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FORUM REVIEW ARTICLE

Induced Pluripotent Stem Cells: Emerging Techniques for Nuclear Reprogramming

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Abstract

Introduction of four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, can successfully reprogram somatic cells into embryonic stem (ES)-like cells. These cells, which are referred to as induced pluripotent stem (iPS) cells, closely resemble embryonic stem cells in genomic, cell biologic, and phenotypic characteristics, and the creation of these special cells was a major triumph in cell biology. In contrast to pluripotent stem cells generated by somatic cell nuclear-transfer (SCNT) or ES cells derived from the inner cell mass (ICM) of the blastocyst, direct reprogramming provides a convenient and reliable means of generating pluripotent stem cells. iPS cells have already shown incredible potential for research and for therapeutic applications in regenerative medicine within just a few years of their discovery. In this review, current techniques of generating iPS cells and mechanisms of nuclear reprogramming are reviewed, and the potential for therapeutic applications is discussed. *Antioxid. Redox Signal.* 15, 1799–1820.

Introduction

Scientists have long experimented with mature cells to determine what factors contribute to their plasticity and differentiation ability. The first somatic cell nuclear-transfer (SCNT) experiments were performed more than 50 years ago by Briggs and King (15) and showed that injection of a nucleus from a differentiated blastula cell into an enucleated frog oocyte could give rise to a tadpole. Later, Gurdon (45, 46) showed that nuclei from even more differentiated frog intestinal cells could give rise to adult animals, although the efficiency was very low. These experiments showed that when placed in the appropriate environment, the nuclei of differentiated adult cells retain nuclear plasticity similarly to those of the early embryo. In 1996, Wilmut and colleagues (18, 195) reported the first cloned animal, Dolly the sheep, by nuclear transfer from adult cells into denucleated eggs. These achievements supported earlier findings that the epigenetic state of nuclei of differentiated cells is changeable, and that nuclei retain the ability to be reprogrammed by factors in oocytes or embryonic stem (ES) cells.

Pluripotency is the ability of the cell to differentiate to all cell types of an adult organism (126). Pluripotency occurs naturally only in early embryos and may be maintained *in vitro* in cultured ES cells harvested from the ICM of blastocysts. Isolated ES cells can maintain their population by proliferating and self-renewing indefinitely, and have the potential to differentiate into every lineage type in the body (38, 108). Self-renewal allows ES cells in culture to undergo numerous cell cycles, including cell division, without losing pluripotency under specific conditions (38, 108). Mouse ES cells require co-culture with a feeder layer of cells that provide unknown but essential factors. The culture medium must also contain leukemia inhibitory factor (LIF) for mouse ES cells, or fibroblast growth factors (FGFs) for human ES cells, to prevent differentiation (176). Without feeders or cytokines, ES cells undergo spontaneous differentiation and lose their pluripotency. Nuclear reprogramming, the process used to make induced pluripotent stem (iPS) cells, is the reverse of differentiation, in which differentiated cells revert to pluripotent cells (35, 63).

One of the major goals of stem cell research is to apply these cells therapeutically for treating patients. Both human ES cell and somatic cell nuclear transfer (SCNT) have technical and ethical problems that make therapeutic use in humans difficult. iPS cell technology circumvents these problems and is regarded as the best method for generating patient-specific pluripotent stem cells for use in regenerative medicine.

Generation of Induced Pluripotent Stem Cells from Somatic Cells

Generation of pluripotent stem cells from adult cells is an artificial manipulation that may not produce cells identical to naturally occurring pluripotent stem cells. However, some aspects of iPS cell generation may parallel the innate genetic processes that occur during embryonic development, including the reprogramming of the gamete pronuclei at

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fertilization under the influence of factors in the oocyte. Although tremendous effort has been put into generating immune-compatible patient-specific stem cells, promising methods were not successful until iPS cell technology with defined transcription factors was developed (172) (Table 1).

Ectopic overexpression of transcription factors

Based on the success and limitations of previous nuclear-transfer and cell-culture experiments, scientists began to experiment with more direct manipulation of cells' genetic information to create developmental plasticity in mature, differentiated cells. The first successful generation of iPS cells from somatic cells was accomplished by ectopic overexpression of pluripotency-related transcription factors (172). Takahashi and Yamanaka (172) introduced a mini-library of 24 candidate reprogramming factors, known as pluripotency-associated genes, which were known to be expressed in ES cells. The genes were introduced into mouse embryonic fibroblasts (MEFs) that carried a fusion of the β-galactosidase and neomycin-resistance genes expressed from the *Fbx15* locus (177). When MEFs were infected with all 24 genes and cultured on feeder cell layers in ES medium in the presence of G418, drug-resistant colonies emerged that had ES cell–like proliferation, gene expression, and morphology. To narrow the factors that are essential for reprogramming, all combinations of the 24 factors were attempted until four factors, *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*, were identified. The resultant cells, which showed pluripotent features indistinguishable from those of ES cells, were referred to as induced pluripotent stem (iPS) cells (172) (Fig. 1). These four essential factors are often referred to as Yamanaka factors in recognition of the inventor of this method, Shinya Yamanaka.

After this discovery, several groups have improved on the original reprogramming method. One group combined *Oct3/4* and *Sox2* with *Lin28* and *Nanog* to derive human iPS cells (211). Remarkably, the same four factors identified in the murine system were able to confer pluripotency in primate cells, indicating that the fundamental transcriptional network governing pluripotency is conserved across species. Several groups have shown that the *c-Myc* gene is dispensable for reprogramming (122, 138), although efficiency was then quite low. Recently, Yamanaka and colleagues (123) showed that *c-Myc* can be replaced with *L-Myc*, another *Myc* family member, for generating human iPS cells, resulting in even higher efficiency. As *c-Myc* reactivation can trigger the tumorigenicity of iPS derivatives, this study is important.

Although most iPS cells were generated from skin fibroblasts with these reprogramming transcription factors (171, 172, 211), expression of the same reprogramming factors also appears to initiate a sequence of stochastic events that eventually lead to generating iPS cells in a variety of other differentiated cells, such as neural cells (72, 73, 154, 156), keratinocytes (19), melanocytes (181), adipose-derived cells (164, 174), amniotic cells (86, 121, 216), pancreatic cells (159), and blood cells (50, 96, 150, 161) (Fig. 1). The forced expression of the four genes is required only temporarily, at the initiation stage of reprogramming, and can then be mostly silenced when endogenous pluripotency-related genes turned on (117).

Methods of delivering transcription factors into cells

Integrating viral vectors. Several types of integrating viral vectors, including retrovirus (132, 138, 172) and lentivirus (14, 159, 190), have been used in iPS cell generation. Retroviral vectors were the first type of vectors used to create iPS cells, and the site of viral integration has been closely studied. Takahashi and Yamanaka (172) noted approximately 20 retroviral integration sites (RISs) per iPS clone in their initial report. In a recent report comparing reprogramming of MEFs with that of murine hepatocytes or gastric epithelial cells, Aoi and colleagues (5) examined the number of RISs for each of the four retroviruses by using Southern blot. They detected one to nine RISs in MEF-derived iPS clones and one to four RISs in gastric epithelium or hepatocyte-derived clones, suggesting that tissues of epithelial origin may be more readily reprogrammed. The integration sites were random and did not show common viral integration sites. Several RISs found by Aoi had previously been identified by retroviral-mediated tumor induction in mice, so the safely of iPS cells is still questionable (2, 52). Bioinformatics analysis revealed no enrichment of any specific gene function, gene network, or canonic pathway by retroviral insertions (184). Retroviruses have a propensity to integrate near transcription start sites and may be more likely to cause malignant transformations.

Lentiviral vectors have a hypothetical safety advantage over retroviral vectors, because they lack the propensity to integrate near transcription start sites (201). No insertion-site analysis has been conducted, and thus the biologic relevance of vector differences remains theoretic. An additional advantage of lentivirus is its ability to transcribe large genetic packages, and two recent publications detailed the use of polycistronic lentiviral vectors that delivered the four reprogramming factors in a single construct, instead of the four separate vectors, each carrying one gene, used previously (19, 157). Both articles demonstrate the derivation of iPS clones from a single vector integration, which may minimize mutagenesis caused by viral insertion, as well as increase efficiency.

Nonintegrating viral vectors. In the first report on germline-competent iPS cells, ~20% of chimeric mice had tumors most likely caused by reactivation of the integrated *c-Myc* proviral transgene in the host genome (132). Another study showed cancer-related mortality in 18 (50%) of 36 of iPS chimeric mice (191). Insertional mutagenesis due to the integration of viral vectors into critical sites of the host genome, leading to malignant transformation, has been observed in preclinical and clinical gene therapy trials (48, 58, 91). Because of these limitations and safety concerns, alternative methods of iPS cell generation have been sought and have focused on eliminating integration of retroviral and lentiviral vectors from the reprogramming procedure.

Accordingly, the potential for using nonintegrating vectors was explored. As it is known that only transient expression of the four original factors is required. Stadtfeld and colleagues (160) used a transiently active and nonintegrating adenoviral vector and succeeded in generating iPS cell lines. These viruses could contribute to the formation of teratomas and chimeric mice, but were unable to pass through the germline (160). By contrast, Okita and colleagues (133) were unable to obtain murine hepatocyte iPS clones when the four reprogramming factors were introduced by adenovirus alone and required additional transfections of *Oct3/4* and *Klf4* or *Oct3/4* and *Sox2* by retrovirus. For unknown reasons, some cell types may be amenable to the safer adenoviral vector transduction, whereas other cell types cannot be made into iPS cells by using

Table 1. The Methods of iPS Cell Generation

Species	*Methods*	*TFs*	*Cell types*	*Other factors*	*Ref.*
Mouse	Retroviral vectors	OSKM	Fibroblasts		(172)
		OSKM	Hepatocytes, epithelial		(5)
		OSKM	BM mononuclear cells		(79)
		OSKM	Amniotic, yolk-sac cells		(121)
		OSKM	Adipose-derived cells		(174)
		OSKM	Adipose-derived stem cells		(164)
		OSKM/OSK	Fibroblasts	5′-azaC, VPA	(59)
		OSKM/OSK	Fibroblasts, keratinocytes	*Ink4a/Arf* shRNA	(87)
		OSKM/OSK	Fibroblasts/T lymphocytes		(56)
		$OSK^{L}M$	Fibroblasts		(123)
		OSK	Fibroblasts		(122)
		OSK	Fibroblasts	*miR-291-3p, 294, 295*	(65)
		OSK	Fibroblasts, $p53^{-/-}$, $Terc^{-/-}$		(105)
		OSK/OS	Fibroblasts, $p53^{-/-}$	*p53, p21, Ink4a/Arf* shRNA	(68)
		OKM/OK	Fibroblasts	RepSox	(61)
		SKM/OK	NPCs	BIX01294	(153)
		OK/OM	NSCs		(72)
		OK	NSCs	PD0325901, CHIR99021	(155)
		OK	Fibroblasts	BIX01294, BayK8644	(152)
		OK	Fibroblasts	CHIR99021	(90)
		O	NSCs		(71)
	Lentiviral vectors	OSKM	Pancreatic β cells		(159)
	Inducible lentiviral vectors (tet)	OSKM	Fibroblasts		(14)
		OSKM	Fibroblasts, B lymphocytes	5′-azaC	(116)
		OSKM	B lymphocytes		(50)
		OSKM/OKM/OSK	Fibroblasts	Alk5 inhibitor	(102)
		OSKM/OKM/OSK	Melanocytes		(181)
		OSK	Fibroblasts	Wnt3a	(107)
		OSM	Fibroblasts	Kenpaullone	(99)
	Lentiviral vectors, single polycistronic	OSKM	Fibroblasts		(151)
		OSK	Fibroblasts		(23)
	Inducible lentiviral vectors (tet), single polycistronic	OSKM	Fibroblasts		(19,157)
		OSKM	Fibroblasts		(181)
	Adenoviral vectors	OSKM	Fibroblasts, hepatocytes		(160)
	Retroviral vectors + adenoviral vectors	OSK	Hepatocytes		(133)
	PiggyBac transposon vectors	OSKM	Fibroblasts	PD173074	(66)
		OSKM	Fibroblasts		(197,212)
	Plasmid vectors	OSKM	Fibroblasts		(133)
	Plasmid vectors, single polycistronic	OSKM	Fibroblasts		(43)
	Recombinant proteins	OSKM	Fibroblasts	VPA	(219)
	ES cell–derived proteins	-	Fibroblasts		(26)
Rat	Retroviral vectors	OSKM	Fibroblasts		(92)
		OSK	Liver epithelial cells	PD0325901, CHIR99021, A-83-01	(69)
Rabbit	Lentiviral vectors	OSKM	Liver/stomach cells		(55)
Dog	Retroviral vectors	OSKM	Fibroblasts	VPA, PD0325901, CHIR99021, A-83-01	(154)
Pig	Retroviral vectors	OSKM	Fibroblasts		(37)
	Inducible lentiviral vectors (tet)	OSKMNL/OSKM	Fibroblasts		(202)
Monkey	Retroviral vectors	OSKM	Fibroblasts		(94)
	Retroviral vectors	OSKMNL	Liver cells		(178)
Human	Retroviral vectors	OSKM	Fibroblasts		(171)
		OSNL	Fibroblasts		(211)
		OSKML	Fibroblasts		(98)
		OSKM	Fibroblasts	*hTERT, Large T*, ROCKi	(138)

(*continued*)

Table 1. (Continued)

Species	*Methods*	*TFs*	*Cell types*	*Other factors*	*Ref.*
		OSKM	Fibroblasts	*p53* shRNA, *p53DD*	(56)
		OSKM	Fibroblasts from patients		(21,29,137, 149,189)
		OSKM	Fibroblasts, oral mucosa		(119)
		OSKM	Amniotic fluid cells		(69)
		OSKM	Amniotic/yolk sac cells		(121)
		OSKM	Cord blood cell, $CD34^+$		(95,206)
		OSKM	Cord blood cells, $CD34^+$	*p53* shRNA	(173)
		OSKM	Blood cell, PB^- and BM–MNC		(79)
		OSKM	Cord blood cells, $CD45^-$		(213)
		OSKM	Hepatocytes		(93)
		OSKM	Adipose-derived cells		(164)
		OSKM/OSK	Fibroblasts	*p53* shRNA, *p53DD*	(68)
		OSKM/OSK	Fibroblasts	*INK4a/ARF* shRNA	(87)
		OSK	Fibroblasts		(122)
		OSK	Fibroblasts	*p53* shRNA	(105)
		OSK	Fibroblasts from patients		(100)
		OSK	Mesenchymal stromal cells		(128)
		OSN	Amnion-derived cells		(216)
		OS	Fibroblasts	VPA	(59)
		OS	Cord blood cells, $CD133^+$		(42)
		OK	Fibroblasts	CHIR99021, Parnate, PD0325901, SB431542	(69)
		OK	Dermal papilla cells		(179)
		OK	NSCs		(54)
		OK/O	NSCs		(71)
	Lentiviral vectors	OSKM/OSNL	Fibroblasts	*Large T*	(104)
		OSNL	Fibroblasts from patients		(33)
		OSNL	Cord blood cells		(47)
	Inducible lentiviral vectors (tet)	OSKM/OKM/OSK	Melanocytes		(181)
		OSKM	Blood cell, T cell/PB–MNC		(96)
	Inducible lentiviral vectors (tet), single polycistronic	OSKM	Keratinocytes		(19)
		OSKM	Blood cell, PB–MNC		(161)
	Lentiviral vectors, Cre-recombinase excisable	OSKM/OSK	Fibroblasts from patients		(156)
	Adenoviral vectors	OSKM	Fibroblasts		(221)
	Sendaiviral vectors	OSKM	Fibroblasts		(40)
		OSKM	Blood cell, T cell		(150)
	PiggyBac vectors	OSKM	Fibroblasts		(197)
	Plasmid vectors	OSKMNL	Fibroblasts		(210)
	Recombinant proteins	OSKM	Fibroblasts		(69)

O, *Oct4*; S, *Sox2*; K, *Klf4*; M, *c-Myc*; N, *Nanog*; L, *Lin28*; ^{L}M, *L-Myc*.

this technique. The biologic basis of this cell-specific plasticity is a fundamental but unanswered question in stem cell biology.

Nonviral reprogramming. Although the partial success in generating iPS cells with adenovirus suggests that this safer vector may be useful, the widespread use of any viral-vector technology in human application is likely impossible. Because of the great potential of iPS cells, investigators have turned to nonviral vector systems for the generation of iPS cells. Yamanaka and colleagues (133) used a polycistronic expression plasmid containing the *Oct3/4*, *Sox2*, and *Klf4* cDNAs linked by the foot-and-mouth disease virus 2A self-cleaving peptide. When this construct, which lacks viral genetic material, was repeatedly transfected into MEFs, together with a separate *c-Myc* cDNA expression vector over a 1 week time period (on days 1, 3, 5, and 7), one to 29 Oct4-positive iPS colonies emerged from 1×10^6 cells in seven of 10 independent experiments, whereas from the same number of retrovirus-infected cells, 100 Oct4-positive iPS colonies were routinely obtained. In six of 10 experiments, no evidence of plasmid integration into the host genome was detected with PCR or Southern blot analysis (133). Although the efficiency of iPS generation by plasmid transfection was greatly decreased, and the oncogenic *c-Myc* viral vector transduction was needed, this report provided proof-of-concept for the generation of iPS cells without transgene integration of viral vector.

Several recent studies have reported the use of a transient viral-vector approach to iPS generation that would increase efficiency and safety (66, 197, 212). This method begins with the incorporation of all four Yamanaka genes into a single *piggyBac* (PB) vector, with viral 2A oligopeptides between adjacent genes allowing synthesis of the four factors as a single transcript followed by posttranslational cleavage of the proteins at the appropriate locations. The most important and unique feature of this approach is that the reprogramming genes are removed from the genome by transient transfection of PB transposase (212). Thus, permanent alteration of the genome is avoided and many safety concerns circumvented. This technique maintains a

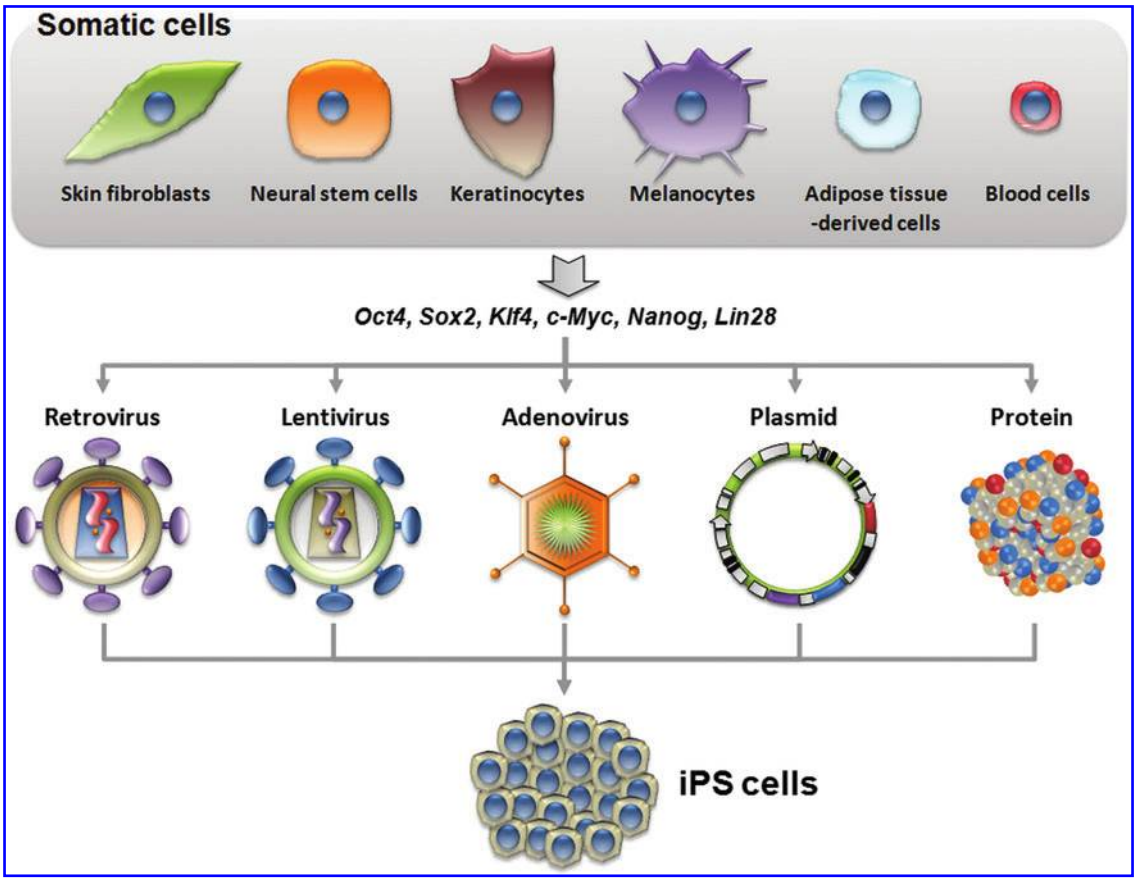

FIG. 1. Various cell sources and delivery methods for generating iPS cells. iPS cells were originally derived from embryonic fibroblasts in mouse and skin fibroblasts in humans. Fibroblasts have been most widely used for reprogramming. Thereafter, other cells, including neural stem cells, keratinocytes, melanocytes, adipose tissue–derived cells, and various blood mononuclear cells, have been successfully used for reprogramming to achieve higher efficiency or to show easier and more convenient accessibility. Advances have occurred in the delivery vectors for reprogramming factors, *Oct4, Sox2, Klf4, c-Myc, Nanog*, and *Lin28*. Initial studies used DNA-integrating viral vectors such as retrovirus and lentivirus, but later studies demonstrated the usefulness of nonintegrating viral vectors, adenovirus, and nonviral systems with plasmids for reprogramming. More recently, delivery methods using recombinant proteins or ES-cell extracted proteins have been shown to reprogram somatic cells into iPS cells, suggesting safer and potentially clinically applicable systems for deriving iPS cells. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

relatively robust reprogramming efficiency. By using a similar idea, the Cre–loxP recombinase system was used to remove the vector-integrated transgene once reprogramming is achieved (66). However, this system is less precise and may leave some residual elements outside the loxP sites, including the transposon repeat that may be mutagenic. These studies confirmed that transient expression of the reprogramming factors is sufficient for reprogramming of somatic cells to acquire pluripotency. They also confirmed that it is possible to remove integrated vector material when it is no longer needed and thus to minimize the risk of late cancer development.

Recombinant proteins of transcription factors. As an alternate approach, the protein products of the reprogramming genes have been delivered directly to cells, without viral DNA at all (69, 219). Two independent groups made proteins in which reprogramming factors were fused to polyarginine, a short basic peptide, known as a cell-penetrating peptide (CPP), which can overcome the cell-membrane barrier and reprogram human (69) or mouse somatic cells (219). Zhou and colleagues (219) used purified recombinant proteins containing 11 arginine residues (11R) at the C-terminus of each reprogramming factor to reprogram OG2/Oct4-GFP reporter MEF cells with four cycles of protein treatments plus the histone deacetylase (HDAC) inhibitor, valproic acid (VPA). Kim and colleagues (69) demonstrated reprogrammed human fetal fibroblasts by treatment of cell extracts from HEK293 cells, which expressed each reprogramming factor fused to eight arginine residues (8R), albeit at a very low efficiency (~0.001%) and with prolonged time (~8 weeks). A recent study by Cho and colleagues (26) also is encouraging, as they reported that a single transfer of ES cell–extracted proteins to mouse fibroblasts with cell permeabilization can successfully reprogram mouse fibroblasts in relatively short time periods (20~25 days after induction), albeit at a still lower efficiency (0.001%). Thus, although in its present form, this protein-based reprogramming technique may fall short of generating patient-specific iPS cells, it is the only promising and realistic technology at the moment to generate virus-free and transgene-free human patient–specific iPS cells.

Nongenetic approaches for reprogramming

Small molecules. Huangfu and colleagues (59, 169) tested the effects of small-molecule chemicals involved in chromatin modification on reprogramming by using Oct4-GFP reporter mouse cells. Treatment of the four factor-infected MEFs with the DNA methyltransferase inhibitor 5′-azacytidine increased the reprogramming efficiency, and three known histone deacetylase (HDAC) inhibitors—suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), and valproic acid (VPA)—also greatly increased the efficiency of reprogramming, with VPA being the most effective compound, showing a >100-fold increase (59). This demonstration of enhanced reprogramming efficiency by HDAC inhibitors suggests that chromatin modification is a key step in defining the pluripotent state of a cell.

Chemical screens have identified two compounds, BIX01294 and BayK8644, which, in combination with two factors (*Oct4* and *Klf4*), enhanced the reprogramming efficiency of mouse neural progenitors (153) and mouse embryonic fibroblasts (152). BIX01294 is an inhibitor of the G9a histone methyltransferases, which methylate histone H3 at the position of lysine 9 (H3K9) (24). G9a histone methyltransferase is reported to silence Oct4 expression during early embryogenesis by subsequent *de novo* DNA methylation at the promoter region, thereby preventing reprogramming (30, 36, 39, 170).

Several other small molecules have been found that indirectly influence the epigenetic state of a cell (Fig. 2). BayK8644 is an L-channel calcium agonist (153) that exerts its effect through upstream signaling pathways rather than direct epigenetic remodeling. Other small molecules involved in signaling pathways also increase the efficiency of reprogramming, such as rho-associate kinase (ROCK) inhibitor, Y-27632, which augments human iPS cell induction by enhancing cell survival (77, 138). Inhibitor of Wnt signaling (89, 107, 155), MEK (89, 155), FGF (66), and TGF-β receptor (61, 89) also had effects on the generation and maintenance of ground-level pluripotency of iPS cells.

microRNAs. Efforts have been made to investigate the potential of microRNA (miRNA) for the production of iPS cells. Pioneering studies revealed the existence of subsets of miRNAs, which are specific for ES cells, referred to as ES cell-specific cell cycle–regulating miRNAs (ESCC miRNAs) (188). Transient transfection of ESCC miRNAs (*miR-291-3p, miR-294,* and *miR-295*), in combination with retroviral infection expressing *Oct4, Sox2,* and *Klf4*, showed enhanced efficiency of iPS cell generation from mouse embryonic fibroblasts (65).

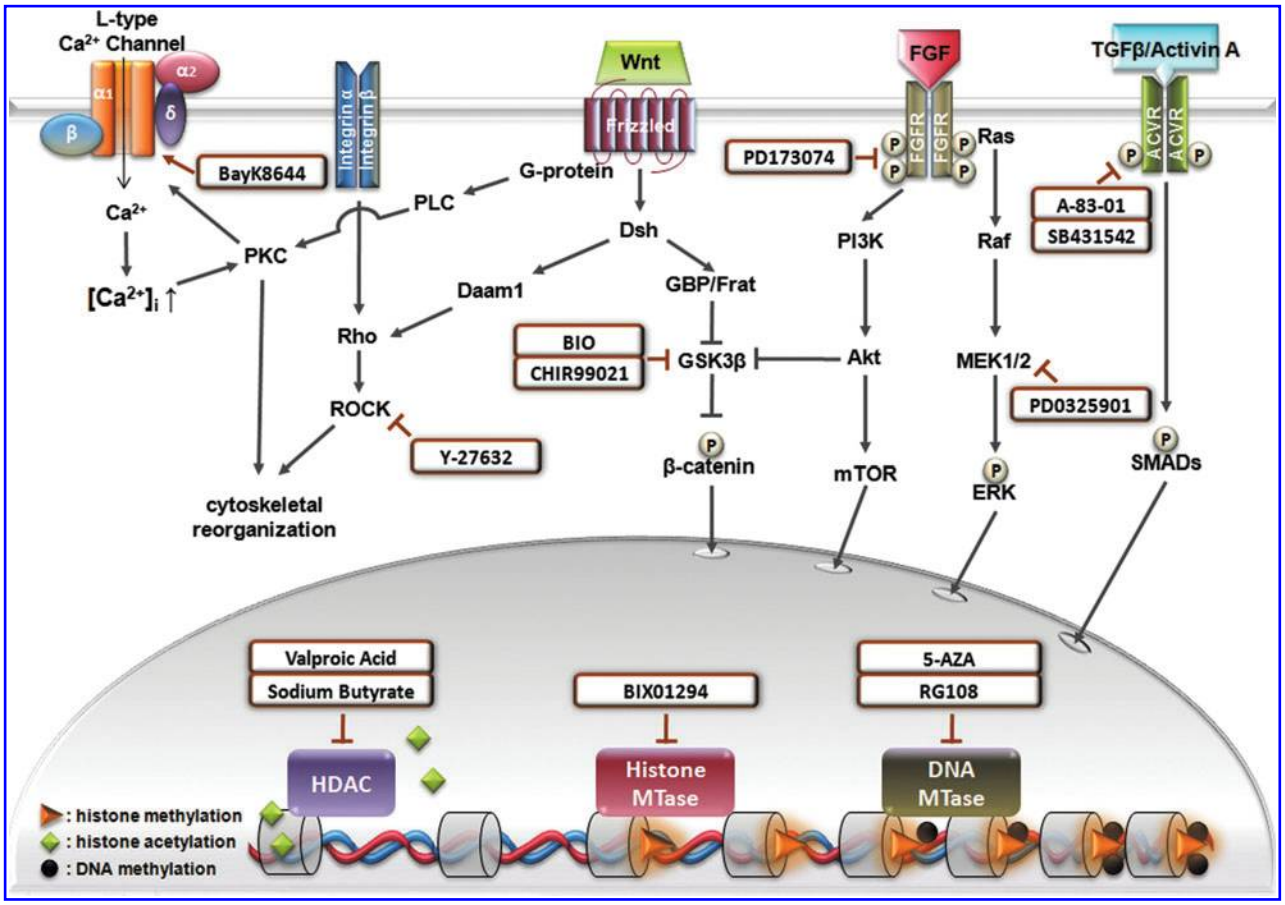

FIG. 2. Small-molecule chemicals for enhancing reprogramming efficiency. DNA methyltransferase inhibitor, histone deacetylase (HDAC) inhibitors, and histone methyltransferase inhibitor were shown to increase the reprogramming efficiency by epigenetic remodeling of pluripotency-related genes. L-channel calcium agonist, rho-associated kinase (ROCK) inhibitor, Wnt signaling inhibitor, and inhibitors of MEK, FGF, and TGF-β receptor can enhance reprogramming efficiency. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Although it remains unclear whether these miRNAs have overlapping targets during reprogramming, studying their regulation during the iPS cell-generation process will enhance understanding of the biology of miRNAs associated with pluripotency. It would also be interesting to test whether the inhibition of miRNAs such as *let-7* and *mir-125* (196), expressed in only differentiated cells but not in stem cells, facilitates reprogramming. Recently, Melton and colleagues (114) showed that introduction of *let-7* inhibitors with retroviral vectors expressing *Oct4, Sox2,* and *Klf4* (minus *c-Myc*) increased the efficiency of reprogramming by 4.3-fold. Together, these findings indicated an important role of opposing miRNA families, ES-specific, and differentiated cell-specific miRNAs, on reprogramming capacity and their potential as reprogramming factors.

Altering cell-cycle signal pathways. Cell-cycle regulatory proteins can also change the efficiency of iPS cell generation in ways that are just beginning to be understood. Inhibition of the tumor-suppressor protein p53–related signal pathway facilitates the generation of iPS cells, suggesting that it represses the dedifferentiation (56, 68, 182). iPS cell technology can be used to study how *p53* modulates the stability of the differentiated state. Inhibition of *p53* directly by *Mdm2* and indirectly by downregulation of *Arf* (87, 182) enhances the progression of normal fibroblasts into iPS cells through its direct target, *p21*, which promotes cellular senescence (56, 68). Deficiency of *p53* improves the efficiency and kinetics of iPS cell generation with only two factors, *Oct4* and *Sox2* (68). Other tumor suppressors, such as *Rb* or *Pten*, are also candidate repressors of dedifferentiation that can be investigated by using iPS cell technology (218).

Advances in the source cells

The choice of appropriate cell types for nuclear reprogramming is not only important but also critical for future clinical therapy with autologous iPS cells; therefore, an easy sampling method with minimal risk and yielding a sufficient quantity of cells for reprogramming must be developed. Originally, skin fibroblasts were chosen as source cells for reprogramming because these cells are relatively easy to collect and expand in *in vitro* culture. However, procuring these cells from patients necessitates a skin biopsy, requiring surgical procedures, such as anesthesia, incision, and suturing, which are inconvenient, painful, and have potential complications, including infection. Notwithstanding this, iPS cells have been established from skin fibroblasts derived from patients with a variety of diseases, including adenosine deaminase (ADA) deficiency, Gaucher disease type III (GD), Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Down syndrome (DS), Parkinson disease (PD), juvenile diabetes mellitus (JDM), Huntington disease (HD), Lesch-Nyhan syndrome (LNS), amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), β-thalassemia, Fanconi anemia (FA), and LEOPARD syndrome (21, 29, 33, 137, 142, 156, 189). Other cell types have advantages over skin fibroblasts. Because they have a higher expression level of endogenous pluripotency-related genes *Sox2* and *c-Myc*, neural stem cells (NSCs) can be reprogrammed by a smaller number of reprogramming factors, two factors (*Oct4* and *Klf4*), or even just one factor (*Oct4*) (54, 71, 72, 153). Keratinocytes derived from human foreskin and plucked hairs, which express high levels of *c-Myc*, showed $\sim$100-fold improvement in reprogramming efficiency over fibroblasts (1). Human melanocytes from skin biopsies express high levels of endogenous *Sox2* and can be reprogrammed with three factors (*Oct4, Klf4,* and *c-Myc*) with higher efficiency (0.05%) and more quickly ($\sim$10 days) (181). Adipose-derived stem cells, which can be obtained from routinely performed lipoaspiration in outpatient clinics, were reprogrammed in a feeder-free system by four factors (*Oct4, Klf4, Sox2,* and *c-Myc*) with $\sim$20-fold higher efficiency and about twofold faster (167). $CD133^+$ cells from frozen banked cord blood can be reprogrammed with only two factors, *Oct4* and *Sox2* (42), or Thomson factors (*Oct4, Sox2, Nanog,* and *Lin28*) (47). In addition, granulocyte colony-stimulating factor (G-CSF) mobilized $CD34^+$ cells from peripheral blood were used as a cell source to generate iPS cells with four factors (95, 206). However, the CD34 fraction represents a very rare population in peripheral blood, and therefore, isolation of a sufficient amount of $CD34^+$ cells requires the use of expensive G-CSF and a large-volume apheresis. Other groups, therefore, have recently developed new methods by using a small amount of whole peripheral blood as a better source of cells for human patient-specific iPS cell generation (96, 150, 161) (Fig. 1).

Each type of source cell has its own advantages and limitations for the generation of iPS cells. In addition, recent studies have shown the significant effects of genetic (41) and epigenetic (73) memory on characteristics and differentiating potency among iPS cell lines, depending on the types of source cells. If these results are further verified by more studies, the choice of the optimal cell source for patient-specific iPS cell generation may be carefully considered and selected for each specific purpose.

Characterization of iPS Cells

iPS cells express ES cell–specific genes that maintain the developmental potential to differentiate into all three primary germ layers: ectoderm, endoderm, and mesoderm. Several functional tests, including *in vitro* differentiation, DNA methylation analysis, *in vivo* teratoma formation, chimera formation, germline transmission, and tetraploid complementation, have been used to define pluripotency of iPS cells. Generally, ES cells and iPS cells seem to have almost identical properties in these assays (67, 217).

Genomic integrity

Maintaining genomic integrity is of crucial importance during the creation of iPS cells, as alterations can cause neoplastic disease and limit therapeutic application. Several groups have investigated the karyotypes of mouse (192) and human iPS cell lines (171, 211) to determine how much genetic alteration is present. One study showed that continuous passaging of human iPS cells resulted in chromosomal abnormalities, starting as early as passage 13 (1). This finding warns that more studies should investigate the exact frequency of culture-induced genetic abnormalities in human iPS cells over the long run.

The use of retroviral and lentiviral vectors to express the reprogramming transcription factors has the inherent risk of insertional mutagenesis. However, Aoi and colleagues (5) found no common insertion sites in hepatocyte- and stomach

cell–derived iPS cells (5). In addition, recent adenoviral and plasmid-based methods have a much lower risk of insertional mutagenesis, because theoretically, the genomic DNA is not perturbed by the virus (133, 160). However, even without viral integration, genetic changes might occur as part of the reprogramming process. Another component reflecting DNA integrity, telomere length, is not altered in mouse (160) and human *hTERT* (138, 171)-derived iPS cells (171). Studies of iPS cells have suggested that DNA integrity is maintained throughout the generation process; however, only long-term studies will show if these cells are truly free of malignant potential *in vivo*. In any case, this risk will have to be weighed against the therapeutic potential.

Gene-expression profiles

Comparative global gene-expression analyses of the ES cell and iPS cell transcriptomes by using microarrays have been performed for human and mouse lines (103, 172, 192). Mikkelsen and colleagues (116) reported that whole-genome expression profiles of iPS cells and ES cells of the same species are no more different than are those of individual ES cell lines. Nonetheless, other groups noted that iPS cells are not identical to ES cells. Takahashi and colleagues (171) compared the global gene-expression profile of human iPS and human ES cells for 32,266 transcripts. Notably, 1,267 (~4%) of the genes were detected with more than fivefold difference in up- or downregulation between iPS cells and human ES cells. Soldner and colleagues (156) compared the transcriptional profiles of human iPS cell lines, in which the Cre-recombinase excisable exogenous viral sequences had been removed (factor-free human iPS cells), with those of human iPS cells before transgene excision. The transcriptomes of factor-free human iPS cells more closely resembled those of human ES cells than the parental human iPS cells with integrated viral sequences. This could be caused by the loss of any downstream gene activation by residual expression of the exogenous transcription factors or by the loss of epigenetic memory of the somatic state after the initial reprogramming event. However, it remains difficult to compare these differences because most groups used genetically unrelated cell lines. More experiments are needed to clarify these discrepancies according to the methods used for reprogramming, sources of iPS cells, disease state of parental cells, ages, and sex.

A well-characterized gene-expression pattern occurs after ectopic expression of the four factors in mouse embryonic fibroblasts, including an initial downregulation of cell-type–specific transcription factors (116, 159) and upregulation of genes involved in proliferation, DNA replication, and cell-cycle progression (116). During the reprogramming process, many self-renewal–related genes are reactivated, including fibroblast growth factor 4 (*Fgf4*) as well as polycomb genes (116, 172). However, a large fraction of pluripotency-related genes are upregulated only during the late stages of reprogramming (14, 116, 159). In a different study, the expression of key pluripotency-related genes, such as *Oct4*, *Sox2*, and *Rex1*, was approximately twofold lower in the iPS cells compared with two human ES cell lines, HSF1 and H9 (98). Pluripotent cells are highly sensitive to the levels of these transcription factors (TFs) (127), and a notable amount of normal transcriptional heterogeneity is found in human ES-cell cultures (135). Therefore, the observed variation could reflect differences in culture conditions rather than incomplete reprogramming. More work on human ES cells is thus required the better to understand the extent of normal transcriptional variation within human and also mouse ES cells and fully to understand how iPS cells compare.

Epigenetic status

As the substrate of transcription, chromatin is subjected to various forms of epigenetic regulation, including chromatin remodeling, histone modifications, histone variants, and DNA methylation. For example, trimethylation of lysine 9 and lysine 27 of histone 3 (H3K9 and H3K27) correlates with inactive regions of chromatin, whereas H3K4 trimethylation, and acetylation of H3 and H4 are associated with active transcription (64), and DNA methylation generally represses gene expression (145).

By regulating chromatin structure, epigenetic modifications play an essential role in controlling access to genes and regulatory elements in the genome (14). The differences in epigenetic status between a somatic cell and a pluripotent stem cell are huge, and dedifferentiation requires global epigenetic reprogramming. For instance, pluripotent stem cells contain bivalent domains that are characteristic chromatin signatures (9, 10). These are regions enriched for repressive histone H3 lysine 27 trimethylation (H3K27me3) and simultaneously for histone H3 lysine 4 trimethylation (H3K4me3) as an activating signal (117). It was assumed initially that bivalent domains might be ES-cell specific because they were first identified by using chromatin-immunoprecipitation (ChIP) followed by hybridization to microarrays (ChIP-Chip) that featured key developmental regulators. All of these resolved either to a univalent (H3K4me3 only or H3K27me3 only) state or lost both marks in differentiated cells (9). With ChIP followed by high-throughput sequencing (ChIP-seq) technology, Mikkelsen and colleagues (112) showed that bivalent domains are more generally indicative of genes that remain in a poised state. Consequently, pluripotent cells were found to contain large numbers of bivalent domains (~2,500) compared with multipotent neural progenitor cells (NPCs) (~200) that still retain multilineage potential but are more restricted than ES cells (112).

Several studies of the murine iPS-cell have identified a small number of representative loci that have consistent chromatin and DNA methylation patterns (103, 172, 192). Maherali and colleagues (103) used ChIP-Chip to investigate the presence of H3K4me3 and H3K27me3 in the promoter regions of 16,500 genes, and results showed that iPS cells were highly similar to ES cells in epigenetic state (103). The H3K4me3 pattern was similar across all samples, indicating that reprogramming was largely associated with changes in H3K27me3 rather than with H3K4me3 (103). Mikkelsen and colleagues (192) used a more-comprehensive ChIP-Seq technique to determine genome-wide chromatin maps in several iPS lines, which are derived with different methods: drug selection by using an *Oct4*–neomycin-resistance gene (192), drug selection by using a *Nanog*–neomycin-resistance gene (192), and by morphologic appearance (113). Overall global levels of repressive H3K27me3 and the characteristic bivalent chromatin structure are retained in the various iPS cell lines. The restoration of repressive chromatin marks appears crucial stably to silence lineage-specific genes that are active in

somatic cells and inactive in undifferentiated pluripotent cells. Failure to establish the repressive marks results in incompletely reprogrammed cells. Activating H3K4me3 patterns are also crucial for complete reprogramming and have been observed to be restored genome-wide, in particular around the promoters of pluripotency-associated genes, such as *Oct4* and *Nanog*, in the fully reprogrammed iPS lines (116).

A second component of the epigenetic machinery is DNA methylation, which is a stable and heritable mark that is involved in gene silencing, including genomic imprinting and X-chromosome inactivation. DNA methylation patterns are dynamic during early embryonic development and are essential for normal postimplantation development (144). Overall, DNA methylation levels remain stable during ES-cell differentiation, although they are not static for any given individual gene (112). The 5′-promoter regions of many transcriptional units contain clusters of the dinucleotide CpG, which are methylated at transcriptionally silent genes and demethylated on activation. In differentiated cells, the *Oct4*, *Nanog,* and *Sox2* promoter regions are highly methylated and in an inactivated state, whereas in ES cells, these promoters are unmethylated to be activated. During reprogramming, almost complete demethylation of these promoters has been observed (103, 116, 132, 192). Therefore, the loss of DNA methylation at the promoters of pluripotency-related genes appears essential for achieving complete reprogramming. Interestingly, loss of DNA methylation at this class of genes seems to be a rather late event in the reprogramming process because cells that have already acquired self-renewing properties still showed high levels of DNA methylation (116).

Developmental potential: pluripotency

Research on the transcriptional and epigenetic state of iPS cells is highly informative, and it might ultimately be possible to characterize newly derived iPS cell lines based on their genomic profiling alone. Before selecting the most informative markers, it is important to use *in vivo* assays to analyze the interplay between transcriptome, epigenome, and developmental potential. Recently, Jaenisch and Young (63) provided a detailed comparison of the different strategies for assessing developmental potential and their stringency. *In vitro* differentiation is the least stringent assay, whereas tetraploid-embryo complementation is the most stringent assay for testing developmental potential (34, 63). These strategies could be used to determine the pluripotency of mouse iPS cells, but only *in vitro* differentiation and teratoma formation could be applied to test human iPS cells. Mouse iPS cells appear to have developmental potential similar to that of ES cells, as confirmed by teratoma formation capability and by a high contribution to chimera formation with germline transmission (103, 172, 192). To show the final step of developmental potential of iPS cells as equivalent to that of ES cells, three separate groups injected mouse iPS cells (2N) into tetraploid blastocysts (4N), which are capable of producing placental and other extraembryonic tissues but not the embryo itself, and have created live mouse (11, 67, 217). The procedure, called tetraploid complementation, is the most stringent test for pluripotency. If the stem cells that are injected into the tetraploid blastocyst differentiate into embryonic tissues that produce a mouse, then the stem cells are considered truly pluripotent.

Mechanism of Reprogramming

Whereas *Oct4, Sox2, Klf4,* and *c-Myc* are known to generate mouse and human iPS cells, another study reported that *Nanog* and *Lin28* (211) can replace *Klf4* and *c-Myc*. Therefore, *Oct4* and *Sox2* appear to be key transcription factors in iPS cell generation, and the complexity of downstream events maintains pluripotency and blocks differentiation (127). Several hundred downstream genes of *Oct4* and *Sox2* exist with complex interactions and signaling networks (12, 70, 97). The other two factors, *Klf4* and *c-Myc,* are known to promote cellular proliferation, chromatin remodeling, and prevention of cell death (205). Studies from Yamanaka and Daley (138, 171) used different combinations of three to six genes to reprogram cells, adding SV40 large T antigen and telomerase (138) or withholding either *Klf4* or *c-Myc* (138). *Lin28* is a regulator of microRNAs previously shown to be downregulated during the differentiation of ES cells (143). *Lin28* appears to function by inhibiting a *let-7* microRNA that in turn inhibits the growth-promoting oncogenes, leading to cellular proliferation (186). Surprisingly, many roads appear capable of leading to the induction of pluripotency. The type of gene appears to be of greater importance than the individual identity of the gene used.

Signal networks

Transcription factor networks. In mammals, it was thought that the differentiation process was irreversible until the successful cloning of Dolly by SCNT (18). This cloning experiment demonstrated that somatic cells can be reprogrammed back to the totipotent zygotic state by cellular factors in unfertilized eggs. The complex signaling interactions took a considerable amount of time and effort to identify. *Oct4* represses itself when overexpressed, whereas *Nanog*, *Sox2*, and *FoxD3* activate its expression (127). Additional repressors may yet be unidentified that provide counterbalance for maintaining *Oct4* expression levels in ES cells.

Based on large-scale data sets, Young and colleagues (12) proposed that *Oct4*, *Sox2*, and *Nanog* collaborate to form regulatory circuitry consisting of autoregulatory and feed-forward loops that dictate pluripotency and self-renewal (Fig. 3). This autoregulatory circuitry suggests that the three factors function collectively to maintain their own expression stably (3). Autoregulatory loops appear to be a general feature of master regulators of cell state (10). Functional studies have confirmed that *Oct4* and *Sox2* co-occupy and activate the *Oct4* and *Nanog* genes (80, 134), and experiments with an inducible *Sox2*-null murine ES-cell line have provided compelling evidence for this interconnected autoregulatory loop and its role in the maintenance of pluripotency (109). The loop formed by *Oct4*, *Sox2*, and *Nanog* also suggests how the core regulatory circuitry of iPS cells might be triggered when *Oct4*, *Sox2*, and other transcription factors are overexpressed in fibroblasts (103, 132, 172, 192). When these factors are ectopically overexpressed, they may directly activate endogenous *Oct4*, *Sox2*, and *Nanog*, the products of which in turn contribute to the maintenance of their own gene expression. Similar approaches were attempted by other groups to identify these networks by high-throughput technologies, and more elaborate mechanisms were proposed (97, 136, 187, 220). *Oct4*, *Sox2*, and *Nanog* co-occupy several hundred genes, often at apparently overlapping genomic sites (12, 97). A large multiprotein complex containing *Oct4* and *Nanog* can be obtained by immunoprecipitation (IP) in

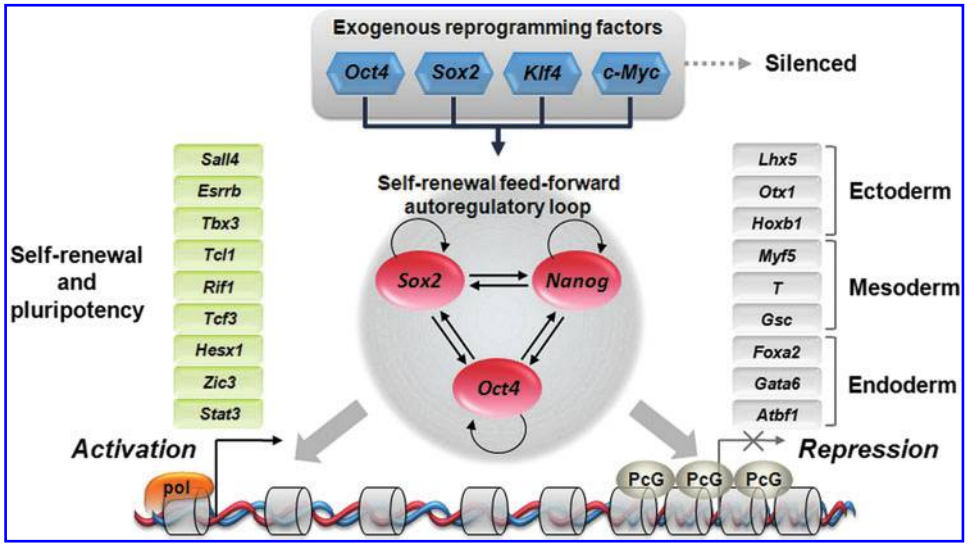

FIG. 3. Core transcriptional circuitry for nuclear reprogramming. The exogenous reprogramming factors, which are eventually silenced by *de novo* DNA methylation, may activate the endogenous *Oct4-Sox2-Nanog* core transcriptional regulatory network, which forms a feed-forward autoregulatory loop that positively activates the RNA polymerase II (pol) to initiate transcription of the self-renewal maintaining genes and negatively to repress the differentiation-promoting genes by the actions of Polycomb family (PcG) proteins for modifying chromosomal structures. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

ES cells (187). Other pluripotency factors may function in complexes to control their target genes, and this phenomenon may explain why efficient iPS cell generation requires combined overexpression of multiple transcription factors. Not all components of this putative complex are required to initiate the process of reprogramming because exogenous *Nanog* is not necessary for iPS generation. It seems that exogenous *Oct4* and other factors induce expression of endogenous *Nanog* to levels sufficient to accomplish full reprogramming.

The master regulators of pluripotency occupy the promoters of active genes encoding transcription factors, signal-transduction components, and chromatin-modifying enzymes that promote ES cell self-renewal (12, 97). However, these transcriptionally active genes consist of only about half of the targets of *Oct4*, *Sox2*, and *Nanog* in ES cells. These master regulators also co-occupy the promoters of a large set of development-specific transcription factors that are silent in ES cells, but whose expression is associated with lineage commitment and cellular differentiation (12, 97). Silencing of these developmental regulators is almost certainly a key feature of pluripotency, because expression of these developmental factors is associated with commitment to particular lineages. *MyoD*, for example, is a transcription factor capable of inducing a muscle gene-expression program in a variety of cells (28). Therefore, *Oct4*, *Sox2*, and *Nanog* likely help maintain the undifferentiated state of ES cells by contributing to repression of lineage-specification factors.

In addition to *Oct4*, *Sox2*, and *Nanog*, many other factors required for pluripotency have been identified, including *Sall4, Dax1, Essrb, Tbx3, Tcl1, Rif1, Nac1,* and *Zfp281* (62, 97, 187). These pluripotency factors regulate each other to form a complicated transcriptional regulatory network in ES cells (220). For example, *Sall4*, a *spalt* family member, interacts with *Nanog* and co-occupies *Nanog* and *Sall4* enhancer regions. Additionally, *Sall4* also regulates *Oct4* expression by binding to the *Oct4* promoter (200, 214). *Essrb* and *Rif1* are primary targets of both *Oct4* and *Nanog* (97).

Besides their DNA-binding activities, these pluripotency-related proteins are extensively interconnected by protein–protein interaction. A protein-interaction network in mouse ES cells has been constructed by tagging Nanog and then purifying Nanog-associated proteins (187). This mini-interactome is highly enriched for proteins that are required for the survival or differentiation of the ICM and for early development. Many of the genes encoding proteins in the interaction network are targets of *Nanog* or *Oct4* or both, suggesting that the transcriptional network might have a feedback mechanism through the protein-interaction network. The protein-interaction network is linked to several cofactor pathways largely involved in transcriptional repression (187). These data support a model wherein essential factors maintain the pluripotent state by simultaneously activating genes involved in pluripotency and repressing genes important for development (Fig. 3).

Extrinsic signal networks for maintaining pluripotency. ES and iPS cells require extrinsic growth factors for maintenance of pluripotency in culture (Fig. 4), suggesting that pluripotency is an inherently unstable cell state and that ES cells are "primed" for rapid differentiation. Historically, ES cells were cultured in the presence of an underlying feeder-cell layer of mitotically inactivated fetal fibroblast cells, which provides an environment capable of supporting pluripotency and blocking spontaneous differentiation. The necessary factor for self-renewal of mouse ES cells is leukemia inhibitory factor (LIF), a cytokine able to maintain mouse ES cells even in the absence of the fibroblast cell feeder layer (194). LIF is not required for pluripotency of the ICM *in vivo* (125) and is unable to maintain pluripotency in human ES cells, suggesting that alternative mechanisms function in the maintenance of pluripotency within these contexts. Serum is also important for mouse ES cell maintenance, although bone morphogenic protein 4 (BMP4) is able to replace this requirement (140, 207). In addition, Wnt signaling has been found to act synergistically with LIF to maintain pluripotency in mouse ES cells and appears to have a role in human ES cells (129, 146). Autocrine loops of Activin/Nodal signaling have also been implicated in the maintenance of mouse ES cells (129).

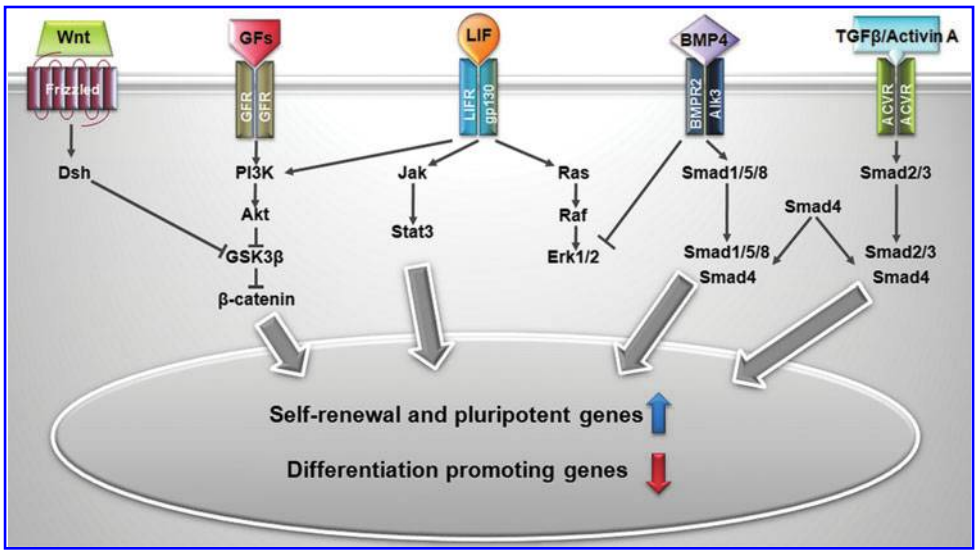

FIG. 4. Extrinsic signal pathways for regulating pluripotency during reprogramming. Extrinsic signaling pathways are required for maintaining the pluripotency of iPS cells as ES cells. Leukemia inhibitory factor (LIF) signaling activates Jak-Stat3 to induce target genes essential for pluripotency and also to inhibit glycogen synthase kinase-3β (GSK3β) to trigger the translocation of β-catenin to the nucleus for activation of pluripotency-related genes. GSK3β also is inhibited by Wnt signaling through the activation of dishevelled (Dsh). PI3K signal can be activated by growth factors (GFs) or LIF, and this PI3K signal also inhibits the GSK3β function of degradation of β-catenin to maintain self-renewal and pluripotency. The extracellular signal-regulated kinase (Erk) pathway, which can also be activated by LIF, triggers the expression of differentiation-promoting genes. For maintaining pluripotency and blocking differentiation, Erk must be inhibited by bone morphogenetic protein (BMP) signals and transforming growth factor (TGF)-β/Activin A signals through Smad protein-complex formation. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Mouse and human ES cells exhibit distinct growth characteristics. Doubling rates for human ES cells are characteristically longer (30 to 40 h) (139) than those for mouse ES cells (17 to 19 h) (172). Human ES cells require maintenance of cell–cell contacts for propagation, and dissociation of human ES cells triggers apoptosis by actomyosin hyperactivation (25, 131). Spontaneous differentiation is initiated from central cells within human ES cell colonies (130), whereas spontaneous differentiation of mouse ES cells occurs at the colony periphery. Human ES cells are routinely cultured on a fibroblast cell feeder layer, but their growth-factor requirements also differ from those of mouse ES cells. The growth factors capable of promoting pluripotency in this system appear to be fibroblast growth factor (FGF2), produced by the feeder cell layer, and insulin-like growth factor (IGF), secreted by human ES cells, which set up interdependent paracrine loops (8). Studies focusing on extrinsic signals required for maintaining human ES cells have reported that FGF2 is sufficient to support growth of these cells on Matrigel, a substrate made up predominantly of laminin and collagen but with additional unknown factors (203). It is likely that extrinsic signals maintaining human ES cells will inhibit BMP signaling to sustain proliferation without differentiation. BMP4 has been shown to regulate pluripotency negatively and to induce trophoblast-like cell formation from human ES cells (204). Activin/Nodal and FGF2 are capable of maintaining human ES cells in the absence of feeder layers and other exogenous factors (183). One role of Activin A in the maintenance of human ES cells has been proposed as inhibition of the BMP4 signaling pathway mediators Smads 1 and 5. Activin A and FGF2 have recently been shown to facilitate derivation and maintenance of pluripotent mouse epiblast-derived cell lines (16, 175).

Although culture conditions and extrinsic growth factors that can support pluripotent cell maintenance have been defined, it is poorly understood how the signaling pathways controlled by these factors maintain the transcription factor network required for pluripotency. In mouse ES cells, LIF activates JAK/STAT signaling and mitogen-activated protein kinase (MAPK) pathways. The choice between pluripotency and differentiation is dependent on a balance between STAT3 and extracellular signal-regulated kinase (ERK) MAPK activity, respectively. In mouse ES cells, BMP4 prevents differentiation through the inhibition of ERK (140) and induction of other inhibitors of differentiation, such as inhibitor of differentiation (Id) proteins (207). STAT3 activates a number of genes that play important roles in pluripotency, including *c-Myc* (20), *Nanog* (22, 27, 118, 168), *Eed* (180), *Jmjd1a* (76), and *GABP*α, which is required for the maintenance of *Oct4* expression (74).

Neither STAT3 nor BMP4 activity is implicated in the pluripotency of human ES cells (60, 204), whereas ERK activity is required for the maintenance of pluripotency (6, 88). Sustained activation of *c-Myc* in human ES cells induces differentiation and apoptosis (166). Thus, consistent with the differing extrinsic requirements, the intracellular signals regulating pluripotency in mouse and human ES cells appeared to be different, despite the conservation of the core transcription-factor networks and functional similarity of the cells. Smith and colleagues (208) reported that the self-renewal of ES cells is maintained by the inhibition of ERK pathway and glycogen synthase kinase 3 (GSK3) after the elimination of extrinsic stimuli, suggesting that ES cells have an innate program for self-replication. More recently, these inhibitions of ERK and GSK3 with treatment of LIF turned human ES cells into a more immature state that shares features of mouse ES cell state, showing that these differences appear to reflect the embryonic origin of mouse and human ES, ICM, and epiblast, respectively, rather than species-specific difference (17, 49).

Epigenetic regulation of chromatin in iPS cells

Given that ES and somatic cells contain almost identical genomic DNA, epigenetic regulation is one of the major influences on their differentiation potential and pluripotency.

To maintain pluripotency in ES cells, differentiation-triggering genes should be inactive. Polycomb group proteins (PcGs) play important roles in silencing these developmental regulators. The PcGs form multiple polycomb PRCs, the components of which are conserved from *Drosophila* to humans (148). The PcG proteins function in two distinct polycomb repressive complexes, PRC1 and PRC2. Genome-wide binding-site analyses have been carried out for PRC1 and PRC2 in mouse ES cells and for PRC2 in human ES cells (13, 84). The genes regulated by the PcG proteins are co-occupied by nucleosomes with trimethylated H3K27. These genes are transcriptionally repressed in ES cells and are preferentially activated when differentiation is induced. Interestingly, the pluripotency factors *Oct4*, *Sox2*, and *Nanog* co-occupy a significant fraction of the PcG protein–regulated genes when acting as transcription factors (13, 84). Most of the transcriptionally silent developmental regulators targeted by *Oct4*, *Sox2*, and *Nanog* are also occupied by the PcG (10, 13, 84), which are epigenetic regulators that facilitate maintenance of the cell state through gene silencing. PRC2 catalyzes H3K27 methylation, an enzymatic activity required for PRC2-mediated epigenetic gene silencing. H3K27 methylation is thought to provide a binding surface for PRC1, which facilitates oligomerization, condensation of chromatin structure, and inhibition of chromatin remodeling activity to maintain silencing. PRC1 also contains a histone ubiquitin ligase, *Ring1b*, whose activity appears likely to contribute to silencing in ES cells (163) (Fig. 5). How the PcGs are recruited to genes encoding developmental regulators in ES cells is not yet understood. Some of the most conserved vertebrate sequences are associated with genes encoding developmental regulators, and some of these may be sites for DNA-binding proteins that recruit PcG proteins.

Recent studies demonstrated that the silent developmental genes that are occupied by *Oct4*, *Sox2*, *Nanog*, and PcG proteins experience an unusual form of transcriptional regulation (44). These genes undergo transcription initiation but not productive transcript elongation in ES cells. The transcription-initiation apparatus is recruited to developmental gene promoters, but RNA polymerase is incapable of fully transcribing these genes, presumably because of repression mediated by the PcG. This explains why the silent genes encoding developmental regulators are generally organized in "bivalent" domains that are occupied by nucleosomes with histone H3K4me3, which is associated with gene activity, and by nucleosomes with histone H3K27me3, which is associated with repression (7, 10, 44).

The presence of inactive RNA polymerase at the promoters of genes encoding developmental regulators may explain why these genes are especially poised for transcription activation during differentiation (13, 84). PcG complexes and associated proteins may serve to pause RNA polymerase machinery at key regulators of development in pluripotent cells and in lineages in which they are not expressed. When the cells are activated, PcGs and nucleosomes with H3K27 methylation are lost (13, 84, 117), allowing the transcription

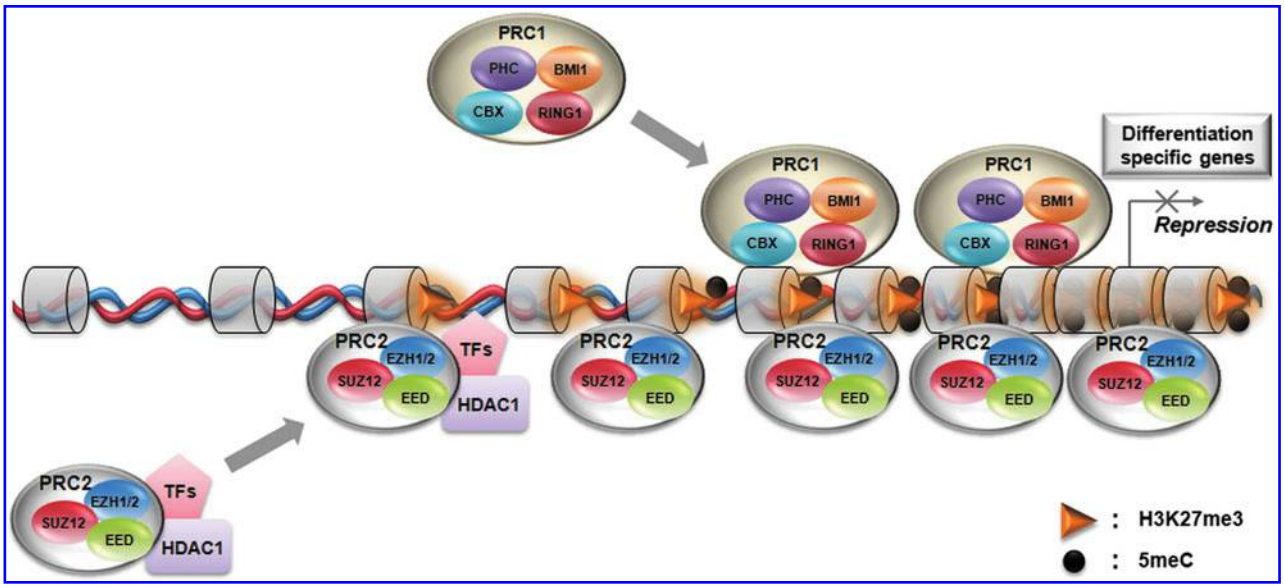

FIG. 5. Polycomb group protein (PcG)-mediated gene silencing during reprogramming. Nuclear reprogramming activates core transcriptional networks, which maintain self-renewal by activating the pluripotent-related genes and by repressing the differentiation-promoting genes by recruitment of polycomb repressive complex (PRC)2, which is responsible for trimethylation of lysine 27 on histone 3 (H3K27me3), in which PRC1 is recruited to repress the target genes, during reprogramming. PRC2 is recruited to specific target sequences by DNA-binding protein, including transcription factors (TFs), and histone deacetylase (HDAC)1 interacts with PRC2. The core PRC2 includes enhancer of zeste homologue 1/2 (EZH1/2), suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED). The modification of H3K27me3 by EZH1/2, which is a H3K27 methyltransferase, in PRC2, provides a binding site recognized by PRC1, which includes B lymphoma MoMLV insertion region 1 (BMI1), Ring1, polyhomeotic C (PHC), and chromobox protein homologue (CBX). For full repression of target genes, binding of both PRC1 and PRC2 at specific sites is required. BMI1 of PRC1 and EZH2 of PRC2 interact with DNA methyltransferase (DNMT), which initiates DNA methylation at CpG sites by converting cytosine to 5-methylcytosine (5meC), and contributes the condensation of the chromosomal structures for blocking the transcription of differentiation-promoting genes. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

apparatus to function and transcribe these genes fully. The mechanisms that lead to selective activation of genes encoding specific developmental regulators are not yet understood (81). Beyond the specific regulation of development-related genes, ES cells maintain chromatin in a highly dynamic and transcriptionally permissive state. Fewer heterochromatin foci are detected in ES cell nuclei compared with differentiated cells. Fluorescence recovery after photobleaching and biochemical analyses reveal that ES cells, compared with differentiated cells, have an increased fraction of loosely bound or soluble architectural chromatin proteins, including core and linker histones and heterochromatin protein HP1. A hyperdynamic chromatin structure is functionally important for pluripotency (115). The status of histone modifications also indicates that the chromatin in ES cells is more transcriptionally permissive than that in differentiated cells. Consistent with the global dynamics of chromatin, ES cell differentiation is associated with a decrease in global levels of active histone marks, such as acetylated histone H3 and H4, and an increase in repressive histone marks, histone H3 lysine 9 methylation (83, 115). Taken together, these unique epigenetic characteristics of ES cells facilitate rapid but regulated transcription, allowing differentiation down different cell-fate pathways as needed by the organism.

MicroRNAs and pluripotency

Noncoding RNA composes a large fraction of vertebrate transcriptomes. Although not all noncoding RNAs are functional, many play important regulatory roles. MicroRNAs (miRNAs) are small noncoding RNAs of ~22 nucleotides in length. They regulate gene expression through at least two distinct mechanisms: degradation of target mRNA transcripts and inhibition of mRNA translation (75). ES cells express a unique set of miRNAs that are downregulated as ES cells differentiate into embryoid bodies. Some of these miRNAs are conserved between human and mouse and are clustered in the genome (57, 165). miRNAs might play a role in the maintenance of pluripotency in iPS cells as well as ES cells (196). *Lin28*, one of the factors used to reprogram human fibroblasts (209), was recently shown to block processing of the *let-7* family microRNA in ES cells (124, 186). *Let-7* family members also have been implicated in the promotion of differentiation of cancer stem cells (78, 209). Thus, *Lin28* may facilitate reprogramming by repressing *let-7*–induced differentiation in fibroblasts. In this sense, inhibition of let-7 miRNAs by transfection of miRNA inhibitors, anti-sense RNAs, increased the efficiency of reprogramming somatic cells into iPS cells (114), whereas introducing of ES-specific miRNAs by transfection of the mature form of miRNAs increased efficiency of iPS cell generation from 0.01% to 0.5% to 0.4% to 0.7%, Recently, Wilson and colleagues (196) showed that *Lin28* is also repressed by *miR-125*, which is another differentiated cell-specific miRNA (196).The inhibitions of both miRNAs, *let-7* and *miR-125*, may have additional effects on reprogramming efficiency. Collectively, these data suggest that miRNAs play important roles in reprogramming cells into iPS cells as well as in maintaining pluripotency.

Other possible mechanisms

Nuclear reprogramming is a complex process that is not fully understood. iPS cells are derived from proliferating somatic cell populations, and reprogramming technology necessitates DNA replication and cell division. Cell-cycle duration of ES and iPS cells is much faster than that of differentiated, cells mainly due to a shortened G_1 phase. In mouse embryonic fibroblasts (MEFs), the G_1 phase lasts 15 to 20 h and temporally accounts for more than 80% of the cell cycle. However, in both mouse and human ES cells, G_1 lasts 2 to 4 h and temporally accounts for only 15% to 20% of the cell cycle. This unique cell-cycle pattern is characterized by hyperphosphorylated RB protein, constitutively high activity of cyclin E and A–associated kinases, and a lack of expression of major CDK inhibitors (162). The role of a shortened G_1 phase in maintaining pluripotency is not clear, although the exclusivity of it among cells that are pluripotent suggests that it is important. On differentiation, the ES cell-cycle pattern quickly switches to an MEF-like pattern (147). Another difference between ES cells and somatic cells is the high level of telomerase activity in ES and many adult stem cells.

Similar to ES cells, iPS cells exhibit a cell cycle with a shortened G_1 phase (103) and elevated telomerase activity (171, 172, 211). During reprogramming, fibroblasts not only become pluripotent, but they also become immortal. Fibroblasts proliferate for a finite period before entering into senescence. In contrast, ES cells and iPS cells do not experience such a limitation. Immortalization requires that at least two barriers be overcome: cellular senescence and telomere shortening (31, 53). *Rb* and *p53* are the key senescence-inducing factors. In ES cells, the *Rb* pathway is constitutively inactivated because of hyperphosphorylation (147), whereas certain aspects of *p53* function are compromised (141). Inhibition of *p53* function and *Ink4/Arf* promotes the reprogramming of somatic cells to pluripotent cells (56, 68, 87, 105, 182).

Cells can enter a so-called replication crisis state in which they undergo apoptosis if their telomere erodes below a critical length (32, 198). To avoid telomere shortening, the activity of telomerase must be upregulated. *c-Myc* directly upregulates the transcription of *Tert*, the gene encoding the enzymatic subunit of the telomerase (199). It is unclear whether elevated telomerase activity in iPS cells is due to ectopic expression of *c-Myc* and how much the resulting change of telomerase activity contributes to reprogramming. It is also unclear how the four factors find ways to inactivate *Rb* and *p53* and to what extent to 11% (4,101). Mouse fibroblasts dedifferentiated *in vitro* from animals made from iPS cells that were established by doxycycline-inducible vectors showed that the efficiency of reprogramming is approximately 2% to 4%, compared with approximately 0.05% in direct reprogramming from fibroblasts (190, 192). One group reported that treatment with VPA increased the reprogramming efficiency up to 11% for mouse fibroblasts (59). However, a different group observed reduced reprogramming when they used the *piggyBac* transposon with VPA treatment (212). The lack of standardization of characterizing iPS cell formation makes comparing reprogramming efficiencies between different laboratories difficult (101). Specific types of somatic cells, such as keratinocytes, stomach cells, and liver cells, are more easily converted than fibroblasts (1, 5). These differences might be caused by variable delivery efficiency of factors or by the cell status, which might be amenable to change. The system of doxycycline-inducible iPS cell generation is useful for measuring the efficiency and kinetics of reprogramming in

murine fibroblasts (14, 159, 190). By withdrawal of doxycycline after various periods, it was shown that transgene expression was essential for a minimum of 10 to 12 days to initiate cellular reprogramming. Longer exposure to doxycycline resulted in an increased number of reprogrammed cells (14, 160). This system revealed the kinetics of pluripotency marker appearance during reprogramming (14, 159). The expression of alkaline phosphatase, which is a key marker for pluripotency, is followed by *Oct4* expression. The stochastic nature of reprogramming could be suggested by the activation of endogenous *Oct4*-driven or *Nanog*-driven reporter genes at different times (113). An interesting recent experiment used "secondary" iPS cells from mice derived from "primary" iPS cells with drug-inducible provirus. The segregation of each factor by germline transmission was identifiable and showed that a single copy of each of the four factors is sufficient to allow optimal reprogramming frequency and kinetics (106). Furthermore, by using the introduction of complementary factors into cell lines carrying single copies of each factor, it has been shown that *Klf4* and *c-Myc* act earlier during reprogramming, conceivably by inducing epigenetic alterations that assist the binding of *Oct4* and *Sox2* to their target genes (106). These conclusions were confirmed and extended by Plath and colleagues (158), showing that the ectopic expression of *c-Myc* is necessary only during the initial stages of reprogramming and can be substituted by VPA. *c-Myc* or VPA might enhance the interaction of *Oct4*, *Sox2*, and *Klf4* with their target genes to repress somatic cell-specific gene expression and initiate the pluripotency-related gene (122, 158, 190).

Two recent studies demonstrated that molecular markers are sequentially expressed during reprogramming of mouse fibroblasts (14, 160). In the first 3 to 5 days after viral transduction, the fibroblast-specific marker THY-1 was downregulated (159), and ES cell-specific marker alkaline phosphatase (AP) was upregulated (14) in a large proportion of fibroblasts. In subsequent days, a population of SSEA-1–positive cells emerged within the previously THY-1–negative or AP-positive cells. Around 10 to 14 days after the initial viral transduction, the endogenous *Oct4* or *Nanog* locus was reactivated in a small percentage of cells within the SSEA-1–positive population. Fully reprogrammed iPS clones that are independent of ectopically expressed factors can be isolated only at this stage.

Clinical Applications of iPS cells

iPS cells, as well as ES cells, can be used as the pluripotent starting material for differentiated cells or tissues in regenerative medicine (85, 120). In a rodent model of Parkinson disease, Wernig and colleagues (193) demonstrated that dopaminergic neurons differentiated from mouse iPS cells could integrate into the host brain and improve symptoms of rats with Parkinson disease. In another study, iPS cells derived from a tail-tip fibroblast of a humanized sickle cell anemia mouse model were generated, and the sickle hemoglobin allele was corrected by introducing the intact human wild-type β-globin gene by homologous recombination (51). These gene-corrected iPS cells provided the basis for the derivation of hematopoietic stem and progenitor cells. Transplantation of gene-corrected hematopoietic stem and progenitor cells into sublethally irradiated mice resulted in robust engraftment and a significant reduction of the sickle cell hemoglobin at 4 and 8 weeks after transplantation. Hemoglobin levels, red cell morphology, and clinical indices of the animals were significantly improved (51).

Although ES cells have pluripotent potential for differentiating into any tissues, ethical considerations delay the establishment of new human ES cells, as we have to destroy early human embryos for the generation of human ES cells, and ES cells do not display the autologous genotypes of patients (38). Human ES cells, however, were recently approved by the United States Food and Drug Administration (FDA) for a human clinical trial. A biotechnology company obtained FDA approval for a phase 1 clinical trial of human ES cell–derived oligodendroglial progenitor cells in subacute thoracic spinal cord injuries (215). Another company received U.S. FDA permission to test its spinal cord stem cells in 12 patients with amyotrophic lateral sclerosis (110, 111, 185).

Human iPS cells provide a suitable system for regenerative therapy by avoiding ethical and immunologic problems while possessing potency similar to that of human ES cells (205). Now that iPS cells can be derived from the patient, human iPS cells might be an ideal cell source for cell therapy. However, numerous challenges remain before iPS cells might be considered for patient-specific therapy: the use of retroviral vectors to introduce reprogramming factors into cells; the use of the oncogene *c-Myc* to achieve reprogramming; and the integration of retroviral vectors into the genome. These manipulations genetically modify the starting cells, and genetically modified cells face significant regulatory hurdles for therapeutic applications. Human iPS cell lines may show significant differences in differentiation potential for specific lineages, as do human ES cells (135). The effects of patient disease on the differentiation potential of patient-derived iPS cells should be addressed.

Another consideration for ensuring the therapeutic effectiveness of cell therapy, regardless of the cell source, will be the environment in which the cells are placed. Cell therapy might not be effective in the context of an autoimmune disease such as type 1 diabetes, because the transplanted cells may be destroyed by the host immune response. Therefore, additional treatments, such as immunosuppressive treatments, might be required. The replaced neural-lineage cells in spinal-cord injury or implanted cardiomyocytes in heart disease may provoke inflammation in the injured spinal cord or damaged heart. In addition, the ischemic environment of the infarcted heart that lacks a normal vascular network and perfusion is a limiting factor for implanted cardiomyocyte survival. Given these complicated microenvironmental changes happening between implanted cells and the diseased host tissues, the development and implementation of iPS-cell–based therapies can be far more complex than simple differentiation into replacement cells. As seen in the use of monoclonal antibodies and the use of viral vectors for gene therapy, novel therapeutics often require many years of development before they come to fruition and deliver results in clinical trials.

Conclusion

iPS cells generated by manipulating pluripotency-related transcription factors shed new light on human regenerative medicine. Although they are limited now in number, a rapid increase of reports outlining potential therapeutic uses of iPS

cells can be expected in the near future. Although it is very promising in early preclinical models, this technology will undoubtedly require further refinement before clinical application will become feasible. To avoid potential drawbacks in clinical applications, such as tumorigenesis of iPS cells generated by Yamanaka's original methods by using retroviral vectors, alternative strategies to generate iPS cells with fewer or absent genome modifications have been successfully developed in a very short period. The potential to create disease- and patient-specific pluripotent cells from fibroblasts or other types of somatic cells of any donor makes it possible to imagine personalized medicine without any immunologic complications. The ability to generate pluripotent cells that differentiate into most cell types will also make possible the generation of *in vitro* models of human disease and drug-screening tools (29, 137, 193). Several human disease models have already been established by using patient-specific iPS cells from patients with Parkinson disease (156), thalassemia (189), Lou Gehrig disease (29), spinal muscular atrophy (33), and familial dysautonomia (82). Moreover, given genetic disorders can be corrected by homologous recombination to restore the functions of affected tissues. This correcting of genetic defects by using iPS cell technology has already been accomplished with Fanconi anemia patient–specific iPS cells (142). iPS technologies now make it possible to create cell lines and differentiated progeny from human donors with different abilities to detoxify and metabolize drugs, suitable for use in the pharmaceutical industry for new drug development.

Although still much remains to be understood about the detail and mechanisms of nuclear reprogramming, and many challenges must be overcome to generate safer iPS cells, the tremendous potential of iPS cell-generating technologies in research and clinical applications will lead to and expand to new and exciting fields of regenerative medicine to treat many incurable diseases. Regenerative medicine is becoming more realistic and closer to clinics by fascinating and emerging new nuclear reprogramming technologies.

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Abbreviations Used

BMP = bone morphogenetic protein
ERK = extracellular signal regulated kinase
ES = embryonic stem
FGF = fibroblast growth factor
GSK = glycogen synthase kinase
ICM = inner cell mass
iPS = induced pluripotent stem
LIF = leukemia inhibitory factor
MAPK = mitogen-activated protein kinase
MEF = mouse embryonic fibroblast
miRNA = microRNA
PcG = polycomb group protein
PRC = polycomb repressive complex
RIS = retroviral integration site
SCNT = somatic cell nuclear transfer
VPA = valproic acid

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