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Generation of pluripotent stem cells via protein transduction

XIA LI, PENGFEI ZHANG, CHAO WEI and YUNHAI ZHANG*

Anhui Provincial Laboratory for Local Livestock and Poultry Genetic Resource Conservation and Breeding, College of Animal Science and Technology, Anhui Agricultural University, Hefei, China

ABSTRACT The development of techniques for reprogramming somatic cells led to the birth of the cloned sheep "Dolly" and the generation of induced pluripotent stem cells (iPSCs). iPSCs hold great promise for *in vitro* disease modeling, new drug screening, regenerative medicine and agricultural production. These cells can differentiate into almost any tissue types and they can be used to produce autografts that will not be rejected by the patient. However, practical application has been limited by the potential for insertion mutagenesis and by the complexity of the associated procedures. A protein-based approach to generation of iPSCs could offer better prospects by avoiding these problems. This review provides an overview of the key processes and mechanism involved in protein-based somatic cell reprogramming, discusses some promising methods for increasing its efficiency and future challenges.

KEY WORDS: *protein, induced pluripotent stem cell, reprogramming, small molecule, immunogenicity*

Introduction

Induced pluripotent stem cells (iPSCs) are somatic cells whose characteristics are reprogrammed, by ectopic expression of defined transcription factors, to resemble those of embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006). Owing to their ability to replicate indefinitely and to differentiate into numerous cells types, generation of iPSCs is thought as one of the important breakthrough in the field of stem cell research (Geoghegan and Byrnes, 2008), along with the earlier isolation of mouse and human ESCs.

Since the first iPSCs were generated by Yamanaka and his colleagues, many types of cells from different animals were successfully induced into the pluripotent state by various methods. However, the reprogramming mechanism is unclear and some problems remain unresolved, including safety issues stemming from the integration of foreign genes into the host genome and from the usage of virus vectors. For example, Yamanaka's initial iPSCs failed to produce germline chimeras, which may have been caused by an insufficient degree of reprogramming in the iPSCs (Takahashi and Yamanaka, 2006). Thereafter, Yamanaka *et al.,* overcame this hurdle and generated murine iPSCs with germline competency. Unfortunately, they found that approximately 20% of the offspring developed tumors attributable to reactivation of the *c-Myc* transgene (Okita *et al.*, 2007).

To introduce reprogramming transcriptional factors into somatic cells while minimizing or avoiding insertion mutagenesis, several techniques using virus-free systems have been developed; these include the use of non-integrating adenoviruses (Stadtfeld *et al.*, 2008) and Sendai viruses (Ban *et al.*, 2011), plasmids (Okita *et al.*, 2010), piggybac transposons (Woltjen *et al.*, 2009) and episomal vectors (Yu *et al.*, 2009). Although these methods reduced genome integration significantly, the DNA of the reprogramming factors (RFs) and short vectors after excision can also cause insertion mutagenesis; therefore, a DNA-free system is highly desirable. To date, virus- and transgene-free iPSCs have been derived through ectopic expression of transcriptional factors using mRNA (Warren *et al.*, 2010, Yakubov *et al.*, 2010), minicircle RNA (Jia *et al.*, 2010), reprogramming proteins (Nemes *et al.*, 2013, Zhang *et al.*, 2012, Zhou *et al.*, 2009) and small molecule compounds (Hou *et al.*, 2013). The most obvious method for avoiding DNA integration into the host-cell genome is delivery of the RFs as recombinant proteins. This method can also provide great control over the concentration, timing, and combination of reprogramming transcription factors.

The critical steps in the process are the production of large quantities of pure bioactive proteins, translocation of these to the nucleus of the somatic cell, and modification of gene expression by the proteins. Large quantities of the required protein can be produced in prokaryotic and eukaryotic cells. They can be trans-

Abbreviations used in this paper: iPSCs, induced pluripotent stem cells; ESCs, embryonic stem cells; RFs, reprogramming factors; PTDs, protein transduction domains; CPPs, cell-penetrating peptides; NLS, nuclear localization signal; R, arginine; SLO, streptolysin O; HIV-TAT, human immunodeficiency virus transactivator of transcription; HS, heparin sulfate; TLR3, toll-like receptor 3; Vc, Vitamin C.

***Address correspondence to:** Yunhai Zhang. College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, China. Tel./Fax.: +86-551-6-5786-357. E-mail: yunhaizhang@ahau.edu.cn

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ported into cells if fused in frame to protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) (Beerens *et al.*, 2003). In this review, we give an overview of protein-based somatic cell reprogramming, analyze the key steps and examine the potential for development of more efficient methods in the future.

Source of reprogramming proteins

In 1997, the birth of cloned sheep Dolly by somatic cell nuclear transfer demonstrated that terminally differentiated cells still have totipotency (Wilmut *et al.*, 1997). In 2000, Kikyo *et al.,* first used egg cytoplasm of *Xenopus laevis* to induce the reprogramming of somatic nuclei, and later extracts of various pluripotent cells, such as carcinoma cells (Freberg *et al.*, 2007, Taranger *et al.*, 2005) and ESCs (Rajasingh *et al.*, 2008) were used to investigate cell dedifferentiation. Recently, many studies have shown that the proteins in these cell extracts are critical to the process of reprogramming somatic cells into iPSCs (Bui *et al.*, 2012, Cho *et al.*, 2010, Ganier *et al.*, 2011, Singhal *et al.*, 2010). However, it is not known which proteins play a decisive role.

In 2009, by using purified and defined recombinant proteins, which were produced in *Escherichia coli* (*E. coli*), Zhou *et al.,* first reported the successful reprogramming of mouse somatic cells to a pluripotent state (Zhou *et al.*, 2009). At present, *E. coli* and mammalian cells have been used to produce defined transcription proteins for iPSCs generation. The *E. coli* expression system can produce a high yield in a short time, and the protein purification procedure is simple. In 2011, Tang *et al.,* demonstrated these features of the *E. coli* expression system and reported that the fusion-protein construct is important for their reprogramming activity (Tang *et al.*, 2011). They found that replacing the Klf4 viral factor with the Klf4 protein fused with trans-activator of transcription (TAT) at the N-terminus showed no reprogramming activity, whereas the fusion-protein with Discosoma red fluorescent protein between TAT and KLf4 caused significant generation of iPSCs. The cost of the *E. coli* expression system is low, once the construct of the protein expression vector has been produced, but it has a major disadvantage in that the recombinant proteins have to be released from inclusion bodies under denaturing conditions. The subsequent steps of protein solubilization, refolding, and purification may also affect the bioactivity of recombinant proteins. These features of the *E. coli* expression system have been reported in the work of Pan *et al.,* (Pan *et al.*, 2010). In their study, the denatured recombinant proteins showed very weak biological activity resulting in ineffective reprogramming of somatic cells. To find and solve these problems relating to biological activity, by using Oct4 recombinant protein replacing *Oct4* viral factor during *Sox2*, *Klf4* and *c-Myc* retroviruses factors mediated reprogramming, Thier *et al.,* demonstrated the biological activity of Oct4 recombinant protein from *E. coli* (Thier *et al.*, 2010). Subsequently, using the same method, the biological activity of Sox2 (Thier *et al.*, 2012) and Klf4 (Tang *et al.*, 2011) recombinant proteins were shown. A dual luciferase reporter system can be used to evaluate biological activity *in vivo* (Pan *et al.*, 2010, Yang *et al.*, 2009, Zhang *et al.*, 2012). iPSCs can also be generated using commercially available pure proteins, also produced by the *E. coli* expression system (Khan *et al.*, 2013, Lee *et al.*, 2012). The *in vitro* activity of these proteins is always determined by the electrophoretic mobility shift assay.

In contrast to *E. coli*, the mammalian cell expression system is capable of producing correct protein folding, assembly and post-translational modification; most recombinant protein pharmaceuticals are produced in mammalian cells (Wurm, 2004). However, the technical and financial costs, associated with purification and large-scale production, impair its application. So far, there has only been a single report of iPSCs being generated by proteins from a mammalian cell expression system (Kim *et al.*, 2009). These researchers first generated stable HEK293 cell lines that could each express one of four RFs; then using extracts of these cells they induced reprogramming of somatic cells. It took six rounds of transduction over at least 8 weeks until iPSCs colonies were observed.

The yeast expression system is another potential option; it could offer large-scale production, eukaryotic protein folding and suitable post-translational modification (Romanos *et al.*, 1992). However, to date, there have no reports of iPSCs generated via proteins produced in yeast.

Methods of transporting proteins across membranes

Another technical obstacle is transportation of proteins across the cell and nuclear membranes to their site of action. So far, streptolysin O (SLO), CPPs and other vectors have been used for this purpose.

For the reprogramming of somatic cells by pluripotent-cell extracts, SLO, a toxic thiol-activated membrane-poration protein, is used as the membrane-permeabilization agent (Bui *et al.*, 2012, Cho *et al.*, 2010, Singhal *et al.*, 2010). In the case of SLO-mediated reversible permeabilization, damaged membranes have been shown to exhibit large, functional holes that allow for the passage of large molecules with molecular diameters exceeding 15 nm (Bhakdi *et al.*, 1985, Taranger *et al.*, 2005). This reprogramming procedure using cell extracts and SLO has the advantage of being particularly simple and efficient. For example, Cho *et al.,* reported that a single transfer of ESC-derived proteins into primary cultures of adult mouse fibroblasts can produce fully reprogrammed iPSCs in 25 days (Cho *et al.*, 2010). So far, there have been no reports of pure, defined proteins being inserted into target cells using SLO-mediated reversible permeabilization methods.

In 1988, Frankel found that the human immunodeficiency virus transactivator of transcription (HIV-TAT) protein could penetrate the cell membrane and activate HIV-specific genes (Frankel *et al.*, 1988, Frankel and Pabo, 1988). The key functional structure of the HIV-TAT protein is TAT, which is composed of a specific 10–20 amino acid sequence. After being added to the culture medium, TAT-fusion proteins can rapidly enter cells and achieve maximal intracellular concentrations in less than 5 min (Becker-Hapak *et al.*, 2001). In 2001, Tyagi *et al.,* reported that heparin sulfate (HS) proteoglycan is a receptor that causes cells to internalize the TAT protein. This was demonstrated by comparing cells with genetically impaired synthesis of HS with wild-type cells, or by using lyases that specifically degrade HS chains (Tyagi *et al.*, 2001). Heparin was used to remove residual proteins attached to the cell surface during assessment of protein transduction (Yang *et al.*, 2009). Then, many groups found TAT-fusion proteins could rapidly enter the cells via lipid raft-dependent macropinocytosis (Murriel and Dowdy, 2006, Wadia *et al.*, 2004, Ziegler *et al.*, 2005). Further studies showed that TAT-PTD sequence-enriched arginine residues and cationic CPPs primarily consist of multiple arginine and lysine

TABLE 1

SUMMARY OF METHODS USED FOR PROTEIN-BASED SOMATIC CELL REPROGRAMMING

Author and time	Parent cell source and delivery vector	Proteins source, factors and small molecules	Treatment procedure Treatment cycles	Differentiation potential of iPSCs
Zhou *et al.* 2009	MEFs 11R	*E. coli* OSK/OSKM VPA	12h proteins 36h without 4 times	Chimeric fetuses
Kim *et al.* 2009	HNFs 9R	293T cells OSKM None	16h proteins 6 days without 6 times	Teratomas EBs
Cho *et al.* 2010	MCFs/MSFs SLO	ESCs-derivedproteins None	1 day proteins 1 times	Chimera offspring
Zhang *et al.* 2012	HFFs TAT	*E. coli* OSKMN VPA	2h proteins 46h without 9 times	No stable iPSCs lines
Lee *et al.* 2012	HFs 11R	*E. coli* OSKM Poly I:C	1-21days proteins 1 times	Teratomas
Khan *et al.* 2013	HFFs Cationic bolaamphiphile	*E. coli* Nr5a2+SKN None	3h proteins None 3times	Teratomas EBs
Je Cho *et al.* 2013	Mouse NSCs Nanotube	*E. coli* OSKMN None	None	No stable iPSCs lines
Nemes *et al.* 2013	MEFs TAT	*E. coli* OSKMN None	12h proteins 2days without 4 times	Chimeric offspring

OSKMNL represent Oct4, Sox2, Klf4, c-Myc, Nanog, and Lin28, respectively; EBs represent embryoid bodies; HFs represent human fibroblasts; HFFs, human foreskin fibroblasts; MCFs represent mouse cardiac fibroblasts; MEF, mouse embryonic fibroblast; MSFs represent mouse skin fibroblasts; NSCs, neural stem cells. Poly I:C, polyinosinic-polycytidylic acid; R, arginine; VPA, valproic acid; None represents no related information in the research.

residues, could deliver various chemical compounds or proteins into cells (Gump and Dowdy, 2007, Wadia *et al*., 2004). This ability of TAT and polyarginine to deliver proteins may be explained by the structure of arginine, which possesses a head group that is known to interact with ions present on the membrane surface via a stable bidentate ion pairing (Chugh *et al*., 2010). However, which transduction system is most suitable for protein-based cell reprogramming remains unknown.

In 2001, Matsushita *et al.,* demonstrated delivery of proteins into neurons in brain slices using eleven arginine (R) residues; among the transduction vectors tested, TAT, 7R, 9R and 11R, the latter showed the greatest efficiency (Matsushita *et al*., 2001). In 2009, Zhou *et al.,* and Kim *et al.,* used 11R and 9R fusion proteins, respectively, to successfully generate iPSCs via recombinant proteins (Kim *et al*., 2009, Zhou *et al*., 2009). Interestingly, Zhang *et al.,* showed that the recombinant TAT-fused RFs are, in general, transcriptionally more active than the corresponding 11R-RFs, as shown by activation of their corresponding reporter genes in dual luciferase reporter assays (Zhang *et al*., 2012). Recently, new delivery proteins have been investigated. In 2012, Khan *et al.,* designed and synthesized a cationic bolaamphiphile for use as a non-viral gene delivery vector (Khan *et al*., 2012) and one year later, they used it to deliver RFs into fibroblasts, generating non-genetic iPSCs (Khan *et al*., 2013). In 2013, Je Cho's group developed a nanotube-mediated protein delivery system, which could activate genes for pluripotency in somatic cells (Cho *et al*., 2013). These technologies may contribute to obtain new breakthroughs in protein transduction and reprogramming efficiency.

Finally, to facilitate nuclear localization of the recombinant protein in cells, the nuclear localization signal (NLS) is always used. NLS contributes to nuclear localization of proteins without further modifications (Pan *et al*., 2004). By using proteins fused with the transactivator transcription-nuclear localization signal polypeptide (NLS) for generating iPSCs without gene integration, Nemes *et al.,* found that the NLS sequence had a double role, supporting the nuclear localization of the proteins, while minimizing the endosomal/lysosomal trapping degradation of the cargo (Nemes *et al*., 2013). On the other hand, the technology of immunocytochemistry always used to exam whether exogenous recombinant proteins transduction into cell nucleus.

Combinations of proteins that permit reprogramming

Various combinations of reprogramming transcription factors and small molecules have been used in a number of recent studies (Table 1). In 2009, Zhou *et al.,* obtained stable iPSCs from OG2/Oct4-GFP reporter mouse embryonic fibroblast (MEF) cells when they were transduced with four proteins (Oct4, Sox2, Klf4 and c-Myc) and treated with valproic acid (VPA). However, they did not obtain stable GFP^+ iPSC colonies by transduction with three (Oct4, Sox2 and Klf4) or all four reprogramming proteins under the same conditions, although GFP-negative cell colonies were observed (Zhou *et al*., 2009). In addition, those GFP-negative cell colonies stained positive with ALP, an early pluripotency marker, suggesting they might be partially reprogrammed cells. In 2012, after failing to obtain iPSC-like colonies from human foreskin fibroblasts (HFFs) using the same four proteins, Zhang *et al.,* added a fifth transcription factor, Nanog, and in the presence of VPA, which allowed for generation of iPSCs from HFFs (Zhang *et al*., 2012). Subsequently, Je Cho *et al.,* used three (Oct4, Sox2 and Nanog) and five (Oct4, Sox2, Klf4, c-Myc and Nanog) transcription proteins to induce reprogramming of neural stem cells from OG2/Oct4-GFP reporter transgenic mice, and only five-protein treatment induced GFP-positive colonies (Cho *et al*., 2013). These results suggest Nanog is important in reprogramming procedure, and previous studies reported that Nanog is the gateway to the pluripotent ground state (Sanges and Cosma, 2010, Silva *et al*., 2009). Recently, Costa *et al.,* also provide an insight into the reprogramming mechanism of Nanog that the function of Nanog depends on TET1 and TET2 in establishment of pluripotency (Costa *et al*., 2013). On the other hand, the research of Li *et al.,* demonstrated that the initiate of mesenchymal-to-epithelial transition (MET) is required for the nuclear reprogramming of mouse fibroblasts (Li *et al*., 2010). Furthermore, Liu *et al.,* reported that a sequential introduction protocol (*Oct4-Klf4* first, then *c-Myc* and finally *Sox2)* outperforms the simultaneous method and introduce the concept of a sequential EMT-MET (epithelial-to-mesenchymal transition, EMT) mechanism for cell reprogramming (Liu *et al*., 2013). Whether this protocol applies to the protein-based reprogramming approach needs further study.

To date, the four factors, Oct4, Sox2, Klf4 and c-Myc have been commonly employed for somatic cell reprogramming, but other factors have also been shown to be important. Zheng *et al.,* found that only recombinant human fibromodulin protein could indcuce conversion of human fibroblasts into multipotent cells (Zheng *et al*., 2012). Compared with virus-based iPSCs, these cells expressed pluripotent markers, formed embryonic bodies and differentiated into all three germ layers *in vitro*; however, they proliferated slowly and did not form teratomas. In 2013, Wang *et al.,* found that Nr5α2 plays an important role in pig iPSCs generation (Wang *et al*., 2013).

Later, Khan and colleagues demonstrated that Nr5a2 protein can replace Oct4 in the reprogramming procedure (Khan *et al.*, 2013).

Conservation of the active proteins is another important issue. *In vitro* degradation and *in vivo* breakdown by cellular machinery need to be accounted for when developing reprogramming methods. Therefore, the concentrations and treatment times for recombinant proteins are critical. Zhou *et al.,* found that 11R-tagged recombinant proteins could translocate into the nucleus at 8 μg/ml within 6 h and they appeared to be stable inside cells for up to 48 h (Zhou *et al.*, 2009). In another study, efficient intracellular translocation of all recombinant proteins was observed within 8 h, and most proteins were translocated into the nucleus (Kim *et al.*, 2009). Zhang and colleagues demonstrated that incubation with 50 nM TAT protein for 2 h was sufficient to obtain the maximum concentration in cells, and primarily nuclear localization (Zhang *et al.*, 2012).

Protein-based reprogramming technology is playing a more and more important role in iPSCs generation. In the development of virus-mediated reprogramming methods, after initially focusing on the quality and quantity of the iPSCs, many researchers switched their attention to the cellular type, small molecule compounds, and the transcription-factor (protein) cocktail. Similarly, for protein-based reprogramming technology, the choice of small molecule and protein cocktail is key to its efficacy.

Small molecule compounds for improved reprogramming

To date, in protein-based reprogramming procedures, only VPA and polyinosinic-polycytidylic acid (poly I:C) have been used by researchers. VPA, a widely used histone deacetylase inhibitor, can significantly enhance reprogramming efficiency (Huangfu *et al.*, 2008b). For the three-factor (Oct4, Sox2 and Klf4) and four-factor (Oct4, Sox2, Klf4 and c-Myc) treatments, addition of VPA for one week can improve the percentage of Oct4-GFP-positive cells by more than 100-fold and 50-fold, respectively (Huangfu *et al.*, 2008a). In addition, germline transmission was achieved using VPA-treated iPSCs from three factors (*Oct4, Sox2, Klf4*) infected MEF cells. In another study, VPA was shown to enable reprogramming of primary human fibroblasts with just two transcription factors, Oct4 and Sox2 (Huangfu *et al.*, 2008b). They found that VPA can greatly increase the efficiency of reprogramming somatic cells to a pluripotent state and replace some of the transcription factors used to reprogram differentiated cells during virus-based reprogramming. Similar results were observed in the procedure of proteins mediated reprogramming. In 2009, Zhou *et al.,* found that GFP-positive colonies were only obtained in the presence of VPA when MEFs were transduced with four or three proteins (Zhou *et al.*, 2009). VPA was also be used in the method of Zhang *et al.,* (Zhang *et al.*, 2012). The effect of VPA on reprogramming may be due to the combined effects of upregulation of ESC-specific genes and downregulation of MEF-specific genes. In seeking to further improve reprogramming efficiency, Lee *et al.,* (2012) demonstrated that the toll-like receptor 3 (TLR3) pathway enables efficient induction of pluripotency by viral or mRNA approaches. Poly I:C is a synthetic analog of double stranded RNA that is recognized specifically by TLR3 (Alexopoulou *et al.*, 2001). After adding this TLR3 agonist to the protein cocktail, Lee *et al.,* found that the reprogramming efficiency of their protein-induction method was increased and that colony formation begun after a shorter time (Lee *et al.*, 2012).

For viral induction of reprogramming, an array of chemical compounds have been investigated for their ability to improve the efficiency of iPSCs generation. For example, the use of two inhibitors, SB431542 and PD325901, can improve efficiency by 100-fold during reprogramming of human fibroblasts; when thiazovivin was also added, this increase in efficiency rose to 200-fold (Lin *et al.*, 2009). Similar results have been reported for other small molecules in somatic reprogramming protocols. In 2010, Mali *et al.,* reported that butyrate could enhance iPSCs formation from 15- to 51-fold when using either retroviral or piggyBac transposon vectors expressing four to five reprogramming genes (Mali *et al.*, 2010). Wang *et al.,* found that treatment with 8-bromoadenosine 3′,5′-cyclic monophosphate and VPA increases the reprogramming efficiency to 6.5-fold compared with the control group (Wang and Adjaye, 2011).

Vitamin C (Vc), an important micronutrient, can also increase reprogramming efficiency (Esteban *et al.*, 2010, Wang *et al.*, 2011). In 2013, Chen and colleagues established direct connections between Vc, ten-eleven translocation (Tet) and DNA methylation, thus putting forward a new pathway for understanding the molecular mechanisms of somatic cell reprogramming (Chen *et al.*, 2013). However, Vc and other chemical compounds have not been used for protein-based reprogramming methods. Further study is needed to find more chemical compounds that could enhance protein-based reprogramming.

The perspective of protein-based iPSCs

Conventional viral- and transgene- mediated methods of generating iPSCs not only pose the risk of viral reactivation, but also may cause insertion mutagenesis. As a result, these iPSCs are potentially tumorigenic. To render the technique clinically applicable, safe methods of generating iPSCs are essential. The newer reprogramming methods based on protein transduction do not involve viruses or genome integration, giving them the potential to produce safe and consistent iPSCs. However, its low reprogramming efficiency was one of the biggest challenges. To our knowledge, only about nine groups have reported obtaining iPSCs via protein transduction, and these studies have been restricted to the cells of mice and humans, with only VPA and Poly I:C being tested as small molecule enhancers of the proteins' efficiency. Therefore, more cellular materials of various species, and new vectors and small molecule compounds should be used to design efficient and robust methods for protein-based cell reprogramming. Besides, to further propel the development of protein-based iPSCs clinical applications, a feeder-independent and serum-free culture system (Totonchi *et al.*, 2010, Yamasaki *et al.*, 2013, Zhang *et al.*, 2014), and a dimethyl sulfoxide-free and no animal-derived components cryopreservation system (Nishigaki *et al.*, 2011) should be tested. While ultimate goals of iPSCs is the treatment of human diseases, the time of obtaining patient-specific iPSCs by protein-based approach needs to be shortened on account of the serious conditions they could treat and the lives they could save.

The immunogenicity of iPSCs is also a critical problem for their clinical application. Zhao *et al.,* reported that mouse ESCs can efficiently form teratomas in the same type mouse without any evident immune rejection, and teratomas formed by the mouse iPSCs by retroviral approach were mostly immune-rejected (Zhao *et al.*, 2011). In addition, the teratomas, which formed by iPSCs by

episomal approach that cause no genomic integration, were small and apparent regression by 40 days after iPSCs implantation. These results showed that iPSCs has higher immunogenicity than ESCs, and iPSCs produced via retroviral methods are more immunogenic than those generated via non-integrating elements. Research has also shown that humans immune system has natural immunity to pluripotency antigen Oct4 (Dhodapkar *et al*., 2010). Therefore, the high immunogenicity of genomic integration iPSCs may be caused by the sustained expression of the exogenous transcription factors. In contrast, the latest researches have reported that iPSCs and the differentiation cells from no genomic integration iPSCs have low immunogenicity (Araki *et al*., 2013, Guha *et al*., 2013). Whether the protein approach leads to lower immunogenicity needs to be studied further.

Conclusion

Taken together, the birth of iPSCs techniques and their rapid development has opened a promising window for human genetic disease modeling, regenerative medicine, drugs screening, and agricultural production. The safety issue of iPSCs leads to the birth of pluripotent stem cells via protein transduction. Although the protein-based iPSCs technique has evolved over more than four years, it is still in the early stages of development. This may attribute to its complexity, involving the information of molecular and stem cell theory, proteomics, epigenetics and immunology. Therefore, we recommend the formation of interdisciplinary collaborations between experts from the fields of protein engineering, molecule, stem cells, epigenetics and immunology, as a means to achieve better and faster development of safe iPSCs. If the various technical difficulties can be resolved, we are confident that the viral- and transgene-free iPSCs by protein-based induction technique will play an important role in the field of medicine and agriculture.

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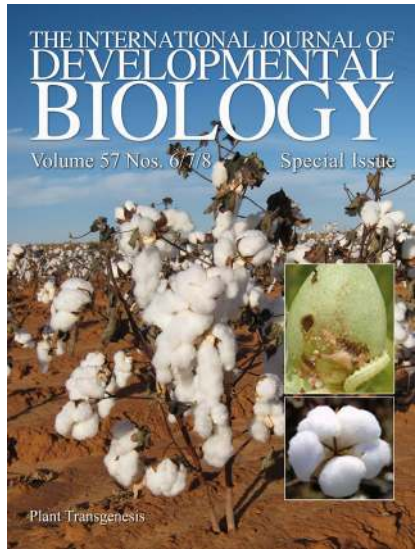

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