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Meeting Report
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Assessing the Safety of Human Pluripotent Stem Cells and Their Derivatives for Clinical Applications

Peter W. Andrews,[1,*] Uri Ben-David,[2] Nissim Benvenisty,[3] Peter Coffey,[4,5] Kevin Eggan,[6,7,8,9] Barbara B. Knowles,[10,11] Andras Nagy,[12,13] Martin Pera,[14,15,16,17,22] Benjamin Reubinoff,[18] Peter J. Rugg-Gunn,[19,20] and Glyn N. Stacey[21]

[1]Department of Biomedical Science, Centre for Stem Cell Biology, University of Sheffield, Sheffield S10 2TN, UK
[2]The Broad Institute, Cambridge, MA 02142, USA
[3]Department of Genetics, The Azrieli Center for Stem Cells and Genetic Research, Silberman Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel
[4]UCL Institute of Ophthalmology, 11–43 Bath Street, London EC1V 9EL, UK
[5]Neuroscience Research Institute, University of California, Santa Barbara, CA 93117, USA
[6]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA
[7]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA
[8]Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
[9]Harvard Stem Cell Institute, Cambridge, MA 02138, USA
[10]The Jackson Laboratory, Bar Harbor, ME 04609, USA
[11]Siriraj Center of Excellence for Stem Cell Research, Mahidol University, Bangkok 10700, Thailand
[12]Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto M5G 1X5, Canada
[13]Monash University, ARMI, Melbourne, Victoria 3800, Australia
[14]Stem Cells Australia, Melbourne Brain Centre, The University of Melbourne, Melbourne, Victoria 3010, Australia
[15]Department of Anatomy and Neurosciences, The University of Melbourne, Melbourne, VIC 3010, Australia
[16]Florey Neuroscience & Mental Health Institute, Melbourne, VIC 3052, Australia
[17]Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC 3052, Australia
[18]Department of Obstetrics and Gynecology, Hadassah Human Embryonic Stem Cells Research Center, The Goldyne-Savad Institute of Gene Therapy, Hadassah-Hebrew University Hospital, Jerusalem 91120, Israel
[19]Epigenetics Programme, The Babraham Institute, Cambridge CB22 3AT, UK
[20]Wellcome Trust – Medical Research Council Cambridge Stem Cell Institute, University of Cambridge, Cambridge, CB2 1QR, UK
[21]UK Stem Cell Bank, Advanced Therapies Division, NIBSC-MHRA, London EN6 3QG, UK
[22]Present address: The Jackson Laboratory, Bar Harbor, ME 04609, USA
*Correspondence: p.w.andrews@sheffield.ac.uk
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Pluripotent stem cells may acquire genetic and epigenetic variants during culture following their derivation. At a conference organized by the International Stem Cell Initiative, and held at The Jackson Laboratory, Bar Harbor, Maine, October 2016, participants discussed how the appearance of such variants can be monitored and minimized and, crucially, how their significance for the safety of therapeutic applications of these cells can be assessed. A strong recommendation from the meeting was that an international advisory group should be set up to review the genetic and epigenetic changes observed in human pluripotent stem cell lines and establish a framework for evaluating the risks that they may pose for clinical use.

It is remarkable that only 20 years after the first report of the growth of human embryonic stem cells *in vitro* and just 10 years after discovering that somatic cells can be reprogrammed to pluripotency as induced pluripotent stem cells (iPSCs), the results of the first clinical trials of pluripotent stem cells (PSCs) to treat macular degeneration have been reported (Mandai et al., 2017; Schwartz et al., 2015; Song et al., 2015). Indeed, clinical trials of PSCs for a number of conditions, including spinal cord injury, diabetes, heart disease, and Parkinson's disease, are already in progress or are just on the horizon (Trounson and DeWitt, 2016). Yet translating these initial trials into routine and safe therapies for the host of conditions for which PSCs offer new opportunities requires a deep understanding of PSC biology. The mechanisms controlling PSC behavior during their *in vitro* isolation, genetic manipulation (if necessary), differentiation, and maintenance are not fully known, and understanding how alterations to these behaviors could have an impact on their fate upon engraftment into humans or the appearance of potentially malignant transformed cells is a requirement for successful stem cell therapeutics.

The central question addressed at this meeting, organized by the International Stem Cell Initiative (ISCI), with support from the UK Regenerative Medicine Platform, was: which of the possible genetic and epigenetic changes that occur in PSCs *in vitro* might compromise the safety and efficacy of PSC-derived products for regenerative medicine? A diverse group of basic scientists ranging from those with expertise in somatic cell and cancer genetics, PSC biology, DNA synthesis and repair, epigenetics and apoptosis, and sensitive systems for variant detection met with those developing and seeking to regulate PSC-based regenerative medicine to discuss this problem.

The genetic changes commonly reported by numerous groups and documented in an ISCI survey of well over

Some participants from the meeting explore Acadia National Park. (Photograph courtesy of Adam Hirst, Stem Cell Technologies).

100 human PSC lines (Amps et al., 2011) are non-random chromosomal alterations, gains of whole, or parts of, chromosomes 1, 12, 17, and 20, as well as losses of regions of chromosome 10, 18, and 22. These changes, which apparently provide a selective growth advantage to the variant cells (Olariu et al., 2010), are readily detected by standard cytogenetic techniques but only if they are present in over 10% of the cells in a PSC line (Baker et al., 2016). Other methods, such as microarray or sequencing analysis, have also uncovered genomic changes, including copy number variants (CNVs) and point mutations that arise during *in vitro* culture. For example, a short region in the proximal part of chromosome 20q was amplified in 22 of 79 PSC lines with an apparently normal G-banded karyotype in the ISCI survey (Amps et al., 2011). This CNV leads to overexpression of *BCL2L1,* with the consequence that these cells can escape apoptosis (Avery et al., 2013). Mutations in *p53,* which were detected by exome sequencing of 117 human PSC lines (Merkle et al., 2017), is also expected to confer protection against apoptosis (Amir et al., 2017), providing a selective advantage to the cells *in vitro*. Recurrent appearance of variants that confer a growth or survival advantage *in vitro* is a worrisome theme, particularly when the changes are known to be associated with human cancer. Epigenetic changes, including erosion of X chromosome inactivation and loss of imprinting, have also been noted in PSCs (Bar et al., 2017; Mekhoubad et al., 2012). The challenge is to understand what impact these epigenetic changes might have on cell phenotype, stability, and growth. One further underexplored consideration is the extent to which epigenetic changes may contribute to the likelihood of acquiring genetic variants in PSCs.

With this background in mind, the assembled scientists at the ISCI meeting set out to discuss three overarching questions regarding the use of PSCs in clinical medicine: (1) How should cells be assayed to detect genetic and epigenetic variants? (2) How can the rate at which variant cells appear in cultures of PSCs be minimized? (3) How can the significance of the presence of particular types of variant cells be assessed as a prelude to ensuring their safety and efficacy *in vivo*?

Detecting and Minimizing the Appearance of Variant Cells

Screening for changes in the genome largely depends on established techniques, including G-band karyotyping, fluorescence in situ hybridization, digital PCR, and microarrays, but the limits of detection for karyotypic changes and CNVs mean that variants may not be noted unless they are present in at least ~10% of the cells in a culture (Baker et al., 2016). Nevertheless, few studies clearly specify the limit of detection of their assays when reporting on

particular PSC lines. Other methods, such as single-cell sequencing, now under development, could improve the sensitivity of detection of abnormalities in mosaic cultures. Several meeting participants pointed out that genetic changes that provide a selective advantage, such as those affecting BCL2L1 or p53 (Avery et al., 2013; Merkle et al., 2017), often become dominant in a culture in a rather short period of time, a factor that might mitigate some concerns over detection sensitivity. However, selective advantage is likely to be context dependent and may come into play only once the cells have been differentiated and/or transplanted, so that a rare variant that does not expand quickly in cultures of undifferentiated PSCs may still pose risk. Minimizing the rates at which variants appear in cultures of PSCs during scale-up and/or differentiation is a goal that might, in part, be achieved by manipulating the culture environment to avoid suboptimal conditions that select for variants. This requires a detailed understanding of the mechanisms and dynamics by which different variant cells gain a growth advantage, information that is lacking in most cases.

An alternative approach is to minimize the rate at which mutations arise in PSCs from errors in DNA synthesis and repair or errors in chromosome segregation at mitosis. Unfortunately, the causes and mutation rates for the common variants in PSCs are unknown and difficult to assess. Although considerable knowledge and expertise exists about the maintenance of genetic and epigenetic integrity in somatic cells and in cancer, we have little knowledge about the extent to which these mechanisms operate in PSCs, or whether other mechanisms may apply in these unique cells. PSCs certainly exhibit distinct cell-cycle regulation and DNA damage responses, probably reflecting the position in development of the early embryonic cells to which they correspond. Indeed, PSCs are an anomaly as they are the only non-transformed diploid cells that can be maintained indefinitely without apparent senescence.

The Potential Significance of Epigenetic and Genetic Variants

It is inevitable, however, that genetic variants will be detected in PSC cultures and, as high-resolution genetic studies continue to accrue, the challenges in interpreting the biological significance of a growing catalog of genetic variation will become more acute. For example, in the earlier ISCI project, we reported that CNVs found in normal, apparently healthy individuals surveyed in the HapMap project, also appeared during the culture of some PSCs, perhaps indicating unstable regions of the genome (Amps et al., 2011). Further, otherwise healthy individuals do harbor subpopulations of cells containing genetic variants of unknown significance, as found in studies of iPSCs derived from skin fibroblasts (Rouhani et al., 2016). A single seemingly pathogenic variant is often not sufficient to lead to cellular transformation, which often requires multiple changes. Nevertheless, since the common recurrent changes seen *in vitro* apparently give a selective growth advantage to the undifferentiated PSCs, they might represent driver mutations capable of promoting teratomas or tumors of differentiated lineages following transplantation (Ben-David et al., 2014).

These issues were discussed side by side with presentations from various groups either conducting or planning PSC-based clinical trials. Although long-term therapeutic strategies with PSCs involve transplanting derivative differentiated cells or adult stem cells, not undifferentiated PSCs, the regulatory requirements for clinical trials discussed at the meeting tended to focus on demonstrating the absence of PSCs in the cell preparations to be transplanted. It is indeed essential to avoid the accidental transplantation of undifferentiated cells, but the more intractable problem is the potential impact on safety caused by variants that lead to malignant transformation, or to some other undesirable change in the particular population of cells required for therapy. In the absence of knowledge about the effects of individual genetic variants on specific adult stem cells or differentiated cell types, existing or proposed strategies for addressing these problems include transplantation to easily resectable sites or encapsulation of the transplanted cells (possible in a few situations such as pancreatic β cells for treating diabetes), or the use of gene editing to introduce conditional suicide genes to provide a fail-safe strategy for eliminating cells after transplantation if a problem arose. On the other hand, representatives of the cancer genomics community pointed out that extensive collaborative studies over the past decade have produced several atlases that document the bulk of the genetic changes that give rise to most types of human cancer. This information should at least provide some perspective on which genetic alterations in PSC lines would be of greatest concern for therapy.

The regulatory implications of these safety issues have been extensively discussed in Japan in relation to trials of iPSC-derived therapeutics, but there is no current international consensus about how they should be addressed. Indeed, it is important to recognize that in many cases we currently lack relevant information to allow an appropriate assessment of potential risks.

Toward an International Consensus on Risk Assessment

The recommendation of this meeting was to establish an international advisory group to collate and curate the information currently in hand while developing an international framework for evaluating the risks posed by genetic and epigenetic variants of PSCs for therapeutic

applications. Assessment in the long run must be context dependent, taking into account patient age, disease severity, the specific cell types to be transplanted, and the sensitivity of the techniques used to characterize the genome and epigenome of the PSCs and their differentiated derivatives. Knowledge from other spheres, such as the cancer genome projects, about the function of genes affected in particular variants, whether they involve copy number changes, or mutations affecting gene regulation or function, should also be considered. The advisory group itself could identify significant knowledge gaps, helping to direct research efforts into the safety assessment of cell therapy. Ideally, it would also curate the genomic information from cells used for clinical trials as the field develops, so that a retrospective review of the phenotypic and functional behavior of the transplanted cells, in particular whether a given variant causes clinical problems, could be achieved. It was noted that the falling costs of genome-wide technologies mean that routine screening of panels of cell lines for subchromosomal genetic variants is now feasible. Ultimately, given the scale of the issues at hand, a coordinated international consensus as to the potential risks posed by the appearance of (epi)genetic variants in PSCs will be necessary to ensure successful realization of the therapeutic potential of PSC-based therapies.

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