INDUCED PLURIPOTENT STEM CELLS AND THEIR IMPLICATION FOR BIOMEDICAL SCIENCES

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ABSTRACT

The groundbreaking discovery, that somatic mammalian cells can be epigenetically reprogrammed to a pluripotent state through the exogenous expression of the transcription factors Oct4, Sox2, Klf4 and c-myc, has yielded a new cell type for potential application in regenerative medicine, the induced Pluripotent Stem Cells (iPSCs). Since the first demonstration of creating iPSCs in 2006 great efforts have been made into improving iPS cell generation methods and understanding the reprogramming mechanism as well as the nature of iPSCs. The iPSCs technique makes it possible to produce patient-specific pluripotent stem cells for transplantation therapy without immune rejection. However, some restriction still remain, including viral vector integration into the genome, the existence of exogenous oncogenic factors and low induction efficiency. In this review we discuss recent advances in methods of generating safer iPSCs lines and their possible use for biomedical applications.

Keywords: Induced Pluripotent Stem Cells, Reprogramming, Gene Therapy, Regenerative Medicine

1. INTRODUCTION

The ability to reprogram a somatic cell into a pluripotent cell has been a goal of regenerative research for many decades. Initial attempts to reprogram cells involved transplanting a somatic nucleus into an enucleated oocyte, called Somatic Cell Nuclear Transfer (SCNT) (Lenger, 2010; Nsair and MacLellan, 2011). The procedure was first successfully accomplished in mammals in 1996 with the birth of a cloned lamb. This discovery caused a paradigm shift in developmental biology. However, the process was very inefficient and the reprogramming incomplete leading to Dolly's premature death and early onset of a number of degenerative disease (Kang *et al.*, 2010; Patel and Yang, 2010). This reprogramming method also has proved successful for several species, but there are technical issues in applying this approach to human cells (Feng *et al.*, 2009). The other major impediment of nuclear transfer-derived Embryonic Stem Cells (ESCs) based therapies in humans involves the moral and ethical dilemma surrounding the requisite blastocyst destruction and oocyte donation necessary to generate patient-specific pluripotent stem cells (Lenger, 2010). Reprogramming of somatic cell by nuclear transfer indicates that unfertilized eggs and ESCs contain factors that can induce pluripotency. Therefore, nuclear reprogramming studies using SCNT have demonstrated that transcriptional factors are essential for the reprogramming of terminally differentiated cells.

Based on this hypothesis, 24 different candidate factors were examined for their ability to induce pluripotency (Ma *et al.*, 2008; Oh *et al.*, 2011). In 2006 Yanamaka´s group demonstrated that viral transduction of murine fibroblasts with only four transcription factors, Oct4, Sox2, Klf4 and c-Myc, could reprogram these cells back to an undifferentiated embryonic state. Human iPSCs were first generated in the same lab by transducing adult human dermal fibroblasts with viral vectors carrying the key pluripotency genes (Yamanaka factors). Thomson's group independently published a similar screening strategy using

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only human materials and identified that Oct4, Sox2, Nanog and Lin 28 were sufficient to reprogram human cells. Since then, alternative strategies for generating iPSCs have been developed. These completely reprogrammed cells are similar to ESCs in capacity for self-renewal *in vitro*, pluripotency, cell morphology, patterns of epigenetic changes, capability to form embryoid bodies, teratoma, viable chimeras and moreover, murine iPSCs, when they are injected into tetraploid blastocyst, can developed into whole organism. On the other hand, several groups have compared the molecular signatures of iPSCs and ESCs in their undifferentiated state. One study showed that while iPSCs were more similar to ESCs than to the paternal somatic cells, they still possessed a distinct gene expression signature. Other great advantage is that iPSCs avoid ethical issues because they do not involve the use of embryonic or fetal material (Cui *et al.*, 2009; Herberts *et al.*, 2011; Li *et al.*, 2010; Medvedev *et al.*, 2010; Nsair and MacLellan, 2011; Takahashi and Yamanaka, 2006). Today, these cells have become the major tools in advancement of personalized medicine (Ferreira and Mostajo-Radji, 2013).

1.1. iPSCs Generation by Viral Vector

Several methods have been used thus far to produce iPSCs (**Fig. 1**), which involve the transfer of genes into the target cell (Deng, 2010). The system of gene delivery system is a key aspect for iPS cell generation. Many researches in this field use viral or nonviral methods to reprogram mature cell and some groups have tried to use nongenetic materials for the generation of efficient and safe iPSCs. However, there are still a number of problems related to current reprogramming methods (Oh *et al.*, 2011).

Most of the iPSCs lines established to date have been generated using integrating retroviral and lentiviral vectors to deliver reprogramming factors. The major advantage of using retroviral vectors is that expression of the pluripotency factors is driven by the retroviral LTR (long terminal repeat). The retroviral LTR becomes silenced in pluripotent stem cells, so upon correct reprogramming of the fibroblast and expression of the endogenous pluripotency genes, the exogenous reprogramming factors are silenced. The potential for oncogenesis due to insertional mutagenesis that is inherent to stable genomic integration has been identified as a limitation. However, it is important to recognize that distinct advantages of the retroviral-based vector systems enabled critical insight into the fundamental mechanisms of nuclear reprogramming (Seifinejad *et al.*, 2010).

Later generations of iPSCs were produced using

lentiviruses. They are less mutagenic than retroviruses but are also not a perfect alternative. Unlike retroviruses, lentiviruses infect both dividing and non-dividing cells, hence could vastly improve the rates of cell transduction and the production of lentiviruses is not overly complicated, therefore, very efficient, stable, reproducible gene expression could be achieved and wide variety of cells could be transduced. Although retrovirally-transduced genes are generally subject to epigenetic silencing during the process of iPSCs induction, reactivation of these genes *in vivo* may lead to a higher risk of tumor incidence and malignant progression. While retro- and lentiviral integration is thought to occur randomly in the genome, certain events may cause activation of nearby oncogene or inactivation of a tumor supresor gene (Duinsbergen *et al.*, 2009; Gardlik, 2012; Wong and Chiu, 2011).

The attempts to make iPSCs more therapeutically applicable have led to the pursuit of non-integrating virus vectors. Adenovirus, which is a non-integrating vector and remains as an epichromosomal form in cells, offers a flexible platform. However, adenovirus is generally rather poor at gene transfer. It is also very difficult to control the level of gene expression infected cell (Lai *et al.*, 2011; Patel and Yang, 2010).

Another viral vector system without genomic integration is Sendaiviral (Sendaivirus) vector, which is a RNA virus that replicates its genome exclusively in the cytoplasm, efficiently generated iPSCs from human somatic cells (Macarthur *et al.*, 2012; Oh *et al.*, 2011).

1.2. iPSCs Generation by Non-Viral Vector

The success of the non-integrating vector with transient gene expression to generate iPSCs has provided an opportunity to potentially develop a non-viral delivery strategy, which is safe, cost-effective, easier to manufacture and manipulate. Non-viral delivery system includes episomal plasmide transfection, minicirle plasmids, piggyBac transposition system, protein transduction and most recently, repeated administration of synthetic mRNA and using of small molecules (Huang and Wu, 2012; Nie *et al.*, 2012; Wong and Chiu, 2011).

Plasmide transfection usually uses plasmids carrying the reprogramming genes encapsulated by lipid or cationic polymers and subsequently trasfected into the cells to be reprogrammed. Episomal plasmid vectors are able to replicate themselves autonomously as extrachromosomal element. They also exhibit prolonged expression of the reprogramming genes in target cells. Nevertheless, the reprogramming efficiency of this approach was extremely low (Lai *et al.*, 2011; Medvedev *et al.*, 2010; Yu *et al.*, 2011).
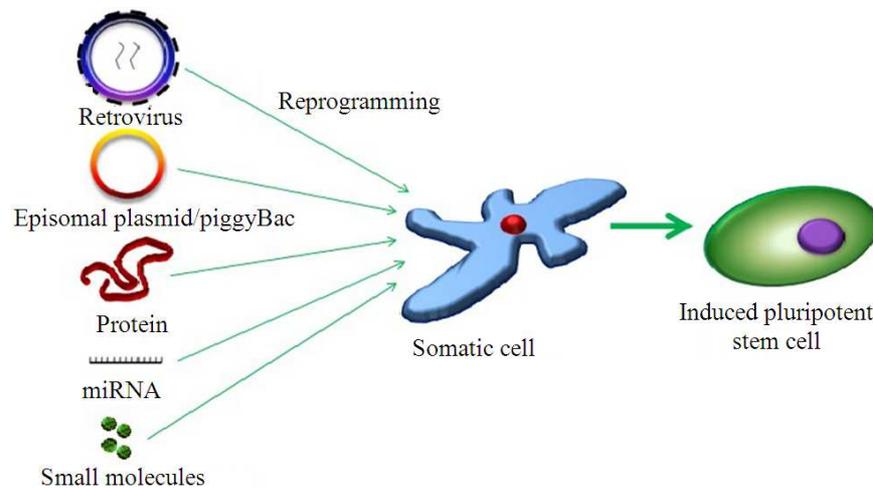
Fig. 1. Reprogramming strategies used to induce pluripotent stem cells from adult somatic cells

The piggyBac system-is promising system used for iPSCs production without any modification of the host genome based on DNA transposons. The transposon includes a mobile genetic element that can be used to integrate transgenes. Successful transposon-based reprogramming of fibroblasts to iPSCs represents a significant advance in current methods of transgene delivery. This system couples enzymatic cleavage with sequence specific recognition using transpon/transponase interaction to ensure high efficiency removal of flanked DNA without footprint. It permits technical simplification and improved accessibility of reprogramming methodology by making use of effortless plasmid DNA preparation and commercial transfection products for delivery. Also the range of somatic cell types that could be used for reprogramming is not limited by a decreased susceptibility to viral infection. Importantly, this study also demonstrated that continued expression of exogenous reprogramming factors is not required for *in vitro* reprogramming (Seifinejad *et al.*, 2010; Woltjen *et al.*, 2009). However, despite the high efficiency of exogenous DNA excision from the genom by this system, the removal of a large number of transposon copies is hardly achievable (Medvedev *et al.*, 2010).

Direct protein transduction system represent significant advance in generating iPSCs and has several major advantages. The observation that some exogenous proteins are capable of being taken up by the cell has resulted in the identification of protein transduction domains, also called "transduction peptides" or "cell-penetrating peptides". When fused to other proteins, these peptides allow the cargo protein to enter the cell, although at different efficiencies (Seifinejad *et al.*, 2010). This method eliminates limitation that may be caused by viral or any other DNA-based reprogramming methods, it means the potential risks associated with chromosomal integration and mutation. Zhou *et al.* (2009) demonstrated that protein-induced Pluripotent Stem Cells (p-hiPSCs) can long-term self-renew and are pluripotent *in vitro* and *in vivo*. These iPSCs exhibited similarity to ESCs in morphology, proliferation and expression of characteristic pluripotency markers. The mouse iPSCs were generated using recombinant proteins of reprogramming factors which were produced in bacteria. However, iPSCs derivation required the addition of the histone deacetylase inhibitor valopronic acid. Their study first demonstrated that somatic cells can be fully reprogrammed into pluripotent stem cells by direct delivery of recombinant reprogramming proteins. However, the generation of p-hiPSCs is very inefficient and requires further optimization. The efficiency of production is around 0,001%, which is one order lower than by using retroviral vectors. In particular, the concentration of the individual factors needs to be calibrated to approximate normal endogenous level (Kim *et al.*, 2009; Lai *et al.*, 2011; Wong and Chiu, 2011).

Yakubov *et al.* (2010) reported the use of *in vitro*-produced mRNA encoding for Oct4, Lin28, Sox2 and Nanog for reprogramming of human foreskin fibroblasts to generate RNA-produced iPSCs (RiPSCs). This procedure also avoids any DNA integration events and the associated genomic damage. The procedure of RNA preparation *in vitro* is simple and easy to scale up

and transfection is very efficient. The production of RiPSCs by RNA overcomes an important hurdle on the way to personalized cell therapy.

Small molecules-another alternative approach to safety improve the reprogramming process for the generation of iPSCs is to use a cocktail of small molecules that are linked with epigenetic modifiers and major signaling pathways. Notably, some of these molecules can also replace individual reprogramming factors, raising the possibility of deriving iPSCs solely with chemicals. These molecules are inhibitors of Histone De A Cetylases (HDACs)-Val Proic Acid (VPA), Tricho Statin A (TSA), Histone De Methylases (HDMs) and Histone Methyl Transferases (HMTs), which regulate chromatin remodeling and act as major players in building up the epigenetic landscape. HDACc inhibitors significantly were shown to improve reprogramming efficiency. Also other small molecules have been reported to be able to replace some transcription factors. For example, a G_{9a} inhibitor, BIX_{01294}, was reported to induce iPS cells from neural stem cells, in place of Oct4. In addition, a Transforming Growth Factor (TGF)-β inhibitor could replace Sox2 during iPSCs generation. Vitamin C also improves the efficiency of reprogramming in both mouse and human somatic cells and promotes the transition of partially reprogrammed iPSCs to fully reprogrammed state likely by reducing reactive oxidant species and senescence.

Because small molecules provide several distinct advantages in controlling protein fusion, they have attracted much interest for control reprogramming toward a faster and more efficient process. Chemical strategies are being used to find additional small molecules that may ultimately allow reprogramming of lineage-restricted cells to pluripotent state in a completely chemically defined condition. However, it should be noted that chemical substitution of a reprogramming factors is, in most cases, associated with a significant decrease in the number of iPSCs clones generated, indicating that no single chemical compound is able to entirely replace the function of a transcription factor (Feng *et al*., 2009; Li *et al*., 2011; Lu *et al*., 2013; Nie *et al*., 2012; Stadtfeld and Hochedlinger, 2010; Oh *et al*., 2011; Shi *et al*., 2008).

1.3. Applications of Human iPSCs

Human iPSCs might be used to treat degenerative and genetic diseases and to study diseases mechanism at the individual patient level (Cui *et al*., 2009). They could generate a limitless source for tissue engineering and regenerative medicine applications (**Fig**. **2**). Another key advantage is the possibility of repairing disease-causing mutation by homologus recombination, a technology that has been used with limited success in adult stem cells because of notorious difficulties in growing them outside the body. Human iPSCs could have also significant impact on drug development and toxicity tests to replace and refine animal experiments and test (Martinez-Fernandez *et al*., 2010; Pappas and Yang, 2008; Stadtfeld and Hochedlinger, 2010; Vitale *et al*., 2011).

Recently, there have been great advancements in the study of iPSCs in the field of regenerative medicine. Organ transplantation among nonrelated individuals is complicated by the limited availability of matched tissues and requirement for life-long treatment with immunosupresive drugs that can have serious side effects. Somatic cells isolated from a patient may be reprogrammed to pluripotent stem cells and then theoretically could be used to replace diseased cells in the same patient avoiding the problem of histocompatibility or immune rejection. Many human diseases, such as myocardial infarction, diabetes, retinal degeneration and spinal cord injury, occur because of cell loss, degeneration and injury. Theoretically, with the transplantation of specific cells created from autologus iPS cells, the cells that lacking can be replenished and replace by cells with the defects corrected, thereby relieving a patient`s symptoms. The mostly use somatic cells are fibroblasts, but different groups generated also iPS cells from other somatic cells providing evidence that is possible to reprogram cells of different origins (Deng, 2010; Ebben *et al*., 2011; Patel and Yang, 2010; Uemura *et al*., 2012; Vitale *et al*., 2011; Walia *et al*., 2012; Wong and Chiu, 2011; Zeng and Zhou, 2011). Other sources of iPSCs that can be easily reprogrammed are human keratinocytes (Aasent *et al*., 2008; Petit *et al*., 2012), oral mucosa fibroblasts (Miyoshi *et al*., 2010), dermal papilla cells (Tsai *et al*., 2010), pancreatic beta cells (Stadtfeld *et al*., 2008), neural stem cells (Kim *et al*., 2009), mature B lymphocytes (Hanna *et al*., 2008), liver and stomach cells (Aoi *et al*., 2008) and cord blood cells (Cai *et al*., 2010; Takenaka *et al*., 2009). For example, human keratinocytes have been shown to be 100-fold more efficient and 2-fold faster than human fibroblasts (Aasent *et al*., 2008). The goal is to find the most accessible, efficient and safest cell to reprogram to future clinical applications.
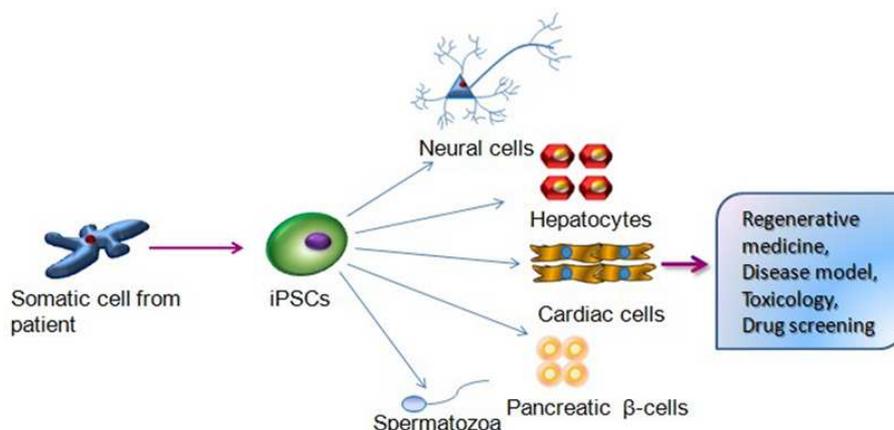
Fig. 2. Applications of iPSCs technology

The potential use of iPSCs as treatments for various disorders has been proposed and tested on *in vitro* and/or *in vivo* animal models with promising results (Patel and Yang, 2010). Proof of principle for iPSCs-based therapy was first demonstrated in mice. iPSCs were generated from tail-tip fibroblasts of humanized sickle cell anemia mice. After the sickle cell gene mutation in the generated iPSCs was corrected via homologous recombination, the cells were directed to differentiate into hematopoetic progenitor cells *in vitro*. After they were transplanted into the affected donor mice, resulting in restoration of the functional hemoglobin protein in the bloodstream and restoring disease parameters such as red blood cell count, hematocrit, weight and breath rate to normal values. Also kidney defects due to the red blood cell destruction in renal tubules with reduction in renal blood flow were rescued upon iPSCs therapy (Cui *et al.*, 2009; Hanna *et al.*, 2007). Thus, the first therapeutic application of iPSCs technology illustrated the advantages of both regeneration of degenerative disease as well as gene-specific correction of an inheritable effect (Nelson *et al.*, 2010).

For example, of particular interest are iPSCs derived from patients suffering from neurodegenerative diseases. These iPSCs lines can be differentiated *in vitro* into the affected neuronal cell type (Lenger, 2010). iPSCs have been generated from patients with amyotryophic lateral sclerosis and they could be differentiated into motor neurons. Greater progress has been made in generating enriched population of ventral midbrain dopaminergic neurons that are relevant for Parkinson disease (Marchetto *et al.*, 2011).

It was also demonstrated that murine iPSCs have ability to differentiate into mesenchymal cells like cells of bone, cartilage and fat *in vitro* and maintain an osteoblast phenotype on a scaffold *in vitro* and *in vivo*. The maintenance of this stable osteoblast phenotype spotlights these cells as a viable source for clinical cell-based therapy to treat musculoskeletal disease (Bilousova *et al.*, 2011).

First endodermal-derived cells differentiated form iPS cells were hepatocytes, which could potentially revolutionize hepatology with respect to the study of hepatitis B and C viruses, alcohol-induced cirrhosis and congenital liver diseases (Gallicano and Mishra, 2010).

Other potential application in near future can be use of iPSCs derived spermatozoa to treat infertile couples, where male suffer from Non-Obstructive Azoospermia (NOA) (caused by testis failure or impaired spermatogenesis). These patient specific iPSCs derived spermatozoa can help patient create their own genetic offspring via ICSI, they also allow the development of novel reproductive engineering afpproaches and advanced studies on pathogenesis and treatment of fertility (Yao *et al.*, 2011).

1.4. Future Perspectives

Despite success in animal models, iPSCs technology is not yet ready for transplanting cells into patients. The main issue is safety concerns, iPSCs, like ES cells, tend to form teratomas and current differentiation protocols cannot efficiently eliminate residual undifferentiated cells. The researches still do not know for each iPSCs clone whether nuclear reprogramming is complete. Aberrant reprogramming may result in an impaired

ability to differentiate and may increase the risk of immature teratoma formation after direct differentiation. Most patient-specific iPSCs have been generated with integrating vectors, which may not get silenced efficiently or could disrupt endogenous genes, which also pose potential impediments for the use of human iPSCs in cell therapy. For diseases that require gene targeting to repair mutant alleles, more efficient targeting strategies need to be developed. However, the recent study showed that even patient´s own iPSCs may induce immune rejection. Moreover, when viral vectors are used to engineer the expression vectors for the various methods for genetic repair, immune rejection may be developed towards the viral vector (Cha and Hwang, 2012; Pawitan, 2012; Stadtfeld and Hochedlinger, 2010; Yamanaka, 2009).

Extensive research has aimed at the generation of human iPSCs using methods that avoid the use of retroviruses and oncogenes. However, all such methods have been found to reprogram cells at low efficiency. To able to reproducibly and efficiently generate iPSCs will require in-depth understanding of the reprogramming process. Because reprogramming is in a sense the opposite of development, involving a return from the adult to embryonic status, it is possible that deeper understanding of developmental pathways may provide further insights into the reprogramming process (Li *et al*., 2010).

Another essential prerequisite for future widespread applications of human iPSCs is the development of efficient cryopreservation methods to facilitate cell banking. Importantly, a simple and effective cryopreservation procedure has been established for single dissociated human iPSCs in feeder-free culture using a Rho kinase inhibitor (Mollamohammadi *et al*., 2009).

2. CONCLUSION

In addition, before using iPSCs for clinical purposes, however, a few issues need to be addressed. Safety remains a critical consideration where stem cells are concerned: the long term effects of the reprogramming process on cells and their progeny must be studied very carefully. The risk of tumor formation after iPSCs treatment needs to be minimized by: reducing genetic disruption to the cell caused by reprogramming using strategies, avoiding prolonged expression of the oncogenic reprogramming factors, ensuring completion of reprogramming process and confirming differentiation capacity of iPSCs, as partial or aberrant reprogramming might be conducive to teratoma formation (Wong and

Chiu, 2011). For human iPSCs to be therapeutically effective in transplantation it needs to be also shown that their progeny will function normally in the intended site for significant periods of time, which will require extensive testing first in animals and then in appropriately designed clinical trials (Sipp, 2010).

3. ACKNOWLEDGEMENT

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