



Editorial

The Current State of Pluripotency Affairs in hiPSCs

Muhammad Farooq^{1*} and Gabrielle M. Hawksworth¹

Embryonic stem cells (ESCs) display the potential for unlimited self-renewal and the capacity to give rise to all somatic cell types. Despite this, the use of human ESCs (hESCs) in research and therapy has been limited due to ethical concerns. Strategies to develop human induced pluripotent stem cells (hiPSCs) by reprogramming somatic cells have presented an alternative to this situation. Despite an impressive progress in the field of cell reprogramming, concerns are consistently raised about the genomic and epigenomic integrity of hiPSCs. A major question remains: are hiPSCs indeed the true equivalent to hESCs?

In humans, development starts when a sperm fertilises an egg to create a zygote, a single totipotent cell. This single cell divides to produce identical totipotent cells that have the ability to produce all the cell types including extra embryonic tissues. After reaching the 16-cell stage, these totipotent cells differentiate into two types of populations that will eventually become either the outer trophoblasts or the blastocyst's inner cell mass. Cells in the Inner cell mass have the potential to differentiate into any of the three germ layers of the developing embryo (endoderm, mesoderm and ectoderm). These cells are termed as "pluripotent" and are source of hESCs.

The first successful isolation of hESCs in 1998 made it possible to study them *in vitro*. Initial studies highlighted the research and therapeutic potential of hESCs. However, derivation of hESCs from the human embryo sparked controversy resulting in the restricted availability of stem cells. A limited number of the stem cell lines were made available to the research community but most of them represented generic cells unsuitable for transplantation purposes due to their potential to elicit an immune response in the recipient [1].

This situation compelled the research community to look for alternatives. Somatic-cell nuclear transfer (SCNT) procedure, a well-established technique that was successfully used to clone "Dolly the sheep" from adult mammary cells, offered an alternative. SCNT involves introduction of genetic material from somatic cells into enucleated oocytes. Resulting nuclear-transfer-generated embryonic stem cells (ntESCs) would capture patient's complete genome making them ideal for cell replacement therapy.

Ethical and political considerations have significantly restricted the use of SCNT for therapeutic applications. However, scientific investigations using this method revealed that differentiation does not involve permanent changes in the DNA sequence of the target

cell. Instead epigenetic factors play a central role in this process. It was also noticed that fertilised eggs as well as oocytes contain factors that are capable of reversing the epigenetic status of mature DNA to re-establish the state of pluripotency.

These observations were successfully translated into a research technique and in 2007 Yamanaka and colleagues transformed human somatic cells into hiPSCs by introducing four transcription factors (TFs): Oct4, Sox2, Klf4 and c-Myc. These hiPSCs seemed to have all of the properties of hESCs. This breakthrough opened up the possibilities to discover disease mechanisms, model diseases and personalised cell therapies using hiPSCs [2].

Initial experiments to reprogramme somatic cells were performed using integrating retroviral and lentiviral vectors as carriers to deliver TFs into the target cells. Random integration of these vectors into the genome of the target cell may result in unwanted mutations. Furthermore, these transgenes may be reactivated after cell reprogramming to produce unwanted results. To eliminate these possibilities safer transgene-free methods have since been developed. However, low reprogramming efficiency remains an issue with all these methods [1].

Once the reprogramming process is complete, the initial assessment of pluripotency in culture is still largely performed manually by identifying distinct morphology of reprogrammed cell colonies. To differentiate between fully and partially reprogrammed cells, selected colonies are further screened for a series of molecular hallmarks including Oct4, Sox2, Nanog and DNA methylation status. Once screened for these markers, resulting cell lines are assessed for functional pluripotency by assessing their differentiation potential *in vitro* along with their ability to develop well differentiated tumours in immunodeficient mice. Both of these assays use hESCs as standards to assess the pluripotency of hiPSCs. Recently, genome wide high-throughput assays have enabled researchers to make more quantitative comparisons between hESCs and hiPSCs [1].

Subsequent to successful reprogramming, hiPSCs qualify as pluripotent cell upon meeting the aforementioned assessment protocols. However, the reprogramming process has been implicated in certain unwanted genomic and epigenomic changes. As usefulness of hiPSCs in research and therapeutic applications relies on their genomic and epigenomic integrity as well as their stability therefore it is important to examine these aspects.

A variety of genomic changes in hiPSCs that are observed as a result of cell reprogramming include: karyotypic and subkaryotypic changes, increased mutation levels, changes in mitochondrial profile, altered DNA damage response pattern and changed gene expression profile as explained below individually.

- Recent large-scale genomic integrity analyses showed no notable differences in chromosomal abnormalities but highlighted differences in subkaryotypic alterations between hESCs and hiPSCs [1,3]. These subkaryotypic changes are acquired by hiPSCs either during reprogramming or during expansion in culture. Deletions in hiPSCs are reported to occur in early passages suggesting that they may be a

*Corresponding author: Muhammad Farooq, Molecular Toxicology Group, Division of Applied Medicine, School of Medicine and Dentistry, University of Aberdeen, Aberdeen, AB25 2ZD, Scotland, E-mail: m.farooq@abdn.ac.uk

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result of reprogramming or an adoptive response to culture conditions. On the other hand amplifications are reported as a later passage phenomenon. An opposite trend of hiPSCs acquiring more deletions and hESCs more gains has also been reported. Copy number variations (CNVs) frequency seems to be higher in early passages of hiPSCs, possibly due to replication stress and occur in the regions of genomic fragility. This situation gradually changes and hiPSCs in later passages display CNVs identical to hESCs [4].

- Earlier studies in hiPSCs reported certain mutations that were thought to be due to transgenes used for cell reprogramming. To eliminate this risk, safer transgene-free methods have since been developed. However, mutation load remains same regardless of the delivery method. Various mutations in protein-coding exons (exomes) of hiPSCs have been reported. These mutations are either inherited from parental somatic cells or developed during reprogramming process and are maintained during the prolonged culture [1,4]. Furthermore, additional mutations are picked up by hiPSCs during prolonged culture. Some of these mutations are thought to be responsible for altered protein functions but no detailed analysis of their downstream effects has been reported to date. It is also important to study the mutation load in prolonged culture of hESCs and compare it with hiPSCs. Nevertheless, higher number of mutations observed in hiPSCs than in the corresponding somatic cells reflect that reprogramming can induce genomic changes that in turn may influence genomic stability of hiPSCs.
- Somatic cells contain much higher number of mitochondria as compared to hESCs. In hESCs number of mitochondria increase with onset of differentiation process and reach its optimal levels when cells are fully differentiated. Mitochondrial function, morphology, distribution and quantity in parental somatic cells change during reprogramming. Fully reprogrammed hiPSCs closely resemble with that of hESCs but upon differentiation they reflect the parental somatic cell mitochondrial profile [4]. This is an indication that hiPSCs retain somatic cell memory that can potentially be exploited for beneficial purposes.
- DNA damage responses such as cell cycle arrest in G2/M, efficient DNA repair through homologous recombination (HR) and non-homologous end joining (NHEJ), high expression levels of genes responsible for DNA damage repair and signalling closely resemble in hESCs and hiPSCs [4].
- Early-passage gene expression profile of hiPSCs displays a distinct signature that becomes increasingly identical to hESCs after extended culture but does not disappear completely [5,6]. These gene expression differences, as suggested by genome-wide analysis, are due to differential promoter binding by the reprogramming factors.

TFs actively engage in cross talk with epigenetic regulators during cell reprogramming. Certain epigenetic changes are necessary for this remodelling process but at the same time cells acquire some inadvertent changes that in turn may influence epigenetic integrity of reprogrammed cells. Although there are many reports that have highlighted the hiPSCs epigenetic aberrations, the biological consequences of these are largely unclear. The major epigenomic

changes that hiPSCs acquire during reprogramming process are as following:

- DNA methylation is one of the key epigenetic mechanisms associated with transcriptional silencing. Cellular reprogramming seems to influence methylation potential in hiPSCs where increased levels of DNA methylation are observed as compared to hESCs. On the other hand partially reprogrammed hiPSCs fail to demethylate pluripotency genes. Although global DNA methylation patterns of hiPSCs and hESCs at both CpG and non-CpG sites are broadly similar, there are some distinct differences between these two cell types. These reported differences in DNA methylome patterns between hESCs and hiPSCs suggest retention of somatic memory by hiPSCs. Furthermore, somatic memory does not necessarily vanish in a passage dependent manner rather, in some cases it was not erasable and had been reportedly transmitted to differentiated progeny [1,4]. This inherited memory may also influence the differentiation pattern of hiPSCs with tendency to differentiate into donor cell type. Nonetheless, this situation can potentially be exploited for beneficial purposes to achieve higher level of differentiation in situations where directed differentiation is difficult to achieve.
- Gene expression levels closely relate to specific post-translational modifications of histone tails. Reprogramming process seems to influence this important epigenetic mechanism. Histone modifications and transcriptional levels of lineage-specific genes in hiPSCs are reported to be more variable than in hESCs. Furthermore, partial reprogramming of hiPSCs is implicated in lower frequencies of permissive and repressive histone marks in the promoters of most genes [1].
- Links between various types of non-coding RNAs (ncRNAs), chromatin organization, gene regulation and development have recently been highlighted by many research groups. ncRNAs are a diverse group of transcripts that play an important part in a variety of crucial cellular functions. MicoRNAs (miRNAs) are members of ncRNAs family and are major players in post-transcription regulation processes. Detailed investigations by different research groups showed that both hiPSCs and hESCs display a distinct miRNA profile [7]. Large intergenic non-coding RNAs (linc-RNAs), another member of ncRNAs family, have been implicated in the maintenance of pluripotency and suppression of lineage specification. Expression levels of linc-RNAs are shown to be higher in hiPSCs as compared with hESCs [8,9].
- During normal human development one X chromosome is randomly inactivated through epigenetic mechanisms in each cell in females. There are controversial reports about the reactivation of X chromosome in hiPSCs. Whether reprogramming reset this epigenetic silencing in hiPSCs remains to be confirmed [1].

As TFs mediated cell reprogramming is achieved through epigenetic remodelling and all the above issues also result from specific epigenetic condition, it will be beneficial to use epigenetic chemicals to optimise reprogramming process. Some of these epigenetic chemicals that are potent inhibitors of DNA methyltransferases, histone methyltransferases, and histone deacetylases have shown to

work synergetically with TFs [10]. Use of these chemicals will not only help reducing epigenetic barriers to improve reprogramming frequency but also will help to improve reprogramming efficiency which prove to be a major problem in materialising the full research and therapeutic potential of hiPSCs.

It is evident that hiPSCs do accumulate genetic and epigenetic changes during reprogramming process and in the prolonged culture. These changes may influence genomic and epigenomic integrity of hiPSCs. Therefore it is important to scrutinise these changes and study their biological consequences in order to ensure potential therapeutic safety of hiPSCs. At the present moment, hESCs are the gold standard with which hiPSCs are compared. Although hiPSCs closely resemble to hESCs, there are important differences between both cell types. A number of factors may contribute to these differences including derivation sources, derivation methods, culture conditions, sample size, and analytical methods including references and platforms. Therefore, there is an urgent need to standardise inter laboratory protocols to address these issues.

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Author Affiliation

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¹School of Medicine and Dentistry, University of Aberdeen, Scotland

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