Variability of human pluripotent stem cell lines

Abstract:

Human pluripotent stem cells derived from embryos (human Embryonic Stem Cells or hESCs) or generated by direct reprogramming of somatic cells (human Induced Pluripotent Stem Cells or hiPSCs) can proliferate almost indefinitely *in vitro* while maintaining the capacity to differentiate into a broad diversity of cell types. These two properties (self-renewal and pluripotency) confers human pluripotent stem cells a unique interest for clinical applications since they could allow the production of infinite quantities of cells for disease modelling, drug screening and cell based therapy. However, recent studies have clearly established that human pluripotent stem cells lines can display variable capacity to differentiate into specific lineages. Consequently, the development of universal protocols of differentiation which could work efficiently with any human pluripotent cell line is complicated substantially. As a consequence, each protocol needs to be adapted to every cell line thereby limiting large scale applications and precluding personalised therapies. Here, we summarise our knowledge concerning the origin of this variability and describe potential solutions currently available to bypass this major challenge.

Introduction

The derivation of human embryonic stem cells (hESCs) [1] opened exciting opportunities for translational research and regenerative medicine. Almost a decade later, direct reprogramming of human somatic cells to pluripotency [2] promised to overcome ethical limitations associated with human embryos and enable personalised therapies. However, the clinical applications of hESCs and hiPSCs continue to be limited by their inherent variability in differentiation capacity [3–5]. Indeed, protocols of differentiation appear to work efficiently only with specific cell lines thereby precluding the use of a broad spectrum of human pluripotent stem cells (hPSCs). This challenge is exacerbated by the lack of a precise test to evaluate the level of reprogramming in hiPSCs. In contrast to mouse ESCs, hPSCs pluripotency cannot be assessed through chimera formation or tetraploid complementation assays. As a consequence, reprogramming of human cells can only be confirmed through relatively crude characterisations such as gene expression profiling, in vitro differentiations or teratoma formation none of which is sufficient to determine the capacity of differentiation of individual hiPSCs into a specific cell types. Thus, the origins of this functional variability remain an active field of investigation within the stem cell community. Some of the previous findings and more recent developments will be discussed here.

Gene expression and signalling variability

There has been a diversity of attempts to find markers characterising hPSC variability and predicting their capacity of differentiation. Indeed, differences in gene expression controlling endogenous signalling are likely to alter cellular behaviour and differentiation potential *in vitro*. This variability in signalling pathway activity may be counteracted by line specific optimisation of differentiation protocols, adapting the concentration of cytokines and inhibitors according to the cell line used [6]. Accordingly, several reports have shown that varying gene expression profiles in hPSCs could be associated with divergent capacities of differentiation. Blauwkamp and colleagues correlated the expression of early lineage markers

in undifferentiated hPSCs [7] with the level of endogenous Wnt signalling resulting in distinct differentiation propensities. Elevated Wnt would thereby lead to enhanced differentiation potential towards mesendoderm, which was confirmed by a separate report [8]. However, wnt-high and wnt-low subpopulations in normal culture seem to change dynamically rather than represent a stable state.

The expression of micro-RNAs was shown to serve as an indicator of neural differentiation propensity [9]. In a recent study, Nishizawa and colleagues compared 35 different hiPSC lines of different origin and four hESC lines in their ability to form hematopoietic cells [10]. This analysis revealed that the differentiation potential of hPSC lines could correlate with the expression of IGF2. This gene is not directly implicated in the hematopoietic differentiation, but seems to serve as an indicator of chromatin accessibility at loci of mesendodermal genes which could render them more amenable for activation. Interestingly, the maturation capacity of different lines was less correlated with varying gene expression patterns, but instead seemed to be influenced by changes in DNA methylation [10] suggesting that epigenetic rather than genetic regulations could be involved. Importantly, this study seems to contradict a previous report showing that the endogenous level of Nodal growth factor in hESCs could predict their capacity to differentiate into blood progenitors [11]. This underlines the difficulty of such experimental comparisons requiring a larger number of independent experimental samples that can be easily influenced by culture conditions, handling skills, methods of analysis and the quality of the hPSC lines used.

DNA methylation and epigenetic memory

Variations in the transcriptome could originate from a diversity of mechanisms. One source could be epigenetic differences induced by either abnormal/incomplete reprogramming [12] or by culture conditions [13–16]. In particular, hiPSCs have been under intense scrutiny as to their similarity with hESCs. Indeed, a methylation analysis of preselected CpG sites concluded that there is a core set of aberrantly methylated genes that can distinguish hiPSCs from hESCs regardless of the donor cell type [17]. This "reprogramming-associated epigenetic signature" was characterised mainly by hypermethylation at specific loci. This is in accordance with an unbiased whole-genome methylome analysis that identified regions of aberrant methylation which also persists in differentiated cell types derived from iPSCs [18]. It was suggested that these epigenetic variations could either be a function of the reprogramming process itself [19] or they could depend on the reprogramming method used to generate these lines [20]. Aberrant CpG methylation demonstrated to be heavily influenced by clone-specific effects and seems to be correlated with MYC binding motifs [21], therefore it may be desirable to omit C-MYC in the reprogramming cocktail, which has been shown in mice to reduce tumorigenicity [22,23]. Furthermore, non-CG methylation can also serve as marker for predicting the potential of hPSCs to differentiate into definitive endoderm, thus linking variability in DNA methylation with differentiation phenotypes [24].

Several studies have instead attributed differences between hiPSCs lines to persistent remnants of epigenetic or transcriptional profiles of the donor cell type [25–28]. This retention of some of the characteristics of the somatic cell type of origin was termed epigenetic memory and was speculated to be at least in part responsible for the variability in differentiation capacity of different hiPSC lines [29–31]. However, the impact of epigenetic memory seems to vary between laboratories, somatic cell types and methods of reprogramming. Indeed, several reports could not detect any major influences of donor cell type on the capacity of differentiation while showing that hiPSCs from different donors are more divergent than hiPSCs originating from different somatic cell types of the same donor [32–34]. Finally,

epigenetic memory appears to decrease after prolonged cell culture *in vitro*. Further reaffirming this notion, conclusive studies of genetically matched lines found hESC and hiPSC to be equivalent and imputed the few recorded differences to transcriptional noise [35,36]. Considered together, these results suggest that epigenetic variation and especially DNA methylation could have a key role in defining the differentiation capacity of hiPSCs. However, the source of this epigenetic variability and its functional impact on differentiation capacity remains controversial.

Genetic factors

Genetic variability could also have a significant influence on the capacity of hPSCs to self-renew and to differentiate. hiPSC lines with different genetic backgrounds could express divergent levels of receptors, transcription factors and growth factors which in turn could affect their capacity to respond to specific culture conditions. Recently even mutations in mitochondrial DNA have been raised as a potential additional source of variability [37]. However, the link between variability and genetic background has proven challenging to demonstrate with hESCs. Indeed, the limited number of hESC lines available precludes large scale studies necessary for genetic analyses. In addition, the variability induced by the process of derivation is difficult to evaluate since production of several hESC lines with the same genetic background is very challenging. Thus, the importance of genetic background has started to be investigated with hiPSCs. Several studies have now revealed that the transcriptome of hPSCs generated from different donors is more divergent than the transcriptome of hPSCs generated from the same donor or even from hPSCs from different tissue types of the same donor [35,38–40]. These reports also showed the existence of pluripotent specific Quantitative Trait Loci (QTL) and that allele specific variations can result in different levels of gene expression. Taken together, these reports suggest that genetic factors could influence key characteristics of hiPSCs including their capacity to differentiate into specific cell types. Nonetheless, functional links of differentiation capacity and molecular mechanisms involved remain to be fully uncovered especially the existence of specific "Differentiation-QTL". The current development of large scale initiatives such as HIPSCI (www. hipsci.org) or the Next Gen consortium (http://www.cell.com/consortium/NextGen) could address this limitation (see table for a more comprehensive list of initiatives). One such study from the Next Gen consortium identified polycomb targets as another non-genetic potential cause of heterogeneity of hPSC lines [39]. Due to the potential to generate iPSCs from various genetic backgrounds these could serve as a good tool to validate GWAS targets in patient and control lines [41]. These projects are deriving a large number of hiPSC lines from healthy donors which could be used to perform detailed genetic analyses to link genomic regions with capacity of differentiation. Such mechanisms have recently been uncovered in drosophila where thousands of developmental specific QTL have been identified in enhancers controlling expression of regulators directing embryonic development [42]. Pashos and colleagues have studied QTLs and allele specific expression in iPSCs and hepatocyte like cells and suggested that hiPSCs are a good model to discover and study genes and functional variants leading to complex human traits [43]. The identification of human "differentiation-QTL" and associated mechanisms could have a major impact on our understanding of development and disease progression. Indeed, such mechanisms are likely to control the activity of key signalling pathways and/or epigenetic networks involved in organogenesis and tissue repair.

X-inactivation and imprinting

Another factor displaying variability in different hPSC lines is the inactivation of the X chromosome. While the usual state of female somatic cells (one X chromosome active and one inactive, XaXi) is largely maintained during reprogramming, XaXa cells have been reported [44]. Furthermore, the inactivation of one X chromosome is frequently eroded with prolonged time in culture [45]. Indeed, a study from the Progenitor Cell Biology Consortium found that one of the major epigenetic differences between 58 different hiPSC lines were differential methylation patterns related to the X-chromosome [46]. The breakdown of X inactivation has been associated with increased expression of oncogenes and reduced developmental potential both of which could affect cell fate decisions in hPSCs [45,47]. DNA methylation is also heavily influencing the allele-specific expression of imprinted genes. Several studies have reported changes in imprinting in mouse and human iPSC lines as a result of either prolonged culture or the reprogramming process [48–50] while others have found imprinting to be relatively stable [51]. Changes in the imprinted Dlk1-Dio3 cluster in mouse iPSCs have been correlated with their level of pluripotency and the ability to contribute to all cell types [52] thereby implying major differences in developmental potential. Finally, global loss of imprinting seems to heavily increase oncogenic potential [53]. Therefore, proper imprinting is essential to avoid functional impairment of PSCs and their derivatives. Most of the aspects mentioned above can be influenced by *in vitro* culture of hPSCs. While aberrant hypermethylation patterns and CNVs seem to decrease over time in iPSCs [54–56], erosion of X inactivation is more likely to occur [45]. Importantly, genetic and epigenetic alterations have been reported in hPSCs by many studies and can heavily influence their functional properties and oncogenic potential [57]. Culture conditions can affect the epigenetic status of hPSCs and constitute a major factor of inter-laboratory variability. Potentially, automated derivation and culture of iPSCs together with standardised culture conditions could decrease this source of variability [58]. It has further been suggested that reverting hPSCs to an earlier developmental state (ground state) could erase epigenetic signatures [59], however it has not been proven experimentally whether this will decrease variability of PSCs. Besides, as mentioned above there can be grave consequences of deleting crucial epigenetic marks such as imprinting. Thus, the details of an ideal hPSC culture minimising variability remain to be determined.

Conclusions

Variability between hPSC lines represents a major challenge for clinical applications since it could impair the development of personalised medicine. However, such variability especially when linked to genetic background could be used advantageously to investigate complex genetic mechanisms involved in disease using hPSC models. Nonetheless, variation induced by abnormal epigenetic profiles needs to be better understood and controlled. Improving methods of reprograming might decrease the background noise induced by such epigenetic mechanisms independent of the genetic background. Precise delivery of reprogramming factors in the right stoichiometry [60] or the inclusion of potential human specific factors may improve the efficiency and reproducibility of iPSC generation. Indeed, a broad number of studies have identified factors able to improve reprogramming efficiency of mouse cells (i.e. increasing the number of reprogrammed cells) however such factors are rarely as efficient with human cells. In addition, the qualitative effect of these factors is difficult to evaluate with human cells due the absence of precise test such as tetraploid complementation. Thus, the discovery of human specific pluripotency factors combined with tailored reprogramming protocols (factors, stoichiometry and timing) could help to derive high quality hiPSC lines. Crucially, not only the number of reprogrammed cells, but also their quality in terms of absence of undesirable genetic or epigenetic variations is paramount and could

also be influenced by the precise reprogramming avenue. To ensure the best quality of pluripotent stem cells, especially for clinical applications, regular and detailed testing is essential to avoid the occurrence of oncogenic mutations or other unwanted alterations [61,62].

Another alternative to bypass the variability between hPSCs consist in purifying proliferative progenitors during the process of differentiation. Such approach has been successfully applied to isolate progenitors from the 3 germ layers endoderm [63,64] or foregut endoderm [65], neural stem cells [66] and mesoderm [67]. Whether this approach could be useful to decrease variability between different lines however remains to be shown. Indeed, variability in the capacity of differentiation is likely to occur at different stages of differentiation and thus could require purification of several intermediates.

Ultimately, the best approach to bypass variability in differentiation and to develop universal protocols of differentiation is to acquire a better understanding of the molecular mechanisms directing hPSCs cell fate decisions and especially how the genetic and epigenetic background can influence these mechanisms. Thus, detailed studies are needed to discover the interplays by which signalling pathways directing differentiation in hPSCs orchestrate transcriptional networks and epigenetic states and how in turn genomic variability can influence the activity of key regulators. This knowledge will allow us to systematically tailor protocols of differentiation to specific genetic backgrounds. Ultimately, some of these mechanisms could also apply to adult somatic stem cells and thus deliver new insights into the mechanisms controlling the individual capacity for tissue repair and organ homeostasis.

Figure 1: Sources of Variability

Figure 2: Signalling pathways in PSC differentiation

Table: Initiatives generating large numbers of iPSCs

Name of the project	Web site	hiPSC lines (Current/ Objective)	Distributor
HISPCI	http://www.hipsci.org/	500 / 800	ECACC (www.pheculturecollections.org.uk/products/celllines/ipsc/)
NYSCF	http://nyscf.org/	21 / ?	NYSCF
WiCELL	www.wicell.org	1200	WiCell
CIRM	www.cirm.ca.gov/researchers/ipsc-repository	1200	Coriell (https://catalog.coriell.org/1/CIRM)
EBISCs	https://www.ebisc.org/	367/10000	EBISCs
NextGen	http://www.cell.com/cell-stem-cell/fulltext/S1934-5909(17)30092-9	? / 2000	WiCell

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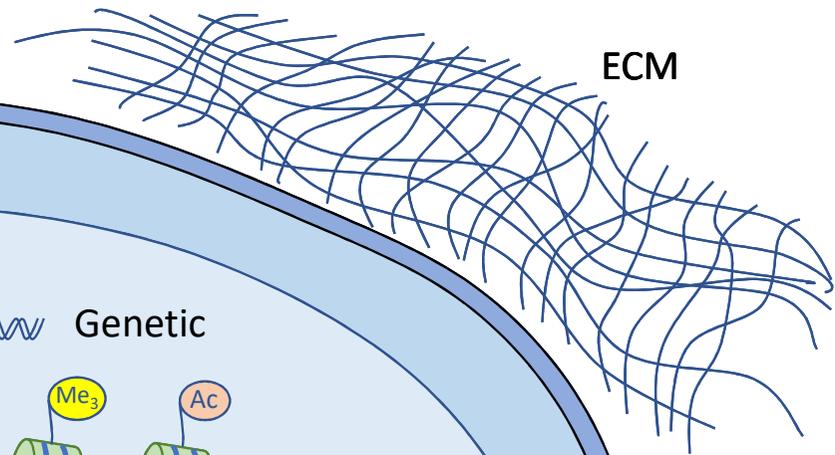
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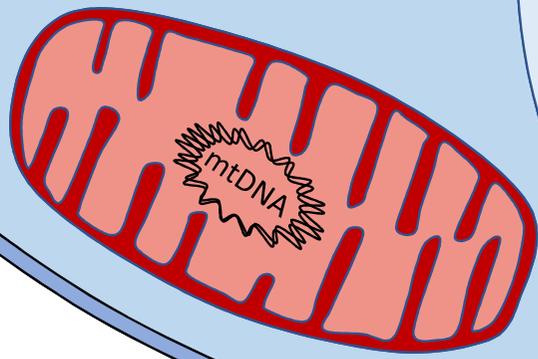Human pluripotent stem cells

Activin/Nodal
FGF

Activin/Nodal

Endoderm

BMP
FGF
Wnt

Mesoderm

Activin/Nodal
BMP
Wnt
FGF

Ectoderm