Pharmaceutical BIOPROCESSING

Exploring standards for industrializing human induced pluripotent stem cells

Popular belief assumes that human pluripotent cells can now be obtained in any lab or company by induced pluripotent stem (iPS) cell reprogramming. However, the difficulties in robustly producing human iPS-derived cells that are fit for drug discovery are becoming increasingly apparent. This is because we still have not come up with a strict definition of pluripotency. Our attempts at prospectively identifying differentiation-defective human iPS cells using teratoma assays or marker expression have clearly failed to date. Here, we will revisit how conventional pluripotency tests have failed in evaluating iPS cells adequately for drug discovery and emphasize two aspects of developmental transitions (what we call here a cell's chronological value and the segregation of factors as it differentiates) to elucidate inherent problems with our current understanding of human iPS cells. Finally, we challenge the field by presenting our perspective on distinguishing good human iPS cells from bad ones.

Hidemasa Kato*[,1], Keiko Hiraki-Kamon[1,2], Masamitsu Eitoku[3], Hidenori Kiyosawa[3], Cindy Neeley[4] & Yasushi Okazaki[1]

[1]Division of Translational Research, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan

[2]Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

[3]Kochi Medical School, Nankoku, Japan

[4]Thermo Fisher Scientific, Rochester, NY, USA

*Author for correspondence: hidekato@saitama-med.ac.jp

Shinya Yamanaka and colleagues have transformed the clinical and pharmaceutical sciences by providing easier access to human cells which at the same time hold the genomic information of their donors. By introducing just a few genes into somatic cells, they have reprogrammed the epigenome of donor cells into one which is equivalent to a much younger stage of development: that of the induced pluripotent stem cells (iPSCs) [1]. This in turn allows us to harness a significant trait of these reprogrammed cells, namely the potential to derive any kind of somatic as well as germline cell, a trait called pluripotency. It should however be kept in mind that the pluripotency of iPSCs has been so far mainly proven for mouse iPSCs (miPSCs) [2] but not sufficiently for human iPSCs (hiPSCs) [3]. The difference between miPSCs and hiPSCs and its consequences for the field of drug discovery will be a major focus of this manuscript.

If one thing could bring hiPSC-based pharmaceutical processes (or any of their downstream applications) to a higher level, it would be a 'prospective marker' of hiPSC *bona fide* pluripotency, if indeed any such marker exists [4]. However, we would claim that we have failed to reproducibly obtain iPSC-derived cells of drug screening standard not because of the absence of gold standard pluripotency markers, but because we do not entirely understand why reprogramming occurs, as reflected by our ongoing inability to unambiguously define pluripotency, the end point of iPSC reprogramming [5]. In an effort to define pluripotency, one proposition has been to equate the presence of the so-called 'pluripotency-related' markers such as OCT3/4, SOX2, NANOG and TRA1–60 as an indication of pluripotency [6]. In this Perspective, we revisit the concept of the 'pluripotency-related marker' and argue that such markers, in principle, do not exist in the strict sense of their meaning. We support our claim by introducing novel viewpoints to the classical view of cell differentiation with regards to pluripotency: namely, a cell's chronological position and the fluidity of its developmental transitions.

Although iPS cell technology has offered us unprecedented opportunities to work with human cells, the technology itself is still in its infancy and has recently been questioned

FUTURE SCIENCE part of fsg

in recent reports, especially regarding the generation of human pluripotent stem cells (hPSCs). Similar doubts were already raised for human embryonic stem cells (hESCs) when Kenji Osafune *et al.* found that most hESC clones they analyzed were skewed in their differentiation propensities [7] and therefore it should not be taken as a surprise if hiPSCs behave similarly or worse [8]. Indeed, recent work has challenged the integrity of pluripotency in both hPSC types (hESCs and hiPSCs) derived from the same somatic cell line and therefore isogenic [9]. The central message of this work was that iPS cell reprogramming protocols have inherent errors vis-à-vis the degree to which they reset the somatic epigenome. We will come back and elaborate on this newly revealed aspect of iPS cell reprogramming in the paragraph entitled 'PSC variability, heterogeneity and developmental potentials.' Also very recently, the pioneers of hiPSCs have proposed a drastic change in their understanding of how hiPSC are specified. Instead of iPSC reprogramming involving the direct reprogramming of a somatic cell to the pluripotent epiblast-like developmental stage, they propose instead that cells take a compulsory detour to the mesendoderm lineage before gradually adapting toward a more epiblast-like developmental stage, which may be (or may not be) achieved after prolonged culture [10]. The direct impact of this is best expressed in the rewritten 'due date' of hiPSCs which has been extended from just a month (in a previous publication [11]) to a limitless 'over a hundred days' [10].

The bottom line is that the definition, the specification or more relevant for the later discussion, the standard for hiPSCs is constantly shifting. The difficulty in setting this standard for hPSCs is again due to our lack of a fuller understanding of the mechanisms underlying pluripotency. Despite its inherent difficulties, we here take on the formidable task of proposing a novel standard for hiPSCs for improved downstream applications based on our current knowledge, which has emerged from experience and insight gained by our research over the last two decades.

Stem cell-derived bioengineered cell materials for drug screening

In this critical review, we will be dealing with pharmaceutical processes which take place before the so-called 'upstream processing' for hiPSC-based drug discovery [12], in other words the acquisition of hiPSC lines, the ensuing culturing and banking of these cells prior to downstream bioprocessing or manufacturing. hiPSC lines are often obtained from dedicated cell banks and only few institutions would make their own hiPSC lines in-house. Still, one should know how to choose decent hiPSC lines and to culture these while maintaining their due pluripotency. To this line, it would be worth revisiting the aim for using these hPSCs in the first place.

Advantages & disadvantages of using stem cell-derived cells

The self-renewing property of pluripotent stem cells (PSCs) is their remarkable asset to be harnessed for drug screening as this offers the chance to provide, in a timely fashion, an unlimited quantity of cells amenable to cell differentiation. Therefore, self-renewal is a major prerequisite to sustain lengthy high-throughput screening (HTS) sessions using live cells [13] and offers a unique opportunity to obtain somatic cells at a controlled purity and at the desired timing, thereby laying the foundation to model postmitotic tissues such as brain, muscle, heart and pancreas for drug screening.

Once a decision has been made to use a certain PSC line for drug discovery, the next step would be to differentiate the cells toward a specific cell type by mimicking the cellular differentiation program that the desired cell types would normally face during development, but in a dish. The ability to faithfully follow a differentiation program is also a strong benefit of PSCs although it is often merely presumed that the properties of the derived cells faithfully match those of their *in vivo* counterparts. Such properties include, for example, the ability of the derived cells to faithfully respond to external stimuli (such as a derived neuron's response to a neurotransmitter) or more implicitly, undergo physiological cell aging. This places PSC-derived cells in opposition to those derived by direct reprogramming (i.e., induced neuronal cells and induced cardiomyocyte-like cells [14]) in which cell age is largely overlooked. The validity of using directly reprogrammed cells for drug discovery is certainly an urgent topic requiring much scrutiny but, given the scope of the perspective, we will leave this discussion for another occasion [15].

Once the desired cell type has been produced by following the appropriate differentiation program, it is ready for HTS or toxicity testing. This procedure represents a common feature for drug discovery bioprocesses using viable human cells. One may of course combine two or more timely tailored cell types in order to more faithfully reproduce the diseased conditions. Another advantage of using PSCs is that these cells are amenable to genetic engineering, thereby easing the readout of the screen (i.e., green fluorescent protein or luc-assay systems) [16].

However, there may also be some drawbacks from using PSCs in drug screening. Although we have seen cases where coculturing cells which co-develop during the development of an organ might be beneficial for

highly efficient cell differentiation [17], most cell differentiation protocols rely on timely applications of bioactive compounds in a hope to mimic the relevant developmental events. This would automatically lead to the differentiation of a limited number of cell types of the same lineage in one go. If this is done successfully, one may end up with a single cell type in a dish. Although this may meet some screening purposes, needless to say, this is however an unnatural situation as we know all too well that a single cell type cannot form an entire organ and therefore cannot logically recreate a diseased condition in a dish. Drug screens performed using these pure-cell-systems cannot for example spot candidate compounds which would exhibit indirect effects such as a drug which primarily targets endothelial cells to allow these cells to better accommodate newborn neural cells which may mitigate damages related to cerebral infarction. Therefore, before any HTS using a PSC-derived pure-cell system can be run, one may need to pilot test the system by comparing it with more complex cell systems such as primary cultures or slice cultures derived from animal models and only choose a PSC-based system if it can outperform the alternative systems. One could also explore the possibility of 'mixing' pure single cell types generated separately from iPS cells and combining them (or not) with specialized biomaterials. This strategy will result in more a comparable system to primary cultures or slice cultures and may enhance the validity of the *ex vivo* system when compared with the *in vivo* physiological niche. Mimicking a tissue environment *ex vivo* would, in turn, require a deeper understanding of the underlying cell physiology, another aspect which requires further basic research.

A situation encountered more frequently is where the differentiation efficiency of the desired cell type does not reach 100% especially when cell sorting is not an option. One may argue that, in some cases, a lower degree of differentiation efficiency can be acceptable, say 30%, when we focus on these cells only. However, this does not take into account that 30% differentiation is more difficult to faithfully reproduce than 100%. Instead of specifying a single cell lineage, a 30% differentiation would require that not only the target cell lineage is induced at exactly 30% every time a differentiation is performed, but that the remaining 70% of the cell population(s) must also be specified at 100% precision! This is the difficulty inherent to drug screening in which you must provide your chemicals with an equal playing field in every single well of cultured cells. Also with a mixed cell population, even with full knowledge of the contaminants, you would have less chance of knowing if the drug effect was direct or not. For drug screening, much more effort should be placed in obtaining high-purity somatic cells than in publishing 'champion' data in journals.

iPS cell innovation

In this section, we will briefly revisit the development of iPS cell technology, with emphasis on what is currently known about the mechanisms underlying hiPSC reprogramming in order to later facilitate defining a standard for hiPSCs of drug testing quality. We will also comment on some recent applications using hiPSCs.

iPS cell reprogramming in short

At the core of iPS cell reprogramming lies the discovery that the transient introduction of several transcription factors into a somatic cell can stably reprogram the cell into a pluripotent state. Kazutoshi Takahashi and Shinya Yamanaka selected these so-called Yamanaka factors based on their expression in the mouse ES cells (mESCs) as model pluripotent cells [18]. What they could not have predicted though is the fact that at least during the reprogramming process, these Yamanaka factors target genes more widely than those targeted in mESCs [2]. This means that Yamanaka factors are engaged in different tasks when expressed in mESCs and during reprogramming. This counterintuitive aspect of reprogramming has been further illustrated by the fact that Yamanaka factors can be replaced to reprogram mouse (Oct3/4 to Gata3/6 or Sox7 and Sox2 to Sox1/3 or Gmnn) [19] and human (OCT3/4 to GATA3 and SOX2 to SOX1/3) [20] somatic cells. Related to this, it is noteworthy that any residual reprogramming factor expression in established iPSC lines hinders them from maintaining a stable undifferentiated cell state which is still amenable to

Key terms

Epigenome: Genome with encrypted epigenetic cell memories such DNA methylation of histone modifications.

Pluripotency: Potential of a cell to differentiate into all are somatic/germ cell lineages. A more detailed picture of its meaning is a topic herein.

Pluripotency-related marker: Transcription factors mainly expressed in pluripotent cells. Its 'existence' is challenged in the text.

Direct reprogramming: Action of reprogramming somatic cells directly toward another cell lineage without passing through a pluripotent state.

Epiblast: Mammalian developmental stage which follows implantation but still contains pluripotent cells as primitive ectoderm cells.

Mesendoderm: Collective nomenclature for mesoderm and endoderm which develop in a cell mass called the primitive streak.

cell differentiation [21]. We will come back to this aspect when considering what 'pluripotency-related' factors mean and also when setting the standards for the stem cells.

For a more accurate discussion about reprogramming, we should now deal separately with mouse and human iPSCs. Full developmental potentials of miPSCs have been demonstrated by their ability to produce, within a single life cycle, live pups consisting entirely of the miPSCs transplanted into tetraploid blastocysts which otherwise would cease development soon after [22]. Although the underlying molecular features of this full pluripotency remain largely a mystery, they appear closely linked to miPSCs' ability to exist in the so-called 'naive ground state' [23], one which faithfully mimics a cellular state most akin to the inner cells of a mouse blastocyst [24] and corresponds to the first instance of pluripotency in the course of development. For a relatively long time, a lab's routine maintenance of mESCs and hence initially of miPSCs as well has been performed in a culture media containing bovine fetal serum and leukemia inhibitory factor. However, it has been recently revealed that mPSCs maintained in this milieu acquire an unusually higher genome-wide DNA methylation status thereby deviating from their natural developmental context [25]. By adding two kinase inhibitors (namely MEK inhibitor and GSK3β inhibitor; collectively coined 2i) to the culture medium, it is now known that the cultured mPSCs exhibit an epigenetic configuration more similar to the inner cells of the blastocyst. We may speculate that this epigenetic 'synchronization' of mPSCs to the relevant developmental stage (that of the inner cells of the blastocyst) allows them to faithfully follow developmental directions and give rise to a live pup. It follows that this epigenetically adjusted pluripotency is better equipped for retrieving the full *in vitro* differentiation potential out of mPSCs. A key message is therefore that the epigenetic status of the PSCs may influence their developmental potential and therefore by definition their pluripotency. Unfortunately, for human PSCs, the naive ground state cultures so far reported are still being scrutinized and warrant further characterization before we can firmly define them as such [26–28]. In particular, the *in vitro* developmental potentials of these naive hPSCs should be demonstrated in direct comparison to the current epiblast-like hPSCs.

The lack of a naive ground state for human PSCs has contributed to hESC science lagging behind its mouse counterpart by 17 years [29]. Instead of being stabilized in the naive stage human PSCs seem to have found another 'safe refuge' for their self-renewal, namely the epiblast primitive ectoderm-like stage, and require different growth factor(s) for cell proliferation. However, this new paradise has come with some costs: hPSCs seem to be vulnerable to single-cell trituration which would otherwise be a routine procedure of cell passaging and they also resist gene introduction. This said, novel technologies to remedy these drawbacks of hPSCs have recently appeared and are summarized elsewhere [30,31].

To sum up, iPS cell reprogramming is achieved by transitioning somatic cells into a pluripotent state followed by stabilizing the resulting PSCs into a (meta) stable state using culture conditions which differ significantly between mouse and human.

Current state-of-the-art

The first iPSC clinical trial has just been launched here in Japan. Masayo Takahashi's trial tackles an intractable retinal disease known as age-related macular degeneration by transplanting a patient-iPSC-derived pigmented retinal cell sheet. The trial is a brave move which should attract further applications of iPSC technology in regenerative medicine and many follow-ups from this study are anticipated.

In the pharmaceutical domain, many drug screening projects have already been performed using hiPSCs-derived cell types and have lead to the discovery of some novel lead compounds [32]. Along this line, another very recent publication in which statins have been repositioned for potential use in skeletal dysplasia with FGFR3 mutations merits attention. The group led by Noriyuki Tsumaki at the CiRA has found that statins can rescue cartilage development from patient-derived iPSCs with hyperactive FGFR3 mutations [33]. Statins appear to destabilize the FGFR3 protein thereby attenuating the downstream cell signaling which jams the normal nascent mesoderm cells' differentiation trajectory toward cartilage and guides them to fibroblast-like cells instead. The currently known major function of statins is their ability to inhibit the rate-limiting enzyme for cholesterol biosynthesis and therefore the drug is mainly prescribed to the elderly with hypercholesterolemia. This work demonstrates how the iPSC drug-testing platform can potentially spot ready-to-go drugs that may be repurposed toward otherwise 'unexpected' disease treatments. It should be kept in mind that drugs can be repositioned not only for their alternative therapeutic uses but also for their applications in assisting biomaterials in various fields such as regenerative medicine and similarly, for the derivation of cells fit for drug screening.

Requirements for stem cells in drug discovery applications

Having considered the current scope for hiPSC applications in drug discovery, it would now be appropriate

to turn to the requirements and specifications of the hiPSCs for their effective use in this process. This naturally necessitates an unambiguous definition of the basic property of these stem cells – their pluripotency. In fact, this is not an easy task. To ease defining pluripotency in the context of deriving cells from iPSCs for drug discovery, in this section, we would like to emphasize two neglected viewpoints of cell differentiation. We will start by summarizing the inherent difficulties of PSCs for their application in drug screens.

Key terms

Blastocyst: Developmental stage of a mammalian embryo just before implantation.

DNA methylation: Covalent modification of mainly cytosines which affects the cell's epigenome.

Teratoma formation: Classical test for pluripotency where stem cells are injected into immune-privileged mice and allowed to differentiate randomly. A trilineage differentiation within the resulting teratoma is used to qualitatively demonstrate pluripotency.

PSC variability, heterogeneity & developmental potentials

Although PSCs hold great promise as platforms for disease modeling and drug discovery, not all PSC lines are equal in their potential to differentiate into the desired cell types *in vitro*. This unfavorable picture is especially true for hPSCs and therefore would merit some discussion here.

Osafune *et al.* were the first to systematically compare the *in vitro* differentiation capacity of various hESC lines [7]. Despite their equivalence in pluripotency by teratoma formation, the 17 hESC lines that were tested exhibit varying propensities to differentiate into specific lineages. Of specific note, none of the lines were able to score top marks in their ability to differentiate into all the somatic lineages tested. Casual critics of this work suggest that the hESC lines used in this study might have been maintained in suboptimal conditions. However, this kind of criticism is not productive when considering the work's important message pertaining to hESCs as a tool for drug discovery. For example, it clouds the benefit of having all hESC lines maintained in a single laboratory, a factor which dismisses interlab differences as the sole explanation for the divergent differentiation tendencies. The comment also depreciates the important message of the paper in that these hESCs all passed the teratoma formation 'litmus' test for pluripotency. If you, in a drug company, were to use hESCs for drug screening, you could only be frustrated to find that most, if not all, cell lines labeled as 'pluripotent,' judged of course by teratoma formation or marker expression which are the current golden standard for this cell entity, have inherent differentiation propensities which would strongly discourage their use as stem cell sources for deriving cells suitable for drug testing. Osafune *et al.* finding was strengthened when another lab subjected six out of the same 17 hESC lines to motorneuron differentiation and again observed significant differences [34]. This had led to a widespread belief that hPSC lines should be selected according to the required cell type.

In another study, 5 hESC lines and 12 hiPSC lines were compared for their propensity for neural differentiation [8]. Generally speaking, the iPSCs showed significantly lower neural differentiation than ESCs, in keeping with the implicit view that hiPSCs have generally lower differentiation potentials than hESCs. An intriguing finding of this work is that SMAD inhibition, and therefore the inhibition of signaling downstream of TGFβ, of several iPSC lines significantly improved their neural differentiation. Ever since, most laboratories have added SMAD inhibition to their hPSC neural induction protocols. It should be noted here, and we will come back to this point soon, that hPSCs more recalcitrant to differentiation often exhibit mesendoderm propensity, the *in vivo* output of SMAD activation during mammalian development.

We are now also starting to understand the molecular underpinnings of differentiation defective PSCs. To our knowledge, the first study to describe a molecular signature of differentiation defectiveness came from Lorenz Studer's group, which identified higher levels of *miR-371–3* expression in hPSCs that resist neural differentiation [35]. Regardless of the derivation methods, the cell sources and lab source, hPSCs expressing higher levels of this microRNA poorly differentiated into neural cells even upon SMAD inhibition. The authors found that the culprit for this miRNA overexpression is KLF4, a Yamanaka factor. By attenuating KLF4 levels in otherwise differentiation-defective cell lines, these cell lines downregulate *miR-371–3* and simultaneously recover their propensity for neural differentiation. Conversely, the introduction of additional KLF4 or *miR-371–3*-mimics into 'good' hPSCs attenuated their differentiation potential. Given this landmark for defining good hPSCs, it may not come as a surprise that Studer's group has been prosperous in the field of neural differentiation out of hPSCs ever since [36–38].

More recently, Yamanaka's group has conducted similar experiments to elucidate gene expression signatures which may prospectively mark differentiation-defective hPSCs [39]. This was probably the result of a big push not only from academic demands for a way to *a priori* validate differentiation-competent hiPSC lines but also from industrial stakeholders who rarely

obtained 'good' hPSCs. Expression microarray comparisons of good versus bad hiPSCs revealed unexpected failures of retrotransposon silencing in bad hPSCs. Through bioinformatic approaches, the group has now narrowed the reason down to a surprising 'overexpression' of KLF4 [40]. Despite the fact that KLF4 is a Yamanaka factor, hiPSCs seem to exhibit abnormalities when this factor is overexpressed. Indeed, KLF4 is only marginally expressed in most hESC lines [40]. In the same study, the authors noticed that this class of retrotransposons is transiently upregulated upon mesendoderm development. And this, reading between the lines, appears to have triggered Yamanaka's group to hypothesize that iPSC reprogramming requires cells to take a compulsory detour to the mesendoderm before the cells can attain an epiblast-like characteristic more akin to hESCs [10]. The group also now recognizes that 'the human reprogramming process takes more time (=more than a hundred days) than we thought it would and the maturation step is important.' By the maturation step the authors denote a step in which mesendodermal cells approach an epiblast-like cell state, for unknown reasons, well after the extinction of the Yamanaka factors. From a practical point of view, the derivation of hiPSCs using the current protocol has turned out to be inconvenient especially for those who plan to use the updated hiPSCs for drug discovery as it becomes extremely painful to schedule a drug screen without knowing at what point after the 100 days of reprogramming the cells will be ready for use. This news struck all of us as recently as in April 2014 [10].

An interesting follow-up in this line came independently from Ali Brivanlou's lab. This group has now found that during development, *miR-371–3* is induced by SMAD signaling and seems to have an inductive effect toward mesendoderm [41]. Thus by pushing hPSCs toward mesendoderm, *miR-371–3* might be preventing neural induction. Likewise, it is intriguing that different labs have independently concluded that an uncontrolled expression of a so-called 'pluripotency-related' factor such as KLF4 in 'pluripotent' cells may deteriorate their 'pluripotency.' Although the reason why KLF4 expression can become rampant in certain hPSCs remains unknown, it is noteworthy that an imprinted antisense noncoding transcript in the gene locus encoding *miR-371–3* has been recently described [42]. Noncoding RNAs have been widely described as affecting not only pluripotency but also functional aspects of the derived somatic cells [43,44].

It is amazing how fast this field is developing and we experience hardly any week without news on the subject. But the paper which appeared last July in Nature would hardly come second in challenging the integrity of iPSC reprogramming. Research teams led by Shoukhrat Mitalipov, the developer of hESCs through somatic cell nuclear transfer (NT-hESCs), compared isogenic NT-hESCs and hiPSCs, two hPSC types which differ only in their reprogramming mechanisms [9]. The report declares NT-hESCs the winner of the 'hardball' match. Although both isogenic NT-hESCs and hiPSCs contained comparable numbers of *de novo* genomic aberrations, DNA methylation and transcriptome profiles of NT-hESCs corresponded closely to those of IVF ES cells, whereas hiPSCs retained substantially more residual DNA methylation patterns typical of parental fibroblasts. So unlike in the mouse, human PSCs can be clearly demarcated by the reprogramming methodologies used. Although not formally discussed in the paper, this work has depicted a similar signature for *bona fide* pluripotency in the mouse and human. An imprinted transcript *Meg3* (also called *Gtl2*) has been previously reported to be differentially expressed between *bona fide* tetraploid-complementing miPSCs and more compromised miPSCs [22]. Now in this paper, the authors also found that the human *MEG3* gene is generally upregulated in NT-hESCs compared with hiPSCs. This is another example where noncoding transcript could be used as a *bona fide* pluripotency marker. We have independently found that *Meg3/Gtl2* is one of the few imprinted genes abundantly expressed in mESCs (our unpublished result). We also know that a significant portion of noncoding transcripts is poly(A)-less, including *Meg3/Gtl2*. Therefore, we reckon that transcriptome analyses to depict the degrees of pluripotency of PSCs should be performed at the total RNA level as well as with allelic discrimination (which would distinguish between maternal vs paternal allele, a prerequisite for showing imprinted expressivity) to take into account the additional layers of the whole transcriptome in gene regulation [9,42].

Next, in an effort to clear this current 'foggy' picture about pluripotency, we would like to introduce some novel guidance in understanding how cell differentiation occurs and how to interpret this.

Key terms

(Genomic) imprinting: Gene expressivity found in plants and mammals where parental allele origin governs the exclusive allelic expression of a gene.

Chronological value: Chronological position during a cell's developmental trajectory which adheres to a strict developmental schedule.

Differentiation by segregation of factors: Mode of cell differentiation where a cell trait must be subtracted in order to inherit the alternative.

The chronological value of cell differentiation

One of the two new ideas introduced here is the chronological value of a cell's differentiation

pathway. An implicit notion in the process of development, illustrated by Waddington in the metaphor of the 'epigenetic landscape' [45], is that as a cell differentiates or goes down a developmental trajectory, its chronological value along the trajectory advances as well (Figure 1A). Although cells of different cell lineages follow different pathways during development, we can imagine that all cells differentiate while keeping pace by sharing the same chronological value throughout as far as they develop within the same organism. The importance of introducing this perspective on cell differentiation can be exemplified by the necessity of synchronizing chronological values between cells of different lineages (Figure 1B). The innervation of a muscle cell by a neuron requires that both cells reciprocally prepare for this developmental event. If a neuron fails to properly find its muscle target, it will die. The same is true for the muscle cell that when not innervated, will eventually wane from loss of contractile inputs.

A direct implication of this view is what we call the chronological constraint, namely the notion that cell differentiation is unlikely to stall or go backward in time as cells would miss their developmental rendezvous with their differentiated partners. It is hard to think that this kind of lazy developmental schedule would go unchecked during evolution if it existed. The chronological constraint would also guide us in understanding PSC behavior. If we included a chronological dimension in our definition of pluripotency we would hypothesize that it represents a cell state where no somatic or germline differentiation has occurred yet, but would be capable of doing so in the future. This automatically places any PSC at the first bifurcating point of Waddington's landscape. We will revisit this aspect very shortly (Figure 2).

Also, in a strict sense of its usage, a chronological value of a PSC should be its value when it is allowed to resume development by joining the Waddington landscape. We must not forget that the PSC is an artificial cell entity: if it occurred naturally it would be in direct violation of the idea that a cell does not stall its chronological value during development. That is to say, a PSC is not necessarily under strict chronological control from the genome as is the pluripotent *in vivo* stem cell, but rather is an arbitrary cell entity which is amenable to human manipulation as far as it keeps its capacity to resume development. We believe that this aspect of pluripotency is currently much disregarded.

Differentiation by segregation of factors

The second proposal here is about the way we look and interpret cell differentiation as cell traits segregate. Cell differentiation would appear in most textbooks like the drawing in Figure 3. Starting from a progenitor cell, the process of cell differentiation will generate more than one cell type (very often two) which will appear different from the original cell. In the narrow context we have here, the trait of this progenitor cell is what we call pluripotency. Depending on how we see this initial pluripotent state, cell differentiation can be viewed from two different perspectives. The prevailing view is that the pluripotent cell bears a trait denoted here as 'O' and upon cell differentiation its offspring produce the different traits 'A' or 'B' (Figure 3A, left panel). Most people suppose that this initial O-trait is gradually lost during differentiation. We would call this type of developmental transition one of '*de novo* acquisition' as cells acquire new traits during differentiation. A contrasting view can also be made here where the traits after differentiation co-exist before differentiation in the pluripotent cells (Figure 3A, right panel). We would call this differentiation by segregation of factors because upon differentiation, at least one of the initial traits is passed on to the descendants.

A recent study by Matt Thomson *et al.* [46] corroborates the concept of differentiation by segregation of factors (summarized in Figure 3B). At the mouse blastocyst stage, pluripotent cells of the inner cell mass express both OCT3/4 and SOX2. Although the prevailing view about the ensuing differentiation was that these two factors gradually extinguish before acquiring discrete traits of the descendants, the authors have found with great surprise that the initial balanced expression only becomes biased (as opposed to extinguished) at the epiblast stage and finally, this marker expression exhibits a mutually exclusive profile in which mesendoderm inherits OCT3/4 and ectoderm SOX2. Thus, a segregation of factors occurs in this early differentiation event during mammalian development and possibly at later bifurcations in fate. From this point of view, we may also describe cell differentiation as an act of subtracting alternative cell fates.

If we accept the view that progenitor factors segregate into offspring cells, we simultaneously obtain a better idea about what should be called pluripotency. Look at any paper which deals with hiPSCs and you will see figures which show the expressivity of the so-called 'pluripotency-related' markers (OCT3/4 and SOX2 being the top two examples). However, under this novel navigation, cell differentiation becomes the subtraction of the alternative fate and the inheritance of the other one. Because pluripotency should prospectively guarantee that the cell in question has the capacity to differentiate into the two alternative pathways, this automatically takes for granted that at least one of the initial traits (i.e., OCT3/4 or SOX2) can be inherited, or the other side of the coin being, that one of these initial traits is 'subtractable' upon differentiation.

To appreciate this new view, we provide here a counterexample of pluripotency when judged by marker expression. As a familiar and relevant example, iPSC reprogrammed using retroviruses may suffer from

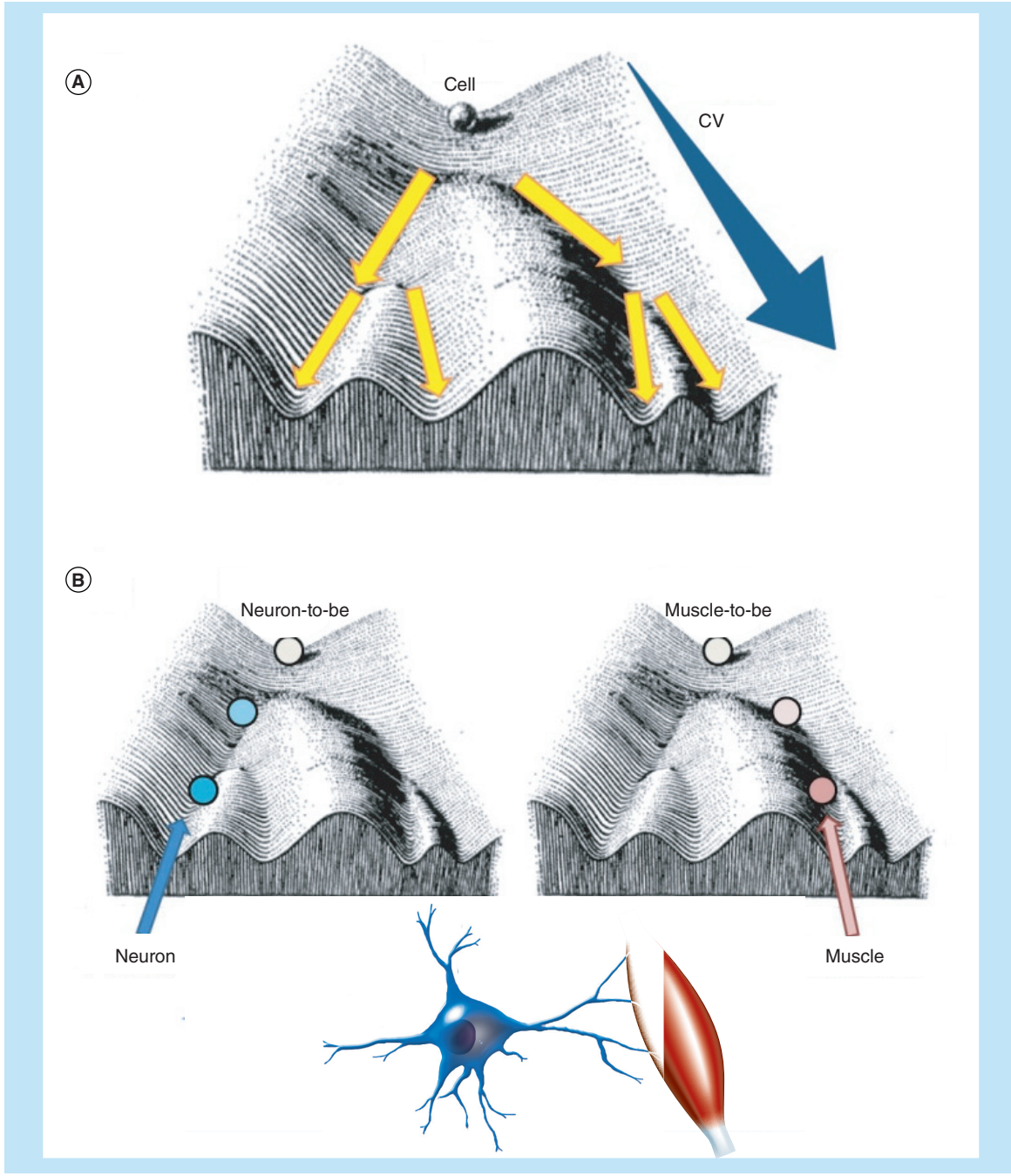

Figure 1. The chronological value of cell differentiation. **(A)** The CV of cell differentiation emphasizes the implicit notion in Waddington's epigenetic landscape that, as a cell differentiates, it advances in time or acquires a chronological position. Although cells of different lineages follow different valleys during development, we can imagine that all cells differentiate while keeping pace with the same clock throughout as far as they develop within the same organism. **(B)** The importance of introducing this chronological dimension to cell differentiation is exemplified here by the necessity in synchronizing chronological values among cells of different lineages. The innervation of a muscle cell by a neuron requires that both cells prepare for this timely developmental event because when a neuron fails to properly find its target, it will die. The same is true for the muscle cell that when not innervated, will eventually wane from loss of contractile inputs.
CV: Chronological value.
Adapted from [45].

residual factor expression, for example SOX2. A hiPSC line made this way will satisfy the current pluripotency test in that it does express both pluripotency-related markers. Under this scenario, by extinguishing OCT3/4 you would probably have no problem for ectoderm differentiation. However, you can no more induce this cell toward mesendoderm, because for this, you would need to completely silence SOX2. So here, SOX2 expression in the original hiPSC *per se* is not indicative of pluripotency. This example also provides a feasible reason why we should avoid integrating the reprogramming vectors into the genome if we cannot be entirely sure that they can be completely inactivated. Also of note, any pluripotency litmus test such as the teratoma assay which cannot predict robust cell differentiation, in our opinion, should be considered as 'obsolete' as it does not provide enough information about the *bona fide* pluripotency of the PSC. Related to this, we are not aware of any case of a PSC line being dismissed because of its failure to produce a teratoma.

Exploring pluripotency standards for drug discovery

Another confounding issue which warrants some additional consideration before we can come up with good practical standards for pluripotency is again about the difference between mPSCs and hPSCs. In particular, we will revisit the idea that hPSCs are epiblast-like before proposing a new list for standardizing hiPSCs.

Naive & epiblast-like pluripotencies: where to place hiPSCs?

Most people normally use the word 'primed' to express what we mean here by 'epiblast-like' [47]. The reason why we avoid its use is because the meaning of 'primed' has recently changed. Austin Smith, who originally defined the word 'primed,' has now changed the word to 'transitional' exactly for the reason we are going to discuss here [48].

hPSCs are considered to have phenotype most akin to the primitive ectoderm cells of an epiblast-stage embryo. We can also derive mouse epiblast stem cells (EpiSCs) when epiblasts are directly cultured in media which supports hPSCs and therefore, EpiSCs were previously considered to be a directly equivalent cell entity to hPSCs [49,50]. This was until Michinori Saitou's group showed that EpiSCs poorly differentiated into germ cells but that mESCs gradually adapted to EpiSC culture condition (which was then called EpiLCs for epiblast-like cells) showed better developmental potentials equivalent to and gene expression profile more similar to epiblasts [51]. This idea that EpiSCs are not epiblast-like was later corroborated by a study from Patrick Tam's lab in which they showed that EpiSCs correspond to the anterior mesendodermal cells but not to the cells of the epiblast stage [52]. What these studies tell us is a surprising message that cell culture conditions used to support hPSCs cannot keep mouse epiblasts at an epiblast-like stage of development but spontaneously advances them to a mesendoderm-like stage. With this revelation that EpiSCs are in a more advanced stage of development, Smith had to change his developmental nomenclature of the epiblast-stage from 'primed' to 'transitional' and to rename a further advanced gastrula-stage embryo as 'primed.' With this confusion, we will not use this familial nomenclature anymore and stick to the less fashionable epiblast-likefor the rest of the manuscript.

If mouse epiblast-like PSCs were not really epiblast-like, what about hPSCs? To the careful readers, similarities of some hiPSC lines to mesendoderm are already

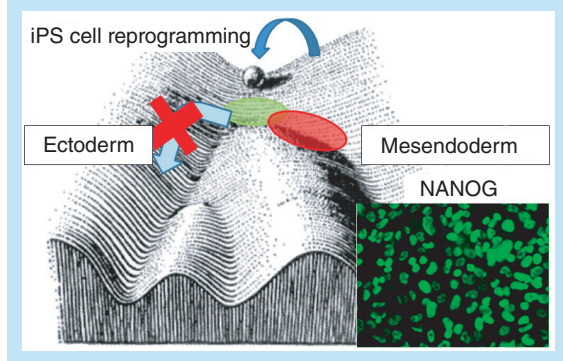

Figure 2. Mesendoderm-propensity of current human pluripotent stem cells. In-house human induced pluripotent stem cells (hiPSCs) stained for NANOG are shown in the bottom right insert. In our hands most hiPSCs made using the Yamanaka protocol display varying levels of NANOG expression. As mouse epiblasts do not express Nanog, we deduce that the cells with strong NANOG expression would represent cells with a more advanced chronological value tilted toward mesendoderm (diagonal red-shaded zone). With the 'no-going-back' rule of a cell's chronological value, it is likely that these cells would resist ectoderm differentiation as was observed for the cell line pictured here (data not shown). The ideal status of an hPSC on this landscape would be the horizontal green area where cells are kept in step with each other sharing the uniform chronological value of the epiblast-equivalent stage. Therefore, the recent finding that hiPSCs pass through a transient mesendoderm stage during reprogramming can also be interpreted that there is a lack of safeguard in keeping the cells in the green area and that the nascent hiPSCs would pass this *bona fide* pluripotent chronological value and enter the red zone instead. The subsequent culture with FGF only would then help to narrow down this diagonal red area closer but rarely converging with the horizontal green area, which can explain the lower developmental potentials of hPSCs. iPSC: Induced pluripotent stem cell. Adapted from [45].

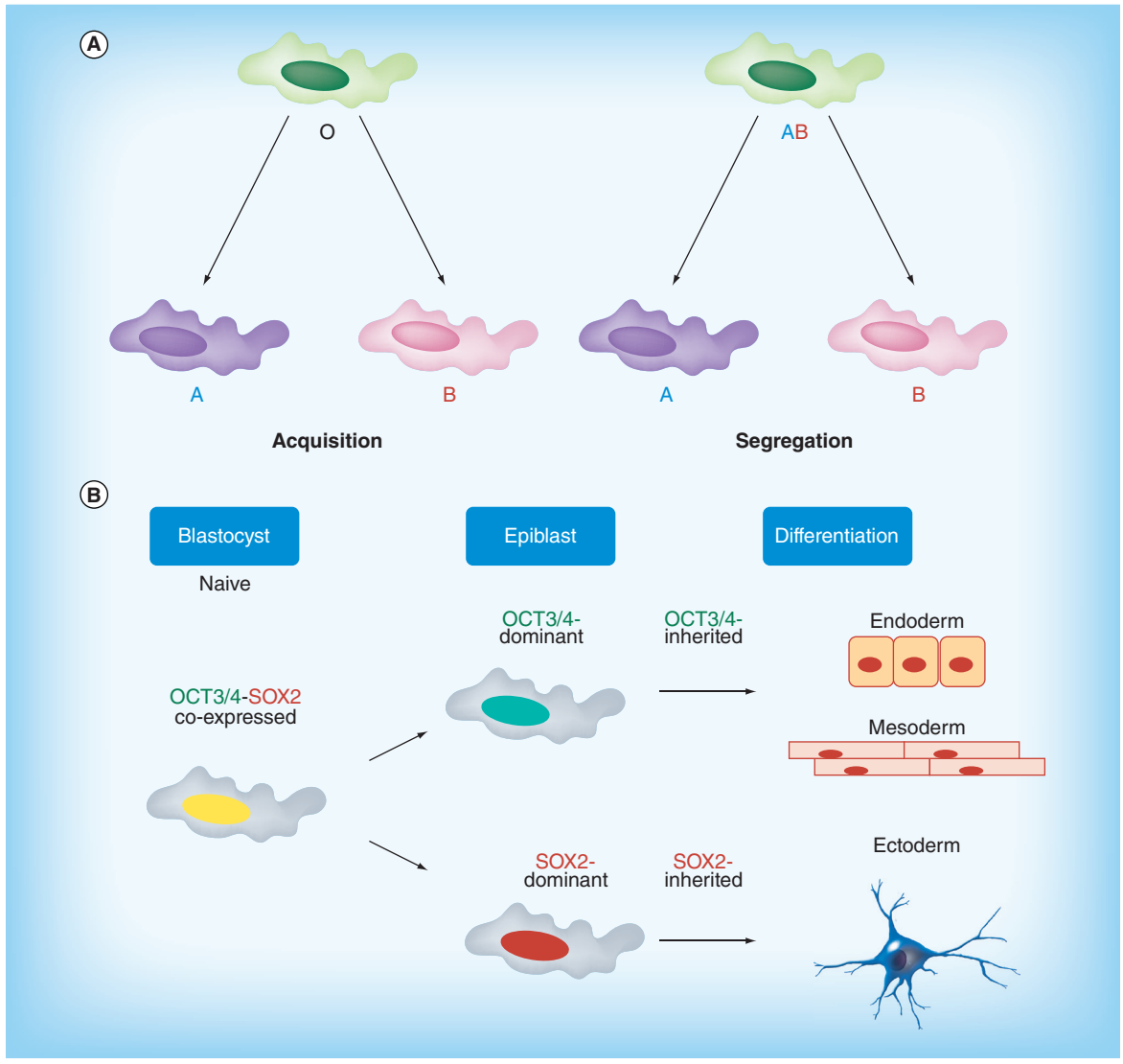

Figure 3. Differentiation by segregation versus *de novo* acquisition of factors. (A) Left: a classical view of cell differentiation. In this mode of cell differentiation, the initial cell trait (denoted here by the letter 'O') is lost upon cell differentiation and new traits (such as 'A' or 'B') are acquired *de novo*. Right: a contrasting view can be made if we suppose that the future traits ('A' and 'B') were already present (but in a less pronounced way) in the progenitor cell (marked here as 'AB'). As this aspect of cell differentiation describes the segregation or acquisition of factors as development proceeds, we will describe these views of differentiation by segregation of factors or differentiation by '*de novo* acquisition.' **(B)** This picture summarizes a recent finding from Thomson *et al.* OCT3/4 and SOX2, two of the Yamanaka factors, are co-expressed in the inner cells of the mouse blastocyst and/or mESCs. The prevailing former view would have dictated that markers of pluripotency disappear from these naive pluripotent cells upon differentiation and the offspring cells would acquire new traits (master regulators) of the relevant cell lineages. In sharp contrast, Thomson *et al.* work show that the balance of these markers' expression becomes unstable when cells reach the epiblast-equivalent developmental stage but more surprisingly, are 'inherited' in a mutually exclusive fashion upon cell differentiation, leaving OCT3/4 for mesendoderm and SOX2 for ectodermal cells.

familiar. As described above, hiPSCs with reduced developmental potentials showed some features of mesendoderm and as recently discovered, all hiPSCs under the Yamanaka protocol were once mesendoderm [10]. As a point of compromise, we may tentatively place a window between epiblast and mesendoderm for human pluripotency. A prediction here is that the closer an hPSC is to epiblast, the more pluripotent it should be. From this viewpoint, the next question is whether all hESCs are epiblast-like and not mesendoderm-like.

Although a definite answer to this question is not at hand yet, there is room for speculation that hESCs have some mesendoderm-like characteristic too. NANOG is generally considered a pluripotency-related marker

for hPSCs. Many studies suggest that its expression is under the control of SMAD signaling [53]. However, recent work from experts of mouse embryology clearly shows that although Nanog protein is abundantly expressed in the blastocyst, it is hardly present in epiblast cells [54]. What was not really fully appreciated in the beginning is the fact also shown in the paper that Nanog soon reappears in the nascent mesendodermal cells of the primitive streak. Now, NANOG is also classified as a marker of the mesendodermal cells of the anterior primitive streak [55] and therefore colocalizes with definitive mesendodermal marker T/BRY during hiPSC reprogramming [10]. Therefore, NANOG has little diagnostic value for marking the pluripotency of hPSCs. This fact alone should be claimed more often and textbooks need revision considering there are probably hundreds of papers which show the expression of NANOG in hESCs and hiPSCs to validate human pluripotency. We strongly believe that NANOG expression just shows that the hPSC line in question is instead closer to the mesendoderm state in the human pluripotency window, which is in direct opposition to the idea that NANOG expression means pluripotency. A testable prediction would then be that strong NANOG protein expression in hiPSCs is another hallmark of differentiation defectiveness as illustrated in Figure 2.

Proposed standards for human pluripotency

We have now reached the point in our discussion at which we are ready to suggest new pluripotency standards of hiPSCs. The propositions made here are not yet in full practice nor do we have enough supporting data to validate all the new recommendations. Instead, these are proposals deduced from the new aspects of cell differentiation explained in this critical review and partly also from recent results obtained in Saitama Medical University forming part of a patent application (WO2014069479). But again, none of the authors of this article are in the position to guarantee that by adhering to these standards, you can detect pure differentiation-competent hiPSC cultures. However, any detailed further discussion would be welcomed if contacted.

We strongly discourage from using teratoma assays as an indication of pluripotency. As mentioned already, these assays only give qualitative results which are not really informative about differentiation defective hiPSC clones. We would also like to relieve you of the burden of looking for pluripotency-related factors. We think these markers do not exist in a strict sense and also, from the hiPSC clones established in Saitama Medical University in the past 3 years, we have never come across hiPSCs, good or bad, which do not express OCT3/4, SOX2 or TRA-1–60. Other markers like surface embryonic antigens (i.e., TRA-1–60/81 and SSEA3/4) are always present in a very heterogeneous manner and many are not functionally validated. Their presence can even be 'harmful' as it has been shown that high SSEA3-expressor hESCs can resist differentiation [56] and TRA-1–60 labels mesendodermal cells in the due course of reprogramming [10]. We took to the habit of using these markers from the very discovery of hESC [29] but in order to discriminate good and bad hiPSCs applicable for drug screenings, these are completely useless.

Instead of the aforementioned 'classical' standard for human pluripotency, we would recommend the following:

- Try to avoid high expressors of mesendoderm markers like T/BRY and SOX17. hiPSCs tend to lean toward mesendoderm particularly under the current methods and we cannot emphasize enough that this is the major drawback of the current hiPSC reprogramming method. We ourselves have seen many examples where high expression of T/BRY indicates defective differentiation especially toward neural fates (data not shown);

- Check the expression of KLF4 and NANOG, if possible at the protein level (not RNA only). These markers were considered 'classical' pluripotency-related markers but there is hardly any logic in using these. Particularly the expression of NANOG in an hPSC context is more likely to be indicative of a mesendodermal, and therefore a differentiated cell;

- Check developmental potential for neural differentiation by avoiding SMAD-inhibition or preferably by performing default neurogenesis [57,58], a tougher but biologically relevant test for early differentiation. Another aspect worth checking is the swift downregulation of NANOG during this process. From frogs to mice, early embryonic cells default to neurogenesis when challenged in a developmentally neutral medium. Only hPSCs so far have shown relatively low differentiation efficiencies when this method is applied. We believe (and some of our results confirm this view) that hiPSCs with full pluripotency can default toward neural cells at high efficiencies;

- Check the directed differentiation of the hiPSCs toward mesendoderm when challenged with BMP4 only. Mouse epiblast primitive ectodermal cells are known to be induced toward T/Bry-positive mesendoderm by Bmp4 emanating from the juxtaposed extraembryonic ectoderm [59]. We see many protocols for inducing mesendodermal cells

by direct addition of Wnt agonists as this is a direct inducer of T/BRY. However, during the mouse development, the first Wnt agonist, Wnt3, is readily induced by Bmp4 alone [59]. We have experienced that the inducibility of WNT3 in hiPSC clones varies extensively and wanes upon prolonged hiPSC maintenance (data not shown), which is an inevitable process given our current understanding that hiPSC revert back from mesendoderm to epiblast-like cells.

Clearly, the current hiPSC reprogramming methods need improvement. Unfortunately, at the present time, it is not possible for us or for anybody else to give quantitative measures in setting this standard. We are not able to define a fixed copy number of *T/BRY* mRNA for example that a hiPSC should not exceed. This is difficult in practice because individual cell character fluctuates in time and an average measurement does not reveal the frequency of this fluctuation which may well have consequences in pluripotency. Nevertheless, we hope this is just a starting point that people in the same industry can share to consistently improve the quality of our common golden tool, the hiPSC.

Conclusion & future perspective

Today, although we have already seen some examples of laboratory successes of drug screening using human iPS cell-derived cells, there are probably only few examples of HTS success using stem cell-derived cells at manufacturing scales. This is not very surprising as it is still technically a formidable task to conquer all the inherent problems of iPSC reprogramming and obtain a manipulable cell population for drug testing as discussed herein. We, through our own experience, could deduce that the reason of thousands of failures in similar attempts is because the initial stem cell 'pluripotency' was ill defined and this situation motivated us to send this message through this critical review. Despite this situation, this field is anticipated to move forward to directly try human iPS cell-derived cells for drug testing. It is there that we will surely face serious problems as several recent reports dismiss full pluripotency for human iPS cells and this can only have serious consequences on its downstream usages as discussed. The pharmaceutical process of drug screening is like choosing good racing car drivers. The company in charge has first to serve as a steel plant, and then also as a car company to manufacture racing cars out of the steel produced. And only after that can it start to think about choosing good drivers (i.e., chemical compounds). Therefore, with drug screens using PSC-derived cells, there are disproportionate needs for efforts toward preupstream processing compared

Executive summary

Background

- Cell differentiation below 100% efficiency is irreproducible and increases noise in the readouts of drug effects.
- The setting of standards for drug discovery-grade stem cells is complicated by constantly shifting definitions of pluripotency.

Induced pluripotent stem (iPS) cell innovation

- iPS cell reprogramming must not only transition somatic cells into a pluripotent state but stabilize the resulting pluripotent stem cells in a (meta)stable cell state.
- A number of drug screens have already been performed using human iPSCs (hiPSCs) as original cell source for and has led to the discovery of some novel lead compounds.

Requirements for stem cells in drug discovery applications

- Many of the current hiPSC lines display various degrees of mesendoderm differentiation and are hence not strictly epiblast-like with full differentiation potential.
- NANOG is neither a pluripotency-related marker nor expressed in epiblasts but labels mesendodermal cells. (Naive and epiblast-like pluripotencies: where to place hiPSCs?).
- Understanding a stem cell's chronological value and its differentiation by segregation of factors help to placing pluripotent stem cells in their developmental context.

Exploring pluripotency standards for drug discovery

- Rules of thumb for identifying good hiPSCs: Avoid mesendoderm-like hiPSCs, choose lower expressors of KLF4/NANOG, and check early developmental potentials by default neurogenesis and by BMP-induced mesendoderm differentiation.

Future perspective

- New standards for hiPSCs should include an updated understanding of differentiation defective iPSCs' tendency toward mesendoderm differentiation.
- Efficient stem cell differentiation for high quality drug discovery applications can't be achieved without a clear understanding of pluripotency.

with the older paradigms. What might be somewhat surprising to the readers but we still hope to have successfully argued the fact that the most difficult part in this whole process is the 'steel plant.' We have to know and acknowledge that stem cells are inherently heterogeneous. This is undoubtedly making these processes far more difficult than just manufacturing homogeneous steel. The heterogeneity of stem cells is also making it difficult to accurately define them and this will enforce continuous basic science to fully understand its nature and to tackle it in the future.

Therefore, one final message we would like to convey is what we need to do as an 'industry' to productively play with these newcomers, the hiPSCs. We can certainly no longer sit on the sidelines of this pluripotency issue and act as idle spectators. We should all join efforts in continuously improving the level of its industrial standard.

Financial & competing interests disclosure

This work was mainly funded by MEXT KAKENHI Grant No. 23111008 and No. 26290063 to H Kato; the cost of the work was partly defrayed from Intramural Research Grant (24–9) for Neurological and Psychiatric Disorders of NCNP awarded to H Kato; M Eitoku is supported by MEXT KAKENHI Grant Number 24790588; H Kiyosawa is supported by MEXT KAKENHI Grant No. 24115703 and No. 26290063. This work was supported in part by the grant of Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) and Grant-in-Aid for Development of New Technology from The Promotion and Mutual Aid Corporation for Private Schools of Japan from MEXT to Y Okazaki. C Neeley is an employee at Thermo Fisher Scientific. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Takahashi K, Tanabe K, Ohnuki M *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131(5), 861–872 (2007).

2 Buganim Y, Faddah DA, Jaenisch R. Mechanisms and models of somatic cell reprogramming. *Nat. Rev. Genet.* 14(6), 427–439 (2013).

3 Zhang B, Krawetz R, Rancourt DE. Would the real human embryonic stem cell please stand up? *BioEssays* 35(7), 632–638 (2013).

4 Young RA. Control of the embryonic stem cell state. *Cell* 144(6), 940–954 (2011).

5 Nichols J, Smith A. Pluripotency in the embryo and in culture. *Cold Spring Harb. Perspect. Biol.* 4(8), a008128 (2012).

6 Theunissen TW, Jaenisch R. Molecular control of induced pluripotency. *Cell Stem Cell* 14(6), 720–734 (2014).

7 Osafune K, Caron L, Borowiak M *et al.* Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat. Biotechnol.* 26(3), 313–315 (2008).

•• **First indication that human embryonic stem cells are not perfectly pluripotent.**

8 Hu BY, Weick JP, Yu J *et al.* Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proc. Natl Acad. Sci. USA* 107(9), 4335–4340 (2010).

9 Ma H, Morey R, O'Neil RC *et al.* Abnormalities in human pluripotent cells due to reprogramming mechanisms. *Nature* 511(7508), 177–183 (2014).

•• **Strong challenge against human induced pluripotent stem cells (hiPSC) reprogramming by showing epigenetic flaws.**

10 Takahashi K, Tanabe K, Ohnuki M *et al.* Induction of pluripotency in human somatic cells via a transient state resembling primitive streak-like mesendoderm. *Nat. Commun.* 5, 3678 (2014).

•• **Important revealation of the mesendoderm nature of current hiPSCs.**

11 Tanabe K, Nakamura M, Narita M, Takahashi K, Yamanaka S. Maturation, not initiation, is the major roadblock during reprogramming toward pluripotency from human fibroblasts. *Proc. Natl Acad. Sci. USA* 110(30), 12172–12179 (2013).

12 Abbasalizadeh S, Baharvand H. Technological progress and challenges towards cGMP manufacturing of human pluripotent stem cells based therapeutic products for allogeneic and autologous cell therapies. *Biotechnol. Adv.* 31(8), 1600–1623 (2013).

13 Grskovic M, Javaherian A, Strulovici B, Daley GQ. Induced pluripotent stem cells—opportunities for disease modelling and drug discovery. *Nat. Rev. Drug Discov.* 10(12), 915–929 (2011).

14 Ladewig J, Koch P, Brustle O. Leveling Waddington: the emergence of direct programming and the loss of cell fate hierarchies. *Nat. Rev. Mol. Cell Biol.* 14(4), 225–236 (2013).

15 Miller J, Studer L. Aging in iPS cells. *Aging* 6(4), 246–247 (2014).

•• **Interesting new issue related the 'age' of the induced pluripotent stem cell-derived cells.**

16 Chakraborty C, Hsu CH, Wen ZH, Lin CS. Recent advances of fluorescent technologies for drug discovery and development. *Curr. Pharm. Design* 15(30), 3552–3570 (2009).

17 Takebe T, Sekine K, Enomura M *et al.* Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* 499(7459), 481–484 (2013).

18 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126(4), 663–676 (2006).

19 Shu J, Wu C, Wu Y *et al.* Induction of pluripotency in mouse somatic cells with lineage specifiers. *Cell* 153(5), 963–975 (2013).

20 Montserrat N, Nivet E, Sancho-Martinez I *et al.* Reprogramming of human fibroblasts to pluripotency with lineage specifiers. *Cell Stem Cell* 13(3), 341–350 (2013).

21 Sommer CA, Christodoulou C, Gianotti-Sommer A *et al.* Residual expression of reprogramming factors affects the transcriptional program and epigenetic signatures of induced pluripotent stem cells. *PLoS One* 7(12), e51711 (2012).

22 Stadtfeld M, Apostolou E, Akutsu H *et al.* Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature* 465(7295), 175–181 (2010).

23 Ying QL, Wray J, Nichols J *et al.* The ground state of embryonic stem cell self-renewal. *Nature* 453(7194), 519–523 (2008).

• **Seminal finding to stabilize mouse pluripotent stem cells.**

24 Marks H, Kalkan T, Menafra R *et al.* The transcriptional and epigenomic foundations of ground state pluripotency. *Cell* 149(3), 590–604 (2012).

25 Leitch HG, McEwen KR, Turp A *et al.* Naive pluripotency is associated with global DNA hypomethylation. *Nat. Struct. Mol. Biol.* 20(3), 311–316 (2013).

26 Gafni O, Weinberger L, Mansour AA *et al.* Derivation of novel human ground state naive pluripotent stem cells. *Nature* 504(7479), 282–286 (2013).

27 Theunissen TW, Powell BE, Wang H *et al.* Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell Stem Cell* 15(4), 471–487 (2014).

28 Takashima Y, Guo G, Loos R *et al.* Resetting transcription factor control circuitry toward ground-state pluripotency in human. *Cell* 158(6), 1254–1269 (2014).

• **New directions of hiPSCs by mimicking mouse naive ground state.**

29 Thomson JA, Itskovitz-Eldor J, Shapiro SS *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391), 1145–1147 (1998).

30 Ohgushi M, Matsumura M, Eiraku M *et al.* Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells. *Cell Stem Cell* 7(2), 225–239 (2010).

31 Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157(6), 1262–1278 (2014).

32 Yang YM, Gupta SK, Kim KJ *et al.* A small molecule screen in stem-cell-derived motor neurons identifies a kinase inhibitor as a candidate therapeutic for ALS. *Cell Stem Cell* 12(6), 713–726 (2013).

33 Yamashita A, Morioka M, Kishi H *et al.* Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. *Nature* 513(7519), 507–511 (2014).

34 Di Giorgio FP, Boulting GL, Bobrowicz S, Eggan KC. Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. *Cell Stem Cell* 3(6), 637–648 (2008).

35 Kim H, Lee G, Ganat Y *et al.* miR-371–3 expression predicts neural differentiation propensity in human pluripotent stem cells. *Cell Stem Cell* 8(6), 695–706 (2011).

•• **Seminal paper to first describe mechanisms behind differentiation deficiency of human pluripotent stem cells.**

36 Kriks S, Shim JW, Piao J *et al.* Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480(7378), 547–551 (2011).

37 Chambers SM, Qi Y, Mica Y *et al.* Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors. *Nat. Biotechnol.* 30(7), 715–720 (2012).

38 Maroof AM, Keros S, Tyson JA *et al.* Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell Stem Cell* 12(5), 559–572 (2013).

39 Koyanagi-Aoi M, Ohnuki M, Takahashi K *et al.* Differentiation-defective phenotypes revealed by large-scale analyses of human pluripotent stem cells. *Proc. Natl Acad. Sci. USA* 110(51), 20569–20574 (2013).

40 Ohnuki M, Tanabe K, Sutou K *et al.* Dynamic regulation of human endogenous retroviruses mediates factor-induced reprogramming and differentiation potential. *Proc. Natl Acad. Sci. USA* 111(34), 12426–12431 (2014).

41 Rosa A, Papaioannou MD, Krzyspiak JE, Brivanlou AH. miR-373 is regulated by TGFbeta signaling and promotes mesendoderm differentiation in human embryonic stem cells. *Dev. Biol.* 391(1), 81–88 (2014).

42 Stelzer Y, Sagi I, Benvenisty N. Involvement of parental imprinting in the antisense regulation of onco-miR-372–373. *Nat. Commun.* 4, 2724 (2013).

43 Rosa A, Brivanlou AH. Regulatory non-coding RNAs in pluripotent stem cells. *Int. J. Mol. Sci.* 14(7), 14346–14373 (2013).

44 Kohama C, Kato H, Numata K *et al.* ES cell differentiation system recapitulates the establishment of imprinted gene expression in a cell-type-specific manner. *Hum. Mol. Genet.* 21(6), 1391–1401 (2012).

• **Paper hinting to the usefulness of analyzing the total transcriptome.**

45 Waddington CH. *The Strategy of the Genes: A Discussion of Some Aspects of Theoretical Biology.* Allen & Unwin, London, UK (1957).

46 Thomson M, Liu SJ, Zou LN, Smith Z, Meissner A, Ramanathan S. Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. *Cell* 145(6), 875–889 (2011).

47 Nichols J, Smith A. Naive and primed pluripotent states. *Cell Stem Cell* 4(6), 487–492 (2009).

48 Leitch HG, Smith A. The mammalian germline as a pluripotency cycle. *Development* 140(12), 2495–2501 (2013).

49 Brons IG, Smithers LE, Trotter MW *et al.* Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448(7150), 191–195 (2007).

50 Tesar PJ, Chenoweth JG, Brook FA *et al.* New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448(7150), 196–199 (2007).

51 Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell* 146(4), 519–532 (2011).

52 Kojima Y, Kaufman-Francis K, Studdert JB *et al.* The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. *Cell Stem Cell* 14(1), 107–120 (2014).

53 Xu RH, Sampsell-Barron TL, Gu F *et al.* NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. *Cell Stem Cell* 3(2), 196–206 (2008).

54 Acampora D, Di Giovannantonio LG, Simeone A. Otx2 is an intrinsic determinant of the embryonic stem cell state and is required for transition to a stable epiblast stem cell condition. *Development* 140(1), 43–55 (2013).

55 Mendjan S, Mascetti VL, Ortmann D *et al.* NANOG and CDX2 pattern distinct subtypes of human mesoderm during exit from pluripotency. *Cell Stem Cell* 15(3), 310–325 (2014).

56 Stewart MH, Bosse M, Chadwick K, Menendez P, Bendall SC, Bhatia M. Clonal isolation of hESCs reveals heterogeneity within the pluripotent stem cell compartment. *Nat. Methods* 3(10), 807–815 (2006).

57 Eiraku M, Sasai Y. Self-formation of layered neural structures in three-dimensional culture of ES cells. *Curr. Opin. Neurobiol.* 22(5), 768–777 (2012).

58 Patani R, Compston A, Puddifoot CA *et al.* Activin/Nodal inhibition alone accelerates highly efficient neural conversion from human embryonic stem cells and imposes a caudal positional identity. *PLoS One* 4(10), e7327 (2009).

59 Aramaki S, Hayashi K, Kurimoto K *et al.* A mesodermal factor, T, specifies mouse germ cell fate by directly activating germline determinants. *Dev. Cell* 27(5), 516–529 (2013).