VIEWPOINT

Induced pluripotent stem cells and reprogramming: seeing the science through the hype

Juan Carlos Izpisúa Belmonte, James Ellis, Konrad Hochedlinger and Shinya Yamanaka

Abstract | No-one can have failed to notice the splash that induced pluripotent stem (iPS) cells have made in the few years since somatic cells were first reprogrammed to pluripotency. But what is their real promise, where should research efforts be focused, and are we at a stage where we can replace embryonic stem cells? Four pioneering iPS cell researchers offer their personal insights into these and other questions of current debate. As well expressing hope for the improved understanding and treatment of human disease, they urge caution over safety and propose the establishment of iPS cell banks.

What has been the most significant recent discovery in induced pluripotent stem (iPS) cell research?

Juan Carlos Izpisúa Belmonte. The recent data obtained by different laboratories establishing a link between reprogramming and the p53 pathway are certainly very interesting[1–7]. One of the barriers that cells need to overcome during reprogramming is the initial stress generated by the ectopic expression of reprogramming factors, which is likely to induce apoptosis, senescence, decreased cell viability, and so on. Activation of the p53 pathway seems to have a major role in this process. Together, these discoveries underscore how stressful reprogramming can be for a cell and highlight how increasing reprogramming efficiency at the expense of removing a major cell damage surveillance system is a price that it might not be wise to pay. These results establish a direct connection between reprogramming and tumorigenesis. As all tumours have a defective p53 pathway, and only two reprogramming factors are needed to reprogram p53-deficient cells, it is tempting to speculate that some of the myriad genetic and epigenetic alterations that are commonly found in p53-deficient cells might phenocopy OCT4 (also known as POU5F1) and SOX2 functions to enable their reprogramming. Therefore, although recent studies raise the possibility that tumours arise from genetic changes in stem cells or proliferative progenitors that transform them into stem-like cells, these data make it reasonable to once again consider the possibility that 'de-differentiation' also contributes to the cellular complexity that is evident in many tumours.

James Ellis. The great near-term potential of iPS cells is to model human disease to identify phenotypes for use in drug screens or toxicity tests. The most important recent discoveries to my mind are those that progress towards these goals. For spinal muscular atrophy (SMA), the neuronal pathology was modelled and reversed with valproic acid, establishing the principle that drug screens could be performed using patient iPS cells[8]. More recently, familial dysautonomia (FD) iPS cell lines were used to identify neurogenic differentiation and migration phenotypes *in vitro*, and these studies demonstrated that the splicing defect in this condition could be corrected with the candidate compound kinetin[9]. Finally, Belmonte's group corrected Fanconi's anaemia by lentivector gene transfer to produce blood progenitors that are disease-free in short-term repopulating assays in mice[10]. These three human iPS cell studies used standard virus-based reprogramming methods and demonstrated that iPS cells can be used for modelling disease. The challenge now is to build on these foundations to improve patient iPS cell models of disease beyond these pioneering neuronal and haematopoietic disorders, and to use *in vitro* phenotypes to discover novel drug treatments that are effective *in vivo*.

Konrad Hochedlinger. I think two recent discoveries have considerably advanced the field of iPS cell research and have brought it one step closer towards modelling and treating disease. The first advance has been the proof-of-principle demonstration that iPS cells can be derived from skin cells from patients with a variety of diseases[8,9,11,12], including Parkinson's disease, diabetes mellitus, amyotrophic lateral sclerosis (ALS), SMA and FD. Some of these cells seem to show the pathological phenotypes that are seen in patients. These are all diseases for which we currently have a poor understanding of the underlying mechanisms and no good cellular models or treatments. Patient-specific iPS cells will help to establish *in vitro* disease models and might lead to the discovery of drugs for treating patients.

The other advance has been the demonstration that iPS cells can be generated without viral integration in mouse and human cells using a variety of approaches, such as adenoviruses, plasmids, transposons or recombinant proteins[13–16]. This discovery eliminated a major roadblock for translating iPS cell technology into a therapeutic setting. Although cell therapy using iPS technology is still quite some distance away, the possibility that transgene-free custom tailored cells can be derived is an important step in this direction and will aid in efforts to direct iPS cells *in vitro* into functional cells for therapeutic purposes.

Shinya Yamanaka. I would like to highlight the findings from one of our own papers — 'Variation in the safety of induced pluripotent stem cell lines'[17] — regarding the future clinical applications of iPS cells. We found that the origin of the iPS cells had a profound influence on the teratoma-forming propensity in a cell transplantation therapy model using mouse secondary neurospheres differentiated from iPS cells. This propensity correlates with the number of undifferentiated cells that persist in the secondary neurospheres. Tail-tip fibroblast-derived iPS cells showed the highest propensity, whereas those from embryonic fibroblasts showed the lowest. In addition, hepatocyte- and stomach-derived iPS cells showed intermediate results. Until now, numerous human iPS cell clones have been generated from a variety of cells, including skin fibroblasts, bone marrow cells, keratinocytes, neural stem cells, peripheral blood cells and fat cells. It is therefore extremely important to examine and elucidate the impact of such origins on both the safety and the properties of human iPS cells.

Q *How far do we need to understand the basic biology of reprogramming and iPS cells to be able to use these cells?*

J.C.I.B. Obviously, the more we know, the better we will be able to control them and use them safely. If our understanding is not complete before we reach the clinic, then we may be treating diseases from a disadvantaged position. Nonetheless, although it is always reassuring to know completely what is going on, if and when (but not before) we are sure a given treatment works, we should use it, even if we do not know why it works. A case in point is the advent of bone marrow transplants, which were developed in the 1950s and 1960s and used successfully for the first time in 1968, long before we gained molecular knowledge of the workings of the haematopoietic stem cell (HSC) system. The HSC system in particular is an area in which clinical applications might be realized the fastest from the basic research that is taking place with embryonic stem (ES) and iPS cells. It is a tissue in which transplanted cells do not seem to require a defined three-dimensional structure to be functional and, moreover, HSCs have already been studied and used successfully in the clinic.

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*Listed in alphabetical order.

J.E. It is important to understand the basic biology in order to simplify the identification of fully reprogrammed iPS cells. Basic research into the intermediate states of reprogramming will help in the development of methods to recognize partially reprogrammed cells more efficiently and to convert them to fully reprogrammed cells. The barriers to reprogramming that block cells in a partially reprogrammed state include: apoptotic or senescence pathways in the p53 network; growth conditions, such as oxygen content and suboptimal media components; and epigenetic modifications, including restoration of the pluripotent DNA methylome and bivalent histone modifications. Although most evidence points to stochastic mechanisms for traversing these barriers, it is an appealing possibility that 'elite' cells, such as blood and neural progenitors, are predisposed to reprogramming by having fewer barriers to efficient reprogramming and by requiring fewer reprogramming factors. By understanding the barriers and, in particular, the predispositions, we can more effectively produce fully reprogrammed cells by methods that might allow iPS cell induction from banked cord blood or other cell types that are available in cell repositories. At the same time, it might be advantageous to generate genuine adult cell types by directly reprogramming them into progenitor cells instead of inducing pluripotency.

K.H. I think the answer depends on the use of iPS cells. If they are used for drug screening of, for example, ALS, it may be sufficient to know that iPS cells can produce motor neurons that die in culture and therefore recapitulate the disease as it occurs in patients. You might not care about the potential of the cells to make the 219 other cell types of the body. However, when considering the possible transplantation of iPS cell-derived neurons into patients, I believe that we need to gather more information on their full differentiation potential and long-term behaviour in animal models. For example, it is still unclear why so many iPS cell lines are not as potent as ES cell lines derived from embryos. Moreover, recent reports claim that there are significant transcriptional differences between ES and iPS cells, the functional consequences of which remain unclear. Therefore, a thorough molecular comparison of ES and iPS cells and an assessment of their full differentiation potential is, in my opinion, key before considering taking these cells into the clinic.

S.Y. There are two distinct types of applications for iPS cells — regenerative medicine and *in vitro* usage. *In vitro* applications — including the development of disease models, drug screening and toxicology — are just around the corner. By contrast, for the use of iPS cells in regenerative medicine, safety remains the highest hurdle still to be overcome. More precisely, we need to double-check that we would not see any tumour formation after transplantation into patients. Two types of tumours should, therefore, be distinguished: tumours caused by transgene integration and teratomas caused by the persistence of undifferentiated cells. To avoid transgene-initiated tumours, the generation of iPS cells without transgene integration is considered to be important. Although several methods, such as those using plasmids or recombinant proteins, have been reported, the efficiency of integration-free iPS cell generation still continues to be too low. We need to elucidate the process of iPS cell generation more fully to increase the efficiency to a more practical level. As for teratomas, we need to understand why each iPS cell clone demonstrates a different proportion of undifferentiated cells after *in vitro* differentiation, as well as why such clones have a propensity to form teratomas.

Q *How far are we from using iPS cells in the clinic?*

J.C.I.B. One must be very cautious when speculating about time frames. The excitement over iPS cells is not without reason, as their potential is clearly enormous. Let me cite an example from our own work: we have recently generated disease-corrected haematopoietic progenitors from Fanconi's anaemia iPS cells[10]. These data offer proof-of-concept that iPS cell technology could be used for the generation of disease-corrected, patient-specific cells, but the enthusiasm of bringing these cells to patients with the disease should be tempered by prudence. I am of the opinion that with so many laboratories and resources dedicated to advancing the field, it is only a matter of time until the basic technology can be translated into specific clinical applications. On the other hand, we should avoid creating overly optimistic expectations in the patients, public, media, funding agencies and even in our own minds. One is reminded of the experience of the gene therapy field in the late 1980s and 1990s, when the technology was hailed as the new cure for many diseases and, in some cases, the rush to the clinic led to some high profile failures that could have been avoided, as could the backlash that followed. I think we will see several years of basic research and technology development that will transition into preclinical research with animal models and preclinical trials in human patients. This might be punctuated with early attempts to use iPS cells directly in humans in particular cases in which the patient's best, or even only, option might be an unproven iPS cell-based therapy. It will take time.

J.E. iPS cells for the clinic must meet the most exacting criteria. Being inherently tumorigenic makes them more dangerous than adult stem cells or somatic cell gene therapy, and even unmodified fetal neural stem cell therapy has transmitted tumours to patients. Some cell sources might have a lower teratoma-forming propensity and might be preferable for clinical-grade reprogramming. For genetic disorders, mutations could be corrected using gene transfer, or zinc finger nucleases could be used to make targeted corrections or to direct transgenes to locations in the genome in which they will not cause adverse effects.

To use iPS cells in therapy, one must make the affected cell type. Sickle cell anaemia was corrected by homologous recombination in mouse iPS cells for the transplantation of HSCs[18]. However, it has not been possible to generate transplantable HSCs from human pluripotent cells. Therefore, using reporter genes, such as ErythRED, to optimize differentiation protocols will be valuable. Likewise, our own early transposon promoter and *OCT4* and *SOX2* enhancer (EOS) pluripotency reporter[19] could monitor the presence of undifferentiated iPS cells, and *in vivo* reporters or suicide vectors could track the overproliferation of transplanted cells and ablate them *in vitro* or in patients. It is essential that the transgenes used for reprogramming are deleted to prevent their reactivation, but there is a strong rationale for using suicide genes to enhance safety. This is the best direction for gene therapy, and we should remain open to this argument for iPS cell therapies too.

K.H. I believe we are still years away from using these cells in a clinical setting for two reasons. First, we do not yet fully understand the biology of iPS cells. Although we know that some iPS cells can do everything that ES cells can do, we still do not understand why some iPS cells are not as versatile

as ES cells. Moreover, we cannot exclude the possibility that some iPS cells show unwanted side effects that ES cells would not normally show — for instance, studies in mice suggest that some of the animals produced with iPS cells succumb to cancer. Even though the technology is available to derive iPS cells without viral integration, which might have caused some of the cancers, there is insufficient data at this point to argue that all virus-free iPS cells are qualitatively equivalent to ES cells. As reprogramming is very inefficient, it may be that rare cells are often selected that carry certain genetic or epigenetic abnormalities that favour their reprogramming into iPS cells.

A second limitation for the use of iPS technology (as well as ES cell technology) in the clinic is the tendency of pluripotent cells to cause teratomas after transplantation. Even a single undifferentiated ES or iPS cell is sufficient, in principle, to grow into a tumour. Therefore, to avoid cancer it will be crucial to eliminate undifferentiated cells from *in vitro* differentiated cultures, either by negative selection approaches or by efficient differentiation protocols. However, I think that this is a purely technical problem that can be solved in the foreseeable future.

S.Y. The clinical application of iPS cells depends on regulatory frameworks, which are different in each country. Some countries, including Japan, have to establish new guidelines for ES cells and iPS cells. The clinical use of iPS cells will also depend on the results of preclinical studies using animals, and many technical challenges still need to be overcome.

We aim to solve the technical challenges discussed above, which are specific to iPS cells, within a few years. However, there are many more challenges that are common to both ES and iPS cells. We need to develop efficient and specific protocols to induce *in vitro* differentiation into specific lineages from ES and iPS cells. We also need to establish safe and effective methods for transplanting those differentiated cells into patients. Thereafter, vigorous preclinical tests would be needed to evaluate the safety and efficacy of pluripotent cell-derived cells in animal models.

Q *What improvements do we need to push iPS cell research forwards?*

J.C.I.B. There are at least three major research directions that need to be pushed forward: the generation of fully pluripotent human iPS cells that are functionally equivalent to human ES cells; the establishment of defined, directed differentiation protocols for obtaining pure populations of various human cell types that are free of animal products and viral methods of gene delivery; and the removal of the threat of cancer. Technically, we will need a common framework with which we can compare and integrate data from different laboratories. We need to agree not only on the criteria and assays we are going to use to compare the different lines that are being generated but also on a common set of parameters by which to assess the identity and functionality of the lines, and these need to be correlated with both the cell type of origin and the method of derivation.

J.E. The major limitations are safety and the labour involved in generating and characterizing iPS cells. One improvement would be to adapt the reprogramming concept to generate adult progenitor cell types rather than iPS cells. Douglas Melton's laboratory has shown the effectiveness of this approach for making pancreatic β-cells for diabetes therapy, and methods for other tissues could be developed. Similarly, there is a real need to improve protocols for the differentiation of iPS cells to the desired pure cell populations. Again, the Melton laboratory has identified chemicals for directing cell differentiation, and the use of growth factors for directed differentiation into cardiac cells is relatively effective. However, most of the cells that are generated from pluripotent cells are types of fetal cell. This may limit modelling studies to early onset or paediatric disease. Approaches for generating adult equivalents are desperately required. Finally, we need *in vitro* phenotype assays and scale-up procedures to translate these discoveries into high-throughput screens and toxicity tests. As these protocols are developed and validated, we need to automate the process of generating iPS cells to increase the throughput of individual facilities from dozens of patient iPS cell lines to hundreds or more per year. Clearly, in this context it will not be possible to fully characterize all lines using strict criteria of whether they form teratomas.

K.H. This again depends on the application. For basic research, a major limitation has been the low efficiency of the reprogramming process. Identifying small molecules and genes that can enhance the efficiency or identifying cell types that are more amenable to reprogramming will be very useful for understanding the mechanism of reprogramming. For example, the identification of several small compounds by the laboratories of Sheng Ding and Doug Melton has been useful in enhancing efficiency and replacing individual reprogramming factors. Moreover, keratinocytes, melanocytes and blood progenitor cells have been reported to reprogram more efficiently and faster than fibroblasts, which had been used previously. I think that we will see many more improvements in this direction in the coming years, and possibly the derivation of iPS cells from somatic cells purely with chemicals.

For disease modelling, it will be crucial to identify diseases that can be recapitulated in a Petri dish. Notably, a recent study by Lorenz Studer's laboratory demonstrated that neural crest cells derived from patients suffering from FD recapitulate many of the disease phenotypes, including decreased neurogenesis, reduced migration of neural crest precursors and a cell type-specific defect in IκB kinase complex-associated (*IKBKAP*) splicing[9]. This validated the use of iPS cells for disease modelling. It will be crucial to identify other disease pathologies that can be mirrored in a Petri dish.

Glossary

Bivalent histone modification
The co-occurrence of histone tail methylation marks that are associated with transcriptional activation and repression. Bivalency is observed in mammalian embryonic stem cells at developmentally important genes.

DNA methylome
The pattern of DNA methylation across the genome. Many DNA methylation marks are set up during early mammalian development and are erased in the germ line.

Neurosphere
A cluster of neurogenic cells that is generated from a single neural stem cell or progenitor cell when it is cultured in a semi-solid medium that contains appropriate neurotrophic growth factors.

p53
A transcription factor encoded by *TP53* that is known to be a tumour suppressor and to be involved in the regulation of many cellular pathways, including the cell cycle.

Reprogramming factors
Transcription factors that enable reprogramming to pluripotency when expressed together in adult somatic cells. The four factors originally used were OCT4, SOX2, Krüppel-like factor 4 and MYC.

Teratoma
A tumour consisting of several cell types.

For cell therapy, improved protocols for differentiation into clinically relevant functional cell types will be important. Reasonable differentiation protocols are available to turn pluripotent cells into neural cells, cardiomyocytes and certain blood cells, but the cells generated are often not as versatile (yet) as their *in vivo* counterparts. Although this should not be an impediment for their use, I think much more research is needed over the next few years to understand how to efficiently make a cell type of interest from ES cells or iPS cells and how to successfully engraft the produced cells *in vivo*. This may be ideally achieved by recapitulating the steps that embryonic cells encounter during normal development, as has been elegantly shown by Tom Jessell and colleagues during the differentiation of motor neurons from ES cells[20].

S.Y. iPS cells are generated from various origins by various methods. In each experiment, multiple iPS cell lines are established. We need to determine the best origins, best induction methods and best evaluation methods.

To further advance iPS cell research towards various applications, we need the following technical improvements: the determination of the best original cells in terms of both safety and efficacy; the development of reproducible, efficient and integration-free methods that can fully reprogram human adult somatic cells; and the establishment of simple but sensitive and reliable methods for evaluating the safety of the myriad iPS cell clones and subclones. To achieve these goals, the mechanism of direct reprogramming must first be further elucidated.

The generation of iPS cells from each patient in accordance with good manufacturing principle (GMP) standards would be very expensive. It would take at least a few months and would not be practical for patients suffering from acute disease and injuries, such as spinal cord injury. We therefore might need to consider the establishment of an iPS cell bank. Previous estimations predict that the collection of 50 unique iPS cell lines that are homozygous for the three major human leukocyte antigen loci would cover ~90% of the Japanese population with perfect matches.

Q *Should we continue to work on ES cells, or have they been replaced by iPS cells?*

J.C.I.B. We should definitely continue to work on ES cells, as they are the 'gold standard' against which we compare iPS cells. ES cells are needed to understand the basic mechanism of pluripotency and self-renewal. As such, it is out of the question to even suggest phasing them out. We will be lost without them. Therefore, in terms of research and funding priorities, ES cells should have at least equal importance to iPS cells as we search for the meaning of pluripotency, the ground state of pluripotency and the mechanisms of self-renewal in an effort to translate this knowledge to the safe generation of iPS cells[21].

J.E. Yes, we need ES research. ES cells are the gold standard for comparison to iPS cells. In particular, heterogeneity is a feature of pluripotent cells. For example, variation in Nanog expression is normal in mouse ES cells and might facilitate their ability to respond to differentiation signals. We need to understand this normal heterogeneity in ES cells to identify iPS cells that differentiate properly. Moreover, ES cells are often karyotypically unstable and even undergo copy number variation during prolonged passage. As many complex disorders, such as autism, have risk factors that are associated with specific genomic copy number variants, we need to understand the normal diversity in ES cells and develop methods for controlling instability in iPS cell cultures. Finally, we need to understand the effect of genetic heterogeneity on *in vitro* phenotypes. It is not yet clear whether iPS cell models of disease will require partially matched parental or sibling control lines for comparison, nor how much independent lines from the same patient will vary in functional assays. Just as ES cell lines have been stored in stem cell banks for distribution, it is equally important that validated patient iPS cell lines be made widely available through such mechanisms.

K.H. I think that we need to continue work on ES cells for several reasons. First, we still do not have a full picture of the molecular and functional properties of iPS cells, and ES cells still are the gold standard to compare them to. Thorough molecular and functional comparisons of several different ES and iPS cell lines (for example, comparisons of gene expression, epigenetic patterns and *in vitro* and *in vivo* differentiation potentials) will reveal whether ES and iPS cells are truly equivalent or whether iPS cells exist in different qualitative states.

Second, ES cells will remain a powerful tool for engineering mutations that model diseases. This is particularly attractive for disorders in which the identity of the gene is known and can be modelled in previously established, well-characterized ES cells. A case in point is the promising work by the laboratories of Kevin Eggan and Fred Gage, which showed that mouse and human ES cell models that carry a mutation of the superoxide dismutase 1 (*SOD1*) gene, as seen in some familial cases of ALS, recapitulate motor neuron loss in culture. Related to this, ES cells can be derived from leftover *in vitro* fertilization embryos that harbour certain disease-specific alleles that are not available in iPS cells.

In summary, once we have a better understanding of what iPS cells can do and what they cannot do — if anything — it will be worthwhile to revisit the question of whether ES cells have become obsolete. At the moment, this is clearly not the case.

S.Y. ES cells are at least a few years more advanced than iPS cells in terms of safety. Therefore, preclinical and clinical trials using ES cells should be continued. A few years means a lot for patients who are urgently waiting for new treatments. I would expect that iPS cells will eventually replace ES cells in most, if not all, applications in the future. Even thereafter, however, ES cells are still expected to have an important role as a control in both experiments and trials. I have a great interest and high expectations in regards to the first clinical trials using human ES cells for patients suffering from spinal cord injuries, which are presently being conducted by Geron, although the US Food and Drug Administration has temporarily postponed these trials.

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Competing interests statement
K.H. declares competing financial interests: see Web version for details.

DATABASES

UniProtKB: http://www.uniprot.org
OCT4 | SOX2

FURTHER INFORMATION

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