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Derivation and application of pluripotent stem cells for regenerative medicine

Jiaqiang Wang & Qi Zhou*

State Key Laboratory of Stem cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

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Pluripotent stem cells (PSCs) are cells that can differentiate into any type of cells in the body, therefore have valuable promise in regenerative medicine of cell replacement therapies and tissue/organ engineering. PSCs can be derived either from early embryos or directly from somatic cells by epigenetic reprogramming that result in customized cells from patients. Here we summarize the methods of deriving PSCs, the various types of PSCs generated with different status, and their versatile applications in both clinical and embryonic development studies. We also discuss an intriguing potential application of PSCs in constructing tissues/organs in large animals by interspecies chimerism. All these emerging findings are likely to contribute to the breakthroughs in biological research and the prosperous prospects of regenerative medicine.

pluripotent stem cells, regenerative medicine, reprogramming, interspecies chimerism

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INTRODUCTION

The life cycle of an organism during development from a zygote to an adult is programmed with gradual loss of differentiation potency at cell level. Regenerative therapy can be achieved by replacing, engineering or regenerating damaged cells, tissues or organs to restore normal function, and holds great promise to cure some untreatable diseases such as spinal cord injury. It is believed that regenerative medicine will play an increasingly important role in medical revolution in the coming decades.

Pluripotent stem cells (PSCs) are the most important seed cells for regenerative medicine due to their unlimited self-renewal *in vitro* and differentiation capacity to form any type of cells *in vivo*. PSCs of different status can be isolated and established at different stages of an embryo or from different tissues of an adult. Apparently, these stem cells have problems of immunological rejection and limited resource. The reversion of a cell into a state with a different gene expression profile (not always with increased potency) is called reprogramming. Reprogramming could generate patient-specific stem cells, making a great prospect for regenerative medicine. Nuclear transfer and overexpression of transcription factors, which includes both transdifferentiation and induced pluripotent stem cell (iPSC) technology, are recognized reprogramming methods.

Establishing animal disease/therapeutic models and animals with "organ niche'' (Rashid et al., 2014) is another concept for regenerative medicine. With the development of functional genomic research and genome-editing technology, many disease models are generated. However, few of them can currently be applied in clinical study. Also, *in vivo* generation of human organs from donor stem cells in a xenogenic environment with "developmental compensation'' or via 3D bio-printing is still a tough task.

*Corresponding author (email: zhouqi@ioz.ac.cn)

PLURIPOTENT STEM CELLS: HIGHER PLURIPOTENCY AND CLOSER TO HUMAN

Embryonic stem cells (ESCs) with highest pluripotency are good for cell-replacement therapies and for *in vitro* generation of organs, thus holding promise in treating degenerative disorders such as Parkinson's disease, Alzheimer's disease, and diabetes. Since the first successful derivations of mouse ESCs were reported in 1981 (Evans and Kaufman, 1981; Martin, 1981), ESCs have been successfully established in many species, including human (Thomson et al., 1998), monkey (Li et al., 2005a; Thomson et al., 1996), rat (Germana, 2011; Iannaccone et al., 1994; Li et al., 2012a), and rabbit (Wang et al., 2007, 2008), and a lot of efforts have been put into improving pluripotency of human ESCs (Gu et al., 2012; Hanna et al., 2010). Since the use of human ESCs for research and therapy must be with deliberation, the international consensus guidance for banking and supply of human ESCs for research purposes (International Stem Cell Banking, 2009) and the points to consider in the development of seed stocks of pluripotent stem cells for clinical applications have been enacted (Andrews et al., 2015).

For regenerative medicine, PSCs with higher pluripotency and lower immunological rejection are always needed. Tetraploid embryo complementation represents the golden standard of demonstration of the highest pluripotency of stem cells. However, only mouse ESCs and iPSCs (Zhao et al., 2009) have been demonstrated to have such developmental potency. For clinic application, transplantable neural progenitor cells from rat ESCs (Herson et al., 2003) and rhesus monkey ESCs (Chen et al., 2009; Li et al., 2005) were established, and the efficacy of neural progenitor-based transplantation therapy in the nonhuman primate was evaluated (Chen et al., 2009; Li et al., 2005). In addition, cynomolgus monkey ESCs with higher pluripotency with which monkey chimeric fetuses were generated have been obtained (Shuai et al., 2015). Human parthenogenetic ESCs (Hao et al., 2009; Shuai et al., 2015) and homozygous human androgenetic ESCs (Ding et al., 2015) were derived, providing important tools for future therapeutic use in a clinical setting.

REPROGRAMMING: SOURCE OF PATIENT-SPECIFIC STEM CELLS

Reprogramming could generate patient-specific stem cells, so it may play a potentially important role in translating stem cells from basic research to clinical application.

Nuclear transfer

The original design of nuclear transfer (NT) experiment was established by Briggs and King in 1952, by injecting a nucleus of a ruptured cell into an enucleated unfertilized xenopus egg (Briggs and King, 1952). The first successful nuclear transfer experiment was accomplished by Gurdon and colleagues in 1958, with developing into adult xenopus (Gurdon et al., 1958). Chinese scientist Dizhou Tong generated the first cloned *Carassius auratus* in 1963, and performed the first interspecies nuclear transfer cloning between *Carassius auratus* and *Rhodeus sinensis* in 1973.

In 1997, Wilmut reported the birth of the first cloned mammal "Dolly" the sheep (Wilmut et al., 1997). Thereafter, mouse (Wakayama et al., 1998), cow (Cibelli et al., 1998; Kato et al., 1998), goat (Baguisi et al., 1999), pig (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), cat (Shin et al., 2002), rabbit (Chesne et al., 2002), mule (Woods et al., 2003), horse (Galli et al., 2003), rat (Zhou et al., 2003), dog (Lee et al., 2005), ferret (Li et al., 2006), and camel (Wani et al., 2010) were successively cloned. In 2006, cloned rhesus monkey blastocysts were generated, yet no cloned monkey have been produced so far (Zhou et al., 2006). In 2004, Hwang established the first nuclear transfer ESCs (ntESCs) (Hwang et al., 2004), being regarded as a milestone for therapeutic cloning. In addition, human oocyte morphology classification (Yu et al., 2009) and therapeutic cloning by xenotransplanted oocytes (Riaz et al., 2011) were also tried. Recently, fertile offspring from Kit^{w}/Kit^{wv} infertile male mice was generated through *in vivo* differentiation of gene corrected ntESCs (Yuan et al., 2015), and spermatid-like cells from mouse ESCs *in vitro* gametogenesis (Zhou et al., 2016) were derived, exploring new paths to rescue male infertility caused by genetic mutations (Yuan et al., 2015).

The reprogramming efficiency of nuclear transfer is very low, so lots of efforts have been made to improve the efficiency. The abnormal development of nuclear transfer embryos is always associated with defects in epigenetic reprogramming (Dean et al., 2001; Kang et al., 2001; Ohgane et al., 2001; Yamazaki et al., 2006). Treatment with histone demethylase inhibitor can increase the reprogramming efficiency (Bui et al., 2010; Dai et al., 2010; Kishigami et al., 2006; Song et al., 2014). Besides, modified culture methods showed that *in vitro* culture environment plays an important role in nuclear transfer reprogramming (Dai et al., 2009). It was also reported that some of the placental abnormality of nuclear transfer embryos can be blamed on the abnormal expression of some genes, such as *HSPC117* (Wang et al., 2010). Furthermore, protein profile of the mouse metaphase-II oocyte was analyzed, which might be valuable in revealing potential mechanisms of epigenetic reprogramming in nuclear transfer (Ma et al., 2008).

Overexpression of transcription factors

As early as in 1987, Davis achieved transdifferentiation of fibroblasts into myocytes via overexpression of MyoD, and it was an innovative progress (Davis et al., 1987). Similarly, overexpression of C/EBPα can change pre-T cells (Laiosa et al., 2006) or pre-B cells (Xie et al., 2004) into macrophagocytes, as pancreatic exocrine acinar cells into liver

cells (Shen et al., 2000). In 2010, Shinya Yamanaka induced mouse fibroblast transdifferentiating into functional neural cells by overexpression of 19 neural-specific transcription factors. In 2012, successful transdifferentiation of Sertoli cells, one type of testis sustentacular cells, into neural stem cells was reported, and the obtained neural stem cells could survive and generate synapses following transplantation into the dentate gyrus. The work has important implications for regenerative medicine (Sheng et al., 2012a, b). In 2008, *in vivo* transdifferentiation of pancreatic acinar cells into pancreas islet β cells with normal insulin secretory function was accomplished by adenovirus-introduced overexpression of three transcription factors (Zhou et al., 2008).

In 2006, Takahashi and Yamanaka induced mouse fibroblasts into ESCs-like cells via overexpression of Oct4, Sox2, Klf4, and c-Myc (OSKM) (Takahashi and Yamanaka, 2006), the cells were named induced pluripotent stem cells (iPSCs). This shed light on regenerative medicine field. The generation of all-iPSCs-mouse "Xiaoxiao" with the method of tetraploid embryo complementation (Zhao et al., 2009, 2010a, b, c), and the verification of iPSCs' capability to generate offspring through nuclear transfer (Zhou et al., 2010), paved the way for iPSCs to be used in regenerative medicine. Notably, however, mice generated from tetraploid complementation competent iPS cells show similar developmental features as those from ES cells but are prone to tumorigenesis (Tong et al., 2011), indicating that more efforts need to be made before iPSCs to be translated from basic research to clinical application.

Pig iPSCs attract a wide attention because pig is an important animal model for *in vitro* generation of human organ and there lacks pig ESCs. It was reported that mouse ESCs-like pig iPSCs were produced efficiently and they processed good cell viability and proliferation capability (Gu et al., 2014), although pig iPSCs with chimaeric ability have yet to be obtained.

Exogenous four transcription factor-coding genes in iPSC technology are the barrier for its application. In 2011, Anokye-Danso obtained OSKM-free iPSCs by transfecting miR-302/367 into fibroblasts (Anokye-Danso et al., 2011). Some other research groups used proteins generated from OSKM genes to induce iPSCs, and obtained satisfying results (Cho et al., 2010; Kim et al., 2009; Lee et al., 2012; Zhou et al., 2009). Furthermore, a chemical reprogramming system without gene-editing (Hou et al., 2013; Zhao et al., 2015) have also been developed.

Enhancing the efficiency of iPSCs reprogramming is important for their application. Adding active small molecules that are associated with epigenetic modification, such as VPA (Huangfu et al., 2008), DNA methylase inhibitors (Huangfu et al., 2008; Mikkelsen et al., 2008), histone methylase inhibitors (Shi et al., 2008), histone deacetylase inhibitor (Hai et al., 2011), protein arginine methyltransferase inhibitor (Yuan et al., 2011), or vitamin C (Esteban et al., 2010), has been reported to be able to increase the iPSCs reprogramming efficiency. Similar to nuclear transfer, some maternal factors, such as TH2A and TH2B (Shinagawa et al., 2014), can promote iPSCs reprogramming. Also, the activity of endogenous retrovirus HERVH was shown to have connection with pluripotency of human iPSCs (Ohnuki et al., 2014). Notably, that the maternal unmethylated Dlk1-Dio3 region would turn methylation during iPSC reprogramming, which may be caused by disorder of c-Myc (Li et al., 2011) and/or Gtl2 (Das et al., 2015), is a key reason for loss of capability of tetraploid embryo complementation (Liu et al., 2010; Stadtfeld et al., 2010). Recently, it was reported that increased N(6)-methyladenosine (m^6A) abundance, which is partially regulated by multiple miRNAs (Chen et al., 2015), promotes the reprogramming of mouse embryonic fibroblasts to pluripotent stem cells; conversely, reduced m^6A levels impede reprogramming (Carette et al., 2009).

Gamete-deriving haploid embryonic stem cells

Gamete-deriving haploid ESCs technology is stemmed from nuclear transfer and has been a novel technology for mammalian genetic study and regenerative medicine (Shuai and Zhou, 2014). Since the 1970s, extensive efforts have been made to generate haploid embryos in the mouse (Tarkowski and Rossant, 1976), but it was not until 2011 that the mouse parthenogenetic haploid ESCs (phESCs) were derived from parthenogenetic haploid embryos via fluorescence-activated cell sorting of haploid cells (Elling et al., 2011; Leeb and Wutz, 2011). Moreover, mouse androgenetic haploid ESCs (ahESCs) which can function as sperm were established in 2012 (Li et al., 2012b; Yang et al., 2012). Subsequently, generations of monkey phESCs (Yang et al., 2013), and rat ahESCs and phESCs (Li et al., 2014) were reported.

As well as valuable tool for genetic screening and drug screening (Shuai and Zhou, 2014), haploid ESCs are also novel resources for regenerative medicine due to the following reasons. (i) Haploid ESCs of mouse and rat have similar pluripotency to diploid ESCs which could produce chimeric animals and contribute to the germline (Leeb et al., 2012). (ii) Both ahESCs and phESCs can generate fertile offspring (Li et al., 2012b, 2014; Wan et al., 2013). (iii) Fused ESCs from ahESCs and phESCs can generate fertile offspring through tetraploid embryo complementation (Li et al., 2015). (iv) Haploid ESCs can differentiate into haploid epiblast stem cells (Elling et al., 2011) and haploid somatic cells (unpublished data). (v) Genome-editing to repair double allele mutations is easier for haploid ESCs. (vi) Haploid ESCs may be induced into gametes by overexpressing transcription factors or adding small drug molecules.

Creatively, mouse-rat allodiploid ESCs (AdESCs) were generated using the mouse and rat haploid ESCs, and the AdESCs have similar pluripotency to mouse and rat ESCs with the ability to differentiate into all three germ layers as well as early stage germ cells while maintaining a stable allodiploid genome (Li et al., 2016a). AdESCs can serve as

a powerful tool for understanding evolution of gene regulation, which would be very useful for uncovering the nature of totipotency of mouse ESCs, and would be helpful for establishing human ESCs with higher pluripotency, especially when based on other possible AdESCs, such as mouse-monkey and even mouse-human AdESCs.

ANIMAL MODELS: DISEASE/THERAPEUTIC MODELS AND ORGAN DEFECT MODELS

Animal disease/therapeutic models and animal models with organ defects are another useful tool for regenerative medicine. Precise genomic modifications at single nucleotide level have promoted the development of animal model construction. For a long period of time, genome editing has largely relied on traditional forward genetic screenings, which are intrinsically limited (Rubin and Spradling, 1982; Solnica-Krezel et al., 1994). With the development of artificial nuclease technology, ZFN (zinc finger nucleases), TALEN (transcription activator like effector nucleases), and CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated) system have boosted the development of genome-editing. Many animal models of human diseases have been established by TALEN or CRISPR/Cas9 system, including mouse (Wang et al., 2014), rat (Li et al., 2013), pig (Hai et al., 2014; Wang et al., 2015), and monkey (Liu et al., 2014; Niu et al., 2014; Wan et al., 2015).

Since blastocyst complementation was first reported by Chen, giving rise of T and B lymphocytes by injecting normal mouse ESCs in Rag2$^{-/-}$ mice which have no mature T or B cells (Chen et al., 1993), the generation of functional rat pancreas in mouse by interspecies blastocyst injection of rat ESCs reported by Kobayashi (Kobayashi et al., 2010) took an initial step toward the future regenerative medicine. Similar successes in organ generation were achieved for kidney of mouse (Usui et al., 2012) and pancreas of pig (Matsunari et al., 2013).

A great development in tissue engineering is represented by 3D bio-printing technology (Gu et al., 2015), which opens a new path for regenerative medicine and holds a great promising future. Nevertheless, it will need parallel advances in biomaterials and cell biology to achieve clinical advances.

OUTLOOK

The 2012 Nobel Prize in physiology or medicine was awarded to Shinya Yamanaka and John Gurdon for their discovery of somatic cell reprogramming, which provides a means to obtain limitless sources of stem cells. Reprogramming marks an instrumental advance for the field of stem cells and regenerative medicine. The mechanistic aspects of reprogramming are currently under extensive investigation, and in-depth and thorough understanding of development and reprogramming will be crucial for developing safe and effective cell therapies to realize the full potential of regenerative medicine.

In the coming decades, to generate stem cells with higher pluripotency especially from large animals and human, to establish animal disease/therapeutic models and animals with "organ niche", and to develop new biomaterials and well-established *in vitro* differentiation system in order to generate human organs through 3D bio-printing, will be major research directions for regenerative medicine.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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