Cell Stem Cell
Review

Strategies and New Developments in the Generation of Patient-Specific Pluripotent Stem Cells

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Generating pluripotent stem cells directly from cells obtained from patients is one of the ultimate goals in regenerative medicine. Two "reprogramming" strategies for the generation of pluripotent stem cells from somatic cells have been studied extensively: nuclear transfer to oocytes and fusion with ES cells. The recent demonstration that, in mouse, nuclear transfer into zygotes can also be effective if the recipient cells are arrested in mitosis provides an exciting new avenue for this type of approach. Patient-specific pluripotent cells could potentially also be generated by the spontaneous reprogramming of bone marrow cells, spermatogonial cells, and parthenogenetic embryos. A third overall type of strategy arose from the demonstration that pluripotent stem (iPS) cells can be generated from mouse fibroblasts by the introduction of four transcription factors (Oct-3/4, Sox2, c-Myc, and KLF4). Recent work has underlined the potential of this strategy by improving the efficiency of the process and demonstrating that iPS cells can contribute to many different tissues in vivo, including the germline. Taken together, these studies underscore the crucial roles of transcription factors and chromatin remodeling in nuclear reprogramming.

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocyst stage embryos, and they have the unique capacity to proliferate extensively while maintaining pluripotency (Evans and Kaufman, 1981; Martin, 1981). ES cell lines can also be generated from human blastocyst embryos (Thomson et al., 1998) and are considered promising donor sources for cell transplantation therapies for diseases such as juvenile diabetes, Parkinson's disease, and heart failure. However, as for organ transplants, tissue rejection remains a significant concern for ES cell transplantation. Another concern is the use of human embryos. One possible means to avoid these issues is by reprogramming the nuclei of differentiated cells to ES cell-like, pluripotent cells.

Currently, three methods have been reported to induce pluripotency artificially in mouse somatic cells (Figure 1). ES-like cells can also be established by long-term culture of bone marrow cells, and pluripotent stem cells can be generated from adult germ cells, either by the in vitro culture of spermatogonial cells or by the parthenogenesis of unfertilized eggs. This review discusses the potential of these strategies to generate tailor-made pluripotent stem cells and the role of transcription factors in the reprogramming process.

Reprogramming by Nuclear Transfer

Successful nuclear transfer was first reported in 1952 by Briggs and King, who showed that nuclei from blastula stage embryos into enucleated Rana pipiens eggs resulted in normal hatched tadpoles (Briggs and King, 1952). Gurdon and colleagues then succeeded in producing fertile adult frogs by transferring tadpole intestinal cell nuclei into enucleated Xenopus laevis eggs in 1996 (reviewed in Gurdon and Byrne [2003]). However, when they transferred the nuclei from adult somatic cells, animals developed to the tadpole but thereafter did not develop further toward the adult stage.

Due to the smaller cell size, nuclear transfer in mammals is more technically demanding. In 1975, Bromhall reported development to the morula stage following the nuclear transfer of rabbit morula cell nuclei into enucleated rabbit eggs, albeit with low efficiency (Bromhall, 1975). The successful nuclear transfer of embryonic donor cell nuclei, which produced adult progeny, was subsequently reported in various mammalian species (Gurdon and Byrne, 2003). However, it proved difficult to generate cloned animals by nuclear transfer from differentiated cells into eggs.

A breakthrough came in 1996, when Wilmut and colleagues produced an adult sheep, famously known as "Dolly," using nuclei derived from follicle cells (Wilmut et al., 1997). Subsequently, somatic cloning was successfully performed in other species, such as the cow, mouse, goat, pig, cat, and rabbit (Gurdon and Byrne, 2003). Furthermore, Jaenisch and colleagues generated mice from B lymphocytes that had undergone immunoglobulin rearrangement (Hochedlinger and Jaenisch, 2002). However, this process required a two-step strategy to obtain

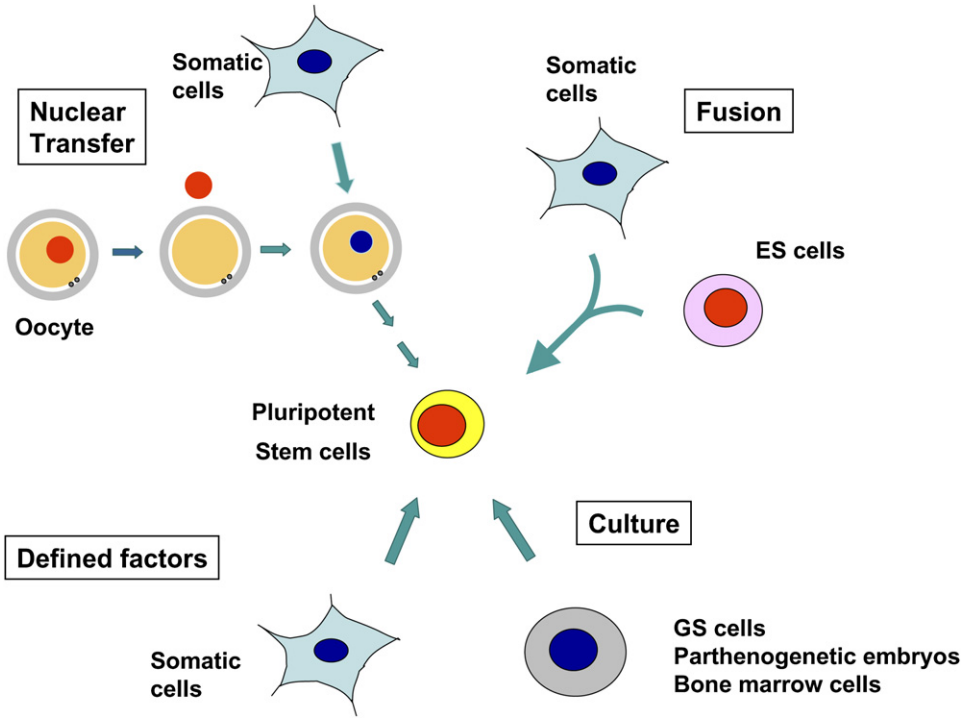

Figure 1. Currently Available Methods to Generate Pluripotent Stem Cells from Adult Somatic or Germ Cells
In mouse models, three methods have been reported to generate pluripotent stem cells from somatic cells: nuclear transfer, fusion, and forced expression of defined factors. Also reported is the generation of chimera-competent pluripotent stem cells after the long-term culture of bone marrow cells. In addition, pluripotent stem cells can be established from mouse adult germ cells: multipotent GS cells and parthenogenetic ES cells.

mice from the terminally differentiated lymphocytes; ES cells were derived from cloned embryos, and mice were then made from those ES cells. The same group observed the highest success rates for cloned animals from ES cells and neural stem cells (Blelloch et al., 2006). Therefore, a reverse correlation between the degree of cell differentiation and the reprogramming efficiency seems to be general phenomena in mammals and amphibians.

In contrast to the extremely low efficiency of obtaining cloned animals, ES cells can be generated from cloned mouse blastocysts with comparable efficiency to those from normal embryos (Wakayama et al., 2001). These nuclear transfer (nt) ES cells might provide a means of avoiding immune rejection after transplantation therapy (Hochedlinger and Jaenisch, 2003), if applicable in human. In 2005, a group in Korea reported that they had successfully generated ntES cells from the skin cells of patients suffering from spinal cord injury and juvenile diabetes (Hwang et al., 2004, 2005). However, their data were later shown to be fabricated, and in fact, they were unable to generate a single ntES cell line from more than 2000 human eggs, thus indicating that generating ntES cells in humans is technically demanding.

A significant issue when considering the potential of nuclear transfer strategies for generating patient-specific human ES cell lines is the availability of human oocytes. However, exciting new work in mouse suggests that it may be possible to devise new strategies that avoid the oocyte requirement. Egli et al. (2007) have found that it is possible to generate pluripotent cells by nuclear transfer using adult somatic cells as donors and zygotes as recipients. Their new protocol involves arresting the recipient zygote in mitosis using drug treatment, removing its chromosomes and replacing them with donor-derived mitotic chromosomes. The mitotic arrest is key, because transfer to interphase zygotes is not effective for donor nuclei beyond the four-cell embryo stage. Using this method, Egli et al. were able to produce embryonic stem cell lines from embryonic and somatic donor cells, and they demonstrated full reprogramming by generating chimeric embryos with germline transmission. Currently, this method has only been demonstrated with mouse zygotes. However, it does raise the possibility that discarded human IVF embryos could potentially be used as recipients for human ntES cell derivation instead of oocytes and even, hypothetically, that mitotic cytoplasm from current hES cell lines might have more effective reprogramming activity than the previously tested interphase extracts.

Several proteins have been shown to play roles in reprogramming in frog oocytes, and their identities may well give clues to the overall requirements for reprogramming in other species as well. These include ISWI, which is involved in protein exchange between the transferred nucleus and the oocyte cytoplasm (Kikyo et al., 2000), and Brg1, which is required for the activation of Oct-3/4, a transcription factor specifically expressed in undifferentiated

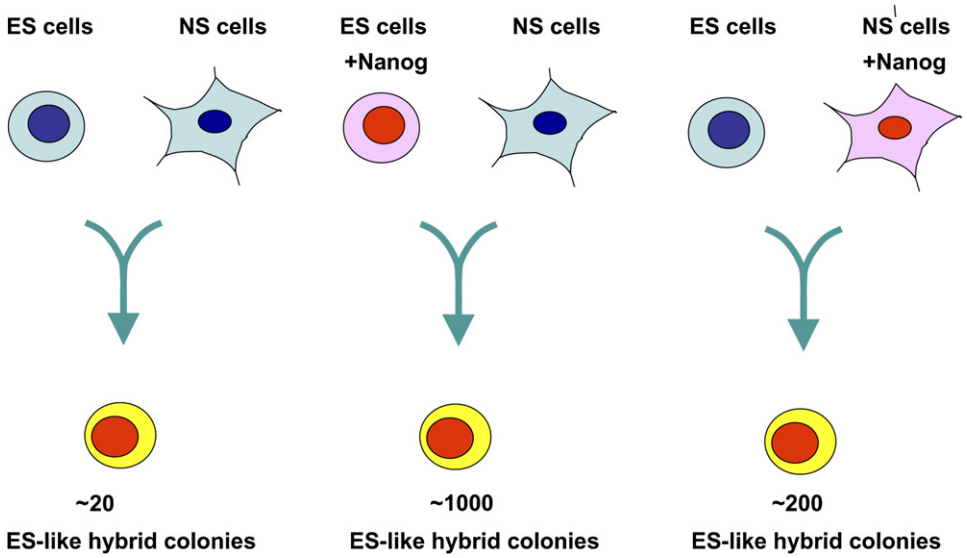

Figure 2. Nanog-Mediated Enhancement of Reprogramming by Fusion with ES Cells
Nanog-overexpressing mouse ES cells showed a marked increase in reprogramming activity after fusion with neural stem (NS) cells. The forced expression of Nanog in NS cells was found to be less effective.

cells (Hansis et al., 2004). Both ISWI and Brg1 are chromatin remodeling ATPases, thus indicating the crucial role of chromatin remodeling in nuclear reprogramming. In addition, the germ cell proteins FRGY2a and FRGY2b reversibly disassemble somatic nucleoli in egg cytoplasm (Gonda et al., 2003), whereas the egg protein nucleophosmin may be involved in chromatin decondensation (Tamada et al., 2006). In devising their new strategy, Egli et al. (2007) reasoned that one difference between oocytes and zygotic cells as potential recipients could be that required factors such as these might become localized to the nucleus during interphase. However, during mitosis the factors could be released and thus available to contribute to reprogramming.

Reprogramming by Fusion with ES Cells

In 1976, Miller and Ruddle demonstrated thymocytes fused with embryonic carcinoma (EC) cells to show pluripotency (Miller and Ruddle, 1976), and similar results were later obtained by electrofusion with mouse ES cells (Tada et al., 2001). Transplantation of these cells into nude mice results in formation of teratomas consisting of various tissues from all three germ layers, confirming the pluripotency of these cells. More recently, reprogramming by fusion with human ES cells was reported (Cowan et al., 2005; Yu et al., 2006).

Whether somatic genomes are fully reprogrammed by fusion remains to be resolved. In thymocyte-ES hybrid cells, the promoter regions of several genes, including Oct-3/4, in the thymocyte genome acquired ES-like epigenetic status, including histone acetylation and methylation (Kimura et al., 2004). Therefore, at least a part of the somatic genome is reprogrammed by fusion. Genome-wide gene expression analyses and chromatin immunoprecipitation analyses will reveal the extent to which somatic genome is reprogrammed by fusion with ES cells. Tada and colleagues recently developed a system to remove a selected chromosome from hybrid cells (Matsumura et al., 2007). They showed that removal of ES cell-derived chromosomes containing *Nanog*, which encodes a transcription factor important for pluripotency (Chambers et al., 2003; Mitsui et al., 2003), did not affect the pluripotency of hybrid cells. The final proof of complete reprogramming would be to show that such hybrid cells remain pluripotent even after removal of all of the ES cell-derived chromosomes.

Rejection upon implantation remains an issue with hybrid cells because of the ES cell-derived chromosomes. Although the selective elimination of specific chromosomes (Matsumura et al., 2007) is an important step to circumvent this problem, removing all of the ES cell-derived chromosomes would be technically challenging. Alternatively, ES cell-derived chromosomes carrying the major histocompatibility complex (MHC) loci could be removed selectively to avoid, or at least reduce, rejection reactions. This possibility should be experimentally investigated. Other groups have attempted to reprogram somatic cells with ES cell extracts (Taranger et al., 2005).

Little is known about the molecular mechanisms underlying reprogramming by fusion with ES cells. The factors responsible may reside in the nucleus (Do and Scholer, 2004) or in cytoplasm (Strelchenko et al., 2006). Smith and colleagues observed marked increase in reprogrammed cell colonies when they fused neural stem cells with ES cells that overexpress the transcription factor Nanog (Figure 2) (Silva et al., 2006). Nanog is a homeobox transcription factor specifically expressed in early mouse embryos and ES cells (Chambers et al., 2003; Mitsui

et al., 2003). Overexpression of Nanog in mouse ES cells enables them to undergo self-renewal in the absence of leukemia inhibitory factor (LIF) (Chambers et al., 2003; Mitsui et al., 2003). Similarly, overexpression of Nanog in human ES cells enabled growth without feeder cells (Darr et al., 2006). *Nanog* null embryos show disorganization of the extraembryonic tissues at E5.5, with no discernible epiblast or primitive ectoderm (Mitsui et al., 2003) (Table 1). ES cells lacking *Nanog* can be derived, but they tend to differentiate spontaneously into extraembryonic endoderm lineages even in the presence of LIF. Another group reported that even heterozygous *Nanog* mutant ES cells were unstable and susceptible to spontaneous differentiation (Hatano et al., 2005). RNAi-mediated knockdown of *Nanog* led to differentiation in both mouse (Ivanova et al., 2006) and human (Zaehres et al., 2005) ES cells. These data underscore the crucial role that Nanog plays in the induction and maintenance of pluripotency.

Spontaneous Reprogramming by Culture

ES cells do not exist physiologically. They are ''transformed'' and ''reprogrammed'' during the course of long-term culture of ICM. Similarly, pluripotent embryonic germ (EG) cells can be generated by long-term culture of primordial germ cells (PGC) (Matsui et al., 1992). Therefore, it might be possible to obtain pluripotent stem cells by culturing other types of cells. In fact, Verfaillie and associates reported the development of pluripotent stem cells after the prolonged culture of bone marrow-derived cells (Jiang et al., 2002). They designated these cells multipotent adult progenitor cells (MAPCs). MAPCs are different from ES cells in that they require a low serum concentration and have to be maintained at a low density. Nevertheless, MAPCs can differentiate into various types of cells in vitro, and in one case, a single MAPC injected into a mouse blastocyst contributed to mouse development and formed chimeras. However, definitively proving the generality and reproducibility of MAPCs still awaits further experiments by other laboratories.

Shinohara and associates demonstrated that pluripotent stem cells can be generated during the course of culture of germline stem (GS) cells from neonate mouse testes, which they designated multipotent germline stem (mGS) cells (Kanatsu-Shinohara et al., 2004). While the culture conditions of GS cells are different from those of ES cells, mGS cells are maintained with ES cell culture condition. mGS cells are similar to ES cells in morphology, proliferation, and teratoma formation and are even competent to form germline chimeras. The efficiency of mGS cell establishment is extremely low and requires GS cells from more than 30 testes. The efficiency may increase by the loss of p53 function. Germline competent pluripotent stem cells were also generated from adult mouse testes, which were designated multipotent adult germline stem (maGS) cells (Guan et al., 2006). Male-specific imprints may result in an impaired differentiation ability and transformation phenotype (Hernandez et al., 2003). Although mGS cells showed a different imprinting pattern from GS cells and chimeric mice from mGS cells seem to be normal (Kanatsu-Shinohara et al., 2004), long-term observations are required to examine the tumorigenicity of mGS cell-derived differentiated cells.

Reprogramming from spermatogonial stem cells cannot apply to females. As an alternative, however, histocompatible ES cells can also be generated by parthenogenesis. Since mammalian embryonic development requires paternal gene expression, parthenogenetic embryos die at early developmental stages after implantation. However, parthenogenetic ES cells have been successfully obtained in mice and primates that showed pluripotency (Allen et al., 1994; Cibelli et al., 2002). Most of the parthenogenetic ES cells, however, show a loss of heterozygosity in the MHC and thus may be rejected by natural killer (NK) cells that recognize the lack of one set of histocompatibility antigens. Daley and colleagues developed methods to maintain both of the maternal MHC loci in mouse parthenogenetic ES cells (Kim et al., 2007). As with mGS cells, imprinting remains a concern with parthenogenetic ES cells, since female-specific imprinting is associated with premature senescence in fibroblasts (Hernandez et al., 2003).

Reprogramming by Defined Factors

Successful reprogramming of somatic cells by fusion with ES cells indicates that ES cells have factors that induce pluripotency. It seemed likely that these pluripotency-inducing factors also play important roles in the maintenance of pluripotency. Based on this hypothesis, 24 different candidate factors were tested for their ability to induce pluripotency. This analysis led to the demonstration that retrovirus-mediated introduction of four transcription factors (Oct-3/4, Sox2, c-Myc, and KLF4) into mouse embryonic or adult fibroblasts and selection for the expression of *Fbx15*, a target of Oct-3/4 and Sox2, resulted in the generation of induced pluripotent stem (iPS) cells, which are similar to ES cells in morphology, proliferation, and teratoma formation (Takahashi and Yamanaka, 2006). Introduction of the three factors excluding Sox2 results in cells somewhat similar to ES cells in morphology and proliferation but lacking pluripotency. Fbx15-selected iPS cells are, however, significantly different from ES cells in gene expression and DNA methylation patterns. When transplanted into blastocysts, iPS cells only give rise to chimeric embryos, but not adult or germline competent chimeras. These data indicate that reprogramming in Fbx15-selected iPS cells is incomplete.

Very recently, however, a significant improvement has been demonstrated (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Three groups generated iPS cells competent for adult and germline chimeras by using a more stringent selection marker, *Nanog*. One also demonstrated germline transmission to progeny mice (Okita et al., 2007). Although both *Fbx15* and *Nanog* are targets of Oct-3/4 and Sox2, the former is dispensable for pluripotency, while the latter plays crucial roles. Nanog-selected iPS cells are almost indistinguishable

Table 1. Comparison of the Five Factors in the Phenotype of Loss-of-Function and Gain-of-Function Experiments

	Knockout ES Cells	Knockout Embryos	Overexpression in ES Cells
Oct-3/4	Cannot be established	No epiblast	Induces differentiation
	Niwa et al., 2000	Nichols et al., 1998	Niwa et al., 2000
Sox2	Cannot be established	No epiblast	Does not induce differentiation
	Masui et al., 2007	Avilion et al., 2003	Does not induce LIF independency
			M. Nakagawa and S.Y., unpublished data
c-Myc	Can be established	Normal epiblast	Does not induce differentiation
	Normal self-renewal		Induces LIF independency
	Davis et al., 1993	Davis et al., 1993	Cartwright et al., 2005
KLF4	Not reported	Normal epiblast	Does not induce differentiation
		Katz et al., 2002	Induces LIF independency
			Y. Tokuzawa, M. Nakagawa, and S.Y., unpublished data
Nanog	Can be established	No epiblast	Does not induce differentiation
	Spontaneous differentiation		Induces LIF independency
	Mitsui et al., 2003	Mitsui et al., 2003	Chambers et al., 2003; Mitsui et al., 2003

from ES cells in global gene expression (Okita et al., 2007), DNA methylation, and histone modification (Maherali et al., 2007; Wernig et al., 2007). Female Nanog-selected iPS cells showed reactivation of a somatically silenced X chromosome and underwent random X inactivation upon differentiation (Maherali et al., 2007). *Oct-3/4* can also be used as a stringent selection marker for iPS cell induction (Wernig et al., 2007). These data demonstrated that full reprogramming can be achieved by expression of the four factors and using an appropriate selection procedure.

The Four Factors

Oct-3/4

Oct-3/4 was identified as a novel Oct family protein specifically expressed in EC cells, early embryos, and germ cells (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990). The Oct family transcription factors contain the POU domain, an ~150 amino acid sequence conserved among Pit-l, Oct-1, Oct-2, and uric-86. Oct-3/4 and other POU proteins bind to the octamer sequence (ATTA/TGCAT). Expression of *Oct-3/4* is restricted in the blastomeres of the developing mouse embryo, the ICM of blastocysts, the epiblast, and germ cells. It is also expressed in pluripotent stem cells, including ES cells, EG cells, EC cells, and mGS cells.

Oct-3/4 null embryos die in utero during the periimplantation stages of development (Nichols et al., 1998). Although these embryos are able to reach the blastocyst stage, in vitro culture of the ICM of homozygous mutant blastocysts produces only trophoblast lineages (Table 1). ES cells can not be derived from *Oct-3/4* null blastocysts (Table 1). Suppression of *Oct-3/4* resulted in spontaneous differentiation into the trophoblast lineages in both mouse (Niwa et al., 2000) and human ES cells (Zaehres et al., 2005). These data demonstrate the essential roles of Oct-3/4 in the maintenance of pluripotency.

Oct-3/4 also plays important roles in promoting differentiation. Only a 50% increase in the Oct-3/4 protein in mouse ES cells resulted in spontaneous differentiation into primitive endoderm and mesoderm (Niwa et al., 2000), which is consistent with the transient increase in *Oct-3/4* expression during the initial stage of primitive endoderm differentiation from ICM (Table 1). Oct-3/4 also plays a role in the neural (Shimozaki et al., 2003) and cardiac (Zeineddine et al., 2006) differentiation from mouse ES cells. Hence, the level of Oct-3/4 expression is an important determinant of the cell fate in mouse ES cells.

Jaenisch and associates showed that activation of Oct-3/4 in gastric epithelial tissues results in dysplastic growth that is dependent on continuous transgene expression (Hochedlinger et al., 2005). Dysplastic lesions show an expansion of progenitor cells and an increased β-catenin transcriptional activity. In the intestine, *Oct-3/4* expression causes dysplasia by inhibiting cellular differentiation. These data indicate that specific adult progenitors may remain competent to respond to key embryonic signals, and they might also be a driving force in tumorigenesis.

Sox2

Sox2 was identified as a Sox (SRY-related HMG box) protein expressed in EC cells (Yuan et al., 1995). The high mobility group (HMG) domain is a DNA binding domain conserved in abundant chromosomal proteins including HMG1 and HMG2, which bind DNA with little or no sequence specificity, and in sequence-specific transcription factors, including SRY, SOX, and LEF-1. All SOX factors appear to recognize a similar binding motif, A/TA/TCAAA/TG. Like *Oct-3/4*, *Sox2* also marks the pluripotent lineage of the early mouse embryo; it is

expressed in the ICM, epiblast, and germ cells. Unlike *Oct-3/4*, however, *Sox2* is also expressed by the multipotential cells of the extraembryonic ectoderm (Avilion et al., 2003). In addition, *Sox2* expression is associated with uncommitted dividing stem and precursor cells of the developing central nervous system (CNS), and it can be used to isolate such cells (Li et al., 1998; Zappone et al., 2000).

Sox2 null embryos die at the time of implantation due to a failure of epiblast (primitive ectoderm) development (Avilion et al., 2003). Homozygous mutant blastocysts appear morphologically normal, but undifferentiated cells fail to proliferate when blastocysts are cultured in vitro, and only trophectoderm and primitive endoderm-like cells are produced (Table 1). The deletion of *Sox2* in ES cells results in trophectoderm differentiation (Masui et al., 2007). Therefore, Sox2, like Oct-3/4, is essential for the maintenance of pluripotency.

Sox proteins, in general, regulate their target genes by associating with specific partner factors (Kamachi et al., 2000; Wilson and Koopman, 2002). Sox2 forms a heterodimer with Oct-3/4 and synergistically regulates *Fgf4* (Yuan et al., 1995), UTF1 (Nishimoto et al., 2003), and *Fbx15* (Tokuzawa et al., 2003). In addition, similar coregulation by Sox2 and Oct-3/4 has been reported in the regulation of *Sox2* and *Oct-3/4* themselves (Chew et al., 2005; Okumura-Nakanishi et al., 2005; Tomioka et al., 2002), as well as *Nanog* (Kuroda et al., 2005; Rodda et al., 2005). Genome-wide chromatin immunoprecipitation analyses demonstrated that Oct-3/4, Sox2, and Nanog share many target genes in both mouse and human ES cells (Boyer et al., 2005; Loh et al., 2006). Surprisingly, *Sox2* deletion in mouse ES cells is rescued by the cDNA introduction of not only *Sox2* but also *Oct-3/4*, thus suggesting that the primary function of Sox2 might be to maintain *Oct-3/4* expression (Masui et al., 2007). The authors speculated that the expression of Oct-Sox target genes, such as *Fgf4* and *UTF1*, can be maintained by other Sox family proteins.

c-Myc

c-Myc is one of the first proto-oncogenes found in human cancers (Dalla-Favera et al., 1982). The N terminus of Myc binds to several proteins, including TRRAP, which are components of the TIP60 and GCN5 histone acetyltransferase complexes, and TIP48 and TIP49, which contain ATPase domains (Adhikary and Eilers, 2005). The C terminus of the Myc protein contains the basic region/helix-loop-helix/leucine zipper (BR/HLH/LZ) domain, through which Myc binds to a partner protein, Max. The Myc-Max dimers bind to a DNA sequence (CACA/GTG), which is a subset of the general E box sequence (CANNTG) that is bound by all bHLH proteins. In addition to binding to DNA, the C terminus of Myc is also involved in transactivation through binding to CBP and p300, which have histone acetylase activities.

Mouse embryos homozygous for a *c-Myc* deletion die between 9.5 and 10.5 days of gestation (Davis et al., 1993). Pathologic abnormalities include the heart, pericardium, neural tube, and delay or failure in turning of the embryo. The lethality of *c-Myc*$^{-/-}$ embryos is also associated with profound defects in vasculogenesis and primitive erythropoiesis (Baudino et al., 2002). In addition, *c-Myc*$^{-/-}$ ES cells are defective in vascular differentiation. However, earlier-stage embryos are apparently normal despite the deficiency of *c-Myc*, and *c-Myc*$^{-/-}$ ES cells show a normal proliferation and self-renewal (Table 1). In contrast, the dominant-negative form of c-Myc induces differentiation in mouse ES cells (Cartwright et al., 2005), thus suggesting that the c-Myc deficiency might be compensated by the related proteins N-Myc and L-Myc.

The most surprising new finding is that there are as many as 25,000 Myc binding sites in vivo in the human genome (Cawley et al., 2004; Fernandez et al., 2003; Li et al., 2003). These studies revealed that only a minority portion of the in vivo binding sites of Myc-Max have the consensus CACA/GTG sequence. The direct binding of the Myc-Max dimer to noncanonical sequences is observed in the human Werner syndrome gene, *WRN* (Grandori et al., 2003). Alternatively, the Myc-Max dimer is recruited to nonconsensus binding sites through an interaction with other transcription factors, such as Miz1 (Peukert et al., 1997). By binding to numerous sites in genome, c-Myc may modify the chromatin structure (Knoepfler et al., 2006) and regulate the expression of noncoding RNAs (O'Donnell et al., 2005).

KLF4

KLF4 belongs to Krüppel-like factors (KLFs), zinc-finger proteins that contain amino acid sequences resembling those of the *Drosophila* embryonic pattern regulator Krüppel (Schuh et al., 1986). *KLF4* is highly expressed in differentiated, postmitotic epithelial cells of the skin and the gastrointestinal tract. *KLF4* is expressed in fibroblasts including MEF and NIH3T3 cells (Garrett-Sinha et al., 1996; Shields et al., 1996). Shields et al. found that, in NIH3T3 cells, *KLF4* mRNA is found in high levels in cells during growth arrest and is nearly undetectable in cells that are in the exponential phase of proliferation (Shields et al., 1996). In addition, *KLF4* is highly expressed in undifferentiated mouse ES cells (Y. Tokuzawa, M. Nakagawa, and S.Y., unpublished data).

KLF4 can function both as a tumor suppressor and an oncogene. In cultured cells, the forced expression of KLF4 results in the inhibition of DNA synthesis and cell cycle progression (Chen et al., 2001; Shields et al., 1996). *KLF4* null embryos develop normally (Table 1), but newborn mice die within 15 hr and show an impaired differentiation in the skin (Segre et al., 1999) and in the colon (Katz et al., 2002), thus indicating that it plays a crucial role as a switch from proliferation to differentiation. A conditional knockout mouse model suggests that KLF4 plays a role as a tumor suppressor in gastrointestinal cancers (Katz et al., 2005). KLF4, however, is overexpressed in squamous cell carcinomas and breast cancers (Foster et al., 2000; Foster et al., 1999). Moreover, the induction of KLF4 in basal keratinocytes blocks the proliferation-differentiation switch and initiates squamous epithelial dysplasia (Foster et al., 2005). Therefore, KLF4 is associated with both tumor suppression and oncogenesis.

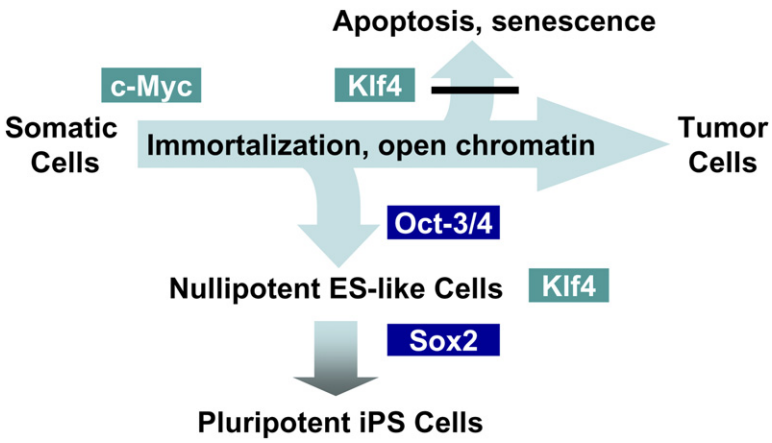

Figure 3. Putative Roles of the Four Factors in the Induction of iPS Cells
Pluripotent stem cells are immortal and have open and active chromatin structure. It is likely that c-Myc induces these two important properties. However, c-Myc also induces apoptosis and senescence, which are probably suppressed by KLF4. Oct-3/4 probably changes the cell fate from tumor cells to ES-like cells. To establish pluripotency, Sox2 is also required.

Recently, the molecular mechanisms underlying the dual functions of KLF4 were partially elucidated (Rowland et al., 2005). They showed that ectopic expression of KLF4 suppresses cell proliferation, but ablation of only one of its target genes, *p21*, is sufficient to rescue the cytostatic effect of KLF4. In *p21* null cells, KLF4 promotes cell proliferation by downregulating p53 (Rowland et al., 2005). Therefore, p21 may function as a switch that determines the outcome of KLF4 signaling (Rowland and Peeper, 2006).

The inactivation of STAT3 in mouse ES cells markedly decreases *KLF4* expression, and forced expression of KLF4 enables LIF-independent self-renewal (Table 1; Y. Tokuzawa, M. Nakagawa, and S.Y., unpublished data). Another group also reported a positive effect of KLF4 in self-renewal of mouse ES cells (Li et al., 2005). In addition, KLF4 cooperates with Oct-3/4 and Sox2 to activate the *Lefty1* core promoter in mouse ES cells (Nakatake et al., 2006).

How Do the Four Factors Induce Pluripotent Stem Cells?

ES cells and other pluripotent stem cells are similar to tumor cells in many aspects. ES cells are immortal and proliferate rapidly. They form tumors (teratomas) when transplanted into immune-deficient mice. Pluripotent stem cells are, in a sense, reversibly "transformed" cells. The transformation takes place during the course of in vitro culture or in their original embryonic cells (ICM for ES cells and PGC for EG cells). Some genes, such as E-Ras (Takahashi et al., 2003), are activated in this process, making ES cells and EG cells distinct from their originating cells.

Taking this into account, it makes sense that the induction of pluripotent stem cells (iPS cells) from somatic cells also requires transformation by the two tumor-associated gene products, c-Myc and KLF4 (Figure 3). The Myc protein can elicit various aspects of transformation (Adhikary and Eilers, 2005). However, it also elicits p53-dependent apoptosis in primary fibroblasts. KLF4 might therefore be required to suppress p53 and c-Myc-induced apoptosis (Rowland et al., 2005). KLF4, in turn, activates *p21* and suppresses proliferation. c-Myc can alleviate this cytostatic effect of KLF4 by suppressing *p21*. Thus, the balance between c-Myc and KLF4 might play a critical role in the transformation process in iPS cells.

It is likely that the function of c-Myc is not confined to the induction of cellular transformation. Pluripotent stem cells have open and active chromatin structures (Meshorer et al., 2006). Myc proteins probably loosen the chromatin structure of somatic cells by binding to numerous sites throughout the genome and by recruiting multiple histone acetylase complexes (Knoepfler et al., 2006). Consistent with this model, even partially reprogrammed Fbx15-iPS cells show hyperacetylated histones in the promoter regions of several ES cell-specific genes (Takahashi and Yamanaka, 2006).

Forced expression of c-Myc and KLF4 alone would result in the generation of tumor cells, but not pluripotent stem cells. It is likely that Oct-3/4 directs the cell fate away from tumor cells toward ES-like cells. The effects of c-Myc on chromatin structure should enable Oct-3/4 to activate or suppress appropriate target genes. Oct-3/4, however, is not sufficient to induce pluripotency. Sox2 is also required to synergistically activate multiple target genes. KLF4 may also function as a cofactor of Oct-3/4 and Sox2 (Nakatake et al., 2006). The finding that a Sox2 deletion in mouse ES cells can be rescued by an Oct-3/4 transgene (Masui et al., 2007) seems to conflict with this finding. However, it is possible that maintenance of pluripotency can be achieved by other Sox proteins that exist at low levels in ES cells, while the induction of pluripotency requires much higher amounts of Sox proteins.

Another key issue is the low efficiency of iPS cell induction. Less that 1% of the cells that have incorporated the four retroviruses can become iPS cells. One possible explanation is that the origin of iPS cells in fact originate from tissue stem or progenitor cells coexisting in the fibroblast culture. An observation consistent with this possibility is that ectopic expression of Oct-3/4 in the stomach and intestine block the differentiation of progenitor cells (Hochedlinger et al., 2005). Another possibility is that, in addition to the four factors, another factor or factors also need to be activated by retroviral insertion. Candidates

Table 2. Pros and Cons of Currently Available Methods to Generate Pluripotent Stem Cells from Adult Cells

	Requirement of Embryos or Donor Oocytes	Report in Human	Chromosome Content	Imprinting	Reference
Nuclear transfer	Yes	No	Normal diploid; no gene transfer	Normal?	Rideout et al., 2000
Fusion with ES cells	Yes	Yes	Tetraploid	Normal?	Tada et al., 2001
iPS cells	No	No	Retroviral integration	Normal?	Takahashi and Yamanaka, 2006
MAPC	No	Yes	Normal diploid; no gene transfer	Normal?	Jiang et al., 2002
mGS cells	No	No	Normal diploid; no gene transfer	Different from ES cells	Kanatsu-Shinohara et al., 2004
Parthenogenetic ES cells	No	No	Normal diploid; no gene transfer	Female specific	Allen et al., 1994

for such factors include the polycomb proteins, which play a critical role in the maintenance of pluripotency (Boyer et al., 2006), and chromatin remodeling factors such as ISWI (Kikyo et al., 2000) and Brg1 (Hansis et al., 2004), which might be involved in nuclear reprogramming in oocytes. The identification of the missing factor(s) may enable more efficient and retrovirus-free generation of iPS cells. Alternatively, iPS cell induction may depend on specific amounts and patterns of the expression of the four factors, which are achieved by chance in a small proportion of the transfected cells. For example, excess Oct-3/4 is detrimental to pluripotency (Niwa et al., 2000). In addition, the balance between c-Myc and KLF4 may also be a crucial factor.

Conclusion

This review has provided an overview of the currently available methods to generate pluripotent stem cells from adult somatic or germ cells. Each method has advantages as well as disadvantages over other methods (Table 2). Nuclear transfer and iPS cells can induce nearly complete reprogramming. In addition, iPS cells are an appealing option, as no embryos or oocytes are required for their generation. However, only fusion with ES cells has been achieved with human cells. Tumorigenicity is a concern for all methods. This issue is especially pertinent for iPS cells, which use retroviruses, and fusion with ES cells, which results in tetraploid cells. In fact, we found that reactivation of c-Myc retrovirus causes tumors in Nanog-iPS cell-derived mice (Okita et al., 2007). At this time it is premature to discuss which method will ultimately be most appropriate for clinical use. It is important to promote thorough and careful basic research on all the methods. Eventually, such studies could potentially even lead to the development of a new, unified technology. It is also important to understand the molecular mechanisms underlying nuclear reprogramming and pluripotency. The factors focused on in this review are likely to play critical roles, but it seems likely that other transcription factors and chromatin-related factors also make important contributions.

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