



This is a repository copy of *Human pluripotent stem cells: tools for regenerative medicine*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/190226/>

Version: Published Version

Article:

Andrews, P.W. orcid.org/0000-0001-7215-4410 (2021) Human pluripotent stem cells: tools for regenerative medicine. *Biomaterials Translational*, 2 (4). pp. 294-300. ISSN 2096-112X

<https://doi.org/10.12336/biomatertransl.2021.04.004>

Reuse

This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike (CC BY-NC-SA) licence. This licence allows you to remix, tweak, and build upon this work non-commercially, as long as you credit the authors and license your new creations under the identical terms. More information and the full terms of the licence here: <https://creativecommons.org/licenses/>

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

Human pluripotent stem cells: tools for regenerative medicine

Peter W. Andrews*

Key Words:

embryonic stem cell; induced pluripotent stem cell; human; pluripotency; differentiation

From the Contents

Introduction	294
On the History of Pluripotency	295
Evaluation of Pluripotency	295
Controlling Pluripotency	296
Pluripotency, Malignancy and Genetic Variability	297
Conclusions	298

ABSTRACT

Human embryonic stem cells and induced pluripotent stem cells, together denoted as pluripotent stem cells have opened up unprecedented opportunities for developments in human healthcare over the past 20 years. Although much about the properties and behaviour of these cells required to underpin their applications has been discovered over this time, a number of issues remain. This brief review considers the history of these developments and some of the underlying biology, pointing out some of the problems still to be resolved, particularly in relation to their genetic stability and possible malignancy.

The Centre for Stem Cell Biology, The School of Bioscience, The University of Sheffield, Sheffield, UK.

*Corresponding author:
Peter W. Andrews,
p.w.andrews@sheffield.ac.uk.

<http://doi.org/10.12336/biomatertransl.2021.04.004>

How to cite this article:

Andrews, P. W.
Human pluripotent stem cells: tools for regenerative medicine. *Biomater Transl.* 2021, 2(4), 294-300.

Introduction

Co-incident with the first description of human embryonic stem (ES) cells by James Thomson and his colleagues in 1998¹ came the recognition that they offer unprecedented opportunities for providing unlimited supplies of almost any somatic cell that could then be used in transplantation to replace diseased or damaged tissues.^{2, 3} The naiveté associated with some of the early enthusiasm for what became known as regenerative medicine was quickly tempered by the recognition of the many hurdles that had to be overcome before clinical applications could become a reality. Not least among these were the ethical concerns felt by some about the probity of deriving and working with cell lines that required the destruction of human embryos, albeit that these were mostly excess to their use in assisted conception, for which they had been created, and would otherwise have been destroyed. The discovery by Kazutoshi Takahashi and Shinya Yamanaka⁴ that murine somatic cells could be easily reprogrammed to an ES cell like state, cells called induced pluripotent stem (iPS) cells, and the subsequent demonstration that human iPS cells could be similarly produced,^{5, 6} removed some of those concerns, and opened up the

field with a subsequent explosion of research. Reprogramming also offered the possibility of matching the genotype of differentiated cells produced for transplantation with that of a prospective patient, so minimizing the chance of immune rejection.

Commonly, ES and iPS cells are grouped together as pluripotent stem cells (PSC) and despite their different origins, they present many of the same issues that need to be addressed by those seeking to use them in different applications such as regenerative medicine. Among these is the definition of pluripotency itself and how the propensity of PSC lines to differentiate into a range of cell types should be characterized. Assessing pluripotency is a question of particular concern in the production of iPS cells and establishing the extent to which reprogramming back to an ES cell-like state has been achieved. But it can also be a problem with ES cells as they adapt to long term culture. The term 'pluripotency' appears to be deceptively simple, but it has evolved over time. It is helpful to have an understanding of that history to appreciate the connotations attached to the term in different contexts. Another issue is understanding how the balance between self-renewal and differentiation



Human pluripotent stem cells

is controlled and how it may be affected by culture conditions. It is likely that subtle variations in key parameters can affect this balance, which, in turn, could affect our ability to produce required differentiated derivatives in both a reproducible and cost-effective manner. However, perhaps one of the biggest problems is not only the inevitability that cell lines acquire mutations during prolonged passaging in culture, but also the propensity of PSC to acquire particular genetic changes recurrently.⁷ A related concern is the potential for malignant transformation and how this may be associated with genetic or epigenetic changes. Finding ways to minimize the rate at which variant cells appear is key. In this review, I will consider some of the issues that must be considered in working with human PSC and developing applications for regenerative medicine.

On the History of Pluripotency

The development of PSC can be traced back over the past 100 years or more to the study of teratomas, peculiar tumours that typically, though not exclusively, arise in the gonads and contain a jumbled array of cell types, sometimes organized into recognizable tissue structures.^{8,9} Though rare, the testicular version of these tumours is highly malignant and was a leading cause of death in young men until the discovery that cis-platinum offers an effective chemotherapy.¹⁰ Nevertheless, teratomas were largely a medical curiosity until Leroy Stevens discovered that they occur spontaneously in the testes of strain 129 mice.¹¹ Importantly some of these tumours are demonstrably malignant since they can be serially transplanted to successive strain 129 mice, and are therefore termed teratocarcinomas. Histopathology suggested that distinct, small, apparently undifferentiated cells, called embryonal carcinoma (EC) cells, are the stem cells of these tumours, responsible for malignancy as well as the source of the differentiated cells comprising the bulk of the tumour. This notion was confirmed in a landmark study published in 1964 by Lewis Kleinsmith and Barry Pierce.¹² They showed that the transplantation of a single EC cell is sufficient to regenerate a complete teratocarcinoma, confirming that an EC cell is capable of generating new EC cells ('self-renewal') as well as differentiating into derivative cell types, both necessary features of a stem cell. At that time, pluripotency of these murine EC cells was defined by the histology of the teratocarcinomas and the presence of cells corresponding to the three germ layers of the embryo, ectoderm, mesoderm and endoderm, as well as EC cells. Teratomas, which are not retransplantable, and so generally not malignant, are distinguished by their absence of EC cells.¹³

The apparent recapitulation of early embryonic cell differentiation in teratocarcinomas attracted the attention of developmental biologists. Clonal lines of murine EC cells were established and shown to be capable of both differentiating *in vitro* or of forming teratocarcinomas when transplanted to host mice.¹⁴⁻¹⁶ These cells exhibited similar characteristics to the cells in the blastocyst, the inner cell mass, from which all the somatic cells of the embryo are subsequently derived. Most remarkably, it was shown that when EC cells were introduced into a blastocyst that was then re-implanted into a pseudo pregnant female mouse and allowed to develop to term, they

would take part in development and contribute to tissues of the resulting chimeric mouse.¹⁷ This evidence of developmental equivalence of EC cells to the inner cell mass led Martin Evans and Gail Martin,^{18,19} independently, to show that the opposite experiment was possible, namely to explant inner cell mass cells *in vitro* and that, under specialized conditions, these could be maintained indefinitely in culture. These cells, which became known as ES cells, retained the pluripotency characteristic of EC cells, evidenced both by their ability to form teratomas when implanted into adult mice, or to form chimeras when introduced into a blastocyst. Importantly, the chimeras formed from ES cells were typically much more extensive than those formed by EC cells, and included functional germ cells derived from the ES cells. Consequently, the preferred definition of pluripotency shifted to the ability of a cell to form germ cell chimeras.

Building on results from the laboratory mouse, experimental studies human PSC began with EC cell lines isolated from human testicular teratocarcinomas.^{20, 21} Although many of these lines had apparently lost the ability to differentiate, and could be classed as 'nullipotent', probably because of extensive adaptation to tumour growth, several lines, notably NTERA2 and GCT27, did retain the ability to differentiate extensively both as xenograft tumours in immunodeficient mice, or *in vitro*.^{22, 23} Using these lines, a number of markers, mostly cell surface antigens, typically expressed by human EC cells were identified and characterized.²⁴ These same markers were then found to be similarly expressed by human ES cells when eventually derived.^{1, 25, 26} In contrast to the developments in characterizing pluripotency in murine ES cells, for obvious reasons, the germ line chimera test cannot be applied to human PSC, so that the ability to form teratomas in immunodeficient mice again became the gold standard for human pluripotency.

Evaluation of Pluripotency

Although the formation of xenografted teratomas remains the most widely accepted tool for confirming the pluripotency of human PSC, it is a complex and expensive assay, taking several weeks and requiring the expert histology.²⁷ Further, in many countries there are strong restrictions on the use of animals, such as mice, for such research. Consequently, a number of other approaches have become widely used, and have been compared in studies by the International Stem Cell Initiative (ISCI) consortium.^{28, 29}

Differentiation *in vitro* is the most obvious alternative to the teratoma assay, and various approaches are widely used. As with mouse PSC, when human PSC are forced to grow in suspension, they tend to form tight clusters of cells, known as embryoid bodies, in which extensive spontaneous differentiation occurs.^{15, 30} Alternatively, differentiation of monolayer cultures of attached cells can be easily induced by altering culture conditions, including the addition of specific growth factors.^{25, 31} In either case, evidence for the appearance of particular cells can be sought by their expression of particular markers. However, a convenient global approach is to assess the expression of a panel of genes that are indicative of cells corresponding to each of the three germ layers, and produce a 'score card' that compares the extent of differentiation along the

ectoderm, mesoderm and endoderm pathways.^{32,33} In a recent, ISCI study of several human PSC lines,²⁹ the results of this score card approach to analyse embryoid body differentiation were compared to the differentiation occurring in xenograft tumours of the same PSC lines, assessed both by histology and a bioinformatic analysis of transcriptome data, called 'Teratoscore'.³⁴ In this ISCI study, the embryoid bodies were allowed to form under 'neutral' conditions, or in growth media designed to promote, respectively, ectoderm, mesoderm or endoderm differentiation. The differentiation occurring in the embryoid bodies growing in neutral conditions was generally comparable to that detected in the xenograft tumours, whether assessed by histology or Teratoscore. However, it was notable that in different lines there were different biases in the extent of apparent spontaneous differentiation and we termed this their 'propensity' for differentiation. Nevertheless, in each of the lines studied marked differentiation to ectoderm, mesoderm or endoderm was detected when the embryoid bodies were grown in conditions promoting each of those lineages, irrespective of the extent of particular germ layer differentiation under neutral conditions. We termed the results of this directed differentiation their 'potential' to differentiate.

Although functional assessment of pluripotency is essential to provide decisive evidence, even *in vitro* assays are time consuming and expensive. Consequently, surrogate assays for the expression of markers that are characteristically expressed by PSC are widely used, for example, the cell surface antigen markers, such as stage specific antigen (SSEA)-3 and -4, and TRA-1-60, TRA-1-81 and GCTM2.²⁸ Similarly, several transcription factors, notably octamer-binding transcription factor 4, NANOG and SRY-box transcription factor 2, as well as other protein markers such as teratocarcinoma-derived growth factor and alkaline phosphatase, are characteristically expressed by human PSC.²⁸ These are often, incorrectly, referred to as 'pluripotency markers': they are, in reality, only markers of the undifferentiated state of human PSC, so their expression may be 'consistent' with a pluripotent state but it is not definitive. For example, nullipotent EC cells are commonly found in germ cell tumours, most likely arising from PSC that have lost the ability to differentiate while adapting to tumour growth.^{21, 27} Such cells typically express all of the common markers of pluripotent cells.²⁴ Further many of these markers are expressed by other cell types that are unrelated to PSC. For example, SSEA-3 and SSEA-4 are strongly expressed by red blood cells and are part of the P-blood group system,³⁵ whereas markers such as TRA-1-60 and TRA-1-81, although down regulated upon differentiation of human PSC, may also be expressed by a range of other cell types.³⁶ Thus, their value as markers of undifferentiated PSC depends upon the context in which they are used.

An extension of the assessment of the expression of a specific set of markers is to use bioinformatic's analysis of the extended transcriptome of pluripotent stem cells. One algorithm for this that has been widely used is 'Pluritest'.^{29, 37} Although providing a convenient rapid initial screen of presumed PSC, it can also only be regarded as providing evidence consistent with a pluripotent state. Functional assays are still required to confirm pluripotency definitively and to evaluate whether

the propensity of a PSC line to differentiate is biased towards particular lineages.

Controlling Pluripotency

For developing applications, such as for regenerative medicine, pluripotency is a two edge sword. While it provides the opportunity to produce almost any somatic cell type, developing methods to produce efficiently just a single cell type, uncontaminated by other unwanted cells presents major challenges. One of the first applications to be developed, to correct age related macular degeneration, took advantage of the propensity of human PSC to spontaneously differentiate to produce retinal pigment cells when allowed to grow to confluence.³⁸ Although these cultures contained many other cell types, the strongly pigmented nature of the retinal pigment cells allowed them be readily isolated manually from the culture. This proved a practical approach because of the small numbers of cells required for transplantation, and several clinical trials with PSC-derived retinal pigment cells have been initiated over the past 10 years.^{39,40}

For most applications, however, spontaneous differentiation is too inefficient to yield specific cells in sufficient quantities, and a wide range of protocols have been developed to promote the differentiation of specific cell types, typically based upon knowledge from the signalling pathways that control different lineages during embryogenesis. Using this approach, applications for treating Parkinson's disease, diabetes, heart disease and spinal cord injury are well advanced and some have begun clinical trials.⁴¹ This has also been greatly facilitated by discoveries about the core signalling mechanisms that promote self-renewal of human PCS, which indicate that human PSC appear to correspond more closely to a primed state of pluripotency rather than the naïve state of murine PSC.⁴² From this work a number of fully defined culture media have been developed, for example, E8,⁴³ which eliminate the use of feeder cells and minimize or eliminate the use of poorly characterized biological additives such as serum or extracellular matrices.

These defined media minimize interference from extraneous ill-defined factors that may interfere with the specific signalling pathways designed to promote particular lineages of differentiation. However, the nature of PSC themselves can also introduce stochastic confounding factors that also interfere with differentiation protocols. For example, in establishing a protocol for the production of neural crest cells from human PSC, we noted considerable variation from one experiment to another. Eventually we identified the cause of the problem as the production of bone morphogenetic protein (BMP) by the PSC cultures themselves, most likely from minor populations of spontaneously differentiated cells that arise in a variable and stochastic manner.⁴⁴ BMP was one of the factors included in the medium to promote neural crest differentiation, but the exact level of BMP signalling is critical. Variable levels of endogenously produced BMP can easily move the total level of BMP outside the range necessary for neural crest differentiation in an unpredictable manner. Our solution was to include a large excess of BMP in the induction medium, together with a BMP signalling inhibitor, dorsomorphin homolog 1, which was titrated down to achieve an exact level

of BMP signalling.⁴⁴ This approach, which we termed ‘top-down inhibition’, proved very effective in greatly reducing the variability in different runs of the neural crest differentiation protocol. In principle the same strategy could be used in other protocols dependent on cytokines that are also produced by the PSC, or their derivatives, themselves. An opposite approach, which we term ‘baseline activation’, could also be used in which the signalling pathway is completely inhibited by an appropriate antagonist binding to an endogenous ligand in the medium, and the reactivated by careful titration of an appropriate with a small molecule that activates the signalling pathway internally.⁴⁴

Another relevant aspect of PSC biology is the notion that during differentiation fate decisions are probabilistic and that within a population of undifferentiated PSC, the ‘stem cell compartment’, individual cells can be classified into different subpopulations, or substates, with different prospective fates when induced to differentiate.⁴⁵ These substates are interconvertible, with cells moving randomly between them, so that averaged over time all the cells have the same set of potential fates, but at a particular time an individual cell will exhibit a bias in its fate if induced to differentiate.⁴⁶ Using cells carrying fluorescent reporters for genes associated with endoderm or mesoderm differentiation, we were able to isolate PSC subpopulations that showed a bias towards these respective lineages when induced to differentiate, but reverted to other pluripotent substates if maintained in standard PSC medium that supported undifferentiated cultures.^{47, 48} In the case of the mesoderm-biased substate we were also able to define culture conditions that trapped the cells in that substate, permitting their prolonged proliferation.⁴⁸ These trapped cells would revert to a fully pluripotent state if returned to PSC culture conditions, but showed enhanced differentiation to particular mesodermal cell types upon appropriate induction. Strategies to trap cells in such a fully proliferative but biased substates of the stem cell compartment might allow for more efficient production of the fully differentiated cells required for a particular application.

Pluripotency, Malignancy and Genetic Variability

Beyond developing strategies for producing particular differentiated cells, one of the major concerns for PSC-based regenerative medicine is its long term safety driven by concerns about potential malignancy and about the appearance genetic variants in PSC cultures. The close relationship of ES and iPS cells to EC cells from germ cell tumours, and the knowledge that they will form teratomas when grown as xenografts has fueled this concern. However, the discussion is often confused and appreciation of the problem requires consideration of the clinical aspects of germ cell tumours.

One point of confusion is that authors often do not distinguish between the terms teratoma and teratocarcinoma: ‘teratoma’ should be used to denote a tumour that contains a wide range of differentiated cell types but lacks any undifferentiated PSC, whereas ‘teratocarcinoma’ should be used to denote similar tumours that also contain persisting undifferentiated

PSC.^{13, 27} Teratocarcinomas are certainly potentially malignant because of the persisting PSC, but the type of tumour that these produce is obviously another teratocarcinoma. Since it is the differentiated derivatives that will be transplanted for regenerative medicine, the starting point for avoiding the unwanted appearance of a teratocarcinoma is to develop strategies to ensure that the transplanted cells are not contaminated by residual undifferentiated cells.

However, the differentiated cells themselves may also present a possible hazard. Here the biology of teratomas can give some insights but the situation is more complicated and less clear. Generally, teratomas in the laboratory mouse tend to be composed of mature differentiated cells and are not malignant but the clinical situation with human teratomas and the differentiated cells of human teratocarcinomas is rather different.²⁷ First, many of the differentiated tissues in these tumours appear to be composed of histologically immature cells which immediately provokes concerns of possible malignancy. The tumours may also contain elements corresponding to extraembryonic tissues, such as the yolk sac. Although evidence of actual malignancy of any differentiated derivatives of human ES or iPS cells is lacking, it is instructive that in clinical cases of germ cell tumours malignant tumours derived from somatic and extraembryonic elements are occasionally observed,⁴⁹ albeit that human germ cell tumours of adults are highly aneuploidy, which might contribute to the development of malignancy. Consequently, for regenerative medicine, assessing the possibility that the transplanted differentiated cells might develop malignant characteristics is a long term problem that needs to be considered.

It is unclear to what extent genetic changes acquired by human PSC during *in vitro* culture may contribute to malignant transformation. When first derived, ES cells are typically diploid and, unless the embryo from which they were derived came from a family known to carry a disease-associated mutation, they present no immediate concerns about their use for developing therapies. Similarly, iPS cells are typically diploid, although they could carry additional somatically-derived mutations that have arisen in the cells used for reprogramming during the life of the donor.⁵⁰ However, on prolonged passage both ES and iPS cells may acquire genetic changes involving major structural chromosomal rearrangements, acquisition of small copy number variations or point mutations.^{51, 52} Some of these occur recurrently, most likely because they offer a selective growth advantage to the cells. Certainly some of these affect genes, notably *BCL2L1* on chromosome 20 and *TP53* on chromosome 17 are often associated with cancers in other situations.^{53, 54} It is notable that in a recent ISCI study, albeit limited, of several human PSC lines, no correlation was observed between some common karyotypic changes and the persistence of undifferentiated PSC in xenograft tumours.²⁹ Nevertheless, the study was too small and not appropriately designed to comment definitively on the effects of these changes on somatic cell malignancy. In principle, of course, cells known to have acquired mutations would not be used in clinical treatments, but this does mean that cell lines must be continuously monitored for the appearance of such mutations.

A further difficulty here is that the means for detecting genetic changes, particularly karyotypic and copy number variations changes, are not very sensitive and genetic variants present at the level of even 10–20% of a population may be easily missed.⁵⁵

Although, the appearance of recurrent genetic variants in human PSC is well documented, the underlying mutation rate is low, maybe even lower than in somatic cells.⁵⁶ On the other hand, it does seem that these cells are particularly susceptible to DNA damage.⁵⁷ These two apparently contradictory observations can be reconciled by the further observation that, in contrast to somatic cells, PSC tend to respond to DNA damage by apoptosis rather than DNA repair.⁵⁸ This might reflect the requirements of early embryogenesis when cell proliferation is rapid and the appearance of a mutation that would be propagated to many cells of the developing embryo could be catastrophic. Several of the common recurrent genetic changes seem likely to raise the apoptotic threshold of the cells, so that cells carry these particular variants are less likely to die in response to genomic insults and so gain a growth advantage, particularly under suboptimal culture conditions.⁷ In this respect it is notable that including additional exogenous nucleosides in the growth medium of human PSC does seem to reduce their susceptibility to DNA damage and improve their growth and survival,⁵⁷ while culture under low oxygen conditions has also been reported to reduce the mutation rate.⁵⁶

Conclusions

It is remarkable that several clinical trials of regenerative medicine based upon transplanting differentiated derivatives of human PSC have begun, even though it is only just over 20 years since human ES cells were first derived. Nevertheless, as highlighted above, much remains to be done to ensure both cost effective and safe treatments can be made available to large numbers of patients. It is notable that some of the first trials, for age related macular regeneration, and Parkinson's disease, have been for conditions that require relatively few cells to be transferred to confined organs and have been directed to older patients for whom other treatments are lacking. Other possible applications, such as for diabetes, will require many more cells and may be for younger patients for whom other treatments are available. In such cases safety standards will inevitably be more stringent.

Continued developments in understanding the mechanisms that control the behaviour of the undifferentiated stem cells and their differentiation will certainly contribute to strategies for producing large numbers of specific cells in a reproducible and cost-effective manner. Strategies for assessing the safety of the products and their potential for developing cancers, however, remain very much in flux without any consensus. Certainly it would be unwise to transplant cells with known karyotypic changes or that have acquired point mutations such as those that occur in *TP53* and are well known to be associated with cancer. Nevertheless, the sensitivity of current assays means that variant cells may remain undetected. Also, although it may be minimised by new culture strategies, genetic variation in cultured cells is inevitable. Indeed, many of the genetic variants that arise in human PSC may in practice have little or no clinical

significance. To identify those that could cause problems is one of the remaining problems for PSC based regenerative medicine. It is unlikely animal models of tumourigenicity will prove useful. More likely, progress will come from a thorough understanding of the consequences of particular mutations for cellular behaviour linked to a bioinformatic analysis of cancer genetics and the role of different mutations for tumourigenesis in humans.

Author contributions

PWA is the sole author, who conceived and drafted the review, and approved the final version of the manuscript.

Financial support

The work was supported by the European Union's Horizon 2020 Research and Innovation program H2020-FETPROACT-2018-01 under grant agreement No. 824070, and by grant MR/V002163/1 from the Medical Research Council, UK.

Acknowledgement

None.

Conflicts of interest statement

The author receives royalty payments from the Wistar Institute from licencing of the hybridomas producing the TRA and VIN series of monoclonal antibodies.

Open access statement

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

1. Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S.; Jones, J. M. Embryonic stem cell lines derived from human blastocysts. *Science*. **1998**, *282*, 1145-1147.
2. Gearhart, J. D. New potential for human embryonic stem cells. *Science*. **1998**, *282*, 1061-1062.
3. Trounson, A.; Pera, M. Potential benefits of cell cloning for human medicine. *Reprod Fertil Dev*. **1998**, *10*, 121-125.
4. Takahashi, K.; Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. **2006**, *126*, 663-676.
5. Takahashi, K.; Tanabe, K.; Ohnuki, M.; Narita, M.; Ichisaka, T.; Tomoda, K.; Yamanaka, S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. **2007**, *131*, 861-872.
6. Yu, J.; Vodyanik, M. A.; Smuga-Otto, K.; Antosiewicz-Bourget, J.; Frane, J. L.; Tian, S.; Nie, J.; Jonsdottir, G. A.; Ruotti, V.; Stewart, R.; Slukvin, I.; Thomson, J. A. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. **2007**, *318*, 1917-1920.
7. Halliwell, J.; Barbaric, I.; Andrews, P. W. Acquired genetic changes in human pluripotent stem cells: origins and consequences. *Nat Rev Mol Cell Biol*. **2020**, *21*, 715-728.
8. Damjanov, I.; Solter, D. Experimental teratoma. *Curr Top Pathol*. **1974**, *59*, 69-130.
9. Andrews, P. W. From teratocarcinomas to embryonic stem cells. *Philos Trans R Soc Lond B Biol Sci*. **2002**, *357*, 405-417.
10. Einhorn, L. H. Treatment strategies of testicular cancer in the United States. *Int J Androl*. **1987**, *10*, 399-405.
11. Stevens, L. C.; Little, C. C. Spontaneous testicular teratomas in an inbred strain of mice. *Proc Natl Acad Sci U S A*. **1954**, *40*, 1080-1087.
12. Kleinsmith, L. J.; Pierce, G. B., Jr. Multipotentiality of single embryonal carcinoma cells. *Cancer Res*. **1964**, *24*, 1544-1551.

13. Damjanov, I.; Andrews, P. W. The terminology of teratocarcinomas and teratomas. *Nat Biotechnol.* **2007**, *25*, 1212; discussion 1212.
14. Evans, M. J. The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratoma cells. *J Embryol Exp Morphol.* **1972**, *28*, 163-176.
15. Martin, G. R.; Evans, M. J. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proc Natl Acad Sci U S A.* **1975**, *72*, 1441-1445.
16. Nicolas, J. F.; Dubois, P.; Jakob, H.; Gaillard, J.; Jacob, F. Tératocarcinome de la souris: différenciation en culture d'une lignée de cellules primitives à potentialités multiples. *Ann Microbiol Inst Pasteur A.* **1975**, *126*, 3-22.
17. Brinster, R. L. The effect of cells transferred into the mouse blastocyst on subsequent development. *J Exp Med.* **1974**, *140*, 1049-1056.
18. Evans, M. J.; Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* **1981**, *292*, 154-156.
19. Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A.* **1981**, *78*, 7634-7638.
20. Hogan, B.; Fellous, M.; Avner, P.; Jacob, F. Isolation of a human teratoma cell line which expresses F9 antigen. *Nature.* **1977**, *270*, 515-518.
21. Andrews, P. W.; Bronson, D. L.; Benham, F.; Strickland, S.; Knowles, B. B. A comparative study of eight cell lines derived from human testicular teratocarcinoma. *Int J Cancer.* **1980**, *26*, 269-280.
22. Andrews, P. W.; Damjanov, I.; Simon, D.; Banting, G. S.; Carlin, C.; Dracopoli, N. C.; Føgh, J. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. *Lab Invest.* **1984**, *50*, 147-162.
23. Pera, M. F.; Cooper, S.; Mills, J.; Parrington, J. M. Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. *Differentiation.* **1989**, *42*, 10-23.
24. Andrews, P. W.; Casper, J.; Damjanov, I.; Duggan-Keen, M.; Giwercman, A.; Hata, J.; von Keitz, A.; Looijenga, L. H.; Millán, J. L.; Oosterhuis, J. W.; Pera, M.; Sawada, M.; Schmoll, H. J.; Skakkebaek, N. E.; van Putten, W.; Stern, P. Comparative analysis of cell surface antigens expressed by cell lines derived from human germ cell tumours. *Int J Cancer.* **1996**, *66*, 806-816.
25. Reubinoff, B. E.; Pera, M. F.; Fong, C. Y.; Trounson, A.; Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol.* **2000**, *18*, 399-404.
26. Draper, J. S.; Pigott, C.; Thomson, J. A.; Andrews, P. W. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat.* **2002**, *200*, 249-258.
27. Damjanov, I.; Andrews, P. W. Teratomas produced from human pluripotent stem cells xenografted into immunodeficient mice - a histopathology atlas. *Int J Dev Biol.* **2016**, *60*, 337-419.
28. International Stem Cell Initiative; Adewumi, O.; Aflatoonian, B.; Ahrlund-Richter, L.; Amit, M.; Andrews, P. W.; Beighton, G.; Bello, P. A.; Benvenisty, N.; Berry, L. S.; Bevan, S.; Blum, B.; Brooking, J.; Chen, K. G.; Choo, A. B.; Churchill, G. A.; Corbel, M.; Damjanov, I.; Draper, J. S.; Dvorak, P.; Emanuelsson, K.; Fleck, R. A.; Ford, A.; Gertow, K.; Gertsenstein, M.; Gokhale, P. J.; Hamilton, R. S.; Hampl, A.; Healy, L. E.; Hovatta, O.; Hyllner, J.; Imreh, M. P.; Itskovitz-Eldor, J.; Jackson, J.; Johnson, J. L.; Jones, M.; Kee, K.; King, B. L.; Knowles, B. B.; Lako, M.; Lebrin, F.; Mallon, B. S.; Manning, D.; Mayshar, Y.; McKay, R. D.; Michalska, A. E.; Mikkola, M.; Mileikovsky, M.; Minger, S. L.; Moore, H. D.; Mummery, C. L.; Nagy, A.; Nakatsuji, N.; O'Brien, C. M.; Oh, S. K.; Olsson, C.; Otonkoski, T.; Park, K. Y.; Passier, R.; Patel, H.; Patel, M.; Pedersen, R.; Pera, M. F.; Piekarczyk, M. S.; Pera, R. A.; Reubinoff, B. E.; Robins, A. J.; Rossant, J.; Rugg-Gunn, P.; Schulz, T. C.; Semb, H.; Sherrer, E. S.; Siemen, H.; Stacey, G. N.; Stojkovic, M.; Suemori, H.; Szatkiewicz, J.; Turetsky, T.; Tuuri, T.; van den Brink, S.; Vintersten, K.; Vuoristo, S.; Ward, D.; Weaver, T. A.; Young, L. A.; Zhang, W. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol.* **2007**, *25*, 803-816.
29. International Stem Cell Initiative. Assessment of established techniques to determine developmental and malignant potential of human pluripotent stem cells. *Nat Commun.* **2018**, *9*, 1925.
30. Itskovitz-Eldor, J.; Schuldiner, M.; Karsenti, D.; Eden, A.; Yanuka, O.; Amit, M.; Soreq, H.; Benvenisty, N. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med.* **2000**, *6*, 88-95.
31. Schuldiner, M.; Yanuka, O.; Itskovitz-Eldor, J.; Melton, D. A.; Benvenisty, N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A.* **2000**, *97*, 11307-11312.
32. Bock, C.; Kiskinis, E.; Verstappen, G.; Gu, H.; Boulting, G.; Smith, Z. D.; Ziller, M.; Croft, G. F.; Amoroso, M. W.; Oakley, D. H.; Gnirke, A.; Eggan, K.; Meissner, A. Reference maps of human ES and iPSC cell variation enable high-throughput characterization of pluripotent cell lines. *Cell.* **2011**, *144*, 439-452.
33. Tsankov, A. M.; Akopian, V.; Pop, R.; Chetty, S.; Gifford, C. A.; Daheron, L.; Tsankova, N. M.; Meissner, A. A qPCR ScoreCard quantifies the differentiation potential of human pluripotent stem cells. *Nat Biotechnol.* **2015**, *33*, 1182-1192.
34. Avior, Y.; Biancotti, J. C.; Benvenisty, N. TeratoScore: assessing the differentiation potential of human pluripotent stem cells by quantitative expression analysis of teratomas. *Stem Cell Reports.* **2015**, *4*, 967-974.
35. Tippett, P.; Andrews, P. W.; Knowles, B. B.; Solter, D.; Goodfellow, P. N. Red cell antigens P (globoside) and Luke: identification by monoclonal antibodies defining the murine stage-specific embryonic antigens -3 and -4 (SSEA-3 and SSEA-4). *Vox Sang.* **1986**, *51*, 53-56.
36. Andrews, P. W.; Banting, G.; Damjanov, I.; Arnaud, D.; Avner, P. Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. *Hybridoma.* **1984**, *3*, 347-361.
37. Müller, F. J.; Schuldt, B. M.; Williams, R.; Mason, D.; Altun, G.; Papapetrou, E. P.; Danner, S.; Goldmann, J. E.; Herbst, A.; Schmidt, N. O.; Aldenhoff, J. B.; Laurent, L. C.; Loring, J. F. A bioinformatic assay for pluripotency in human cells. *Nat Methods.* **2011**, *8*, 315-317.
38. Vugler, A.; Carr, A. J.; Lawrence, J.; Chen, L. L.; Burrell, K.; Wright, A.; Lundh, P.; Semo, M.; Ahmado, A.; Gias, C.; da Cruz, L.; Moore, H.; Andrews, P.; Walsh, J.; Coffey, P. Elucidating the phenomenon of HESC-derived RPE: anatomy of cell genesis, expansion and retinal transplantation. *Exp Neurol.* **2008**, *214*, 347-361.
39. Schwartz, S. D.; Regillo, C. D.; Lam, B. L.; Elliott, D.; Rosenfeld, P. J.; Gregori, N. Z.; Hubschman, J. P.; Davis, J. L.; Heilwell, G.; Spirn, M.; Maguire, J.; Gay, R.; Bateman, J.; Ostrick, R. M.; Morris, D.; Vincent, M.; Anglade, E.; Del Priore, L. V.; Lanza, R. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet.* **2015**, *385*, 509-516.
40. da Cruz, L.; Fynes, K.; Georgiadis, O.; Kerby, J.; Luo, Y. H.; Ahmado, A.; Vernon, A.; Daniels, J. T.; Nommiste, B.; Hasan, S. M.; Gooljar, S.

- B.; Carr, A. F.; Vugler, A.; Ramsden, C. M.; Bictash, M.; Fenster, M.; Steer, J.; Harbinson, T.; Wilbrey, A.; Tufail, A.; Feng, G.; Whitlock, M.; Robson, A. G.; Holder, G. E.; Sagoo, M. S.; Loudon, P. T.; Whiting, P.; Coffey, P. J. Phase 1 clinical study of an embryonic stem cell-derived retinal pigment epithelium patch in age-related macular degeneration. *Nat Biotechnol.* **2018**, *36*, 328-337.
41. Kimbrel, E. A.; Lanza, R. Pluripotent stem cells: the last 10 years. *Regen Med.* **2016**, *11*, 831-847.
 42. Rossant, J.; Tam, P. P. L. New insights into early human development: lessons for stem cell derivation and differentiation. *Cell Stem Cell.* **2017**, *20*, 18-28.
 43. Chen, G.; Gulbranson, D. R.; Hou, Z.; Bolin, J. M.; Ruotti, V.; Probasco, M. D.; Smuga-Otto, K.; Howden, S. E.; Diol, N. R.; Propson, N. E.; Wagner, R.; Lee, G. O.; Antosiewicz-Bourget, J.; Teng, J. M.; Thomson, J. A. Chemically defined conditions for human iPSC derivation and culture. *Nat Methods.* **2011**, *8*, 424-429.
 44. Hackland, J. O. S.; Frith, T. J. R.; Thompson, O.; Marin Navarro, A.; Garcia-Castro, M. I.; Unger, C.; Andrews, P. W. Top-down inhibition of BMP signaling enables robust induction of hpscs into neural crest in fully defined, xeno-free conditions. *Stem Cell Reports.* **2017**, *9*, 1043-1052.
 45. Enver, T.; Pera, M.; Peterson, C.; Andrews, P. W. Stem cell states, fates, and the rules of attraction. *Cell Stem Cell.* **2009**, *4*, 387-397.
 46. Tonge, P. D.; Olariu, V.; Coca, D.; Kadiramanathan, V.; Burrell, K. E.; Billings, S. A.; Andrews, P. W. Prepatterning in the stem cell compartment. *PLoS One.* **2010**, *5*, e10901.
 47. Allison, T. F.; Smith, A. J. H.; Anastasiadis, K.; Sloane-Stanley, J.; Biga, V.; Stavish, D.; Hackland, J.; Sabri, S.; Langerman, J.; Jones, M.; Plath, K.; Coca, D.; Barbaric, I.; Gokhale, P.; Andrews, P. W. Identification and single-cell functional characterization of an endodermally biased pluripotent substate in human embryonic stem cells. *Stem Cell Reports.* **2018**, *10*, 1895-1907.
 48. Stavish, D.; Böiers, C.; Price, C.; Frith, T. J. R.; Halliwell, J.; Saldaña-Guerrero, I.; Wray, J.; Brown, J.; Carr, J.; James, C.; Barbaric, I.; Andrews, P. W.; Enver, T. Generation and trapping of a mesoderm biased state of human pluripotency. *Nat Commun.* **2020**, *11*, 4989.
 49. Ahmed, T.; Bosl, G. J.; Hajdu, S. I. Teratoma with malignant transformation in germ cell tumors in men. *Cancer.* **1985**, *56*, 860-863.
 50. Rouhani, F. J.; Nik-Zainal, S.; Wuster, A.; Li, Y.; Conte, N.; Koike-Yusa, H.; Kumasaka, N.; Vallier, L.; Yusa, K.; Bradley, A. Mutational history of a human cell lineage from somatic to induced pluripotent stem cells. *PLoS Genet.* **2016**, *12*, e1005932.
 51. International Stem Cell Initiative; Amps, K.; Andrews, P. W.; Anyfantis, G.; Armstrong, L.; Avery, S.; Baharvand, H.; Baker, J.; Baker, D.; Munoz, M. B.; Beil, S.; Benvenisty, N.; Ben-Yosef, D.; Biancotti, J. C.; Bosman, A.; Brena, R. M.; Brison, D.; Caisander, G.; Camarasa, M. V.; Chen, J.; Chiao, E.; Choi, Y. M.; Choo, A. B.; Collins, D.; Colman, A.; Crook, J. M.; Daley, G. Q.; Dalton, A.; De Sousa, P. A.; Denning, C.; Downie, J.; Dvorak, P.; Montgomery, K. D.; Feki, A.; Ford, A.; Fox, V.; Fraga, A. M.; Frumkin, T.; Ge, L.; Gokhale, P. J.; Golan-Lev, T.; Gourabi, H.; Gropp, M.; Lu, G.; Hampl, A.; Harron, K.; Healy, L.; Herath, W.; Holm, F.; Hovatta, O.; Hyllner, J.; Inamdar, M. S.; Irwanto, A. K.; Ishii, T.; Jaconi, M.; Jin, Y.; Kimber, S.; Kiselev, S.; Knowles, B.; Kopper, O.; Kukharensko, V.; Kuliev, A.; Lagarkova, M. A.; Laird, P. W.; Lako, M.; Laslett, A. L.; Lavon, N.; Lee, D. R.; Lee, J. E.; Li, C.; Lim, L. S.; Ludwig, T. E.; Ma, Y.; Maltby, E.; Mateizel, I.; Mayshar, Y.; Mileikovsky, M.; Minger, S. L.; Miyazaki, T.; Moon, S. Y.; Moore, H.; Mummery, C.; Nagy, A.; Nakatsuji, N.; Narwani, K.; Oh, S. K.; Oh, S. K.; Olson, C.; Otonkoski, T.; Pan, F.; Park, I. H.; Pells, S.; Pera, M. F.; Pereira, L. V.; Qi, O.; Raj, G. S.; Reubinoff, B.; Robins, A.; Robson, P.; Rossant, J.; Salekdeh, G. H.; Schulz, T. C.; Sermon, K.; Sheik Mohamed, J.; Shen, H.; Sherrer, E.; Sidhu, K.; Sivarajah, S.; Skottman, H.; Spits, C.; Stacey, G. N.; Strehl, R.; Strelchenko, N.; Suemori, H.; Sun, B.; Suuronen, R.; Takahashi, K.; Tuuri, T.; Venu, P.; Verlinsky, Y.; Ward-van Oostwaard, D.; Weisenberger, D. J.; Wu, Y.; Yamanaka, S.; Young, L.; Zhou, Q. Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage. *Nat Biotechnol.* **2011**, *29*, 1132-1144.
 52. Merkle, F. T.; Ghosh, S.; Kamitaki, N.; Mitchell, J.; Avior, Y.; Mello, C.; Kashin, S.; Mekhoubad, S.; Ilic, D.; Charlton, M.; Saphier, G.; Handsaker, R. E.; Genovese, G.; Bar, S.; Benvenisty, N.; McCarroll, S. A.; Eggan, K. Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. *Nature.* **2017**, *545*, 229-233.
 53. Sillars-Hardebol, A. H.; Carvalho, B.; Beliën, J. A.; de Wit, M.; Delis-van Diemen, P. M.; Tijssen, M.; van de Wiel, M. A.; Pontén, F.; Fijneman, R. J.; Meijer, G. A. BCL2L1 has a functional role in colorectal cancer and its protein expression is associated with chromosome 20q gain. *J Pathol.* **2012**, *226*, 442-450.
 54. Baugh, E. H.; Ke, H.; Levine, A. J.; Bonneau, R. A.; Chan, C. S. Why are there hotspot mutations in the TP53 gene in human cancers? *Cell Death Differ.* **2018**, *25*, 154-160.
 55. Baker, D.; Hirst, A. J.; Gokhale, P. J.; Juarez, M. A.; Williams, S.; Wheeler, M.; Bean, K.; Allison, T. F.; Moore, H. D.; Andrews, P. W.; Barbaric, I. Detecting genetic mosaicism in cultures of human pluripotent stem cells. *Stem Cell Reports.* **2016**, *7*, 998-1012.
 56. Thompson, O.; von Meyenn, F.; Hewitt, Z.; Alexander, J.; Wood, A.; Weightman, R.; Gregory, S.; Krueger, F.; Andrews, S.; Barbaric, I.; Gokhale, P. J.; Moore, H. D.; Reik, W.; Milo, M.; Nik-Zainal, S.; Yusa, K.; Andrews, P. W. Low rates of mutation in clinical grade human pluripotent stem cells under different culture conditions. *Nat Commun.* **2020**, *11*, 1528.
 57. Halliwell, J. A.; Frith, T. J. R.; Laing, O.; Price, C. J.; Bower, O. J.; Stavish, D.; Gokhale, P. J.; Hewitt, Z.; El-Khamisy, S. F.; Barbaric, I.; Andrews, P. W. Nucleosides rescue replication-mediated genome instability of human pluripotent stem cells. *Stem Cell Reports.* **2020**, *14*, 1009-1017.
 58. Desmarais, J. A.; Hoffmann, M. J.; Bingham, G.; Gagou, M. E.; Meuth, M.; Andrews, P. W. Human embryonic stem cells fail to activate CHK1 and commit to apoptosis in response to DNA replication stress. *Stem Cells.* **2012**, *30*, 1385-1393.

Received: November 13, 2021

Revised: December 12, 2021

Accepted: December 20, 2021

Available online: December 28, 2021