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REVIEW

Mechanism and methods to induce pluripotency

Peizhe Wang, Jie Na ✉

School of Medicine, Tsinghua University, Beijing 100084, China
✉ Correspondence: jie.na@tsinghua.edu.cn

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ABSTRACT

Pluripotent stem cells are able to self-renew indefinitely and differentiate into all types of cells in the body. They can thus be an inexhaustible source for future cell transplantation therapy to treat degenerative diseases which currently have no cure. However, non-autologous cells will cause immune rejection. Induced pluripotent stem cell (iPSC) technology can convert somatic cells to the pluripotent state, and therefore offers a solution to this problem. Since the first generation of iPSCs, there has been an explosion of relevant research, from which we have learned much about the genetic networks and epigenetic landscape of pluripotency, as well as how to manipulate genes, epigenetics, and microRNAs to obtain iPSCs. In this review, we focus on the mechanism of cellular reprogramming and current methods to induce pluripotency. We also highlight new problems emerging from iPSCs. Better understanding of the fundamental mechanisms underlying pluripotenty and refining the methodology of iPSC generation will have a significant impact on future development of regenerative medicine.

KEYWORDS reprogramming, induced pluripotent stem cells, pluripotency

INTRODUCTION

Pluripotency refers to the ability of a cell to differentiate into all types of cells, including germ cells, in the body. Only a few kinds of mammalian cells are pluripotent, including the inner cell mass (ICM) cells of the blastocyst, embryonic stem cells (ESCs), embryonic germ cells (EGCs) and some embryonic carcinoma cells (ECs). There is great interest in cellular reprogramming to pluripotency because this allows us to generate patient-specific pluripotent stem cells, differentiate them into functional cells such as neurons and cardiomyocytes, and use them to study human diseases or replace degenerated and damaged tissue. Several approaches have been developed to reprogram somatic cells to pluripotent state, including somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997), fusing with ESCs or EGCs, or exposing them to ESC or EC extracts (Taranger et al., 2005). In 2006, an important breakthrough was achieved by Takahashi and Yamanaka (Takahashi and Yamanaka, 2006). They showed that by stably expressing four transcription factors, one can convert fibroblast cells to ESC-like cells. These reprogrammed cells expressed ESC marker genes and can contribute to all types of tissue in chimeric animals; hence they are called induced pluripotent stem cell (iPSC) (Takahashi and Yamanaka, 2006). iPSCs circumvented the ethical issues associated with human ESCs and SCNT and opened a whole new era for regenerative medicine. In this review, we discuss our current understanding of the mechanism of reprogramming and various methods to induce pluripotency.

MOLECULAR MECHANISM OF REPROGRAMMING TO PLURIPOTENCY

The function of reprogramming factors

The first four reprogramming factors—Oct4, Sox2, Klf4, and cMyc—all play vital roles in early embryogenesis and ESCs (Takahashi and Yamanaka, 2006). Oct4 is a POU homeodomain transcription factor. It is an essential factor in most reprogramming experiments and is required for the pluripotency of ICM cells and ESCs (Nichols et al., 1998). Although the nuclear receptor Nr5a2 can lead to reprogramming instead of Oct4, Nr5a2 was found to bind to the promoters of Oct4 and Nanog and upregulated their expression. Thus, its reprogramming ability was still directly dependent on Oct4

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(Heng et al., 2010). Oct4 cooperates with many other factors to establish pluripotency. In mouse ES cells, proteomic studies revealed that Oct4 binds to as many as 92 proteins (Wang et al., 2006; Pardo et al., 2010), which implies that intracellular context can have a significant impact on reprogramming. When fusing a somatic cell with an ESC, or transferring its nucleus into an oocyte (where many Oct4 binding partners are naturally present), endogenous pluripotency genes can be turned on in two days, much more rapidly than reprogramming with a few defined factors (Cowan et al., 2005; Egli et al., 2007). Adult cell types (neural stem cells or melanocytes) that express a key Oct4 interacting partner, Sox2, a Sry-related high mobility group box transcription factor, can be converted to iPS cells with a significantly higher efficiency (Kim et al., 2009b; Utikal et al., 2009a).

The second reprogramming factor, Sox2, forms a complex with Oct4 to regulate the transcription of key pluripotency genes including Oct4, Sox2 and Nanog (Masui et al., 2007). However, it is not absolutely required for pluripotency or reprogramming. In Sox2 null ES cells, overexpression of Oct4 was able to maintain the undifferentiated state (Masui et al., 2007). During reprogramming of mouse fibroblast cells, TGFβ inhibitors can replace Sox2 by inducing Nanog and cMyc expression (Ichida et al., 2009; Maherali and Hochedlinger, 2009). This suggests that Oct4 can work with other key factors in the pluripotency network to regulate reprogramming albeit with a lower efficiency.

The Krüppel-like zinc finger transcription factor Klf4 is enriched in ES cells and plays a central role in the pluripotency circuitry by regulating the expression of Sox2 and Nanog (Kim et al., 2008; Niwa et al., 2009). Overexpressing Klf4 prompted mouse epiblast derived stem cells (EpiSCs) to enter the naïve ES cell state (Guo et al., 2009). Similarly, human ES cells and iPS cells with elevated levels of Klf4 and Oct4 or Klf4 and Klf2 exhibiting mouse ESC characteristics were able to grow in a medium containing leukemia inhibitory factor (LIF) and 2i (ERK1/2 and GSK3 inhibitors), and had both X chromosomes activated (Hanna et al., 2010a).

cMyc is an oncogene and can significantly increase the efficiency of iPS cell generation (Nakagawa et al., 2008). In ESCs, cMyc was found to occupy promoters of active genes and ESC specific microRNAs (miRNAs) such as miR-291-3p, miR-294, miR-295, miR-141, miR-200, and miR-429 (Kim et al., 2008; Judson et al., 2009; Lin et al., 2009). Overexpression of these miRNAs either promoted iPSC formation or decreased mouse ESC differentiation (Judson et al., 2009; Lin et al., 2009). It is likely that cMyc creates a permissive cellular state by recruiting multiple chromatin remodelers, such as histone acetyltransferase GCN5 and histone demethylase Lid. This allows the ectopically expressed ESC transcription factors to activate their target genes more easily (Laurenti et al., 2009). But there is a negative side to cMyc's action. Chimeric iPSC mice frequently developed tumors when cMyc was used for reprogramming (Nakagawa et al., 2008). Thus, it is better either to exclude this oncogene from the reprogramming mix or remove it from the iPSCs upon successful reprogramming.

Yu and colleagues used Nanog and Lin28 instead of Klf4 and cMyc to reprogram human fibroblast cells (Yu et al., 2007). Nanog is a core member of the pluripotency circuitry (Kim et al., 2008). Its constitutive expression was sufficient to prevent mouse ESC differentiation in the absence of LIF (Chambers et al., 2003) and it is required for the development of germline (Chambers et al., 2007). Although ectopic Nanog expression is not absolutely required for reprogramming, it increased the frequency of iPSC formation (Hanna et al., 2009).

Lin28 is an ESC-specific RNA binding protein whose major role lies in the degradation of *let-7* miRNAs (Heo et al., 2008; Viswanathan et al., 2008). The *Let-7* family of miRNAs is expressed ubiquitously in somatic cells, but not in ESCs, and is up-regulated upon ESC differentiation. Their targets include many cell cycle regulators such as CDC25A and cyclinD1 (Roush and Slack, 2008). The mechanism of Lin28's action appears to promote cell cycle progression by suppressing the Let-7 miRNAs, and hence accelerate the reprogramming process (Hanna et al., 2009).

Recently, after screening more than a thousand transcription factors, Yamanaka group discovered a new reprogramming factor, Glis1, a GLI family transcription factor. It is enriched in unfertilized eggs and zygotes, and could replace cMyc to produce iPSCs from adult cells with a higher efficiency and decreased tumorigenicity (Maekawa et al., 2011). Mechanistic studies revealed that the Glis1 protein interacts with Oct4, Sox2, and Klf4. The gene targets of Glis1 include several Wnt ligands, Lin28a (a homologue of Lin28), Nanog, Mycn, Mycl1, and Foxa2. They propose that Glis1 facilitates iPSC formation by activating multiple pro-reprogramming pathways (Maekawa et al., 2011).

In summary, the reprogramming factors are core members of either the pluripotency circuitry or their upstream regulators. These factors initiate a cascade of events that ultimately lead to reactivation of the endogenous pluripotency genes and the interconnected autoregulatory loop which sustains the pluripotent state (Hanna et al., 2010b). As most master regulators of pluripotency are present in oocytes, preimplantation embryos, ESCs and germ cells, elucidating the mechanism maintaining pluripotency in these systems will help to discover novel inducing factors and improve the efficiency of iPSC generation.

Change of epigenetic landscape

The epigenetic landscapes of somatic cells and ESCs are dramatically different. In differentiated cells, the promoter regions of lineage-specific transcription factors are labeled either with the repressive histone H3 lysine 27 trimethylation

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(H3K27me3) mark or the activing histone H3 lysine 4 trimethylation (H3K4me3) mark, while the promoters/enhancers of pluripotency genes are heavily methylated (Bernstein et al., 2006). In contrast, the epigenetic landscape of ESC is characterized by the demethylation of the promoters of the pluripotency genes Oct4, Sox2, and Nanog, and a bivalent chromatin modification (containing both H3K4me3 and H3K27me3) in the promoter region of some differentiation transcription factors such as Nkx2.2, Sox21, and Zfpm2 (Bernstein et al., 2006). This ensures a high level expression of the core factors that maintain pluripotency, and in the mean time, cells rest in a poised state, ready for differentiation in response to appropriate developmental signals (Bernstein et al., 2006). A detailed comparison of the epigenetic status of fully reprogrammed, partially reprogrammed, and fibroblast cells revealed that only fully reprogrammed cells erased the DNA methylation at promoters of pluripotency genes (Mikkelsen et al., 2008). In partially reprogrammed cells, although gene loci related to stemness and cell proliferation had changed to activating histone modifications, the core pluripotency gene loci (Oct4, Nanog…) were still repressed (Mikkelsen et al., 2008). These observations emphasize that establishment of a real ESC-like epigenetic landscape is essential for a cell to enter the pluripotent state.

The epigenetic state of a somatic cell can have significant impact on its reprogramming potential. Eminli and colleagues discovered that in the hematopoietic lineage, stem and progenitor cells can be converted to iPSCs much more easily than terminally differentiated B and T cells (Eminli et al., 2009). This implies that the relatively flexible epigenetic state of tissue-specific stem cells makes them more amenable for reprogramming.

Since the DNA and histone modifications are largely dependent on the activity of the corresponding regulator enzymes, appropriately manipulating the activity of these enzymes can greatly facilitate iPSC formation. To this end, several inhibitors of DNA and histone methyltransferase, as well as histone deacetylase (HDAC) inhibitors have been found to dramatically improve reprogramming efficiency (Huangfu et al., 2008a; Shi et al., 2008).

Overcoming the DNA damage response

The dramatic changes in a cell's epigenome during reprogramming will inevitably trigger DNA damage response. This often leads to cell cycle arrest and senescence. Only a few cells can overcome this barrier and become iPSCs. A number of studies demonstrated that when key components (such as p53 and p21) of the DNA damage pathway were deleted, the percentage of iPS cell generation significantly increased (Zhao et al., 2008a; Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009b). Suppression of the p53 pathway may compromise a cell's genome stability. For instance, adding SV40 large T into the reprogramming mix accelerated the speed of iPSC formation; however, many of the iPSCs gained chromosomal abnormality (Mali et al., 2008). Thus, during reprogramming, one must take extra care to maintain the intricate balance between safeguarding genome integrity and change of cell fate.

METHODS TO INDUCE PLURIPOTENCY

Viral transduction of defined factors

To date, the most efficient method to generate iPSCs is through viral transduction of reprogramming factors (Takahashi et al., 2007; Yu et al., 2007). Genetically modified retro or lenti virus can infect mammalian cells with almost 100% efficiency. It is their intrinsic property to integrate into the host genome, so that stable expression of the reprogramming factors can be achieved. Upon entry into a pluripotent state, viral promoters are silenced, while the reprogrammed cells activate the endogenous pluripotency genes. Despite the high efficiency, the viral approach has serious drawbacks. Kane and colleagues reported an alarming case: human iPSC-like cells formed when fibroblast cells were transducted with lentivirus that did NOT contain any reprogramming factors. These "pseudo" iPSCs were created by viral insertion triggered changes in host genome and microRNA expression, and they were also karyotypically abnormal (Kane et al., 2010). Moreover, viral transgenes may not be silenced completely or become reactivated during prolonged culture or differentiation. As mentioned previously, chimeric mice generated from viral iPSCs frequently developed tumors, which was due to reactivation of the cMyc expression virus (Nakagawa et al., 2008). Thus, for iPSC use in clinical settings, a viral- and transgene-free reprogramming approach is needed.

Viral-free approaches that do not cause genetic alteration

Obtaining iPSCs without genetic alteration is necessary for regenerative medicine. To meet this need, several non-integration systems and removable systems have been developed. These include episomal vectors (Yu et al., 2009), the piggyBac transposon system (Kaji et al., 2009; Woltjen et al., 2009; Yusa et al., 2009), and minicircle vectors (Jia et al., 2010).

Compared with most viral systems, transposons and episomal vectors can carry much larger DNA fragments (more than 10 kb) into the host cell. To achieve simultaneous expression of reprogramming factors, cDNA encoding as many as five reprogramming factors connected by the 2A peptide sequence from the foot-and-mouth disease virus was cloned in a single vector. Individual proteins were produced through a "ribosomal skipping" mechanism mediated by the 2A peptide (Szymczak et al., 2004; Yusa et al., 2009). In the

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case of the piggyBac system, after successful generation of iPS cells, the reprogramming cassette can be removed by piggyBac transposase without footprint and transgene-free iPS cells were obtained (Yusa et al., 2009). However, the removal efficiency of piggyBac transposon was only 1 in 10^5 (Yusa et al., 2009), and such a low efficiency limits wider application of this technology in reprogramming and gene therapy.

Episomes can self-replicate and in most circumstances do not integrate into the host genome (Yu et al., 2009). Compared with plasmid-mediated gene expression, episomal vectors sustain longer and more stable expression, and thus facilitate generation of iPS cells that require a prolonged presence of ectopic reprogramming factors (Yu et al., 2009). Minicircle vectors are supercoiled DNA molecules that have higher transfection efficiency and longer ectopic expression window than plasmid DNA (Chen et al., 2003; Chen et al., 2005) due to reduced activation of the silencing mechanisms against foreign DNA. However, they cannot self-replicate and thus the expression time is not as long as for episomal DNA (Jia et al., 2010). The non-integration DNAs will be diluted upon cell division and lost with time, so that the resulting iPSCs were free of any foreign DNA contamination (Yu et al., 2009; Jia et al., 2010). However, the reprogramming efficiency of these non-integration systems was low compared to the viral approach.

Generation of iPS cells using proteins or mRNAs of defined factors

Although iPSCs can be obtained by removable transposon or the non-integration episomal system, all of these methods still involve introducing foreign DNA into the cell so that the resulting cell lines need to be sequenced to verify that they are indeed free of genome alteration (Kaji et al., 2009; Woltjen et al., 2009; Yu et al., 2009). Thus, a reprogramming approach that does not use any DNA is highly desirable. Delivery of reprogramming factors in their protein form is the first obvious choice. In 2009, two groups showed that they were able to obtain transgene-free iPSCs with proteins of reprogramming factors (Kim et al., 2009a; Zhou et al., 2009). In these studies, reprogramming factors were fused with cell-penetrating peptides. After repeated exposure of fibroblast cells to these proteins, they were transformed into an ESC-like morphology, expressed undifferentiated cell markers, and gained differentiation ability. However, these protein-based reprogramming experiments have not been widely successfully repeated.

More recently, Warren and colleagues demonstrated that modified mRNA encoding reprogramming factors could generate iPSCs with high efficiency if the innate immune response was properly suppressed (Warren et al., 2010). mRNAs could be an ideal vehicle for reprogramming for several reasons: they are much smaller than DNA, so they can enter cells with high efficiency; mRNAs will be translated into proteins by the host cell, and hence they are properly modified and have higher activity than proteins produced in bacteria. Moreover, mRNAs are easy to multiplex and the amount of each factor can be precisely controlled. Therefore, mRNA can be a versatile tool both for reprogramming and to study the mechanism of this process (Plews et al., 2010). However, mRNA also has a number of serious drawbacks: the expression window of mRNA is only 2–3 days, so that repeated transfection is needed to achieve reprogramming and innate immune response must be suppressed alongside mRNA transfection. Although currently these problems make mRNA reprogramming experiments expensive and difficult to repeat, with the discovery of more reprogramming factors and means to accelerate this process, it may be possible to induce pluripotency within 2–3 days using an optimal mRNA and small molecule “cocktail.” Such speed is similar to the reprogramming speed of somatic nuclear transfer, and therefore should be achievable in theory using defined factors.

Generating iPS cells by microRNAs

Three recent studies reported that miRNAs alone are sufficient to induce pluripotency in mouse and human cells. The miR302 cluster is a direct target of Oct4 and Sox2 (Card et al., 2008) and abundantly present in mouse and human ES cells (Suh et al., 2004). In 2008, Lin and colleagues reported the transformation of human skin cancer cells into an ES-like state by overexpression of the miR302 cluster (Lin et al., 2008). Very recently, two independent groups announced highly efficient generation of mouse and human iPSCs either by lentiviral delivery of miR302 into fibroblast cells or by transfection of mature miR200c, miR302, and mir369 (Anokye-Danso et al., 2011; Miyoshi et al., 2011). These microRNA iPSCs were indistinguishable from OSKM-iPSCs in pluripotency marker expression, teratoma formation, and germline transmission (in mice). Compared to coding gene based reprogramming approaches, microRNA reprogramming offers several clear advantages. It completely avoids using oncogenic transcription factors such as cMyc and Oct4 and does not need to introduce genetic changes into cell genomes. However, this new reprogramming approach needs to be successfully repeated by other laboratories and the molecular mechanism of how miRNAs activate the entire pluripotency network is yet to be revealed.

Chemical compounds

Obtaining iPSCs by chemical compounds alone would be the most convenient approach, since they are easy to apply and this does not involve any genetic modification. The first compounds used in reprogramming experiments were DNA methyltransferase inhibitor 5′-azacytidine and the HDAC

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inhibitor valproic acid (VPA), which increased the reprogramming efficiency by five fold and more than a hundred fold respectively (Huangfu et al., 2008a, 2008b; Mikkelsen et al., 2008). Some small molecules could replace certain reprogramming factors. For example, the G9a histone methyltransferase inhibitor, BIX-01294, has been shown to be able to substitute cMyc and improve the reprogramming efficiency of neural stem cells by Oct4 and Klf4 (Shi et al., 2008). A-83-01, a TGFβ kinase/activin receptor-like kinase (ALK5) inhibitor can replace Sox2 through inducing Nanog and cMyc expression (Ichida et al., 2009; Maherali and Hochedlinger, 2009) and promoting mesenchymal-to-epithelial transition (Li et al., 2010). Signaling pathways have significant influence on the state of pluripotency. Silva and colleagues showed that neural stem cells could be rapidly converted to iPSCs with only two factors, Oct4 and Klf4, while application of the ERK inhibitor PD0325901 and GSK3 inhibitor CHIR99021 promoted these iPS cells to enter a more authentic ES state, reflected by the reactivation of both X chromosomes and ability to contribute to the germline (Silva et al., 2008).

As the DNA damage pathway poses a major barrier towards regaining pluripotency (Zhao et al., 2008b; Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009b), chemical compounds that alleviate the stress of DNA damage response may help cells to overcome this barrier. To this end, Vitamin C has been shown to significantly increase the formation of both mouse and human iPSC by suppressing p53 induced cell senescence (Esteban et al., 2010).

Although chemical compounds are able to greatly facilitate the reprogramming process, they still cannot replace all reprogramming factors. Zhu and colleagues successfully obtained human iPSCs with only one master reprogramming factor, Oct4, and a combination of small molecules including HDAC inhibitor sodium butyrate (NaB), PDK1 activator PS48, ALK 5 inhibitor A-83-01 and ERK inhibitor PD0325901 (Zhu et al., 2010). Thus, optimizing non-integrating reprogramming vehicles and application of chemical compounds may enable us to obtain transgene-free human iPSCs with high efficiency and in a much shorter time frame.

FURTHER CHALLENGES

iPSCs have opened a new door to understand pluripotency and greatly expanded our ability to study human diseases and develop regenerative medicine. However, a number of significant hurdles remain before these cells can be used in clinical settings. The most important is whether the differentiated derivatives of iPSCs are safe to be transplanted into patients. Early in 2011, several groups reported that many iPSC lines contain somatic coding mutations, copy number variations, and aberrant epigenomic reprogramming (Gore et al., 2011; Hussein et al., 2011; Lister et al., 2011). These genetic and epigenetic changes may increase the tumorigenesity of iPSCs. Another issue is their immunogenicity. Zhao and colleagues found that in mice, most teratoma formed from iPSCs but not ESCs were immune rejected by the recipient, suggesting that some abnormal gene products from iPSCs had triggered a serious immune response (Zhao et al., 2011). Finally, iPSCs were found to retain epigenetic memories of their cell type of origin and had preferred differentiation lineage choice (Bar-Nur et al., 2011). Moreover, iPSCs from fragile X syndrome patients failed to reactivate the fragile X mental retardation 1 (FMR1) gene while the ESCs derived from human fragile X syndrome blastocysts had active FMR1 gene loci (Urbach et al., 2010). This suggests that iPSCs may not be an appropriate model to study certain human genetic diseases. The above problems imply that the quality of iPSCs must be rigorously checked before their differentiated derivatives can be used in any clinical settings. However, these issues also provide many exciting challenges and opportunities. By elucidating the functions of pluripotency transcription factors, epigenetic machineries and signaling pathways as well as their regulatory network in various types of pluripotent stem cells, it should be possible to devise methods to significantly improve the reprogramming efficiency, and obtain safe iPSCs for regenerative medicine.

ABBREVIATIONS

ECs, embryonic carcinoma cells; EGCs, embryonic germ cells; EpiSCs, epiblast derived stem cells; ESCs, embryonic stem cells; ICM, inner cell mass; LIF, leukemia inhibitory factor; SCNT, somatic cell nuclear transfer

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