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Review

Understanding the roadmaps to induced pluripotency

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Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by ectopic expression of transcription factors Oct4, Sox2, Klf4 and cMyc. Recent advancements have shown that small-molecule compounds can induce pluripotency, indicating that cell fate can be regulated by direct manipulation of intrinsic cell signaling pathways, thereby innovating our current understanding of reprogramming. The fact that lineage specifiers can induce pluripotency suggests that the pluripotent state is a fine balance between competing differentiation forces. Dissection of pluripotent roadmaps indicates that reprogramming is a process of reverse development, involving a series of complicated and distinct reprogramming stages. Evidence from mouse iPSC transplantation studies demonstrated that some certain but not all cells derived from iPSCs are immunogenic. These studies provide new ways to minimize reprogramming-induced abnormalities and maximize reprogramming efficiency to facilitate clinical development and use of iPSCs.
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Facts

- Reprogramming is not only a simple process of reverse development but also a very complicated procedure with different reprogramming stages.
- Binding of both facilitators and inhibitors by reprogramming factors simultaneously at early stage directly contributes to low reprogramming efficiency.
- The fact that lineage specifiers can induce iPSC production suggests that pluripotency is a balance between competing differentiation forces.
- Induced pluripotency by small-molecule compounds indicates that cell fate decision is regulated by intracellular signaling pathways.
- Evidence from different research groups supports that some certain but not all cells derived from iPSCs are immunogenic.

Open Questions

- What are the critical molecular events involved in reprogramming of somatic cells to iPSCs?
- How do reprogramming factors orchestrate such a complicated de-differentiation process?
- Are there any ways to selectively activate facilitator genes without co-activation of inhibitory genes during initiation of reprogramming?
- How do the complicated signal transduction networks inside a cell control its fate?

The concept of totipotent differentiated vertebrate cells was first proposed by the German embryologist Spemann in 1938, who reported that the nucleus from an embryo retained the ability to develop into a salamander after undergoing four divisions.[1] In 1952, Briggs and King[2] successfully generated tadpoles by transferring a cell nucleus derived from an embryo in the blastocyst stage into an enucleated oocyte using a technique called somatic cell nuclei transfer (SCNT; Figure 1). Using this breakthrough technology, Briggs and King[3] tried to determine whether aging cells are still totipotent and found that as cells develop they become more difficult to clone. Gurdon extended these experiments by using nuclei from matured intestinal and keratinized skin cells of frogs as donors.[4–6] His research indicated that differentiated and even matured cells do indeed retain the genetic information needed to develop into a life, and the cytoplasm of oocytes contains certain factors that can reprogram mature nuclei into pluripotency.

By fusion of mouse pluripotent embryonic carcinoma cells with thymocytes, Miller and Ruddle[7] generated hybrids that form carcinomas after transplantation into nude mice, indicating that the differentiated thymocytes were reprogrammed into a pluripotent state. Two further reports showed that fusion of embryonic stem cells (ESCs) with somatic cells resulted in the formation of pluripotent hybrids, with

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Abbreviations: iPSC, induced pluripotent stem cell; ESC, embryonic stem cell; MET, mesenchymal-to-epithelial transition; EMT, epithelial -to-mesenchymal transition; SCNT, somatic cell nuclei transfer; GSK3, glycogen synthase kinase-3; OSKM, Oct4, Sox2, Klf4 and cMyc
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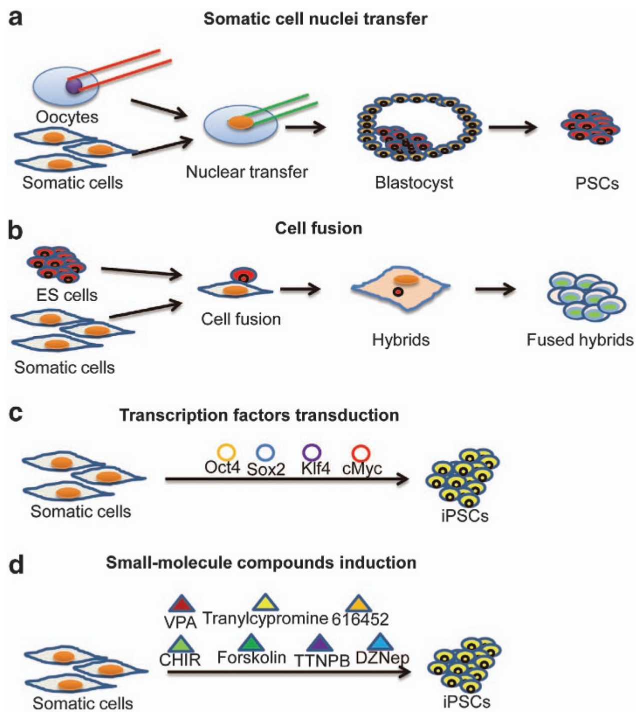

Figure 1 Different strategies to induce pluripotency. (**a**) Somatic cell (SC) nuclei transfer (SCNT). Pluripotency can be achieved by transfer of somatic nuclei to enucleated oocytes. (**b**) Cell fusion. Fusion of ESCs with SCs generates pluripotent fusion hybrids. (**c**) Transcription factor induction. Ectopic expression of four transcription factors (Oct4, Sox2, Klf4 and cMyc) in SCs can reverse them to an ESC-like state; these cells are called iPSCs. (**d**) Small-molecule treatment. SC treatment with a combination of small molecules (Valproic acid, Tranvlcypromine, 616452, CHIR, Forskolin, TTNPB, DZNep) can reprogram differentiated cells into iPSCs

characteristics similar to parental ESCs (Figure 1).[8,9] These proof-of-concept experiments together with successful nuclear reprogramming by SCNT indicate the presence of reprogramming factors in the ESCs and/or oocytes.[10]

In 2006, by screening a panel of genes specifically expressed in ESCs, Takahashi and Yamanaka[11] showed that Oct4, Sox2, Klf4 and cMyc could reprogram mouse somatic cells into pluripotency, called induced pluripotent stem cells (iPSCs; Figure 1). This same molecular cocktail can successfully reprogram human somatic cells to iPSCs.[8,9] Using a similar strategy, Thomson's group found that Oct4, Sox2, Lin28 and Nanog were able to reprogram human somatic cells into iPSCs.[12] Like ESCs, iPSCs can undergo self-renewal and differentiate into all three germ layers *in vitro*. They can also contribute to chimeric mice and go germ line transmission. Most importantly, iPSCs can directly develop into mice by 4n complementation assay, indicating that iPSCs are totipotent.[13–15]

Advancements in Reprogramming Methods

Since the discovery of iPSCs, reprogramming technology has developed rapidly. To date, they are three generation of iPSCs in general. First, iPSCs can be generated by overexpression of transcription factors in target cells infected by retrovirus or lentivirus.[11,12,16] However, viral integration into the host genome poses serious cancer risks, which significantly hinders the clinical development of this type of iPSC technology.[10,11,17]

To resolve potential cancer risks associated with viral integration, integration-free iPSC production techniques were widely developed as the secondary generation of reprogramming approaches. So far, iPSCs without exogenous DNA integration have been generated using different strategies, including *piggyBac* transposition,[18,19] episomal vectors,[20–25] and microRNA, which were initially demonstrated to enhance reprogramming efficiency and later used to generate iPSCs.[26,27] Furthermore, by delivering reprogramming proteins directly, mouse and human somatic cells were shown to be successfully reprogrammed but with extremely low efficiency.[28–30] All of these reprogramming methods avoid using viral delivery of transcriptional factors, thereby significantly improving their safety and use in clinical settings. Most recently, a third method to generate iPSCs was successfully developed, by addition of small-molecule compounds into mouse fibroblast cultures, raising hopes of generating human iPSC for therapeutics without tedious genetic manipulations (Figure 1).[31]

Understanding the Mechanisms of Reprogramming

Induced pluripotency was traditonally achieved by SCNT and cell fusion before the discovery of iPSC. It is now widely accepted that different cells have distinct transcriptional repertoires correlated with their own epigenetic signatures. The identification of master transcription factors, which execute reprogramming, represents significant progress in the understanding of mechanisms of induced pluripotency. However, how reprogramming factors orchestrate epigenetic remodeling is still largely unknown.

Facilitators and inhibitors of efficient reprogramming. iPSCs are generated by forced overexpression of transcription factors in targeted somatic cells, followed by multiple divisions over a long period of time during the reprogramming process. However, SCNT and cell fusion can induce pluripotency in a shorter time by using natural proteins inside the cytoplasm of eggs or ESCs.[32] This process is similar to mammalian fertilization: soon after entering the egg, sperm chromatin rapidly switch their conformation and coordinately undergo mitosis together with the chromosomes of eggs, suggesting that the cytosolic proteins of eggs can efficiently change the epigenetic state of sperm. After introduction of a sizeable amount of reprogramming factors (Oct4, Sox2, Klf4 and cMyc; that is, OSKM), somatic cell chromosomes are forced to bind these foreign factors in either ESC physiologically relevant or irrelevant regions.[33] The extensive regionally irrelevant binding of OSKM to targeted somatic cell chromatin during initial stages impedes successful reprogramming and might be a major cause of low reprogramming efficiency. Although Oct4, Sox2 and Klf4 (that is, OSK) bind targeted chromatin, cMyc enhances chromatin binding by OSK. This feedback loop is thought to facilitate successful reprogramming. On the other hand, a large scale of chromatin domains spanned by H3K9me3 that inhibit OSKM binding are known to hinder efficient reprogramming.[33]

Moreover, the tumor suppressor p53 is proposed to prevent induced pluripotency, as p53 knockouts have been shown to significantly increase reprogramming efficiency.[34–38] p53 has also been shown to bind the Nanog promoter and regulate its expression;[39] suppressing Nanog expression leads to differentiation of ESCs. Most interestingly, knockdown of p53 downstream effectors Puma and/or p21 can significantly increase iPSC production efficiency, indicating that apoptosis and/or cell-cycle arrest function of p53 significantly inhibits efficient reprogramming.[40]

Reprogramming roadmaps. It has been shown that a mesenchymal-to-epithelial transition (MET) is required for reprogramming,[41,42] and Vitamin C can enhance reprogramming efficiency throughh H3K36 demethylation.[43] A recent study identified an unexpected sequential epithelial-to-mesenchymal (EMT)–MET transition during the initiation of reprogramming,[44] while another suggested that reprogramming is not simply a process of reversed development.[45] By analysis of the expression of novel cell-surface markers CD44 and ICAM1, Malley *et al.*[45] defined four stages of reprogramming: (1) mesenchymal, characterized by CD44+/ICAM1+ and high expression of N-Cadherin, Snail, Slug, Zeb1 and 2; (2) epidermal, characterized by CD44+/ICAM−, with high expression of Krt6a, Krt17, Ehf, Ngfr, Sfn and Evp1; (3) early pluripotent, characterized by CD44-/ICAM1− and expression of Oct4, Sall1, Sall4, Zfp296, Tcfcp2l1 and Etv5; and (4) late pluripotent, characterized by CD44−/ICAM+, with high expression of Nanog, Dppa4, Dppa5a, Sox2, Esrrb and Klf2.

More and more studies aimed to embody reprogramming roadmaps. Using genome-wide analysis, Polo *et al.*[46] defined an intermediate cell population poised to become iPSCs and showed that two transcriptional waves were elicited by reprogramming factors; the first wave was driven by cMyc/Klf4 and the second by Oct4/Sox2/Klf4. Cells experiencing the first transcriptional wave were refractory to reprogramming and could be rescued by elevated expression of all four OSKM factors (Figure 2).[46] Meanwhile, low efficiency reprogramming (normally $<3\%$ cells expressing OSKM give rise to iPSCs) complicates the dissection of its molecular mechanisms. Furthermore, a study profiling the expression of 48 pluripotent genes at the single-cell level during the reprogramming process revealed an early stochastic and a late deterministic phase.[47] Intriguingly, a recent study proposed that cellular reprogramming is a deterministic process, as nearly 100% reprogramming efficiency was achieved when using Mbd3 knockout cells as the initiating somatic cells.[48]

Seesaw model for reprogramming. The fact that cell fate is controlled by a series of master transcription factors (OSKM) overwhelms demonstration of reprogramming mechanisms.[11] Most interestingly, a recent study showed that mesendodermal (GATA3, GATA6, SOX7, PAX1, GATA4, CEBPa, HNF4a, GRB2) and ectodermal specifiers (Sox1, Sox3, RCOR2, GMNN) can replace Oct4 and Sox2 to induce pluripotency, respectively.[49] Most Oct4 substitutes are not enriched in ESCs and normally function in the early stages of mesendodermal differentiation. Oct4 and its substitutes can inhibit the upregulation of a group of

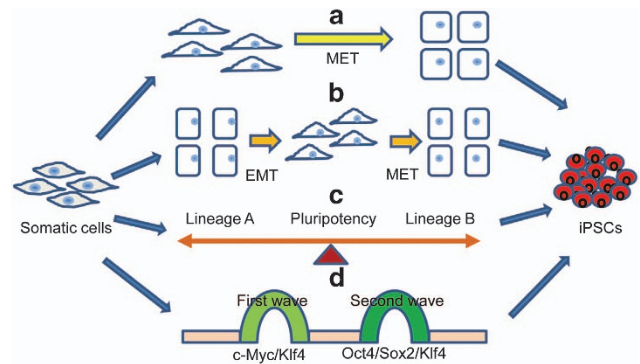

Figure 2 Roadmaps to induced pluripotency. (**a**) A MET is required for reprogramming of somatic cells to iPSCs. (**b**) An epithelial-to-mesenchymal transition (EMT) before MET is required during reprogramming. (**c**) Induced pluripotency is a balance between competing differentiation forces. (**d**) Reprogramming elicits two transcriptional waves, which are driven by c-Myc/Klf4 (first wave) and Oct4/Sox2/Klf4 (second wave)

ectodermal genes, such as the ectodermal lineage-specifier Dlx3, triggered by SKM during reprogramming. Conversely, Sox2 and its substitutes can attenuate expression of mesendodermal genes induced by OKM. The competition between mesendodermal and ectodermal specifiers promotes successful reprogramming without the two most critical reprogramming factors Oct4 and Sox2. These innovative findings have resulted in a completely new way in which to explain reprogramming, called the ‘see-saw’ model wherein cell fate is dependent on the balance between pluripotency factors and/or counteracting lineage specifiers.[49,50]

Pluripotency by modification of intracellular signaling. Small-molecule compounds were originally shown to enhance reprogramming efficiency, introducing the possibility of generating iPSCs using only these compounds.[51,52] Inhibition of both mitogen-activated protein kinase and glycogen synthase kinase-3 (GSK3) signaling pathways have been shown to promote ground pluripotent reprogramming.[53,54] Furthermore, the concept of pluripotency being a balance between competing differentiation forces also suggests that it is possible to achieve pluripotency by modifing signaling networks. Most recently, by adding seven small-molecules (CHIR, 616452, Forskolin, DZNep, Valproic acid, Tranylcypromine, TTNPB) into mouse somatic cells, Hou *et al.*[31] successfully generated completely compound-derived iPSCs. The small-molecule compounds used for reprogramming target GSK3,[55–57] transforming growth factor-beta, cAMP, S-adenosylhomocysteine hydrolase, histone deacetylase, lysine-specific demethylase 1 and retinoic acid signaling (Figure 3, Table 1). This finding not only provides a new approach to induce pluripotency, avoiding tedious genetic manipulation, but also revolutionizes our understanding of molecular mechanisms of reprogramming, raising the possibility to modulating cell fate by simply modifying intrinsic cell signaling pathways alone. In support of this hypothesis, a recent report claimed a transient low-pH stress treatment could induce pluripotency in CD45+ cells.[58] However, how small-molecule compounds and

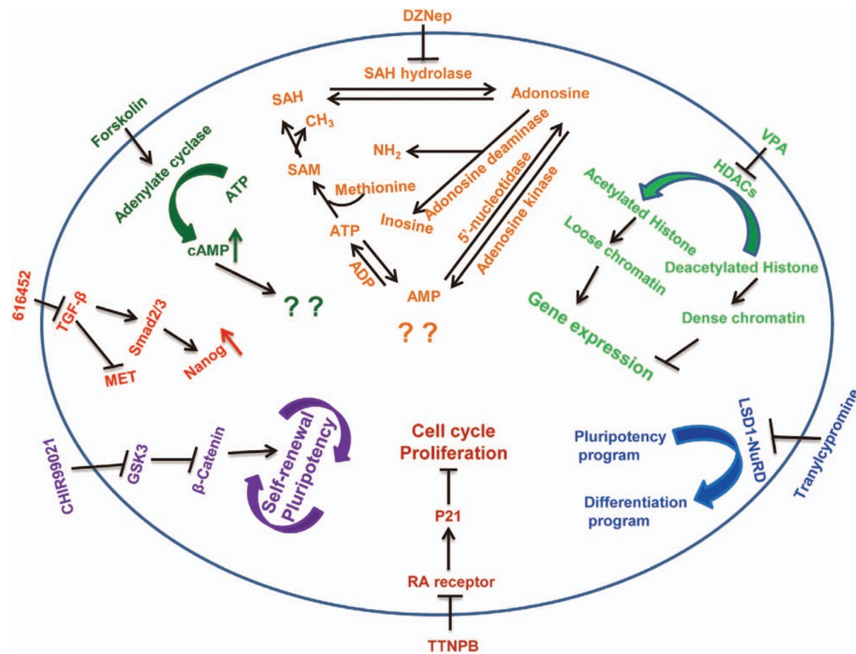

Figure 3 Small-molecule signaling pathways in reprogramming. Seven small-molecule compounds synergistically induce pluripotency. CHIR99021 blocks GSK3 signaling, promoting ESC self-renewal and pluripotency. Compound 616452 inhibits TGF-β activity, which promotes MET and Nanog expression. Forskolin activates adenylate cyclase to increase cAMP expression, and DZNep inhibits S-adenosylhomocysteine hydrolase (SAH) activity and trimethylation of lysine 27 on histone H3, but how these molecules promote reprogramming is unclear. Valproic acid (VPA) looses chromatin through histone deacetylase (HDAC) inhibition and activates gene expression, while tranylcypromine inhibits lysine-specific demethylase 1 (LSD1) activity and blocks differentiation. Finally TTNPB competitively binds retinoic acid (RA) receptors and enhances proliferation

external stimuli like low pH activate and integrate intracellular signal transduction pathways to form completely new regulatory networks is still largely unknown.

Immunogenicity of iPSC Derivatives

A major reason for the wide-spread increase in iPSC studies is their clinical application, providing potentially unlimited autologous cells for regenerative medicine. Patient-specific iPSCs can conceivably provide autologous cells that do not induce an immune response underlying rejection in human body. Unexpectedly, a previous report using a teratoma transplantation model showed that some certain iPSC-derived cells can elicit immune rejection responses.[25] However, as opposed to allografts, only some certain but not all iPSC-derived cells caused immune rejection responses, due to the differential presentation of abnormalities induced by reprogramming.[25,59] For example, Abe's group recently found that iPSC-derived cardiomyocytes, but not skin cells, can induce significant immune rejection responses.[60] Although, compared with allogeneic iPSC derivatives, rejection responses induced by syngeneic iPSC-derived cells are significantly lower, endodermal cells differentiated from syngeneic iPSCs can induce immune rejection, whereas cells derived from syngeneic ESCs do not.[61] Thus, these data support that only certain cell types differentiated from iPSCs are immunogenic (Figure 4).

As the guardian of genome, p53 has an important role in maitaining genomic stability in somatic cells and ESCs.[10] If inhibition of p53 pathway is required for successful reprogramming, this raises concerns regarding the genomic stability of iPSCs and their derivatives.[34–38] Recent studies have shown that reprogramming itself can induce both genetic and epigenetic abnormalities,[62–66] fostering additional concerns regarding the safety of iPSCs in clinical use. Two minor antigens were identified to be abnormally expressed in the teratomas derived by syngeneic iPSCs, but not ESCs, leading to immune rejection, suggesting that reprogramming-induced epigenetic abnormalities can be passed to their progeny.[67] To facilitate clinical application of iPSCs, it is important to develop new techniques that expedite clinical production of iPSCs. Furthermore, immunogenic evaluation of therapeutically valuable cells for improved patient tolerance is of utmost importance.

Conclusions and Perspectives

Long induction periods and only small initiating populations of reprogrammable cells are two major hurdles in understanding the detailed molecular mechanisms of reprogramming. New reports of lineage specifiers and small-molecule compounds that can induce pluripotency have begun to transform our comprehension of reprogramming mechanisms. However, the most critical molecular events are still unclear. In addition, considering suppression of Mbd3 has been shown to significantly increase reprogramming efficiency to almost 100%, manipulation of this gene/protein in future provides a very promising system to dissect reprogramming mechanisms. Meanwhile, with the quick development on single-cell profiling techniques and omics, our understanding of reprogramming will significantly expand. Furthermore, given the urgent clinical need, reprogramming techniques need to be optimized to

Table 1 Molecular structures and functional mechanisms of small-molecule compounds in reprogramming

Chemicals: Molecular structures/Functional mechanisms[refs]
CHIR99021: Glycogen synthase kinase 3 (GSK-3) inhibitor/blocks the activity of GSK-3β, inhibits β-Catenin degradation and enables the ESC self-renewal and pluripotency[68–70]
616452: Transforming growth factor-beta (TGF-β) inhibitor/inhibits the TGF-β signaling pathway and induces nanog expression[56]
Forskolin: Adenylate cyclase agonist/activates cAMP/PKA signal pathway, which acts as a negative regulator of the hedgehog signaling pathway[71,72]
DZNep: S-adenosylhomocysteine hydrolase (SAH) inhibitor/also a Lysine methyltransferase inhibitor, inhibits trimethylation of lysine 27 on histone H3 and regulates cell-cycle arrest and apoptosis[73,74]
Valproic acid: Histone deacetylase inhibitor (HDAC)/looses chromatin and activates gene expression[75,76]
Tranylcypromine: Lysine-specific demethylase 1 (LSD1) inhibitor/inhibits LSD1 activity and makes the ESC-specific enhancers fail to undergo the histone demethylation events associated with differentiation[31,70,77]
TTNPB: Retinoic acid receptor ligand/binds to RA receptor competingly with RA and inhibits differentiation[39,78]

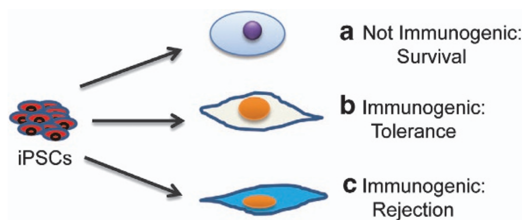

Figure 4 Different fates of cells derived from iPSCs after transplantation. (**a**) Most cells derived from iPSCs are not immunogenic and can survive after transplantation. (**b**) Some cells differentiated from iPSCs with minor immunogenicity can be tolerated by the host. (**c**) Some abnormal iPSC progenies with immunogenicity will be rejected

minimize potential reprogramming-induced abnormalities in iPSCs.

Conflict of Interest

The authors declare no conflict of interest.

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