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Review

Cellular reprogramming to reset epigenetic signatures

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ABSTRACT

The controlled differentiation of induced pluripotent stem cells (iPSC) towards clinically-relevant cell types has benefitted from epigenetic profiling of lineage-specific markers to confirm the phenotype of iPSC-derived cells. Mapping epigenetic marks throughout the genome has identified unique changes which occur in the DNA methylation profile of cells as they differentiate to specific cell types. Beyond characterizing the development of cells derived from pluripotent stem cells, the process of reprogramming cells to iPSC resets lineage-specific DNA methylation marks established during differentiation to specific somatic cell types. This property of reprogramming has potential utility in reverting aberrant epigenetic alterations in nuclear organization that are linked to disease progression. Since DNA methylation marks are reset following reprogramming, and contribute to restarting developmental programs, it is possible that DNA methylation marks associated with the disease state may also be erased in these cells. The subsequent differentiation of such cells could result in cell progeny that will function effectively as therapeutically-competent cell types for use in regenerative medicine. This suggests that through reprogramming it may be possible to directly modify the epigenetic memory of diseased cells and help to normalize their cellular phenotype, while also broadening our understanding of disease pathogenesis.

Contents

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1. Introduction

Development of technologies for the generation of induced pluripotent stem cells (iPSC) is an exciting and rapidly-evolving field with great potential for clinical translation. Adult somatic cells can be reprogrammed by a variety of methods to a cellular state that is phenotypically similar to human embryonic stem cell lines (ESC) isolated from the inner cell mass of human embryos (Takahashi et al., 2007; Yu et al., 2007). The unlimited expansion potential and ability to generate therapeutically-relevant and patient-specific cell types makes these pluripotent cells valuable progenitors for the regeneration of diseased cells and tissues (Robinton and Daley, 2012; Yamanaka, 2009). Before iPSC can be implemented for therapeutic applications, rigorous phenotypic analysis and thorough testing of function and biological potential of cells differentiated from them is needed (Yamanaka, 2009). Despite the immense opportunities that iPSC technologies have provided to study development and disease-progression, there is still considerable debate over the extent of epigenetic changes associated with reprogramming (Lister et al., 2011) and implications of this for human therapies remain largely unknown.

In this review, we will discuss the contribution of epigenetic profiling, particularly the use of DNA methylation profiling, for characterization of iPSC- and ESC-derived cell populations and cells differentiated from them to specific lineage fates, and assessing the therapeutic safety and efficacy of iPSC-derived cells. In addition, there is growing evidence that reprogramming may serve to reset the cellular "biological clock" through epigenetic rearrangements (Marion and Blasco, 2010; Suhr et al., 2010). This intriguing concept could lead to vastly-improved therapies to replace cells or repair tissues that have been damaged due to degenerative diseases and/or aging. Furthermore, since several diseases are now linked to an altered epigenetic profile (Leung et al., 2012), there may be opportunities to correct these disease phenotypes through reprogramming approaches.

2. Epigenetics of pluripotency

A critical step in further developing pluripotent cell sources for future cellular therapies and for disease modeling is elucidating the underlying epigenetic mechanisms that establish and maintain the pluripotent state. Sophisticated tools have been developed to screen the epigenetic profile from a wide range of cellular phenotypes (Spivakov and Fisher, 2007) allowing assessment of how differences in epigenetic status distinguish between cell lineage fates as well as direct and maintain their cellular phenotype (Sant et al., 2012).

2.1. Pluripotency

Within the nucleus of pluripotent stem cells, the epigenetic state of DNA is in an open configuration, in which lineage-specific genes are not expressed, yet have not been repressed, by epigenetic rearrangement (Bernstein et al., 2006; Spivakov and Fisher, 2007). This orientation allows for rapid differentiation and lineage specification, facilitated by global changes in nuclear organization, DNA methylation, and histone modifications. Genome-wide mapping of this epigenetic state has identified a signature of pluripotent cells that distinguishes them from differentiated cell types (Bibikova et al., 2006; Doi et al., 2009). The chromatin structure of pluripotent cells contains a bivalent chromatin structure marking key developmental genes, such as Sox1, Pax3 Msx1, Irx3 that are not expressed in pluripotent cells (Bernstein et al., 2006). The chromatin pattern of these lineage-associated genes have been epigenetically modified with a combination of activating and repressive histone modifications that prime them for expression or repression upon induction of cell lineage specification (Gan et al., 2007).

2.2. DNA methylation

One extensively studied epigenetic mechanism that maintains differentiation potential in pluripotent cell types is DNA methylation. DNA methylation primarily occurs at cytosine residues throughout the genome, and the majority of DNA methylation events occur at the 5 position of cytosine residues at a CG dinucleotide (or CpG site) (Adams, 1990). Since spontaneous deamination of 5-methylCytosine (5mC) to thymidine is known to happen frequently throughout the mammalian genome, these dinucleotides are relatively rare and are thought to be evolutionarily-conserved gene regulatory regions (Wiench et al., 2011). Methylation events at these dinucleotide pairs can be localized into regions known as CpG islands, or are dispersed throughout the genome. Often, the CpG sites are localized near gene promoters and high levels of methylation at these promoters have been typically associated with repression of gene expression (Trowbridge and Orkin, 2010). The mechanism of DNA methylation-mediated repression involves interference with binding of activating transcription factors, as well as recruitment of repressors and realigning chromatin structure (Khavari et al., 2010; Nagae et al., 2011). The catalysis of DNA methylation is carried out by DNA-methyltransferases (DNMTs), and the expression and localization of these enzymes are determinants for whether CpG methylation is lost, gained, or stably maintained on the newly-synthesized strand following DNA replication and cell division (Chen et al., 2003).

Several whole-genome methods for DNA methylation profiling (Bisulfite NextGen sequencing, MeDIP sequencing, RRBS, etc.) have increased the depth of profiling data and enabled the accessibility of these technologies into novel experimental systems. By mapping the genomic distribution of methylated sequences, or methylome, it is possible to generate a detailed

profile of DNA methylation within specific subsets of cells (Wilson et al., 2006). This has allowed the study of DNA methylation changes in a wide variety of cell types, and large datasets have begun to be compiled that compare the methylome between different cell types; between pluripotent cells and cell differentiated from them, and between pluripotent-derived and adult-derived cell types (Bock et al., 2012; Fernandez et al., 2011). Profiling of the dynamics of genomic regions that change their DNA methylation status during cellular differentiation have revealed critical insights into the mechanisms of lineage fate decisions (Aranda et al., 2009). Further understanding the epigenetic mechanisms that establish and maintain the pluripotent state, is an important prerequisite to the goal of differentiating therapeutic cell types from pluripotent cells for regenerative medicine.

2.3. Reprogramming

Cellular reprogramming techniques, such as generation of iPSC, has definitively shown that terminal differentiation of many cell types is reversible and that nuclei in mature tissues can be reset to support embryonic development (Hochedlinger and Jaenisch, 2002). This reprogramming process is inherently epigenetic, as it involves the reversal of many of the changes in nuclear organization that occurred initially upon differentiation to specific somatic cell types. Cells reprogrammed to pluripotency have a similar epigenetic profile to ESCs, including a dramatic reconfiguration of DNA methylation. Treatment of cells with demethylating agents, such as 5-azacytidine, during reprogramming has been shown to enhance the speed and efficiency of this process (Mikkelsen et al., 2008). This provides evidence that DNA methylation is involved in maintaining the phenotype of differentiated cells, and is a critical barrier that must be reversed in order for cells to be reprogrammed to the pluripotent state. Interestingly, while demethylation appears to be critical for reprogramming, de novo DNA methylation is not necessary for reprogramming, as DNMT-knockout cells are still capable of successful reprogramming (Pawlak and Jaenisch, 2011).

While ESC and iPSC have very similar epigenetic profiles, large-scale methylation comparisons have identified differences between these pluripotent cell types, as well as variability between individual iPSC clones (Chin et al., 2009; Hu et al., 2010; Robinton and Daley, 2012). Overall, CpG methylation levels in iPSC are higher than in ESC cell lines (Deng et al., 2009). Whole-genome screening of the methylome comparing a number of iPSC and ESC lines has revealed that large, differentially-methylated regions (DMRs) exist within iPSC that are resistant to reprogramming and may lead to aberrant gene expression in cells differentiated from them (Lister et al., 2011). Such DMRs found in iPSCs are thought to harbor a residual DNA methylation signature related to their cell-of-origin, termed ‘‘epigenetic memory’’, that predisposes certain iPSC clones to differentiate along lineages related to the cell type from which the iPSC were initially derived, thus restricting differentiation to alternative cell fates (Kim et al., 2010; Polo et al., 2010). Epigenetic memory has also be correlated with a residual transcriptional profile in iPSC that is related to their cell-of-origin (Ghosh et al., 2010), contributing to differences in their phenotype when compared to ESC. Knockdown of several of the genes associated with these DMRs has determined that some of these incompletely reprogrammed genomic regions may be functionally-relevant during reprogramming (Ohi et al., 2011). These regions may be useful in determining whether a reprogrammed cell will be capable of differentiation to specific functional lineages, and may help to identify iPSC clones that may be incompletely reprogrammed and not safe for patient transplantation.

3. Epigenetics of differentiation

The directed differentiation of human pluripotent cells to specific cell types, by following many of the fate decisions which occur during development, can be recapitulated *in vitro* using tenets established in developmental biology (Hu et al., 2010; Murry and Keller, 2008). Lineage fate decisions are based, in part, on a range of diverse signaling gradients which are required to regulate the temporal and spatial segregation of cells observed during gastrulation and subsequent developmental stages (Gadue et al., 2005; Murry and Keller, 2008). Human ESCs and iPSCs can be rapidly differentiated using defined cell culture techniques to cells with a wide range of functionalities. To realize the potential of these pluripotent stem cells in regenerative medicine or other therapeutic applications, it essential to predictably and reproducibly control their differentiation to specific cell types, in a way that also maintains the stable lineage fate and functional properties of pluripotent-derived cells.

3.1. Differentiation

The stem cell state is often described as ‘‘poised’’, as it awaits signals to differentiate down a specified lineage path. Genome-wide changes in the epigenome occur in pluripotent stem cells as they differentiate (Bock et al., 2012). The step-wise differentiation of specific cell types from pluripotency, first to multipotency and then to more mature cell types, is characterized by a progressive restriction of differentiation potential and an increase in lineage-specific gene expression. On a molecular level, differentiation to specific cell types from ESC and iPSC cells is associated with alterations in nuclear arrangement, including remodeling of chromatin structure, histone modifications and DNA methylation, that restrict expression of certain genes according to cell function and phenotype (Aranda et al., 2009). During differentiation, the genome becomes compacted within the nucleus, with clusters of chromatin at the nuclear periphery, while regions of the genome that contain

tissue-specific promoters are preferentially localized internally (Meister et al., 2011). This differentiation is accompanied by increased DNA methylation of silenced chromatin and decreased methylation at sites of active transcription. Overall, these mechanisms serve to allow rapid differentiation of pluripotent stem cells, and direct the sequential maturation of cell types towards specific and stable lineages. Profiling these changes can provide valuable information as to the differentiation status, and lineage stability of resultant, differentiated cell populations. Such mechanistic insights into cell specification using pluripotent stem cell sources can enhance our understanding of human development, as well as provide insight into the controls that regulate cell fate and lineage specification.

The establishment of reproducible protocols for deriving specific cell types from ESCs and iPSC has allowed for epigenetic comparisons between these pluripotent cells and adult, somatic cells. Characterization of the methylation profile of cells differentiated from iPSC is a necessary step to begin to delineate the phenotype of these cells when compared to ESC-derived cells and to their adult counterparts. Cell types differentiated from pluripotent cells are commonly characterized based on marker expression, however these markers are not always sufficient for predicting cellular behavior and functional properties in more complex environments (Boquest et al., 2007; Egles et al., 2010). Adult cell types, such as fibroblasts, which demonstrate a wide range of cellular functions in a tissue-specific context, are often indistinguishable in cell culture based on traditional markers (Shamis et al., 2011). Furthermore, the lack of physical separation of tissues during *in vitro* differentiation makes it more difficult to directly link pluripotent-derived cells to specific tissue types and predict their behavior in functional assays and potential therapeutic applications. Therefore, it is essential to develop more sophisticated profiling tools, based on epigenetic markers, which more accurately predict lineage fate and functional properties of cells derived from pluripotent sources. Overall, however, these studies have shown remarkable similarities when the profile of cells derived from ESC are compared to those derived from iPSC and to adult cells (Deng et al., 2009; Hewitt et al., 2011; Mikkelsen et al., 2008). Since the goal of ESC and iPSC differentiation is recapitulation of development and generation of stable cell phenotypes, epigenetic analysis provides a critical baseline for studying cellular changes that occur *in vitro* during developmental processes that mimic human development *in vivo*.

3.2. DNA methylation profiling

True to the premise that epigenetic mechanisms control specific differentiation events, the pattern of DNA methylation varies in different cell types following differentiation, and whole genome mapping of DNA methylation provides a signature for identification of cell types and helps to establish their degree of commitment to specified lineage fates (Bibikova et al., 2006; Hogart et al., 2012). The DNA methylome can be useful in generating a "fingerprint" that is unique to a particular tissue-type or disease state (Fernandez et al., 2011). On a whole-genome level, detailed methylation sequence comparisons between pluripotent versus differentiated cell types have identified global differences in the patterns of DNA methylation, including differential methylation at non-CG sites (Lister et al., 2009). Overall CpG methylation is higher in pluripotent cell types than in somatic fibroblasts that are typically used for reprogramming (Deng et al., 2009). DNA methylation patterns have also proven to be of great utility in distinguishing the potency of particular cell types. For example, by determining the methylation status at only 23 CpG sites in the genome, it is possible to predict the level of pluripotency in embryonic stem cells versus that of differentiated cells (Bibikova et al., 2006). These unique signatures provide a valuable tool for stem cell researchers that can aid in identifying specific cell types derived from pluripotent sources and better assess their phenotypes when compared to mature, adult-derived cells.

Beyond global differences in DNA methylation, specific methylation changes that occur within gene promoters following lineage specification are known to have a predictable effect on cell-type-specific gene expression (Wiench et al., 2011).

Table 1
Examples of gene-specific methylated regions in development and disease. Selected DMRs are consistently methylated or demethylated following differentiation from various stem cell sources. In some diseases, aberrant methylation has also been identified associated with specific gene regions.

	Tissue/disease type	Gene region/location of methylation change	Specific change	References
Differentiation	*Mesenchymal*	PDGFR-β	Demethylated in fibroblasts	Hewitt et al. (2012)
		Leptin	Demethylated in MSC	Sorensen et al. (2010)
		MyoG	Methylated in fibroblasts	Sorensen et al. (2010)
	Epithelial	Epha2	Demethylated in term. differentiated skin	Bock et al. (2012)
		Krta5	Demethylated in skin	Bock et al. (2012)
	Hematopoietic	Meis1	Methylated in erythrocytes	Hogart et al. (2012)
		Gata-1	Demethylated in erythrocytes	Broske et al. (2009)
		Gata-2 (−3.9 kb, −77 kb)	Demethylated in erythrocytes	Hogart et al. (2012)
	Neuronal	FMR1	Demethylated in neurons	Sheridan et al. (2011)
Disease	*Diabetes*	FTO Region	Hypomethylated	Bell et al., 2010a
		PDX-1	Hypermethylated	Yang et al. (2012)
	Cancer	HIC-1	Hypermethylated	Wales et al. (1995)
		RASSF1	Hypermethylated	Dammann et al. (2000)
	Fragile X syndrome	FMR1	Hypermethylated	Sheridan et al. (2011)
	Schizophrenia	MB-COMT	Hypomethylated	Abdolmaleky et al. (2006)

Following differentiation, cells acquire methylation of CpG sites within promoters known to be associated with pluripotency, thereby restricting their differentiation potential and making de-differentiation back to pluripotency more difficult. Hypomethylation of the Oct-4 gene promoter, for example, is known to be associated with pluripotency in ESC and iPSC, while these CpG sites are hyper-methylated in differentiated cells (Mikkelsen et al., 2008). Conversely, demethylation following differentiation is localized to promoter regions that regulate cell type-specific differentiation (Nagae et al., 2011). Gene promoters associated with adult cells committed to specific lineage fates have been shown to be predictably demethylated following differentiation, and the identification of specific gene promoters involved in the process of lineage specification have helped to clarify the role of DNA methylation during differentiation (Andersen et al., 2012; Hewitt et al., 2011). Outlining such markers has shown that consistent changes in DNA methylation following differentiation occur at gene promoters with key functional significance (Table 1). For example, the promoter region of PDGFRβ is demethylated concomitant with the differentiation of fibroblasts (Hewitt et al., 2012). In addition, the adipocyte-specific gene LEP is methylated in ESCs and demethylated in adipocyte-precursor cells following differentiation (Sorensen et al., 2010). By comparing the methylation profiles of cells differentiated from pluripotent cell types to their adult cell counterparts, it is possible to predict the behavior of these differentiated cells and to reveal novel mechanisms involved in the establishment of stable somatic cell type (Aranda et al., 2009). Overall, epigenetic changes and the establishment of a methylation signature early in the process of differentiation, are essential in defining and maintaining the lineage identity and cell phenotype.

4. Disease-associated epigenetic profiles

In adult cells and tissues, analogous epigenetic events that allow for diverse cellular differentiation also play important roles at the interface between genetic and environmental information (Bell and Beck, 2010). Whole-genome profiling of DNA methylation has provided powerful tools to begin defining unique epigenetic markers of a diverse range of cellular phenotypes found in specific disease conditions (Leung et al., 2012). DNA polymorphisms that predispose onset of diseases in some individuals have shown the potential to influence DNA methylation in specific regions and may lead to disease progression (Bell et al., 2010a,b). In addition, environmental effects such as maternal behavior and diet have been correlated with changes in the epigenomic landscape, and may be important in mediating disease progression at certain loci (Anderson et al., 2012; Rodriguez-Cortez et al., 2011). Modifications of the H3 histone tail of lysine 4 and 9 have been found to be associated with gene expression linked to hyperglycemia in diabetics (Cooper and El-Osta, 2010). Intriguingly, there is an increasing body of evidence that such aberrant epigenomic alterations in genomic organization can influence the progression of disease (Bell and Spector, 2011; Feil and Fraga, 2011). Specific changes in DNA methylation have also been found at functionally-relevant promoters associated with complex diseases such as diabetes and cancer (Table 1).

As mentioned earlier, epigenetic analyses have shown that the process of reprogramming faithfully resets methylation marks established prior to the differentiation of somatic cells. Since reprogramming activates a cascade of epigenetic rearrangements to reset the epigenome and reverse terminal differentiation, it is possible that reprogramming will also erase the environmentally-induced DNA methylation marks that are associated with specific disease states. These events during reprogramming could have potential utility in reverting aberrant epigenetic alterations that are associated with a variety of disease conditions (Lang et al., 2012).

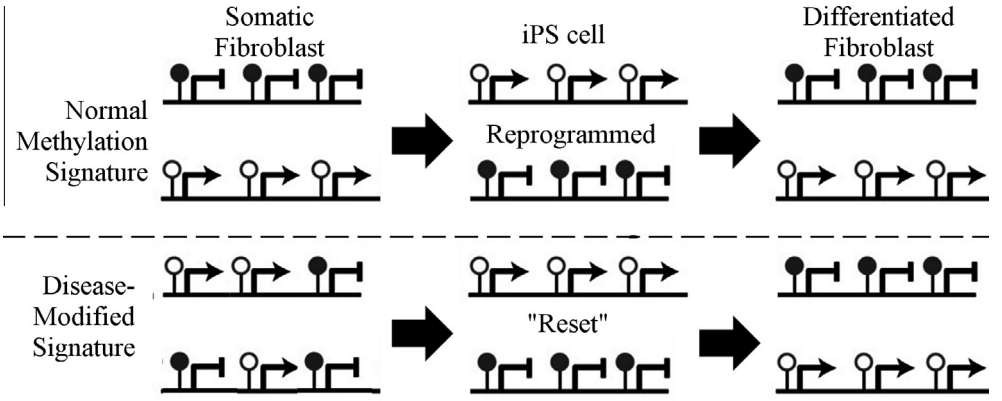

Fig. 1. Schematic model for reversing changes in diease-associated DNA methylation. Somatic fibroblasts have a characteristic methylation signature that consists of gene promoters that are methylated and repressed (e.g. OCT-4), as well as other promoters that are unmethylated and expressed (e.g. PDGFRβ). Following reprogramming, these methylation marks are reversed, and upon differentiation they are re-established to resemble the original somatic fibroblasts. In a diseased state there are changes in methylation that allow expression of repressed genes and repress genes that should be expressed, resulting in aberrant phenotypes. Upon reprogramming, methylation is "reset" to allow for differentiation to fibroblasts with a normalized epigenetic signature. Methylation is marked by dark circles, and unmethylated is marked by open circles. Arrows indicate active transcription, and blocks indicate repression.

The differentiation of cells from iPSC derived from patients with environmentally-induced diseases could give rise to mature cells that have reacquired a "normalized" epigenetic profile, and will allow these cells to reacquire healthy cellular function. A schematic of these changes, where a disease-associated methylation signature is "reset" through reprogramming, is shown in Fig. 1. Evidence for this comes from reprogramming studies using malignant cells, which have shown that tumor-derived iPSC can be differentiated to well-differentiated, functional types with a normalized phenotype (Zhang et al., 2012). This suggests that it may be possible to erase disease-specific epigenetic changes and revert their cellular phenotype to more normal state, providing a more direct link between disease progression and cellular, epigenetic alterations. Beyond this, these findings could have implications for disease modeling using iPSC-derived sources, as the reversion of epigenetic marks that define specific diseases may no longer be present following reprogramming (Onder and Daley, 2012). The prospects surrounding reprogramming efforts to reset epigenetic signatures could have broad influence on regenerative medicine efforts to normalize and correct cells that have acquired altered epigenetic states, with the longer term application to reintroduce those cells in a patient-specific manner as therapeutic relief.

5. Limitations and perspective

Despite their potential, the application of pluripotent stem cells for human therapy has not been widely implemented due to concerns related to their safety, purity, and immunogenicity, as well as technical limitations in differentiating specific cell types (Belmonte et al., 2009; Miura et al., 2009). Due to the necessity for maintaining these cells in culture for prolonged periods, it has been demonstrated that they accumulate genetic mutations over time (Gore et al., 2011). Additionally, while pluripotent cell types have generally been shown to be resistant to chromosomal abnormalities, some ESC and iPSC lines have been shown to acquire aberrant karyotypes (Taapken et al., 2011). At the same time, epigenetic alterations have also been identified in some iPSC lines. For example, aberrant DNA methylation found in iPSCs can also maintained in cells differentiated from iPSC, and may affect their ability to differentiate and their stability as mature cell types (Lister et al., 2011). Thus, extensive genetic and epigenetic screening should be a necessary step in ensuring the safety of iPSC lines prior to clinical use. A better understanding of the epigenetic events that regulate the reproducible derivation of a broad spectrum of stable, differentiated cell lines from ESC and iPSC will provide a significant boost to efforts designed to offer safe and improved therapeutic outcomes with these cells.

Due to the known epigenetic variability in iPSC clones and differentiated cells, the established methods for characterizing differentiating cells from both ESC and iPSC will need to be improved to include more detailed epigenetic analysis before their promise in regenerative medicine can be realized. In addition, more thorough functional analysis of iPSC-derived cells needs to be performed in order to better predict how these cells will behave *in vivo*. For instance, using 3D tissue reconstructs to assess cell functionality, will markedly improve upon existing methods to determine the therapeutic safety and utility of iPSC-derived cells. Mature cells differentiated *in vitro* from pluripotent cells sources carry a large burden-of-proof to show their association with specific tissue types, and even greater stringency is needed to define biologically-meaningful parameters for functionally assaying the cells in disease models. Future findings that further define the profiles of specific cell and tissue types with distinctive epigenetic features, will serve as parameters to screen and identify optimal cells for human therapy. We can thus begin to compare the epigenetic profiles of ESC- and iPSC-derived cells, to more precisely define their identity when compared to expression profiling techniques that are dependent on tissue context. With such an enormous variety of stem cells and differentiated cell types available, including patient-specific and disease-specific iPSC and iPSC-derived cell types, epigenetic profiling will be an essential screening tool for future work to ensure the quality and establish the identity of specific lineages derived from iPSC. Furthermore, defining specific epigenetic changes that occur during differentiation will be useful in delineating mechanisms of lineage specification, and identifying aberrant profiles may help elucidate the pathogenesis of a broad spectrum of diseases. Through continued refinement of the mechanisms that are barriers to cell differentiation from pluripotent cells, we can further understand the critical epigenetic changes needed to derive specific functional cell types, and these findings will likely shed light on their therapeutic benefits or limitations.

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