STEM CELLS AND DEVELOPMENT
Volume 22, Number 8, 2013

DOI: 10.1089/scd.2012.0668

Advancing Pluripotent Stem Cell Culture: It Is a Matter of Setting the Standard

Peter Sartipy

Human pluripotent stem cells (hPSCs), defined by their ability to proliferate indefinitely and the capacity to differentiate into all tissue cell types of the adult, represent a platform for the realization of breakthrough technologies for industrial and regenerative medicine applications. We have witnessed tremendous developments over the last decade related to methods for establishment, maintenance, differentiation, and applications of hPSCs and their derivatives. Despite all progress made in the hPSC field, there are still fundamental issues yet to be resolved. For example, our understanding of the pluripotent state remains limited, which in turn may have substantial consequences on how we interpret and communicate scientific data concerning hPSCs. This brief commentary aims to highlight recent important findings that demonstrate additional levels of complexity to the current assessment of pluripotent stem cell cultures. In addition, these data may help to provide some explanations for the challenges in reproducing hPSC differentiation protocols across laboratories.

The techniques for generation of human pluripotent stem cells (hPSCs) are nowadays procedures that most laboratories master, and the reagents needed can be obtained from commercial sources relying on standardized and quality controlled processes [1,2]. Thus, creating an hPSC line no longer represents a major obstacle to enter into this research field. In addition, if there is a preference to work on already established cell lines, there is a wide access to various cell lines available from academic and industrial institutions. Furthermore, a range of specific culture media and support matrices for propagation and expansion of the undifferentiated cells are also available [3]. Thus, by combining feeder-free culturing systems and taking advantage of enzymatic or nonenzymatic passaging, researchers can now grow hPSCs with relative ease [4]. Over the years, much effort has been dedicated to the development and agreement on a set of standard tests that should be used to characterize and classify hPSCs [5]. These tests were initially defined using human embryonic stem cells (hESCs) and have later been expanded to also include induced PSCs. Such tests include gene and protein expression analysis of pluripotency markers (e.g., Oct3/4, Nanog, Sox2, and Lin28), alkaline phosphate activity, analysis of genomic integrity, and evaluation of differentiation capacity in vitro and in vivo (teratoma analysis). Together, the tests have proven useful to be able to demonstrate the pluripotent phenotype of the undifferentiated cells at the time of their procurement. However, it is important to emphasize that a continuous monitoring of the hPSCs is needed during extended in vitro culture since genomic aberrations or other types of culture adaptations can create a drift in the cultures over time [6]. Spending precious time and resources to implement and uphold rigorous quality routines in the hPSC laboratories is sometimes viewed as less exciting and scientifically less rewarding and is therefore at risk to be down-prioritized in the day-to-day activities. Nevertheless, for all applications of hPSCs, it is obvious that a standardized source of high-quality hPSCs is critical. Self-renewing cultures of the hPSCs most often also contain fractions of differentiated cells that may affect the pluripotent state and the down-stream performance of the cultures. Unfortunately, the vast majority of published articles using hPSCs contain very limited data, which describe the detailed properties of the starting cultures used in the investigations. This sometimes makes it quite difficult for a reader to interpret and assess the findings and conclusions made in the articles. Specifically, reproducing published directed differentiation protocols is a challenging exercise, since even subtle differences between the starting hPSC cultures between laboratories may have a substantial impact on how the cells respond to the differentiation signals [7,8].

Variation between individual hPSCs is often attributed to their having different genetic backgrounds, but there are many other parameters that can influence the behavior and characteristics of the cells. Typically, investigators point to the fact that there are variations in the culture systems used, media formulations, and epigenetic differences between the cells lines. Nevertheless, there is seldom experimental evidence to support that these factors are indeed the

Cellectis Stem Cells, Cellartis AB, Göteborg, Sweden.

explanation for the discrepancies in the data obtained. The standard set of markers and functional tests that are used to characterize the undifferentiated cells are certainly informative and useful for a basic evaluation of the hPSC cultures [5]. However, with regard to being able to predict the hPSC's capacity to differentiate, either spontaneously or in a guided manner to one or a few specific cell types using a directed differentiation approach, a more detailed analysis of the hPSC culture is required.

Recent studies have illustrated that, although the hPSCs display a pluripotent gene profile, the cells may be primed to respond to differentiation cues in a substantially differential manner. In this regard, Blauwkamp et al. elegantly demonstrated that cultures of undifferentiated hESCs were composed of subfractions of cells expressing high or low Wnt activity [8]. Interestingly, the hESC populations displayed comparable expression levels of the typical pluripotency markers *OCT4*, SSEA-4, and TRA-1-60, indicating that these marker expressions were relatively blunt and imprecise in this setting. The authors separated Wnt^{high} from Wnt^{low} hESCs and showed differences between these populations with regard to clonogenic potential, epigenetic status, and differentiation propensity. The Wnt^{high} hESCs seemed to be primed to differentiate toward mesoderm/endoderm, and expressed higher levels of early differentiation markers, such as Brachyury, Goosecoid, Sox 17, and CXCR4. On the other hand, the Wnt^{low} hESCs expressed higher levels of Pax6, a neuroectodermal marker. The gene expression profiles correlated also with lineage-specific differentiation, and the Wnt^{high} hESCs differentiated more efficiently to mesoderm and endoderm derivatives and the Wnt^{low} hESCs were more prone to generate ectoderm. Nevertheless, the Wnt^{high} and Wnt^{low} hESCs generated teratomas when injected into immunocompromized mice demonstrating their maintained pluripotency despite the different levels of Wnt activity. Interestingly, reculturing pure Wnt^{high} hESCs resulted in a heterogeneous population containing both the Wnt^{high} and Wnt^{low} hESCs within 1 passage, indicating a high degree of plasticity of the state of the Wnt activity levels in the cells. This study is an excellent example that illustrates that there is a great need to investigate the heterogeneous nature of hPSC cultures in much more detail. Taken together, these data may help to begin to explain why different laboratories can obtain widely different results when employing the same differentiation protocol on seemingly similar and pluripotent hPSCs.

The complexity of the molecular network regulating pluripotency was also recently illustrated by Wang and colleagues who investigated in detail the roles of *OCT4*, *SOX2*, and *NANOG* in lineage specification of hESCs [7]. Of particular note, and of relevance for the present discussion, the authors demonstrated that the level of *OCT4* expression significantly affected the response of the cells toward BMP4 treatment. Specifically, high levels of *OCT4* in combination with BMP4 resulted in mesendoderm differentiation, while low levels of *OCT4* in combination with BMP4 resulted in extraembryonic lineage differentiation. As indicated above, standard cultures of hPSCs are not homogenous and cells displaying different levels of *OCT4* are most likely present to various degrees in a dynamic manner during extended in vitro culturing and passaging. This is important to consider, especially when subjecting the cells to various differentiation regimes. Accordingly, specific subfractions of cells may respond substantially different to the induction signals, and thus, offer an explanation for the variability in results obtained when subjecting the cells to directed differentiation protocols.

Conclusions

It is clear that our current understanding of the pluripotent state remains limited. The assays and markers that are commonly used to characterize hPSCs are certainly useful, but they also have shortcomings especially in light of the recent studies demonstrating the presence of subfractions of cells with diverse differentiation propensities, which are present in hPSC cultures [7,8]. To be able to efficiently implement hPSC technologies in industries and clinics, it will become increasingly important to be able to transfer protocols for culturing and differentiating hPSCs across laboratories and production facilities worldwide. The success of these efforts will ultimately rely heavily on standardization and quality control of the cells and the processes involved in their handling and differentiation. However, the value of such quality control is set by the specificity and sensitivity of the markers and functional tests that are being carried out, and from a cost-effective perspective, the key issue is to determine a minimum number of tests that can provide the required information. Based on the recent studies [7,8], it may be reasonable to suggest that monitoring and declaration of the Wnt activity as well as, for example, *OCT4* levels should be an integral part of future published studies related to directed hPSC differentiation. Although significant progress have been made, there is a great need for more in-depth research to identify additional critical processes affecting lineage specification as well as studies that will improve our understanding of the pluripotent state. Such studies will pave the way for setting the standard for future developments of hPSC-based technologies in the industrial and clinical settings.

Acknowledgment

This work was supported by the EU-COLIPA funded project SCR & Tox (Grant Agreement No. 266753).

Author Disclosure Statement

Peter Sartipy is employed by Cellectis Stem Cells, Cellartis AB.

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Address correspondence to:
Dr. Peter Sartipy
Cellectis Stem Cells
Cellartis AB
Arvid Wallgrens Backe 20
Göteborg SE-413 46
Sweden

E-mail: peter.sartipy@cellectis.com

Received for publication November 30, 2012
Accepted December 4, 2012
Prepublished on Liebert Instant Online January 18, 2013