences in the transcriptome of human ES cell and ICM cells have been shown, demonstrating that the process of ES cell derivation significantly alters gene expression [133] and providing evidence for this selective process. Adaptation may also involve modification to the metabolome of cells. ES cells, pluripotent by all standard measures, can display disparate metabolic profiles, suggesting metabolic adaptation can occur. It will be important to assess whether these changes in the metabolome impact, in turn, the pluripotent epigenome, eliciting changes in differentiation potential and/or cell function. These studies are not complete, and next-generation sequencing is required to establish a comprehensive characterization of the ES cell epigenome in culture to identify the impact of culture adaptation on epigenetic integrity.

8.1. The Transition from Differentiated to Pluripotent Cell State Is Accompanied by Changes in Metabolism. The introduction of pluripotency transcription factors to somatic cells brings about progressive loss of the somatic phenotype and the acquisition of a pluripotent-like cell state (induced pluripotent stem; iPS cells). Reprogramming to a pluripotent-like state requires the remodelling of both metabolism (reviewed by [9]) and chromatin organization (reviewed by [134]). Genome-wide chromatin remodeling is initiated in response to reprogramming factor expression [135] establishing an epigenetic profile similar to that of embryonic stem cells, where key developmental genes remain poised in a bivalent (repressed but activatable) state [136, 137]. Acquisition of a pluripotent-like state necessitates the upregulation of glycolysis and downregulation of oxidative phosphorylation [77, 78, 138]. Appropriately modulating metabolism is essential to establish the pluripotent cell state, evidenced by the ability to enhance or reduce reprogramming efficiency through metabolite modulation, including through modulation of oxygen concentrations [77, 139, 140]. Despite the capacity to acquire a number of pluripotent characteristics, iPS cells are not physiologically equivalent to their ES cell counterparts.

Differences in metabolism between ES and iPS cells have been described. Increased levels of SAM pathway metabolites and differences in unsaturated fatty acids are seen in iPS cells [141], suggesting that the reprogramming of metabolism is incomplete or perturbed in these cells. We have documented alterations in the capacity of iPS cells to regulate metabolism in response to oxygen (Harvey et al, unpublished). iPS cells have been shown to retain an epigenetic memory of their somatic origin [142–145], which is perpetuated through differentiation [144–146]. Conceivably, the higher level of global DNA methylation in iPS cells is evidence of the inappropriate regulation of metabolism during reprogramming. Several factors used to reprogram somatic cells are known regulators of metabolism. For example, Lin28a has been shown to enhance the translation of mRNAs for several metabolic enzymes and thereby to regulate glycolysis and OXPHOS [147]. These findings raise questions on the potential roles these factors will play, if any, in modulating pluripotent stem cell metabolism and how their impact on iPS cell metabolism is reflected in the epigenome. Epigenetic modifications could conceivably impact the physiology of iPS disease models or the utility of these cells in drug discovery or regeneration.

9. Conclusions

Many factors impact the relative activity of metabolic pathways and the composition of metabolite pools within the cell, including the extracellular milieu, the regulation of the cell: environment interface, cell identity and function, and the stress imposed on the cell by extracellular and intracellular regulators. Metabolites, such as acetyl-CoA and SAM, connect metabolism to signaling and gene expression. The availability of these compounds also impacts epigenetic modifications in the cell, with low levels resulting in reductions in acetylation and methylation, respectively. Evidence, as reviewed here, suggests that the metabolome plays a defining role in the epigenetic regulation of the cell, including

cells of the pluripotent lineage. What is equally clear from the available evidence is that there is much more work needed to describe the role of metabolism in the epigenome and then to understand the biological programs regulated by metabolically controlled epigenetic mechanisms. Integration of how metabolism changes with cell states is needed, as the majority of studies to date fail to delineate between cell states or, more specifically, address the transitions between them.

In culture, the ability of a cell to *adapt* to its environment may be reflected in changes to the epigenome. These changes are selected to promote survival in an environment defined by nutrient availability and effected through the activity of metabolic pathways and are *perpetuated* within the cell population. Despite this knowledge, the impact of different media used for pluripotent stem cell maintenance and iPS cell generation is poorly appreciated, where the quality of cells within a medium is generally evaluated without metabolic analysis. As with the blastocyst/inner cell mass, an inappropriate nutrient composition for the culture of pluripotent cells may compromise "metabolic fidelity" and have significant downstream impacts on development and viability. It is likely that these impacts are mediated through epigenetic regulation, and, in culture, alterations will be perpetuated with cell division. Selected changes in metabolism could limit the availability of cofactors, like SAM and acetyl-CoA with long term, heritable alterations to the epigenome. These could impact the identity of daughter cells, bias differentiation potential, or compromise the function of differentiated derivatives, potentially in subtle but important ways.

Elucidating the dynamics of, and mechanisms that control, cellular responses to metabolite availability will provide opportunities to manipulate cell fate. Establishing the appropriate balance of nutrients to support ongoing development requires a clearer understanding of the regulation of the pathways modulating metabolic control and identification of mechanisms that are perturbed by specific environmental conditions. Future studies should address the impact of metabolic adaptations of pluripotent stem cells to various culture conditions in the absence of changes to the base formulation, the metabolic regulation of differentiation, and how differences in metabolism impact cell function. As epigenetic landscapes can impact disease states, including cancer and neurodegenerative disorders, the appropriate regulation of these enzymes established through metabolic pathways will rely on the establishment of physiologically relevant conditions to support the continuum of pluripotent stem cell states. The impact of culture protocols on downstream epigenetic profiles and differentiated cell function will need to be investigated to inform of any deleterious conditions that negatively alter cell physiology.

Abbreviations

ac: Acetylation
ACL: ATP citrate lyase
αKG: Alpha-ketoglutarate
ATP: Adenosine triphosphate
F-6-P: Fructose-6-phosphate
G-6-P: Glucose-6-phosphate
G6PD: Glucose-6-phosphate dehydrogenase
Glu: Glutamate
GSH: Glutathione
HATs: Histone acetyltransferases
HMT: Histone methyltransferases
HCY: Homocysteine
JMJ: Jumonji demethylases
5mC: Methylcytosine
5hmC: Hydroxymethylcytosine
LDH: Lactate dehydrogenase
me: Methylation
Met: Methionine
NAD^+: Nicotinamide adenine dinucleotide
NADH: Reduced form of NAD^+
$NADP^+$: Nicotinamide adenine dinucleotide phosphate
NADPH: Reduced form of $NADP^+$
O_2: Oxygen
O-GlcNAc: O-linked *N*-acetyl glycosylation
OXPHOS: Oxidative phosphorylation
PDH: Pyruvate dehydrogenase
PKM2: Pyruvate kinase M2
PPP: Pentose phosphate pathway
Pro: Proline
SAH: S-Adenosylhomocysteine
SAM: S-Adenosyl methionine
SIRTs: Sirtuins
TCA: Tricarboxylic acid cycle
TET: Ten-Eleven Translocation demethylases
Tdh: Threonine dehydrogenase
THF: Tetrahydrofolate
5-CH_3-THF: 5-Methyltetrahydrofolate
Thr: Threonine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Integration of Signaling Pathways with the Epigenetic Machinery in the Maintenance of Stem Cells

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Stem cells balance their self-renewal and differentiation potential by integrating environmental signals with the transcriptional regulatory network. The maintenance of cell identity and/or cell lineage commitment relies on the interplay of multiple factors including signaling pathways, transcription factors, and the epigenetic machinery. These regulatory modules are strongly interconnected and they influence the pattern of gene expression of stem cells, thus guiding their cellular fate. Embryonic stem cells (ESCs) represent an invaluable tool to study this interplay, being able to indefinitely self-renew and to differentiate towards all three embryonic germ layers in response to developmental cues. In this review, we highlight those mechanisms of signaling to chromatin, which regulate chromatin modifying enzymes, histone modifications, and nucleosome occupancy. In addition, we report the molecular mechanisms through which signaling pathways affect both the epigenetic and the transcriptional state of ESCs, thereby influencing their cell identity. We propose that the dynamic nature of oscillating signaling and the different regulatory network topologies through which those signals are encoded determine specific gene expression programs, leading to the fluctuation of ESCs among multiple pluripotent states or to the establishment of the necessary conditions to exit pluripotency.

1. Introduction

Stem cells balance their self-renewal and differentiation potential by integrating environmental signals with the transcriptional regulatory network (TRN) [1–4]. Adult stem cells are generally long-lived quiescent cells, which, upon prodifferentiation stimuli, would give rise to progenitors that will further differentiate into postmitotic mature cells. Controlling the equilibrium between stem cell self-renewal and cell fate specification is indispensable for maintaining tissue homeostasis and the deregulation of these processes would lead to loss of cell identity and tumor initiation [5–7]. In the early embryo, the inner cell mass (ICM) cells are pluripotent and progressively restrict their developmental potential in response to local cues, which direct the formation of the three germinal layers. Defining the molecular mechanisms that govern the establishment of a defined epigenetic program in response to transient signals is fundamental to understand the basis of stem cell specification and reprogramming. The feasibility of isolating and propagating in culture both embryonic and adult stem cells, which can self-renew or differentiate in response to specific signals, allows delineating how extrinsic signals are integrated with the TRN [5, 8–10]. Signaling pathways crosstalk fine-tunes the correct pattern and timing of gene expression by modulating downstream effectors such as transcription factors (TFs), cofactors, and histones modifiers. These modulations are achieved through different mechanisms including differential DNA binding affinities, protein shuttling, posttranslational modifications, and protein-protein interactions. Importantly, the combinatorial DNA binding action of cell type-specific TFs and signal effectors on *cis*-regulatory elements is strongly influenced by the chromatin landscape of a given cell, thus resulting in the establishment of multiple transcriptional programs. In this regard, the dynamic interplay between signaling pathways, TFs, and epigenetic machinery plays a major role in integrating multiple inputs and switching a transient signaling event into a long-lasting phenotypic change.

In this review, we will discuss regulatory mechanisms through which signaling cascade can directly regulate histone modifications, nucleosome occupancy, and chromatin

modifying enzymes. We will highlight how these chromatin modifications triggered by extrinsic signaling may affect the TFs binding, the epigenetic state, and the consequent gene expression program of stem cells. Finally, we would underline the critical role of these regulatory circuits to control the cell identity and how their misregulation may initiate pathological events such as tumorigenesis.

2. Mechanisms of Signaling to Chromatin

2.1. Signaling Mechanisms Regulating Histone Modifications. The coordinated activation of signaling pathways impacts the epigenetic landscape by targeting TFs, chromatin regulators, or nucleosome occupancy or by directly modifying nucleosomes (Table 1).

Histones are subject to a large set of posttranslational modifications (PTMs) including phosphorylation, acetylation, methylation, ubiquitination, sumoylation, and citrullination, which influence the chromatin structure [11, 12]. The possible combinations of histone modifications differently affect the chromatin accessibility to TFs and determine molecular platforms for recruiting regulatory complexes, which would further modify the chromatin state. Importantly, histone modifications are reversible as opposing modifying enzymes, writers and eraser, introduce or remove the same modifications in response to specific signals [13–16]. Among them, kinases are activated mainly by upstream signaling cascade and they transiently phosphorylate both histone and nonhistone nuclear proteins [17, 18]. The temporal pattern of a certain pathway combined with the cell type-specific chromatin state strongly affects the resulting transcriptional outcome [19]. A large body of data shows that histone phosphorylation influences the deposition of other histone modifications facilitating the recruitment of Histone Acetyltransferases (HATs) while opposing the maintenance of repressive marks. Phosphorylation of histone H3 on serine 10 (H3S10ph) is accomplished in response to different signaling cascades, which activate the downstream kinases such as Rsk2, MSK1/2, IKKα, Aurora B, and PIM1 [14, 20–23]. Although the stimulus-induced H3S10ph is transient, it could cooperate with histone acetylation in blocking the binding of the chromodomain containing protein HP1γ, thus promoting chromatin remodeling and transcription activation [23]. At enhancers, H3S10 phosphorylation drives the recruitment of MOF, which, by acetylating histone H4, establishes a nucleosome binding platform for the BRD4/P-TEFb complex, thereby stimulating transcription elongation [24]. Other histones phosphorylation is involved in controlling transcriptional switch by mediating histone crosstalk. For example, during androgen receptor- (AR-) dependent gene activation, PKCβ-mediated H3T6 phosphorylation switches the LSD1 demethylation activity from H3K4 towards H3K9 methyl group [25]. Similarly, the epidermal growth factor- (EGF-) activated tumor-specific pyruvate kinase M2 (PKM2) phosphorylates histone H3 at T11, which triggers dissociation of HDAC3, thus favoring H3K9ac and transcription activation [26].

Taken together, these results illustrate how a kinase-mediated short-lived signal activates a cascade of events, which determines a long-standing output by inducing chromatin modifications and impacting gene expression.

2.2. Signaling Mechanisms Regulating Nucleosome Occupancy. Nucleosome organization and higher order chromatin structures package genomic DNA, limiting its accessibility to most of the nuclear factors. Chromatin remodelers are multisubunit complexes that utilize ATP hydrolysis to mobilize nucleosomes and their positioning on the eukaryotic DNA, thereby being essential for modulating chromatin accessibility to transcription factors and RNA Polymerases [27, 28]. In addition, histone chaperones and DNA helicases facilitate histone exchange and the insertion of histone variants into nucleosomes surrounding *cis*-regulatory elements such as promoters and enhancers [29–32]. Many sophisticated mechanisms involve the crosstalk between signaling pathways and chromatin remodelers in order to alter nucleosome occupancy, as a requisite for gene regulation. The steroid hormone receptors interact and recruit SWI/SNF complexes to render the chromatin more accessible. In breast cancer cell, progesterone-activated ERK1/2 phosphorylates both the progesterone-receptor (PR) and the downstream kinase MSK1, forming an active ternary complex, which mediates the phosphorylation of histone H3 at serine 10. This initial step triggers the recruitment of histone modifiers and chromatin remodeling complexes, which ultimately leads to local displacement of histones H1 and H2A/H2B. In this setting, chromatin remodeling is responsible for transcriptional activation of progesterone responsive genes [33–35]. Another study linked nucleosome occupancy at enhancers to androgen receptor (AR) signaling [36]. Apart from nuclear receptors, other signaling pathways have been recently involved in modulating nucleosome occupancy. Specifically, it has been shown that the downstream effectors of the Hippo pathway YAP/TAZ promote transcriptional repression of numerous target genes by stimulating chromatin remodeling. YAP/TAZ interact with the TEAD transcription factor and recruit the NuRD complex on target genes, causing histones deacetylation and increased H3 histone occupancy, thus leading to chromatin compaction [37].

These data illustrate how chromatin remodelers are influenced by environmental signals, which in turn modulate nucleosome occupancy, thereby affecting transcription regulation.

2.3. Signaling Mechanisms Regulating Chromatin Modifiers. Signaling pathways can also impact the chromatin state by targeting chromatin modifying proteins. The activation of the Jak2/STAT5 pathway leads to Jak-dependent phosphorylation of STAT5, which causes its dimerization, nuclear translocation, and binding to *cis*-regulatory elements. In addition, Jak2 functions as histone tyrosine kinase by phosphorylating H3Y41 and perturbing HP1α binding [38].

Another example of linking signaling pathways with chromatin modifications is represented by the Polycomb and Trithorax group of proteins which act antagonistically in maintaining a specific gene expression state [39, 40]. The H3K27 methyltransferase enzyme EZH2 is the catalytic

Table 1: Summary of different mechanisms of signaling to chromatin.

Mechanism of signaling to chromatin	Signaling pathway	Chromatin target	Functional outcome	Reference
Histone posttranslational modifications	Serum stimulated PIM1 kinase cascade	H3S10 phosphorylation	Recruitment of MOF, which acetylates H4, thus in turn recruiting the BRD4/P-TEFb complex and stimulating transcription elongation	[14]
	Epidermal growth factor (EGF) induced Rsk2 kinase signaling	H3S10 phosphorylation	Recruitment of HAT complexes and rapid acetylation of phosphorylated H3S10	[20]
	Mitogen- and stress-induced MSK1/2 cascade	H3S10 and S28 phosphorylation	Reduced efficiency in inducing mitogen- and stress-induced IE genes	[20]
	Cytokine stimulated IKKa kinase cascade	H3S10 phosphorylation	Regulation of NF-κB-dependent gene expression after cytokine exposure	[22]
	Mitotic Aurora B kinase signaling	H3S10 phosphorylation	Displacement of HP1 from mitotic heterochromatin and gene activation	[23]
	Androgen dependent PKCβ kinase signaling	H3T6 phosphorylation	Androgen-stimulated gene expression activation, through modulation of LSD1 demethylating activity	[25]
	Epidermal growth factor (EGF) activated PKM2 kinase cascade	H3T11 phosphorylation	Dissociation of HDAC3 from CCND1 and MYC promoters, introduction of H3K9ac, and induction of transcription activation	[26]
	Jak2/STAT5 signaling pathway	H3Y41 phosphorylation	Jak2 acts as histone tyrosine kinase, which phosphorylates H3Y41 and excludes HP1a from chromatin	[38]
Modulation of nucleosome occupancy	Progesterone-activated ERK1/2 signaling	Histones H1 and H2A/H2B	ERK1/2 mediated phosphorylation of the progesterone-receptors, MSK1 and H3S10, which recruit chromatin remodeling complexes leading to the displacement of H1 and H2A/H2B and transcriptional activation of progesterone responsive genes	[33–35]
	Androgen signaling pathway	Nucleosomes	Induction of a nucleosome-depleted state at androgen receptor enhancers, leading to recruitment of histone modifiers, chromatin remodelers, and ultimately gene activation	[66]
	Hippo signaling pathway	Histones H3	The YAP/TAZ/TEAD ternary complex recruits NuRD complex on target genes, leading to histones deacetylation, increased H3 histone occupancy and reduction of chromatin accessibility	[37]
Regulation of chromatin modifiers	Stress-activated p38α kinase cascade	EZH2 Thr372 phosphorylation	PRC2-mediated repression of Pax7 during regeneration	[41]
	PI3K-AKT signaling pathway	EZH2 Ser21 phosphorylation	Suppression of EZH2 methyltransferase activity by reducing its binding to histone H3 and derepression of silenced genes	[42]
	p38 MAPK signaling pathway	MLL complexes	The signaling cascade leads to phosphorylation of Mef2d, which interacts with MLL complex, targeting it to specific genes that are activated during myogenesis	[43]

subunit of the polycomb repressive complex 2 (PRC2) and is targeted by different signals, which can promote or inhibit its enzymatic activity, respectively [41, 42]. The stress-activated p38α kinase phosphorylates EZH2 on Thr372 in muscle satellite cells and promotes PRC2-mediated repression of Pax7 during myogenesis. Instead, the prosurvival PI3K-AKT signaling pathway targets EZH2 by inducing Ser21 phosphorylation, which causes the reduction of PRC2 affinity for histone H3. At the same time, AKT-mediated phosphorylation of P300 increases its H3K27-specific acetyltransferase activity, thus participating in switching from a methyl (repressive) towards an acetylated (active) K27 state.

On the other hand, Myeloid/Lymphoid or Mixed-Lineage Leukemia (MLL) group of proteins mediates the trimethylation of histone 3 at lysine 4 (H3K4me3) and are core components of the Trithorax complexes. Multiple MLLs are targeted in response to signaling leading to their PTMs. For example, during the commitment of myoblasts into multinucleated myotubes, p38 MAPK signaling pathway leads to phosphorylation of Mef2d and its interaction with MLL2 complex. This signaling cascade promotes MLL2 targeting to muscle-specific genes leading to their H3K4 trimethylation and transcriptional activation [43].

Overall, the reported examples clearly show that signaling cascades not only influence the activity of transcription factors but also perturb the chromatin state by driving dynamic chromatin changes that impact on the transcriptional program.

3. Outcomes of Integrated Signals on Stem Cells Transcriptional and Epigenetic State

Beside the examples described so far, developmental signaling pathways are also interconnected with the TRN and influence the chromatin state of stem cells (Figure 1). The developmental signaling, which includes the Wnt/β-catenin, Notch, Nodal/Activin, Hippo pathways, and the circadian clock, is involved both in the maintenance of stem cell homeostasis and in inducing cell lineage commitment. In general, their activation triggers the stabilization and the nuclear accumulation of their downstream effectors, which finally influence the expression of their target genes. The downstream effectors, which are activated in a controlled spatiotemporal manner by the external stimuli, provide the competence for a stem cell to adopt a particular cell fate by cooperating with the cell type-specific TFs. This concept is particularly relevant in pluripotent embryonic stem cells (ESCs), in which the same signaling pathways play a key role in the maintenance of self-renewal capacity but are also involved in lineage differentiation. This divergent stem cell responsiveness depends on the fact that signaling pathways target both TRNs and chromatin landscapes. Besides that, the integration of multiple extrinsic signals determines different transcriptional program, thus influencing the cellular response.

3.1. Signaling to the Transcriptional Regulatory Network of Embryonic Stem Cells. Both mouse and human ESCs (mESCs and hESCs) are isolated from the transient pluripotent cells of the inner cell mass (ICM) [44, 45]. The two major features that define ESCs consist in their ability to self-renew as well as to differentiate into all the cell lineages in response to developmental cues. This balance is regulated by a specific transcription program, which is centered on the cooperative action of the pluripotency transcription factors Oct4, Sox2, and Nanog (OSN) [46]. OSN targets have been mapped and showed an extensive cobinding in both mESCs and hESCs, suggesting the existence of a common core transcriptional regulatory network (TRN) [47, 48]. Oct4 is a member of the POU family of homeodomain proteins and it is essential for the establishment and maintenance of pluripotency both *in vivo* and *in vitro*. Perturbation of Oct4 transcript level abrogates formation of the ICM [49] and promotes ESCs differentiation [50]. Oct4 heterodimerizes with the high-mobility group box (HMG) family member Sox2 and they cobind distal regulatory elements, thus activating the expression of many pluripotency factors and repressing lineage-specific genes. The synergic action of Oct4/Sox2 in the regulation of key pluripotency factors is underlined by the similar phenotype observed both during blastocyst formation and in cultured ESCs upon the knock-out of the respective genes [51, 52]. Although Nanog is not essential for deriving and maintaining ESCs, it is required for the formation of the ICM. Functionally, Nanog cooccupies most sites with Oct4/Sox2, thus playing a key role in controlling pluripotency in ESCs [53–55]. These core transcription factors control the ESCs transcriptional program by establishing an interconnected regulatory loop in which they influence the gene expression level of each other. This self-sustained transcription regulatory network generates a bimodal transcriptional state of ESCs, which is characterized by the coexistence of transient and exchangeable cellular states. Appropriate levels of the core transcription factors ensure a residence state in which ESCs self-renew. On the contrary, transient perturbation of the positive feedback transcriptional program produces a window of opportunity to exit pluripotency and to initiate cell lineage commitment [55–57]. The ability of OSN to maintain mESCs state is influenced by additional transcription factors such as Klf4, Klf2, Dax1, Nac1, Zfp281, Essrb, Sall4, Tbx3, and Prdm14, which cobind enhancers occupied by OSN [3, 58–60]. Importantly, the OSN-centered regulatory network includes also Stat3, Smad1, and Tcf3, which are the downstream effectors of the LIF, BMP4, and Wnt signaling pathways [3, 61, 62]. These observations underline how the extracellular signals converge on the core TRN, thus participating in the modulation of the stem cell transcriptional program (Figures 1(a) and 1(b)). While LIF leads to phosphorylation of Stat3, which is required to promote self-renewal, BMP4 suppresses differentiation through Smad1-mediated activation of Id genes. Wnt signaling counteracts the transcriptional repressive activity of Tcf3 on pluripotency genes by stabilizing β-catenin [63, 64] (Figure 1(a)). However, both hESCs and mouse postimplantation epiblasts derived stem cells (EpiSCs), collectively referred to as "primed" pluripotent stem cells, depend on different signaling pathways for self-renewal, such as FGF/ERK and Activin A/Smad [65, 66] (Figure 1(b)). In particular, both the Wnt/β-catenin pathway and the BMP/Smad signaling cascades, which are required to promote mESCs pluripotency, once activated in primed stem cells trigger mesoendoderm lineage commitment. In hESCs, nuclear β-catenin cooperates with SMAD2/3, leading to the activation of differentiation genes, thus inducing exit from pluripotency [67]. On the other hand, it has been shown that BMP4/TGF-β stimulation induces hESCs and EpiSC to differentiate towards mesoderm [68].

3.2. Signaling to Chromatin in Embryonic Stem Cells. Signaling-mediated gene regulation in ESCs could be directly

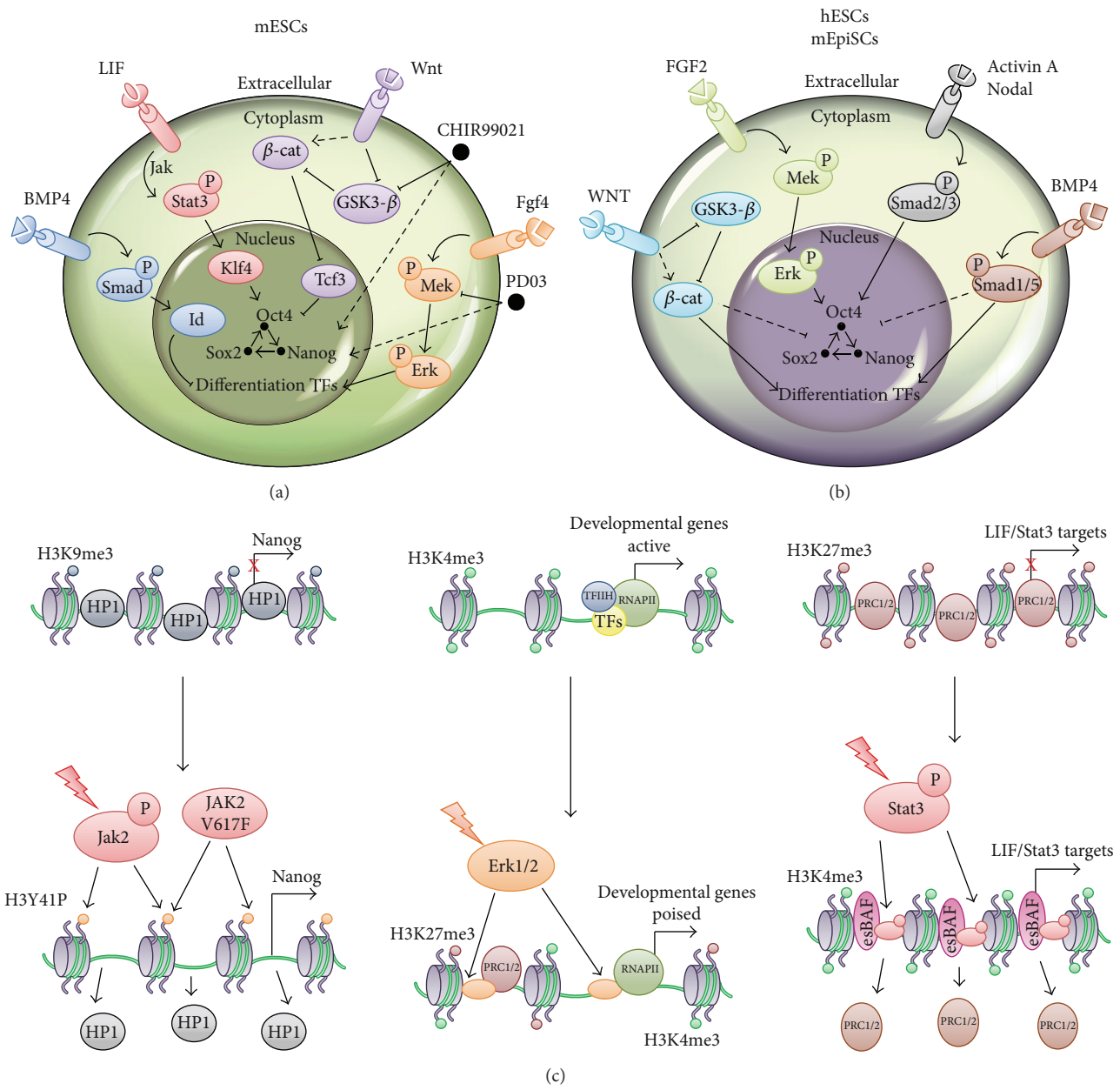

Figure 1: Signaling affecting stem cells identity and their interplay with chromatin. Key signaling pathways and relative factors contributing to the maintenance of mESCs (a) or hESCs/EpiSCs (b) identity or to their differentiation (see details in the main text). Black circles in (a) indicate the two chemicals used in the 2i culturing medium (CHIR99021 and PD03). Solid black arrows and lines indicate positive or negative modulation, respectively. Dashed black lines indicate indirect effects. Colored circles with "P" indicate phosphorylation. (c) Key examples of signaling to chromatin in ESCs. The upper panels are relative to a more differentiated state in which the LIF/Stat3 and Nanog targets are repressed while developmental genes are active. Lower panels, instead, describe embryonic stem cells chromatin features. On the right, effect of Jak2, or its constitutive active form Jak2V617F, on H3Y41P and HP1 loading on chromatin. In the middle, interconnection between Erk1/2 and the loading of PRC2 and RNA polymerase II activity at developmental genes. On the left, interplay between the esBAF complex and Stat3 in regulating LIF/Stat3 signaling pathway targets. Details of each example are reported in the main text.

achieved through the modulation of chromatin players and the epigenetic machinery (Figure 1(c)).

In mESCs, a LIF-independent role for Jak signaling has been demonstrated and consists in the phosphorylation of histone H3 on tyrosine 41 (H3Y41). This event leads to a reduction in the binding of heterochromatin protein 1α (HP1α) on pluripotency genes [69]. In hematopoietic stem cells, mutations leading to the activation of Jak2 correlate with myeloproliferative neoplastic and leukemic transformation. One such mutation is represented by the Jak2V617F allele, which turns on the Jak/STAT pathway without the requirement of activating cytokines [70, 71]. Interestingly,

the expression of Jak2V617F in mESCs leads to their cytokine independent self-renewal and is associated with the direct Jak2 signaling to the chromatin. Chemical inhibition of the Jak/STAT pathway in Jak2V617F mESCs leads to the decrease of H3Y41ph levels coupled with increased association of HP1α to Nanog promoter, thereby inducing its transcriptional repression. These findings underline the critical role of the direct Jak2 signaling to the chromatin in sustaining self-renewal of both embryonic and hematopoietic stem cells and how its deregulation may cause tumorigenesis [69]. In the same oncogenic setting, mutated Jak2 may also phosphorylate and inhibit PRMT5 preventing histone arginine methylation and favoring uncontrolled haematopoietic progenitor cell expansion [72]. Finally, both Jak2V617F and Jak2K539L, other oncogenic forms of Jak2, cooperate with the histone demethylase JMJD2C in lymphomas, by promoting MYC overexpression [73]. Contrary to the role of Jak2 on chromatin, MAP kinases signaling favors mESCs differentiation through the JNK-mediated H3 Ser10 (H3S10) phosphorylation of its target genes [74].

Other mechanisms of signaling to chromatin involve the modulation of the targeting of chromatin complexes. ERK pathway regulates PRC2 deposition at developmental genes, by phosphorylating the RNA polymerase II at serine 5 and establishing poised domains [75]. The chromatin remodeling complex esBAF is, instead, interconnected with the LIF/Stat3 signaling pathway. Brg1, the ATPase subunit of esBAF, favors the correct targeting of Stat3 onto chromatin by stimulating chromatin remodeling at Stat3 target genes, thus supporting mESCs pluripotency [61]. Similar mechanisms are shared between ESCs and cancer cells and are relevant for tumorigenesis. In ESCs, the pluripotency genes Myc and LIN28 counteract the action of Let-7, which inhibits self-renewal genes [76, 77]. Among others, HMGA2 represents a DNA binding and chromatin modifying protein which regulates both differentiation and stem cell self-renewal [78, 79]. Misregulation of the components of this regulatory circuit has been associated with a wide range of malignancies [80, 81]. Interestingly, in breast cancer cells, inhibition of the MAPK signaling by the Raf kinase inhibitory protein (RKIP) is transduced onto the chromatin where the HMGA2 activity is inhibited, leading to inactivation of proinvasive and prometastatic genes [82]. In hESCs, the Activin A/Smad pathway has been demonstrated to be involved in the correct deposition of H3K4me3 on key developmental genes, through its effectors SMAD2/3, which cooperate with NANOG to recruit DPY30, a subunit of the COMPASS methyltransferase complexes, contributing to the capacity of stem cells to differentiate into specific lineages [83].

3.3. Gene Expression Heterogeneity of ESCs and Fluctuating Signaling. Single-cell studies on mESCs showed substantial gene expression heterogeneity with subpopulations of ESCs, which express variegated levels of pluripotency-associated factors [55, 56, 84–87]. The discovery of fluctuating expression levels of pluripotency regulators, which supports the existence of interconvertible ESCs states with different potency to self-renew or differentiate, highlights the key role of sustaining a dynamic transcriptional program in pluripotent cells [87]. Fluctuations in gene expression may depend on multiple factors, which include the structure of the cell TRN, sequential and combinatorial epigenetic regulations, and the integration of signaling pathways.

The TRNs are characterized by recurring regulatory circuits, named network motifs, which define a particular pattern of interconnections, leading to a certain transcriptional outcome [88]. Among them, negative feedback loops and type 1 incoherent feedforward loops may generate oscillatory responses of TFs. Specifically, the ESCs regulatory circuit is characterized by dynamic TFs that regulate each other and autoregulate their own expression through both feedforward and negative feedback loops, thus determining fluctuating states of transcript levels within the ESC population [1, 48, 59, 60, 89–92]. At the posttranscriptional level, microRNAs (miRNAs) play a central role in modulating TRN as the core pluripotency factors OSN and Tcf3 directly bind their loci, thus influencing their expression [93]. Mechanistically, the ES cell-specific cell cycle-regulating (ESCC) miRNAs indirectly activate several self-renewal genes including c-Myc and Lin28 which, by inducing degradation of pre-Let-7 transcripts, inhibit Let-7 opposing effects on ESCs self-renewal [76, 77]. These results suggest that let-7 and ESCC miRNAs act in self-reinforcing loops to sustain the ESCs transcriptional network. The finding that many miRNAs target the pluripotency TFs in ESCs suggests that they may be involved in controlling their fluctuating transcript levels.

Recently, it has been shown that impairment of miRNAs production in ESCs (Dgcr8$^{-/-}$ and Dicer$^{-/-}$ ESCs) resulted in a more homogenous expression of pluripotency factors [87]. In terms of transcription heterogeneity, the Dgcr8$^{-/-}$ ESCs manifest features similar to the so-called 2i ESCs, which mirror the "naïve" or "ground state" of preimplantation epiblast cells [2, 94]. The 2i ESCs are grown in a chemically defined medium which comprises the Mek inhibitor PD03 (PD0325901) and the GSK3 inhibitor CHIRON (CHIR99021), which shield ESCs from prodifferentiation autocrine signaling and reinforce for pro-self-renewing pathways [2] (Figure 1(a)). The Fgf4/Erk cascade drives the transition from naïve pluripotency to a primed state, which is responsive to lineage-specific differentiation signals [95]. GSK3 inhibition reinforces the Wnt pathway by stabilizing β-catenin [64, 96–98]. In 2i ESCs, the fluctuating expression of the pluripotency-associated transcription factors is strongly reduced, thus highlighting the crucial role of signaling pathways in modulating transcriptional pulsing in ESCs.

These observations could be explained by considering the intrinsic feature of signaling pathways, which is the *dynamics*. This represents an additional mode of transmitting information, meaning that signaling pathways encode information in the frequency, amplitude, and duration of the signals into the cells [99]. Importantly, cells are able to decode the signaling dynamics by executing different biological responses. Fox example, studying the ERK pathway revealed that different upstream signals trigger divergent dynamic patterns of the same signaling cascade leading to two different cellular fates [100]. In this case, the EGF treatment of PC-12 neural precursors drives a transient ERK

activation, which induces cell proliferation, whereas the NGF stimulus triggers a sustained ERK response, culminating in differentiation. The differences in ERK dynamics, in response to those alternative growth factors, depend on the network structure, which encodes the stimulus into a dynamic change of ERK activation. In the EGF pathway, the stimulus activates a SOS-dependent negative feedback loop. Instead, the NGF signal induces a PKC-centered positive feedback loop, thus sustaining ERK activation [101, 102]. The molecular mechanisms through which cells decode the temporal pattern of a certain signaling are poorly understood. Regarding the ERK dynamics, it has been proposed that the interpretation of the temporal signals is depending on network motifs that sense the spatiotemporal changes of the upstream signaling [103–105]. In this case, transient ERK activation leads to the expression of the immediate early gene c-Fos that is rapidly degraded. On the contrary, a persistent nuclear ERK signal drives the accumulation of the effector, which is directly phosphorylated by ERK itself, thus increasing its protein stability.

In pluripotent stem cells (PSCs), the ERK pathway triggers opposite cellular response: in naïve ESCs, it induces cell lineage commitment while it sustains self-renewal of primed EpiSCs (Figures 1(a) and 1(b)). This striking difference could depend on the dissimilar cellular and epigenetic context of these PSCs but it may also be caused by diverse signaling dynamics, which are decoded differently, thus leading to divergent cellular fates (Figure 2). Few studies addressed this specific point in ESCs but the obtained results clearly showed a link between ERK signaling pathway and fluctuating transcriptional response [2, 94, 106, 107]. The attenuation of the autocrine Fgf4/MAPK signaling induced by either the chemical inhibition of Mek or the genetic targeting of the heparin sulfate proteoglycans reduces the transcriptional fluctuations of pluripotent transcription factors. Although the dynamics of autocrine FGF signaling has not been studied in mESCs, computational modeling, based on the well-studied EGF signaling in other systems, postulates that the oscillatory pattern of Nanog could depend on the dynamics of the regulatory system, including individual cell-specific changes in parameters of FGF autocrine feedback loop and crosstalk with other signaling pathways. The interconnection between MAPK, PI3K/AKT, BMP4/Nodal, and Wnt signaling plays a major role in the maintenance of hESCs [67]. Of note, the dynamics of these signaling pathways have been described to play a major role in controlling ESC pluripotency and reprogramming [108–112]. Among them, the Wnt/β-catenin pathway is particularly interesting as its periodic activation favors cell fusion-mediated reprogramming while its sustained stimulation inhibits it [112]. Mechanistically, the activation of the Wnt signaling in the early stage of reprogramming causes a TCF1-dependent inhibitory effect, while its stimulation in the late phase reinforces reprogramming towards PSCs [110, 113]. It would be interesting to evaluate whether a similar fluctuating Wnt signaling pattern may support the maintenance of the naïve state in ESCs. However, recent data showed that β-catenin fluctuates in both "primed" (serum + LIF maintained) and naïve (2i + LIF maintained) mESCs [111]. These results are of particular interest considering that in the 2i condition the inhibition of GSK3-β should stabilize the endogenous β-catenin, thus suggesting that other regulatory circuits may modulate the dynamics of Wnt/β-catenin pathway.

More broadly, the observed transcriptional dynamics of pluripotency-associated TFs may reflect the integration of input at the chromatin level including histone modification, chromatin accessibility, the topology of the transcriptional regulatory networks, and activity of autocrine signaling pathways. Despite their importance, the effects of these fluctuating signaling pathways at the transcriptional and chromatin level have not been investigated so far. For example, there are no data regarding the dynamic response of the downstream effectors of the fluctuating signaling pathways in stem cells, nor on the impact on histone modifications at the target genes. Although understanding how cells decode the different dynamical patterns at the molecular level is currently a challenging goal, it is mandatory to better define these regulatory mechanisms in order to clarify their contribution to the maintenance of stem cell identity and pluripotency.

3.4. Transcriptional Dynamics in Neural Progenitor Cell Fate Choice. The importance of the integration between signaling dynamics, TRN, and the epigenetic state is well exemplified during cell lineage choice of pluripotent Neural Progenitor Cells (NPCs) in developing nervous system. In the developing telencephalon, the neuroepithelial cells, which represent the earliest NPCs, proceed towards the formation of Radial Glial (RG) cells by the oscillating Notch signaling [114–116]. Asymmetric cell division of polarized RG cells gives rise to immature neurons which would further differentiate into mature neurons and to intermediate progenitors, which go towards cell division in the subventricular zone (SVZ) before fully differentiating. During neurogenesis, it is essential to maintain a certain balance between self-renewing NPCs, proliferating intermediate progenitors, and their commitment towards postmitotic differentiated cells [114]. This goal is achieved, at least in part, by integrating the fluctuating Notch signaling and the transcriptional regulatory circuit of NPCs. In particular, the Notch pathway induces the expression of the bHLH transcription factors Hes1 and Hes5, which are required for the specification of RG cells [117, 118]. Of interest, in neural progenitors, these factors are expressed in an oscillatory manner in response to the fluctuating expression of the Notch ligand Dll1, as well as a consequence of their negative feedback loop. The Hes transcription factors maintain the precursors' multipotency by inhibiting the proneural bHLH factors Ascl1 and Ngn2 [118]. The two bHLH transcription factors Olig1/2 are required to specify the formation of oligodendrocyte progenitor cells and their subsequent differentiation and maturation. Astrocytes fate determination is the result of the interplay between transcription factors, epigenetic modifiers, and environmental signals. Specifically, during neurogenesis, NSCs become responsive to Jak/STAT and BMP signaling pathways, which support astrocyte differentiation, as a consequence of transcription factors-dependent DNA and histone demethylation of the astrocyte-specific genes [119].

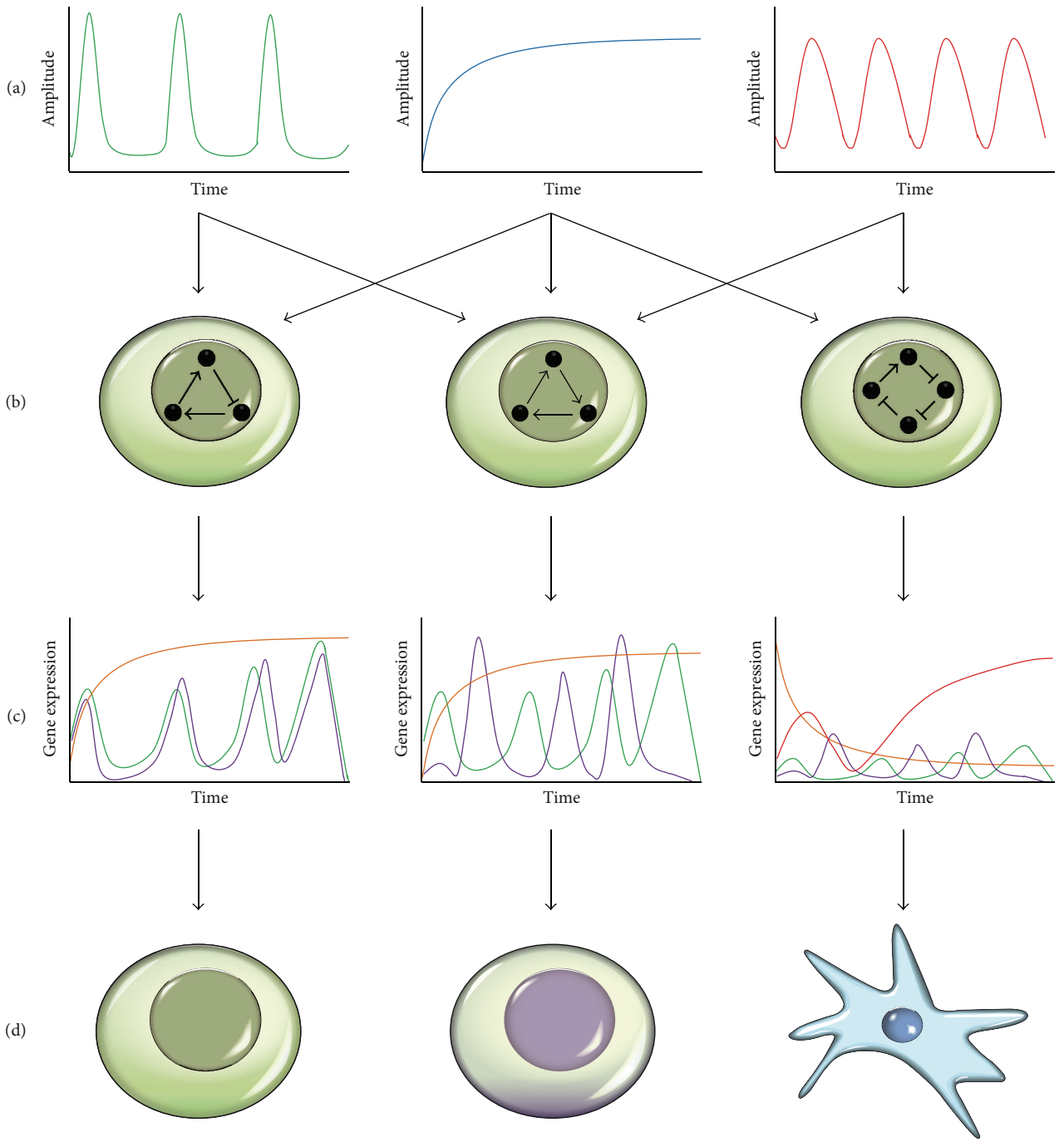

Figure 2: Emergence of gene expression heterogeneity in ESCs and cell fate determination. Gene expression heterogeneity of ESCs is determined by complex multistep mechanisms. (a) Multiple spatiotemporal restricted signals are differentially sensed and integrated by ESCs, leading to signaling pathways activation, which ultimately converges both onto the TRN and directly onto the chromatin. (b) Specific regulatory networks, which involve both TFs and epigenetic regulators, are established in the cell according to their transcriptional and epigenetic landscape and transduce the signals. Arrows and lines indicate positive and negative regulation between factors (black circles), respectively. Negative feedback loops (left) or incoherent feedforward loops (right) may generate oscillatory responses to signals. (c) The result of this integration is the fluctuation of genes expression profiles among cells, which permits ESCs to fluctuate in a continuum of interconvertible pluripotent states and may generate the suitable condition to exit pluripotency and differentiate. (d) The final biological outcome of this process is the establishment of a heterogeneous population of ESCs captured at different pluripotent states (green and purple cells) or the eventual differentiation toward committed cell (blue cell).

The lineage-commitment factors Ascl1, Hes1, and Olig2 play opposite function in sustaining proliferation and cell differentiation of NPCs [120–123]. This contradictory function can be explained by considering their dynamic pattern rather than their relative transcriptional level. Live cell imaging studies have shown that the Notch-dependent fluctuating pattern of Hes1 causes oscillation of Ascl1 and Ngn2 in neural precursors [124–126]. Of importance, by adapting an optogenetic approach to mimic the spatiotemporal pattern of Ascl1 expression in NPCs, it has been demonstrated that periodic oscillations of this TF induce cell proliferation, while its prolonged transcriptional activation triggers lineage commitment towards the formation of neurons [126]. The molecular mechanism through which NPCs differentially interpret the dynamics of Ascl1 gene expression is currently undefined. In addition, it has not been determined which are the different targets that are responsive to this encoded information. Moreover, it has not been investigated so far whether this expression dynamics may be integrated into the chromatin, giving rise to different pattern of histone modifications in the two opposite settings (fluctuating versus sustained transcription).

4. Conclusions and Future Perspectives

Over the recent past years, the massive utilization of systems biology techniques and functional genomics increased dramatically our knowledge on the regulatory networks, which control both the maintenance of cell identity and the lineage commitment. Nonetheless, a better understanding of how cells integrate multiple environmental signals and transduce them onto chromatin, in order to modulate gene expression, is still needed.

In this review, we provide multiple evidences, demonstrating how different pluripotent stem cells rely on specific extrinsic cues, which converge on transcriptional and epigenetic networks, thereby determining their cell fate (Figures 1 and 2). Pluripotency is not an invariant state, but rather represents a continuum of states between which cells can fluctuate in response to both extrinsic and intrinsic signals. This concept is supported by both the heterogeneous expression profile of pluripotency factors registered between ESCs subpopulations and the fact that we can capture *in vitro* multiple pluripotent states, hanging on different regulatory networks. Oscillating, spatiotemporal restricted signaling pathways represent a first causative layer for this heterogeneity (Figure 2(a)). Although their role has not been fully addressed yet, the reported example of the cell fate choice in NPCs clearly supports their potentiality in determining fluctuation of downstream TFs activity. The same oscillating signals can affect the choice of cell fate in different manners, according to the topology of the TRNs they are encoded by (Figure 2(b)). Importantly, we reviewed here multiple data, showing how these TRNs in ESCs collectively involve TFs and chromatin players. Therefore, both the transcriptional and epigenetic landscapes should always be taken into consideration when studying how a signal is transduced to regulate genes expression. Integration of multiple oscillating signaling onto different network motifs is responsible for alternative patterns of genes expression (Figure 2(c)), which then determine the cell fate (Figure 2(d)). Since subtle fluctuations of pluripotency factors expression may reflect fluctuations of stem cells through different pluripotent states, similar mechanisms may also favor the exit from pluripotency and cell lineage commitment. Accordingly, mESCs grown in 2i are less heterogeneous in terms of pluripotency genes expression, partially because these fluctuations are reduced by the chemical control of the signaling required to maintain the self-renewal.

Major interest arises from the observations that the same mechanisms may take place during the onset of cell transformation. Cancer stem cells share unique biological features with embryonic and adult stem cells, such as the ability to self-renew, to indefinitely proliferate, and to give rise to aberrant multiple cell progenies. Therefore, the signaling pathways that are important to define stem cell identity have also been demonstrated to play an important role in tumor formation and maintenance. In addition, ESCs specific signatures related to epigenetic features and TFs activity have been found to be common to multiple tumors [127–130], suggesting that regulatory networks, similar to the ones discussed above in ESCs, may be aberrantly activated during tumorigenesis. Their deep understanding is fundamental to unravel the multistep processes that lead to tumor formation and held promise for novel therapeutic targets.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Direct Reprogramming of Mouse Fibroblasts to Neural Stem Cells by Small Molecules

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Although it is possible to generate neural stem cells (NSC) from somatic cells by reprogramming technologies with transcription factors, clinical utilization of patient-specific NSC for the treatment of human diseases remains elusive. The risk hurdles are associated with viral transduction vectors induced mutagenesis, tumor formation from undifferentiated stem cells, and transcription factors-induced genomic instability. Here we describe a viral vector-free and more efficient method to induce mouse fibroblasts into NSC using small molecules. The small molecule-induced neural stem (SMINS) cells closely resemble NSC in morphology, gene expression patterns, self-renewal, excitability, and multipotency. Furthermore, the SMINS cells are able to differentiate into astrocytes, functional neurons, and oligodendrocytes *in vitro* and *in vivo*. Thus, we have established a novel way to efficiently induce neural stem cells (iNSC) from fibroblasts using only small molecules without altering the genome. Such chemical induction removes the risks associated with current techniques such as the use of viral vectors or the induction of oncogenic factors. This technique may, therefore, enable NSC to be utilized in various applications within clinical medicine.

1. Introduction

Recently, fibroblasts have been reprogrammed into induced neural stem cells (iNSC) by transcription factors [1–5], which makes the neural stem cell (NSC) therapy for neurodegenerative disease feasible. However, clinical utilization of patient-specific NSC for the treatment of human diseases remains elusive, mainly due to the risks associated with viral transduction vectors used for induction. Several studies have shown that some small molecules can directly modify epigenetics and improve somatic cell reprogramming by regulating signaling pathways. For example, valproic acid (VPA) inhibits histone deacetylase and improves the efficiency of reprogramming mouse embryonic fibroblasts (MEF) into induced pluripotent stem (iPS) cells [6]. RG108 is a DNA methyltransferase inhibitor, which improves the efficiency of MEF into iPS cells [7]. Vitamin C (VC) is a cofactor in reactions driven by dioxygenases including collagen prolyl hydroxylases, hypoxia-inducible factor (HIF), prolyl hydroxylases, and histone demethylases, which has been found to enhance the generation of mouse and human iPS [8]. BIX01294, a G9a HMTase inhibitor, has been found to improve the efficiency of cell reprogramming [9]. A83-01 strongly inhibits ALK4, 5, and 7 and only weakly inhibits ALK1, 2, 3, and 6 and appears to inhibit TGF-β-induced epithelial-to-mesenchymal transition via the inhibition of Smad2 phosphorylation [10]. CHIR99021 is an inhibitor of glycogen synthase kinase 3β (GSK3β) that prevents the phosphorylation of beta catenin by GSK3β and activates Wnt signaling [11, 12]. MEK inhibitor PD0325901 can inhibit the MAPK/ERK signaling pathway to promote mouse embryonic stem cell (ESC) self-renewal [11, 13, 14]. Furthermore, iPS cells were induced from mouse fibroblasts by eight small molecules without using any transcription factors [15].

NSC have a strong potential to repair neurodegenerative diseases and enhance the regeneration of the damaged nervous system [7, 16], however there is still not a viral transduction vector free method to obtain a sufficient number of NSC for individualized therapies. Here, we set out to determine whether using only small molecules, in place of potentially hazardous transduction vectors, could induce mouse fibroblasts into NSC.

2. Materials and Methods

The animal ethics had been approved by the Flinders University Animal Ethic Committee and South Australia Pathology Animal Ethic Committee.

2.1. Cell Culture. Mouse embryonic fibroblasts (MEF) and tail-tip fibroblasts (TTF) were isolated from C57/BL6 mice as described previously [17]. MEF and TTF were cultured in DMEM (Life Technologies) containing 10% FBS (Life Technologies), 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies).

2.2. Induction of SMINS Cells. MEF or TTF were seeded at 1.4×10^5 per 35 mm dish coated with feeder cells before induction. MEF (Passages 1–3) were treated with mitomycin C (10 μg/mL) for 2.5 hours and then washed three times with 1 × PBS and finally cultured in stem cell culture medium overnight for feeder cells. The stem cell signaling pathway modulator small molecules PD0325901, CHIR99021, and A83-01 were used to start the induction. The epigenetic modulator small molecules valproic acid, Bix01294, and RG108 were selected to improve the induction efficiency and the cell senescence modulator small molecule vitamin C was used to reduce cell death during the induction [18]. The cells were induced in 6 cycles. On the first day, the cells were induced in stem cell culture medium (SCM) (DMEM supplemented with 15% FBS, 1% nonessential amino acids (Life Technologies), 1% L-glutamine (Life Technologies), 50 units/mL penicillin, 50 μg/mL streptomycin, 0.1 mM β-mercaptoethanol (Life Technologies), and 1,000 units mL^{-1} leukaemia inhibitory factor (LIF) (Millipore)) containing small molecules (valproic acid, 1 μM; Bix01294, 1 μM; RG108, 0.04 μM; PD0325901, 1 μM; CHIR99021, 3 μM; vitamin C, 25 μM; A83-01, 2.5 μM). The cells were cultured in SCM for the next two days. Then, the cycle was repeated 5 times. Next, the cells were passaged and suspended in 1 mL SCM (as for per 35 mm dish) and then did a drop of 20 μL for suspending culture in petri dishes as shown in Supplementary Figure S1 available online at http://dx.doi.org/10.1155/2016/4304916. Finally, the cells were cultured in the neural stem cell medium (DMEM/F12 (Life Technologies) supplemented with B-27 (1 : 50, Life Technologies), 50 units/mL penicillin, 50 μg/mL streptomycin, 8 mM HEPES buffer, 20 ng/mL EGF, and 10 ng/mL bFGF) in petri dishes for two weeks. As for feeder-free induction, the cells were seeded at 5×10^5 cells per 35 mm dish coated with PDL (10 μg/mL) (Sigma) at 37°C for two hours. The cells were cultured in SCM containing Bix01294, RG108, and PD0325901 for 2 weeks; the medium was changed on the other day. The colonies appeared during the induction process. The colonies were cultured in petri dishes in NSC medium for another two weeks. Native NSC were cultured from brain of new born mouse in the NSC medium as positive controls as described previously [19]. All the small molecules were from Stemgent.

2.3. RT-PCR and RT Profiler PCR Array. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) with on-column DNA digestion. Total RNA (500 ng) was converted to cDNA by Superscript III Direct cDNA Synthesis System (Life Technologies). PCR was performed by 30 cycles using the primers described in Supplementary Table 3. The RT profiler PCR array was carried out using the Mouse Neurogenesis and NSC PCR Array (Qiagen).

2.4. Alkaline Phosphatase (ALP) and Immunofluorescence Staining. ES culture medium was added to NS and SMINS cells overnight. Alkaline phosphatase staining was carried out according to the manufacture's protocol (Roche). For the immunocytochemistry staining, cells were washed with 1 × PBS and then fixed with 4% paraformaldehyde for 10 min. After washing twice with 1 × PBS, cells were permeabilized with 0.1% Triton X-100 for 20 min. Cells were then washed twice and blocked in a solution of PBS containing 1% FBS and 4% BSA for 1 hour. Primary antibodies were diluted in blocking buffer and applied for 1 hour at room temperature or overnight at 4°C. Primary antibodies were used at the following dilution: Sox2 (Millipore, 1 : 200, mouse), Olig2 (Millipore, 1 : 500, rabbit), GFAP (Dako, 1 : 400, rabbit), Map2 (Osenses, 1 : 1000, rabbit), Nestin (Santa Cruz Biotechnology, 1 : 300, mouse), Oct4 (N-19) (Santa Cruz Biotechnology, 1 : 500, goat), Vamp2 (Osenses, 1 : 2000, rabbit), NeuN (Biosensis, 1 : 500, mouse), Alpha-tubulin (Sigma, 1 : 1000, mouse) and O4 (Millipore, 1 : 200, mouse), and Ki67 (Abcam, 1 : 100, rabbit). Cells were washed three times with 1 × PBS and then applied with secondary fluorescent antibodies (1 : 1000, Cy3 or Alexa-488) and 10 μg/mL DAPI for 1 hour at room temperature.

2.5. FACS Analysis. TTF cells were dissociated and incubated in 2% FBS-PBS solution with antibody P75 conjugated with FITC (Biosensis, 1 : 6, mouse) on ice for a half hour. The cells were washed three times with ice-cold 2% FBS-PBS before running FACS. The positive fraction was evaluated by FACS (Beckman Coulter Epics Altra HyperSort, using Expo MultiComp Software version 1.2B (Beckman Coulter, Miami, FL, USA)) comparing with a blank control.

2.6. In Vitro Differentiation of SMINS Cells. Cells were seeded at 0.5×10^4 on a PDL (10 μg/mL)/laminin (10 μg/mL) (Sigma) coated 4-well plate. For spontaneous differentiation, cells were cultured in NS cell culture medium containing N2 (Life Technologies) without EGF and bFGF for one or three weeks. For the differentiation of mature neuron, the single SMINS cells were cultured in neurobasal medium (Life Technologies) containing B27 (2%) (Life Technologies), GlutaMAX (2 mM) (Life Technologies), and dibutyryl cAMP (0.5 mM) (Sigma) for four weeks. As for specific astrocyte differentiation, the cells were cultured in neurobasal medium

containing 1 × N2, 1 × B27, and 1% FBS for 3 weeks. For neuron differentiation, the cells were cultured in neurobasal medium containing 1 × N2, 1 × B27, 1% FBS, 5 μM forskolin, and 1 mM retinoic acid for 2 weeks and then in neurobasal medium containing 1 × N2, 1 × B27, 1% FBS, 10 ng/mL BNDF, and 10 ng/mL GDNF for 2 weeks. The cells were cultured in DMEM/F12 containing 1 × N2, 10 ng/mL bFGF, 10 ng/mL PDGF-AA, and 5 μM forskolin for 5 days and then in 0.2 mM vitamin C and 30 ng/mL T3 for 3 weeks for specific oligodendrocyte differentiation.

2.7. Differentiation of SMINS Cells In Vivo. Dissociated SMINS cells were labeled with lentiviral EGFP vectors and a total volume of 3 μL ($10^5/\mu$L) was injected into the lateral ventricle of brain in nude pups (6 pups) at the age of 3 days. Brains were collected at 6 weeks after injection following a saline perfusion, fixed in periodate-lysine-paraformaldehyde for 24 hours [20], washed with PBS, soaked in 30% sucrose for 48 hours, and sectioned into 30 μm coronal sections. Brain sections were immunostained for Sox2 (for neural stem cells), ki67 (for proliferating cells), GFAP (for astrocytes), Map2 (for neurons), NeuN (for neurons), and Olig2 (for oligodendrocytes) using our methods described previously [21].

2.8. Electrophysiology. Whole cell patch clamp was performed on differentiated cells using a HEKA EPC-10 patch clamp amplifier and Patch Master software (HEKA Electronik, Lambrecht/Pfalz, Germany). Patch pipettes were pulled from borosilicate glass and fire polished, with resistance of 3–5 MΩ. Internal solution contained the following (mM): NaCl, 10; KCl, 145; HEPES, 10; $MgCl_2$, 1; and EGTA, 1, adjusted to pH 7.3. External solution contained the following (mM): NaCl, 135; KCl, 2.8; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 2; and Glucose, 10, adjusted to pH 7.4 with NaOH. Measurement of Na^+ and K^+ currents was performed in voltage-clamp mode, utilising a protocol with voltage steps of −70 to +70 mV (10 mV increments), for 20 ms or 100 ms, from a holding potential of −80 mV. Series resistance was compensated at least 70%. Action potentials were recorded in current-clamp mode, with injection of 20–50 pA of current if required. Voltages shown were not adjusted for liquid junction potential.

3. Results

3.1. Small Molecule-Induced Neural Stem (SMINS) Cells Can Be Obtained from MEF by a Combination of 7 Small Molecules. We selected a number of candidate small molecules to reprogram fibroblasts into NSC. A combination of small molecules (valproic acid, 1 μM; Bix01294, 1 μM; RG108, 0.04 μM; PD0325901, 1 μM; CHIR9901, 3 μM; vitamin C, 25 μM; A83-01, 2.5 μM) is found to induce mouse embryonic fibroblasts (MEF) into NSC. Considering that too much expression of transcription factors is detrimental to the self-renewal of pluripotent cells [17], we designed a 6-cycle protocol for the induction process (Supplementary Figure S1). Fibroblasts were cultured alternatively in small molecule-containing stem cell culture medium (SMSCM) for 1 day and in stem cell culture medium (SCM) without small molecules for 2 days as cycle 1 and the cycle was repeated for additional 5 times. After the 6th cycle, the cells were cultured in suspension for 2 days and then in NSC culture medium for 2 weeks. There are no colonies in the induction process before suspending culture. There is one colony in each drop after suspending culture. And then the colonies were cultured in NSC medium for two weeks. MEF were negative for Sox2, Nestin, and SSEA-1 after several passages (Supplementary Figure S2). In order to eliminate the possibility of neural crest stem cells from mouse skin [22], only MEF that are negative to Sox2, SSEA-1, and Nestin were used for induction. Using 7 small molecules for induction, SMINS (SMINS-MEF-7) cells were able to be stably and homogenously expanded more than two years without a significant reduction in the self-renewal capacity and are morphologically indistinguishable from classic NSC either suspending culture in petri dishes or attaching on poly-D-lysine (10 μg/mL)/laminin (10 μg/mL) or matrigel coated cell culture dishes at high density ($1 \times 10^5/cm^2$) (Supplementary Figures S3A and B and Figure S4). Firstly, we stained the colonies with ALP, and they appeared positive (Supplementary Figure S5). And then we tested the typical NSC markers Sox2 and Nestin; they were also positive (Figures S3C and D).

Next we tested the expression of NSC marker genes by the reverse transcription PCR (RT-PCR). Compared to fibroblasts, SMINS-MEF-7 cells expressed NSC marker genes including *Sox2*, *GFAP,* and *Olig2* (Supplementary Figure S3E). Just like NSC, SMINS-MEF-7 cells did not express the pluripotent genes *Oct4* and *Nanog* (Figure S3E). In order to further assess the expression profiles of genes relevant to NSC, we carried out an analysis of 84 genes which are related to mouse neurogenesis and NSC utilizing RT profiler PCR arrays. Compared with MEF, 23 genes were upregulated by 3- to 1543-fold and 13 genes downregulated by at least 3-fold in SMINS-MEF-7 cells (Figure S6A and Supplementary Table 1). Notch [23, 24], Wnt [25], BMP [26, 27], and Shh [28] signaling pathways are known to regulate NSC properties. Among the upregulated genes, *Dll1*, *Notch2*, *Hey1,* and *Pou3f3* are involved in the Notch signaling pathway, *Shh* in the Shh signaling pathway, and *Bmp2* and *Bmp15* in the BMP signaling pathway. Among the downregulated genes, *Hey2* and *Heyl* are involved in the Notch signaling pathway, *Nog* in the BMP signaling pathway, and *Ndp* in the Wnt signaling pathway. Ten genes including *Notch2*, *Shh,* and *Fgf2* in SMINS-MEF-7 were upregulated in comparison with native NSC (Figure S6B and Supplementary Table 2).

To confirm the multipotency of the SMINS cells, we performed *in vitro* differentiation assays. SMINS-MEF-7 cells were able to spontaneously differentiate into astrocytes (GFAP-positive cells, 20 ± 2%), neurons (Map2-positive cells, 31 ± 3%), or oligodendrocytes (O4-positive cells, 36 ± 1%) (Supplementary Figures S7, 8, and 4). Moreover, SMINS-MEF-7 cells were able to express mature neural markers VAMP2 and NeuN in mature neuron differentiation medium (Figure S7). These results indicate that, like native NSC, SMINS cells are multipotent *in vitro*.

3.2. SMINS Cells Can Be Obtained from Tail-Tip Fibroblasts (TTF) by a Combination of 3 Small Molecules. Next we examined which small molecules are important for the

generation of SMINS cells by withdrawal of individual small molecules from the combination. We found that the small molecules Bix01294, RG108, and PD0325901 are important for the induction to occur. To further confirm the validity of the protocol to obtain SMINS cells from fibroblasts and to eliminate potential contamination from skin-derived neural crest stem cells, we isolated TTF from adult mouse tails which had been stripped of skin. In order to further eliminate the possible contamination of the neural crest cells in TTF, TTF were sorted by FACS with fluorescence-labelled antibody to p75. Only 0.1% TTF cells are p75-positive cells after 3 passages (Supplementary Figure S9). Only p75-negative TTF cells were used for induction. Just like MEF, TTF could also robustly form neurospheres after the 6 cycles' induction protocol with these three small molecules, Bix01294, RG108, and PD0325901. These SMINS (SMINS-TTF-3) cells resemble native NSC in morphology (Figures 1(a) and 1(b)). SMINS-TTF-3 cells also express the NSC markers Sox2, Nestin, and ALP (Figures 1(c)-1(d) and Supplementary Figure S5). Next we tested the expression of NSC genes by reverse transcription PCR (RT-PCR). SMINS-TTF-3 cells expressed NSC marker genes including *Sox2*, *GFAP*, *Olig2,* and *Gli2* (Figure 1(e)) compared to fibroblasts which did not show this expression. Similar to NSC, SMINS-TTF-3 cells did not express the pluripotent genes *Oct4* and *Nanog* (Figure 1(e)). Furthermore, SMINS3 cells did not show pluripotent marker Oct4 by ICC (Supplementary Figure S10). Finally, we performed *in vitro* differentiation assays. SMINS-TTF-3 cells were able to differentiate into astrocytes (GFAP-positive cells, 24±1%), neurons (Map2-positive cells, 36±2%), or oligodendrocytes (O4-positive cells, 30±2%) (Figure 2). Furthermore, SMINS-TTF-3 cells were able to express mature neuronal markers Vamp2 and NeuN in mature neuron differentiation medium (Figure 3(a)). To check whether the SMINS cells contain feeder cells, the SMINS cells after passage 5 were stained for fibroblast marker Alpha-tubulin. We did not find any Alpha-tubulin positive cells in the SMINS cells (Supplementary Figure S11), suggesting that there was no feeder cell contamination in SMINS cells after induction.

3.3. SMINS Cells Can Differentiate into Functional Neurons. Next, we checked whether the SMINS cells can differentiate into functional neurons. The differentiated SMINS-TTF-3 cells display positive mature neuron markers (Figure 3(a)). Furthermore, a small subset of differentiated SMINS3 cells displays a unique phenotype similar to that of mature neurons. Electrophysiological analysis demonstrated a resting membrane potential of -57.7 ± 5.2 mV ($n = 5$) in these cells which contained fast inactivating inward Na^+ currents in addition to slowly inactivating outward K^+ currents (Figures 3(b) and 3(c)). Action potentials either were spontaneous or were able to be evoked in these cells by injecting current pulses injection (Figure 3(d)). The majority of neural-like differentiated cells displayed a different phenotype, with a more positive resting membrane potential, only K^+-like outward currents with no inward Na^+ currents or evoked action potentials (Supplementary Figure S12). This indicates that the SMINS cells are able to differentiate into functional neurons.

3.4. SMINS Cells Can Differentiate to Neural Cell Lineages In Vivo. To assess whether the SMINS cells are able to survive and differentiate into neural cell lineages *in vivo*, SMINS-TTF-3 cells were labeled with green fluorescence protein (GFP) with lentiviral EGFP vector and were transplanted into lateral ventricle of nude pups. Only few GFP^+ cells were Sox2 (Figure 4(a)) and Ki67 (Figure 4(b)) positive at 6 weeks after transplantation, which means almost all of SMINS cells differentiate to neural cell lineages *in vivo*. The GFP^+ cells migrated with a long distance from lateral ventricles into the parenchyma and were well integrated with the host brain tissues (Supplementary Figure S12). Furthermore, the SMINS cells were positive to astrocyte marker GFAP (Figure 4(c)), neural markers Map2 and NeuN (Figures 4(d) and 4(e)), and oligodendrocyte marker Olig2 (Figure 4(f)). These data indicate that the SMINS cells are able to differentiate to neural cell lineages *in vivo*.

3.5. Induction Efficiency. It is difficult to calculate the induction efficiency because of the special induction process. No colony appeared before the drop suspending culture. Each drop formed one colony after suspending drop culture. So we calculated how many cells could form one colony per drop. We found the minimum cell number is 50 to form one colony. Therefore, the efficiency of induction is up to 2%. The induction efficiency of 2% is a relative number compared with suspending culture cells as described in the method section. A better way for the induction efficiency is to use of Sox2-EGFP fibroblasts for induction in the future.

3.6. Feeder-Free SMINS3 (FF SMINS3). Our SMINS cells contain feeder cells in the first several passages, which can affect the NSC application in the future. So we tried to remove the feeder cells with some dish substrates. It was found that the poly-D-lysine (PDL) could replace feeder cells during the induction. Moreover, the induced cells formed colonies during the induction process on PDL, so the feeder free protocol does not need to perform suspending drop culture for the colony formation. The feeder-free SMINS3 were positive for NSC markers Nestin and Sox2 (Figure 5(a)). Furthermore, the cells were able to differentiate into astrocytes (Figure 5(b)), neurons (Figures 5(c) and 5(d)), and oligodendrocytes (Figure 5(e)) in specific differentiation medium. All these data indicate that the feeder-free SMINS3 are NSC.

4. Discussion

Reprogramming somatic cells to iNSC makes the NSC therapy feasible. iNSC also have great value as models of disease pathogenesis, drug screening, and toxicity tests. Although NSC can be generated from ESCs or iPS [29, 30], there are still ethic and safety problems when these cells are used for cell therapy in patients [30–32]. To overcome these problems, some scientists have successfully induced somatic cells to iNSC by overexpressing transcription factors [1–5]. However, they all used viral vectors to introduce transcription factor genes into the host cells, which brings the safety concern on the NSC therapy. Moreover, the c-Myc oncogene can cause

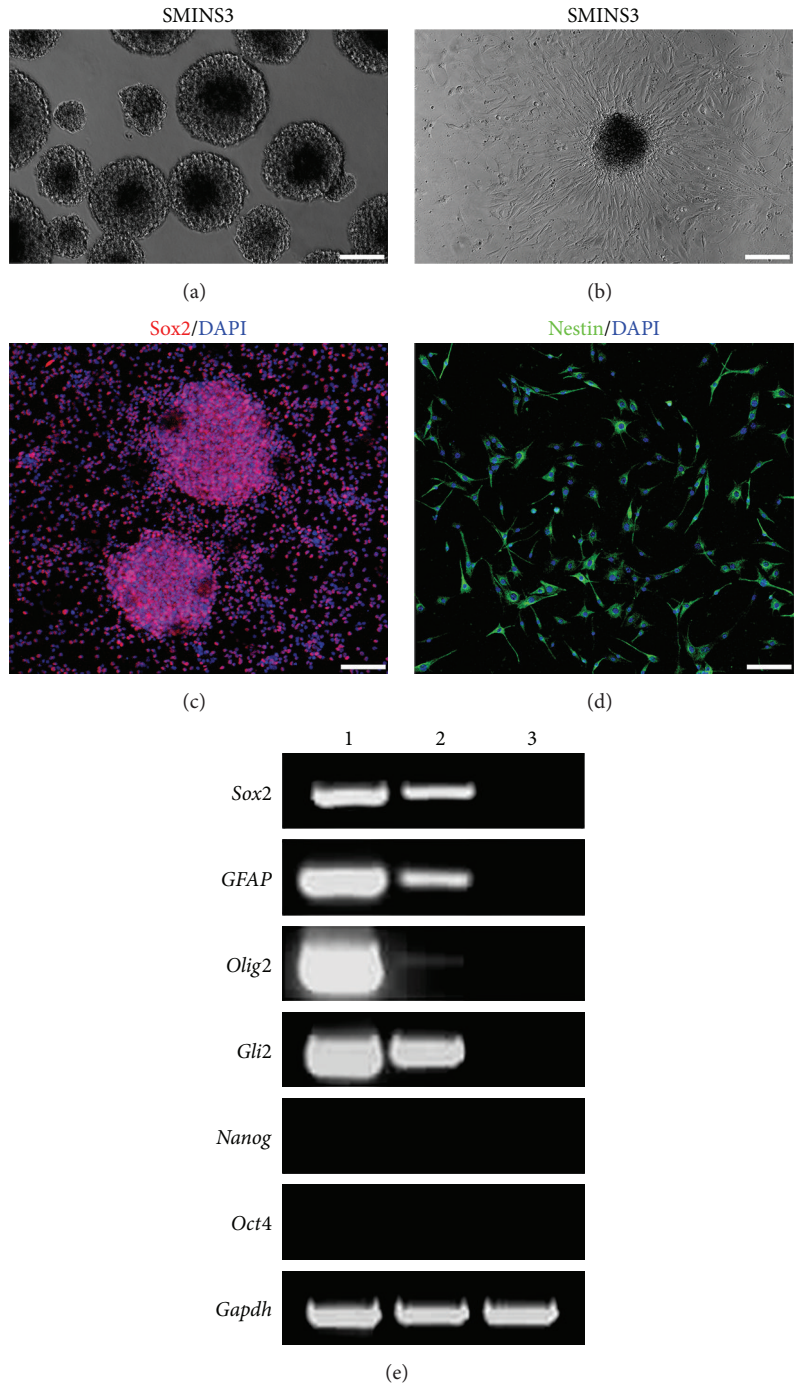

Figure 1: SMINS-TTF-3 cells induced from mouse tail-tip fibroblasts utilizing three small molecules (Bix01294, RG108, and PD0325901). (a) SMINS-TTF-3 neurospheres were cultured in petri dishes under bright field in suspending culture. (b) A SMINS-TTF-3 neurosphere was cultured on matrigel coated dishes under bright field in attaching culture. (c-d) SMINS-TTF-3 neurospheres were dissociated and stained by typical neural stem cell markers Sox2 (red) and Nestin (red) examined by immunocytochemistry. DAPI was used for nuclei counterstaining (blue). (e) Analysis of typical neural stem cell gene expressions by RT-PCR, (1) NS (native neural stem cells), (2) SMINS-TTF-3 (small molecule-induced neural stem cells from TTF with 3 small molecules), and (3) TTF (tail-tip fibroblasts). Scale bar: 100 μm.

brain tumorigenesis from transplanted iPS-derived NS cells [4]. Our study demonstrates that mouse fibroblasts can be efficiently induced into NSC using only small molecules. This is the first report that multipotent stem cells can be induced from fibroblasts without using any exogenous transcription factors. In our experiment, the SMINS cells were passaged for more than two years and still continue proliferating in either attaching or suspending culture. Thus,

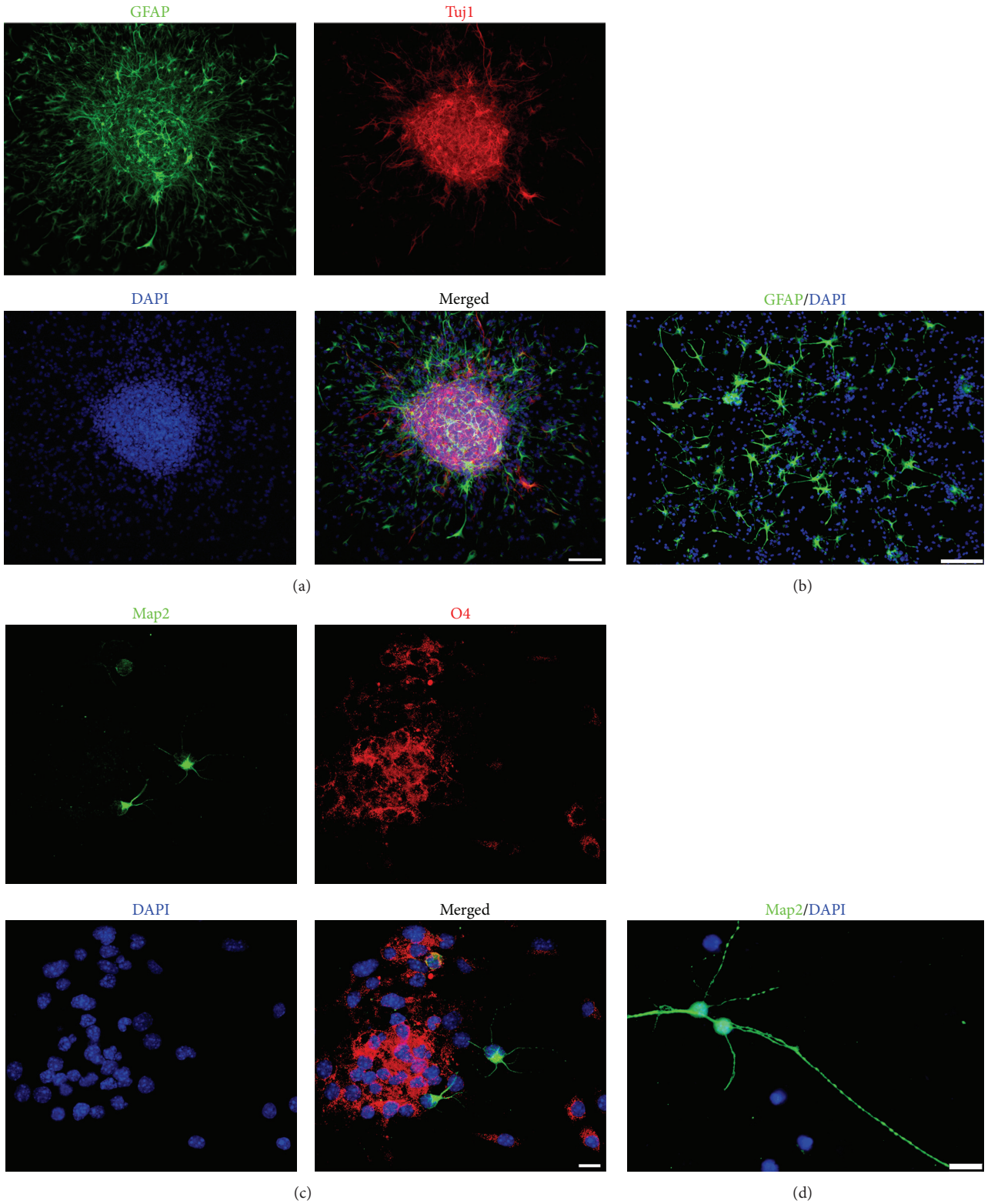

Figure 2: Differentiation of SMINS-TTF-3 cells *in vitro*. (a-b) SMINS-TTF-3 cells spontaneously differentiated into astrocytes marked by GFAP (green) and neurons marked by Tuj1 (red) in spontaneous medium for one week. (c-d) SMINS-TTF-3 cells spontaneously differentiate into neurons marked by Map2 (green) and oligodendrocytes marked by O4 (red) in spontaneous medium for three weeks. DAPI was used for nuclei counterstaining (blue). Scale bar: 100 μm (a and b) and 10 μm (c and d).

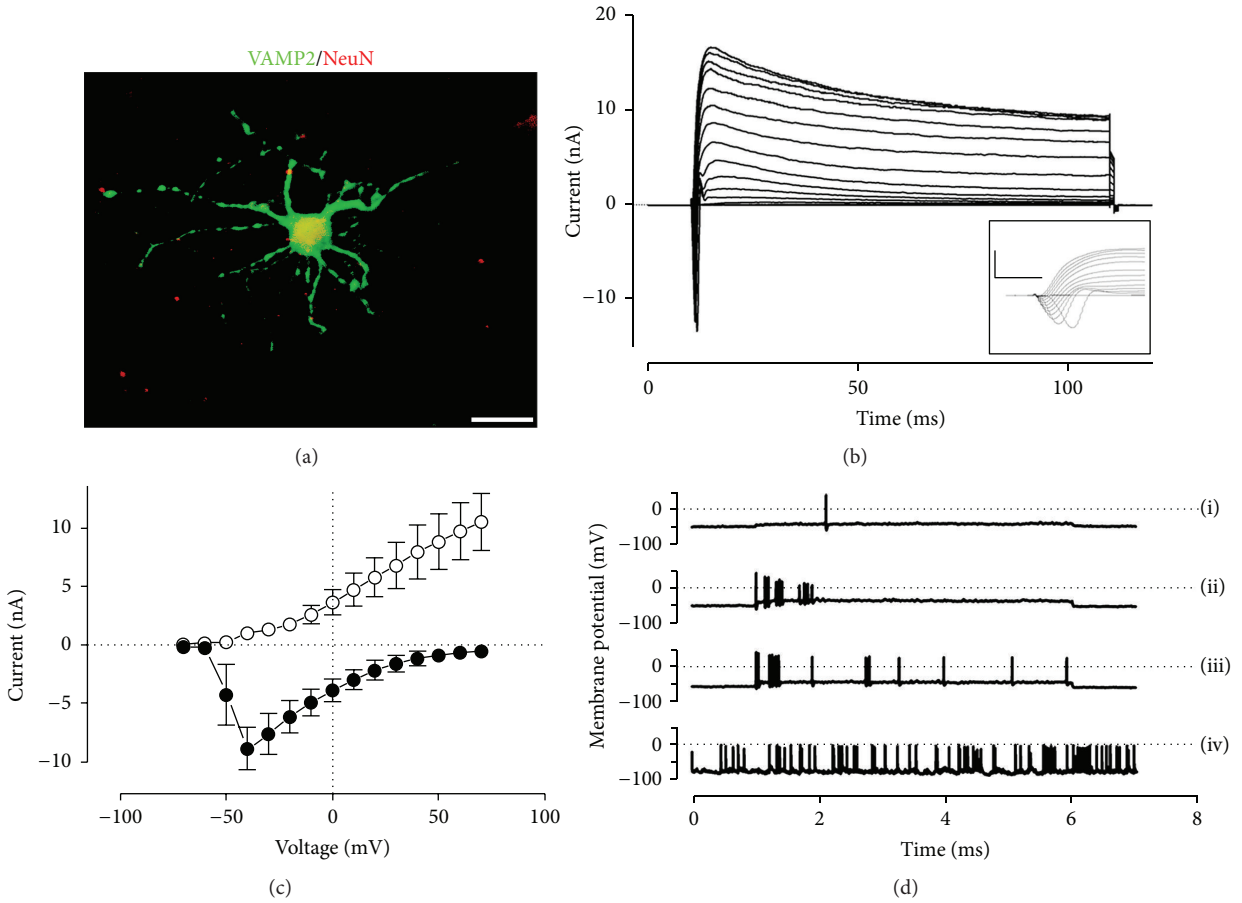

Figure 3: Mature neurons from SMINS-TTF-3 cells. (a) SMINS-TTF-3 cells were cultured in mature neural medium for one month and stained by mature neuron markers VAMP2 (green) and NeuN (Red). (b) Electrophysiological investigations of a subset of long-term differentiated cells showed the presence of both inward Na^+ currents and outward K^+ currents in response to electrical stimulation with steps from −70 to +70 mV (10 mV increments) from a holding potential of −80 mV. Representative trace with 100 ms steps, inset with 20 ms steps (inset scale bars represent 5 ms on x-axis, 10 nA on y-axis). (c) Mean ± SEM maximal Na^+ (closed circles) and K^+ currents (open circles, $n = 4$). (d) These cells demonstrated action potential firing in response to current injection ((i) 20 pA for 5 s, (ii) and (iii) 50 pA for 5 s) or spontaneously (iv). Scale bar: 10 μm (a).

the SMINS cells are able to form stable cell lines for stem cell therapy. Our studies have made an important step forward towards tailoring individualized therapies for patients with neurodegenerative diseases and other neurological disorders, as our method eliminates the concerns of potentially harmful genome integration by viral transduction vectors or the introduction of oncogenic transcription factors. Thus, these SMINS cells may have a direct potential in clinical treatment of neurological disorders.

One issue that is concerned is the origin source of SMINS cells from MEF and TTF. We used two methods to eliminate possible neural crest contamination. By FACS method, only 0.1% TTF cells after 3 passages are p75-positive. These positive cells are most likely derived from p75-positive Schwann cells or blood vessel cells such as endothelial cells and smooth muscle cells [33]. Secondly, skin-derived neural crest cells were eliminated by stripping off the skin before the TTF preparation with enzymatic digestion. As the induction efficiency is 2%, it is unlikely that the SMINS cells are from the p75-positive neural crest cells, which only occupied 0.1% and was not used for the induction after FACS. Taken together, these results do not support the assumption of neural crest cells as the original source of the SMINS cells. Another issue to be considered is whether the SMINS cells pass by the pluripotent stage. Based on the present data, Oct4 and Nanog expression could not be detected in SMINS cells. Therefore, our data does not support the notion. However, we speculate that the SMINS cells may come through a partial pluripotent stage and become NSC when they were cultured in the NSC culture medium.

Although the mechanism of reprogramming is still unknown, it is related to DNA demethylation, histone demethylation, and acetylation [34–38]. The small molecules, such as VPA (histone deacetylase inhibitor), BIX01294 (G9a HMTase inhibitor), and RG108 (DNA methyltransferase inhibitor), can enhance reprogramming. It is reasonable to reprogram fibroblasts to NSC by these small molecules in proper conditions. It is reported that MEK inhibitor PD0325901 can inhibit the MAPK/ERK signaling pathway to promote mouse ESC self-renewal [11, 13, 14]. Our data also

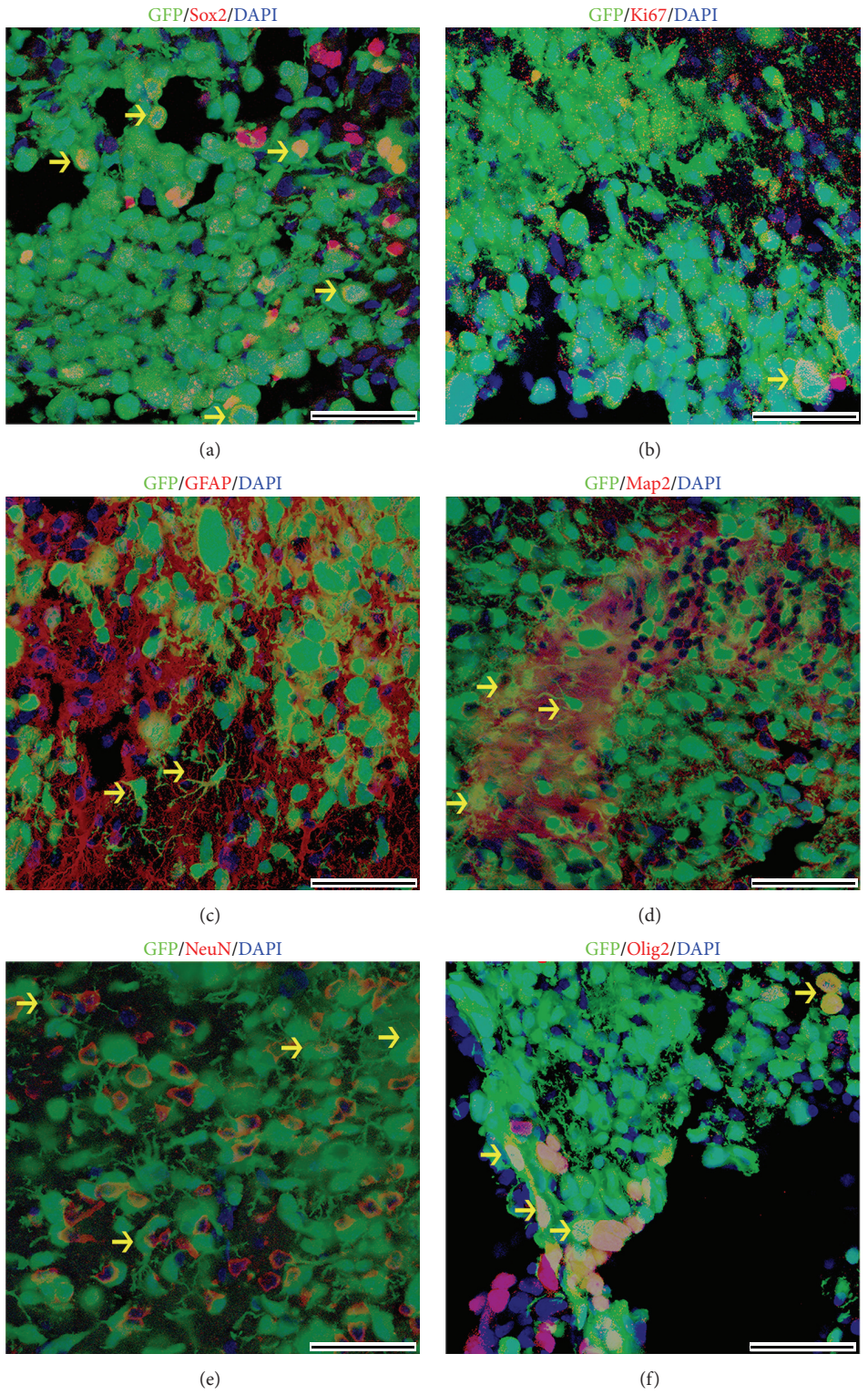

Figure 4: Differentiation of SMINS-TTF-3 cells *in vivo*. SMINS-TTF-3 cells infected with lentiviral EGFP vectors were injected into the lateral ventricle of brain in nude pups at the age of 3 days and the brains were collected at 6-week point. (a-b) Some injected SMINS-TTF-3 cells remained as neural stem cells, as indicated by neural stem cell marker Sox2/GFP$^+$. Some cells kept the ability of proliferation, as shown by Ki67/GFP$^+$ staining. (c–f) The injected cells differentiated into astrocytes (GFAP/GFP$^+$), neurons (Map2/GFP$^+$ and NeuN/GFP$^+$), and oligodendrocytes (Olig2/GFP$^+$) *in vivo*. DAPI was used for nuclei counterstaining (blue). Scale bar: 50 μm. The arrows direct positive cells.

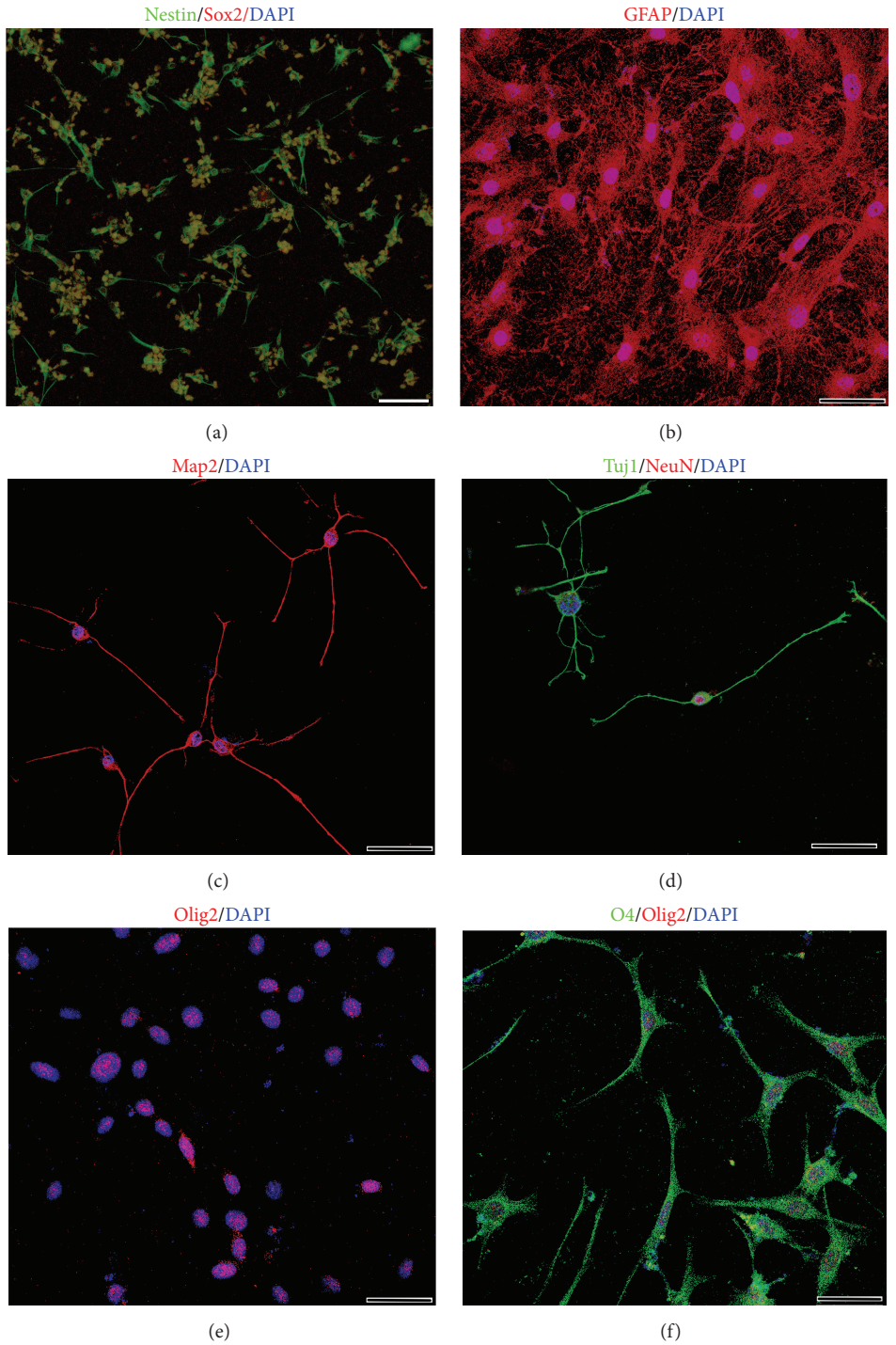

Figure 5: Feeder-free SMINS-TTF-3 cells (FF SMINS3). TTF were seeded on PDL coated dishes in stem cell medium containing Bix01294, RG108, and PD0325901 for two weeks and then transferred into NSC medium in petri dishes for two weeks. (a) The FF SMINS3 cells were stained by neural stem cell markers Sox2 and Nestin. (b–f) The FF SMINS3 cells differentiated to astrocytes (GFAP), neurons (Tuj1, Map2, and NeuN), and oligodendrocytes (Olig2 and O4) in specific differentiation medium. DAPI was used for nuclei counterstaining (blue). Scale bar: 100 μm (a) and 50 μm (b–f).

support the report that mouse pluripotent stem cells were differentiated to neuroectoderm by blocking MAPK/ERK signaling pathway [39]. Furthermore, our studies suggest that signaling pathways such as Notch, Shh, BMP, and Wnt are likely involved in the reprogramming of fibroblasts by these small molecules. It will be valuable in the future to understand how the small molecules affect each of the Notch, Shh, BMP, and Wnt pathways. SMINS cells may

also provide a novel model for studying the mechanisms of reprogramming of somatic cells into adult stem cells. Moreover, it still remains unknown whether these small molecules could induce human fibroblasts to NSC.

Conflict of Interests

There is no conflict of interests.

Authors' Contribution

Yan-Chuang Han and Xin-Fu Zhou conceived the idea and designed all the experiments. Yan-Chuang Han performed the majority of the experiments and wrote the paper. Yoon Lim and Hua Li performed the RT-PCR experiment. Jia Liu constructed lentiviral vectors and built the EGFP cell lines; Nimshitha Pavathuparambil Abdul Manaph and Miao Yang performed cell culture, FACS, and performed some immunocytochemistry. Michael D. Duffieldl performed and analyzed the electrophysiology experiments. Damien J. Keating conceived the electrophysiology experiments, analyzed the electrophysiology data, and revised the paper. Xin-Fu Zhou supervised the project and revised the paper.

Acknowledgments

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Research Article

Dynamic Changes in Occupancy of Histone Variant H2A.Z during Induced Somatic Cell Reprogramming

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The development of induced pluripotent stem cells (iPSCs) has enabled study of the mechanisms underlying cellular reprogramming. Here, we have studied the dynamic distribution of H2A.Z during induced reprogramming with chromatin immunoprecipitation deep sequencing (ChIP-Seq). We found that H2A.Z tended to accumulate around transcription start site (TSS) and incorporate in genes with a high transcriptional activity. GO analysis with H2A.Z incorporated genes indicated that most genes are involved in chromatin assembly or disassembly and chromatin modification both in MEF and Day 7 samples, not in iPSCs. Furthermore, we detected the highest level of incorporation of H2A.Z around TSS in Day 7 samples compared to MEFs and iPSCs. GO analysis with only incorporated genes in Day 7 also displayed the function of chromatin remodeling. So, we speculate H2A.Z may be responsible for chromatin remodeling to enhance the access of transcription factors to genes important for pluripotency. This study therefore provides a deeper understanding of the mechanisms underlying induced reprogramming.

1. Introduction

The development of induced pluripotent stem cell (iPSC) technology that was first reported in 2006 by Takahashi and Yamanaka [1] has facilitated great advancements in the field of stem cell biology. The ability to reprogram somatic cells to a pluripotent state has enhanced our understanding of the mechanisms underlying nuclear reprogramming and the regulation of cell stemness and spawned new approaches for regenerative medicine and disease therapy [2, 3]. However, while iPSC technology offers unprecedented opportunities, the precise molecular steps by which donor cells regain pluripotency remain somewhat unexplained [4]. Furthermore, a number of issues [5] surrounding the efficiency and stability of the induced reprogramming process need to be resolved.

Some studies have provided insights into changes in gene expression patterns during reprogramming [6–8]. However, differences between donor cells and iPSCs, and embryonic stem cells (ESCs) and iPSCs exist at the epigenetic level as well as in gene expression [9, 10]. Dynamic chromatin structures affect transcriptional patterns by altering the accessibility of transcription factors or other regulatory proteins to DNA through permissive remodeling machinery. The unique epigenetic state of the ESC is characterized as a higher order chromatin structure [12], suggesting that the hyperdynamic plasticity of chromatin proteins may be responsible for maintaining pluripotency. Furthermore, both remodeling complexes and histone variation can affect chromatin structure and cell fate. For example, brahma-associated factor (BAF) complexes can contribute to stable, tissue-specific memory of cell fate [11, 13], and histone variant replacement can regulate

Table 1: Clean data output from H2A.Z ChIP-Sequence.

Sample ID	Length of read (bp)	Total number of reads	Output (bp)
MEFs	49	12,807,843	627,584,307
Day 7 cells	49	12,910,314	632,605,386
iPSCs	49	12,779,646	625,908,654

Table 2: Mapped reads output from H2A.Z ChIP-Sequence.

Sample ID	Total reads	Mapped		Unique mapped	
		Reads	Percentage	Reads	Percentage
MEFs	12,807,843	12,458,074	97.27%	11,216,366	87.57%
Day 7 cells	12,910,314	12,575,751	97.41%	10,961,715	84.91%
iPSCs	12,779,646	12,412,347	97.17%	10,577,813	82.81%

stem cell differentiation through critically determining the gene expression profile [14]. However, whether a dynamic chromatin state leads to changes in cell fate during iPSC development remains unclear.

The incorporation of specific histone variants underlies changes in chromatin structure which are crucial for transcriptional control during remodeling. One of the most highly evolutionarily conserved histone variants is H2A.Z, and this variant is responsible for unique structural features of chromatin. H2A.Z possesses a tetramer-dimer docking domain [15], is usually enriched at the transcriptional start site (TSS) of genes, and plays critical roles in gene regulation through transcriptional activation [4, 16]. H2A.Z has also been implicated in chromatin regulation processes as well as in the establishment of chromatin boundaries for nucleosome exchange and polycomb repression [4, 17]. However, the relationship between these seemingly disparate functions and induced reprogramming of pluripotent cells remains obscure. Understanding exactly how different histone variants influence gene expression patterns and ultimately cell fate will enhance our understanding of induced reprogramming.

To this end, we explored the H2A.Z genome-wide deposition profile of three cell samples representing different stages of reprogramming from murine embryonic fibroblasts (MEFs) to iPSCs using chromatin immunoprecipitation (ChIP) coupled with high-throughput sequencing (ChIP-Seq). We identified drastic enrichment of H2A.Z deposition in the transition period from Day 7 to characterization as an iPSC. Examination of H2A.Z dynamics during this transition period has enhanced our understanding of the roles of histone variants in facilitating induced reprogramming. Our results find that H2A.Z may contribute to the phenotypic plasticity of chromatin structure, thereby enhancing transcription factor access to DNA and contributing to the reprogrammed cell state.

2. Results

2.1. Dynamic Occupancy of H2A.Z Variant during iPSC Development. To investigate whether the histone variant H2A.Z is involved in reprogramming from MEFs to iPSCs, we utilized ChIP-Seq technology to examine the dynamics of chromatin-bound H2A.Z in the process of induced reprogramming. Our previous report showed mouse iPSCs (m-iPSCs) were successfully generated under ectopic expression of Oct3/4, Sox2, and Klf4 (OSK) in the presence of Vc [18]. Importantly, there were aggregated cells that appeared at Day 7 and always became the iPSC clone in the end. Here, the initial cell MEF, Day 7 cells (MEFs were induced for 7 days, and aggregated cells were used in subsequent analysis), and iPSCs (clones from MEF undergoing induction for 14 days) representing different stages of the reprogramming course were collected to fulfill the ChIP-Seq.

To avoid the false positive data due to nonspecificity from the H2A.Z antibody, we checked the antibody with an ordinary experiment fulfilled by IgG antibody ChIP. The DNA deposited with H2A.Z antibody fulfilled by ChIP was quantized by qRT-PCR. Special affinity genes hoxa11, hoxb1, zfpm2, lrx2, pax5, neurog1, and hoxb2 selected from UCSC database and report [19] were immunoprecipitated with H2A.Z and IgG antibody independently and then tested by qRT-PCR with the same primers (Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3162363).

We achieved over 20 Mb output clean dataset per cell sample (Table 1). Reads were aligned to a reference genome that combines mouse genome mm9. Using SOAP 2.21 software, we selected effectively mapped reads as detailed in Table 2. Further selection of reads that were mapped to unique genomic regions based on the precise mapping annotations "promoter," "UTR," "CDS," and "intergenic" refined the dataset (Table 2). Employing MACS 1.4.0 [20] software, we identified 23,818, 13,744, and 14,768 peaks among the MEF, Day 7, and iPSC cell samples, respectively (Table 3). Peaks are preferentially dropped in intergenic, promoter, intron, and 5′UTR regions both in MEF and iPSC cell samples (Figures 1(a) and 1(c)). Distinctively, 31.55% of the peaks were found on promoters in Day 7 cell samples more than in intergenic regions (23.64%) and any other genomic regions. Certainly, the percentage of peaks on promoter regions in Day 7 (31.55%) was higher than MEF (26.55%) and iPSC (25.04%).

We know that different genetic regions contain a different number of bases. In order to eliminate the influence of nonspecific incorporation, we evaluated the relative enrichment of peaks in genetic elements depending on ratio

Table 3: Output of peaks from H2A.Z ChIP-Sequence.

Sample ID	Total number of peaks	Length of peaks (bp)			Percentage
		Total	Mean	Median	
MEFs	23,818	32,163,617	1,350	1,581	1.18%
Day 7 cells	13,744	17,951,826	1,306	1,516	0.66%
iPSCs	14,768	12,532,924	849	942	0.46%

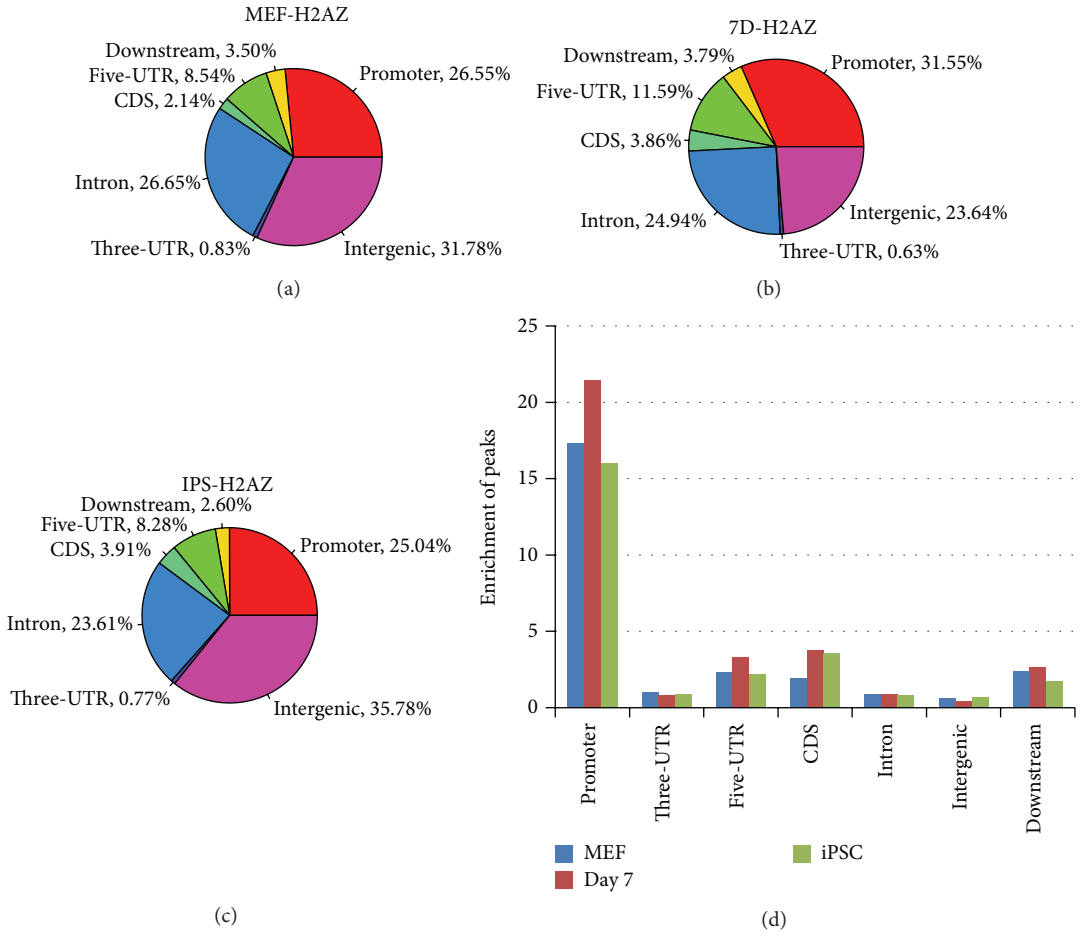

Figure 1: Distribution and enrichment of H2A.Z in different gene regions. (a–c) Mapping of peaks representing H2A.Z occupancy on a genome-wide scale relative to RefSeq mouse genes. "Promoter" and "downstream" are defined as 2 kb of 5′ or 3′ flanking regions. The "intergenic" region refers to all locations other than the promoter, 5′UTR, CDS, intron, 3′UTR, or "downstream" (d). Relative enrichment of H2A.Z peaks in gene elements defined as (number of ChIP sequencing peaks in one region/total number of ChIP sequencing peaks in genome)/(number of whole genome bases in one region/total number of whole genome bases).

[(number of ChIP sequencing peaks in one region/total number of ChIP sequencing peaks in genome)/(number of whole genome bases in one region/total number of whole genome bases)]. Figure 1(d) demonstrates that most peaks specifically were mapped to promoters preferentially, whereas small sets of peaks were mapped in regions including "downstream," 5′UTR, and CDS while some peaks were mapped to others, including intron, 3′UTR, and intergenic regions. Interestingly, H2A.Z occupied more DNA sequences in the promoter during the period of development from MEF to Day 7 but departed from these sites with iPSC formation (Figure 1(d)).

2.2. H2A.Z Is Deposited at the TSS Region during Induced Reprogramming. To deeply investigate the deposition of H2A.Z at promoter regions across the entire genome, we compared the presence of H2A.Z at annotated gene regions 5 kb upstream and 5 kb downstream TSSs with normalized reads in each of the three cell types. We found that H2A.Z was enriched on both sides of the TSS, with a small drop over the TSS site (Figure 2(a)). However, the average normalized reads have unmarked changes near the transcriptional end sites (TESs) (Figure 2(b)). These double peak curves and the relatively depleted distribution style of H2A.Z are in agreement with another report [21, 22], which proved that the result

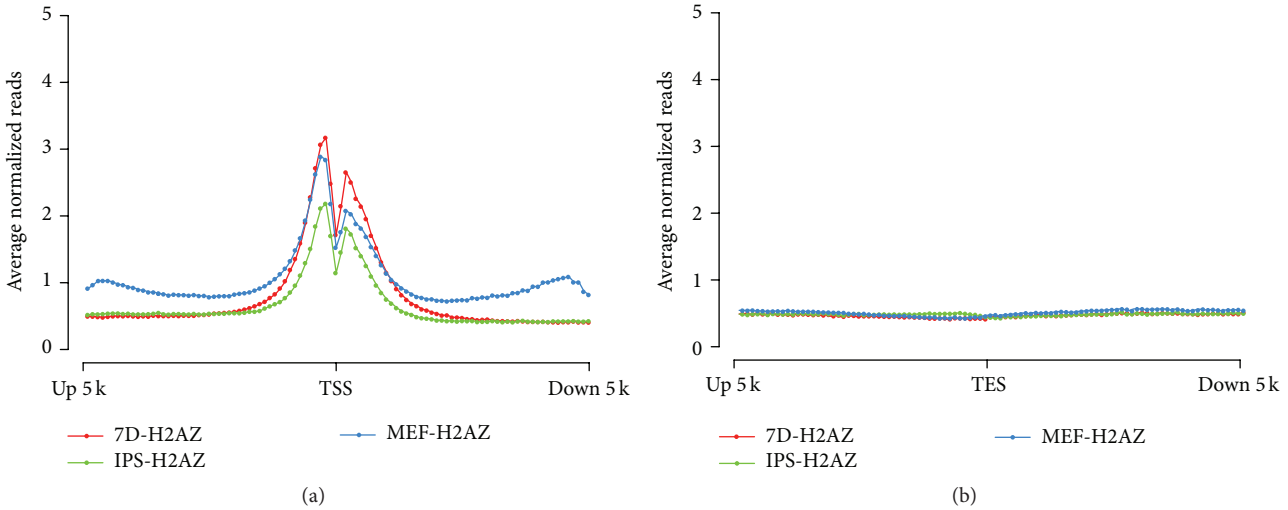

FIGURE 2: The distribution of average normalized reads in TSS and TES gene regions. The regions 5 kb upstream and downstream the TSS (a) and TES (b) are divided into 40 windows, and H2A.Z average normalized reads coverage is assessed in each window.

is believable and indicated H2A.Z preferentially deposits upstream TSSs. Consistent with the above result, Day 7 cells have the highest average level of H2A.Z occupancy, while the iPSCs have the lowest.

In mRNA expression level, such immune response genes (gene expression level: the highest at Day 7 compared to MEF and iPSCs) were noticeable [18]. However, gene related to acetylation, chromatin assemblies, and cell cycle was deposited with higher H2A.Z occupancy (Figure S2). These data indicate that cells induced by infection by OSK viral vectors change their cell fate through the help with H2A.Z's accessing to the epigenetics factors.

To examine the relationship between the enrichment of H2A.Z on chromatin and gene expression levels, we compared H2A.Z occupancy and the gene expression data from our previous report [18] in different genes. We then separated genes into four classes based on expression levels and displayed H2A.Z levels around TSSs. This revealed that, for all three samples, H2A.Z displayed high occupancy flanking TSSs in high expression and middle expression genes and decreases with decreasing transcription genes (Figure 3). This suggested a correlation between the incorporation of H2A.Z around TSS and gene expression in the development of iPSC.

Noticeably, transcription factors Oct4, Nanog, Klf4, cMyc, sox2, and Lin28 were usually employed to fulfill the induced reprogramming. In their DNA sequence, the enrichment of H2A.Z was dynamic and complex (Figure S3). However, the data showed that there was the highest occupancy of H2A.Z in the upstream region of Nanog and Lin28 individually. And on the wide whole sequence, the peak was shifted from downstream to upstream during the induced reprogramming. As the expression level of pluripotent genes was upregulated in the process of induced reprogramming, it indicates that the H2A.Z benefits the reactivation of these genes by the dynamic occupancy. Likely, the enrichment of H2A.Z on cMyc was dynamically shifted from upstream to TSS. Strikingly, the occupancy kept a higher level in these regions during the whole induced reprogramming. For Nanog and Oct4, the peaks always appeared in the CDS regions and more deposited H2A.Z at Day 7. This data supports that binding between Oct4 and H2A.Z downstream [23]. Unlike these transcriptional factors, the enrichment on Sox2 did not have the highest level on the TSS region at Day 7; however, there were continues occupancy both at the TSS and TES regions at Day 7. It indicted that regulation of these pluripotent genes' expression by H2A.Z may rely on the different behavioral style of dynamic occupancy.

2.3. H2A.Z Occupancy Contributes to Induced Reprogramming through Chromatin Remodeling. H2A.Z nucleosome composition at promoters and gene body influences nucleosome stability and transcriptional state [16], and the above results have indicated that H2A.Z preference incorporation to the high expressed genes around TSS during somatic cell reprogramming (Figure 3); we speculate the incorporation of H2A.Z can regulate the expression of binding genes and then promote reprogramming. To investigate the function of H2A.Z occupancy during induced somatic cell reprogramming, we selected genes which detected H2A.Z deposition in the promoter or gene body regions in three cell samples (Tables S1–S3). We identified 12281 genes that were occupied by H2A.Z in MEFs, 9598 genes in Day 7 cells, and 7944 genes in iPSCs. By biological processes analysis performed on H2A.Z related genes, there were 116 biological processes in iPSCs, 168 in Day 7 cells, and 237 in MEFs (Tables S4–S6). These genes which H2A.Z occupied in MEFs were more various in gene ontology than both Day 7 cells and iPSCs. It indicated that H2A.Z deposited more widely at the first time and eventually located on limited scale. For this biological processes analysis, the terms gene expression, regulation of gene expression, transcription, and regulation of transcription ranked much higher in iPSCs than in Day 7 cells and MEFs. None of the genes occupied by H2A.Z were involved in chromatin modification, assembly, or disassembly in iPSCs, but genes involved in

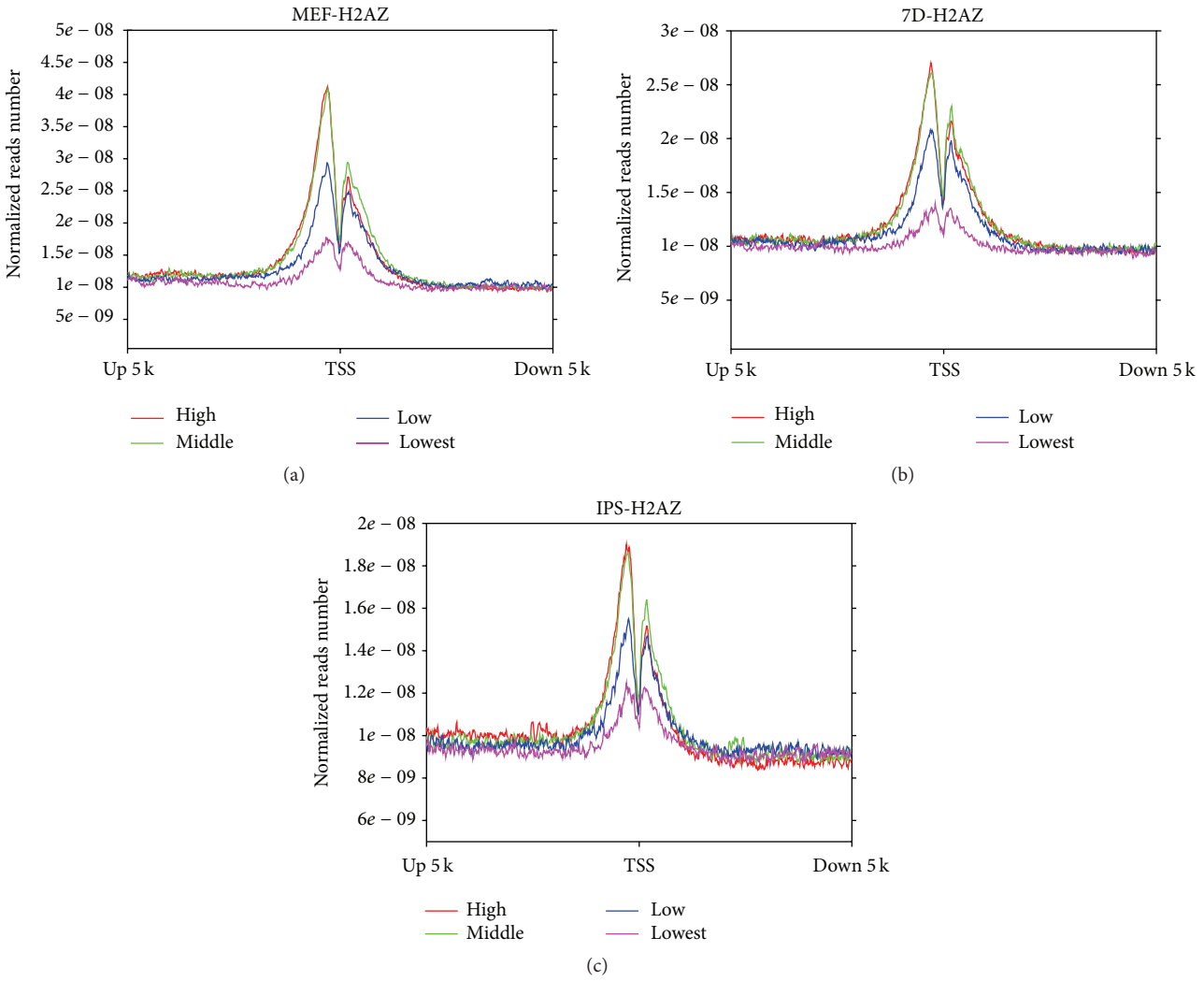

Figure 3: Comparison of microarray expression data and enrichment of H2A.Z in MEFs, Day 7 cells, and iPSCs. Genes are aligned at the TSS and divided into four parts (high, middle, low, and lowest) by decreasing expression level. Statistical H2A.Z normalized reads number in MEFs (a), Day 7 cells (b), and iPSCs (c).

these processes were occupied by H2A.Z in Day 7 cells and MEFs (Figure 4).

Most genes that were occupied by H2A.Z in all three cell types are involved in metabolic processes. While the same biological processes were largely present in the top 10 across the cell types, the label "gene expression" was present here in Day 7 cells. This indicates that genes occupied by H2A.Z at Day 7 are more involved in gene expression (Figure 5). Further analysis found that 2200 genes were occupied only in MEFs, 478 genes only in Day 7 cells, and 1067 genes only in iPSCs (Figure 6(a)). H2A.Z enhances specific incorporation in Day 7 (Figures 1 and 2). So, we speculate that the increased enrichment of H2A.Z in Day 7 may change the expression of specific genes and then promote reprogramming. Biological processes analysis was performed with 478 genes with H2A.Z incorporation only in Day 7 cells. We found they were primarily involved in macromolecular complex assembly and organization, including DNA packaging, chromatin assembly or disassembly, and protein-DNA complex assembly (Figure 6(b)). This indicated that H2A.Z tended to occupy specific genes, being especially involved in the regulation of epigenetic factor genes that affect chromatin structure after Day 7.

3. Discussion

H2A.Z is evolutionarily conserved in both molecular sequence and structure [24], altering chromatin structure through its deposition at DNA binding sites. In addition to the alternative chromatin structure induced by binding of histone variant H2A.Z, the access of other regulatory elements can be regulated by the kinetics status of the nucleosome. Our data have demonstrated that histone variant H2A.Z is always present in the promoter of active genes during induced somatic cell reprogramming (ISCR) (Figure 3). Recent reports [25] support the idea that H2A.Z can deposit independently of replication, and it is preferentially enriched in active gene promoters. And

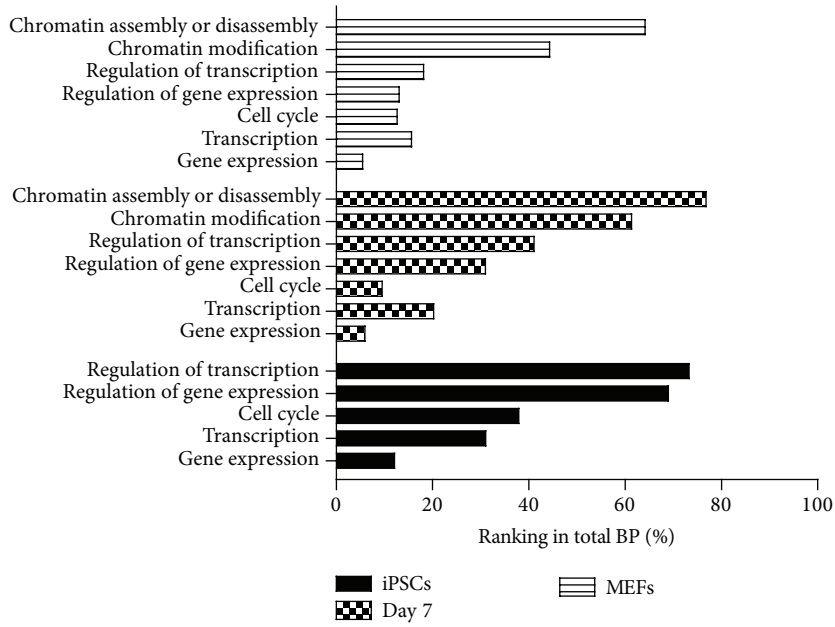

Figure 4: Rank of the same biological process in different samples. Ranking depicts biological processes in order of changes to expression of genes in which H2A.Z was incorporated. The total number of biological processes in distinct samples was normalized as 100, and the enrichment of selected processes is presented as a percentage. The shorter bar indicates the higher order in GO categories, which means higher activity genes.

this access of TSS may be responsible for the feature of active genes [26] and offer the inactivated gene a feasible nucleosome with active promoters [27]. Previously reported data show that active genes were highly occupied by H2A.Z, which face-lifted self-renewal of stem cells [23]. This is consistent with a role for H2A.Z in facilitating the generation of iPSCs through its deposition at promoters across the genome during ISCR.

H2A.Z is believed to facilitate the assembly or disassembly of chromatin and to increase transcriptional activity. In yeast, H2A.Z is often located at inactive promoters within nucleosomes where it aids in providing the correct architecture for transcription activation [28]. Our data have shown that many H2A.Z occupied genes are involved in biological processes related to gene expression in all three of the cell samples examined. Previous reports have established that acetylated H2A is often present in active nucleosomes [29] and that these acetylated proteins can recruit protein complexes for regulation of gene transcription [30]. It is apparent that genes occupied by H2A.Z can be easily marked as active and this combination between H2a.z and DNA regions may be signals for recruitment of reassemble acetyl enzyme complexes. Furthermore, H2A.Z is a coactivator required for the complete acetylation of histones, which facilitates access to gene promoters for transcription factors [30]. Our data indicate that H2A.Z may have a strong influence on the regulation of gene expression through its deposition at TSSs during ISCR. It strongly supports the notion that H2A.Z acts as a marker of active transcription throughout its distribution across the genome where it promotes and participates in the regulation of chromatin structure [31].

Increased binding of H2A.Z was detected in genes that were expressed at higher levels during ISCR (Figure 3). This suggests that the presence of H2A.Z in the chromatin structure may assist in the recruitment of transcriptional machinery, which maybe not only aids exogenous transcriptional factors in binding to genes required for pluripotency but also maintains the epigenetic status of stem cells [32]. Previous data have revealed that a stress response occurs at the beginning of reprogramming process [8]. Here, we have found that H2A.Z was enriched in genes involved in the biological processes of response to hypoxia, response to oxygen levels, response to steroid hormone stimulus, regulation of apoptosis, and regulation of cell death at the beginning of ISCR. Similarly, a particular set of occupied genes which only appeared in Day 7 cells were related to chromatin assembly. Our data showed that the total number of occupied genes in Day 7 cells was decreased and the number of genes occupied solely in these cells was the lowest (Figure 6(a)). However, the enrichment of H2A.Z in gene promoter regions in Day 7 cells was the highest (Figures 1(d) and 2(a)). Furthermore, the number of occupied genes present in both MEF and Day 7 cells was greater than the number present in both Day 7 cells and iPSCs. This indicates that H2A.Z tends to occupy a smaller and specialized subset of genes during reprogramming and that H2A.Z related genes received the largest amount of enrichment at the promoter in Day 7 cells.

Reprogramming is a process involving changes to both gene expression and epigenetic modification. Previous data have shown that MEFs and Day 7 cells share a similar transcription profile [18], whereas the transcription profile of iPSCs is reversed and transcription factor overexpression

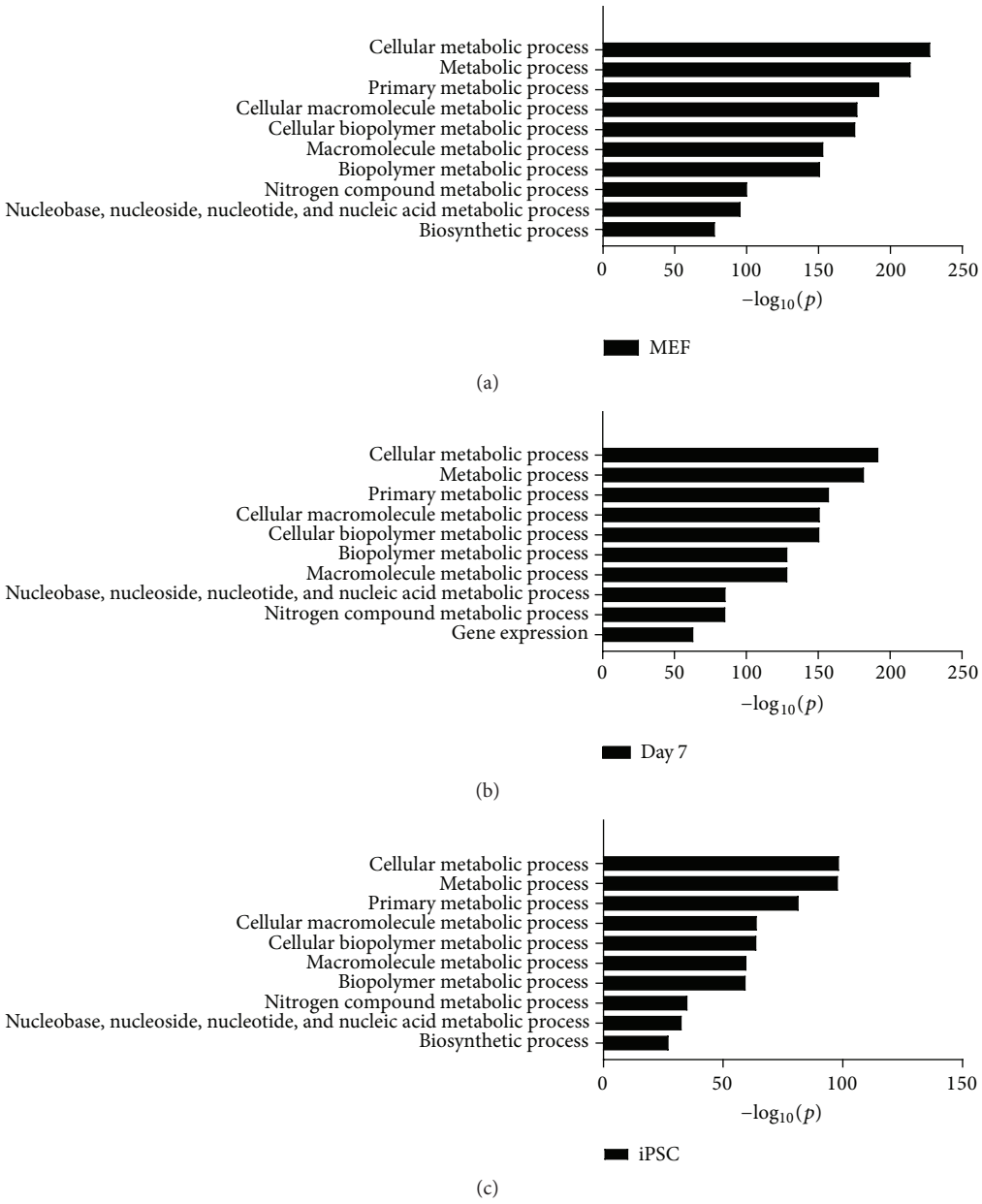

Figure 5: Top 10 biological processes undertaken by H2A.Z-associated genes in MEFs (a), Day 7 cells (b), and IPSCs (c). p values for revaluation of GO categories among genes show evidence for enrichment of H2A.Z.

can favor the upregulation of epigenetic factor genes [18]. H2A.Z can act as marks on open chromatin at promoters; it can recruit by PRC2/MLL complexes [33] or be recruited as OCT4 coactivators [23]. And histone acetylation may also act as active gene markers in genome-wide setting to recruit RNA polymerase II promoter [34] to reestablish gene expression pattern. As H2A.Z occupied p300-bound enhancers to support a global change of pluripotent genes [23], it showed that H2A.Z was predicted as the active markers for chromatin remodeling and supplied the accessible binding sites to epigenetic factors or transcriptional factors. We speculate that the enrichment of H2A.Z in the promoter regions of a small number of specialized genes in Day 7 cells may represent the initiation of a changeover phase during reprogramming. Firstly, the enhanced incorporation of H2A.Z around specific genes' TSS in Day 7 cells can change chromatin structure on the local scale [31] and supplies an accessible binding site for epigenetic or transcription factors to exert their effects. And then, the expression of the specific genes has been changed which can alter chromatin structure of the whole genome (Figures 4 and 6(b)). At last, a large number of genes' expression changed to promote reprogramming, accompanied with incorporation in chromatin enrichment to fulfil epigenetic reprogramming (Figure S4). However, all of these predictions are merely a starting point for the study of H2A.Z in ISCR, and the exact functions are still waiting for more experimental evidence to be verified.

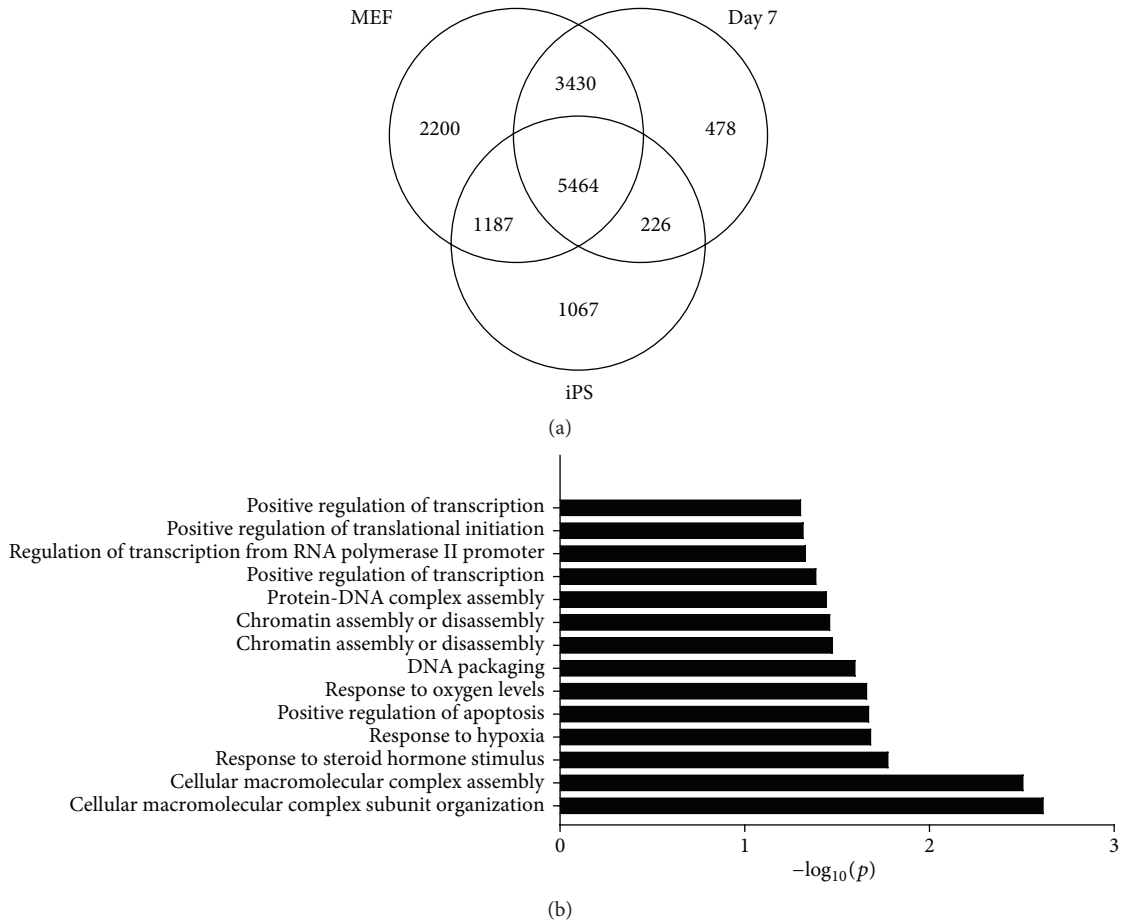

Figure 6: Distribution of H2A.Z during reprogramming and special functions of H2A.Z in Day 7 cells. (a) Venn diagram depiction of the number of genes occupied with H2A.Z in different cell samples. (b) Biological processes involved with the special genes occupied with H2A.Z in Day 7 cells.

4. Materials and Methods

4.1. Ethics Statement. Animal studies were carried out in a specific pathogen-free animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All animal protocols were approved by the Animal Care and Use Committee of the Model Animal Research Center, the host for the National Resource Center for Mutant Mice in China, Nanjing University.

4.2. Cell Culture. C57BL6 MEFs were derived from embryonic day 13.5 mice embryos [35] and maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) containing 10% fetal bovine serum. ESCs and iPSCs were cultured on γ-irradiated MEFs in ES medium containing Knockout DMEM supplemented with 20% Knockout Serum Replacement, 0.1 mM NEAA, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 50 U and 50 mg/mL penicillin/streptomycin, and 1,000 U/mL leukemia inhibitory factor (Millipore, Billerica, MA, USA). All other reagents were purchased from Invitrogen Corp. (Carlsbad, CA, USA).

4.3. iPSC Generation. Plat-E cells were transfected at 80% confluence with PMX-based retroviral vectors (Addgene, Cambridge, MA, USA) containing murine Oct4, Sox2, and Klf4 cDNAs using Lipofectamine 2000 (Invitrogen). Virus supernatant was harvested 48 hours after transfection. MEFs at passage 2 or 3 were seeded at a density of 3,500–5,000 cells/cm^2 and incubated with filtered viral supernatants containing equal parts of the three transcription factors (Oct4, Sox2, and Klf4) [36] and 5 μg/mL polybrene. Twelve hours later, the medium was gradually replaced with ESC medium supplemented with 50 μg/mL Vc (Sigma-Aldrich, St. Louis, MO, USA) using a 4-day stepwise process [37, 38]. And all the induced cell clones were alkaline phosphatase (AP) positive and can develop to teratoma with three primary kind germ cell layers in vivo. In addition, the m-iPSC clones still keep a normal karyotype and can express typical pluripotent gene markers such as SSEA-1, Nanog, and Oct4 [18].

4.4. ChIP-Seq. Anti-H2A.Z antibody (mAb) was used for immunoprecipitation. Cells (1×10^7) were fixed with 1% fresh formaldehyde at 37°C for 10 min and lysed in SDS lysis buffer. The nuclei were resuspended and sonicated on ice to chromatin of 200–1000 bp DNA. A total of 50 μL of Dynal protein G beads, 5 μg of antibody, and the sonicated chromatin were incubated at 4°C overnight. Precipitated

immunocomplexes were treated with proteinase K at 65°C for 2 h and DNA was purified with Qiagen quick polymerase chain reaction (PCR) purification kit (Qiagen, Dusseldorf, Germany). ChIP DNA was amplified, ligated with adaptor, and re-paired at the DNA end. The 150 bp DNA fragments were isolated from agar gel and sequenced with a Solexa Illumina 2G genetic analyzer.

4.5. ChIP-Seq Analysis. Solexa pipeline analysis was performed as described [39]. ChIP-Seq data were processed and aligned to the reference genome mm9. SOAP 2.21 software was used to map the 36 nucleotide sequence tags to the mouse genome with two mismatches allowed. Firstly, the raw data were obtained from the sequencing machine Illumina HiSeq2000, and clean data were extracted through quality control and filtering analysis process, and then the unique mapped reads were fulfilled with align analysis. MACS 1.4.0 software was used to identify the enriched peaks of the H2A.Z occupied sites. The program parameters were set as follows: p value of $<10^{-4}$, model fold of 32, and band width of 300 bp for the mouse genome. Promoters were defined as 2 kb upstream all annotated TSSs and "downstream" refers to the region 2 kb downstream a TES site. Composite plots were generated by averaging values in each of the 200 bp windows. Statistical significance of enrichment ($p < 10^{-4}$) was determined based on background distribution of randomized reads specific for each independent genome-wide ChIP analysis.

4.6. Gene Ontology. Gene ontology analysis was performed with DAVID (http://david.abcc.ncifcrf.gov/). EntrezGene IDs were used for the generation of enrichment statistics for the biological process category on the basis of a background list of all represented genes on the promoter microarray design.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Fulu Dong, Zhenwei Song, and Jiali Yu are equal contributors.

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Review Article

Long Noncoding RNA Regulation of Pluripotency

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Pluripotent stem cells (PSCs) represent a unique kind of stem cell, as they are able to indefinitely self-renew and hold the potential to differentiate into any derivative of the three germ layers. As such, human Embryonic Stem Cells (hESCs) and human induced Pluripotent Stem Cells (hiPSCs) provide a unique opportunity for studying the earliest steps of human embryogenesis and, at the same time, are of great therapeutic interest. The molecular mechanisms underlying pluripotency represent a major field of research. Recent evidence suggests that a complex network of transcription factors, chromatin regulators, and noncoding RNAs exist in pluripotent cells to regulate the balance between self-renewal and multilineage differentiation. Regulatory noncoding RNAs come in two flavors: short and long. The first class includes microRNAs (miRNAs), which are involved in the posttranscriptional regulation of cell cycle and differentiation in PSCs. Instead, long noncoding RNAs (lncRNAs) represent a heterogeneous group of long transcripts that regulate gene expression at transcriptional and posttranscriptional levels. In this review, we focus on the role played by lncRNAs in the maintenance of pluripotency, emphasizing the interplay between lncRNAs and other pivotal regulators in PSCs.

1. Introduction

The term long noncoding RNAs (lncRNAs) refers to a heterogeneous class of RNA polymerase II (Pol II) transcripts greater than 200 nucleotides in length with no evident protein-coding capacity [1, 2]. They are generally spliced from multiexonic precursors, capped, polyadenylated, and localized to the nucleus, cytoplasm, or both [2, 3]. Based on the anatomical properties of their transcription loci and the relationship with the adjacent genes, lncRNAs can be classified in intronic, intergenic, or overlapping (in sense or antisense orientation) transcripts. Even though lncRNAs are less conserved than mRNA and small noncoding RNAs [4, 5], lack of conservation does not imply a lack of function [6]. Indeed, both the transcript length and the versatility of RNA to base-pair let these molecules fold into complex secondary structures [7, 8], which are interspersed with longer and less conserved stretches of nucleotide sequences. As highlighted by pioneering studies [1, 6], these structures allow lncRNAs to simultaneously interact with multiple complexes, thereby coordinating their activities.

Even though only a part of lncRNA transcripts have been mechanistically characterized, several studies have shown the participation of lncRNAs in different processes related to normal physiology and/or disease [4, 6]. As they are Pol II transcripts, lncRNA expression can be tightly regulated. Indeed lncRNA transcripts are globally more tissue specific than protein-coding genes suggesting potential roles in specifying cell identity [9, 10].

The intracellular localization of lncRNAs is predictive of their mode of action [6, 10]. Usually, nuclear lncRNAs can guide chromatin modification complexes to specific genomic loci and/or serve as molecular scaffolds that tether together distinct functionally related complexes [11, 12]. Due to their intrinsic ability to base-pair with other nucleic acids, both *cis*-acting (on neighboring genes) and *trans*-acting (on distant loci) lncRNAs can exert either repressive or promoting activities on target genes by coordinating protein and RNA interactions [12–14]. Based on known examples [12], nuclear lncRNAs can exert their regulatory function as decoys by simply titrating transcription factors and other proteins

away from chromatin [15–17]. As a paradigm, depletion of the lncRNA PANDA substantially increased target genes occupancy by NF-YA, a nuclear transcription factor that triggers apoptosis upon DNA damage [17]. lncRNA binding on DNA can initiate the formation of heterochromatin by recruitment of DNA or histone methyltransferases (such as the histone H3 lysine 27, H3K27, and methyltransferase complex PRC2), resulting in repression of gene expression. Conversely, transcriptional activation can be induced by recruitment of different chromatin modifiers, such as the H3 lysine 4, H3K4, and methyltransferase MLL1, or by changing the 3D chromatin conformation [12, 13]. Among the *cis*-acting species, the enhancer-associated ncRNAs (eRNAs) are functional transcripts participating in many programs of gene activation. In particular, they play fundamental roles in targeting chromatin-remodeling complexes to specific promoters and to assist the formation of chromatin loops [18]. Using an integrated epigenomic screening, Ounzain and colleagues recently established a catalogue of enhancer-associated noncoding RNAs dynamically expressed in ESCs during cardiac differentiation [19]. The expression of these transcripts correlated with the expression of target genes in their genomic proximity. Interestingly, the expression of the eRNAs was inhibited when the target mRNAs reached maximal levels. Overall, these data gave an important contribution to the functional impact of cardiac eRNAs on heart development and cardiac remodeling after injury.

Some other lncRNAs are localized in the cytoplasm, where they can regulate gene expression through base-pairing complementary regions on target RNAs. In human, several cytoplasmic lncRNAs transactivate Staufen1-mediated mRNA decay by duplexing with 3′UTRs via Alu elements [20]. Another example is represented by the β-site APP-cleaving enzyme 1 BACE1-AS antisense RNA, which binds to BACE1 mRNA inducing its stabilization. By regulating BACE1 expression, the noncoding RNA plays a role in controlling the boundaries between physiology and pathology driving Alzheimer's disease pathology [21]. Base-pairing is also the principle that applies to the competing endogenous RNA (ceRNA) activity of lncRNAs [22]. In this case, lncRNAs can indirectly enhance protein translation by sequestering, or "sponging," miRNAs that otherwise would inhibit their target mRNAs. This mechanism has been shown to be involved in differentiation and cancer [22, 23]. Finally, a peculiar class of sponging lncRNAs is represented by circular RNAs (circRNAs) [24, 25], whose unusual circular structure confers increased stability. Altogether, these different properties engender lncRNAs to operate through distinct modes of action and to exert a wide range of functions across diverse biological processes.

Embryonic Stem Cells (ESCs) are the *in vitro* counterpart of the pluripotent epiblast of the blastocyst and constitute a useful system to study the molecular mechanisms at the basis of pluripotency. A group of transcription factors (TFs), comprising OCT4, NANOG, and SOX2, has been proposed as the core regulatory circuitry in ESCs [26]. These are pluripotency factors that ensure the proper expression of genes involved in the maintenance of the undifferentiated state. At the same time, they repress many genes that play a role during subsequent development. Such developmental genes, however, are often kept in a silent but "poised" state by the establishment of bivalent chromatin domains, where histone repressive marks coexist with marks related to active transcription [27]. It is now becoming clear that the core pluripotency TFs operate in concert with miRNAs and lncRNAs [28–30]. One example of a miRNA family that plays a role in the crossroad between pluripotency and differentiation is the miR-302 family [31]. Among other activities, miR-302 regulates the balance between agonists and antagonists of the TGFβ/BMP signalling, which is a crucial pathway for the choice between maintenance of pluripotency and differentiation [32]. In ESCs, the activity of miR-302 is counteracted by let-7, an opposing miRNA family that plays a prodifferentiative role [33]. Other miRNAs also facilitate differentiation by targeting pluripotency factors or chromatin modifiers [28]. In this review, we focus on recent evidence suggesting that lncRNAs also play an important role in the maintenance of pluripotency.

ESCs have represented for a long time the only system to model human early development. More recently, the Nobel Prize-awarded derivation of induced Pluripotent Stem Cells (iPSCs) provided an alternative source of pluripotent cells [34]. iPSCs can be derived from human somatic adult cells through a reprogramming process consisting in the ectopic expression of defined factors. As their derivation requires a simple skin biopsy (or blood sampling), human iPSCs overcome ethical and legislative issues that limit the research based on human ESCs (hESCs). Importantly, iPSCs generated from human patients with genetic disorders represent a promising tool for both regenerative medicine and *in vitro* disease modeling.

2. The lncRNA Signature in Embryonic Stem Cells

As for protein-coding genes and miRNAs [31], Pluripotent Stem Cells express a characteristic set of lncRNAs. The lncRNA signature of mouse ESCs (mESCs) has been defined by microarray analysis [35] and genome-wide mapping of chromatin marks of actively transcribed genes, such as trimethylation of lysine 4 of histone H3 (H3K4me3) in the promoter coupled with trimethylation of lysine 36 of histone H3 in the transcribed region (K4-K36 domain) [36]. Work by Dinger et al. [35] identified several lncRNAs that are differentially expressed in proliferating mESCs and upon induction of hematopoietic differentiation. Analysis of K4-K36 domains located outside the known protein-coding loci allowed Guttman et al. [36] to identify over a thousand novel lncRNAs in mESCs and somatic cells. The catalogue of mESC lncRNAs was then expanded by including a substantial fraction of species transcribed from genes not marked by a K4-K36 domain, identified by a computational method that allowed the reconstruction of the whole transcriptome from RNA-Seq data (Scripture) [37]. A significant subset of these lncRNAs may be regulated at the transcriptional level by the ESC core TFs [29, 38].

As in the case of mouse ESCs, K4-K36 domains analysis allowed the initial identification of a characteristic set of

lncRNAs genes expressed in human ESCs [39]. This list was then further extended by integrating data from RNA-Seq analysis [4]. A more detailed characterization has shown that some human lncRNAs could be under the direct control of the core pluripotency TFs [40, 41].

3. lncRNAs Play a Role in the Maintenance of Pluripotency in ESCs

Increasing evidence points to a crucial role for lncRNAs in the maintenance of ESC self-renewal (pluripotency), thus preventing their differentiation. In a large-scale functional study, the individual knockdown of more than 90% of lncRNAs (out of 147 tested) caused a significant perturbation of the transcriptome, often resulting in the loss of mESC pluripotency [29]. Interestingly, lncRNAs involved in the maintenance of ESC self-renewal are often transcriptionally regulated by core pluripotency TFs and act in regulatory networks. Examples of this mechanism include AKO28326/GOMAFU/MIAT (OCT4-activated) and AK141205 (NANOG-repressed) lncRNAs that when altered lead to robust changes in OCT4 and NANOG levels and affected pluripotency of mESCs [38]. The lncRNA TUNA/MEGAMIND is required for mESCs proliferation and maintenance of self-renewal [5]. TUNA binds a complex comprising several RNA-binding proteins and activates transcription of NANOG and SOX2 upon binding on their promoters [42]. The interplay between core TFs and lncRNAs has been reported also in hESCs for lncRNA_ES1, lncRNA_ES2, and lncRNA_ES3 [40]. Taken together, these examples indicate that lncRNAs are involved in the maintenance of the undifferentiated state and the repression of genetic programs that direct lineage commitment during differentiation.

The challenge now is to dissect the molecular mechanisms underlying the functions of these ESC lncRNAs. Mechanistically, nuclear lncRNAs may exert their function by binding and regulating the activity and/or target specificity of chromatin-modifying factors. It has been shown that ESC lncRNAs interact with all classes of histone modifiers (writers, readers, and erasers), as well as other chromatin-associated proteins [29]. This is in line with a possible role of these long transcripts as molecular scaffolds that bridge together different chromatin modification complexes [11]. Recent examples support the hypothesis that lncRNAs may be pivotal regulators of the activity of crucial chromatin modifiers, which play an essential role in the epigenetic regulation of ESCs pluripotency and differentiation. Genome-wide analysis identified a multitude of potential lncRNA interactors of PRC2 in mESCs and a somewhat promiscuous RNA-binding activity of this complex has been suggested [43, 44]. Recent work proposed that lncRNA binding might be important to modulate the interaction of PRC2 with its cofactors, thus modulating its activity and/or specificity. One of such cofactors is JARID2, belonging to the JUMONJI family of lysine demethylases (KDMs). JARID2 is peculiar as its KDM catalytic domain is inactive and it is particularly enriched in ESCs where it regulates PRC2 activity and genome occupancy [45, 46]. It has been recently shown that JARID2 contains RNA-binding region and directly interacts with about 100 previously annotated lncRNAs in mESCs [47]. Particularly interesting, among these interactors are MEG3 (also known as GTL2), RIAN, and MIRG, lncRNAs that are encoded within an imprinted locus on chromosome 12qF1, referred to as the Dlk1-Dio3 gene cluster. Proper expression of these lncRNAs is required for embryonic development [48, 49] and to achieve full pluripotency during reprogramming, as iPSCs carrying aberrantly silenced Dlk1-Dio3 cluster genes are unable to fulfill stringent pluripotency tests, such as contribution to chimaeric mice development and complementation of a tetraploid blastocyst [50]. Functionally, by binding JARID2, MEG3 and other Dlk1-Dio3 gene cluster lncRNAs may modulate the activity of PRC2 in Pluripotent Stem Cells. Genome-wide analysis indeed showed that Meg3 stimulates PRC2 occupancy *in trans* at genomic loci encoding for factors involved in differentiation and development [44]. These genes are derepressed in human iPSC lines expressing low levels of MEG3, suggesting evolutionary conservation of the MEG3-JARID2 axis. Mechanistically, MEG3 and other lncRNAs work as scaffolds to increase the interaction between JARID2 and the PRC2 core component EZH2 and, therefore, PRC2 assembly on chromatin at JARID2 target sites. Moreover, it has been suggested that these lncRNAs may also guide the initial recruitment of PRC2/JARID2 at specific target sites in pluripotent cells via RNA-DNA base-pairing [47] (Figure 1).

Trithorax group (TrxG) factors, including mammalian MLL complexes, positively regulate transcription via the H3K4me3. This activity is required to maintain pluripotency in ESCs. In particular, the WDR5 member of the MLL complex directly interacts with the core transcriptional regulatory circuitry and its depletion causes loss of self-renewal [51]. By taking advantage of an RNA-binding deficient mutant, Yang and coworkers recently demonstrated that the interaction with RNA is essential for WDR5 activity [52]. The half-life of the WDR5 mutant protein in the nucleus is reduced compared to wild-type, indicating that RNA binding positively regulates protein stability. Over 1000 RNAs might bind WDR5 in ESCs, including 23 previously annotated lncRNAs. Among these interactors, six were previously identified as lncRNAs required to maintain pluripotency in mESCs [29], providing a mechanistic explanation of their function. WDR5 also binds the promoters of two of these interacting lncRNAs, lincRNA-1592 and lincRNA-1552, suggesting a *cis* regulatory mechanism [49]. lincRNA-1552 expression may be under the direct control of many pluripotency transcription factors, including OCT4, NANOG, and KLF4 that bind its promoter, and its knockdown leads to misexpression of OCT4 and NANOG, among other mRNAs [29]. This evidence, together with the impairment of self-renewal in cells expressing the RNA-binding deficient WDR5 mutant [52], suggests that lncRNAs interacting with the Trithorax complex play a crucial role in the maintenance of ESC pluripotency (Figure 1).

The interplay between lncRNAs and Trithorax complexes may also direct specification towards specific cell fates upon ESCs differentiation. The homeotic genes *Hoxa6* and *Hoxa7* are involved in the specification of mesoderm derived tissues and organs [53, 54]. Bertani and colleagues demonstrated that the lncRNA MISTRAL (MIRA) mediates the transcriptional

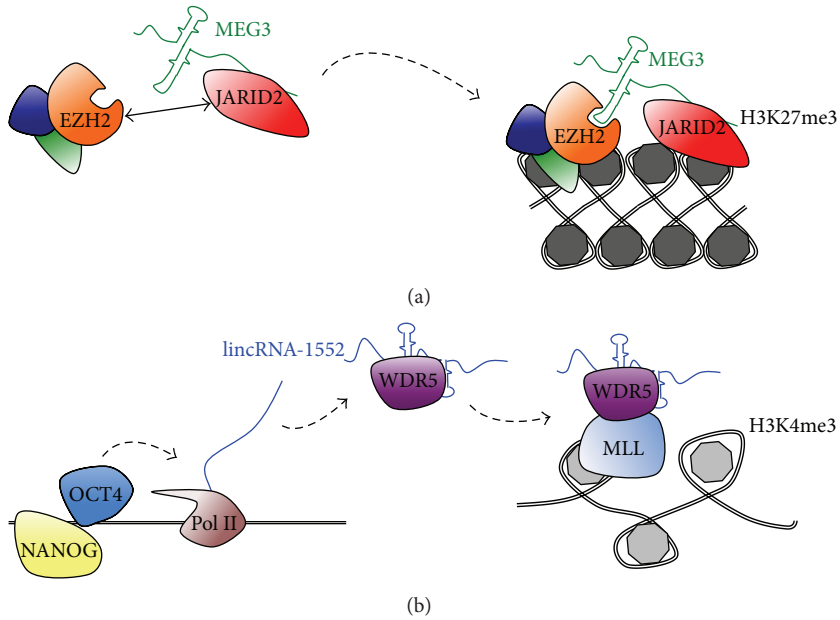

Figure 1: Examples of nuclear lncRNAs interacting with chromatin regulators in ESCs. (a) The lncRNA MEG3 promotes the interaction between the EZH2 subunit of PRC2 and JARID2, thus guiding PRC2 recruitment and H3K27me3 deposition at JARID2 target sites [47]. It has also been proposed that lncRNAs specify the target site recognition of PRC2/JARID2 via RNA-DNA base-pairing (not shown) [47]. (b) lincRNA-1552 transcription is promoted by core pluripotency factors and is required for ESC self-renewal [29]. This and other transcripts positively regulate TrxG activity by binding and stabilizing the WDR5 cofactor [52].

activation of *Hoxa6* and *Hoxa7* genes by recruiting MLL to chromatin [55]. MIRA-mediated activation of *Hoxa6* and *Hoxa7* culminates in the expression of genes involved in early germ layer specification in differentiating mESCs. Another interesting example of lncRNA involved in mESC differentiation is pRNA, which is localized in the nucleolus [56]. In Pluripotent Stem Cells, chromatin is globally in a transcriptionally permissive open state and becomes increasingly condensed and transcriptionally repressed upon differentiation (reviewed in [57]). Chromatin condensation occurs also at ribosomal genes and is promoted by pRNA, which guides the nucleolar repressor factor TIP5/BAZ2A to ribosomal DNA (rDNA) [56]. Interestingly, pRNA overexpression caused an increase of heterochromatin also outside rDNA, initiating the global epigenetic remodeling normally observed during differentiation.

In addition to nuclear ESC lncRNAs, on the other side of the coin, fewer examples exist for cytoplasmic lncRNAs that regulate pluripotency. During the initial steps of the reprograming process, cells initiating their conversion to pluripotency must elude inhibitory hurdles, such as cell cycle arrest, senescence, and apoptosis, raised by p53 activation by the overexpression of the reprogramming factors [58]. Thus, any change in p53 activity is predicted to affect the efficiency of reprogramming by limiting the number of cells entering the process. In this context, the cytoplasmic linc-RoR (Regulator of Reprogramming) was initially identified as lncRNA able to promote the reprogramming process [41] by acting as a negative regulator of p53 [59]. Subsequently, Wang and colleagues showed that endogenous linc-RoR also plays a key role in the maintenance of hESC self-renewal by acting as a ceRNA [60]. Previous work had shown that a single miRNA, miR-145, inhibits translation of core TFs during ESC differentiation [61]. According to the model by Wang et al., in human ESCs linc-RoR would trap miR-145, derepressing the translation of the core pluripotency transcription factors OCT4, SOX2, and NANOG and ensuring proper levels of expression in undifferentiated hESC. Upon differentiation, the disappearance of linc-RoR releases miR-145, allowing it to repress the translation of core pluripotency factors [41]. Thus, this work strongly supports the idea that linc-RoR acts as a miRNA sponge. Since OCT4, at the transcriptional level, represses miR-145 and activates linc-RoR, these studies unraveled an interesting network comprising TFs, long and short regulatory RNAs which act at the crossroad between self-renewal and differentiation (Figure 2).

More recently, Bao and colleagues [62] showed that lincRNA-p21, a nuclear noncoding transcript previously characterized as a global repressor of the p53-dependent transcriptional cascade [63], represents another example of lncRNA regulating pluripotency. Interestingly, in the context of somatic cell reprogramming, lincRNA-p21 inhibits this process without inducing apoptosis or impairing cell proliferation. It was identified in a functional screening performed in mouse to examine events accompanying the pre-iPSCs to iPSCs conversion. This is a late step, required to achieve a self-sustaining fully reprogrammed status, in which the cells become independent of the activity of the exogenous reprogramming factors and turn on the expression of endogenous pluripotency regulators [64].

Table 1: lncRNA transcripts expressed in ESCs and controlling pluripotency.

lncRNA	Species	Proposed role in ESCs pluripotency	References
AK028326 (GOMAFU/MIAT)	m	Oct4-activated lncRNA which controls Oct-4 expression by a feedback loop.	[38]
AK141205	m	Nanog-repressed lncRNA; AK141205 positively regulates Oct4 expression.	[38]
ES1, ES2, ES3	h	Oct4 and Nanog transcriptional targets. ES1-3 act as modular scaffold for PRC2 and SOX2.	[40]
MEG3/GTL2	m, h	Facilitates PRC2/JARID2 complex recruitment on target genes.	[47]
lincRNA-1592, lincRNA-1552	m	Bind WDR5/MLL1 complex and are necessary for Nanog and Oct4 expression.	[52]
MIRA	m	Facilitates WDR5/MLL1 complex recruitment on chromatin.	[55]
TUNA/MEGAMIND	m	Activates transcription of Nanog, Sox2, and Fgf4.	[42]
pRNA	m	Recruits TIP5 on rDNA upon differentiation.	[56]
linc-RoR	h	Acts as a sponge for miR-145.	[60]
lincRNA-p21	h	Interacts with HNRNPK to form a repressive complex at the promoter of key pluripotency regulators.	[62]
Dum	m	Favours DNA methylation at CpG sites at the promoter of the pluripotency-associated Dppa2.	[65]

Species: m = mouse; h = human.

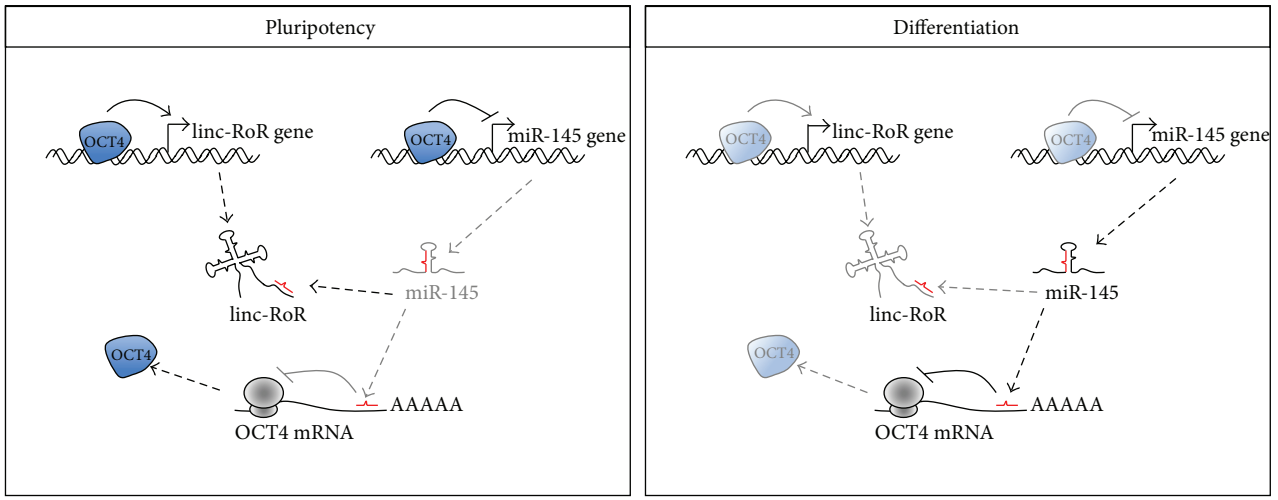

Figure 2: linc-RoR as a paradigm for lncRNA regulation of pluripotency in the cytoplasm. In pluripotent cells, the levels of linc-RoR are controlled by OCT4. In a positive feedback loop, linc-RoR sponges miR-145, thus derepressing its targets, including OCT4. OCT4 also negatively controls miR-145 at the transcriptional level [41, 59–61]. See text for details.

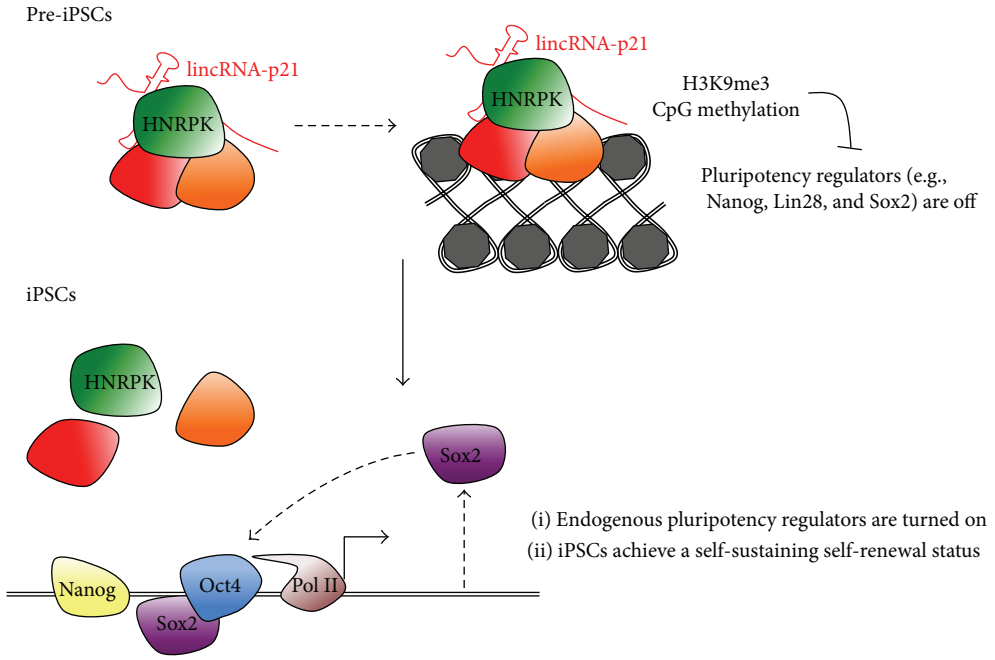

Figure 3: lincRNA-p21 regulates the transition of pre-iPSCs to iPSCs. During reprogramming, the induction of endogenous pluripotency genes is necessary to achieve a self-sustaining status in which the core regulatory factors act in a positive feedback loop on their own expression. lincRNA-p21 hinders this transition by recruiting an inhibitory complex, which deposits repressive marks such as H3K9me3 and CpG methylation on the promoters of pluripotency regulators [62].

Three lncRNAs, including lincRNA-p21, had a negative effect in pre-iPSCs to iPSCs conversion. Mechanistically, lincRNA-p21 has been suggested to sustain the heterochromatic state at pluripotency gene promoters by interacting with HNRNPK. HNRNPK and lincRNA-p21 together would form a repressive complex able to preserve H3K9me3 and CpG methylation at the promoter of key pluripotency regulators such as Nanog, Sox2, and Lin28 [62] (Figure 3). Besides lincRNA-p21, there are only limited examples of nuclear lncRNAs regulating gene expression by controlling DNA methylation. In a more recent paper, Wang and colleagues reported the identification of Dum, a Developmental pluripotency-associated 2 (Dppa2) Upstream binding Muscle lncRNA [65]. LncRNA Dum was found to silence its neighboring gene Dppa2 in *cis* by recruiting Dnmt1, Dnmt3a and Dnmt3b on its promoter. Although the cited work was mainly focused on myogenic differentiation, it is tempting to speculate that a similar regulatory mechanism might play a role in pluripotent

cells as well. Dppa2 is highly enriched in pluripotent cells and activation of endogenous Dppa2 during late steps of reprogramming specifically marks the small subset of cells that will achieve full pluripotency, in which Dppa2-mediated induction of Nanog transcription is a crucial event [66]. Therefore, it will be extremely interesting in the future to assess whether the lncRNA Dum regulates critical steps of reprogramming through modulation of Dppa2.

4. Concluding Remarks

Pluripotency is a unique property of ESCs and iPSCs, which are the only cell types able to undergo indefinite self-renewal and differentiation into derivatives of the three germ layers. Pluripotent cells therefore represent both ideal candidates for dissecting the mechanisms of early embryonic development and potential therapeutic tools for regenerative medicine. Patient-specific iPSCs also provide *in vitro* platforms to model human disease and to test drugs in preclinical studies. Such potential applications, however, are subordinated to a deep understanding of the molecular mechanisms underlying pluripotency.

An orchestra of transcription factors, chromatin regulators, signaling transducers, miRNAs and lncRNAs play coordinately in pluripotent cells. Each of them cannot be considered a solo player. Complex networks and feedback loops exist, which comprise members of each class of regulatory factors. A huge increase of transcriptome-wide analyses, facilitated by recent advancements in next-generation sequencing technologies, uncovered a universe of long noncoding transcripts. While there is no general consensus on the extent of the global impact of lncRNAs on the regulation of cell identity and differentiation, few examples in which selected lncRNAs have been more deeply analyzed exist. As discussed in this review, at least a subset of known lncRNAs are as important as previously defined "core transcription factors" in the context of pluripotent cells (Table 1). The paucity of functional studies is in striking contrast with the number of annotated lncRNAs (thousands) that are specifically enriched in ESCs and/or described as interactors of crucial pluripotency regulators, such as Polycomb and Trithorax complexes. We expect, in the near future, a substantial increase of functional studies describing new examples of lncRNAs acting in network with other master regulators in the definition of the pluripotent state.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Epigenetic Research of Neurodegenerative Disorders Using Patient iPSC-Based Models

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Epigenetic mechanisms play a role in human disease but their involvement in pathologies from the central nervous system has been hampered by the complexity of the brain together with its unique cellular architecture and diversity. Until recently, disease targeted neural types were only available as postmortem materials after many years of disease evolution. Current in vitro systems of induced pluripotent stem cells (iPSCs) generated by cell reprogramming of somatic cells from patients have provided valuable disease models recapitulating key pathological molecular events. Yet whether cell reprogramming on itself implies a truly epigenetic reprogramming, the epigenetic mechanisms governing this process are only partially understood. Moreover, elucidating epigenetic regulation using patient-specific iPSC-derived neural models is expected to have a great impact to unravel the pathophysiology of neurodegenerative diseases and to hopefully expand future therapeutic possibilities. Here we will critically review current knowledge of epigenetic involvement in neurodegenerative disorders focusing on the potential of iPSCs as a promising tool for epigenetic research of these diseases.

1. Introduction

A major challenge to model neurodegenerative disorders has been the inaccessible nature of the specific neural cell types targeted by disease which are usually available only in postmortem state. Recent somatic cell reprogramming protocols have contributed to overcome such a difficulty. Reprogramming of somatic cells to pluripotency can be currently achieved by different methods including somatic cell nuclear transfer (SCNT), fusion of somatic and pluripotent cells, included ectopic expression of defined sets of pluripotency transcription factors (TF) in adult somatic cells to generate induced pluripotent stem cells (iPSCs), and direct reprogramming of adult somatic cells to induced neurons (iN) by empirically determined cocktails of neurogenic factors [1–5]. In neurodegenerative disorders where animal models have not been able to entirely recapitulate key disease pathological aspects [6], reprogramming of human fibroblasts into iPSC has become a widely used technique permitting the generation of patient-specific disease-relevant cells in virtually limitless amounts with implications for the elucidation of disease mechanisms [7].

Parkinson disease (PD) is a neurodegenerative disorder associated with the progressive loss of dopaminergic neurons (DAn) in the substantia nigra pars compacta (SNpc) resulting in the cardinal motor symptoms of bradykinesia, rigidity, tremor, and postural instability [8, 9]. Due to their potential applicability for cell-based therapies, midbrain DAn were one of the first cell types generated by somatic cell reprogramming [10]. The resemblance of iPSC-derived DAn with midbrain DAn has centered various studies [11–13]. The hallmark of amyotrophic lateral sclerosis (ALS) is the selective death of motoneurons (MN) in the motor cortex, brain stem, and spinal cord leading to the progressive wasting

and weakness of limb, bulbar, and respiratory muscles [14, 15]. Similar to DAn in PD, the high specialization and relative reduced number of MN in ALS also hold great potential for the use of somatic cell reprogramming in ALS cell-based therapies. Huntington disease (HD) is a monogenic neurodegenerative disorder triggered by trinucleotide expansions in the huntingtin gene causing corticostriatal dysfunction and leading to abnormal muscle coordination (choreic movements), mental decline, and behavioral symptoms [16, 17]. Alzheimer disease (AD) is a progressive neurodegenerative disorder characterized by global cognitive decline involving memory, orientation, judgment, and reasoning deficits which are associated with the deposition of amyloid plaques and neurofibrillary tangles in different brain areas including the hippocampus [18]. For these diseases and similar age-related neurodegenerative disorders, cell reprogramming has appeared as a promising tool to investigate the molecular and cellular processes related to the pathophysiological process in a subject-personalized manner.

Whereas only 5–10% of cases with neurodegenerative disorders such as AD, PD, or ALS are Mendelian disorders caused by pathogenic mutations in disease-associated genes, the vast majority of cases are considered sporadic resulting from the complex interplay of genetic risk factors and largely unknown environmental conditions [19, 20]. However, cumulative evidence has demonstrated that both monogenic and sporadic cases can share common pathogenic mechanisms [19]. To date, iPSC-derived neural models of PD [21–30], ALS [31–37], HD [38–41], and AD [42–45] have proved instrumental to model in vitro molecular alterations involved relevant to disease. Yet whether reprogramming of adult somatic cells constitutes a truly epigenetic reprogramming [7], detailed epigenomic characterization of patient-specific iPSC-based disease models and the role of epigenetic changes in the pathophysiology of these diseases remain underexplored. While it is well established that epigenetic alterations contribute to the physiopathology of human disease [46] including also neurodegenerative disorders [47], it should be accordingly expectable that iPSC-derived neural models may represent valid tools to investigate epigenetic changes involved in neurodegenerative disorders.

Here we will review the potentiality and current challenges of iPSC-based models to investigate epigenetic regulation of neurodegenerative disorders in the context of other existing patient specimens and disease models.

2. Epigenetic Mechanisms Relevant to the Nervous System

There are three major categories of epigenetic modifications including DNA methylation, histone posttranslational modifications, and noncoding RNAs (ncRNAs) which encompass microRNAs (miRNA), small noncoding RNAs (sncRNA), and long noncoding RNAs (lncRNA) [48]. The first two categories involving chemical changes in the DNA or histones will be discussed in this review. Regarding posttranslational modifications, which can mainly occur via acetylation, methylation, phosphorylation, and ubiquitination of histone residues, we will focus on histone acetylation and methylation since these two marks are relatively better characterized.

2.1. Epigenetic Definitions. "Epigenetics" can be defined as mitotically and/or meiotically heritable changes in gene expression which occur without changes in the DNA sequence. These epigenetic modifications mediate the execution of cell-type specific genomic programs activated in response to interoceptive as well as environmental stimuli. Conceptually, epigenetic mechanisms include long-term silencing, transcription, posttranscriptional RNA processing, translation, X chromosome inactivation, genomic imprinting, DNA replication and repair, and the maintenance of genomic integrity [49]. These epigenetic mechanisms play a crucial role in the regulation of gene expression by organizing the disposition of chromosomes in the nucleus, restricting or facilitating the access of TFs to DNA, and preserving a memory of past transcriptional activities [50]. A more operational definition of epigenetics is the study of heritable changes in gene activity caused by direct modification of the DNA sequence without altering the nucleotide sequence, namely, DNA methylation and posttranslational histone modifications [51]. For extension, the "epigenome" can be defined as the overall genomic collection of the DNA methylation states and covalent modification of histone proteins along the genome which is characteristic of each cell type [52]. Similarly, the specific epigenetic signature of each cell type is often designated as "epigenetic landscape." The epigenetic signature of a particular cell, albeit normally stable, can also be dynamic over time [53] and thus dynamic changes in the epigenetic signature are known as "epigenetic plasticity." According to the epigenetic theory, the genome and the environment can work synergistically impacting the regulatory mechanisms controlling gene expression by modification of the DNA epigenetic marks [54] which can occur throughout lifespan [55]. In monozygotic twins, epigenetic differences from the original epigenome have been shown to accumulate over time [56] and such divergences are known as "epigenetic drift." This apparently stochastic accumulation of epigenetic changes has been associated with aging [57] and also with sporadic neurodegenerative disorders [58] in which, to date, aging is the major known risk factor [20].

2.2. DNA Methylation and Hydroxymethylation. DNA methylation is an important regulatory mechanism of gene expression in eukaryotes. In mammals and humans DNA methylation mostly affects the cytosine (C) base when it is followed by a guanine (G). These CpG sites can be clustered in the so-called "CpG islands" (GCI) but DNA methylation can also be present in non-CG contexts (mCH where H = A, C, or T). Both CpG and non-CG methylation are detected in the mouse and human brain [59, 60] but non-CG methylation is rare or absent in other differentiated cell types [61, 62]. Normally, DNA methylation follows a bimodal distribution ranging from unmethylated to fully methylated loci but intermediately methylated transition loci also exist. The methylation status of specific CpGs can be variable across individuals but stable over time within the same

individual [63]. The biochemical process of DNA methylation involves the covalent modification of cytosines by addition of methyl groups ($-CH_3$) to the $5'$ position resulting in 5-methylcytosine (5mC). This reaction is catalyzed by specific enzymes termed de novo DNA methyltransferase (DNMT) and it occurs at expense of ATP and S-adenosylmethionine as methyl donor.

DNMT is expressed during neural development and in adult brain in a tissue- and cell-specific manner including areas of active neurogenesis [64] and adult stem cell niches [65] where they have been involved in neural survival and plasticity [66]. DNMT1 is the maintenance enzyme of DNA methylation sustaining methylation after DNA replication [67] whereas DNMT3a and DNMT3b have the capacity for methylating DNA de novo [68]. Of these, DNMT3b has been specifically involved in the specification of the neural crest [69]. Once methylation is established, proteins of the methyl-CpG-binding domain (MBD) family are recruited to methylated loci to elicit the recruitment of histone modulatory factors such as histone deacetylases (HDAC) [70, 71] indicating a synergistic coordination of different epigenetic marks [48]. The MBD proteins have also been involved in developmental and adult brain functions [72]. The most common consequence of DNA methylation is the silencing of genes and noncoding genomic regions, especially when affecting gene promoters. But DNA methylation can also be associated with enhanced expression by mechanisms that yet remain poorly understood [72, 73]. Recent studies have shown that about 75% of the DNA methylation affecting the gene body is associated with gene expression downregulation whereas the remaining 25% is associated with upregulation [74].

Other enzymes such as the oxidoreductases of the Ten-Eleven Translocation (TET) family are responsible for the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) [75]. Members of this group such as TET1, TET2, and TET3 have been shown to counterbalance the effect of 5mC by inhibiting the binding of MBP proteins. Whereas 5mC correlates positively with age and, in general, negatively with gene expression in the brain [76], 5hmC despite of correlating also positively with age [77] has been shown to associate positively with expression [61, 78]. In addition, the 5hmC mark seems to be particularly abundant in tissues with low cell renewal rates such as the cerebellum and cortex [79] where it has been shown to be highly dynamic and susceptible to age-related changes [80, 81]. The process of DNA demethylation and the enzymes catalyzing this reaction remain less well known although DNA demethylases such as the activation-induced cytidine deaminase (AID) [82] or the DNA demethylating activity of TET1 [75] have been identified. In neurons, the global balance among DNA methylation, demethylation, and hydroxymethylation determines neurobiological processes such as neural plasticity, memory, or learning, and their deregulation can be associated with neurodegenerative disorders [58].

2.3. Histone Modifications. In addition to DNA methylation, the conformation of the chromatin is also regulated by histone posttranslational modifications. In eukaryotic chromatin, the genomic DNA is packed around histone proteins forming the so-called nucleosome, which consists of 147 base pairs of DNA wrapped around a histone octamer containing 2 copies each of the core histones H2A, H2B, H3, and H4 [83]. The nucleosome represents the fundamental unit of eukaryotic chromatin which folds through a series of successively higher order structures to form the chromosome. Thus, the nucleosome compacts DNA and creates an added layer of regulatory control to ensure correct gene expression by determining the three-dimensional structure of DNA and its accessibility to TFs, RNA polymerases, and other DNA sequences [84]. Ultimately, the nuclear organization of the chromatin is given by the balance between condense inactive heterochromatin and open active euchromatin [85]. Ultimately, the transcriptional regulation of genes is primarily controlled by physical access of the RNA polymerase II to promoter regions. Nonetheless, gene expression is also regulated by cis-elements termed enhancers which can be distally located upstream or downstream of promoters and whose epigenetic regulation is required for gene expression [86–88]. Thus, in addition to methylation, posttranslational modifications of histones at both promoters and enhancers critically regulate the conformation of the chromatin and the transcriptional state of specific genes [89].

There are more than 100 different histone posttranslational modifications which can affect different histone amino acid residues including lysine (K), arginine (R), serine (S), threonine (T), and glutamate (E) [48]. Of these, acetylation and methylation of lysine residues are the most well-known histone modifications [90]. Enzymatically, the chemical reactions of histone acetylation/deacetylation are catalyzed by histone acetyltransferases (HAT)/deacetylases (HDAC) whereas histone methylation/demethylation are mediated by histone methyltransferases (HMT)/demethylases (HDM) which typically form chromatin-modifying complexes [91]. These histone marks are also specifically recognized by chromatin-binding proteins involved in transcriptional activation or repression and DNA replication and repair.

For instance, methylation of H3K4 can inhibit the binding of HDAC therefore favoring acetylation whereas acetylation of H3K18 facilitates the engagement of HAT [92]. Methylation and specially trimethylation of histone 3 at lysine 27 (H3K27me3)/lysine 9 (H3K9me3) have been associated with gene repression. Conversely, methylation of H3K4 normally marks active enhancers whereas acetylation of H3K4, H3K9, and H3K27 correlates with transcriptional activation [93–95]. In addition, the H3K27ac mark has been found to specifically distinguish active enhancers from poised enhancers in embryonic stem cells (ESCs) in genes which are relevant during development [86]. In general, acetylation of ε-amino groups of lysine residues of histones neutralizes their positive charge thereby relaxing chromatin structure [91] commonly favoring the protein binding of transcriptional activators [96]. Per contrary, histone deacetylation favors chromatin compaction and transcriptional repression [97]. Histone marks affecting H3 lysine residues have recently been associated with functional chromatin states including, in a summarized comprehensive manner, repressed regions (H3K27me3 and H3K9me3), promoter (H3K4me3), promoter/enhancer

Table 1: Comprehensive summary of histone epigenetic marks and corresponding functional states of the chromatin.

Histone epigenetic mark	Chromatin state
H3K27me3	Repressed
H3K9me3	Repressed
H3K4me3	Promoter
H3K4me1	Promoter and enhancer
H3K27ac	Promoter and enhancer
H3K36me3	Transcriptional elongation
CTCF-binding sites	Insulator

(H3K4me1 and H3K27ac), and transcriptional elongation sites (H3K36me3) [98] (Table 1). Recently, reference functional chromatin states have been defined in humans for a wide variety of tissues including the central nervous system (CNS) providing a valuable resource for future epigenetic studies [99]. In the CNS, these histone modifications have been associated with neural stem cell (NSC) maintenance, neural and glial cell type specification, homeostasis, neural plasticity, learning, memory, and aging [48].

2.4. Epigenetic Mechanisms during Neural Cell Differentiation. During development, the progression from pluripotent stem cells through progenitors to differentiated cells occurs through an increase of repressive histone marks, DNA methylation, and chromatin compaction [100]. These repressive epigenetic marks limit the phenotypic plasticity properties of the developing cells and therefore are essential for acquiring a differentiated cell identity [101]. Little is known about the epigenetic pattering during the development of the human brain but efforts towards its characterization are being conducted including methylome studies for at least certain cell types. Thus, a pioneer work has identified differentially methylated CpG regions associated with synaptogenesis during brain development in mouse and humans which seem to be enriched in key regulatory regions indicating their putative functional relevance [61]. In addition, this study revealed that 5hmC marks are present in fetal brain at regions that become activated by losing CG methylation and also that non-CG methylation accumulates in neurons but not in glia during this process. On the contrary, histone marks of the developing brain [102, 103] or global transcriptome alterations involved in the cell-type specification remain poorly explored [104, 105]. Yet once the neural fate program is activated, the remodeling of the chromatin is driven by cell specification signals such as TFs that interact with target sequences [106] showing binding site enrichment of the specific TFs whose activity regulates gene expression [53, 107]. Conceptually, multiple TFs acting in a coordinated manner orchestrate the remodeling of the epigenome of the differentiating neural cell to acquire specific cell phenotypes [108, 109]. These core "pioneer" TFs influence the chromatin environment by increasing the DNA accessibility to additional TFs [110] which promote cellular specification [111]. Core TFs such as OCT4, SOX2, and NANOG have been shown to be major regulators in the maintenance of pluripotency state in human embryonic stem cells (ESCs) [112, 113]. Of these, OCT4 has been shown to control the expression of H3K9me3 demethylases contributing to preserve the epigenetic marks needed for self-renewal of ESC [114]. Thus, genes transcriptionally active in ESC such as OCT4 or NANOG are characterized by H3K27ac and H3K4me3 active marks. In contrast, most key developmental genes remain inactive during ESC self-renewal and carry simultaneously bivalent chromatin marks including repressive H3K27me3 and active H3K4me1/H3K4me3 marks [115]. Among genes with bivalent marks are the HOX clusters which are master regulators of embryonic development [116] and are silenced until cell fate commitment by polycomb repressive complexes (PRC). These PRC promote chromatin condensation by adding H3K27me3 [117] while keeping a poised state of transcription. The bivalent marks become univalent active ones during ESC commitment towards neural lineage [118] by the action of specific H3K27me3 [119] and H3K4me3 demethylases [120]. In mouse NSC, bivalent marks have been shown to resolve into active H3K4me3 monovalency upon differentiation in GABAergic neurons and into repressive H3K27me3 in non-GABAergic neurons [121] indicating that genes carrying bivalent marks may lose one type of mark and become active or silenced depending on the direction of the differentiation. In general, during differentiation, a progressive closure of the chromatin occurs at loci required for differentiation [115] by a depletion of open chromatin histone marks, mainly H3 and H4 acetylation, and a simultaneous increase of repressive marks such as H3K9me3 [122, 123].

As part of the Epigenome Roadmap Project, a recent study has shown that cell specification into the three-germ layer derivatives involves dynamic changes of TFs which work coordinately in specific and sequential TF modules which are integrated by individual TFs showing similar binding preferences for common sequences [124]. Thus, specific loss of DNA methylation has been detected at target sequences due to binding of the lineage-specific TFs as well as increments of the promoter/enhancer H3K27ac mark. In the nervous system, another study characterized the TFs neural regulatory networks involved in differentiation from ESC through neuroepithelial progenitors to radial glial cells [125]. This study found that different neural stages are characterized by different epigenetic states in which distinct TFs are associated with stage-specific epigenetic changes as observed by shRNA inhibition. Thus, early stage-transition from ES to neuroepithelial progenitor showed enrichment for the promoter/enhancer H3K4me1 and H3K27ac marks whereas later transition to radial glial cell showed abundance of the promoter mark H3K4me3 [125].

2.5. Epigenetic Mechanisms during iPSC Reprogramming. Whereas the process of cell reprogramming means a truly epigenetic reprogramming [7], the precise epigenetic mechanisms underlying this process are only partially known. A defined set of pluripotency TFs including only four or even three reprogramming TFs, namely, OCT4, SOX2, KLF4, and MYC, have been shown to be sufficient to generate the induced pluripotent stem cell (iPSC) state [2]. These

TFs are commonly known as OSKM factors (or as OSK when not including c-MYC). The expression of OSKM is needed to overcome epigenetic barriers such as the histone repressive mark H3K9m3 during cell reprogramming [126]. Once the OSKM factors are expressed and the epigenetic barriers are overcome, pluripotency is stably maintained without the need of further ectopic TF expression. Shortly after the expression of the OSKM factors, human fibroblasts initially downregulate specific markers of their somatic state to subsequently activate genes associated with pluripotency [127, 128].

To adopt the epigenome characteristic of a stem cell, the somatic cell has to erase and reorganize its chromatin epigenetic signature [129]. This process involves the genomewide resetting of histone marks which occurs immediately after the induction of OSKM factors [2, 130–132]. Subsequently, the DNA demethylation of promoter regions of pluripotency genes such as NANOG, SOX2, and OCT4 is mediated by activation-induced cytidine deaminases (AID) which are required at later stages of the reprogramming process [82, 130, 133]. Yet DNA demethylation can also occur early since AID is needed to demethylate the OCT4 promoter in fibroblasts and to initiate the process of nuclear reprogramming towards pluripotency [82]. Recent studies have suggested that the OSK TFs act as pioneer factors in loosening the chromatin into more open accessible forms and allowing the activation of genes relevant to the establishment and maintenance of the induced pluripotent state [134]. The initial histone posttranslational changes induced by OSKM include acetylation, methylation, phosphorylation, and ubiquitination of histones. These histone posttranslational changes are catalyzed by HAT and HMT (also known as the "writers") and HDAC and HDM (known as the "erasers") [52] which act, respectively, as coactivators or corepressors of the OSKM factors [126]. Among the earliest processes, an increase of the H3K4me2 mark occurs at promoter and enhancer regions of the genes involved in pluripotency which are enriched for binding sites of the OSKM factors and lack the H3K4me1 and H3K4me3 active marks [130]. To achieve pluripotency induced by OSKM, recent studies have shown that there are three groups of epigenetic targets. First, somatic genes with open chromatin states showing DNase I hypersensitivity and active histone marks H3K4me2 and H3K4me3 are readily accessible to OSKM to be downregulated [134]. Second, distal regulatory elements showing DNase I hypersensitivity and the enhancer mark H3K4me1 act as permissive enhancers that, after the binding of OSKM, are associated with promoters eliciting nucleosome depletion, chromatin relaxation, and transcriptional activation of lineage-specific genes [135]. A third group of OSKM targets encompasses core pluripotency genes containing heterochromatic regions enriched for the repressive mark H3K9me3 in which the binding of OSKM leads to the repression of non-lineage-specific genes [136].

The epigenetic remodeling of chromatin during reprogramming towards pluripotency also requires changes in DNA methylation. Although DNA methylation is considered as the most stable epigenetic modification conferring permanent gene silencing during development [126], histone modifications have been shown to typically antedate changes in DNA methylation during development [109] and consistently this hierarchy of events has also been observed in reprogramming [133]. Demethylation of pluripotency genes is crucial for faithful reprogramming, and although demethylation can occur either by passive or active mechanisms [137], active demethylation catalyzed by specific enzymes has been shown to play a more important role in the induction of pluripotency [126]. In addition, a progressive decrease of DNA methylation and of the H3K27me3 repressive mark at promoters of genes relevant to conversion occurs throughout reprogramming [133]. Although these changes take place almost exclusively at CpG islands of initiating loci at the beginning of reprogramming process, they later expand outside CpG islands to affect other regions [138]. During reprogramming, inefficient DNA demethylation or remethylation has been associated with "epigenetic memory"; that is, the partial retention in iPSC of epigenetic and transcriptional patterns of the somatic cell type of origin which as consequence may limit the differentiation properties to generate specific cell-type derivatives favoring the generation of certain cell types over others [139]. This epigenetic memory has been linked to the failure to reverse repressive epigenetic marks associated with cell fate commitment [101]. To date, epigenetic memory has been regarded as intrinsic limitation of iPSC permitting pluripotency but not totipotency.

3. Epigenetic Research of Neurodegenerative Disorders Using iPSCs

From a technical point of view, ESC represents an ideal tool to investigate development and model human disease as they provide a virtually endless resource of cells of interest given their high self-renewal and differentiation capacity. However, the use of ESC has been limited by ethical issues since current isolation protocols of ESC from the blastocyst inner cell mass imply the destruction of the embryo. In this scenario, in vitro generation of iPSC has contributed to overcome at least in part such an obstacle. Here, we will review the potential of iPSC models as promising cell systems to perform epigenetic research of neurodegenerative disorders in the context of human postmortem brain tissues and animal models which can also implement this new venue of research.

3.1. Genomewide Methylation Studies in Patient Postmortem Brain Tissues. A recent study investigated the methylome of AD in cortex tissue grey matter using a large number of prospectively collected autopsied brains from patients and controls [140]. This study identified differential DNA methylation in 11 CpGs which correlated with AD pathology as assessed by the burden of neuritic amyloid plaques and with RNA expression. Six of the identified differentially methylated genes connected to a known genetic network of AD susceptibility. Among these, methylation differences in the *ANK1* gene were further confirmed in an independent analysis of entorhinal cortex, which is a primary site of AD pathology, as well as in other affected areas including the superior temporal gyrus and the prefrontal cortex [141]. In PD, one genomewide association study (GWAS) identified new genetic variants

associated with disease and, for a subset of genes, it also found differential methylation levels in PD frontal cortex and cerebellum which overlapped with previously reported genetic associations [142]. Another genomewide DNA methylation study in PD frontal cortex also identified distinct methylation patterns in PD affecting genetic polymorphisms associated with PD and, interestingly, these differential methylation patterns correlated in brain and blood samples [143]. Altogether, these studies in AD and PD provide the proof-of-concept that epigenetic deregulation occurs in neurodegenerative disorders and encourage the use of iPSC-based models to conduct epigenetic research in these diseases. However, DNA methylation changes from these studies were detected despite of the heterogeneous mix of brain cell types, and therefore it is possible that overall epigenetic differences may be underestimated. Similarly, it would be expectable that epigenetic changes associated with disease could be potentially identified using iPSC-based models albeit of the cell population heterogeneity which is inherent to these models. Yet in this scenario, iPSCs models offer the opportunity to characterize the epigenetic profiles of specific cell populations by using techniques such as fluorescence-activated cell sorting (FACS) as recently shown for the transcriptome characterization of mouse iPSC-derived DAn [144].

3.2. Lessons from Epigenetic Studies in Mouse Models. A recent RNA-seq study in the Ck-p25 mouse model of AD identified gene expression upregulation of immune system genes and downregulation of genes associated with neuronal function [145]. Similar findings were also reported in human AD postmortem hippocampus [146]. These expression changes correlated with the epigenomic status of promoters, enhancers, and polycomb-repressed regions which showed a specific depletion of neuronal promoter and enhancer marks. Interestingly, this study demonstrated a strong conservation of gene expression and epigenomic signatures between human and mouse with a specific enrichment of AD-associated loci in enhancer orthologs. Similarly, in PD the epigenomic [147] and transcriptomic [144] signatures of a mouse model of iPSC-derived DAn have been deeply characterized but similar studies in PD human iPSC-based models are still missing. Another study in PD compared the transcriptome and the methylome of primary embryonic mesodiencephalic DAn from the $Pitx3^{Gfp/+}$ knock-in mouse as well as iPSC-derived DAn generated upon embryonic fibroblast reprogramming [148]. PITX3 is a highly specific maker of DAn of the substantia nigra and FACS analysis based on the Pitx3-GFP reporter revealed that although mouse iPSC-derived DAn largely adopted highly similar global gene expression and DNA methylation patterns as their in vivo counterparts, they also showed deviations including intermediately methylated neural loci (40–60% methylation) whose role yet remains to be elucidated. Altogether, these studies in AD and PD illustrate a scenario in which epigenetic research relevant to neurodegenerative diseases is more advanced in iPSC-based models from mouse than humans due to availability reasons. Yet achievements of these mouse epigenetic studies can be useful for epigenetic research using patient-derived iPSC-based models since mouse studies can provide valid technical data which may help to prevent pitfalls in designing experiments using human iPSCs as well as to generate novel epigenetic knowledge to be explored in human models genuine to the patients.

3.3. iPSC Models for Epigenetic Research of Neurodegenerative Disorders. Recently, well-established protocols have been elaborated to generate patient-derived disease-relevant cell types upon iPSC reprogramming (comprehensively reviewed in [149]). The specific cell types obtained by these methods include iPSC-derived DAn in PD [21–30], MN in ALS [31–37], striatal medium spiny neurons in HD [31–37], or neurons in AD [42–45]. Although these protocols have been steadily improved by increasing reprogramming and differentiation efficiencies, cell heterogeneity accompanying disease-relevant cell types is still inherent to current iPSC models. This accompanying cell heterogeneity can act as a potential confounder in epigenetic research but, yet, if affecting in an equal manner to iPSC from patients and controls, it may also lead to an underestimation of the observed epigenetic differences, as recently suggested in postmortem epigenetic studies analyzing heterogeneous mix of brain cells [140, 141]. Still this cell heterogeneity should be appropriately controlled for epigenetic studies by performing FACS isolation to deliver pure cell populations prior to the epigenetic analyses [144, 148]. Alternatively, it could also be possible to control the variability caused by cell heterogeneity by studying the epigenetic profile of iPSC-derived neural types nonenriched in the specific neural type of interest, for example, iPSC-derived neural cultures nonenriched in DAn as a control population for a DAn study in PD. Thus, if differences appear only in DAn but not in other cultures nonenriched in DAn, the identified epigenetic differences would be attributable to DAn (Fernández-Santiago et al., unpublished data). Yet despite of current technical challenges, cellular reprogramming provides conceptually a unique opportunity to generate in vitro human models that will permit to investigate epigenetic regulation and alterations of functional states of the chromatin related to neurodegenerative diseases [52]. Recently, the epigenetic signature from 111 human tissues has become publically available including multiple brain regions such as the hippocampus or the substantia nigra which are relevant to AD and PD, respectively [99]. This large multicentre study has implemented the reference human genome sequence and is expected to set the basis for future studies on epigenetic variation and its role in human disease by providing reference maps of histone modifications and DNA methylation, as well as global RNA expression data. This information will prove instrumental to investigate specific epigenetic alterations and to model in vitro novel epigenetic disease mechanisms using currently available patient-derived iPSC-based models of neurodegenerative diseases which up to date have not been epigenetically characterized [150]. Interestingly, iPSC-derived neural models preserve the genetic background of the patient and this is relevant since the disease-associated genetic variants were previously shown to be enriched in tissue-specific epigenomic marks suggesting an overlap of

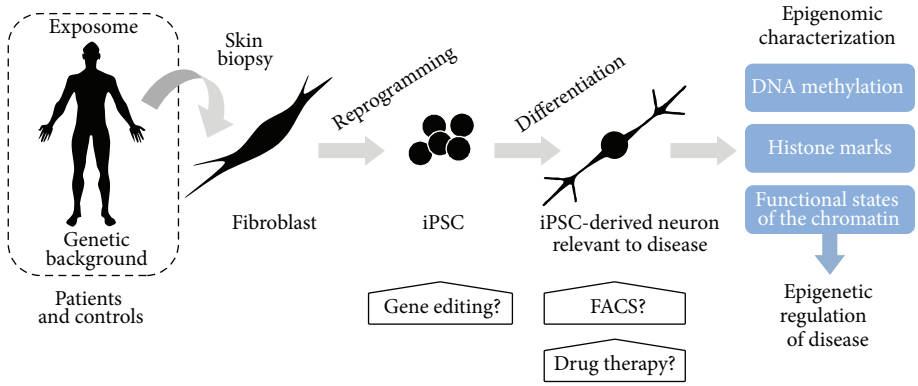

Figure 1: Possible experimental design of epigenomic characterization of neurodegenerative diseases using patient-specific iPSC-derived neural models.

genetic and epigenetic alterations which may be associated with human disease [99]. In addition, iPSC-derived neural models can virtually offer the opportunity to recapitulate the exposome or environmental history of the individual that may be relevant complex diseases with an expected large environmental contribution such as AD, PD, or ALS and also to their monogenic forms (Figure 1).

3.4. iPSCs Models for Epigenetic Research in Monogenic versus Sporadic Forms of Neurodegenerative Disorders. With the exception of HD which is a largely monogenic disease, most of the patients with other neurodegenerative disorders such as AD, PD, or ALS are considered sporadic or idiopathic. In these cases, the disease is expectably driven by the cumulative and/or synergistic effect of genetic risk variants together with largely unknown environmental conditions [151–153] whose effect could eventually be reflected in the epigenome of the patients. To date, although iPSC-based disease modeling has been preferentially performed in mutation-caused monogenic forms of neurodegenerative disease, recent studies in AD and PD have set the proof-of-concept that iPSC-derived models from sporadic patients can exhibit molecular alterations similar to those changes detected in monogenic patients [28, 42, 43]. In monogenic cases, iPSC-based systems offer the attractive possibility to perform gene editing [29] contributing to the elucidation of the molecular events triggering disease through the analysis of the effect of specific pathogenic mutations. Unfortunately, this approach is not to be feasible for sporadic forms due to the polygenic effect of the multiple genetic risk factors which are expected to be involved in the sporadic disease. Despite this inconvenience, iPSC-derived systems have proved efficient to model sporadic disease as, for example, in PD [154] but, yet, it has not clarified the underlying mechanisms by which these iPSC-derived models from sporadic patients can develop disease phenotypes. It can be hypothesized that since iPSC-based neural models preserve the genetic background from the patient, derived neurons also carry the specific set of susceptibility genetic variants which could ultimately trigger the disease initiating pathogenic changes. Alternatively, biological alterations and damages could already be present in the primary fibroblast as consequence of the interaction of the genetic background and environmental factors but their full pathogenic effect might only be observed in the appropriate context of the disease-relevant neural cell types. Supporting this view, biological alterations have been recently described in fibroblast from sporadic cases with PD or AD [155, 156], thus reinforcing the idea of latent molecular defects which can be present in the somatic cells. Yet the genetic or epigenetic nature of these potentially latent molecular defects in the somatic cells from sporadic cases has so far not been explored into detail in neurodegenerative diseases. In addition, interactions of genetic and epigenetic factors represent an important field of investigation in complex disorders [157]. In this scenario, patient-specific iPSC-derived neural cells could represent useful models able to not only capture the subject genetic background but also potentially recapitulate the environmental exposome of the individual through the epigenome (Figure 1). Accordingly, iPSC-based neural models are expected to be helpful for investigating epigenetic changes of the sporadic forms of neurodegenerative diseases where the environment is supposed to play a more prominent role. However, the complexity of these multifactorial diseases is expected to be high, especially when taking into account the presence of possible interactions between genetic risk variants and their methylation status that could ultimately modify their pathogenicity [157]. Under this view, iPSC-derived neural models open new research venues to investigate epigenomic changes associated with neurodegenerative diseases and most especially with their sporadic forms.

3.5. Environmental Epigenomics in Complex Neurodegenerative Diseases. Environmental conditions include the exposition of an individual to drugs, toxins, metabolites, or other external stimuli. However, the environment can also be considered as the single cell microenvironment encompassing external cellular stimuli, inflammatory responses, or signaling from nearby cells. Yet both of these macro- and microenvironmental conditions have been shown to contribute to the modification of the epigenome by ultimately inducing interoceptive cell signaling cascades [158]. These

environmental conditions have also been shown to contribute to the epigenetic drift observed in monozygotic twins who accumulate diverging epigenomic changes over time [56]. Among metabolites modulating the epigenome, folic acid has been shown to remodel the chromatin conformation at neural promoters during neural tube development indicating that environmental exposition to chemical cues can be associated with epigenetic regulation relevant to the nervous system [159]. In addition, cumulative evidence has shown that other compounds interfering with epigenetic control during early development are suspected to consequently cause other neural defects later in life [160]. Thus, epigenetic research could provide novel mechanistic paradigms for developmental toxicology studies in late-onset diseases like AD or PD [161, 162] where epigenetic changes could mediate the transition from an early insult caused by chemical compounds to an adverse effect on the developing nervous system [160]. Several works have also revealed associations between early-life exposure to pesticides and PD [163] but the epigenetic involvement in this pathogenic process is yet unclear. As examples of environmental factors potentially triggering neurodegenerative diseases such as PD later in life, cell culture studies have shown that exposure to several neurotoxicants such as methyl mercury (MeHg) impairs the formation of DAn or reduces their neuritic growth [164, 165]. In adults, pesticide-induced hyperacetylation of histones leading to chromatin decondensation and nonspecific transcriptional upregulation has been linked to PD [166]. For example, Paraquat another pesticide acting as neurotoxin in PD has been associated with hyperacetylation of histones [167]. In addition, time-dependent increase of H3 and H4 hyperacetylation induced by environmental toxins such as the insecticide dieldrin has also been associated with the pathophysiology of PD [168]. Moreover, recent studies have demonstrated the mediation of specific epigenetic mechanisms in promoting axonal regeneration after spinal cord injury providing further evidence of the influence of environmental cues in the epigenome of the individual [169]. In this context, patient-derived iPSC-based cellular models of neurodegenerative disease could represent a valid tool to explore the effect on the subject epigenome of candidate environmental factors identified in epidemiologic studies. Results from these studies may ultimately contribute to deciphering the pathophysiological processes associated with environmental conditions in neurodegenerative disorders by the identification of specific underlying epigenetic mechanisms.

4. Epigenetic Therapeutic Targets in Neurodegenerative Disorders

In principle, iPSC models are ideally suited for drug development due to their limitless self-renewal capacity allowing the production of large quantities of cells and to their high differentiation properties into disease-specific cell types. However, iPSC models have not yet been extensively used in large-scale drug screenings in neurodegenerative disorders due to the clonal variation associated with stochastic gene mutation [170] and also due to the difficulties in controlling for correct efficiency differentiation when using large amounts of clones. Thus, although studies of several thousands of compounds have been published in ALS [171, 172] or AD [173], large-scale drug studies using iPSC are still an ongoing area of development. On the contrary, strategies using a limited number of candidate therapeutic drugs have been successfully tested in other diseases [174]. This approach seems to be more feasible at present for neurodegenerative diseases as recently shown in iPSC models of AD [175] and ALS [35].

Epigenetic drugs currently explored in human disease models include most prominently histone deacetylation inhibitors, DNA methylation inhibitors, and histone acetylation activators [158]. Conceptually, HAT and HDAC maintain the balance of correct acetylation marking of histone lysine residues upon acetyl-coenzyme A as donor of acetyl groups. HAT act by enhancing the DNA accessibility for TF binding and increasing gene expression while on the contrary HDAC have the opposite effect by attenuating transcription. These acetylation balances determine cell survival and homeostasis whereas imbalances are related to pathological conditions [176, 177]. In neurodegenerative disorders, recent reports have suggested that the deregulation of histone acetylation levels could be modulated by epigenetic drugs [178, 179]. Thus, drugs activating HAT [180] as well as HDAC inhibitors have been shown to improve neuroprotection and synaptogenesis [181, 182]. Moreover, epigenetic drugs modulating HAT or HDAC activity have been shown to alleviate pathological symptoms in experimental models of PD, AD, and HD by reverting abnormal gene repression associated with disease [183–186]. Yet different drugs may be needed for different aspects of disease [158] since, for example, in ALS a HAT inhibitor called anacardic acid has been proved effective in downregulating abnormal gene expression and rescuing ALS MN phenotype [35]. Valproic acid (VA) is one HDAC inhibitor enhancing H3 acetylation which has been shown to be neuroprotective against MPTP-induced neurotoxicity in PD mouse models [187]. The neuroprotective effect of VA has been demonstrated to be mediated by glial cell-derived factor (GDNF) and brain-derived neurotrophic factor (BDNF) signaling in DAn models from rats [188]. ESCs retinoic acid (RA), which is a determinant for anteroposterior patterning of the developing CNS, has been shown to have a similar effect as HDAC inhibitors by increasing histone acetylation levels and upregulating gene expression of its targets [189].

Specifically in PD, other studies illustrate that epigenetic drugs can be useful to modulate disease aspects related to epigenetic deregulation. Thus, a recent study in a human DAn model and mouse organotypic brain slice cultures has shown that the treatment with the HDAC inhibitor sodium butyrate (NaBu) upregulates the expression of oxidative stress-sensitive protein kinases (PKCs) and augments DAn apoptotic cell death [184]. Since the effect of this HDAC inhibitor directly leads to H4 hyperacetylation, this study supports the role of HDAC deregulation in PD and identifies novel potential epigenetic therapeutic targets. In addition, DNMT inhibitors such as 5-aza-2′-deoxycytidine (5-aza-dC) have been shown to induce the expression of tyrosine hydroxylase (TH), the synthesis of dopamine, and also

the expression of alpha-synuclein [190]. Moreover, levodopa-induced dyskinesia which is a major side effect of the levodopa treatment in PD patients has been associated with histone deacetylation in PD animal models [191] suggesting a possible role of histone acetylating drugs for the treatment of dyskinesias [192]. In general, if levodopa is proved to act through an epigenetic pathway, traditional treatments should be revisited to elucidate the novel epigenetic aspects and to design novel and more specific medications [158].

In summary, let alone that epigenetic research of neurodegenerative disorders is an emerging field, the identification of epigenetic therapeutic targets for AD, PD, or ALS is at its infancy. In this scenario, iPSC-based models may be useful not only to detect epigenetic changes associated with these diseases but also to explore the ability of candidate epigenetic drugs to correct epigenetic alterations and to design novel therapeutic strategies. To date, this goal seems to be technically feasible only for small or medium scale drug studies or for very specific drugs. Yet future patient-specific iPSC-based systems using improved cell reprogramming protocols are expected to pave the way out for epigenetic research ultimately intending the delivery of personalized epigenetic therapies.

5. Future Perspectives and Open Questions

Although recent works have provided evidence about the involvement of epigenetic changes in neurodegenerative diseases and supported the use of iPSC-derived neural models to explore epigenetic alterations in these disorders, several questions remain to be answered.

A first question is whether iPSC-derived neurons really mimic the epigenetic and expression features occurring in the affected brain areas of the patients. Epigenetic changes relevant to neurodegenerative diseases are expected to reflect the complex interaction of genetic background, environmental factors, and gene expression in the context of the brain. Accordingly, it would be necessary to assess whether iPSC-derived neurons obtained from patient fibroblasts do faithfully recapitulate the molecular events occurring in the complex cell microenvironment of the brain thus representing good models of disease. A direct approximation would encompass the comparison and determination of the level of coincidence of epigenetic marks in iPSC-derived neurons and in their patient brain cell counterparts as investigated in a recent study [148]. However, defining the epigenetic alterations related with disease remains challenging since usually postmortem brain tissues are only available after many years of disease evolution and also after important cell loss of the neurons targeted by disease. In addition, it is also essential to determine whether changes in the epigenomic profile, the gene expression patters, the protein composition, and the overall neuron performance could represent on themselves initial changes triggering disease or alternatively secondary physiological changes of the neurodegenerative process [193]. Fairly, this is an important application of iPSC-derived cells models of neurodegenerative diseases which could help to identify early alterations occurring in nervous tissues [194]. These cells can show neural networks and are functional in terms of biochemistry, electrophysiology, and synaptic transmission as previously described [28, 42]. Yet it remains to be demonstrated whether the epigenetic alterations detected in iPSC-derived neurons could represent pathological epigenetic changes associated with disease or alternatively whether they could be beneficial compensatory changes in response to disease injury caused by other molecular mechanisms.

Second, assuming that iPSC systems represent good disease models for neurodegenerative diseases, a new venue would be open to explore epigenetic changes in specific genetic loci by using high resolution whole-genome methodologies, including commercially available methylation arrays, whole-genome bisulphite sequencing, or whole-genome histone marks analyses in patients and controls. In order to implement our comprehension of disease mechanisms, these data could be analyzed by integrative biology methods interrogating the epigenome, the transcriptome, and the known risk genetic loci detected in genomewide association studies (GWAS) [195–197]. In this way, GWAS have identified hundreds of genetic risk variants for neurodegenerative diseases like AD or PD [198–200] which are located in different genes loci affecting aetiological pathways involved in disease but with subtle effects on disease susceptibility. However, it is important to note that more of the risk polymorphisms identified until present by GWAS has not immediately provided functional insights and also that most of the risk variants cannot always be clearly assigned to target genes since many variants are located in noncoding or intergenic regions [201]. Thus, to improve our understanding of disease risk mechanisms, it is possible to correlate cis- or trans-located genetic risk polymorphisms, gene activity as determined by transcripts quantification [202, 203], and CpGs methylation differences at specific loci [204]. These genetic variants can affect gene expression activity by altering the affinity of DNA binding TFs [205] leading to differential methylation patterns and if located in enhancers regions they can also alter the expression of distal target genes [204]. Thus, this combined approach associating specific candidate genetic polymorphisms, gene expression changes, and differential methylated CpGs could help to gain insight into the functional consequences of genetic variants associated with disease risk and to facilitate the interpretation of data from GWAS studies [206], for example, by restricting the analysis of risk candidate polymorphism in GWAS to those associated with differences in methylation levels in disease-targeted cells [197]. Thus, we anticipate that epigenetic and expression alterations detected in iPSC-derived models of neurodegenerative diseases will serve as a functional system to reinterpret the genetic risk loci associated with these diseases by implementing the knowledge of the pathogenic mechanisms associated with the risk genetic loci.

Third, assuming that these neurons recapitulate causative epigenetic alterations, a new opportunity will be offered to explore the capacity of different epigenetic-modifying drugs to modulate pathologic epigenetic patterns and then observe their respective functional effects and establish cause-effect mechanisms. In this way, iPSC-derived neurons

could represent a valid tool to test potential therapeutic drugs reversing the pathological epigenetic changes and to monitor disease-relevant processes such as alpha-synuclein or amyloid deposition, autophagy alterations, or mitochondrial dysfunction. In summary, iPSC-derived neurons models have promising perspectives and open new avenues for biological mechanistic studies, drug discovery and testing, and clinical therapy of neurological disorders related with epigenetic changes.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

The authors contributed equally to the conception of the paper, review of data, and writing of the paper.

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Research Article

Cardiac Niche Influences the Direct Reprogramming of Canine Fibroblasts into Cardiomyocyte-Like Cells

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The Duchenne and Becker muscular dystrophies are caused by mutation of dystrophin gene and primarily affect skeletal and cardiac muscles. Cardiac involvement in dystrophic GRMD dogs has been demonstrated by electrocardiographic studies with the onset of a progressive cardiomyopathy similar to the cardiac disease in DMD patients. In this respect, GRMD is a useful model to explore cardiac and skeletal muscle pathogenesis and for developing new therapeutic protocols. Here we describe a protocol to convert GRMD canine fibroblasts isolated from heart and skin into induced cardiac-like myocytes (ciCLMs). We used a mix of transcription factors (GATA4, HAND2, TBX5, and MEF2C), known to be able to differentiate mouse and human somatic cells into ciCLMs. Exogenous gene expression was obtained using four lentiviral vectors carrying transcription factor genes and different resistance genes. Our data demonstrate a direct switch from fibroblast into ciCLMs with no activation of early cardiac genes. ciCLMs were unable to contract spontaneously, suggesting, differently from mouse and human cells, an incomplete differentiation process. However, when transplanted in neonatal hearts of SCID/Beige mice, ciCLMs participate in cardiac myogenesis.

1. Introduction

Ischemic heart disease is one of the leading causes of death worldwide and so far therapeutic approaches are limited [1].

Because of the negligible regenerative ability, the heart has been considered for nearly a century as a terminal differentiated postmitotic organ [2]. Although this concept is currently outdated, the heart is not able to heal itself after injury

by any native processes, and fibrotic scars replace necrotic tissue. This stiffens the heart tissue and prevents the normal contractility of cardiomyocytes. Cardiac fibroblasts (CFs) are widely involved in the heart healing process and represent one of the largest cell populations in the myocardium [3]. For this reason, CFs have been identified as ideal cell source for *in vivo* direct conversion approaches [4].

The discovery of MyoD, as master gene for skeletal muscle differentiation [5], generated a broad interest in cell reprogramming by using defined factors. Unfortunately, for cardiac differentiation, a single master gene such as MyoD is not known yet. Ieda et al. reported that the forced expression of three exogenous transcription factors (Gata4, Mef2c, and Tbx5) in neonatal cardiac and dermal fibroblasts is sufficient for the conversion to cardiomyocyte-like cells *in vitro* [6]. After this first elegant study, several groups reported similar results using different transcription factors and microRNA [7–10]. Recently, two groups reported the conversion of fibrotic scar tissue into induced cardiomyocytes-like cells *in vivo* through retroviral delivery of GATA4, MEF2C, and TBX5 (GMT) transcription factors [11, 12]. In the same year, Olson's group demonstrated an improvement of the cardiac differentiation rate combining the GMT with Hand2 both *in vitro* and *in vivo* [10]. Similar results have been achieved lately on human fibroblasts using the same protocol [13, 14].

These discoveries hold a great promise for the treatment of heart chronic diseases where the invading fibrotic tissue could be replaced by contractile cardiomyocytes. Muscular dystrophies (MDs) are a group of inherited diseases caused by mutations in the Dystrophin Glycoprotein Complex. Patients affected by MDs, in particular Duchenne and Becker muscular dystrophy, who survived to the third decade of life are affected by cardiomyopathy and heart failure is the main cause of death for these patients [15–17]. The heart degeneration and remodelling lead to the formation of subepicardial fibrosis of the inferolateral wall [18] that could represent the ideal target for direct lineage reprogramming to cardiomyocyte lineage. Among the widely used animal models of DMD, the golden retriever muscular dystrophy (GRMD) dog is considered the closest model to the human disease in terms of size and pathological onset of the disease. In fact, the clinical course of GRMD dogs is characterized by progressive muscle wasting, degeneration, fibrosis, and shortened lifespan [19–21]. Cardiac involvement in GRMD dogs has been demonstrated by electrocardiographic studies, revealing a progressive cardiomyopathy similar to DMD patients [22–24]. In this respect, GRMD dog is a useful model for the development of new therapeutic protocols to improve cardiac function [25].

In this study we aim to evaluate the direct lineage conversion strategy (GATA4, MEF2C, TBX5, and HAND2) on cardiac and skin fibroblasts isolated from a large animal model of Duchenne muscular dystrophy (GRMD). Canine-induced cardiac-like myocytes (ciCLMs) expressed late cardiac markers genes, immature sarcomeric structures, and engrafting ability *in vivo*.

2. Material and Methods

2.1. Isolation and Culture of Skin Canine Fibroblasts. Canine skin samples from GRMD dogs were obtained from fresh abdominal skin biopsies and isolated as previously shown [26]. Obtained canine fibroblasts were kept in culture in DMEM supplemented with 15% FBS and antibiotics. The medium was replaced every two days. Cells were frozen, after being cultured for one week, and then plated for lentiviral transduction.

2.2. Isolation and Culture of Cardiac Canine Fibroblasts. Left ventricular heart biopsy from a deceased GRMD dog was chopped into small pieces and plated on a tissue culture dish in DMEM : 199 (4 : 1), 15% FBS and antibiotics. Cardiac fibroblasts spread out from the minced heart after few days and one week later cells were frozen. The medium was replaced every two days.

2.3. Cell Culture. Bj-1 and canine cardiac and skin fibroblast were maintained in DMEM supplemented with 5 mM L-glutamine, 5 mM sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL streptomycin (all from Invitrogen), and 20% fetal bovine serum (Gibco). Neonatal rat cardiomyocytes were used for the preparation of the induction medium (IM) and for coculture with transduced fibroblasts. Neonatal rat cardiomyocytes were isolated using the Neonatal Cardiomyocyte isolation kit from Worthington Biochemical Corporation (Lakewood, NJ, USA) and cultured in DMEM 10% FBS (Invitrogen, Carlsbad, CA, USA). Medium was replaced and frozen every day and subsequently used in the preparation of the induction medium (IM). Primary cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2.

2.4. Plasmid Cloning and Expansion. Lentiviral vectors were prepared by using standard procedures. Bacterial stocks were grown in Luria-Broth medium ON at 37 degrees. All vectors preparations were obtained using a GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma) and Plasmid DNA was quantified using Nanodrop system.

The plasmid carrying the human transcription factors (GATA4, MEF2C, TBX5, and HAND2) and α-MyHC-GFP cardiac differentiation reporter gene were subcloned into the lentiviral vector pCHMWS carrying different resistance genes (Figure 2(a)).

2.5. Lentivirus Production. HEK 293 cells were seeded at density of 6×10^6 in a 10 cm tissue culture dish and at the day after being transfected, using Lipofectamine 2000 (Invitrogen), with nine micrograms of the individual transfer vectors. Cells were kept in OptiMem; after six hours the medium was replaced to 10 mL of fresh DMEM with 10% FBS and antibiotics. The medium containing lentiviral particles was harvested after 36 hours of transfection, filtered with 0,45 μm cellulose filter.

2.6. Viral Transduction and Differentiation. Skin, cardiac, and Bj-1 fibroblasts were plated at density of $1 \times 10^5/\text{cm}^2$ on Nunc

culture dishes (ThermoFisher Scientific) and transduced with fresh lentiviral supernatant (MOI = 50) mixed with polybrene at final concentration of 4 μg/μL (Sigma). The day after transduction with the mix of transcription factors, medium was replaced with induction medium (IM), containing 10% conditioned medium (obtained from neonatal rat cardiomyocyte culture), DMEM/199 (4 : 1), 10% FBS, 5% horse serum (HS), antibiotics, nonessential amino acids, essential amino acids, B-27, insulin-sodium selenite-transferrin, vitamin mixture, and sodium pyruvate (Invitrogen) [10]. Eukaryotic selection antibiotics (Blasticidin 5 μg/mL, Hygromycin 100 μg/mL, and Zeocin 200 μg/mL) were added to the IM the second day after transduction and kept in culture for one week. Induction medium was replaced every two days.

2.7. Real Time PCR. Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) and 1 μg was reverse transcribed to cDNA by Superscript III Reverse Transcriptase (Life Technologies). Real Time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG and Mastercycler ep realplex (Eppendorf). qPCR experiments were performed using the following primers:

hGATA4: Fw5′-tctcagaaggcagagagtgtg; Rv5′-ggttgatgccgttcatcttgt;

hMEF2C: Fw5′-aagaagggccttaatggctgt; Rv5′-atctcgaagttgggaggtgga;

hTBX5: Fw5′-gcatggagggaatcaaagtg; Rv5′-cttcgttttgggattaaggcc;

hHAND2: Fw5′-atgagtctggtaggtggttttcc; Rv5′-catactcggggctgtaggaca;

dGATA4: Fw5′-cgggctatcatcccactatg; Rv5′-gcgactggctgacagaagat;

dMEF2c: Fw5′-cccgatgcagacgattca; Rv5′-caaaattgggaggtggaaca;

dTBX5: Fw5′-ccttctaccgctcaggctac; Rv5′-caggctgggcacaggctca;

dHAND2: Fw5′-ccgctaaccgcaaggaga; Rv5′-cgtcttgatcttggagagtttg;

ACTC1: Fw5′-tcccatcgagcatggtatcat; Rv5′-ggtacggccagaagcataca;

CNX43: Fw5′-ccagttgagattccactcagt; Rv5′-gttgagtaccacctccactga;

TNNT2: Fw5′-atgtctgacgtagaagaggag; Rv5′-atcaccttccgcgttggtctc;

MYH6: Fw5′-gccctttgacattcgcactg; Rv5′-ggtttcagcaatgaccttgc.

2.8. Western Blotting. Cells were collected 14 days after transduction and resuspended in Ripa buffer (Sigma) supplemented with 10 mm NaF (Sigma), 0.5 mm NaOrthovanadate (Sigma), 1 : 100 Protease Inhibitor Cocktail (Sigma), and 1 mm PMSF (Sigma). Western blot analysis was performed on 40 μg of cell lysate, using anticardiac-TnnI (Tnni3; Abcam; ab19615) 1 : 100 as primary antibody and secondary HRP-conjugated antibodies (Santa Cruz), 1 : 500. Bands were detected and pictured at Bio-Rad GelDoc by means of Pico substrate (Thermo Fisher Scientific; Dura substrate for DYS analysis); densitometry analyses were carried on gels loaded, blotted, and detected in parallel by means of QuantityOne software (Bio-Rad).

2.9. Immunocytochemistry and Flow Cytometry Analyses. Cells were fixed 14 days after transduction in 2% PFA, permeabilized with 0.2% Triton, and blocked with donkey serum (Sigma). Cells were then stained overnight at 4°C with different primary antibodies. Antibody dilutions were the following: anti-Vimentin (Sigma; V6630) 1 : 200, anti-Ki67 (sigma; ab15580) 1 : 200; anti-GFP (Abcam; ab545) 1 : 500; anti-sarcomeric α-actinin (αSA; Abcam; ab9465) 1 : 200; anti-connexin 43 (Santa Cruz; sc-9059) 1 : 300; MyHC (MF20; Hybridoma) 1 : 3; cardiac troponin T (Abcam; ab10214) 1 : 200.

Secondary fluorescents antibodies (Alexa-Fluor; 1 : 500) were used for detection. Nuclei were stained with Hoechst 33342 (Sigma).

Flow cytometry analyses were performed according to standard procedures with the following antibodies: anti-CD90/Thy1 antibody (FITC) (Abcam; ab124527); anti-cardiac troponin T (Abcam; ab10214).

Cardiac troponin T expression was analyzed by intracellular staining; cells were fixed with 2% PFA, blocked with donkey serum (Sigma), and permeabilized by fixation/permeabilization (BD Cytofix/Cytoperm).

2.10. In Vivo Xenotransplant Experiment. GHMT GFP positive cells were sorted 7 days after transduction using AriaIII FACS and 5×10^4 were injected directly in the heart of 2-day-old SCID/Beige-mice pups. After 2 weeks, mice were sacrificed and the whole heart was embedded in OCT (Sakura, Tissue-Tek) and cut as 10 μm sections using a cryostat (Leica). Sections were fixed in 4% PFA, permeabilized by solution PBS-BSA1%-Triton X-100, and immunostained by antibodies against dystrophin (NCL-DYS2-NCL-DYS3; Novocastra; 1 : 50), myosin heavy chain (MF20; Hybridoma; 1 : 3), and GFP (Abcam; ab5450).

2.11. Statistical Analysis. Values are expressed as mean ± SD, and when two groups were compared, unpaired Student's t-test was used. When more than two groups were compared, a two-way analysis of variance (ANOVA) was used and a probability of less than 5% ($p < 0.05$) was considered to be statistically significant (Bonferroni post hoc test). Statistical significance of the differences between the percentage values was assessed by the Kruskal-Wallis one-way ANOVA on ranks, with a Bonferroni-Dunn post hoc test, and significance was scored when $p < 0.05$ for both tests.

3. Results

3.1. Characterization of Fibroblast Populations. In the last years, several groups reported the possibility to induce direct reprogramming from adult somatic cells to other lineages through the overexpression of a set of transcription factors

or miRNA [6, 10, 27–31]. In this study we tested whether the same four cardiac transcription factors, GHMT, shown previously to direct the reprogramming on neonatal human foreskin fibroblasts [14], were able to reprogram fibroblasts isolated from GRMD dogs. According to those previous studies, canine fibroblasts were considered for the reprogramming protocol due to their easy access and ability to proliferate *in vitro*. We characterized canine fibroblasts for their morphology (Figures 1(a)–1(c)), expression of Vimentin as mesodermal marker, and Ki67 as proliferative marker (Figures 1(d)–1(f)) and cell growths have been analysed (Figure 1(g)). In addition, Bj-1 human fibroblasts, a commercially available adult human dermal fibroblast cell line, were used as control. The three fibroblast populations resulted 100% positive for Vimentin and fibroblasts derived from skin and cardiac biopsies were, respectively, 79% and 65% positive for Ki67, slightly lower than Bj-1 (87%). In order to avoid possible contamination of progenitor cells, known to be more susceptible to reprogramming, we employed cell-sorting strategy in our freshly isolated primary cultures to isolate homogenous fibroblast populations. FACS analysis showed that cells were homogenously positive for CD90 (Thy1) (Figure 1(i)), similarly to Bj-1 cell line. CD90 positive cells were subjected to the reprogramming protocol.

3.2. Transcription Factors Overexpression. To determine the best set of transcription factors able to convert fibroblast to cardiac-like cells, we generated lentiviruses carrying different genes involved in cardiac differentiation including GATA4, HAND2, MEF2C, TBX5, MESP1, and BAF60c. A cardiac muscle specific promoter (alpha myosin heavy chain, αMyHC) was used to drive expression of GFP in our cell system, as gene tracer for cardiac differentiation. We infected Bj-1 cells with different mix of lentiviral vectors and we evaluated the conversion potential by GFP expression. In our hand, the best set of genes resulted in being GHMT (data not shown). Indeed, the overexpression of GHMT has been shown to be sufficient for the reprogramming of tail tip and cardiac fibroblast [10] and human fibroblasts [14] to cardiac-like cells. In the following experiments, coding sequence genes for GATA4, HAND2, MEF2C, and TBX5 (GHMT) were cloned into lentiviral vectors carrying different gene resistance (Figure 2(a)) and used to infect CD90+ fibroblasts from skin (SF) and cardiac (CF) GRMD biopsies and Bj-1 human cell line. Cells were infected with a MOI of 50 and after 2 days few GFP positive cells were detected in all samples (Figure 2(b)). Then the medium was replaced with the differentiation medium and cells were maintained in culture up to 28 days. GFP positive cells underwent drastically morphological changes, resulting in flattened and binucleated cells similar to foetal cardiomyocytes (Figure 2(c)). GFP positive GHMT CFs were sorted and counterstained, alone (Figure 2(d), upper left panel) or in coculture with rat cardiomyocytes (Figure 2(d), lower left panel), for myosin heavy chain in order to confirm the specificity of our differentiation protocol. GFP and myosin heavy chain (MyHC) colocalized in the majority of the cells (Figure 2(d), left upper panel). However, we could also find occasionally GFP positive cells negative for MyHC (Figure 2(d), right panel), suggesting possible leakiness of the αMyHC promoter. At day 1 from the infection, a small population of skin and cardiac canine fibroblasts died; however, they maintained a comparable proliferation rate to GHMT Bj-1 fibroblasts in the following days (Figure 2(e)). With the prospective of a possible cell therapy and functional tests after cell conversion, we transduced GHMT CFs and GHMT SFs with lentiviral vectors carrying microdystrophin (μdys) gene, and μdys expression was evaluated via qRT-PCR in transduced cells (Figure 2(f)). As shown in Figure 2(g), infected cells maintained a high and comparable expression of GHMT transcription factors over the 28 days of differentiation.

3.3. Evaluation of the Conversion of Fibroblast to ciCLMs. To evaluate the conversion of fibroblasts into cardiac myocyte-like cells, we analysed the expression of early (MEF2c, NKX2.5, GATA4, TBX5, and HAND2) and late (CNX43, TNNT2, ACTC1, and MYH6) cardiac markers at different time points (Figure 3). After 14 days in differentiation medium, we detected an increase of the expression of late cardiac markers. On the contrary, we did not detect a consistent fold change in the expression of endogenous early cardiac markers. It is likely that due to the constitutive expression of the exogenous transcription factors genes involved in the first steps of cardiomyogenesis were repressed. Interestingly we found that CNX43, TNT and ACTC1 were significantly more highly expressed in GHMT CFs compared to GHMT SFs and Bj-1, suggesting that fibroblasts derived from a cardiac niche could retain a higher conversion potential to cardiac myocytes-like cells. Moreover, confirmatory results were obtained with FACS (Figure 4(a)) and Western blot analysis (Figure 4(b)). When cells were stained for the cardiac marker Tnnt2 and evaluated via FACS analysis (Figure 4(a)), we detected 17.3% and 12% Tnnt2+ cells, respectively, in GHMT CFs and GHMT SFs. In addition, Western blot analysis with anti-TnnI3 antibodies revealed a higher amount of troponin I protein in GHMT CFs compared to GHMT SFs. On the contrary, troponin I was not detected in the negative control (Figure 4(b)). Consistent with the upregulation of cardiac specific genes during the conversion, we found an important decrease in the expression level of the fibroblast markers HSP47 (Figure 4(c)) and CD90 (Figure 4(d)) in GHMT CFs and GHMT SFs compared to controls. Although between 20 and 30% of cells still expressed Thy1 (CD90), we cannot exclude that the reprogramming protocol altered their fibroblast phenotype. Up to 95% of converted cardiomyocytes survive and we managed to keep them in culture for 30 days. Immunofluorescence analysis was then performed (at day 14) to further elucidate the protein organization in the ciCLMs. GHMT CF, GHMT SF, and Bj-1 cells stained positive for alpha sarcomeric actinin (αSA), connexin 43 (CNX43), myosin heavy chain (MyHC), and cardiac troponin T (TNNT2) (Figures 5(a) and 5(b)). The results obtained from immunofluorescence analysis seem to represent a spectrum of cardiac reprogramming, where ciCLMs induced from adult cardiac and skin fibroblasts are composed of different cell types, previously referred to as types A, B, and C [10]. However, we could not observe more organized sarcomeres in

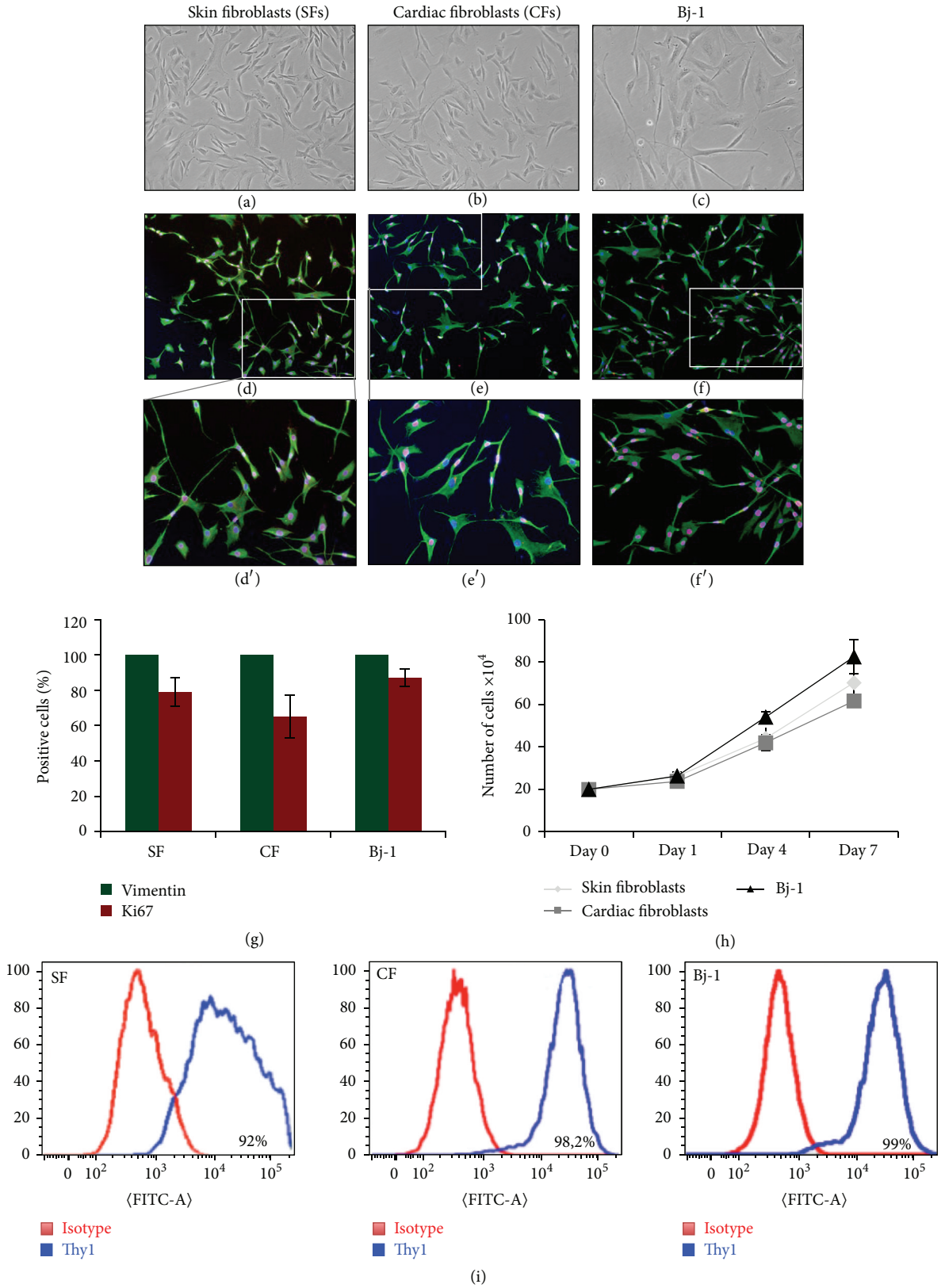

FIGURE 1: Canine primary culture characterization. Skin (a) and cardiac (b) canine fibroblasts showed similar morphology to human Bj-1 cells (c) and express in similar fashion Vimentin (in green) and Ki67 (in red) (d–f). The percentage of Vimentin and Ki67 positive cells was similar in the three cell populations (g). Growth curves of canine fibroblasts (n = 5/fibroblast type) showed comparable proliferation ability with respect to Bj-1 fibroblasts (h). (i) Examples of FACS analysis revealing that canine fibroblast primary cultures highly expressed Thy1 (CD90), similarly to Bj-1 fibroblasts.

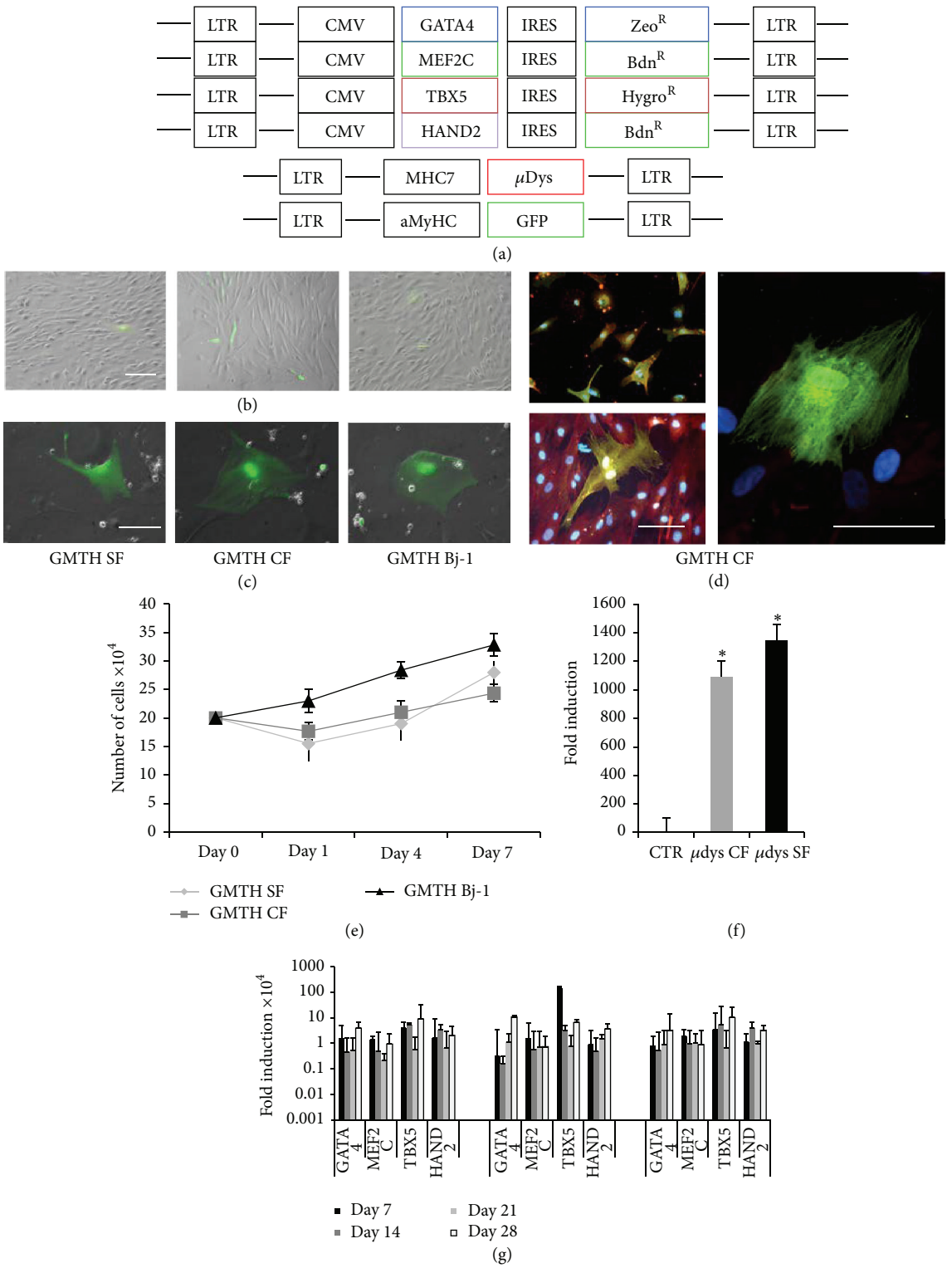

Figure 2: Viral transduction and exogenous gene expressions. (a) Schematic representation of the lentiviral vectors carrying the human sequence of the transcription factor genes (GATA4-MEF2C-TBX5-HAND2), the microdystrophin gene, and the GFP reporter gene. (b) The GFP reporter gene under the control of the α-MyHC promoter was already expressed 48 hours after transduction. (c) Fibroblasts underwent morphology changes 7 days after transduction and become flattened and binucleated cells expressing GFP. (d) Immunofluorescence analysis on GHMT CF alone (top left panel) and in coculture with neonatal rat cardiomyocytes (bottom left panel), with antibodies against GFP (green) and myosin heavy chain (red), shows double positive cells and single positive cells for GFP (right panel). (e) Cell growth curve after transduction shows a decrease of cell proliferation ($n = 5$/cell type). (f) Fold induction of microdystrophin (μdys) mRNA expression in transduced cells compared to the control; $n = 5$ $^*p < 0.01$, CF and SF versus controls (CF and SF transduced with empty vector). (g) Exogenous transcription factors expression ($n = 5$) after transduction shows high induction levels during the 28 days of differentiation.

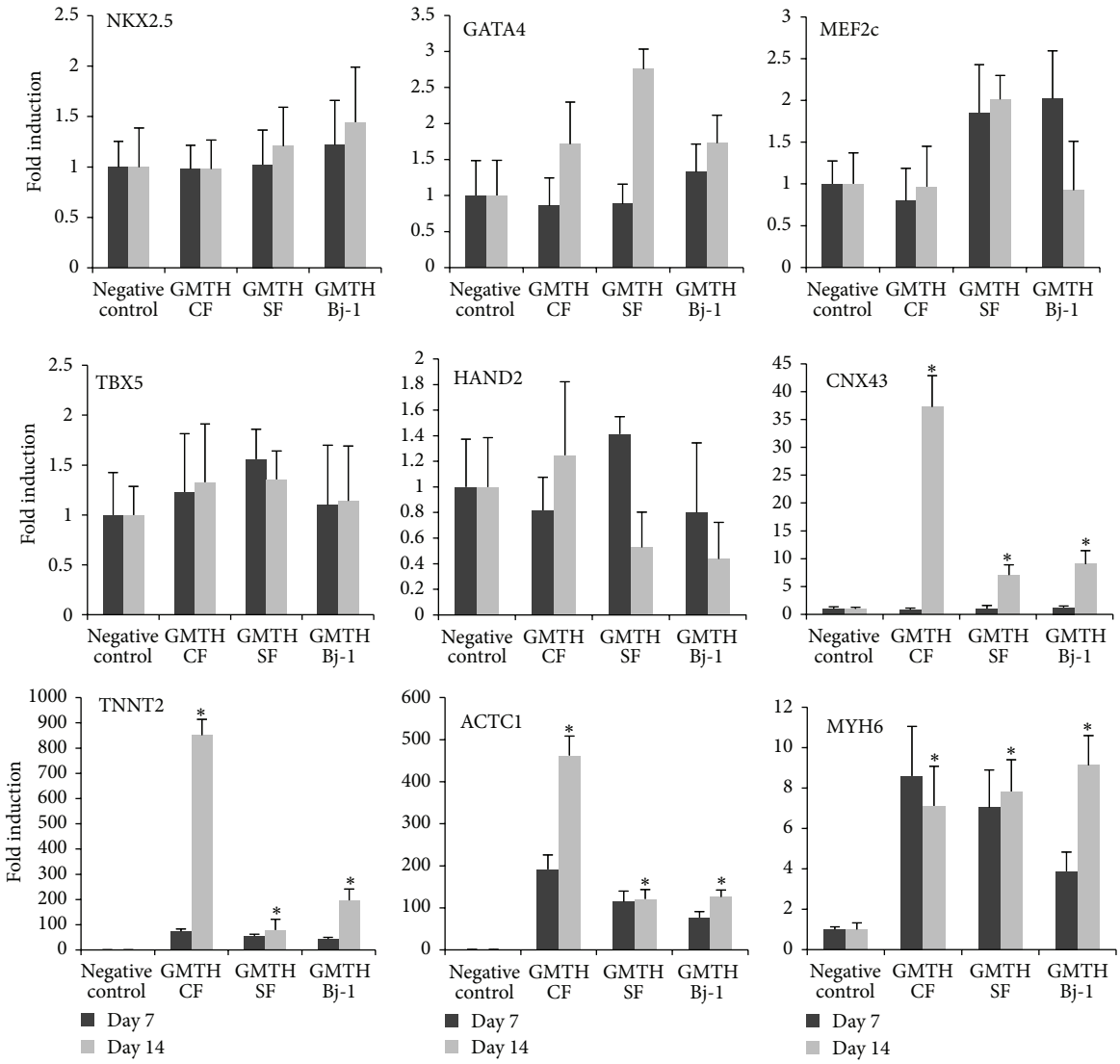

Figure 3: Characterization of GHMT transduced cells. qRT-PCR was performed at day 7 and at day 14 from transduction. Early endogenous cardiac marker genes (Nkx2.5, Gata4, and Mef2c-Tbx5-hand2) and late cardiac marker genes (CNX43, TNNT2- ACTC1 and MYH6) were analysed. Fold induction of mRNA expression in transduced cells compared to the controls (cells transduced only with α-MHC-GFP vector). The expression of late cardiac markers identified canine-induced cardiac-like myocytes (ciCLMs) in the transduced cell populations; $n = 5$, 2-way ANOVA test, $^{*}p < 0.01$.

ciCLMs at day 30 compared to day 14 and indeed no beating cells were detected.

3.4. Xenotransplantation of ciCLMs in SCID/Beige Mice. In order to evaluate the ability to contribute to the heart development, we set up a xenotransplant model of the GHMT cells into developing heart of 1-day-old SCID/Beige pups, as previously reported [32]. GFP positive cells were sorted after 7 days from GHMT transduction (Figure 6(a)) and 50.000 cells of each cell type were injected directly in the heart of 12 pups. Three weeks after the injection, mice were sacrificed and hearts were explanted and immunostained for GFP and dystrophin. Immunofluorescence analysis revealed that ciCLMs were able to engraft in the growing heart as shown in Figure 6(b). Interestingly, we found that ciCLMs generated from CF have a higher engraftment capacity (Figures 6(c) and 6(d), left panel) compared to those generated from SF. On the contrary, injected GFP positive CF cells, used as negative control, were confined in the interstitial area (Figure 6(d), right panel).

4. Discussion

Duchenne and Becker muscular dystrophies are a group of inherited diseases that affect both skeletal and cardiac muscles. In particular, adolescence patients tend to develop dilated cardiomyopathy that could result in arrhythmia, fatigue, shortness of breath, and swelling of the legs and feet, and that could lead to heart failure and sudden death [33].

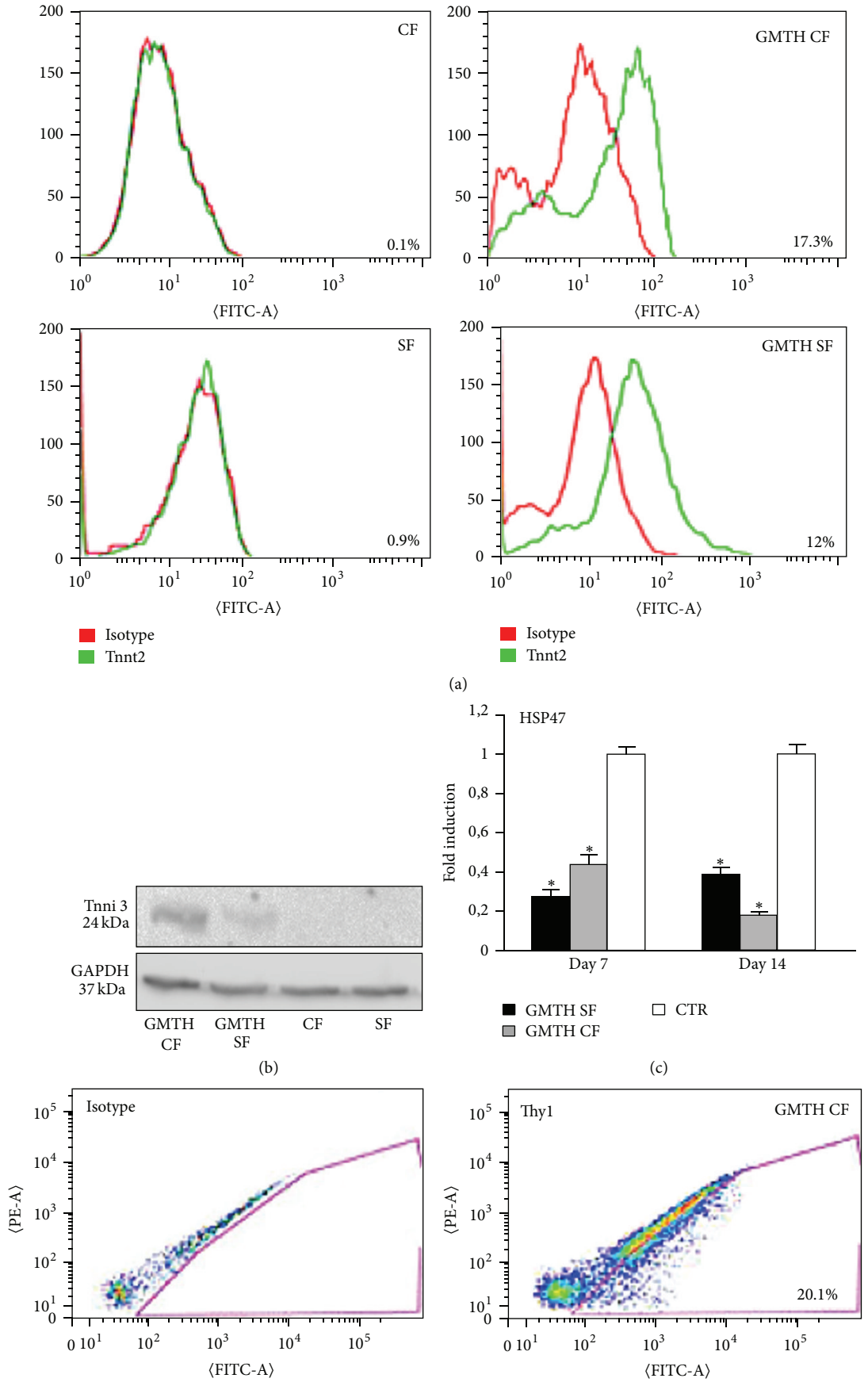

Figure 4: Continued.

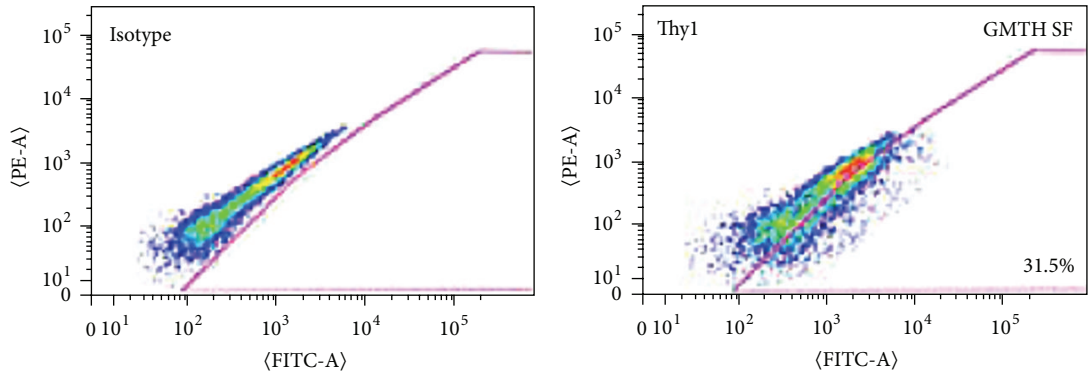

Figure 4: Expression of cardiac and fibroblast markers in ciCLMs. (a) Examples of intracellular FACS analysis for the isolation of Tnnt2+ cells (green) and isotype controls (red) in ciCLMs after 14 days of differentiation. (b) Example of Western blot analysis after 14 days of differentiation showed the presence of cardiac troponin I as a late cardiac marker protein in ciCLMs derived from both GHMT CF and GHMT SF cells. Densitometry quantification performed with ImageJ software showed that Tnni3 was 3,2-fold more expressed in GHMT CF compared to GTMH SF. Troponin I was absent in CF and SF transduced with α-MHC-GFP vector. (c) Expression of the fibroblast marker HSP47, in transduced cells compared to control cells (transduced with only α-MHC-GFP vector). HSP47 expression strongly decreases in ciCLMs after 7 and 14 days of differentiation compared to the controls; $n = 5$, 2-way ANOVA test, $^{*}p < 0.01$. (d) Examples of FACS analysis on ciCLMs confirmed a reduced number of Thy1 (CD90) + cells after 7 days of differentiation.

Cardiac fibroblasts (CFs) are involved in the healing and remodeling process of the heart resulting in scar tissue formation with limited contractile properties. A possible therapeutic approach would be to convert CFs into functional cardiac myocytes. Indeed several studies showed the possibility to generate cardiomyocytes by direct reprograming with specific transcription factors [10–14]. In this context, we isolated cardiac and skin fibroblasts from GRMD dogs, a large animal model for Duchenne muscular dystrophy, and attempted to reprogram them into canine-induced cardiac-like myocytes (ciCLMs) using a conversion protocol recently described [6, 14]. Our results showed that the overexpression of four cardiac transcription factors (GHMT) is sufficient to convert cardiac and skin canine fibroblasts into ciCLMs. In order to generate a reliable protocol, we employed cell-sorting technology to obtain CD90+ cells as a starting cell population from fresh isolated cells. This cell population was up to 100% positive for Vimentin and HSP47. As already described in literature [34], the healthiness and freshness of the starting population represent a critical step for obtaining good rate and quality of conversion to ciCLMs. After isolation, cells were highly proliferative as demonstrated by cell growth curves and the Ki67 staining. However, after some passages in culture, some signs of senescence were observed. To preserve proliferative potential, cells were expanded for 10 days after isolation and then frozen for later reprogramming experiments. In our study, we included Bj-1 fibroblast cells as controls and they showed comparable conversion efficiency with canine fibroblasts. Nevertheless, the use of human transcription factors could represent a further limitation in our reprogramming method. GFP+ cells were already detectable 48 hours after transduction with GHMT and αMHC-GFP and at 14 days cells become flattened and binucleated, expressing late cardiac markers such as CNX43, TNNT2, ACTC1 and MYH6. Early endogenous cardiac genes did not show evident modulation during the reprograming protocol, highlighting the fact that cardiac differentiation occurred differently from cardiac embryonic development. In all our attempts, no beating cells were detected from cells subjected to the direct conversion protocol as previously reported for human fibroblasts [35]. We speculate that this could be due to two different issues in our direct reprogramming protocol. First, we used human transcription factor cDNA, although the human transcription factor sequences used for transduction are well conserved among mammalian species and the similarity between canine and human sequences is between 96% and 98%. Second it is well recognized that isolated cells from newborn pups retain a higher plasticity and regeneration potential compared with cell derived from adult animals. However, in our experiments we used fibroblasts isolated from skin and heart biopsies of adult deceased GRMD dogs, since we could not obtain cardiac fibroblasts from pups due to ethical restrictions for large animal models. We speculate that those limitations could have affected the efficiency of our reprogramming protocol. Another important issue emerging in the recent literature is the stoichiometric ratio of reprogramming factors.

Indeed, using a mixture of viruses expressing individual factors has heterogeneous and unmanageable ratio of reprogramming factor expression among infected fibroblasts, which results in variable and low reprogramming effectiveness.

To further investigate the conversion potential efficiency of canine fibroblasts subjected to reprogramming, we analysed the expression of late cardiac markers by qPCR, the percentage of Tnnt2+ cells via FACS, and quantified Tnni3 protein via Western blot. Interestingly our results showed for the first time that GRMD canine cardiac fibroblasts

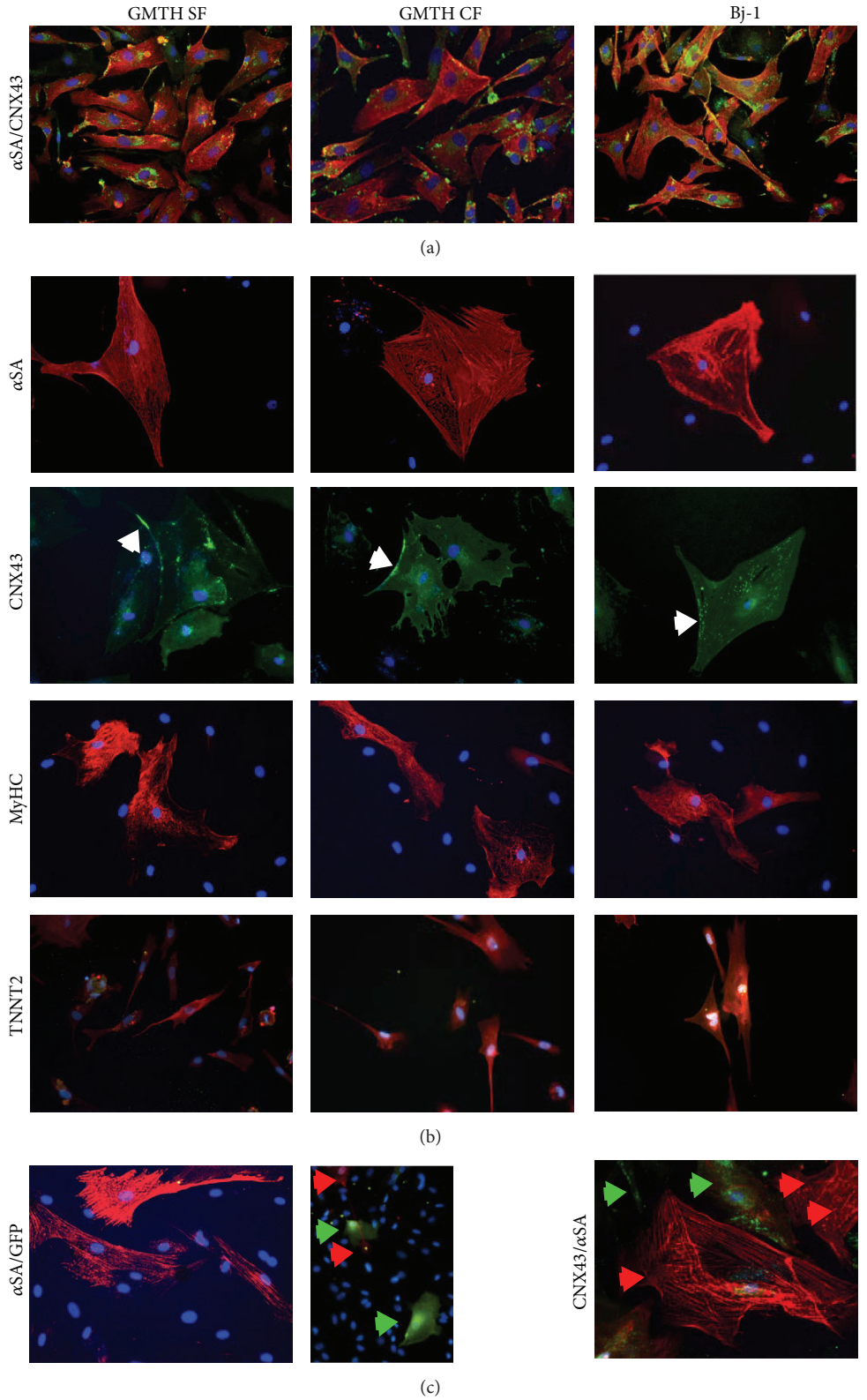

Figure 5: Localization of cardiac markers in ciCLMs. (a) Alpha sarcomeric actinin (red) and connexin 43 (green) were present in ciCLMs in similar amount to reprogrammed Bj-cells. (b) Immunofluorescence analysis revealed the expression of alpha sarcomeric actinin (αSA, red), connexin 43 (CNX43, green; white arrows indicate CNX43 expression on the cell membrane), myosin heavy chain (MyHC, red), and cardiac troponin T (red) in ciCLMs after 14 days under differentiating conditions. (c) ciCLM cells did not express cardiac markers homogenously. A few cells expressing αSA were GFP negative and vice versa (left panels) and occasionally CNX43 (green arrows) and αSA (red arrows) were mutually exclusively expressed (right panel).

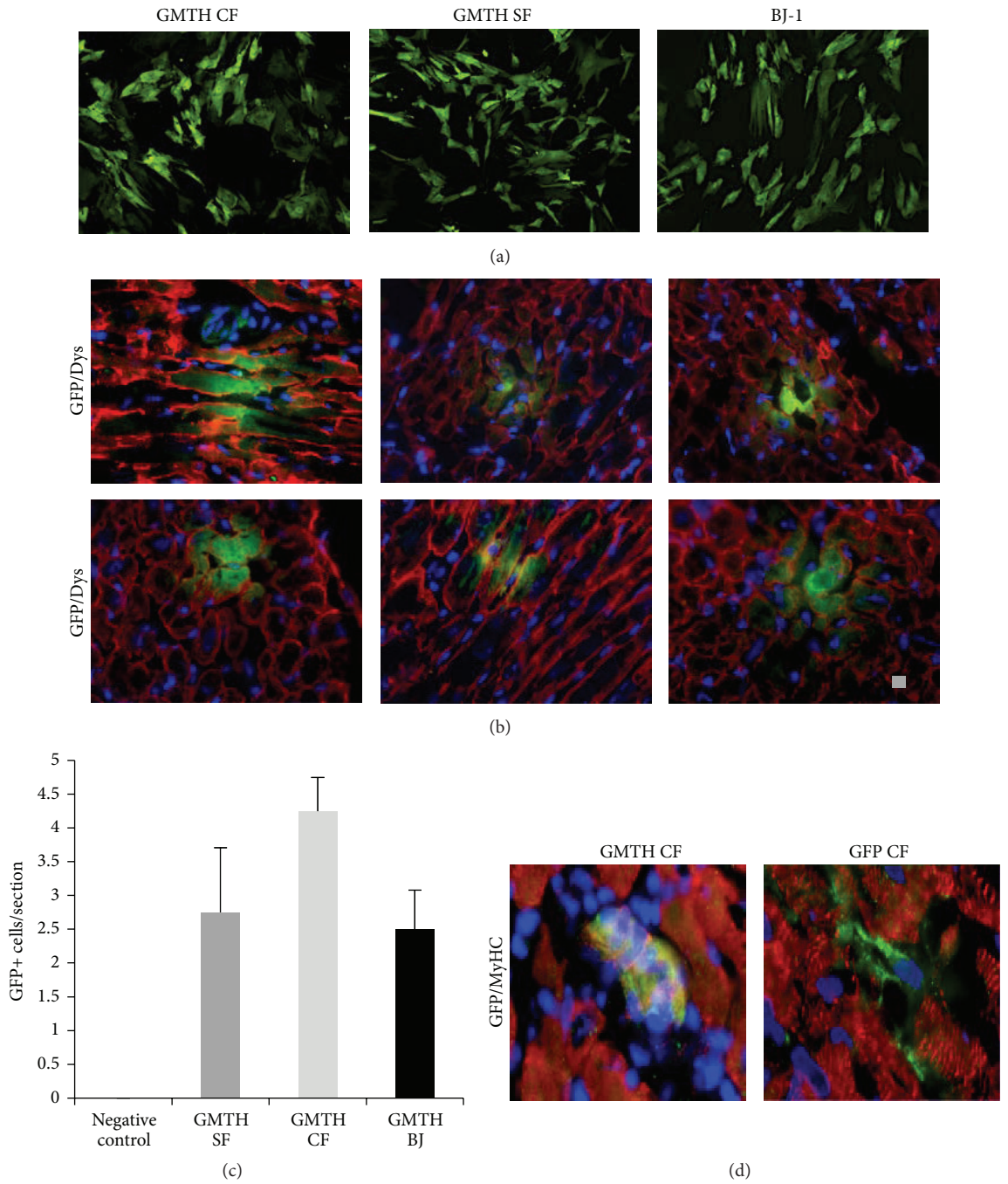

Figure 6: ciCLMs *in vivo* injection. (a) Immunofluorescence analysis for GFP expression ciCLMs after cell sorting for GFP and before injections. (b) 2×10^5 GFP positive cells were injected directly in the heart of 2-day-old pups. After 14 days pups were sacrificed and heart sections were prepared and double stained with antibodies against dystrophin (red) and GFP (green). (c) Average number of ciCLMs GFP positive cells per section; $n = 3$. (d) Double staining with antibodies against myosin heavy chain (red) and GFP (green).

have higher conversion ability to ciCLM cells compared to skin and dermal human fibroblasts. Indeed, qPCR analysis showed that GHMT CFs expressed higher level of CNX43, TNNT2 and ACTC1 after 14 days of cardiac differentiation. In addition, 17.3% of GHMT CFs and 12% of GHMT SFs were positive for Tnnt2. Densitometry quantification showed that Tnni3 was 3,2-fold more expressed in GHMT CF compared to GTMH SF. Furthermore, both GHMT CF and GTMH SF downregulated the fibroblast markers, HSP47 and CD90, after 14 days of conversion. Consistent with literature, IF analysis revealed that ciCLM cells do not express all the cardiac markers homogenously, since they could represent a spectrum of cardiac differentiation [10].

Finally, sorted GFP+ ciCLM cells at day 7 of conversion, injected into the heart of SCID/Beige newborn pups, were able to engraft the developing heart and expressed dystrophin

and MyhC. On the contrary, injected GFP-CFs remain confined in the interstitial area. Although the mechanisms and stoichiometric effects in cell fate specification are still largely unknown, this latter result highlights that GHMT forced expression is sufficient to generate cardiac progenitors from somatic adult canine cells able to integrate into the cardiac niche.

5. Conclusions

In this study we provided evidences that canine fibroblasts are converted to immature cardiac myocytes (ciCLMs) with the GHMT reprogramming protocol. We showed that ciCLMs express cardiac late markers and ability to engraft in a developing heart in xenotransplantation experiment. Moreover, we found that fibroblasts isolated from cardiac tissue are more prone to cardiac conversion than skin fibroblasts, suggesting that an epigenetic regulation shapes cardiac cell fate. Polycistronic constructs to express homogenously each factor as recently reported for human fibroblasts [36] could improve this methodology to obtain a large number of canine ciCLMs.

Nonstandard Abbreviations and Acronyms

αSA:	α-sarcomeric actinin
Actc1:	α-cardiac actin
Bdn^r:	Blasticidin resistance gene
ciCLMs:	Canine-induced cardiac-like myocytes
CF:	Cardiac fibroblasts
DMD:	Duchenne muscular dystrophy
DMEM:	Dulbecco's modified eagle medium
Ki67:	Antigen identified by monoclonal antibody Ki67
FACS:	Fluorescent activated cell sorting
FBS:	Fetal bovine serum
GATA4:	GATA binding protein 4
GMT:	Mix of 3 transcription factors, GATA4, MEF2C, and TBX5
GHMT:	Mix of 4 transcription factors, GATA4, HAND2, MEF2C, and TBX5
GFP:	Green fluorescent protein
GRMD:	Golden retriever muscular dystrophy
HS:	Horse serum
Hygro^r:	Hygromycin resistance gene
IF:	Immunofluorescence
IM:	Induction medium
MDs:	Muscular dystrophies
MEF2C:	Myocyte enhancer factor type 2C
MYH6:	Cardiac myosin heavy chain α
MyoD:	Myogenic differentiation
Nkx2.5:	NK2 transcription factor related, locus 5
qPCR:	Quantitative polymerase chain reaction
SCID:	Severe combined immunodeficient disease
SFs:	Skin fibroblasts
TBX5:	T-box transcription factor
TnnI3:	Cardiac troponin I
WB:	Western blot
Zeo^r:	Zeocin resistance gene.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Epigenetics and Shared Molecular Processes in the Regeneration of Complex Structures

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The ability to regenerate complex structures is broadly represented in both plant and animal kingdoms. Although regenerative abilities vary significantly amongst metazoans, cumulative studies have identified cellular events that are broadly observed during regenerative events. For example, structural damage is recognized and wound healing initiated upon injury, which is followed by programmed cell death in the vicinity of damaged tissue and a burst in proliferation of progenitor cells. Sustained proliferation and localization of progenitor cells to site of injury give rise to an assembly of differentiating cells known as the regeneration blastema, which fosters the development of new tissue. Finally, preexisting tissue rearranges and integrates with newly differentiated cells to restore proportionality and function. While heterogeneity exists in the basic processes displayed during regenerative events in different species—most notably the cellular source contributing to formation of new tissue—activation of conserved molecular pathways is imperative for proper regulation of cells during regeneration. Perhaps the most fundamental of such molecular processes entails chromatin rearrangements, which prime large changes in gene expression required for differentiation and/or dedifferentiation of progenitor cells. This review provides an overview of known contributions to regenerative processes by noncoding RNAs and chromatin-modifying enzymes involved in epigenetic regulation.

1. Introduction

Aristotle was captivated by the observation that lizards were capable of regrowing a tail after having it cut [1]. Regeneration—the ability to redevelop lost body parts—has been displayed in myths and folktales for centuries. Today, accumulating evidence shows that regenerative events that may seem fictitious are a reality in a wide range of organisms, from unicellular ciliates to large plants and animals (Figure 1). The regenerative capacities of different organisms vary immensely, as some are restricted to specific tissues or periods of time during development (e.g., the *Xenopus* tadpole tail), while others are capable of regenerating their entirety over uncountable occasions (e.g., planarian flatworms) [2, 3]. The mechanisms involved in regeneration have mystified observers throughout history and left them wondering whether a cellular permit forgiving the loss of a limb or an eye could be uncovered and shared with us, the unlucky humans who seem obligated to get through life with only one set of body parts.

Over 300 years ago, the famous French entomologist René-Antoine Ferchault de Réaumur reported detailed observations of crayfish claw regeneration [4]. Réaumur's detailed accounting of the regenerative process is often credited for creating awareness about this topic amongst the scientific community. Since, descriptions of regeneration events in vertebrates have been reported widely, ranging from limbs, tails, and retinas of Urodele amphibians (i.e., newts and salamanders) [5–10] to hearts and fins of fish [11, 12], deer antlers [13], and skin of spiny mice [14]. The analysis of cellular and molecular mechanisms involved in natural regenerative phenomena is of great interest to improve medical applications for replacement of lost or damaged tissue in humans.

2. Mechanistic Similarities of Regeneration Processes

Even though the study of vertebrates and crustaceans has uncovered regenerative capabilities that surpass the expectations of past and present scientists, their capacity for

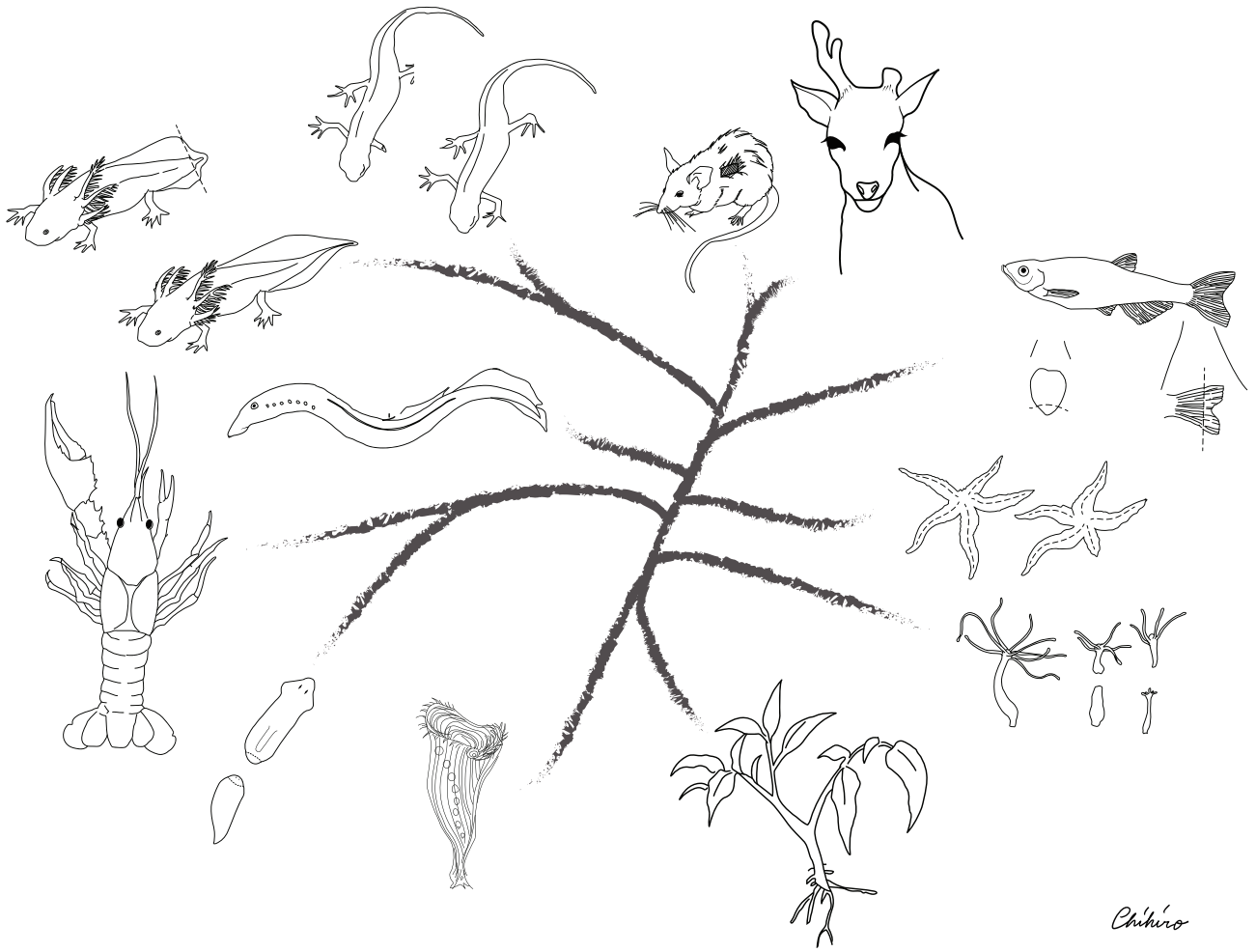

Figure 1: Phylogenetic distribution of regenerative organisms. Regenerative abilities tend to decline as complexity increases through evolution. For instance, *Hydra* and planarians can regenerate their whole bodies, whereas regeneration in deer or African spiny mice is limited to certain parts of their body such as antlers or skin, respectably. The following representatives from different phyla are illustrated: plants, *Stentor* (Ciliophora), *Hydra* (Cnidaria), planarian (Platyhelminthes), crayfish (Crustacea), starfish (Echinodermata), lamprey, fish, axolotl, and newt (Urodela), as well as deer and spiny mouse (Mammalia). Phylogenetic distances and organisms are not drawn to scale. Illustration contributed by Chihiro Uchiyama Tasaki.

regeneration remains relatively modest when compared to a collection of invertebrates that rely (at least partially) on asexual reproduction. Freshwater organisms belonging to the genus *Hydra* (named after the mythological multi-headed monster futilely decapitated by Hercules) can reproduce asexually through "budding," which involves the development and detachment of an individual from somatic tissue of the "parent." Similarly, planarian flatworms can reproduce asexually through "fission," which involves separation of a tail piece from the body of the "parent" followed by regeneration of missing structures by both anterior and posterior fragments. These organisms are not only able to develop their entire anatomy from somatic tissue during asexual reproduction but also capable of regenerating their entire body from a small piece of tissue upon injury. Slicing a planarian into 20 different fragments could result in the formation of 20 completely functional descendents. Early reports describing the regenerative potential of these organisms [15, 16] were followed by decades of experimental investigation based on amputations, dissections, transplantations, and microscopic analyses. Ultimately, these studies were the foundation of current investigations using modern molecular techniques to identify the genes and cellular processes that drive regeneration [17–19]. The revival of regeneration research in the era of molecular genomics, RNA-interference, and modern microscopy has resulted in detailed experimental accounts of the regenerative processes in a wide range of organisms. Altogether, these studies have illustrated fundamental mechanistic commonalities and differences involved in regeneration of complex structures; a few of these are detailed below.

2.1. Distalization Followed by Intercalation. Agata et al. (2007) proposed that a common phenomenon shared amongst complex regenerative events, be it a newt limb or the entire head of a polyp or a planarian, was the initiation of regenerative

deployment by establishment of the most distal structure first (distalization) followed by a subsequent expansion of the structures in between (intercalation) [20]. This view contrasts with previous models in which the regenerative process was thought to take place as a progression from proximal to distal, akin to mason laying bricks to build a wall. Normally, complex tissue regeneration establishes the identity of the furthest end of the missing tissue and gradually develops the regions in between. Although perplexing at first sight, distalization and intercalation seem logical if one considers that embryonic development constitutes a continuously morphing and moving mass of cells that follow signaling gradients and not a linear progression from one end to the organism to the other. Regeneration does not reinvent development; it applies preexisting mechanisms utilized during embryogenesis.

2.2. Programmed Cell Death and Cellular Proliferation. Analyses of the initial events that follow tissue loss and wound healing in flies, planarians, frogs, and mice have revealed that signals released by dying cells induce a proliferative response in progenitor cells of regenerating tissue [21]. There are two major modes by which cells die: "necrosis," which occurs when cells are exposed to unusual conditions or ruptured, and "apoptosis," in which the cell actively participates in its own demise. It is still unclear whether necrotic cells that arise from tissue damage release any molecules that specifically induce downstream regenerative events. On the other hand, studies in varied regenerative contexts support that a burst in apoptosis occurs following tissue amputation [21]. Apoptotic cells near the wound site release signaling factors (e.g., Wnt3 in *Hydra*; [22]) that induce the increased proliferation of progenitor cells that are needed to support the redevelopment of missing tissue. Apoptosis also plays a role in later steps of the regenerative process, during which preexisting tissue rearrangement guides the functional connection and proportionality of new and old parts of the organism [23].

2.3. The Futile Search for the "Regeneration Gene". It may seem unsatisfactory to propose that cellular events that occur during regeneration are not exclusive to this phenomenon. Wound healing is a common process that occurs in regenerative and nonregenerative tissue. Growing limbs, retinas, or heads are events that take place during normal embryonic development. The surprise is that so many organisms are capable of replicating embryonic processes as adults by reactivating developmental genetic pathways within the context of differentiated, previously grown tissue. So what then is the secret to regeneration? One key component is the availability of proliferative cells with the potential to differentiate into the cellular makeup of the missing tissue, whether these are obtained from reservoirs of stem cells or reactivation and reprogramming of differentiated cells in response to injury (see below). At the same time, a wealthy accumulation of stem cells does not guarantee that regeneration. This concept is beautifully demonstrated recently in studies of planarians with decreased regenerative capabilities from three different continents [24–26]. These studies showed that the evolutionary loss of head regeneration observed in some planarian species was not due to insufficient populations of stem cells, but by differences in expression levels of components in the conserved Wnt/β-catenin developmental pathway. The influence of the Wnt/β-catenin pathway on regeneration is not unique to invertebrates such as planarians and hydra; this pathway also controls digit regeneration in mice [27]. Another developmental signaling circuit that can dictate mammalian digit regeneration outcomes is the Noggin/Bone Morphogenic Protein (BMP) pathway. Yu et al. (2010) demonstrated that Noggin inhibits capable digit regeneration, whereas the fate of nonregenerating amputation wounds was reversed by BMP treatments that reinitiate digit tip development at the wound [28]. The identity of the signals driving regeneration after wound healing may vary throughout evolution, as long as activating proliferation and differentiation of progenitors in the correct spatiotemporal context is achieved.

2.4. "Stemness" and Cellular Sources for Regeneration. The presence of regenerative abilities in a wide range of organisms distributed throughout the animal kingdom suggests the evolutionary conservation of mechanisms involved in regeneration [29, 30]. A difference that has become apparent amongst the mechanisms that drive regeneration in different organisms is the source of cells used when redevelopment of missing tissue is needed. "Simpler" organisms such as *Hydra*, planarians, acoels, sponges, and plants rely on reservoirs of somatic stem cells classified as pluripotent, or highly multipotent, which continuously proliferate and differentiate to provide for regeneration, growth, and homeostatic maintenance. On the other hand, regenerative events in more complex organisms, such as regeneration of a vertebrate limb, heart, or retina, depend on cells with limited lineage differentiation potential, which often arise from dormant or dedifferentiated cells [31]. Humans continuously repair their intestinal epithelium through use of small reservoirs of intestinal stem cells that continuously proliferate and differentiate into a handful of epithelial cell types [32]. These cells, however, would be expected to fail at restoring damaged tissue in the heart or kidney, due to their limited potency. On the other hand, amphibian limb regeneration does not rely on reservoirs of stem cells, but rather on the partial reprogramming and proliferation of cells with restricted identities (e.g., muscle cells come from muscle) [33]. Surprisingly, the cell-type within each tissue that serves as the source for specific tissues during limb regeneration varies even in closely related species of salamanders [34]. These observations suggest that regenerative constraints are not established by the absence of stem cells or a specific cell type and that there are different possible avenues to achieve regeneration of complex structures.

In summary, regeneration of complex structures depends on the ability of cells to undergo significant changes in proliferative activity followed by activation of specific differentiation programs. In some cases, proliferation and differentiation are preceded by partial trans- or dedifferentiation. Each of these processes requires large changes in gene expression profiles, which could be facilitated by extensive changes in

chromatin structure. Consequently, one would predict that epigenetic regulators have broad—and possibly conserved—contributions to regenerative phenomena. The remainder of this short review describes recent evidence for involvement of epigenetic regulation during regenerative events observed in different phyla.

3. Observations of Epigenetic Regulation in Regenerative Processes

Much effort has been invested in describing the contributions of epigenetic regulators to maintenance and differentiation of stem cells *in vitro*. Regeneration is the ultimate stage to analyze the molecular mechanisms that regulate adult cell "stemness" and differentiation. Recent evidence describing the contributions of three major modes of epigenetic regulators to regenerative phenomena is described below.

3.1. DNA Methylation. An abundant epigenetic tag in plant and vertebrate genomes is the methylated form of the DNA base cytosine known as 5-methylcytosine. This modification occurs within CpG dinucleotide repeats and has a role in silencing gene expression by blocking access of transcription factors and other proteins to DNA [35]. Although detection of this epigenetic mark has been negligible in the genomes of yeast, nematode, fly, and flatworm model systems [36, 37], it is abundant in algae, moss, plants, and vertebrates, as well as in *Ciona*, honeybees, and beetles [36, 38, 39]. The enzymes responsible for deposition of methyl groups on the C-5 position of cytosine are known as DNA methyltransferases (DNMTs). Homologs of DNMT3 are responsible for establishment of *de novo* DNA methylation patterns, whereas DNMT1 homologs reiterate such molecular arrangements overtime [40].

Plants retain profound regenerative abilities that have proven advantageous not only to survive damage in their natural habitats but also in their propagation from "cuttings" by horticulturists, as well as production of whole plants from cultured transgenic cells in biotechnology and agriculture. Initial observations of epigenetic regulation during plant regeneration came from studies of a crown gall tumor line derived from Transfer DNA (T-DNA) delivery through Agrobacterium infection of tobacco plants [41]. Phenotypic variation was observed in clonal cell lines expressing different T-DNA transcripts, some of which appeared normal due to silenced T-DNA that was heavily methylated. More recently, it has been established that differences in cytosine methylation distribution are not limited to T-DNA but extend throughout the genome of plants regenerated from *in vitro* culture systems [42]. Additionally, changes in methylation and activity of corresponding loci have been reported in response to physical stress [43]. Although many of the methylated loci correspond to silenced transposable elements, there is clear evidence for regulation of cellular pathways crucial to the regenerative potential of plants via cytosine methylation. Such is the role of MET1, a DNA methyltransferase that modulates expression of factors involved in transduction of auxin signaling. This hormone is largely responsible for the regulation of plant regenerative potential [44].

Mammalian cells lines can be reprogrammed into stable induced pluripotent stem (iPS) cells through a process that involves gradual demethylation of important pluripotency loci [45, 46]. Incomplete loss of methylation during dedifferentiation contributes to somatic "memory" in iPS cells [47]. Interestingly, similar cellular "memory" is illustrated in various vertebrate regenerative events, such as the aforementioned example of axolotl limb amputation, where cells giving rise to the regenerating limb retain tissue identity (new muscle comes from old muscle cells; new skin comes from old skin cells) [33]. Thus, one would suspect that partial changes in DNA methylation patterns occur in dedifferentiating and differentiating cells during regeneration of specific tissues. Although comprehensive analyses of changes in DNA methylation signatures during vertebrate limb regeneration remain to be analyzed, it has been observed that activation of *shh* during limb regeneration (which is required for proper limb development) correlates with the DNA methylation status of a conserved enhancer region required for expression of *shh* in limbs [48]. Expression of *shh* during limb regeneration was found to correlate with methylation of the enhancer region known as Mammalian Fish Conserved Sequence 1 (MFCS1). Interestingly, MFCS1 was found to be hypermethylated in adult *Xenopus* limbs, when regenerative abilities are limited, but hypomethylated during developmental stages when full limb regeneration is possible [48]. Additionally, Yakushiji et al. (2007) showed that methylation of MFCS1 was low in adult limbs of amphibian species with high regenerative potential (i.e., axolotl and newt). Thus, it appears that the methylation status of crucial developmental genes may not only change to allow for regeneration, but, as this case suggests, it is rather the preestablished patterns of low methylation that may be crucial to allow flexibility and reactivation of important loci during regeneration. This idea is supported by studies in zebrafish, where Müller glia transition from quiescent supportive cells to progenitor cells for lens regeneration. It was observed that although genes encoding for DNA demethylation and methylation machinery are activated at early and later times during lens regeneration, respectively [49], it was also found that DNA methylation of promoters of genes encoding for important pluripotency factors is already low in quiescent Müller glia, suggesting a "primed" state for genes contributing to stemness during regeneration precursor cell reprogramming.

3.2. Histone Modification. The study of posttranslational modification of histone tails by methylation and acetylation may be the most general and extensive type of epigenetic regulation observed in eukaryotes. Regulation of gene expression through modification of histones is also tightly connected to other mechanisms of epigenetic regulation discussed in this review, since preceding DNA methylation and noncoding RNA targeting often result in silenced chromatin structures via histone methylation. Events influenced by factors involved in histone modifications have been identified in various regenerative contexts. Perhaps the earliest evidence for methylation of histones during regeneration came from observations during liver regeneration in rats, in which highest levels of histone methylation were observed after

cellular proliferation in response to amputation and likely in the process of new cell differentiation [50, 51].

The catalytic component of the Polycomb Repressive Complex 2 is encoded by *enhancer of zeste 2* (EZH2) orthologs, a family of methyltransferases that modify histone 3 at position Lysine 27 (H3K27me3) and mark important developmental loci for transcriptional repression [52]. This form of histone methylation regulates the differentiation of embryonic and adult stem cells in a stepwise fashion [53, 54]. H3K27me3 levels have been shown to decrease during zebrafish fin regeneration leading to reactivation of loci important for the regenerative process [55]. Similarly, global studies of chromatin showed that H3K27me3 was the only histone modification differentially established between cells of the ventral and dorsal iris during newt lens regeneration [56]. This is interesting because removal of the lens leads to an initial regenerative response through transdifferentiation from both dorsal and ventral irises; however ventral cells cease in their response and only cells from the dorsal iris end up contributing to regeneration [57]. Maki et al. (2010) showed that H3K27me3 stayed constant in cells of the dorsal iris but increased in the ventral iris, suggesting that increased silencing through the Polycomb Repressive Complex may inhibit contributions of cells from the ventral iris to lens regeneration [56]. A negative effect on regeneration by increased H3K27me3 methylation, more directly EZH2 activity, was reported in muscle regeneration in mice [58]. It was shown that inflammation-activated signaling in muscle satellite stem cells lead to increased PRC2-mediated inhibition of *Pax7* expression, a gene whose function is required for stem cell proliferation during muscle regeneration [58]. Similarly, EZH2 inhibition also increased transdifferentiation during imaginal disc regeneration in flies [59] and wound healing in mice [60]. Altogether, several lines of research show that the flexibility of precursor cells to display "stemness" in regenerative processes of different organisms depends on maintaining low levels of H3K27 methylation or, alternatively, increasing the activity of H3K27me3 demethylases [55, 61].

Planarian flatworms have become a fruitful model for identification of molecular mechanisms that take place during regeneration. Given their exceptional ability to undergo whole-body regeneration, as well as the experimental amenability for high-throughput analyses of gene expression and function in these organisms [17–19], it has become possible to test the contributions of numerous molecular pathways to the process of regeneration. Planarian regeneration is fueled by a large population of adult pluripotent stem cells, which are present in the mesenchyme throughout the life of these organisms. A number of recent reports have begun to uncover how chromatin regulation contributes to adult stem cell driven regeneration of the planarian body. Detection of histone modifications using commercial antibodies raised against well-conserved sequence epitopes from other organisms indicated the presence of H3K9K14ac, H3K4me2, and H3K9me3 in planarian cells [62]. Surveys of epigenetic histone modification enzymes by sequence conservation have identified dozens of putative chromatin regulators in the genome of the widely utilized planarian *Schmidtea mediterranea* [62–64]. From the identified enzymes related to chromatin regulation, a histone deacetylase homolog (HDAC-1) has been shown to be required for planarian regeneration and stem cell maintenance [63, 65]. Planarian homologs of the SET1/MLL family of histone methyltransferases, which catalyze H3K4 methylation, as well as members of the associated COMPASS and COMPASS-like complexes, have also been characterized [63]. SET1/MLL family homologs are expressed in both the stem cell population and differentiated planarian tissues [63]. Most importantly, inhibition of *set1* homolog expression was shown to lead to stem cell depletion and loss of regeneration [63]. Given the experimental ability to separate planarian stem cells and differentiated cells by fluorescence-activated cell sorting (FACS) [66], it will be interesting to identify loci with different patterns of histone marks between these cell types and during regeneration. It will also be intriguing to analyze how tinkering with the function of chromatin regulators affects the distribution of epigenetic modifications in the planarian genome and whether there is any conservation with loci targeted for regulation during regeneration of other genomes.

3.3. Noncoding RNA. It has become evident from work fueled by transcriptomic sequencing that the presence of regulatory noncoding RNAs (ncRNAs) is extensive in both eukaryotic and prokaryotic genomes. Some of the pioneer studies that demonstrated the importance of ncRNAs in regulating gene expression came from the analysis of 21–25 nucleotide (nt) length molecules known as microRNAs (miRNAs) crucial to nematode development [67, 68]. Although miRNAs work mainly at the posttranscriptional level by base-pairing with sequence at the 3′UTR of target mRNAs and blocking their translation, their discovery fueled sequencing expeditions that uncovered other types of ncRNAs. Three classes of ncRNAs shown to direct epigenetic regulation of chromatin include endogenous small-interfering RNAs (which are of ~21 nt length and are mainly found in plants), long (>200 nt) noncoding RNAs (lncRNAs), and PIWI-interacting RNAs (piRNAs) [69–72]. Of these, lncRNAs and piRNAs have surfaced as regulators in complex tissue regeneration contexts.

The identification of lncRNAs has increased substantially due to advances in Next-Generation RNA sequencing technologies. Thousands of lncRNAs are expressed in mammalian genomes, some of which have been shown to have tissue-specific, temporal, or disease-specific distribution [73, 74]. Although characterization of lncRNA functions has just begun, a few are thought to affect regeneration of skeletal muscle through possible epigenetic mechanisms. The *Dppa2* Upstream Binding Muscle lncRNA (Dum) is expressed in skeletal myoblast cells and promotes damage-induced muscle regeneration [75]. Dum was shown to work by repressing expression of the pluripotency factor *Dppa2* through recruitment of DNMTs [75]. Another lncRNA, the Myo-D Upstream Noncoding (MUNC) lncRNA, was shown to be required for murine muscle regeneration [76]. MUNC is transcribed from a region upstream of the Myo-D gene, but it was proposed to regulate promoters of other genes through mechanism yet to be identified [76]. As stated above, the characterization of lncRNA functions has just begun and there is already a strong

indication that this category of molecules will play important roles in regeneration.

The PIWI subfamily of proteins belongs to the ARGONAUTE/PIWI family and was first characterized for the ability of its members to silence transposable element gene expression in the germline of popular animal model organisms [77, 78]. PIWI proteins work in association with piRNAs (24–31 nt length) at the posttranscriptional level to destroy target mRNAs and also silence loci of homologous sequence through epigenetic mechanisms [77]. Although the vast majority of identified PIWI/piRNA targets contain transposon sequence, there are a few known genes with cellular and developmental functions regulated by this pathway [77, 79]. Interestingly, several groups have observed strong expression of PIWI homologs in stem and/or progenitor cells that contribute to regeneration of invertebrate species of exceptional regenerative capacities [80]. These include planarians [81–84], acoels [85], sponges [86], ctenophores [87], and hydra [88, 89]. These observations suggest that expression of PIWI homologs may be a characteristic shared with ancestral somatic stem cells [80, 90]. Given that genomic integrity of adult somatic stem cells is paramount to the homeostatic maintenance and asexual reproduction of many of these organisms, one would expect that expression of PIWI homologs is required to serve that function. However, several lines of evidence suggest that the function of the PIWI/piRNA pathway goes beyond protecting the genomic integrity of stem cells from transposable events. First, planarian flatworms subjected to PIWI homolog *smedwi-2* RNAi fail to regenerate regardless of stem cell availability, which suggests that SMEDWI-2 may play a role in differentiation [84]. Secondly, some have estimated that only 20–30% of sequenced planarian piRNAs comprise transposable element sequence [91], which raises obvious questions regarding the function of the 70–80% of remaining piRNAs. Finally, activation of expression of PIWI homologs has been observed during regeneration of vertebrate limbs [92] and mammalian liver [93], which suggests that PIWIs have functions outside of stem cells during regeneration.

How does the PIWI/piRNA pathway influence the process of regeneration outside its role in maintaining genomic stability? One possibility is that important specific mRNAs must be destroyed by PIWIs during processes of differentiation. However, a more appealing possibility is that epigenetic mechanisms guided by piRNAs mediate extensive changes in chromatin structure necessary for cellular reprogramming during regeneration. piRNAs direct epigenetic silencing through recruitment of DNA methylation, H3K9 methylation, and/or direct interactions between PIWI and Heterochromatin Protein 1 (HP1) [77]. Since piRNAs target hundreds to thousands of loci for epigenetic silencing throughout the genome, their effect on chromatin covers not only transposable element sequence loci and direct target loci but also may extend to neighboring genes. This approach not only makes piRNAs the center of a major mechanism for epigenetic reprogramming as recently proposed [94], it suggests an active role for transposable element sequences as docks for regulation in activity of large chromosomal regions.

Planarian flatworms represent a system in which PIWI function during regeneration has been (and continues to be) extensively studied [81–84]. Although DNA methylation has failed to be detected in planarians [37], both H3K9 methylation and a requirement for HP1 function in stem cells of these organisms have been reported [62, 64]. Additionally, piRNAs and PIWI proteins have been shown to be associated with sequences representing transposable elements and genes with indispensable cellular functions, such as those coding for core histones [81, 91, 95]. Interestingly, genes and pseudogenes with homology to histone sequence are represented by hundreds of loci in the genome of the planarian *Schmidtea mediterranea* [62, 81], which is comparable to numbers of loci representing transposable element sequences. Expression of histone genes shuts down quickly in the process of planarian stem cell differentiation, so it is possible that piRNAs targeting these transcripts are activated to destroy histone mRNAs and silence respective loci for successful reprogramming during differentiation. This is one mechanism by which PIWI proteins and piRNAs could contribute to major changes in differentiation/reprogramming of cells during regeneration.

4. Closing Statements

Advances in genomic sequencing, manipulation, and stem cell biology have reinvigorated the study of regeneration. Now more than ever we are able to learn about the different ways in which a multitude of organisms overcome loss of tissue. The study of regeneration not only reveals the secrets of this fascinating phenomenon, but it also uncovers developmental pathways of differentiation, molecules that influence the longevity and memory of cells, as well as the control of cellular proliferation. Uncovering the function of machineries of epigenetic regulation in the context of regeneration will demonstrate how changes in chromatin drive differentiation and dedifferentiation of stem and progenitor cells *in vivo*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

A Small RNA-Based Immune System Defends Germ Cells against Mobile Genetic Elements

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Transposons are mobile genetic elements that threaten the survival of species by destabilizing the germline genomes. Limiting the spread of these selfish elements is imperative. Germ cells employ specialized small regulatory RNA pathways to restrain transposon activity. PIWI proteins and Piwi-interacting RNAs (piRNAs) silence transposons at the transcriptional and posttranscriptional level with loss-of-function mutant animals universally exhibiting sterility often associated with germ cell defects. This short review aims to illustrate basic strategies of piRNA-guided defense against transposons. Mechanisms of piRNA silencing are most readily studied in *Drosophila melanogaster*, which serves as a model to delineate molecular concepts and as a reference for mammalian piRNA systems. PiRNA pathways utilize two major strategies to handle the challenges of transposon control: (1) the hard-wired molecular memory of prior transpositions enables recognition of mobile genetic elements and discriminates transposons from host genes; (2) a feed-forward adaptation mechanism shapes piRNA populations to selectively combat the immediate threat of transposon transcripts. In flies, maternally contributed PIWI-piRNA complexes bolster both of these lines of defense and ensure transgenerational immunity. While recent studies have provided a conceptual framework of what could be viewed as an ancient immune system, we are just beginning to appreciate its many molecular innovations.

1. Mobile Genetic Elements Threaten Genomic Integrity

Transposons are mobile genetic elements that can move into novel locations within the genome. These genetic parasites have long colonized large portions of all eukaryotic genomes [1]. Transposons are classified based on their movement strategies: retrotransposons move through a "copy and paste" mechanism, involving reverse transcription of initial RNA copies and consecutive insertion into novel genomic regions. This mechanism does not alter the original genomic insertion and results in amplification of the element. In contrast, DNA transposons employ a "cut and paste" mechanism. Transposition to a new genomic location leaves a gap at the donor site that upon repair either results in restoration or in loss of the original insertion [2–7]. While the journey of transposons is often neutral to the host, novel insertions can cause severe damage, or, in rare cases, beneficial changes.

During evolution, host genomes accumulated scars while eliminating deleterious insertions and selecting for advantageous mutations. Above all, genomes devised mechanisms to repress transposon activity. Germline genomes constitute a crucial battleground for the arms race between transposons and their hosts. To ensure vertical transfer and amplification of a mobile element, transposition has to take place in germ cells. Transposons continuously adapt to thrive in this particular environment and germ cells in turn have devised specialized strategies to guard their genomic integrity and thus the continuation of a species [6, 8]. With defense mechanisms in place, host genomes seem to have come to equilibrium with their parasites. Most current transposon insertions are defunct, representing defeated fragments rather than powerful insurgents [1, 9].

Tamed transposons become part of the host's evolutionary toolkit and serve as a rich source of coding and noncoding sequences that allow for genetic innovation [3, 4].

A prominent example of such domestication is Telomere reverse transcriptase, which probably evolved form an ancient retrotransposon [10]. In *Drosophila melanogaster*, retroelements themselves have colonized telomeric regions and directly maintain chromosomal ends without the need for an active telomerase [11, 12]. In addition to domestication of coding sequences, transposon fragments have shaped gene regulatory networks by providing an arsenal of noncoding building blocks [2, 13, 14]. Despite these important positive contributions that have been selected for during evolution, transposons are intrinsically selfish and their activity must be tamed or it will threaten the integrity of host genomes.

Controlling the activity of transposable elements presents two major challenges: (1) to recognize transposons as "nonself" and to (2) mount an efficient defense selectively against active elements. Recognizing transposons as "nonself", thereby discriminating them from host genes is not trivial. Transposons have become integral parts of our genomes and their mastery of camouflage enables them to hijack host machineries for transcription and translation. Additionally, their sequence diversity prevents recognition of specific motifs and their many mechanisms to transpose do not share vulnerable cofactors. In addition to direct genomic damage though transposition, transcription of certain elements can be toxic to the cell through the immense amount of produced RNA transcripts [15]. Adapting to the immediate threat of active transposons requires a prompt and specific defense. Small noncoding RNAs rise to the challenges posed by mobile genomic parasites [16–19]. Specialized small RNA pathways recognize transposons through the molecular memory of individual mobile sequences and have devised an elegant adaptive response against active elements.

2. Small RNA Pathways Are Prevalent in Eukaryotes

RNA interference (RNAi) was first observed as transgene cosuppression in plants [20]. Subsequent studies in *Caenorhabditis elegans* identified double-stranded RNA as the trigger of homology dependent gene silencing with corresponding small RNA products (~20–30 nt in length) serving as executive guides [21, 22]. Conserved small RNA pathways play crucial roles in development and disease [23–27]. These pathways can silence expression of target genes at transcriptional (i.e., recruiting histone and DNA methylation) or posttranscriptional level (i.e., promoting RNA degradation, inhibiting translation) [28–32]. At the heart of all RNA silencing pathways resides an RNA induced silencing complex (RISC), which in essence consists of a small noncoding RNA and its associated Argonaute protein partner [33, 34]. Within RISC, the small RNA determines target specificity by complementary base pairing, while its Argonaute partner governs effector mechanisms. Argonaute proteins are defined by a PAZ (Piwi-Argonaute-Zwille), a MID (middle), and a PIWI domain. The PAZ and MID domains specifically interact with the small RNA partner, anchoring its 3′ and 5′ termini, respectively [35]. The PIWI domain structurally resembles an RNase H fold and harbors RNA-guided endoribonuclease activity [36]. Phylogenetically Argonaute proteins segregate into two conserved subfamilies: the Ago-clade, similar to *Arabidopsis thaliana* AGO1, and the PIWI-clade, named after *Drosophila piwi* (P-element induced wimpy testis [37]) [38]. Members of the Ago-clade are ubiquitously expressed and associate with microRNAs (miRNAs) and small interfering RNAs (siRNAs). In contrast, the PIWI-clade is mostly restricted to germ cells in animals. PIWI proteins associate with a less well-understood class of small RNAs, piRNAs. PIWI-piRNA complexes silence transposons at the transcriptional and posttranscriptional level to guard the integrity of germline genomes [39–41].

3. Strategies of piRNA Pathways to Guard Genomic Integrity in *Drosophila melanogaster*

PiRNAs greatly differ from miRNAs and siRNAs in their biogenesis, Argonaute protein partners, and expression patterns [42, 43]. In contrast to miRNAs and siRNAs, biogenesis of piRNAs does not depend on the RNase III enzyme Dicer [44]. PiRNAs are thought to be processed from long single stranded precursors that get parsed into an army of small RNAs with the potential to cooperatively target individual transposons. Owing to their sequence diversity and the lack of unique molecular characteristics, piRNAs are best defined by their physical and functional association with PIWI proteins [44–48]. Mechanisms of piRNA biogenesis and effector functions are best understood in the female germline of *Drosophila melanogaster*. It should be noted that piRNA pathways also operate in the male germline of flies, but their mechanisms are less well characterized [49, 50]. Three PIWI proteins, Piwi, Aubergine (Aub), and Ago3, are expressed during oogenesis in *Drosophila* and associate with piRNAs to form piRNA-silencing complexes (piRISCs) (Figure 1). Aub- and Ago3-piRISCs reside in the cytoplasm to degrade transposon transcripts [45, 51, 52]. In contrast, Piwi-piRISC localizes to the nucleus and induces transcriptional silencing at transposon loci [53–55]. Loss of either *PIWI* gene results in sterility of the animals, presumably as a consequence of uncontrolled transposon activity in the germline [37, 56–60].

PiRNAs can be grouped into two classes that represent molecular answers to the two major challenges of transposon control: "nonself" discrimination and selective adaptation to an immediate threat. "Primary piRNAs" and their generative loci, so-called piRNA clusters, achieve "nonself" recognition through extensive genetic memory of individual transpositions. "Secondary piRNAs" defend against transposon activity through selective amplification of sequences that target transposon transcripts. Primary and secondary piRNAs require distinct processing enzymes and cofactors, but they function collaboratively to protect the integrity of germline genomes [32, 61–63].

3.1. Memory of Previous Transpositions Enables "Nonself" Recognition Guided by Primary piRNAs. Most primary piRNAs originate from a limited number of discrete genomic regions termed piRNA clusters that are defined as genomic

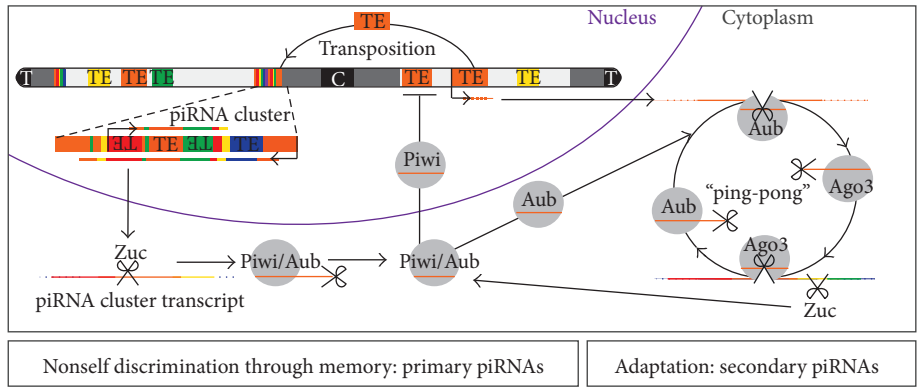

Figure 1: Mechanisms of "nonself" discrimination and adaptation in piRNA-guided defense against transposons in *Drosophila*. PiRNA clusters are genomic intervals that accumulate defunct fragments of transposable elements (TE) as a record of prior mobile activity. (1) Cluster regions are unidirectionally or bidirectionally transcribed and give rise to long presumably single stranded transcripts. PiRNA cluster transcripts are specifically processed into primary piRNAs by the consecutive action of at least two nucleases. The endonuclease Zucchini (Zuc) generates the 5′ terminus of a primary piRNA that is loaded into Piwi or Aubergine (Aub) and then trimmed by a 3′-5′ exonuclease. Piwi-piRNA complexes enter the nucleus and recruit chromatin modifying enzymes to silence transposon loci. In contrast, Aub-piRNA complexes initiate an adaptive response against transposon transcripts in cytoplasmic germ granules. (2) Aub and Ago3 engage in a feed-forward adaptation mechanism—the "ping-pong" cycle—that degrades transposon transcripts and concomitantly produces secondary piRNAs to selectively enhance the response against active elements. Through a feedback mechanism secondary piRNAs can initiate further Zuc-dependent piRNAs that are loaded into Piwi and Aub (Centromere (C), Telomere (T)).

intervals with a high density of uniquely mapping piRNAs [45] (Figure 1). Some of these ~140 genomic regions were previously linked to transposon control [64–67]. PiRNA clusters act as transposon traps, accumulating numerous transposon insertions over time and retaining a collection of densely packed defunct fragments. Insertion of novel sequences into piRNA clusters adds information to the repository, marks corresponding elements as "nonself" and confers resistance in trans [68, 69]. piRNA clusters are a fossil record of transposition activity, reflecting the mobile heritage of genomes and providing a molecular database for "nonself" recognition. How piRNA clusters are formed and how their transcripts are specifically marked for processing into piRNAs are major outstanding questions in the field.

Most piRNA clusters contain transposon insertions in mixed orientation and generate transcripts from both genomic strands. Transcriptional regulation of these dual-strand piRNA clusters differs from genic transcription in respect to interpretation of chromatin marks and cotranscriptional processing of nascent transcripts. In contrast to active genes, piRNA clusters preferentially reside in pericentric or subtelomeric regions that mark the boundaries of constitutive heterochromatin and euchromatin [70]. Cluster transcription requires recruitment of Rhino, a fast evolving heterochromatin protein 1 (HP1) family member, to trimethylated lysine 9 of histone H3 (H3K9me3), which otherwise typically marks silent genes [71–74]. Rhino associates with the adaptor protein Deadlock to recruit Cutoff, a homolog of the Rai1/Dom3Z decapping enzyme, which has lost its enzymatic activity. The Rhino-Deadlock-Cutoff complex is suggested to protect the 5′ end of nascent cluster transcripts and to suppress both canonical splicing and transcriptional termination [74–78]. Moreover, Rhino recruits the RNA helicase UAP56 to specify cluster transcripts for transport to their processing site [78]. Using a positive feed-back mechanism, Piwi-piRNA complexes themselves maintain transcriptional activity at cluster loci through recruitment of H3K9me3 [79–81].

PiRNA clusters give rise to long single-stranded transcripts without known structural or sequence determinants or significant formation of double-stranded RNA. These cluster transcripts are processed into a large body of diverse small RNAs by the consecutive action of at least two nucleases. An endoribonuclease generates the 5′ monophosphorylated end of a new piRNA that is consecutively loaded into PIWI's MID pocket. Consecutively, the 3′ end of the PIWI-bound pre-piRNA is trimmed by a 3′ to 5′ exonuclease. The endonucleolytic activity that likely generates the 5′ terminus of a primary piRNA can be assigned to the conserved nuclease Zucchini (Zuc) [82–84]. In contrast, the identity of the 3′ trimming exonuclease remains elusive [85]. The final length of a mature piRNA is marked by 2′-O-methylation and likely represents a footprint of its associated PIWI protein [85–87]. Little is known about the initial processing of piRNA cluster transcripts. While primary processing intermediates or degradation fragments have been observed, the precise mechanisms remain obscure [88]. Further characterization of piRNA cluster transcripts and identification of the factors that specify transcripts for processing into piRNAs are required to understand how hardwired memory of past transpositions is parsed into small RNA guides for "nonself" recognition.

3.2. An Adaptive Mechanism That Produces Secondary piRNAs Defends Selectively against Mobile Elements That Have Evaded Silencing at the Site of Transcription. PiRNAs engage in a feed-forward adaptive response to specifically eliminate cytoplasmic transposon transcripts and reinforce piRNA production. This robust strategy relies on removal of transposon transcripts through piRNA-guided cleavage

and simultaneous production of select secondary piRNAs [45, 51, 52]. In contrast to primary piRNAs, which represent all indexed transposons irrespective of their transcript abundance, secondary piRNAs shape the overall piRNA pool toward recognition of active elements. Biogenesis of secondary piRNAs relies on the nuclease activity of two PIWI proteins, Aub and Ago3, that collaborate in the so-called "ping-pong" cycle [45, 52]. Processing of secondary piRNAs is believed to be triggered by either primary or maternally contributed Aub-piRNAs. These piRNAs guide Aub to cleave complementary transposon transcripts. Target RNA cleavage by Aub generates the 5′ end of a new piRNA that is loaded into Ago3. Because Ago3-piRNAs originate from transposon transcripts themselves, they carry selective information about these potentially harmful active elements. Ago3-piRNAs in turn guide cleavage of complementary stretches within cluster transcripts to generate additional Aub-piRNAs and complete the "ping-pong" cycle [57] (Figure 1). Multiple rounds of selection alternate between transposon and cluster transcripts as substrates for secondary piRNA production and mold piRNA populations towards preferentially target active elements. During "ping-pong," transposon-triggered processing of cluster transcripts selects for sequences that are genetically determined as "nonself." This mechanism could act as a protective measure to prevent amplification of piRNAs that accidentally target host genes and thus could cause autoaggression, analogous to the phenomena of autoimmunity.

Coordination of this elegant adaptation mechanism is achieved in specialized perinuclear germ granules called Nuage [89]. Members of the Tudor protein family are key-components of these germ granules and play crucial roles in piRNA biology. Tudor proteins are defined by the presence of one or multiple Tudor domains that recognize methylated Arginine residues and facilitate protein interactions [90]. PIWI proteins are methylated at N-terminal Arginines and various Tudor proteins regulate interactions between PIWI proteins and cofactors to ensure efficient heterotypic processing of secondary piRNAs [91]. Dynamic orchestration of these interactions requires periodic remodeling of PIWI-RNA complexes by the DEAD box helicase Vasa, which chaperones the transfer of piRNA intermediates between PIWI "ping-pong" partners [92, 93].

Additionally, secondary piRNAs have the potential to trigger Zucchini-dependent processing of adjacent piRNA cluster regions into piRNAs that are loaded into Aub and Piwi. This feedback mechanism not only results in diversification of piRNA sequences, but also enables transmission of information from the adaptive defense to the transcriptional silencing machinery [94–96]. Additional genes involved in piRNA pathways emerged from genome-wide screens and further characterization of these factors will add to our mechanistic understanding of this elaborate small RNA based immunity [97–99].

3.3. Maternally Inherited PIWI-piRNA Complexes Bolster Trans-Generational Immunity. Genetic observations have long suggested the role for a maternally contributed factor in transposon control in *Drosophila*. Crosses of males carrying a specific transposon to females naïve to this element result in a loss of germ cells and sterility in the offspring. Interestingly, despite genetic identity, the reciprocal crosses do not exhibit defects in germ cell development. Germ cell defects in the dysgenic progeny are associated with mutations and chromosomal rearrangements attributed to unleashed transposon activity [100–105]. This phenomenon of hybrid dysgenesis suggests the requirement of nongenetic, maternally contributed factors in transposon defense [106, 107]. The nature of these factors remained mysterious until PIWI-piRNA complexes were identified as molecular determinants of maternal immunity [81]. Maternally contributed PIWI-piRNA complexes transmit immunity in two ways: (1) they reinforce memory through recruitment of H3K9me3 to piRNA clusters, thus bolstering noncanonical transcription at these loci, and (2) they directly initiate adaptive processing of secondary piRNAs through the "ping-pong" cycle [55, 80, 81, 108, 109].

4. Small RNA Pathways Guard Genomic Stability in Mammalian Germ Cells

Transposon sequences and active transposon families vary significantly between different species [110]. Considering coevolution between transposons and the host's defense system, adaptive variation in molecular mechanisms is expected. Yet, principles of RNA interference have seemingly proven efficient for defense. Like flies, mouse piRNA pathways employ three PIWI-like (PWIL) proteins: Piwil1/Miwi, Piwil2/Mili, and Piwil4/Miwi2 [46, 47, 111]. Removal of either PIWI gene results in germ cell defects accompanied by a burst in transposon activity in the male germline [112–115]. PiRNA pathways operate at two distinct stages during mouse spermatogenesis. In embryonic testes, Piwil2/Mili collaborates with Piwil4/Miwi2 to protect primordial germ cells. Similar to *Drosophila* piRNAs, mouse embryonic piRNAs mainly correspond to transposon sequences and engage in selective generation of secondary piRNAs in response to transposon transcripts. In contrast to flies, the "ping-pong" cycle in mouse involves the catalytic activity of only one PIWI partner, Piwil2/Mili [116]. Intra-Piwil2/Mili "ping-pong" generates secondary piRNAs that are loaded into Piwil2/Mili and Piwil4/Miwi2. Interaction with piRNAs licenses Piwil4/Miwi2 to transfer to the nucleus and silence transposon loci [113, 115, 116]. Recruitment of Piwil4/Miwi2-piRNA complexes results in histone and DNA methylation to full-length, potentially active transposon-loci [117, 118]. Interestingly, full-length transposons seem to evade a prior piRNA-independent wave of heterochromatinization in the embryonic gonad and require piRNA-guided control [117]. A second phase of piRNA silencing takes place in adult testis during entry into meiosis. Two cytoplasmic PIWI proteins, Piwil2/Mili and Piwil1/Miwi, are involved in this pathway. The functions and precise mechanisms of adult piRNAs remain enigmatic. Adult piRNAs mainly originate from relatively few intergenic clusters with little potential to target cellular transcripts but their own [111, 119]. Although

this class of piRNAs is not enriched in transposon sequences and does not exhibit "ping-pong" activity, loss of Piwil1/Miwi, Piwil2/Mili, or either nuclease activity results in derepression of transposons [120, 121]. How adult piRNAs guide transposon silencing without obvious enrichment in complementary sequences remains elusive. Recent studies suggest a function for these piRNAs in posttranscriptional regulation beyond transposon silencing [122–124].

Curiously, while piRNAs are present in mouse oocytes, they are not required for fertility. In the female germline of mice transposon defense relies on endogenous siRNAs [125, 126]. These siRNAs originate from dsRNA precursors that are generated by the repetitive nature of transposon sequences or antisense transcription at corresponding loci. Long dsRNA substrates are processed into ~21-nt long siRNAs by the RNase III enzyme Dicer and guide Ago2 to slice transposon transcripts. Transposon loci produce piRNAs and siRNAs in a seemingly redundant fashion in the developing oocyte. However, siRNAs dominate piRNAs in abundance and function. It is puzzling how and why mouse oocytes chose siRNAs over piRNAs in transposons control. An unexpected recent finding shed some light on this paradox: mice express an oocyte-specific isoform of Dicer—Dicer^{O}—that harbors enhanced activity on processing dsRNA into siRNAs [127]. Interestingly, transcription of Dicer^{O} is driven by an intronic transposon insertion. Deletion of this insertion causes loss of Dicer^{O} and female sterility and resembles a maternal Dicer null phenotype [128]. This observation emphasizes the tight relationship between transposons and their host even in establishing a transposon defense system. Transposon driven Dicer^{O} expression is specific to mice and rats. Thus, siRNA-mediated transposon silencing seems to be an exception rather than a rule in the mammalian germline, which prompts a reevaluation of piRNA function in the female germline of other mammals [129, 130].

5. Conclusion

Germ cells have devised small RNA mechanisms to master the challenges of transposon control. PiRNA clusters establish and maintain memory of "nonself" and generate mobile guides to induce an adaptive defense against active transposons. Maternally transmitted PIWI-piRNA complexes add an additional layer to transgenerational immunity against mobile genetic elements in flies. Over the past several years RNA-based immunity against genomic invaders has been discovered in all branches of life ranging from the CRISPR-Cas systems in bacteria to piRNAs in animals [18]. RNA-based strategies for "nonself" discrimination and adaptation have seemingly proven efficient to protect the integrity of genomes. Insights into the elegant mechanisms of these RNA-based immune systems will further our appreciation of the complex relationship between parasitic nucleic acids and their host genomes.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Epigenetic Control of Haematopoietic Stem Cell Aging and Its Clinical Implications

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Aging, chronic inflammation, and environmental insults play an important role in a number of disease processes through alterations of the epigenome. In this review we explore how age-related changes in the epigenetic landscape can affect heterogeneity within the haematopoietic stem cell (HSC) compartment and the deriving clinical implications.

1. Introduction

Aging is associated with alterations in the heterogeneous haematopoietic stem cell (HSC) compartment including changes in clonal composition and lineage contribution. Recent data shows that these changes in functional potential within the HSC population may be modulated by a drift in the epigenome that occurs with increasing age and may ultimately lead to a transcriptional change within the HSC pool. Here we describe the current state of knowledge of haematopoietic stem cell heterogeneity and its changes with age, discuss the evidence for changes in the epigenetic landscape as a potential driver, and propose a model by which these changes may explain some of the pathological consequences of aging.

2. Haematopoietic Stem Cell Heterogeneity

The haematopoietic system relies on a small population of HSCs resident in the bone marrow to generate ~10^{11} new cells every day. HSCs have the capacity of self-renewal and differentiation through a cascade of progressively committed and lineage restricted progenitors to ultimately generate all mature circulating myeloid and lymphoid cell types [1]. Originally it was thought that HSCs were a single homogenous cell population with the same proliferation and multipotent differentiation capability [2]. However in the last 10 years it has become clear that the HSC compartment is in fact made up of a number of subsets each distinguished by its own self-renewal capacity (long-term and short-term HSCs) [3–5] and lineage differentiation potential [6].

The first evidence of HSC compartment heterogeneity came from mouse spleen CFU assays; these showed a high degree of variability in numbers and types of colonies produced, challenging the idea of a single homogenous population. However, direct evidence on HSC heterogeneity came from methods that allow assessment of mature cell outputs from limiting numbers and even single HSCs [7–10]. These include the ability of purified single HSCs to repopulate a secondary myeloablated host and cellular barcoding whereby lentiviral gene transfer is used to uniquely label individual HSCs allowing their progenies to be tracked within the transplanted host. This work has led to HSC subsets being distinguished according to their mature cell output. The existence of analogous HSC subsets in humans has been suggested by evidence from therapeutic transplantation in the setting of β-thalassemia [11].

Although currently defined HSC subsets are able to produce all the mature cell progeny, the ratio of myeloid to lymphoid progeny varies markedly. HSC compartment

Table 1: Currently defined HSC subsets in mouse and their definition by mature cell output and cell surface markers.

HSC subset	Predominant mature cell population	Cell surface markers that have been used for prospective identification
Mouse HSC		Lin^- $Sca\text{-}1^+$ $cKit^+$ $CD150^+$ $CD48^-$ $CD34^-$
Myeloid-biased/lymphoid-deficient (α)	Myeloid	$+CD150^{High}$, $CD41^+$
Lymphoid-biased/myeloid-deficient (γ/δ)	Lymphoid	$+CD229^+$
Balanced (β)	Myeloid and lymphoid	
Platelet-biased	Platelet and myeloid	$+VWF^+$, $CD41^+$
Human HSC		Lin^- $CD34^+$ $CD38^-$ $CD90^+$ $CD45RA^-$ $CD49f^+$

Lin: lineage markers; VWF: von Willebrand factor.

subsets have been described as myeloid-biased, lymphoid-biased, or balanced [3, 12] or by others as lymphoid-deficient (α), myeloid-deficient (γ and δ), and balanced (β), respectively [13] (Table 1). While the majority of accumulated evidence comes from mouse models there is also support for myeloid-biased and lymphoid-biased HSC subsets in humans [14, 15]. While serial transplantation experiments are able to define HSC subsets by their progeny of mature cells *in vivo* there is not yet an effective method to prospectively distinguish HSC subsets at molecular level. An attempt to prospectively enrich the myeloid- and lymphoid-biased subsets has been made by defining these populations based on CD150 cell surface level. Myeloid-biased HSC subsets express higher levels of this surface marker compared to lymphoid-biased HSCs [16–18]. However, this is not considered an ideal marker as the cell populations separated are not pure and expression of CD150 changes when cells are manipulated and transplanted [19, 20]. Recently lymphoid-biased HSCs have been shown to have higher expression of the surface marker CD229 [21].

A further HSC subset recently identified is the megakaryocytic or platelet-biased HSC subset (Table 1). This has been prospectively defined by reporter gene expression and/or surface markers that are highly expressed in the megakaryocyte/platelet lineage: VWF [22] and CD41 (ITGA2B) [23]. Approximately 60% of mouse HSCs have been shown to coexpress VWF and when these are serially transplanted in limiting numbers they effect highly platelet-biased reconstitution [22]. Interestingly, this population also has a strong myeloid lineage bias whereas VWF-HSC contribution to the myeloid lineage is minimal [22]. It has therefore been suggested that previous studies that identified a myeloid-biased HSC subset [3, 12, 13] may have identified both platelet-biased and myeloid-biased HSCs in the absence of methods to evaluate platelet output. Furthermore, the platelet-biased HSC subset has been hierarchically placed at the apex of the haematopoietic tree, due to its ability to give rise to the lymphoid-biased HSC subset [22]. Similarly, a platelet- and myeloid-biased HSC subset has been identified by the expression of the megakaryocyte/platelet cell surface marker and part of the glycoprotein IIb/IIIa fibrinogen receptor: CD41, a population that may possibly be phenotypically analogous to that expressing VWF [23]. CD41 had been known to be expressed in embryonic HSCs but then switched off after birth [24–27]. Its expression has now been demonstrated on a subset of mouse HSCs which show a platelet and myeloid bias on serial transplantation with a knockdown of CD41 resulting in reduced levels of all mature blood cell lineages [23]. A platelet-biased HSC subset has not yet been defined in the human.

While the majority of evidence on HSC lineage commitment to date, including that presented here, derives from transplantation studies, transplantation creates an artificial environment that is limited by engraftment-associated inflammation. The use of novel *in situ* inducible labelling techniques has enabled the study of physiological haematopoiesis in a healthy bone marrow environment. Two recent mouse studies using this approach have proposed a model of haematopoiesis that while supporting data from transplantation studies suggest that classical long-term HSCs have a limited contribution to steady-state haematopoiesis [28, 29]. Rather HSC heterogeneity is produced by thousands of multipotent clones within a reservoir of cells traditionally defined as short-term HSCs and multipotent progenitors [29], which are shown to be longer-lived than previously thought and have considerable self-renewal capability. These novel techniques while still in their early stages are anticipated to provide further insight into haematopoietic lineage commitment in more physiological conditions.

3. Aging within the HSC Compartment

Multiple studies have established that aging, both in mouse and in human, leads to a myeloid-skewed haematopoietic system, with diminished representation of lymphoid cell populations and an increased representation of myeloid progenitors that has been shown to be associated with a myeloid-biased HSC population [17, 30–32]. Furthermore, it has been shown that serial transplantation of young mouse HSCs into young secondary hosts selectively expands a myeloid-biased HSC population independent of a nonaging microenvironment which suggests that HSC lineage bias is intrinsic to the cell itself [32]. In addition, there is also evidence in the mouse of an age-related increase in platelet-biased HSCs

defined by their expression of CD41. When transplanted these cells show a predominantly platelet- and myeloid-biased reconstitution, which suggests that the myeloid-biased HSC population seen in elderly mice may also be platelet-biased [23]. Although age-related changes in VWF+ HSCs have not been evaluated it could be speculated that this population would also expand with age since this subset leads to a platelet- and myeloid-biased reconstitution. This is supported by similarities in gene expression profiles between VWF+ HSCs and aged HSCs, both demonstrating significant upregulation of megakaryocyte-lineage genes such as *Selp* and *Clu* as well as the upregulation of *VWF* in aged HSCs [33]. Expansion of the VWF+ HSC population with age does not, however, support the hierarchical positioning of this population above the lymphoid-biased HSC subset as this population is known to decrease with age.

In humans, age-related haematopoietic changes include decreased bone marrow cellularity [34], attenuated lymphoid potential [35], increased incidence of myeloproliferative disorders and myeloid malignancies [36], and increased incidence of thrombosis [37]. As in mouse, these findings have been correlated with an accumulation of HSCs within the aged human bone marrow, which, while being able to generate both lymphoid and myeloid progeny in culture and in xenotransplant, showed significant myeloid skewing compared with young HSCs [14]. The existence of an age-related platelet-biased HSC subset in humans has not yet been investigated.

Postulated mechanisms that may lead to a decline in lymphoid differentiation with age include a gradual erosion of lymphopoietic potential within the HSC compartment over time, a conversion of lymphoid-biased to myeloid-biased HSCs, and a gradual dominance of myeloid-biased HSCs either due to their slower turnover leading to increased survival or a higher self-renewal capacity leading to clonal dominance with time [12, 38]. How these changes are controlled and regulated is still unclear. Aging within the HSC compartment has been shown to be associated with decreased functionality due to elevated levels of reactive oxygen species [39] and accumulation of DNA damage [40] which may account for some of the differences observed with age. It is clear, however, that these mechanisms do not account for all of the cellular and molecular attributes that are associated with aging of the HSC compartment, indicating that other mechanisms must be involved. There are a number of lines of evidence showing that HSC aging is transcriptionally regulated with differences in gene expression between young and aged HSC populations [14]. This suggests that alteration in gene expression by changes in the epigenetic landscape may play a key role in modulating age-related changes in the HSC compartment.

4. Epigenetic Regulation of HSC Aging

The term epigenetic encompasses all heritable changes in gene expression that are not due to changes in DNA sequence. These are modifications of the genome or of DNA-associated proteins. They include changes in DNA methylation, histone modifications, and changes in chromatin structure that impact on the accessibility of genetic loci for transcription machinery. Noncoding RNAs also play a critical role in epigenetic regulation. It is because of epigenetic regulation that a cell retains its identity and its gene expression profile through cell division and differentiation without altering its DNA sequence. However, epigenetic marks can also change over time [41–43] due to aging and environment, which may be related to mutations in epigenetic regulators, although the underlying molecular mechanisms are still unclear. It is this change in the epigenetic landscape of the HSC compartment that has been suggested to lead to age-related changes.

While the coding potential of the genome lies in the arrangement of the four nucleotides, additional information affecting phenotype is stored in the distribution of methylated cytosine (5-methylcytosine). DNA methylation occurs at CpG motifs that are interspersed within the genome in clusters called CpG islands. Densely methylated promoter regions are associated with compacted chromatin structure and therefore transcriptional shutdown; conversely demethylation leads to chromatin opening and therefore gene expression.

Aging in somatic tissues has been associated with global hypomethylation [44] where the majority of cells are post-mitotic. In contrast in aged HSCs, which are characterised by mitotic potential often longer than the organism lifespan, a significant degree of global DNA hypermethylation is observed. However, if HSCs are taken to the end of their proliferation potential by serial transplantation, although not a physiological condition, similar patterns of global hypomethylation are observed [33, 45]. Young HSCs gain DNA methylation in regions associated with nonhaematopoietic lineages and significant losses of DNA methylation in genomic regions associated with blood cell production. Conversely, aged HSCs display gains of DNA methylation in genomic regions associated with lymphoid and erythroid lineages; both lineages decline in number during aging. Interestingly, the majority of genes differentially methylated during HSC aging were associated with lineage potential and highly expressed downstream of the HSC in the haematopoietic tree [45]. Furthermore, age-related hypermethylated regions were enriched for targets of the Polycomb group of proteins, known to establish repressive chromatin [33, 45].

Regulators of DNA methylation include DNA methyltransferases (DNMTs) that drive methylation of CpG motifs and the ten-eleven translocation (Tet) enzymes that regulate demethylation. Functional studies implicate these epigenetic regulators in the aging process within the HSC compartment. Genetic alteration studies demonstrate that DNMT1 is responsible for maintaining methylation and its loss in the HSC compartment leads to myeloid skewing and self-renewal defects [46, 47]. Furthermore, loss of both DNMT3A and DNMT3B leads to a severe arrest in HSC differentiation [48]. Loss of Tet2 in mice attenuates differentiation and leads to myeloid transformation and myeloid malignancies [49–51]. Somatic mutations in Tet2 have also been shown in normal elderly human subjects [52]. Importantly, there is now

evidence of differential expression of both DNMT and Tet2 enzymes in aged HSCs compared with young HSCs [33, 45].

Histone posttranslational modifications including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination can change chromatin structure and therefore DNA accessibility to transcriptional machinery. These modifications may act separately or synergistically to regulate gene expression. Priming DNA in such a way precedes lineage commitment in the HSC population as seen by histone modifications associated with gene expression in committed mature cell populations already present within the HSC compartment [53, 54]. This observation is similar to that of differential DNA methylation of genes that are expressed downstream described above. HSCs also utilise Polycomb (PcG) genes to regulate aging, prevent premature aging, and maintain HSC function by forming PcG repressive complexes (PRC). While the PRC1 complex possesses H2AK119 ubiquitin ligase activity, PRC2 acts as a H3K27 methyltransferase.

Age-related changes in histone modifications also provide mechanisms that may contribute to changes seen in the aged HSC compartment. Aged HSCs show methylation of H3K4me3, a mark of active chromatin, which correlate with increases in gene expression of HSC identity and self-renewal genes [33]. Differential methylation of the repressive mark H3K27me3 has also been shown in aged HSCs, with increased H3K27me3 on a number of promoters [33]. HSC aging is also associated with low levels of H4K16ac activation mark [55]. Furthermore, interdependency between DNA methylation and histone modification exists and it might be relevant to HSC aging; however its full understanding is reliant on the development of assays that require smaller cell numbers to detect both epigenetic marks in the same samples.

Functional studies, where lysine-specific demethylases that drive H3K4 demethylation and regulate chromatin accessibility have been genetically modulated, show their critical role in stem cell differentiation. Kdm3a and Kdm5a have also been implicated in regulating stem cell aging, a notion supported by the fact that these proteins' expression decreases with age [33, 56, 57]. Knockdown of the lysine demethylase Kdm5b (Jarid1b) leads to increased HSC activity [58] and is also known to be differentially expressed with aging [45]. Knockout studies of the H3K27me3 demethylase Kdm6a (UTX1) have shown it to be a key regulator of haematopoiesis [59] and knockdown in *C. elegans* extends their lifespan [60]. Furthermore, HSCs deficient in the Bmi1 component of PRC1 [61–63] as well as the Ezh1 [64] and Eed [65] components of PRC2 show a severe defect with marked derepression of the tumour suppressor and aging-associated complex Ink4a/Arf.

Noncoding RNAs are RNAs that are not translated into protein but are known to play an important epigenetic regulatory role. While the direct impact of noncoding RNAs on HSC aging requires further investigation, there is evidence to suggest that noncoding RNAs are both highly expressed and regulate HSC survival and function. The microRNA miR-125b is highly expressed in HSCs and plays a role in regulating survival [66, 67] whereas miR-126 knockdown is associated with a myeloid-biased HSC compartment [68]. The long noncoding RNA Xist is essential for HSC survival [69].

5. Consequences of Age-Related Changes in the HSC Epigenetic Landscape

It is not clear if there is a physiological benefit of a progressive myeloid bias within the HSC compartment. However, it is clear that the epigenetic drift that leads to this phenotype correlates with the increased incidence with age of myeloproliferative disorders and myeloid malignancies as well as increased risk of infection and thrombosis (Figure 1).

Myeloproliferative disorders, myelodysplastic syndromes, and haematological malignancies [36] are attributed to accumulation of mutations in the aging HSC compartment, many of which are involved in epigenetic regulation of the HSC population such as Tet2 and DNMT3 [52, 70–73]. DNA methylation plays an important role in the pathogenesis and progression of myelodysplastic syndromes where DNA hypermethylation and methyl silencing are implicated as the pivotal mechanism [74–76]. In accordance with this, clinical trials for agents that inhibit DNA methylation are ongoing in myelodysplastic syndromes [77–80]. Acute myeloid leukaemia is typically associated with hypomethylation caused by deregulation of DNMT1 or possibly overexpression of Tet family genes [81, 82].

The myeloid skewing that occurs with advancing age may also be associated with the decline in adaptive immune response with a resultant increased risk of infection that confers high levels of morbidity and mortality in the elderly population. There is a known reduction in numbers of circulating naïve lymphocytes that could be a direct effect of a changing epigenetic landscape within the HSC compartment leading to a reduced mature lymphoid lineage generation [35, 83–85].

Age is also an important risk factor for coronary and cerebrovascular platelet thrombosis [37], conditions that have been causally linked to increased platelet activity and increased platelet mass [86]. The setting of acute arterial thrombosis has been likened to states of increased haemostatic demand. While only correlative and chronological data exists, it is reasonable to hypothesise that as in states of increased haemostatic demand, when functional circulating platelet mass drops, there is a consequential regulation of megakaryocyte activity and platelet production. The nature of this control system and how megakaryocyte activity is regulated is still unknown; however, increases in platelet mass have been correlated with increased megakaryocyte ploidy [87–89]. It is also possible that this feedback may be at the level of the HSC by an expansion of a platelet-biased HSC subset. With an age-related increase in both platelet mass [90, 91] and platelet-biased HSCs [23] it could be suggested that the recently discovered megakaryocyte- or platelet-primed HSC subset may have thrombotic implications in the elderly. It is reasonable to hypothesise that age-related changes in the epigenetic landscape with age lead to a platelet-biased HSC subset that is primed to generate a transcriptionally distinct megakaryocyte phenotype due to defined epigenetic marks that leads to increased platelet mass and activity. In support of this increasing megakaryocyte ploidy is associated with significantly increased expression of VWF and CD41 [92], both markers of a platelet-biased HSC subset.

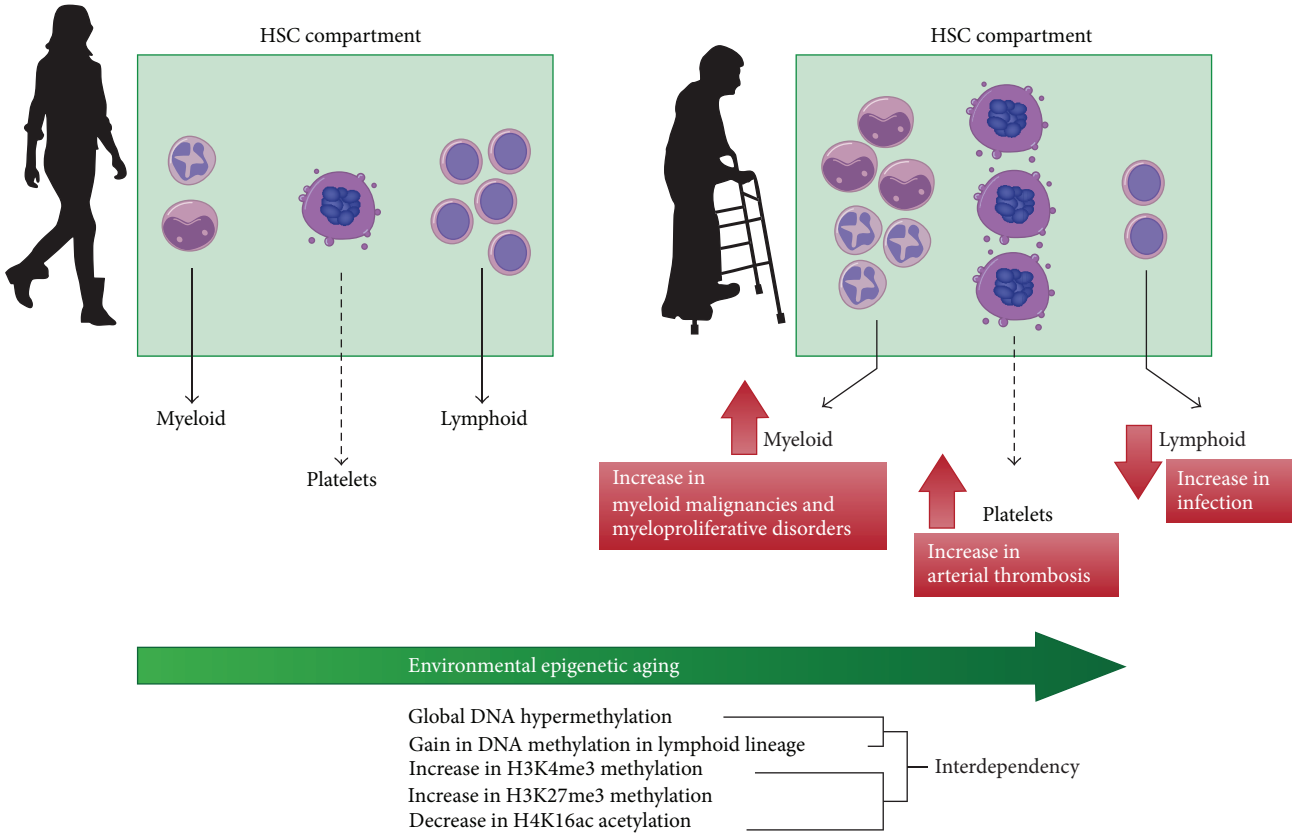

Figure 1: Drift in the epigenetic landscape that occurs with environmental and biological factors associated with aging leads to transcriptional differences between the HSC compartments in the young population compared with the elderly population. This is proposed to give rise to expansion of particular clones within the heterogeneous HSC pool to produce a myeloid- and platelet-skewed haematopoietic system. These changes may play an important role in driving the increased incidence of myeloproliferative disorders, myeloid malignancies, infection, and acute arterial thrombosis observed in the elderly.

A complex relationship exists between megakaryocytes and HSCs, with a number of phenotypic and molecular similarities including surface markers (CD41 and VWF), thrombopoietin (TPO), its receptor (MPL) and CXCR4, transcription factor dependence (RUNX-1, GATA-2, Evi-1, SCL/TAL-1, and Ets family transcription factors), signalling pathways, and proximity within the bone marrow niche [93]. Furthermore, maintenance of both megakaryocytes and HSCs crucially depends on TPO [94–96]. While the functional relevance of these similarities remains unclear there is now a body of evidence that supports the existence of tight homeostatic control mechanisms along the HSC-megakaryocyte-platelet axis. Recent reports demonstrate a critical role for megakaryocytes in maintaining HSC quiescence through either release of CXCL4 [97] and TGFβ [98] or indeed CD41 expression [23]. In mouse acute depletion of megakaryocytes leads to HSC expansion and proliferation implying a critical regulatory feedback mechanism between megakaryocytes and the HSC compartment [97]. Moreover, platelets may also regulate HSC quiescence through effects on circulating TPO concentrations [99]. Further work is required to gain a full understanding of this complex relationship.

6. Conclusions

Epigenetic changes in the HSC compartment lead to the phenotypic and functional changes that are seen in the mature cell output of the haematopoietic system with advancing age. Although these epigenetic changes are not directly pathological they produce an environment that is conducive to pathological processes that are seen in prevalence in the elderly population. In some disease processes age-related epigenetic changes may be more directly pathogenic but in other complex diseases such as coronary artery disease they may indeed account for the missing heritability determinants that have not been accounted for to date by genetic studies of sequence variation [100]. Our understanding of the role of epigenetic changes in stem cell regulation, though quickly expanding, is only at its beginnings. However, as epigenetic marks are potentially reversible this opens up the possibility

of manipulating epigenetic states and ultimately changing the way the genome functions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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