Tinkering with Transcription Factors Uncovers Plasticity of Somatic Cells

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Abstract

The advent of induced pluripotent stem cells (iPSCs) has brought the goal of using patient-derived cells for tissue repair closer to reality. However, the mechanisms involved in reprogramming to a pluripotent state are still not clear. It is understood that reprogramming to pluripotency involves epigenetic remodeling and the reactivation of “core” pluripotency factors. However, little is known about the mechanisms involved in overcoming senescence while avoiding oncogenesis, the maintenance of self-renewal, and the regulation of the balance between pluripotency and differentiation. Here, we review recent advances in reprogramming technology and what is currently known about the mechanism of reprogramming to pluripotency. Work with patient-derived iPSCs is already providing new insights into the cellular and molecular mechanisms involved in human disease. Further advances in reprogramming technology should result in efficient methods to reprogram patient-derived cells into iPSCs for use in regenerative medicine.

Keywords: reprogramming, induced pluripotent stem cells (iPSCs), transcriptional regulation, senescence, epigenetic mechanisms

Foreword

When writing this review, inspired by the memory of Hidesaburo Hanafusa, we cannot help but come back time and again to the gigantic scope of his contributions and foresight. His work on RNA tumor viruses was instrumental in showing how these viruses have captured many previously unknown regulatory genes. In classic experiments, he discovered many of the cellular and molecular mechanisms by which these mysterious genes alter the morphology and growth properties of cultured cells and cause tumors in animals. His highly original work on recovered viruses showed that recombination of the cellular *src* proto-oncogene with transformation-defective isolates of Rous sarcoma virus caused the oncogenic activation of *c-src* in an animal host. This was a very elegant demonstration of the cellular origin of oncogenes, which was recognized with a Lasker Award. With his colleagues, HH, as we affectionately called him, made one seminal discovery after another, including the identification of many new oncogenes, tyrosine protein kinases, and the elucidation of their mechanisms

of action. His work on the SH2/SH3-containing *v-crk* oncogene provided the first glimpse into a new family of adapter proteins, which are essential for signal transduction. It is fair to say that H. Hanafusa’s legacy extends well beyond the field of oncogenes and signal transduction and that some of the topics discussed below owe much to his profound insight. It is fitting to point out that the advent of induced pluripotent stem cells (iPSCs), an unexpected discovery with broad implications for regenerative medicine, was made possible using retroviral vectors that encode familiar oncogenes.

Introduction

Normal development entails the differentiation of cells through a series of developmental stages, culminating with the myriad of specialized cell types that make up an organism. While this process is normally unidirectional and irreversible, nuclear transplantation experiments carried out more than a half century ago demonstrated that cell fate could be manipulated experimentally. The first indication that cellular differentiation can be reversed, was the generation of a

healthy tadpole following the transplantation of blastula cell nuclei from a frog into enucleated eggs of the same species.¹ This demonstrated that unknown factors present in the cytoplasm of the recipient egg were capable of providing reprogramming instructions to the transplanted nucleus. The subsequent discovery that the transfer of a nucleus from a fully differentiated frog intestinal cell into unfertilized enucleated eggs could lead to the development of a normal frog,² further demonstrated that cell differentiation was not as unidirectional or irreversible as originally thought. Instead, differentiation must be the result of changes in nuclear gene expression as opposed to the permanent inactivation or loss of genes.³

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This suggested the existence of cellular factors that were capable of governing cell fate. Cell fusion experiments supported this idea and further demonstrated that differentiated cells contain cytoplasmic regulatory molecules that can alter gene expression patterns in the nuclei.³ In 1987, it was shown that forced expression of the muscle-specific gene MyoD was sufficient to convert mouse fibroblasts into myoblasts.⁴ These experiments were the first to demonstrate that transcription factors were capable of acting as master regulators of cell fate by altering gene expression patterns. The realization that cells could be forced to change lineages through the exogenous expression of cell type-specific master regulators foretold the advent of reprogramming technology.

Induced Pluripotent Stem Cells

In 2006, Yamanaka and colleagues at Kyoto University showed that adult somatic cells could be dedifferentiated to a primordial embryonic pluripotent state by ectopic expression of 4 transcription factors, namely Oct3/4, Klf4, Sox2, and c-Myc. The retroviral delivery of these factors was sufficient to convert both mouse and human fibroblasts into induced pluripotent stem cells (iPSCs).^{5,6} These iPSCs exhibited similar colony morphology to embryonic stem cells (ESCs) and expressed ESC-specific markers. The global gene expression profiles of iPSCs clustered with ESCs and were distinct from the expression profiles of the parental fibroblasts. Pluripotency was assessed *in vitro* by the ability of iPSCs to differentiate into cell lineages of all 3 germ layers. Their *in vivo* pluripotency was determined by the formation of teratomas containing derivatives of all 3 germ layers in nude mice. The pluripotent nature of mouse-derived iPSCs was further confirmed by the formation of germline-competent chimeras⁷ and by the generation of viable, fertile progeny by tetraploid complementation.⁸ For obvious reasons, the pluripotency of human iPSCs cannot be assessed based on the ability to generate chimeras. Instead, the

assessment of human iPSC pluripotency relies on *in vitro* differentiation assays and their ability to form teratomas *in vivo*. However, it is important to note that teratoma formation is not indicative of complete reprogramming as some mouse iPSCs are capable of forming teratomas but are unable to produce germline chimeras.⁹ In the future, it will be important to develop assays for the selection of fully reprogrammed human iPSCs.

The generation of iPSCs is now a very active and exciting area of research as these cells have substantial implications for both basic research and regenerative medicine. The ability of patient-derived iPSCs to self-renew extensively and to differentiate into virtually any cell type makes them suitable for modeling somatic and inherited diseases. Diabetes, heart disease, retinal disease, Parkinson disease, Alzheimer disease, and inherited disorders are just a few examples of the types of ailments that could benefit from iPSC technologies.⁹ While iPSCs are unique reagents for use in disease modeling, drug discovery, and assessment of drug toxicities,¹⁰ the most promising application of patient-derived iPSCs is their potential use for autologous tissue repair.

Are ESCs and iPSCs Indistinguishable?

When defining fully reprogrammed iPSCs, it is common to compare these cells to ESCs. Reprogrammed iPSCs are characterized based upon their global gene expression profile, chromatin configuration, the methylation status of pluripotency-associated promoters, reactivation of the silent X chromosome, and the expression of pluripotency markers.¹¹ While iPSCs and ESCs appear to be nearly identical in many of these properties, some studies have suggested that these 2 cell types may not be indistinguishable. Analysis of the global gene expression profile of early- and late-passage human iPSCs determined that, although similar, iPSCs retain a unique gene expression signature. While the continued culturing of early-passage human iPSCs partially corrects the signature,

late-passage human iPSCs, even those generated under different protocols and by different laboratories, are more similar to each other than to human ESCs. The unique gene expression profile of iPSCs extends to the differential expression of over 100 microRNAs (miRNAs).¹² However, reanalysis of the gene expression microarray data generated from 7 different laboratories found that there is a significant correlation between the gene expression signatures of iPSCs and their laboratory of origin. These laboratory-specific signatures may be due to differences between *in vitro* microenvironment conditions.¹³ Based upon these findings, it was suggested that the small variations in gene expression between human iPSCs and ESCs reported by Chin *et al.*¹² were either stochastic or the result of laboratory-specific differences. A second study confirmed the finding that iPSC and ESC expression data cluster together on the basis of the laboratory of origin, as opposed to distinct iPSC and ESC clusters. These results suggest that the small variations in chromatin structure and gene expression profiles between iPSCs and ESCs do not represent a consistent signature distinguishing iPSCs from ESCs.¹⁴ On the other hand, a recent report brings a note of caution on the use of iPSCs for regenerative purposes. Laurent *et al.*¹⁵ showed that the iPSC reprogramming process and extended time in culture are associated with genomic aberrations, which may contribute to oncogenesis. In addition, the use of a polycistronic lentiviral vector for delivery of the reprogramming factors was associated with chromosomal instability and trisomy of chromosome 8.¹⁶ These findings underscore the close scrutiny iPSCs must receive before they can be used in regenerative medicine applications.

Somatic Cells Are Quite Amenable to Reprogramming

iPSC generation from fibroblasts is a very inefficient process, with only approximately 0.02% of the cells showing successful reprogramming.⁶ This prompted investigation into the mechanisms

involved in reprogramming and a search for alternative cell types that may be reprogrammed at higher efficiencies. In general, it was found that when a somatic cell already expresses endogenous reprogramming factors, iPSC generation is easier and may require fewer ectopic factors. For instance, adult mouse neuronal stem cells (NSCs), which express high levels of Sox2, can be reprogrammed by Oct4, and either c-Myc or Klf4 alone, albeit at lower efficiency (0.1%) compared to the 4-factor reprogramming of these same cells (3.6%).¹⁷ Human NSCs isolated from human fetal brain tissue were also reprogrammed using 2 factors (Oct4 and Klf4) and 1 factor (Oct4), although the efficiencies of reprogramming were low.¹⁸ A more readily accessible alternative to NSCs are human melanocytes, which are of the same neuroectoderm origin as NSCs and also express high levels of endogenous Sox2. Melanocytes isolated from the skin can be reprogrammed by exogenous expression of Oct4, c-Myc, and Klf4.¹⁹ The plasticity and regenerative capacity of the human endometrium, which already expresses Oct4, Sox2, Myc, and Nanog, make these cells particularly permissive to reprogramming. Upon retroviral transduction of c-Myc, Klf4, Oct4, and Sox2, these cells were reprogrammed with 10-fold higher efficiency and improved kinetics over human neonatal fibroblasts.²⁰

In general, adult somatic cells appear to be more difficult to reprogram than younger cells or fetal cells.²¹ In particular, fetal and neonatal cells have proven to be very amenable to reprogramming. Human cord blood-derived endothelial cells have been reprogrammed to pluripotency with high efficiency,²² and human amniotic fluid-derived cells exhibit 100-fold higher efficiency of reprogramming to iPSCs than human fibroblasts.²³ Human umbilical vein endothelial cells (HUVECs), which express high levels of endogenous Klf4, can be reprogrammed by Oct4 and Sox2 alone with efficiencies comparable to those seen with 4-factor reprogramming of human adult fibroblasts.²⁴

While fetal and neonatal cells are very desirable sources for reprogramming, these types of tissues are not always available. Human peripheral blood cells, which can be obtained through a minimally invasive procedure, have been successfully reprogrammed to pluripotency. Terminally differentiated circulating T cells appear to be particularly receptive to reprogramming.²⁵⁻²⁷ Other potential sources of accessible cells for reprogramming include dermal papilla cells and human adipose stem cells (hASCs). Dermal papilla cells, which express high endogenous levels of Sox2 and c-Myc, are readily reprogrammed by Oct4 and Klf4 alone.²⁸ hASCs have been reprogrammed with improved efficiencies compared to fibroblast reprogramming, which may be due to their multipotent nature and their high endogenous levels of Klf4, Esrrb, and c-Myc.²⁹ The oral cavity offers another source of readily obtainable somatic cells that are particularly receptive to reprogramming. Dental pulp cells isolated from human wisdom teeth have a high reprogramming efficiency, possibly due to endogenous expression of Klf4.³⁰ Mouse gingival fibroblasts exhibit 7-fold higher reprogramming efficiencies compared to mouse tail-tip fibroblasts.³¹ As gingival fibroblasts are routinely excised during dental treatments, these cells could serve as a good source for efficient reprogramming. Mesenchymal-like stem and progenitor cells isolated from human dental tissue are also reprogrammed with higher efficiency than human fibroblasts.³² The relative ease by which multiple cell types can be reprogrammed to a pluripotent state suggests that pluripotency may be a default epigenetic state.³³

Pluripotency and Epigenetic Memory

Reprogramming to pluripotency is only the first step in generating a desired cell type. In most cases, iPSCs must be differentiated into the cell types of interest before they can be used for disease modeling, drug discovery, or

for regenerative medicine purposes. A potential hurdle in the directed differentiation of iPSCs is the discovery that these cells have different propensities to differentiate, depending upon their parental cell type of origin. It is believed that this is a result of a failure to completely reprogram the epigenetic signature within somatic cells.³⁴ iPSCs express distinct transcriptional profiles and maintain residual DNA methylation patterns, which are characteristic of their somatic tissue of origin. This residual epigenetic memory contributes to variation in differentiation capacity, such that iPSCs appear to be biased to differentiate along lineages related to the cell type of origin.^{35,36} Epigenetic memory has also been found to influence gene expression following somatic cell nuclear transfer experiments, further demonstrating the stability of epigenetic modifications.³⁷ However, epigenetic memory can be erased following prolonged passaging of iPSCs or by treatment with chromatin-modifying drugs.^{35,36} This suggests that demethylation occurs as a passive process, whereby somatic epigenetic marks are lost during replication.³⁵ While it appears that epigenetic memory may hinder reprogramming to complete pluripotency, it may also facilitate the differentiation of iPSCs along particular lineages based upon the somatic cell type of origin.³⁸ The existence of this epigenetic memory may weigh in the decision of which cells to use for reprogramming, according to the intended use of the iPSCs.

Methods for Generating iPSCs

The first iPSCs were generated using retroviral vectors encoding each of the 4 reprogramming factors.^{5,6} The genomic integration of the provirus allows for prolonged transgene expression, which is a requirement for reprogramming. Epigenetic modifications during reprogramming contribute to silencing of the retroviral promoter in iPSCs,³⁹ and this is essential for subsequent differentiation of iPSCs.⁴⁰ However, reprogramming to iPSCs requires high titer virus and multiple integrations of the reprogramming genes into the host chromosomes.

The presence of multiple genomic integrations, approximately 20 per infected cell,⁵ is problematic as it may activate proto-oncogenes or disrupt tumor suppressor genes, increasing the risk of oncogenic events. The integration of the reprogramming factors also entails the risk of reactivating the oncogenes c-Myc and Klf4, leading to tumorigenesis.⁷ Another potential disadvantage of retroviral vectors is that proviral integration requires cell division, making more differentiated cells or slowly dividing cells more difficult to infect and reprogram. The ability of lentiviruses to infect both proliferating and nonproliferating cells⁴¹ led to the development of a variety of lentiviral vectors for use in reprogramming. However, there are reports that lentiviral vectors may be incompletely silenced,⁴² which could interfere with subsequent differentiation of the iPSCs. To reduce the number of integrated proviruses that need to be silenced, and to decrease the possibility of insertional mutagenesis, polycistronic vectors that encode all of the reprogramming factors in a single cassette have been developed.¹⁶ Interestingly, even when different multiplicities of infection were used for reprogramming, most of the recovered iPSCs contained single integration sites.⁴³ This suggests that in this lentiviral system, the relative stoichiometries of the reprogramming factors, which is important for the efficient generation of iPSCs,⁴⁴ were optimal when a single copy of the provirus was integrated. Another feature of this vector was the presence of LoxP sites flanking the reprogramming factors, enabling Cre-mediated excision of the vector from iPSC clones.¹⁶ Excision of the ectopic genes would facilitate subsequent differentiation of iPSCs, even when there is incomplete silencing of the lentiviral LTR, as well as reduce the risk of reactivating oncogenic transgenes.

Nonviral Methods of Reprogramming

While viral vectors are convenient reagents for generating iPSCs, the genetic modification of the reprogrammed cells is problematic for use in regenerative

medicine. This has prompted a vigorous search for alternative methods that avoid genetic alteration of the reprogramming cells. The LoxP/Cre recombinase system has also been employed for nonviral generation of iPSCs. In this case, iPSCs were generated by transfection of a single plasmid containing a 2A peptide-linked reprogramming cassette flanked by LoxP sites. The transient transfection of Cre into the resulting iPSCs effectively eliminated the exogenous factors.⁴⁵ Alternative excision strategies include the piggyBac transposon system for delivery of the reprogramming factors followed by transgene removal by transposase expression.⁴⁶ iPSCs were also generated following the repeated transfection of expression plasmids encoding the 4 reprogramming factors under the control of the constitutively active CAG promoter.⁴⁷ The single transfection of an oriP/Epstein-Barr nuclear antigen-1-based (EBNA) episomal vector was also successful in generating iPSCs. EBNA vectors are lost in the absence of drug selection, generating vector- and transgene-free human iPSCs.⁴⁸ iPSCs have also been obtained by repeated transfection of recombinant proteins fused to a cell-penetrating peptide.^{49,50}

Although these nonviral methods resulted in iPSC generation, their low efficiency of reprogramming greatly limits their utility. Most recently, synthetic modified mRNAs encoding the 4 reprogramming factors were used to generate iPSCs with high efficiency and improved kinetics.⁵¹ The use of modified mRNAs circumvents issues of insertional mutagenesis, and the transient nature of protein expression following mRNA transfection minimizes the risks of oncogene reactivation.⁵¹ Although generating the modified mRNAs for reprogramming is technically difficult, this novel technology has great potential for regenerative medicine.

Chemical Reprogramming

Chemical screens have identified compounds that can increase the efficiency of iPSC reprogramming or replace one

or more of the reprogramming factors. As we discuss below, epigenetic modifications are a requirement for complete reprogramming, and many of the chemicals used for reprogramming are epigenetic modifiers. Histone deacetylase inhibitors and DNA methyltransferase inhibitors have both been found to increase the efficiency of reprogramming.⁵² For example, the chemical inhibition of G9a methyltransferase improves the efficiency of reprogramming fetal mouse neuronal stem cells and allows for their efficient reprogramming by 2 factors, Oct4 and Klf4.⁵³ While epigenetic modifiers can improve the efficiency of reprogramming and/or replace some of the reprogramming factors, the possibility of off-target effects due to their lack of specificity of action will require careful examination.⁵⁴ Genome-wide epigenetic modifications raise the possibility of inappropriately modifying the expression of genes leading to diseases such as cancer, neurodegenerative disorders, and autoimmune diseases, all of which have been associated with aberrant epigenetic regulation.⁵⁵ To avoid such genome-wide modifications, chemicals that target specific signaling pathways governing stem cell fate are also being investigated. Chemical screens have identified numerous molecules that alter signaling pathways involved in reprogramming. These include activators of the Wnt/ β -catenin pathway⁵² and inhibitors of the MAPK/ERK pathway^{52,56} that increase the efficiency of reprogramming. The latter can also be used as a potential selection mechanism for reprogrammed iPSCs.⁵³ Recently, a small molecule cocktail that included a MAPK/ERK inhibitor, a TGF β receptor inhibitor, a histone deacetylase inhibitor, and an activator of 3'-phosphoinositide-dependent kinase-1 (PDK1), in combination with ectopic expression of Oct4, was sufficient for reprogramming neonatal human epidermal keratinocytes to iPSCs.⁵⁷ The successful reprogramming to iPSCs reported in these studies suggests that chemical reprogramming will continue to be a very active area of research.

Mechanisms of Reprogramming to Pluripotency

Clinical application of iPSC reprogramming technology requires that the efficiency of reprogramming be substantially improved and that the frequency of incompletely reprogrammed cells be minimized. Somatic cell nuclear transfer (SCNT) and cell fusion are still more efficient methods of reprogramming to pluripotency than iPSC technologies. In addition, SCNT does not produce partially reprogrammed intermediates, as seen with iPSC reprogramming.³³ Thus, there is still considerable room for improvement in the methods used to generate fully reprogrammed, homogeneous iPSCs. This will require a better understanding of the molecular mechanisms involved in this process. Below, we summarize what is known about the molecular mechanisms leading to reprogramming.

Reprogramming to pluripotency involves the establishment of a unique transcriptional network to regulate the balance between the pluripotent state and differentiation. The molecular mechanisms by which exogenous expression of reprogramming factors induces the activation of this transcriptional network remain largely unknown. However, it is understood that the reprogramming process involves the establishment of a permissive epigenetic state, whereby the endogenous pluripotency-associated genes Nanog, Sox2, and Oct4 are reactivated.⁵⁸

Reprogramming Must Overcome a Senescence Block

Pluripotent stem cells are characterized by 2 distinct properties: self-renewal and the ability to differentiate into cell lineages of all 3 germ layers. The ability to undergo unlimited self-renewal requires that iPSCs overcome the intrinsic property of somatic cells to undergo cellular senescence. This involves bypassing senescence induced by the progressive shortening of telomeres following cell

division, as well as senescence induced in response to the activation of the cyclin-dependent kinase inhibitors, p21 and p16^{INK4a}.⁵⁹

The ability of iPSCs to avoid terminal growth arrest due to progressive telomere shortening is mediated by increasing the expression of the catalytic subunit of telomerase, TERT. Telomerase is responsible for maintaining telomere length by catalyzing telomeric elongation following the incomplete replication of telomeres during cell division.⁶⁰ Both iPSCs and ESCs express elevated levels of TERT compared to differentiated somatic cells.^{6,61} The increase in telomerase activity results in increased telomere length typical of both human and mouse ESCs and iPSCs.^{60,62-64} It has been suggested that increased TERT expression in iPSCs is regulated by c-Myc, which has been found to directly upregulate the transcription of TERT.⁵⁸ However, the generation of iPSCs with similar levels of TERT expression and telomere elongation following reprogramming in the absence of c-Myc indicates that additional mechanisms are involved in the upregulation of TERT expression.⁶² One such mechanism may be the loss of repressive histone methylation marks, which interfere with the ability of telomerase to interact with the telomeres, preventing elongation. The telomeres of ESCs are characterized by a decrease in these repressive epigenetic modifications.⁶⁰ Reprogramming during iPSC generation also involves similar derepressive epigenetic modifications at the telomeres. Thus, it appears that the high expression of TERT and the subsequently high telomerase activity within iPSCs contribute to their ability to undergo prolonged self-renewal.

Terminal growth arrest can also be induced in response to stress or aberrant cell signaling.⁶⁵ For proper immortalization to occur, iPSCs must also overcome this “induced senescence.” It is believed that induced senescence is the result of the induction of the cyclin-dependent kinase inhibitors, p21 and p16^{INK4a}.

These inhibitors are products of the *INK4a/ARF* locus and mediate cell cycle arrest via the activation of the p53 and Rb pathways.⁶⁵ p21 is a downstream target of p53, and these 2 molecules are upregulated in senescent cells. The mechanism governing the upregulation of p16^{INK4a} in senescent cells is less understood but may involve signaling through the MAPK cascade.⁵⁹ In addition, Polycomb group proteins have also been found to regulate the induction of p16^{INK4a},⁵⁹ which suggests that the Polycomb group proteins may play additional roles in reprogramming besides the silencing of developmental genes.¹¹ To avoid senescence induced by the expression of p21 and p16^{INK4a}, the *INK4a/ARF* locus of iPSCs and ESCs is effectively silenced. This silencing of the *INK4a/ARF* locus is mediated through epigenetic modifications during the reprogramming process that establish a bivalent, silent chromatin configuration.⁶⁶ Repression of *INK4a/ARF* may serve to avoid senescence and increase cell proliferation, which may lead to enhanced reprogramming efficiency and kinetics. The experimental knockdown of the *INK4a/ARF* locus by short hairpin RNA improved the efficiency of reprogramming in both mouse and human fibroblasts,⁶⁶ further suggesting that reprogramming requires overcoming p21- and p16^{INK4a}-induced senescence.

Role of Reprogramming Factors in iPSC Generation

Currently, Oct4 is the only factor whose requirement for reprogramming human somatic cells is absolute. Alternative family members of Sox2, Klf4, and c-Myc have been used to generate iPSCs.^{67,68} iPSCs can also be generated in the absence of c-Myc, decreasing the tumorigenicity of iPSCs by eliminating the risk of reactivating this oncogene.⁷ However, the efficiency of reprogramming in the absence of c-Myc is substantially reduced.⁶⁸ Nanog and Lin can replace c-Myc and Klf4 for reprogramming, suggesting that c-Myc and Klf4

Figure 1. Putative mechanism for the induction of pluripotency in somatic cells. Downregulation of p21 by c-Myc promotes the oncogenic activity of Klf4, which serves to inhibit p53-dependent apoptosis induced by c-Myc overexpression. The coordinated actions of c-Myc and Klf4 suppress apoptosis and senescence and promote cell cycle progression. Through interactions with epigenetic modifiers, c-Myc and Klf4 contribute to chromatin decondensation and promoter demethylation. These epigenetic changes may represent the rate-limiting step in the induction of pluripotency. This permissive chromatin conformation allows Oct4 and Sox2 to bind to the promoters of target genes. Genes associated with pluripotency are expressed, including the endogenous “core” pluripotency factors: Oct4, Sox2, and Nanog. These 3 factors form an autoregulatory feed-forward loop that regulates the pluripotent transcriptional network and suppresses genes associated with differentiation, leading to the induction of pluripotency and the formation of iPSCs.

may not be essential for reprogramming but instead may function to enhance this process.⁶⁹

The conversion of somatic cells to pluripotency is a gradual process that requires expression of the exogenous reprogramming factors for 10 to 16 days.^{40,61} Through the coordinated actions of Oct4, Sox2, Klf4, and c-Myc, changes in gene transcription and epigenetic status cumulate in the dedifferentiation of somatic cells. While the roles of Oct4 and Sox2 in the maintenance of ESC pluripotency have been well established,⁷⁰ the function of c-Myc and Klf4 in pluripotency is less well understood.

The *c-myc* proto-oncogene plays a critical role in regulating cell growth, cell cycle progression, and apoptosis. A common feature of most human cancers is the deregulation of Myc.⁷¹ Both the activation of target genes and the negative regulation of growth-arrest genes, particularly the repression of p21,

contribute to the oncogenic nature of c-Myc.⁷² On the other hand, Myc overexpression activates p53 and triggers apoptosis.⁷³ The bifunctional transcription factor Klf4 can act as a tumor suppressor or as an oncogene in a cell context-specific manner.⁷⁴ Through interaction with β -catenin, Klf4 serves to inhibit the transcription of Wnt/ β -catenin target genes, leading to the inhibition of tumorigenesis in gastrointestinal cancers.⁷⁵ In addition, Klf4 acts as a tumor suppressor through the induction of the cell cycle inhibitor, p21.⁷⁶ In contrast, Klf4 is overexpressed in breast cancer and squamous cell carcinoma, where it acts as an oncogene by blocking apoptosis through the inhibition of p53.⁷⁶ The switch between the tumor suppressor and oncogenic functions of Klf4 appears to be dependent upon the functionality of p21. In normal cells, Klf4 acts as a p21-dependent suppressor of proliferation. However, when p21 is inactivated, as often occurs in cancers,

the antiproliferative effect of Klf4 is neutralized. Under these conditions, Klf4 instead acts as an oncogene by inhibiting p53-dependent apoptosis, leading to tumorigenesis.⁷⁶

It has been suggested that it is precisely these oncogenic properties of c-Myc and Klf4, which when properly balanced and combined with Oct3/4 and Sox2, lead to reprogramming.⁵ By suppressing p21, c-Myc can tip the balance towards the oncogenic properties of Klf4. In the absence of p21, Klf4 may serve to block p53-dependent apoptosis induced by the overexpression of c-Myc (Fig. 1). The hypothesis that dual inhibition of p21 and p53 contributes to reprogramming is supported by recent work, demonstrating that the p53-p21 pathway suppresses iPSC generation.⁷⁷ Underscoring the importance of the cell cycle as an important parameter in reprogramming is the finding that reprogramming is a stochastic process, which is accelerated by increasing the rate of cell division. The

inhibition of the p53/p21 pathway is one way to increase cell proliferation.⁷⁸ This would also suggest a potential mechanism through which the stimulation of Wnt/ β -catenin signaling leads to increased reprogramming.⁵² It is possible that this activation of Wnt/ β -catenin signaling may overcome the inhibition of β -catenin by Klf4, promoting proliferation and facilitating reprogramming.

Avoiding senescence and apoptosis itself is not sufficient to induce pluripotency and could lead to tumorigenesis. Together, Oct3/4 and Sox2 serve to direct reprogramming cells away from the tumor phenotype and towards the pluripotent state.⁷⁹ Oct4 is a transcription factor specifically expressed in pluripotent ESCs. By regulating the expression of genes involved in self-renewal and lineage specification, Oct4 specifies the self-renewal state of ESCs.⁶⁷ Sox2 is also essential for the maintenance of pluripotency and is critical for proper differentiation.⁷⁹ The heterodimerization of Sox2 and Oct4 allows for the regulation of target genes required for specification of the pluripotent state.⁶⁷

The actions of c-Myc and Klf4 most likely play a role in immortalization through the inhibition of p53-dependent apoptosis and senescence. Additionally, the ability of c-Myc and Klf4 to interact with coactivators or corepressors of transcription involved in epigenetic modifications may serve to increase the accessibility of chromatin (Fig. 1).^{6,80,81} In support of this idea, it was found that sites of c-Myc occupancy are highly enriched for chromatin modifications, including the activating H3K4me3 mark.⁸² iPSCs and ESCs share a similar epigenetic status, with an abundance of histone modifications associated with transcriptional activation.⁶⁷ ESCs and iPSCs also share bivalent chromatin modifications such that there are both activating and repressive methyl group additions to genes important for lineage specification.⁷⁰ It is suggested that this bivalent modification is required to maintain the balance between stem cell self-renewal and differentiation.⁵⁸ Bivalent modifications

may also serve to poise ESCs and iPSCs for differentiation.⁶⁷

In addition to histone modifications, a distinct DNA methylation pattern is shared between ESCs and iPSCs. The establishment and maintenance of pluripotency require the demethylation of pluripotency-associated genes.¹¹ The slow kinetics of the reprogramming process suggests that this demethylation is a passive process, whereby methylation is lost during DNA synthesis.⁵⁸ This suggests a potential mechanism that may explain the observations that increased cell proliferation aids in reprogramming.⁷⁸ It is possible that increasing cell proliferation leads to the formation of favorable epigenetic modifications that serve to enhance reprogramming efficiency. Together, the relaxed state of the chromatin and the demethylation of pluripotency-associated genes may allow Oct3/4 and Sox2 to access the promoters of target genes and regulate their expression.

An important step in reprogramming is the activation of the endogenous “core” pluripotency factors: Oct4, Sox2, and Nanog. Together, the interaction between these 3 factors and other protein binding partners regulates the pluripotent transcriptional network.⁸² The rate-limiting step in reprogramming may be related to the establishment of a permissive epigenetic status, which allows reactivation of these core pluripotency factors (Fig. 1).³³ The activation of the core factors leads to the formation of an autoregulatory loop, whereby Oct4, Sox2, and Nanog bind to their own and each others’ promoters in order to maintain and enhance their own gene expression (Fig. 1).¹¹

For the establishment of true pluripotency, it is important to repress genes involved in differentiation and lineage specification.⁵⁸ The transcriptional start sites of developmental regulators are also occupied by the pluripotency factors Oct4, Sox2, and Nanog, which in this case lead to the repression of gene transcription. The difference between the activation of transcription and its repression by the same factors may depend on

the combinations of factors that are involved. Transcriptionally active target sites are occupied by multiple factors, while the binding of individual factors seems to repress gene transcription.⁸² It is possible that the binding of single transcription factors may serve to recruit the Polycomb group proteins, which act as epigenetic regulators, leading to gene silencing through H3K27 methylation and the condensation of chromatin. These complexes are known to associate at the transcriptionally silent promoters of ESCs, although the mechanism of their recruitment is unknown.¹¹

Role of miRNAs in Reprogramming

In addition to the actions of transcription factors, regulatory miRNAs also play a fundamental role in normal stem cell self-renewal and cellular differentiation. The importance of miRNAs in stem cell biology is highlighted by the slow proliferation rates and defective differentiation of ESCs lacking mature miRNAs.⁸³ It is now understood that the pluripotency factors Oct4, Sox2, Nanog, and Tcf3 bind to and regulate the expression of miRNAs. These factors both promote the expression of ESC-specific miRNAs as well as silence the expression of miRNAs involved in differentiation.⁸⁴ It is likely that miRNAs play a crucial role in maintaining ESC identity through the fine-tuning of the expression of transcripts induced by the pluripotency factors.⁸³ The role of miRNAs in reprogramming is not completely understood, but miRNAs are likely to play an important role in the establishment of pluripotency in somatic cells. The transfection of ESC-specific, cell cycle-regulating miRNAs improved the efficiency of reprogramming mouse embryonic fibroblasts by Oct4, Sox2, and Klf4.⁸⁵ In addition, the finding that Lin28 in combination with Oct3/4, Sox2, and Nanog is sufficient to generate iPSCs⁶⁹ further suggests that miRNAs could play an important role in reprogramming. Lin28 is known to prevent the processing of the let-7 family of

miRNAs, which are expressed predominantly in somatic cells and thus may play a role in the differentiation of ESCs.⁸³ miRNAs may also play a role in mediating the actions of the p53 pathway in hindering reprogramming efficiency. p53 enhances the processing of miRNA-145, which can act to silence c-Myc and augment the actions of p21 to block cell cycle progression. Interfering with particular miRNAs involved in the p53 pathway may serve as a safer alternative to enhance reprogramming efficiency, as the direct inhibition of p53 is associated with increased DNA damage.⁸⁶ Further investigations into the dynamic roles of miRNAs in the mechanism of reprogramming will determine whether they can be used to improve reprogramming protocols.

Direct Conversion of Cell Types across Lineages

Pluripotent cells are extremely oncogenic, and even a small number of transplanted pluripotent cells are capable of giving rise to tumors.⁹ Directly converting one somatic cell type to another would help minimize the risks associated with the use of pluripotent cells in regenerative medicine. As in the case of iPSC generation, lineage conversion has been achieved through the forced expression of specific transcription factors. The instrumental role of transcription factors in governing cell fate was demonstrated when ectopic expression of MyoD converted mouse fibroblasts into myoblasts.⁴ Since then, many genes that play essential roles in the specification of cell lineages, and whose ectopic expression can prompt lineage conversion, have been identified. For instance, the introduction of the myeloid-specific factors, PU.1 and C/EBP- α/β , is sufficient to convert mouse fibroblasts and B cells into macrophage-like cells.^{87,88} Mouse fibroblasts have been reprogrammed to functional neurons through the forced expression of the neural lineage-specific transcription factors Ascl1, Brn2, and Myt1l.⁸⁹ A combination of 3 development

factors, Gata4, Mef2c, and Tbx5, converted both mouse cardiac and dermal fibroblasts into cardiomyocyte-like cells capable of engrafting *in vivo*.⁹⁰ The ectopic expression of Oct4 was able to induce the conversion of human fibroblasts into CD45⁺ hematopoietic progenitors capable of differentiating into several hematopoietic lineages.⁹¹ Adult pancreatic exocrine cells were reprogrammed into insulin secreting β cells *in vivo* through the ectopic expression of 3 transcription factors, Ngn3, Pdx1, and Mafa, which are important in the embryonic development of pancreas and β cells.⁹² The success of this *in vivo* transdifferentiation suggests that, in the future, the targeted delivery of reprogramming factors to specific organs may mediate lineage conversion for applications in regenerative medicine. Targeted *in vivo* reprogramming in humans is far off but should be a long-term goal of regenerative medicine.

There are still some caveats to the use of direct reprogramming for clinical applications. In some cases, stable transdifferentiation requires continued expression of the ectopic factors,^{87,88} and direct conversion usually results in incomplete reprogramming. For instance, although exogenous MyoD expression induced the activation of muscle-specific genes in multiple cell types, endogenous MyoD and additional muscle-specific genes were not induced.⁹³ Another obstacle to the use of direct conversion in regenerative medicine relates to the inability of differentiated cells to proliferate. This failure to proliferate makes expansion of the reprogrammed cells difficult. Until this hurdle is overcome or high efficiencies of conversion are achieved, the therapeutic potential of these cells is limited.⁹⁴ One way to solve this problem would be to develop reprogramming strategies for conversion of adult differentiated cells into adult stem cells. Like embryonic pluripotent cells, adult stem cells are self-renewing. They are also multipotent, and once engrafted, they can potentially become a life-long source of cells for regenerative purposes, as is the case of hematopoietic stem cells used in

bone marrow transplantation. The potential benefits of bypassing the iPSC state and directly generating long-term self-renewing, nontumorigenic adult stem cells for transplantation are likely to stimulate much effort to generate these types of adult stem cells in the future.

Disease Modeling Using iPSCs

iPSCs are already showing great utility for disease modeling. One of the first demonstrations of the ability to successfully derive disease-specific iPSCs was the generation of iPSCs from somatic cells of patients with amyotrophic lateral sclerosis (ALS). These disease-specific iPSCs were subsequently differentiated into motor neurons, the affected cell type in ALS, providing an *in vitro* cell-based model of ALS.⁹⁵ At about the same time, iPSCs were also generated from cells representing 10 different genetic diseases including Duchenne muscular dystrophy, Parkinson disease, and Down syndrome.⁹⁶ A number of studies discussed below have shown the utility of disease-specific iPSCs to model disease.

In one instance, iPSCs derived from mouse models of lysosomal storage diseases were used to study the molecular mechanisms governing pathogenesis. These studies have provided new insights into the effects of defective lysosomal enzymes on embryogenesis.⁹⁷ Another example is the successful generation of iPSCs from dyskeratosis congenita (DC) cells, which exhibit shortened telomeres due to decreased levels of telomerase RNA component (TERC). The dependence of the generation of iPSCs on the upregulation of telomerase leading to telomere elongation and self-renewal suggested that somatic cells from DC patients would not be susceptible to reprogramming. Utilizing these disease-specific iPSCs, it was discovered that the expression of multiple telomerase components is restored during the reprogramming process. The upregulation of TERC during the establishment of pluripotency was sufficient to restore telomere

maintenance in DC-iPSCs. This suggests that therapeutics targeting the TERC locus may be beneficial in the treatment of DC, as well as in bone marrow failure where the telomerase activity of hematopoietic stem cells is often limited.⁶⁴ The establishment of an *in vitro* model of Rett syndrome, an X-linked autism spectrum disorder, through the reprogramming of patient fibroblasts into iPSCs has allowed elucidation of the role of the methyl-CpG binding protein, MeCP2, in contributing to the disease phenotype. Neurons derived from Rett iPSCs were employed to screen the effectiveness of current treatments and can be used in large-scale drug screens to identify new therapeutics.^{98,99}

iPSC technology has also been used to demonstrate that correction of a genetic defect prior to iPSC reprogramming can yield patient-specific, phenotypically normal cells for potential applications in therapy. To correct the disease phenotype, fibroblasts from patients with Fanconi anemia were first infected with lentivirus encoding a wild-type copy of the mutated protein. The reconstituted Fanconi fibroblasts were then reprogrammed with Oct4/Sox2/KLF4/c-Myc to generate disease-corrected iPSCs. These iPSCs were then differentiated to hematopoietic progenitors, which were capable of undergoing further differentiation into phenotypically normal myeloid and erythroid lineages.¹⁰⁰ Autologous disease-corrected iPSCs have also been used to successfully treat sickle cell anemia in an *in vivo* model. In this instance, fibroblasts from a mouse model of sickle cell anemia were first reprogrammed to pluripotency and were then genetically corrected via homologous recombination with the wild-type globin gene. The disease-corrected iPSCs underwent directed differentiation into hematopoietic progenitors. Transplantation of the corrected progenitors into irradiated sickle cell mice led to reconstitution of the hematopoietic system and reversal of the disease phenotype.¹⁰¹ This illustrates the potential utility of patient-specific iPSCs to generate disease-corrected cells for possible use in cell therapy.

Although human iPSCs have not been used to treat patients, there are a number of clinical trials for human ESC-based therapies in progress. Geron Corporation (Menlo Park, CA) received U.S. Food and Drug Administration (FDA) approval for the first human ESC-based clinical trial in January 2009, and patients were enrolled starting in October 2010. This study involves the injection of oligodendrocytes derived from human ESCs into the site of spinal cord injury to improve spinal cord function. Advanced Cell Technology (Santa Monica, CA) also received FDA approval for 2 clinical trials using human ESC-derived stem cell products. One trial involves the use of human ESC-derived retinal cells to treat Stargardt macular dystrophy, an inherited form of juvenile macular degeneration. Then, in January 2011, these same human ESC-derived retinal cells were approved for clinical trials for the treatment of age-related macular degeneration.

Concluding Remarks

The ability to induce pluripotency in somatic cells avoids the ethical dilemmas associated with the use of human embryonic stem cells. It can be predicted that further understanding of the molecular mechanisms governing the establishment and maintenance of pluripotency will eventually lead to the use of iPSCs for autologous tissue repair. Another area of investigation that should see considerable progress will be the development of reprogramming strategies for direct conversion of patient-derived cells into adult stem cells. Bypassing the pluripotency step would alleviate safety concerns associated with the use of pluripotent cells. Much work still needs to be done for the field of reprogramming to reach its full potential, but the enormous promise of somatic cell reprogramming for regenerative medicine validates the necessity of this work.

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References

1. Briggs R, King TJ. Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc Natl Acad Sci U S A*. 1952;38:455-63.
2. Gurdon JB, Uehlinger V. "Fertile" intestine nuclei. *Nature*. 1966;210:1240-1.
3. Gurdon JB, Melton DA. Nuclear reprogramming in cells. *Science*. 2008;322:1811-5.
4. Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*. 1987;51:987-1000.
5. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663-76.
6. Takahashi K, Tanabe K, Ohnuki M, *et al*. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861-72.
7. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature*. 2007;448:313-7.
8. Zhao XY, Li W, Lv Z, *et al*. iPS cells produce viable mice through tetraploid complementation. *Nature*. 2009;461:86-90.
9. Yamanaka S. A fresh look at iPS cells. *Cell*. 2009;137:13-7.
10. Laustriat D, Gide J, Peschanski M. Human pluripotent stem cells in drug discovery and predictive toxicology. *Biochem Soc Trans*. 2010;38:1051-7.
11. Jaenisch R, Young R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell*. 2008;132:567-82.
12. Chin MH, Mason MJ, Xie W, *et al*. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell*. 2009;5:111-23.
13. Newman AM, Cooper JB. Lab-specific gene expression signatures in pluripotent stem cells. *Cell Stem Cell*. 2010;7:258-62.
14. Guenther MG, Frampton GM, Soldner F, *et al*. Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. *Cell Stem Cell*. 2010;7:249-57.
15. Laurent LC, Ulitsky I, Slavin I, *et al*. Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. *Cell Stem Cell*. 2011;8:106-18.
16. Sommer CA, Sommer AG, Longmire TA, *et al*. Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector. *Stem Cells*. 2010;28:64-74.

17. Kim JB, Zaehres H, Wu G, *et al.* Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature*. 2008;454:646-50.
18. Kim JB, Greber B, Arauzo-Bravo MJ, *et al.* Direct reprogramming of human neural stem cells by OCT4. *Nature*. 2009;461:649-3.
19. Utikal J, Maherali N, Kulalert W, Hochedlinger K. Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *J Cell Sci*. 2009;122:3502-10.
20. Park JH, Daheron L, Kantarci S, Lee BS, Teixeira JM. Human endometrial cells express elevated levels of pluripotent factors and are more amenable to reprogramming into induced pluripotent stem cells. *Endocrinology*. Epub 2011 Jan 5.
21. Park IH, Zhao R, West JA, *et al.* Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008;451:141-6.
22. Haase A, Olmer R, Schwanke K, *et al.* Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell*. 2009;5:434-41.
23. Li C, Zhou J, Shi G, *et al.* Pluripotency can be rapidly and efficiently induced in human amniotic fluid-derived cells. *Hum Mol Genet*. 2009;18:4340-9.
24. Ho PJ, Yen ML, Lin JD, *et al.* Endogenous KLF4 expression in human fetal endothelial cells allows for reprogramming to pluripotency with just OCT3/4 and SOX2--brief report. *Arterioscler Thromb Vasc Biol*. 2010;30:1905-7.
25. Loh YH, Hartung O, Li H, *et al.* Reprogramming of T cells from human peripheral blood. *Cell Stem Cell*. 2010;7:15-9.
26. Seki T, Yuasa S, Oda M, *et al.* Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell*. 2010;7:11-4.
27. Staerk J, Dawlaty MM, Gao Q, *et al.* Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell*. 2010;7:20-4.
28. Tsai SY, Clavel C, Kim S, *et al.* Oct4 and klf4 reprogram dermal papilla cells into induced pluripotent stem cells. *Stem Cells*. 2010;28:221-8.
29. Sun N, Panetta NJ, Gupta DM, *et al.* Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci U S A*. 2009;106:15720-5.
30. Tamaoki N, Takahashi K, Tanaka T, *et al.* Dental pulp cells for induced pluripotent stem cell banking. *J Dent Res*. 2010;89:773-8.
31. Egusa H, Okita K, Kayashima H, *et al.* Gingival fibroblasts as a promising source of induced pluripotent stem cells. *PLoS One*. 2010;5:e12743.
32. Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. *Stem Cells Dev*. 2010;19:469-80.
33. Hanna JH, Saha K, Jaenisch R. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. *Cell*. 2010;143:508-25.
34. Sullivan GJ, Bai Y, Fletcher J, Wilmot I. Induced pluripotent stem cells: epigenetic memories and practical implications. *Mol Hum Reprod*. 2010;16:880-5.
35. Polo JM, Liu S, Figueroa ME, *et al.* Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotechnol*. 2010;28:848-55.
36. Kim K, Doi A, Wen B, *et al.* Epigenetic memory in induced pluripotent stem cells. *Nature*. 2010;467:285-90.
37. Ng RK, Gurdon JB. Epigenetic memory of active gene transcription is inherited through somatic cell nuclear transfer. *Proc Natl Acad Sci U S A*. 2005;102:1957-62.
38. Zwaka TP. Stem cells: troublesome memories. *Nature*. 2010;467:280-1.
39. Okita K, Yamanaka S. Induction of pluripotency by defined factors. *Exp Cell Res*. 2010;316:2565-70.
40. Brambrink T, Foreman R, Welstead GG, *et al.* Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell*. 2008;2:151-9.
41. Patel M, Yang S. Advances in reprogramming somatic cells to induced pluripotent stem cells. *Stem Cell Rev*. 2010;6:367-80.
42. Ellis J. Silencing and variegation of gammaretrovirus and lentivirus vectors. *Hum Gene Ther*. 2005;16:1241-6.
43. Somers A, Jean JC, Sommer CA, *et al.* Generation of transgene-free lung disease-specific human iPS cells using a single excisable lentiviral stem cell cassette. *Stem Cells*. 2010;28:1728-40.
44. Papapetrou EP, Tomishima MJ, Chambers SM, *et al.* Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation. *Proc Natl Acad Sci U S A*. 2009;106:12759-64.
45. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature*. 2009;458:771-5.
46. Woltjen K, Michael IP, Mohseni P, *et al.* piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*. 2009;458:766-70.
47. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science*. 2008;322:949-53.
48. Yu J, Hu K, Smuga-Otto K, *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. *Science*. 2009;324:797-801.
49. Kim D, Kim CH, Moon JI, *et al.* Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell*. 2009;4:472-6.
50. Zhou H, Wu S, Joo JY, *et al.* Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell*. 2009;4:381-4.
51. Warren L, Manos PD, Ahfeldt T, *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell*. 2010;7:618-30.
52. Feng B, Ng JH, Heng JC, Ng HH. Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. *Cell Stem Cell*. 2009;4:301-12.
53. Shi Y, Do JT, Despons C, Hahm HS, Scholer HR, Ding S. A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell*. 2008;2:525-8.
54. Solanki A, Lee KB. A step closer to complete chemical reprogramming for generating iPS cells. *Chembiochem*. 2010;11:755-7.
55. Haluskova J. Epigenetic studies in human diseases. *Folia Biol (Praha)*. 2010;56:83-96.
56. Lluís F, Cosma MP. Somatic cell reprogramming control: signaling pathway modulation versus transcription factor activities. *Cell Cycle*. 2009;8:1138-44.
57. Zhu S, Li W, Zhou H, *et al.* Reprogramming of human primary somatic cells by OCT4 and chemical compounds. *Cell Stem Cell*. 2010;7:651-5.
58. Zhao R, Daley GQ. From fibroblasts to iPS cells: induced pluripotency by defined factors. *J Cell Biochem*. 2008;105:949-55.
59. Herbig U, Sedivy JM. Regulation of growth arrest in senescence: telomere damage is not the end of the story. *Mech Ageing Dev*. 2006;127:16-24.
60. Marion RM, Blasco MA. Telomere rejuvenation during nuclear reprogramming. *Curr Opin Genet Dev*. 2010;20:190-6.
61. Stadtfeld M, Maherali N, Breault DT, Hochedlinger K. Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. *Cell Stem Cell*. 2008;2:230-40.
62. Marion RM, Strati K, Li H, *et al.* Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. *Cell Stem Cell*. 2009;4:141-54.
63. Banito A, Gil J. Induced pluripotent stem cells and senescence: learning the biology to improve the technology. *EMBO Rep*. 2010;11:353-9.
64. Agarwal S, Loh YH, McLoughlin EM, *et al.* Telomere elongation in induced pluripotent stem cells from dyskeratosis congenita patients. *Nature*. 2010;464:292-6.
65. Drayton S, Peters G. Immortalisation and transformation revisited. *Curr Opin Genet Dev*. 2002;12:98-104.
66. Li H, Collado M, Villasante A, *et al.* The Ink4/Arf locus is a barrier for iPS cell reprogramming. *Nature*. 2009;460:1136-9.
67. Loh YH, Ng JH, Ng HH. Molecular framework underlying pluripotency. *Cell Cycle*. 2008;7:885-91.
68. Nakagawa M, Koyanagi M, Tanabe K, *et al.* Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*. 2008;26:101-6.
69. Yu J, Vodyanik MA, Smuga-Otto K, *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007;318:1917-20.
70. Boyer LA, Mathur D, Jaenisch R. Molecular control of pluripotency. *Curr Opin Genet Dev*. 2006;16:455-62.
71. Soucek L, Evan GI. The ups and downs of Myc biology. *Curr Opin Genet Dev*. 2010;20:91-5.
72. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res*. 2005;65:3980-5.
73. Murphy DJ, Junttila MR, Pouyet L, *et al.* Distinct thresholds govern Myc's biological output in vivo. *Cancer Cell*. 2008;14:447-57.

74. Evans PM, Liu C. Roles of Kruppel-like factor 4 in normal homeostasis, cancer and stem cells. *Acta Biochim Biophys Sin (Shanghai)*. 2008;40:554-64.
75. Zhang W, Chen X, Kato Y, *et al*. Novel cross talk of Kruppel-like factor 4 and beta-catenin regulates normal intestinal homeostasis and tumor repression. *Mol Cell Biol*. 2006;26:2055-64.
76. Rowland BD, Bernards R, Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat Cell Biol*. 2005;7:1074-82.
77. Hong H, Takahashi K, Ichisaka T, *et al*. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature*. 2009;460:1132-5.
78. Hanna J, Saha K, Pando B, *et al*. Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature*. 2009;462:595-601.
79. Yamanaka S. Strategies and new developments in the generation of patient-specific pluripotent stem cells. *Cell Stem Cell*. 2007;1:39-49.
80. Evans PM, Zhang W, Chen X, Yang J, Bhakat KK, Liu C. Kruppel-like factor 4 is acetylated by p300 and regulates gene transcription via modulation of histone acetylation. *J Biol Chem*. 2007;282:33994-4002.
81. Kim J, Woo AJ, Chu J, *et al*. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. *Cell*. 2010;143:313-24.
82. Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell*. 2008;132:1049-61.
83. Mallanna SK, Rizzino A. Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells. *Dev Biol*. 2010;344:16-25.
84. Marson A, Levine SS, Cole MF, *et al*. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell*. 2008;134:521-33.
85. Judson RL, Babiarz JE, Venere M, Belloch R. Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol*. 2009;27:459-61.
86. Sun X, Fu X, Han W, Zhao Y, Liu H. Can controlled cellular reprogramming be achieved using microRNAs? *Ageing Res Rev*. 2010;9:475-83.
87. Bussmann LH, Schubert A, Vu Manh TP, *et al*. A robust and highly efficient immune cell reprogramming system. *Cell Stem Cell*. 2009;5:554-66.
88. Feng R, Desbordes SC, Xie H, *et al*. PU.1 and C/EBPalpha/beta convert fibroblasts into macrophage-like cells. *Proc Natl Acad Sci U S A*. 2008;105:6057-62.
89. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*. 2010;463:1035-41.
90. Ieda M, Fu JD, Delgado-Olguin P, *et al*. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*. 2010;142:375-86.
91. Szabo E, Rampalli S, Risueno RM, *et al*. Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature*. 2010;468:521-6.
92. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature*. 2008;455:627-32.
93. Weintraub H, Tapscott SJ, Davis RL, *et al*. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc Natl Acad Sci U S A*. 1989;86:5434-8.
94. Graf T, Enver T. Forcing cells to change lineages. *Nature*. 2009;462:587-94.
95. Dimos JT, Rodolfa KT, Niakan KK, *et al*. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science*. 2008;321:1218-21.
96. Park IH, Arora N, Huo H, *et al*. Disease-specific induced pluripotent stem cells. *Cell*. 2008;134:877-86.
97. Meng XL, Shen JS, Kawagoe S, Ohashi T, Brady RO, Eto Y. Induced pluripotent stem cells derived from mouse models of lysosomal storage disorders. *Proc Natl Acad Sci U S A*. 2010;107:7886-91.
98. Marchetto MC, Carromeu C, Acab A, *et al*. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell*. 2010;143:527-39.
99. Walsh RM, Hochedlinger K. Modeling Rett syndrome with stem cells. *Cell*. 2010;143:499-500.
100. Raya A, Rodriguez-Piza I, Guenechea G, *et al*. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature*. 2009;460:53-9.
101. Hanna J, Wernig M, Markoulaki S, *et al*. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science*. 2007;318:1920-3.