Genomics Proteomics Bioinformatics

REVIEW

Pluripotency of Induced Pluripotent Stem Cells

Chunjing Feng [1,2,3], Yun-Dan Jia [1,2], Xiao-Yang Zhao [1,2,*]

[1] State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[2] Key Laboratory of Translational Stem Cell Research, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[3] University of Chinese Academy of Sciences, Beijing 100049, China

Abstract Induced pluripotent stem (iPS) cells can be generated by forced expression of four pluripotency factors in somatic cells. This has received much attention in recent years since it may offer us a promising donor cell source for cell transplantation therapy. There has been great progress in iPS cell research in the past few years. However, several issues need to be further addressed in the near future before the clinical application of iPS cells, like the immunogenicity of iPS cells, the variability of differentiation potential and most importantly tumor formation of the iPS derivative cells. Here, we review recent progress in research into the pluripotency of iPS cells.

Introduction

Induced pluripotent stem (iPS) cells can be derived from mouse somatic cells via the ectopic expression of four defined factors, Oct4, Sox2, Klf4 and c-Myc (also known as Yamanaka factors) [1]. The mouse iPS cells express pluripotency markers and both X chromosomes are reactivated, allowing differentiation into various cell types of three germ layers when injected into a blastocyst. iPS technology makes reprogramming much easier [2,3] in comparison to early reprogramming methods such as somatic cell nuclear transfer (SCNT) [4,5], iPS technology also circumvents the ethical problems arising from the use of human oocytes. In addition, the generation of

patient-specific iPS cells could be used to screen new drugs [6,7]. However, there are currently several limitations in applying iPS cells clinically. Efficiency of converting somatic cells to iPS cells is still very low. In particular, only approximately 0.1% to 1% of somatic cells experience changes at the transcriptional level and finally become pluripotent stem cells when non-integration approaches are used [8]. Moreover, compared to embryonic stem (ES) cells, the developmental potential and differentiation capacity of iPS cells is significantly reduced and there is increased variability among all iPS cell lines [9]. In mice, only small proportions of these cells are fully reprogrammed based on the most stringent tetraploid complementation assay for evaluating pluripotency [10–13]. Therefore, it is necessary to establish a strict molecular standard system to distinguish fully reprogrammed iPS cells from those partially reprogrammed, as we currently lack suitable *in vivo* pluripotency tests for human iPS cells.

In this review, we mainly focus on recent progress on rodent, non-human primate and human iPS cells, and point out some key questions which need to be addressed in the near future, such as the pluripotency level of human iPS cells and

* Corresponding author.
E-mail: xyzhao@ioz.ac.cn (Zhao XY).

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the establishment of a new standard to assess the pluripotency level of human iPS cells.

Generation of non-integration iPS cells

Takahashi and Yamanaka reprogrammed mouse embryonic fibroblasts by the ectopic expression of four reprogramming factors using retroviral vectors, and finally produced iPS cells which resemble ES cells [1]. This original iPS reprogramming approach used viral vectors, including retrovirus and lentivirus which possess high reprogramming efficiency [14,15]. The genome may be mutated by integrating other gene sequences, thus raising concerns on the safety issue. In addition, the insertion of oncogenes, like c-Myc, increases the risk of tumor formation [16,17]. Subsequently, several modified methods were used to obtain much safer iPS cells, for instance, piggyBac transposon [18], adenovirus [19], sendai virus [20], plasmid [21], episomal vectors [22] and minicircle vectors [23]. However, the reprogramming efficiency is significantly decreased and it takes longer to reactivate the key pluripotency markers to achieve full reprogramming. Therefore, efficient generation of non-integrated iPS cells by new approaches may promote their clinical application.

Recent studies have described several reprogramming methods using proteins, RNAs and small-molecule compounds to derive safe iPS cells [24–26]. Zhou et al. obtained iPS cells induced by recombination of the proteins of the four Yamanaka factors obtained by fusing the C-terminus of the proteins with poly-arginine (11R) [24]. A recent study reported that mouse and human iPS cells can be efficiently generated by miRNA mediated reprogramming [25]. Miyoshi et al. [26] successfully generated iPS cells by direct transfection of human somatic cells using mature miRNA. iPS cells can also be generated by synthetic RNAs, which bypass the innate response to viruses [27]. Recently, Houet et al. [28] showed that pluripotent stem cells can be generated from mouse somatic cells at an efficiency of 0.2% by using a combination of seven small-molecule

compounds. Compared to traditional viral methods, the aforementioned approaches can be used to generate qualified iPS cells (Table 1) without the risk of insertional mutagenesis. Nonetheless, some familiar drawbacks exist, such as a longer and less efficient reprogramming process. In other words, what we need to do next is to optimize non-integration induction systems in order to resolve these drawbacks.

The pluripotency of mouse iPS cells

Pluripotency of mouse ES and iPS cells can be detected by a series of testing standards. These standards include the expression of pluripotency markers, alkaline phosphatase (AP) staining, teratoma formation in vitro, the formation of diploid chimera, and tetraploid complementation. The first-generation of iPS cells resemble ES cells in morphology and express some pluripotency markers, but are not able to produce live chimeric mice [1], indicating that the original iPS cells were not fully reprogrammed to pluripotent stem cells. Subsequently, modified protocols were used for reprogramming to create improved qualified iPS cells, which resulted in the generation of live chimeric mice with germline transmission [16,29].

Whether fully reprogrammed pluripotent stem cells have the ability to generate iPS-mice through tetraploid complementation has been questioned for a long time until 2009, when live pups were finally generated in two independent research laboratories [12,13]. Since then, no matter reprogrammed by one or three factors [30,31], using adult or fetal cells [13,32], the iPS cells are able to generate iPS-mice, even after genetic modification.

However, not all mouse iPS cells are able to pass the most rigorous tetraploid complementation assessment. Therefore it is important to identify molecular markers which can predict the pluripotency level of iPS cells, which will help future mechanistic studies. A small number of transcriptionally active genes within the imprinted Dlk1-Dio3 gene cluster on chromosome 12qF1, particularly Glt2 and Rian, are aberrantly

Table 1   Summary of different reprogramming methods for the generation of iPS cells

Viral or nonviral	Type of vector	Genomic integration	Advantages	Disadvantages	Ref
Viral	Retrovirus	Yes	Stably integrated into the host genome; high efficiency	Too risky because of their insertional tendencies; cause tumor formation	[1,16]
	Lentivirus	Yes	Reduces the risk of transgene expression; high efficiency	Too risky because of their insertional tendencies	[14]
	Adenovirus	No[*]	Lacking viral integration; high efficiency	Tend to carry the virus genome	[19]
	Sendai virus	No[**]	Lacking viral integration; high efficiency	Tend to carry the virus genome	[20]
Nonviral	piggyBac transposon	No[***]	Virus-free	A labor-intensive process	[18]
	Plasmid	No[*]	Virus-free; no integration of the plasmid into the host genome	Lower efficiency; four rounds of transfection	[21]
	Episomal vector	No[*]	Virus-free; a single transfection	Lower efficiency	[22]
	Minicircle vector	No[*]	Virus-free; higher transfection efficiency	Longer ectopic expression	[23]
	Protein	No	Virus-free	Lower efficiency	[24]
	RNA	No	Virus-free; high efficiency	Labor-intensive procedures	[25–27]
	Small molecule	No	Virus-free	Lower efficiency	[28]

Note: [*] Lack of genomic integration can be examined; [**] lack of virus RNA genome can be examined; [***] transposon vector can be removed from the genome.

silenced in most iPS cell lines. These iPS cell lines poorly contribute to chimeras and fail to support the development of iPS cell-derived embryos generated by tetraploid complementation [33,34]. In contrast, in fully pluripotent iPS cell lines these genes are expressed at levels comparable to those in embryonic stem cells.

The pluripotency of human iPS cells

Human iPS cells produced via somatic cell reprogramming have opened up another new territory for regenerative medicine. Human iPS cells generated from adult human fibroblasts express hES cell-specific surface antigens, including SSEA-3, SSEA-4, tumor-related antigen (TRA)-1–60, TRA-1–81 and NANOG protein, while displaying high telomerase activity and multiple differentiation potential [35–37]. In addition, human iPS cells can differentiate into cells of all three germ layers. However, unlike the mouse situation, there are no suitable *in vivo* testing standards for human ES/iPS cells available that can be applied to test the *in vivo* functions in embryonic development and pluripotency. As a result, the failure to distinguish pluripotent cell lines will hinder clinical application in the future (**Table 2**).

The pluripotency of naïve iPS cells

Pluripotency can be defined as the ability of a single cell to differentiate into all types of somatic cells in an adult organism. Rodent pluripotent stem cells can be considered to exist in two distinct states: naïve and primed [38]. Rodent naïve pluripotent stem cells can be derived through expansion of the inner cell mass (ICM) of the blastocyst, the reprogramming of somatic cells, or the reversion of primed pluripotent cells. Rodent primed pluripotent stem cells can be accessed through harvesting the post-implantation epiblast or pre-implantation blastocyst [39–41]. Naïve and primed iPS cells have some common characteristics: indefinite self-renewal, tri-germ layer differentiation potential and reliance on the core transcription factors Oct4, Sox2 and Nanog. However, naïve pluripotent stem cells are distinguished from primed cells in that they rely mainly on leukemia inhibitory factor (LIF) signaling and MEK/GSK3 inhibition to maintain their self-renewal, two active X chromosomes in female cells and pluripotency to generate high-grade chimeras, even tetraploid complementation animal [11,42–44]. Primed pluripotent stem cells, however, depend on the Activin-nodal signaling pathway. They generate chimeric mice with low efficiency and fail to contribute to the germline of chimeric mice [40].

Human embryonic stem cells are derived from pre-implantation blastocysts and more closely resemble mouse epiblast stem cells in pluripotency level, when compared with rodent counterparts [45]. In fact, several attempts to capture human naïve pluripotent stem cells have been carried out. Naïve-like female human ES cells (with two active X chromosomes) were derived in 5% oxygen and conventional human ES culture conditions containing bFGF [46]. NANOG-positive cells can be harvested from human pre-implantation embryos and maintained *in vitro* at physiological oxygen concentrations when supplemented with FGF inhibitor or 2i, which is used to stabilize naïve rat ES cells. This suggests that some transient naive cells may exist in early human embryos [47,48]. Though we have witnessed exciting progress in the field of naïve human pluripotent stem cells research, definitive evidence for naïve

Table 2   Pluripotency levels of ES/iPS cells vary among different species

Cell type	Species	Pluripotency markers	AP staining	Teratoma formation	Diploid chimera/germline	Tetraploid complementation	Ref
ES/iPS	Mouse	Positive	Positive	Yes	Yes/Yes	Yes	[1,16]
ES/iPS	Rat	Positive	Positive	Yes	Yes/Yes	Unknown	[47]
ES/iPS	Human	Positive	Positive	Yes	\	\	[35,45]
ES/iPS	Rhesus monkey	Positive	Positive	Yes	Unknown/Unknown	Unknown	[49–51]

Note: \ indicates that chimera assay cannot be used in human ES/iPS pluripotency test.
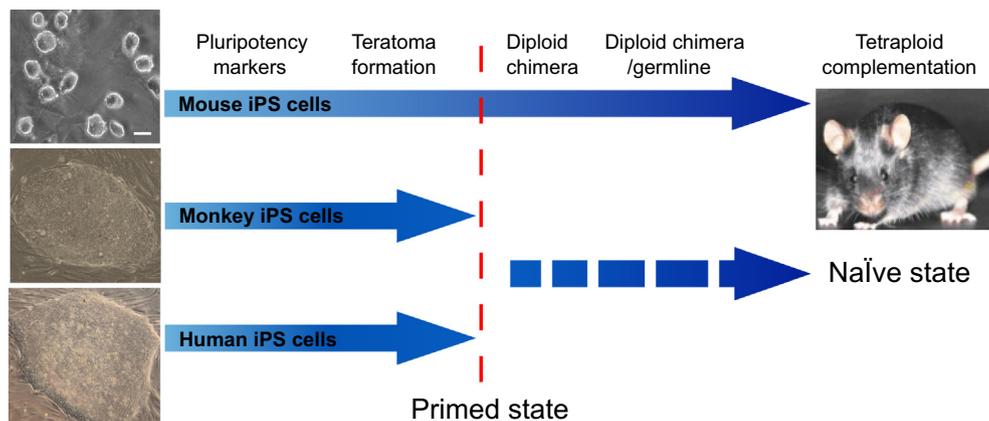
Figure 1   Comparison of pluripotency levels of iPS cells from mouse, human and monkey

human pluripotent stem cell state is lacking. Although reprogramming mouse embryonic fibroblast using the four Yamanaka factors in mouse ES culture medium yields naïve mouse iPS cells, similar endeavor in reprogramming human embryonic fibroblasts by applying naïve culture condition generates human iPS cell lines that lack characteristic qualities seen in *bona fide* mouse ES/iPS cells [1,15]. This suggests a critical question: whether human naïve-specific pluripotent stem cells can feature characteristics seen in rodent cell lines.

Non-human primate ES cells and iPS cells share very similar characteristics with regard to pluripotency markers, gene expression and ability to differentiate into all three germ layers [49,50]. The chimera assay can be used to distinguish primed and naïve rodent pluripotent stem cells, which cannot be used on human cells. Non-human primate cells may be the most ideal substitute to test the rodent paradigm (Figure 1). Similar to mouse primed stem cells (epiblast stem cells), a report shows that rhesus monkey ES cells cannot produce high-grade chimeric embryos or blastocysts [51], although ES cells can be observed in the ICM of blastocyst transiently when introduced to four-cell stage embryos. These results suggest that non-human primate embryonic stem cells are therefore primed stem cells. Primates, as the model animal most similar to human, can be used for chimera arrays to screen Naïve pluripotent stem cells. Additionally, development of new methods to functionally evaluate non-human pluripotent stem cells, such as by establishing a fetal chimera array, may help to evaluate the pluripotency level of human pluripotent stem cells in the future.

Competing interests

The authors declared that no competing interests exist.

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