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REVIEW

Epigenetic reprogramming: roads to pluripotency

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Abstract Epigenetic reprogramming provides valuable resources for customized pluripotent stem cells generation, which are thought to be important bases of future regenerative medicine. Here we review the commonly used methods for epigenetic reprogramming: somatic cell nuclear transfer, cell fusion, cell extract treatment, inducing pluripotency by defined molecules, and briefly discuss their advantages and limitations. Finally we propose that mechanisms underlying epigenetic reprogramming and safety evaluation platform will be future research directions.

Keywords Epigenetic reprogramming, pluripotency, regenerative medicine

1 Introduction

Animal development begins with a fertilized egg and goes on to from an adult organism. Cells in early embryos before gastrulation are pluripotent and hold the potential to differentiate into any cell in the body. Adult cells, including adult stem cells, have very limited developmental capacity and does not turn into or give rise to cells of other germ layer naturally. It was once believed that the adult nuclei could never re-gain the embryonic state because of some unknown lost during development. However, several groundbreaking experiments based on a technology called nuclear transfer proved that the adult nuclei still had the full programming ability to convert the cell into any fate. During the past half century, nuclear reprogramming has been expanded from amphibians (Gurdon and Uehlinger, 1966), to livestock (Wilmut et al., 1997), and to primates including human. Meantime, pluripotency from various species and developmental stages have been successfully captured in dish by deriving the pluripotent stem cell lines such as embryonic stem (ES) cell lines and embryonic germ cell lines. In 1981, Evans et al. established ES cell lines from mouse blastocyst (Evans et al., 1981). In 1998, James Thomson group established the first human embryonic stem cell lines (Thomson et al., 1998). The human embryonic stem cell, like embryonic stem cell from other species, could be differentiated into many kinds of cells *in vitro*, including neurons, cardiac muscles and insulin-making cells. This potential provides great promise to future regenerative medicine and cell replacement therapy. Specially, the combination of epigenetic reprogramming of somatic cells and pluripotent stem cell derivation and directed differentiation propose a customized therapy based on regeneration and replacement. It is to derive patient-specific pluripotent stem cell line and differentiate into specific cells and tissues, which could be used for replacement therapy without immunorejection. This potential therapy has driven a big exploration on creating new methods for epigenetic reprogramming of a somatic cell into pluripotency. Here we review these methods and briefly discuss their advancements, limitations and prospects.

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2 Somatic cell nuclear transfer

Development is accompanied with more and more restricted potential of cells and this process cannot be reversed naturally. It was once a very big question that whether adult cell nuclei still hold totipotency. While nuclear transfer, which is transferring a nucleus into an enucleated egg or oocyte, provides an experimental platform to test it, the first successful nuclear transfer experiment was carried out in frogs by Briggs and King (Briggs and King, 1952). They transferred the nuclei of early embryo cells into enucleated eggs and generated normal tadpoles. In 1966 Gurdon group transferred nuclei of intestinal epithelium cells into enucleated Xenopus eggs and generated both male and female frogs. The result clearly demonstrated that nuclei hold pluripotency during development and cell differentiation could be fully

reversed. This led to the birth of Dolly, the sheep, in 1996, which was generated by transferring mammalian gland cells into enucleated sheep eggs. Dolly's birth showed that the fully epigenetic reprogramming could also happen in mammalians. Soon after that, explorative success yielded in somatic nuclear transfer in species such as mouse (Wakayama et al., 1998), bovine (Kato et al., 1998), pig (Polejaeva et al., 2000) and rat (Zhou et al. 2003), which were useful experimental models or economic livestock. In 1998, Thomson group established the first human embryonic stem cell line which could differentiate into many cell kinds in the body. The therapeutic potential of human embryonic stem cells evokes great interest in therapeutic cloning of human beings. Therapeutic cloning aims to produce human blastocyst by somatic cell nuclear transfer (SCNT) and therefore to derive embryonic stem cell line which can differentiate into specific cells needed for transplantation therapy. Therapeutic cloning was first successfully practiced in mice (Rideout et al., 2002). A genetic-deficient mouse was nuclei donor for somatic cell nuclear transfer and embryonic stem cell lines were then derived from cloned blastocyst. Genetic deficiency of these ES cells was modified by gene recombination. Then they were directed to differentiate into hematopoietic precursor cells followed by transplantation into the diseased mouse for cure. The whole process was like an annotation of therapeutic cloning and the results proved it worked well in mice.

However, it is not an easy turning from mice to primates. Later success in monkey SCNT embryonic stem line derivation suggested that it would be much harder to generate both the cloned blastocyst and SCNT ES line in humans (Byrne et al., 2007). Human SCNT blastocyst rate was much lower than that achieved in mice. Till now, to generate an embryonic stem cell line for the human cloned blastocysts is still under trying. To obviate the needs of human unfertilized eggs, human therapeutic cloning using animal eggs has also been widely studied. The generation of full term mice by transferring adult nuclei into enucleated zygote suggested reprogramming capability existed not only in oocyte (Egli et al., 2007). Actually we found that two cell stage embryo could also reprogram adult nuclei beyond blastocyst stage (Amjad et al., in preparation). These studies will broader the resources for epigenetic reprogramming other than fresh oocyte.

Human therapeutic cloning has some limitations making it unlikely to be widely used in future regenerative medicine. There are too much ethical concerns about this technique such as oocyte donation and destroying embryos. Besides the low efficiency, difficult manipulation of SCNT also limits its wide use. However, reprogramming by SCNT may serve as a golden control for other reprogramming methods mainly for two reasons. First, the great success of SCNT in so many species suggests it is a faithful reprogramming method. Second, the SCNT embryonic stem cells have been proved indistinguishable from embryonic stem cells (Brambrink et al., 2006; Zhao et al., 2007). Thus, it is still much valuable for further study of human therapeutic cloning.

3 Reprogramming by cell fusion or cell extract

The enucleated oocyte can reset the adult nuclei to a totipotential state. This demonstrates that cytoplasm of one cell can influence the fate of the other. However, normal cells cannot be used as recipients as the size is too small for manipulation. Cell fusion makes it possible to exert the function of cytoplasm to each other's nuclei. Tada et al. fused the adult thymocytes with ES cells and found some hybridized cells were reprogrammed with the following characteristics: activation of the inactivated X chromosome in thymocyte, expression of pluripotential genes from thymocyte genome and contribution to three germ layers *in vivo* (Tada et al., 2001). But the reprogramming was not complete. For example, the imprinting gene methylation still held somatic pattern but not embryonic pattern. This was the first evidence that cell fusion with embryonic stem cells could reprogram adult cells. The result was reproduced in human cell later (Cowan et al., 2005). Cell extract can also exert functions on nuclei. Hakelien et al. used the cell extract of primary human T cells to treat 293T fibroblasts (Hakelien et al., 2002). They found the 293T cells could uptake the extract and be reprogrammed towards primary T cell state. The same group also treated somatic cells with the cell extract of embryonic stem cells and found epigenetic reprogramming of Oct4 and Nanog regulatory region (Freberg et al., 2007). We also found mouse fibroblast cells could be partially reprogrammed by mouse ES cell extract. The reprogrammed cells could be sequentially passaged with a similar morphology with ES cells and expressed pluripotent genes; however, they could not give rise to teratomas in SCID mice (personal communication).

Reprogramming by cell fusion or cell extract raises little ethical concern and is very easy to handle. But cells reprogrammed by cell extract only achieve low-grade pluripotency with much less differentiation ability than ES cells. And hybridized cells reprogrammed by cell fusion are tetraploidy. So generally, these two reprogramming methods cannot be used for clinical application. However, these methods clearly demonstrate that the epigenetic reprogramming can be induced by *in vitro* systems without oocyte. Their easy handling also provides a convenient system for reprogramming mechanism study. Meanwhile, the reprogramming ability of embryonic stem cells proves that they also contain the "mysterious" reprogramming factors existing in the oocyte. While the easy culture of ES cells will provide massive materials for selection of these factors.

4 Induced pluripotency by defined molecules

It was never thought that epigenetic reprogramming into pluripotency would be so easy before Yamanaka's milestone discovery: pluripotent stem cells could be induced from somatic cells by ectopic expression of four factors: Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006). From 24 candidate genes, after several rounds of selection, Takahashi and Yamanaka finally defined four factors of those were enough to induce pluripotency. Soon after this, they and other groups generated induced pluripotent stem (iPS) cells capable of germline transmission through more stringent selection (Maheral et al., 2007; Okita et al., 2007; Wernig et al., 2007). Induction of germline competent iPS cells broke the doubt and more groups shifted into this field with no hesitate. Soon later this, Takahashi et al. and Yu et al. quickly transferred this technique to human cells (Takahashi et al., 2007; Yu et al., 2007). However, there are still two hindrances for clinical translation of iPS cell technology. First, the original method to generate iPS cells has big safety concern. In the Yamanaka factors, c-Myc and Klf4 are famous oncogenes. Meantime, the gene delivery method by retrovirus and integration of exogenous genes may cause gene mutations or genome rearrangement. So there are extensive studies focusing on this. One choice is to reduce the inducing factor number. Nakagawa et al. found that without Myc gene, iPS cells could also be generated albeit at lower induction rate. Very soon, Shi et al. found that Sox2 and c-Myc could be avoided in the addition of small molecules (Shi et al., 2008). Till now, Kim et al. found that only OCT4 gene was needed when reprogramming human neural stem cells (Kim et al., 2009b). The other choice is to use gene delivery systems that have no integration. Yu et al. generated human iPS cells free of vector and transgene sequences (Yu et al., 2009). The third choice is to use small molecules or proteins that will not change the genome. Zhao et al. and Kim et al. separately established proteins induced pluripotent stem cells from mouse and human (Zhao et al., 2007; Kim et al., 2009a). The other hindrance comes from a basic question: whether only four factors are enough to induce fully reprogramming. Before iPS, the only way to generate full reprogramming is to utilize oocyte. Compared to the unknown complex oocyte system, the four factors may not be sufficient to yield full reprogramming. Even the germline competent iPS cell cannot eliminate the doubt. The most stringent method for test of pluripotency is tetraploid complementation. The tetraploid mice all come from injected pluripotent cells while original tetraploid cells only contribute to extra-embryonic tissues. Till now only ES cells and nuclear transfer embryonic stem (NTES) cells can produce healthy adult mice. Recently, Zhao first reported their research on producing tetraploid mice from iPS cells, while other two groups also achieved success later (Boland et al., 2009; Kang et al., 2009; Zhao et al., 2009). The results clearly show that fully epigenetic reprogramming can be induced by just ectopic expression of four defined factors. The first tetraploid mice Zhao generated was named Xiao Xiao. Since this report published online in Nature, worldwide media gave the quake tremendous covers. "Most research progresses in small steps. Xiao Xiao represents a jump forward," said Bruce Whitelaw, head of the Division of Developmental Biology at the Roslin Institute, and editor-in-chief of the journal *Transgenic Research*. Whitelaw also said, "The world must now believe that iPS cells can be truly pluripotent". "Yes, Xiao Xiao received the torch lit by Dolly," Whitelaw commented while reviewing the history of clone.

Epigenetic reprogramming by iPS technology is very easy to get access to, thus it exerts a profound effect on this field and generates explorative achievements. iPS cells have little ethical concerns and provide a better model system for reprogramming mechanism study. They can also be used to mimic the pathogenesis process *in vitro* and for drug selection. There are still some problems existing, such as safety concern. Miura et al. found that derivatives from some iPS cell lines had high teratoma-forming propensity (Miura et al., 2009). It is reasonable to believe these problems will be fixed in the near future.

5 Perspectives

Epigenetic reprogramming to pluripotency provides us valuable resources for derivation of customized pluripotent stem cell lines, which is considered the bases for regenerative medicine. However, it is still a long journey before clinical translation. At least two aspects need extensive and deeper studies. First, the mechanisms of epigenetic reprogramming are still largely unknown even though the first SCNT cloned frog was generated in 1966. The mechanism study will help to decipher the existing defects in the reprogramming process. It can also help to explore new reprogramming pathways that are safer and more convenient. Though it is not directly related to therapeutic purpose, such studies deserve more time and effort. Second, a safety evaluation platform is needed for clinical translation. Pluripotent stem cell lines generated by reprogramming contain some known and unknown safety problems such as tumorigenicity. It is very important to set up such a platform to evaluate the safety of different customized pluripotent stem cell lines and to set up golden standards to discriminate the safer lines from the danger lines. Considering much more ethical concerns for human pluripotent cell research, a platform with fast and convenient test is especially needed.

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