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REVIEW

Induced pluripotency: history, mechanisms, and applications

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The generation of induced pluripotent stem cells (iPSCs) from somatic cells demonstrated that adult mammalian cells can be reprogrammed to a pluripotent state by the enforced expression of a few embryonic transcription factors. This discovery has raised fundamental questions about the mechanisms by which transcription factors influence the epigenetic conformation and differentiation potential of cells during reprogramming and normal development. In addition, iPSC technology has provided researchers with a unique tool to derive disease-specific stem cells for the study and possible treatment of degenerative disorders with autologous cells. In this review, we summarize the progress that has been made in the iPSC field over the last 4 years, with an emphasis on understanding the mechanisms of cellular reprogramming and its potential applications in cell therapy.

the specialized functions of somatic tissues. For example, only zygotes and blastomeres of early morulas retain the ability to give rise to all embryonic and extraembryonic tissues (Kelly 1977), and are therefore called “totipotent,” while cells of the inner cell mass (ICM) of the blastocyst can give rise to all embryonic but not all extraembryonic tissues, and are hence called “pluripotent.” Cells residing in adult tissues, such as adult stem cells, can only give rise to cell types within their lineage and are called either “multipotent” or “unipotent,” depending on the number of developmental options they have. Upon terminal differentiation, cells entirely lose their developmental potential.

During the 1950s, Briggs and King (Briggs and King 1952; King and Briggs 1955) established the technique of SCNT, or “cloning,” to probe the developmental potential of nuclei isolated from late-stage embryos and tadpoles by transplanting them into enucleated oocytes. This work, together with seminal experiments by Gurdon (Gurdon 1962; Gurdon et al. 1975), showed that differentiated amphibian cells indeed retain the genetic information necessary to support the generation of cloned frogs. The major conclusion from these and subsequent findings was that development imposes reversible epigenetic rather than irreversible genetic changes on the genome during cellular differentiation. The cloning of Dolly the sheep (Wilmut et al. 1997) and other mammals from adult cells, including terminally differentiated cells (Hochedlinger and Jaenisch 2002a; Eggen et al. 2004; Li et al. 2004; Inoue et al. 2005), showed that the genome of even fully specialized cells remains genetically totipotent; i.e., can support the development of an entire organism. However, most cloned animals exhibit subtle to severe phenotypic and gene expression abnormalities, suggesting that SCNT results in faulty epigenetic reprogramming (Wakayama and Yanagimachi 1999; Hochedlinger and Jaenisch 2002b; Humpherys et al. 2002; Ogonuki et al. 2002; Tamashiro et al. 2002; Gurdon et al. 2003).

History of cellular reprogramming

The discovery of induced pluripotency represents the synthesis of scientific principles and technologies that have been developed over the last six decades. These are (1) the demonstration by somatic cell nuclear transfer (SCNT) that differentiated cells retain the same genetic information as early embryonic cells; (2) the development of techniques that allowed researchers to derive, culture, and study pluripotent cell lines; and (3) the observation that transcription factors are key determinants of cell fate whose enforced expression can switch one mature cell type into another. In this section, we briefly summarize these three areas of research and the influence they had on the generation of induced pluripotent stem cells (iPSCs).

Nuclear transfer and the cloning of animals

During mammalian development, cells gradually lose potential and become progressively differentiated to fulfill

Pluripotent cell lines and fusion hybrids

While SCNT is a powerful tool to probe the developmental potential of a cell, it is technically challenging and not well suited for genetic and biochemical studies. Thus, another major advance toward isolating iPSCs was the

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establishment of immortal pluripotent cell lines from teratocarcinomas, tumors of germ cell origin. These cell lines were called embryonal carcinoma cells (ECCs) (Stevens and Little 1954; Kleinsmith and Pierce 1964) and could be clonally expanded in culture while retaining pluripotency (Finch and Ephrussi 1967; Kahan and Ephrussi 1970). Importantly, when ECCs were fused with somatic cells, such as thymocytes, the resulting hybrid cells acquired biochemical and developmental properties of ECCs and extinguished features of the somatic fusion partner (Miller and Ruddle 1976, 1977). The dominance of the pluripotent state over the somatic state in hybrids suggested that soluble *trans*-acting factors must exist in ECCs that can confer a pluripotent state upon somatic cells, and that these factors should be identifiable.

Most ECC lines are aneuploid and contribute only poorly to adult somatic tissues (Brinster 1974) and rarely to the germline (Stewart and Mintz 1982; Bradley et al. 1984) upon injection into blastocysts and subsequent transfer into foster females, a key assay to probe the ability of nonhuman pluripotent cell lines to functionally integrate into tissues of the developing embryo (Table 1; Gardner 1968). The finding that ECCs can also be derived from teratocarcinomas experimentally induced by the transplantation of implantation-stage mouse embryos to extrauterine sites in histocompatible hosts motivated attempts to isolate pluripotent cells directly from such embryos. These efforts led to the derivation of embryonic stem cells (ESCs) from the ICM of mouse blastocysts (Evans and Kaufman 1981; Martin 1981) and, subsequently, also from human embryos (Thomson et al. 1998). In contrast to ECCs, ESCs are karyotypically normal and contribute efficiently to all adult tissues, including the germline. In fact, ESCs are capable of producing entirely ESC-derived animals after injection into tetraploid blastocysts (Nagy et al. 1990; Eggan et al. 2001), which by themselves develop into extraembryonic tissues only but fail to give rise to the embryo proper, thereby passing the most stringent developmental assay available in mice (Table 1). Notably, ESC lines can also be derived from cloned blastocysts generated by SCNT (Cibelli et al. 1998; Munsie et al. 2000; Wakayama et al. 2001), generating so-called NT-ESCs. In contrast to the abnormalities seen in directly cloned animals, NT-ESCs are transcriptionally and functionally indistinguishable from fertilization-derived ESCs, presumably because of a selection of faithfully reprogrammed cells in culture (Brambrink et al. 2006; Wakayama et al. 2006).

Pluripotent cell lines have also been derived from other embryonic and adult tissues upon explantation in culture. For example, epiblast-derived stem cells (EpiSCs) (Brons et al. 2007; Tesar et al. 2007) have been isolated from post-implantation embryos, embryonic germ cells (EGCs) (Matsui et al. 1992; Resnick et al. 1992) have been derived from primordial germ cells (PGCs) of the mid-gestation embryo, and multipotent germline stem cells (mGSCs) have been generated from explanted neonatal (Kanatsu-Shinohara et al. 2004) and adult (Guan et al. 2006; Seandel et al. 2007; Ko et al. 2009) mouse testicular cells. Common to all of these cell types is their origin from either early embryos or germ lineage cells, which

appear to be the only cells that harbor an epigenetic conformation that is permissive for spontaneous conversion into a pluripotent state. A molecular commonality among these cell types is the expression of endogenous Oct4, which may thus serve as a valuable predictive marker for whether or not a cell can give rise to pluripotent cell lines. While ESCs, ECCs, mGSCs, and EGCs are pluripotent, only ESCs pass the most stringent developmental assay: tetraploid embryo complementation. This is because ESCs carry balanced parental imprints that are critical for normal development, whereas EGCs and mGSCs have erased imprints or paternal-only imprints, respectively, as a result of germline development (Hochedlinger and Jaenisch 2006). However, all of the pluripotent cell lines tested so far (ESCs, ECCs, and EGCs) have been shown to induce pluripotency in somatic cells after cellular fusion, demonstrating that they harbor dominant reprogramming activities (Tada et al. 1997, 2001; Cowan et al. 2005).

Transcription factors and lineage switching

The third principle that contributed to the discovery of induced pluripotency was the observation that lineage-associated transcription factors—which help to establish and maintain cellular identity during development by driving the expression of cell type-specific genes while suppressing lineage-inappropriate genes—can change cell fate when ectopically expressed in certain heterologous cells. This idea was first demonstrated by the formation of myofibers in fibroblast cell lines transduced with retroviral vectors expressing the skeletal muscle factor MyoD (Davis et al. 1987). Subsequently, Graf and colleagues (Xie et al. 2004; Laiosa et al. 2006) discovered that primary B and T cells could be converted efficiently into functional macrophages upon overexpression of the myeloid transcription factor C/EBP α . More recently, researchers have identified sets of transcription factors that induce the conversion of pancreatic acinar cells into insulin-producing β cells by overexpressing the pancreatic factors *MafA*, *Pdx1*, and *Ngn3* (Zhou et al. 2008); the conversion of fibroblasts into neurons by the activation of the neural factors *Ascl1*, *Brn2*, and *Myt1l* (Vierbuchen et al. 2010); and the conversion of fibroblasts into cardiomyocytes by the cardiac factors *Gata4*, *Mef2c*, and *Tbx5* (Ieda et al. 2010). Of note, these experiments proved that lineage conversions are not restricted to cell types within the same lineage or germ layer, since fibroblasts are mesodermal in origin, whereas neurons are derived from ectoderm. Some of the early transdifferentiation experiments provided the intellectual framework for a more systematic search for transcription factors that could induce the conversion of differentiated cells to a pluripotent state, which is discussed below.

iPSCs

To identify transcriptional regulators that can reprogram adult cells into pluripotent cells, Yamanaka and Takahashi (Tokuzawa et al. 2003) devised an elegant screen for factors within a pool of 24 pluripotency-associated candidate

Table 1. Common assays to evaluate the developmental potency of iPSCs

	Assay	Time	Advantages	Disadvantages
<u>Molecular</u>	Morphology Alkaline phosphatase staining Pluripotency markers	Minutes ~1 h 1–2 d	Rapid and simple Straightforward colorimetric assay Detection of endogenous pluripotency genes using immunofluorescence, PCR, or reporter alleles	Not specific to pluripotent cells Not specific to pluripotent cells Some genes also activated in partially reprogrammed cells (e.g., <i>Fbxo15</i>)
	Retroviral silencing	1–2 d	Hallmark of pluripotent state; used as surrogate for global epigenetic reprogramming	Requires delivery of factors by retroviral vectors or addition of “indicator” viral vector; nonspecific
	DNA demethylation	1–2 wk	Promoter demethylation of pluripotency genes (e.g., <i>Oct4</i>) is good indicator for epigenetic remodeling.	Some somatic cells show demethylation of pluripotency genes (e.g., <i>Nanog</i> in melanocytes)
	Factor independence	4–7 d	Self-renewal in the absence of dox-inducible <i>trans</i> -genes is good indicator of faithful reprogramming	Requires use of inducible transgenes
<u>Functional</u>	In vitro differentiation	Weeks	Controlled differentiation into cell type of interest	Differentiation protocols and functional assays for only few cell types available
	Teratoma formation	Weeks (mice), months (humans)	Gives information about spontaneous differentiation potential into three germ layers; assay with highest stringency for human cells	Not quantitative; cannot detect abnormal cells
	Chimera development	Weeks	Tests potential to contribute to normal development and adult tissues upon blastocyst injection	Subtle abnormalities may be masked and complemented by host blastocyst-derived cells
	Germline transmission	Months	iPSC-derived offspring document potential to form functional germ cells, indicating genomic integrity	Readout for single, very specialized, nonessential cell type
	Tetraploid complementation	Weeks	Measures potential to direct normal development of an entire mouse, including all cell types	Subtle developmental or postnatal phenotypes may be missed; does not assess the capacity of cells to form extraembryonic tissues

genes that could activate a dormant drug resistance allele integrated into the ESC-specific *Fbxo15* locus. The combination of 24 factors, when coexpressed from retroviral vectors in mouse fibroblasts, indeed activated *Fbxo15* and induced the formation of drug-resistant colonies with characteristic ESC morphology (Takahashi and Yamanaka 2006). Successive rounds of elimination of individual factors then led to the identification of the minimally required core set of four genes, comprising *Klf4*, *Sox2*, *c-Myc*, and *Oct4*. iPSCs generated by selection for *Fbxo15* activation expressed markers of pluripotent stem cells such as SSEA-1 and Nanog, generated teratomas when injected subcutaneously into immunocompromised mice, and contributed to different tissues of developing embryos upon blastocyst injection (Takahashi and Yamanaka 2006), thereby fulfilling some criteria of pluripotency (Table 1). However, these iPSCs expressed lower levels of several key pluripotency genes compared with ESCs, showed incomplete promoter demethylation of ESC regulators such as *Oct4*, and failed to generate postnatal chimeras or contribute to the germline (Takahashi and Yamanaka 2006). These “first-generation” iPSCs therefore appeared to be only partially reprogrammed. Soon after this study, several laboratories, including Yamanaka’s (Okita et al. 2007), were able to reproduce and improve upon these findings. For example, by selecting for the reactivation of the essential pluripotency genes *Nanog* or *Oct4* instead of *Fbxo15*, iPSCs were generated that molecularly and functionally more closely resembled ESCs (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007). More recently, rare iPSC lines have been identified that are even capable of generating “all-iPSC” mice upon injection into tetraploid blastocysts (Boland et al. 2009; Kang et al. 2009; Zhao et al. 2009; Stadtfeld et al. 2010b), suggesting that at least some iPSC clones have a developmental potency equivalent to ESCs.

iPSCs have also been derived from a number of different species—including humans (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008b), rats (W Li et al. 2009a), and rhesus monkeys (Liu et al. 2008)—by expression of the four Yamanaka factors (Table 2), demonstrating that fundamental features of the transcriptional network governing pluripotency remain conserved during evolution. Similarly, iPSCs have been derived from other somatic cell populations, such as keratinocytes (Aasen et al. 2008; Maherali et al. 2008), neural cells (Eminli et al. 2008; JB Kim et al. 2008), stomach and liver cells (Aoi et al. 2008), and melanocytes (Utikal et al. 2009a), as well as from genetically labeled pancreatic β cells (Stadtfeld et al. 2008a) and terminally differentiated lymphocytes (see also Table 2; Hanna et al. 2008; Eminli et al. 2009), further underscoring the universality of induced pluripotency.

Technical advances in iPSC generation

iPSC derivation is ethically and legally less problematic and technically more feasible than SCNT. In order to use iPSCs as efficient research tools and ultimately translate this technology into clinical applications, suitable techniques of factor delivery and efficient identification of

faithfully reprogrammed cells are crucial. Thus, recent advances in the area of iPSC generation and identification are discussed in the following section.

Factor delivery into target cells

A number of different approaches have been devised to shuttle reprogramming factors into somatic cells (Table 3), which can affect the efficiency of reprogramming and the quality of resultant iPSCs. For example, the first studies on iPSCs used constitutively active retroviral vectors that stably integrated into the host cell genome to introduce *c-Myc*, *Klf4*, *Oct4*, and *Sox2* (Takahashi and Yamanaka 2006; Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007). While retroviral transgenes are usually silenced toward the end of reprogramming (Stadtfeld et al. 2008b), due to the activation of both DNA (Lei et al. 1996) and histone (Matsui et al. 2010) methyltransferases, this process is often incomplete, resulting in partially reprogrammed cell lines that continue to depend on exogenous factor expression and fail to activate the corresponding endogenous genes (Takahashi and Yamanaka 2006; Mikkelsen et al. 2008; Sridharan et al. 2009). In addition, residual activity or reactivation of viral transgenes in iPSC-derived somatic cells can interfere with their developmental potential (Takahashi and Yamanaka 2006) and frequently leads to the formation of tumors in chimeric animals (Okita et al. 2007). This issue becomes exacerbated when constitutively active lentiviral vectors are used to produce iPSCs, which are even less efficiently silenced in pluripotent cells than retroviral vectors and can thus cause a differentiation block (Brambrink et al. 2008; Sommer et al. 2010). The use of inducible lentiviral vectors, whose expression can be controlled by the inert drug doxycycline, diminishes the risk of continued transgene expression and allows for the selection of fully reprogrammed iPSCs, since cells that depend on exogenous factor expression readily stop proliferating upon doxycycline withdrawal (Brambrink et al. 2008; Stadtfeld et al. 2008b). Lentiviral vectors are also more efficient than retroviral vectors at infecting different somatic cell types and can be used to express polycistronic cassettes encoding all four reprogramming factors, thus increasing reprogramming efficiency (Carey et al. 2009; Sommer et al. 2009).

Inducible vector systems have been employed to generate so-called “secondary” reprogramming systems, which do not rely on direct factor delivery into target cells. These systems entail differentiating “primary” iPSC clones, generated with doxycycline-inducible lentiviral vectors or transposons, into genetically homogeneous somatic cells using either in vitro differentiation (for human cells) (Hockemeyer et al. 2008; Maherali et al. 2008) or blastocyst injection (for mice) (Wernig et al. 2008a; Woltjen et al. 2009). These somatic cells are then cultured in doxycycline-containing media, thus triggering the formation of “secondary” iPSCs at efficiencies that depend on the specific cell type used but are generally several orders of magnitude higher than the efficiencies obtained after primary infection. Secondary systems therefore (1) allow for the reprogramming of large quantities of genetically

Table 2. *iPSCs derived from different species and somatic cell types*

Species	Germ layer	Cell type	Factors	Efficiency	Reference
<u>Mouse</u>	MS	Fibroblasts	OKSM	0.02	Takahashi and Yamanaka 2006
			OKS	0.002	Nakagawa et al. 2008; Wernig et al. 2008b
			OSE	ND	Feng et al. 2009
			KSNr	0.002	Heng et al. 2010
			OKSM + C	3%	Hanna et al. 2008
			OKSM	0.02%	Eminli et al. 2009
			OKSM	25%	Eminli et al. 2009
			OKSM	13%	Eminli et al. 2009
			OKSM	0.2%	Sugii et al. 2010
			OKM	1.4%	Tsai et al. 2010
	OK	0.02%	Tsai et al. 2010		
	EN	Pancreatic β cells Hepatic endoderm	OKSM	0.1%	Stadtfield et al. 2008a
			OKS	ND	Aoi et al. 2008
	EC	Neural stem cells Melanocytes	OK	<0.1%	JB Kim et al. 2008
			O	<0.01%	JB Kim et al. 2009a
OKM			0.2%	Utikal et al. 2009a	
<u>Human</u>	MS	Fibroblasts	OKSM	0.02%	Takahashi et al. 2007
			OSLN	0.02%	Yu et al. 2007
			OKS	0.002	Nakagawa et al. 2008
			OKSM	0.01%	Loh et al. 2009
			OSLN	<0.01%	Haase et al. 2009
			OKSM	ND	Eminli et al. 2009
			OS	<0.01%	Giorgetti et al. 2009
			OKSM	0.5%	Sugii et al. 2010
			OKS	<0.1%	Aoki et al. 2010
			EN	Hepatocytes	OKSM
	EC	Keratinocytes Neural stem cells	OKSM	ND	Aasen et al. 2008
			OKS	ND	Aasen et al. 2008
	O	<0.004%	JB Kim et al. 2009b		
	EX	Amniotic cells	OKSM	0.05%–1.5%	C Li et al. 2009
			OSN	0.1%	Zhao et al. 2010
<u>Rat</u>	MS	Fibroblasts	OKSM	0.05% ^a	Liao et al. 2009
			OKS	0.01% ^b	Chang et al. 2010
	EN	Liver progenitor cells	OKS	ND ^c	W Li et al. 2009a
EC	Neural progenitor cells	OKS	0.01% ^b	Chang et al. 2010	
<u>Pig</u>	MS	Embryonic fibroblasts	OKSM	ND	Esteban et al. 2009
<u>Rhesus monkey</u>	MS	Ear skin fibroblasts	OKSM	ND	Liu et al. 2008
<u>Marmoset</u>	MS	Skin fibroblasts	OKSM	0.1%	Wu et al. 2010

Listed are (1) the first demonstration a given cell type has been reprogrammed, (2) alternative factor cocktails, and (3) the smallest combination of factors reported. Minimum criteria of listed references are teratoma formation (primates) and contribution to chimeras (nonprimates). iPSCs generated with chemicals are not included. (MS) mesoderm; (EN) endoderm; (EC) ectoderm; (EX) extraembryonic; (C) *C/EBP α* ; (E) *Esrrb*; (K) *Klf4*; (L) *Lin28*; (M) *c-Myc*; (Nr) *Nr5a2*; (O) *Oct4*; (S) *Sox2*; (ND) not determined.

^aContinued expression of lentiviral transgenes might have allowed expansion without inhibitors.

^bContinued culture requires chemical inhibition of GSK3 and MEK.

^cContinued culture requires chemical inhibition of GSK3, MEK, and TGF β signaling.

homogeneous cells for biochemical studies and cells that are difficult to culture or transduce, and (2) facilitate the comparison of genetically matched iPSCs derived from different somatic cell types.

In a modification of the conventional secondary system, mouse strains lacking individual reprogramming trans-

genes have been generated as a screening platform for the identification of small molecules that can substitute for a given reprogramming factor (Markoulaki et al. 2009). Because lentiviral transgenes, however, often exhibit heterogeneous expression patterns in secondary cells, several primary iPSC clones need to be screened to identify the

Table 3. Factor delivery methods for iPSC derivation

Vector type	Integrating			Excisable			Nonintegrating			DNA-free	
	Retroviral	Lentiviral	Inducible lentiviral	Transposon	Floxed lentiviral	Adenoviral	Plasmid	Protein	RNA	Protein	RNA
Cell types	Fibroblasts, neural stem cells, liver cells, keratinocytes, amniotic cells, blood cells, adipose cells	Fibroblasts, keratinocytes	Fibroblasts, β cells, keratinocytes, blood cells, melanocytes	Fibroblasts	Fibroblasts	Fibroblasts, liver cells	Fibroblasts	Fibroblasts	Fibroblasts	Fibroblasts	Fibroblasts
Species	m, h, rm, p, r	m, h	m, h, p	m, h	m, h	m, h	m, h	m, h	m, h	m, h	h
Efficiency	~0.01%-0.5%	~0.1%-1% ^a	~0.1%-1% ^a	~0.1%	~0.1%-1% ^a	~0.001%	~0.001%	~0.001%	~0.001%	~0.001%	~1%
Pros	Reasonable efficiency	Reasonable efficiency	Reasonable efficiency; controlled factor expression	Reasonable efficiency; no integration	Reasonable efficiency; no integration	No integration	No integration	No integration; no DNA	No integration; high efficiency	No integration; no DNA	No integration; high efficiency
Cons	Multiple integrations; incomplete silencing	Multiple integrations; incomplete silencing	Multiple integrations; requires transactivator expression	Screening of excised lines laborious	Screening of excised lines laborious, loxP sites remain in genome	Low efficiency	Low efficiency, occasional vector integration	Low efficiency	Multiple rounds of transfection needed	Low efficiency	Multiple rounds of transfection needed

[h] Human; (m) mouse; (p) pig; (rh) rhesus monkey; (r) rat.

^aOne percent efficiency can be achieved when using concentrated lentiviral vectors encoding all factors in one polycistronic transcript.

ones that efficiently reactivate the factors, a process that can be quite cumbersome. The recent development of “reprogrammable” mouse strains, which contain a single inducible polycistronic transgene in a defined genomic position, has solved this issue, and also enables the breeding of animals into desired mutant backgrounds for mechanistic studies (Carey et al. 2010; Stadtfeld et al. 2010a).

Integration-free iPSCs

Approaches to derive iPSCs free of transgenic sequences are aimed at circumventing the potentially harmful effects of leaky transgene expression and insertional mutagenesis. This is particularly important when considering iPSC technology in a therapeutic setting. Techniques to generate integration-free iPSCs can be subdivided into three categories: (1) those that use vectors that do not integrate into the host cell genome, (2) those that use integrating vectors that can be subsequently removed from the genome, and (3) those that do not use nucleic acid-based vectors at all (Table 3).

The first integration-free iPSCs were generated from adult mouse hepatocytes using nonintegrating adenoviral vectors (Stadtfeld et al. 2008c), and from mouse embryonic fibroblasts (MEFs) transfected with plasmids (Okita et al. 2008). These experiments provided the proof of principle that transient expression of the four classical reprogramming factors is indeed sufficient to induce pluripotency in somatic cells. The absence of common integration sites in iPSCs produced with retroviruses (Varas et al. 2009) or lentiviruses (Winkler et al. 2010) corroborated this conclusion, and showed that insertional mutagenesis at best plays a supportive role during iPSC formation. Human fibroblasts have also been reprogrammed into iPSCs with adenoviral vectors (Zhou and Freed 2009) and Sendai virus (Fusaki et al. 2009), as well as with polycistronic minicircle vectors (Jia et al. 2010) and self-replicating selectable episomes (Yu et al. 2009), albeit the latter system required the simultaneous overexpression of additional factors, including another potent oncogene (Oct4, Sox2, c-Myc, and Klf4, together with Nanog, Lin28, and SV40LT).

Reprogramming efficiencies with current nonintegrating methods are several orders of magnitude lower (~0.001%) than those achieved with integrating vectors (0.1%–1%) (Table 3), most likely because factor expression is not maintained for a sufficient length of time to allow complete epigenetic remodeling. To avoid this issue, several laboratories have developed integration-dependent gene delivery vectors with incorporated loxP sites that can be subsequently excised from the host genome by transient expression of Cre recombinase (Kaji et al. 2009; Soldner et al. 2009). This approach enables the efficient generation of iPSCs from different cell types, especially if polycistronic vectors are used (Chang et al. 2009; Sommer et al. 2010). It remains to be seen, however, whether short vector sequences, which inevitably remain in the host cell DNA after excision, affect cellular function. Transgene-free iPSCs can also be generated with *piggyBac* transposons, mobile genetic elements that can be intro-

duced into and removed from the host genome by transient expression of transposase (Woltjen et al. 2009; Yusa et al. 2009). The low error rate of this process allows for a seamless excision, but requires characterization of integration sites in iPSCs before and after transposon removal. It also remains unclear if transposase expression can induce nonspecific genomic alterations in iPSCs (Stadtfeld and Hochedlinger 2009).

Successful reprogramming has been achieved recently without the use of viral or plasmid vectors at all. Specifically, iPSCs have been derived from both mouse and human fibroblasts by delivering the reprogramming factors as purified recombinant proteins (Zhou et al. 2009) or as whole-cell extracts isolated from either ESCs (Cho et al. 2010) or genetically engineered HEK293 cells (D Kim et al. 2009). While the use of purified proteins represents an attractive approach for the generation of transgene-free iPSCs, its efficiency is extremely low and, in the recombinant protein approach, required the addition of the histone deacetylase (HDAC) inhibitor valproic acid (VPA) to the culture media. A more efficient and safer way of producing integration-free iPSCs may be the introduction of modified RNA molecules encoding for the reprogramming factors into somatic cells, which has been validated recently (Warren et al. 2010).

To improve the overall low efficiencies of generating iPSCs with most nonintegrating approaches, screens for chemical compounds that promote reprogramming have been performed. This led to the identification of a number of molecules that significantly increase reprogramming efficiencies in the context of Oct4, Klf4, Sox2, and c-Myc overexpression (for review, see Desponts and Ding 2010; Li and Ding 2010). Notably, some of these molecules can also replace individual reprogramming factors, raising the possibility of deriving iPSCs solely with chemicals (Desponts and Ding 2010; Li and Ding 2010). However, it should be noted that chemical substitution of a reprogramming factor is, in most cases, associated with a significant decrease in the number of iPSC clones generated, indicating that no single chemical compound is able to entirely replace the function of a transcription factor. Another potential caveat of chemical reprogramming approaches is the introduction of genetic or epigenetic abnormalities into resultant iPSCs, especially since many of the reported compounds are potent modulators of DNA and chromatin modifications.

Identification of iPSC colonies

The inherently low efficiency of iPSC derivation benefits from selection approaches that distinguish successfully reprogrammed clones from partially reprogrammed or simply transformed colonies. The reactivation of endogenous pluripotency-associated genes—such as *Fbxo15* (Takahashi and Yamanaka 2006), *Nanog* or *Oct4* (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007), and *Utf1* (Pfannkuche et al. 2010)—linked to drug selection cassettes has been successfully employed for this purpose. As mentioned above, *Fbxo15* selection generates partially reprogrammed cells (Takahashi and

Yamanaka 2006), likely because activation of this gene occurs early in the reprogramming process when the majority of cells are not yet faithfully reprogrammed (Stadtfeld et al. 2008b). A general limitation of any drug selection approach is that it requires genetic engineering of cells or mice. To circumvent this problem, lentiviral vector systems have been developed that carry promoter fragments of pluripotency genes whose activity can be selected for, and that, in principle, can be applied to a wide range of murine and human cell types (Hotta et al. 2009).

For human iPSCs, expression of surface markers such as TRA-1-81 has been shown to enrich for reprogrammed cells (Lowry et al. 2008). A more stringent approach to identify faithfully reprogrammed human iPSCs without the use of drug selection combines the detection of surface markers with that of “indicator retroviruses” expressing fluorescent proteins, which become silenced upon acquisition of pluripotency (Chan et al. 2009).

Importantly, high-quality iPSCs can be derived from unmodified somatic cells without drug selection or fluorescent reporters at all by simply using morphological criteria (Blelloch et al. 2007; Maherali et al. 2007; Meissner et al. 2007), although this approach requires careful characterization of the resultant cell lines. This “no selection” approach is therefore most powerful when combined with doxycycline-inducible vectors, as cells that have entered a self-sustaining pluripotent state can be easily selected for by removal of doxycycline (Brambrink et al. 2008; Stadtfeld et al. 2008b), even though, in rare cases, doxycycline-independent partially reprogrammed cells have been reported (Mikkelsen et al. 2008).

For mouse iPSCs, the expression status of the imprinted *Gtl2* gene has been described recently as a refined marker that allows for the prospective identification of clones that support the development of tetraploid embryo complementation mice and therefore appear developmentally indistinguishable from ESCs (L Liu et al. 2010; Stadtfeld et al. 2010b). Whether the human homolog *MEG-3* or any other gene has similar predictive value in human iPSCs remains to be tested.

Mechanisms underlying iPSC formation

In the following section, we introduce models that have been developed to explain the low efficiency of reprogramming at a cellular level. We then discuss key molecular events that may act as barriers during the reprogramming process, and speculate on the role of the individual reprogramming factors as well as on supporting and antagonizing factors during epigenetic remodeling. This is followed by a discussion of different pluripotent states that have been identified recently and that can be interconverted by some of the same transcription factors. Finally, we address the question of whether iPSCs are molecularly and functionally equivalent to fertilization-derived ESCs.

Elite vs. stochastic models

The derivation of iPSCs from most studied somatic cells is extremely inefficient (0.01%–0.1%) and occurs at a

slow speed (~2 wk) (Brambrink et al. 2008; Stadtfeld et al. 2008b). Even in the context of secondary systems, in which somatic cells homogeneously express the factors, the efficiency of fibroblast reprogramming generally does not exceed 1%–5% (Maherali et al. 2008; Wernig et al. 2008a; Stadtfeld et al. 2010a), although one report documented an efficiency of up to 20% (Woltjen et al. 2009). Two opposing, but mutually nonexclusive, models have been put forward to explain these observations (Yamanaka 2009).

The so-called “elite” or “deterministic” model proposes that the efficiency of iPSC derivation is low because only a few cells in a somatic cell culture are susceptible to reprogramming. Somatic stem or progenitor cells, present in most adult tissues and possibly also in explanted cell populations, are the most obvious candidate cells, as they are rare and developmentally closer to pluripotent cells than differentiated cells. In contrast, the “stochastic” model poses that all somatic cells are equally amenable to factor-mediated reprogramming, but have to go through a series of stochastic epigenetic events to acquire pluripotency. Only a few cells may pass all of these roadblocks, resulting in the overall low efficiency.

The elite model by itself is difficult to sustain, since iPSC can be derived from several defined somatic cell types, including fully differentiated B and T lymphocytes (Hanna et al. 2008; Eminli et al. 2009) as well as pancreatic β cells (Stadtfeld et al. 2008a). Moreover, when following clonal populations of early B cells and monocytes expressing the reprogramming factors, almost all cell clones ultimately give rise to daughter cells that form iPSCs, even though this process requires several weeks to months (Hanna et al. 2009b). The latter observation suggests that continuous cell proliferation allows rare cells in a homogeneous cell population to acquire stochastic changes that facilitate their conversion into a pluripotent state (Hanna et al. 2009b). In an independent set of experiments, however, clonally plated hematopoietic stem and progenitor cells were shown to give rise to iPSCs with significantly higher efficiencies and within a shorter time frame than mature lymphocytes and myeloid cells (10%–40% vs. 0.01%–1%), regardless of the proliferative state of the cells at the time of factor expression (Eminli et al. 2009), suggesting that the differentiation state itself may also influence the susceptibility of cells to form iPSCs. These seemingly contradictory results may be reconciled if one assumes that adult progenitor and stem cells require fewer stochastic events to induce pluripotency than terminally differentiated cells (Fig. 1A). Hence, reprogramming of progenitor or stem cells may occur in a shorter period of time, which is consistent with the observed fast up-regulation of pluripotency markers upon factor expression (Eminli et al. 2009). It would be important to perform similar long-term clonal assays as described above for monocytes and B cells with defined immature cell types to test this hypothesis. It should also be informative to follow the reprogramming of defined mature and immature cell populations at single-cell resolution by using, for example, time-lapse microscopy, as has been done recently for fibroblasts (Araki et al. 2010; Smith et al. 2010).

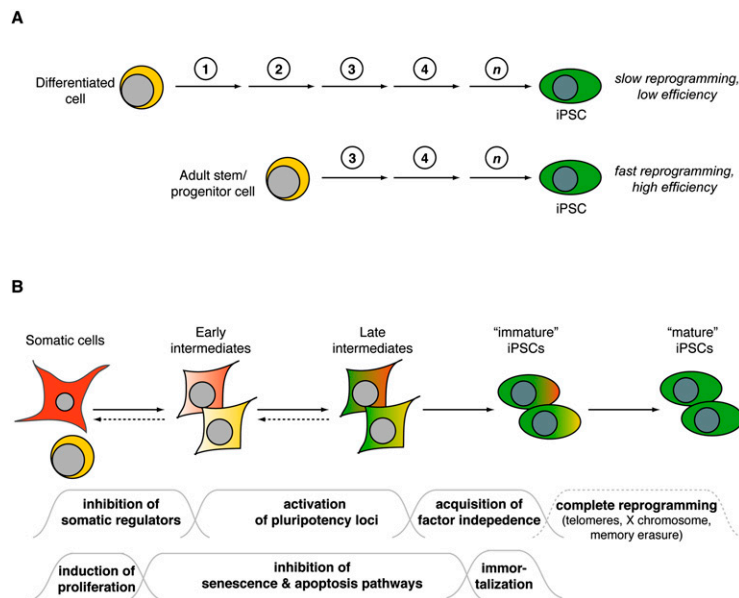


Figure 1. Models of cellular reprogramming. (A) Mature cells, such as lymphocytes, reprogram into iPSCs at lower efficiencies than immature cells, such as hematopoietic stem cells. This may be due to a lower number of stochastic epigenetic events (represented by circled numbers and arrows) that are required in immature cells to acquire pluripotency. The precise number and nature of such changes is unclear (represented by "n"). (B) Scheme summarizing major changes that characterize the transition of somatic cells into iPSCs. The early steps are reversible, as indicated by the dashed reverse arrows. "Immature iPSCs" are defined as cells that have already acquired pluripotency but still retain an epigenetic memory of their cell type of origin, while "mature iPSCs" have lost this memory. The wavelines below indicate assumed reprogramming roadblocks that cells are facing at different stages. Failure to pass any of these roadblocks may result in cells that arrest at that stage or, alternatively, undergo senescence or apoptosis.

In conclusion, a modified stochastic model that integrates an elite component might be most accurate to explain the low efficiency of reprogramming. Consistent with this interpretation is the recent observation that subpopulations of fibroblasts give rise to iPSCs much sooner than the bulk population when following individual cells with live cell imaging (Smith et al. 2010). Similarly, a low expression status of the tumor suppressor gene *Arf* in fibroblast populations marks cells that are more amenable to reprogramming than cells with high *Arf* expression (Utikal et al. 2009b), suggesting that "elite" cells may also exist in cell populations with no proven differentiation hierarchy. Whether these elite populations represent fibroblasts that have undergone fewer numbers of cell division, contaminating nonfibroblast cell types, or immature mesenchymal cells remains to be tested.

Reprogramming roadblocks

The low efficiency and slow kinetics of iPSC derivation is in contrast to somatic cell lineage switching triggered by transcription factor overexpression, such as the conversion of B cells into macrophages induced by C/EBP α , which occurs at efficiencies of up to 100% and within 48 h (Xie et al. 2004; Bussmann et al. 2009). This suggests that the induction of pluripotency by defined factors faces more barriers than lineage conversion, possibly because of a higher degree of transcriptional and epigenetic similarity among mature cell types than between mature cells and pluripotent cells. Thus, what are the major molecular changes a somatic cell has to undergo during reprogramming into an iPSC?

Studies in fibroblasts suggest that reprogramming follows an organized sequence of events, which begins with the down-regulation of somatic markers (Stadtfield et al. 2008b) and morphological changes reminiscent of a mesenchymal-to-epithelial transition (MET). In accordance, interference with genes involved in MET, such as E-cadherin

and BMP receptor signaling, abrogate reprogramming (Li et al. 2010; Samavarchi-Tehrani et al. 2010). These events are followed by the activation of the early pluripotency markers SSEA-1, alkaline phosphatase, and *Fbxo15* before bona fide pluripotency genes such as *Nanog* or *Oct4* become expressed and cells gain independence from exogenous factor expression (Brambrink et al. 2008; Stadtfield et al. 2008b). Notably, the acquisition of pluripotency may not be complete upon independence of exogenous factor expression and the activation of endogenous pluripotency genes but may continue for several rounds of cell divisions, as suggested by the finding that early- and late-passage iPSCs exhibit discernible differences in telomere length (Marion et al. 2009b) as well as global transcriptional and DNA methylation patterns (Chin et al. 2009; Polo et al. 2010). The transition to a pluripotent state is also accompanied by genome-wide remodeling of chromatin modifications, such as DNA and histone tail methylation from a somatic to an ESC-like state (Maherali et al. 2007; Mikkelsen et al. 2008), although the temporal sequence of these changes remains elusive.

Only a subset of fibroblasts expressing reprogramming factors down-regulates somatic markers and activates pluripotency genes (Wernig et al. 2008a; Stadtfield et al. 2010a), indicating that many cells are refractory to reprogramming or become so as a consequence of factor expression. Consistent with this observation, reprogramming intermediates isolated based on combinations of the aforementioned markers have an increased probability of forming iPSC colonies (Stadtfield et al. 2008b), suggesting that these cells have overcome several transcriptional and epigenetic barriers that normally prevent the induction of pluripotency. Of note, refractory fibroblast populations do not give rise to iPSCs even after prolonged culture, despite homogeneous factor expression (M Stadtfield and K Hochedlinger, unpubl.), which is in contrast to observations made in pro-B cells (Hanna et al. 2009b) and may reflect cell type-specific responses to reprogramming factors.

Collectively, these results suggest that the extinction of the somatic program and the subsequent activation of endogenous pluripotency genes may be roadblocks during iPSC formation (Fig. 1B).

A case in point for the importance of endogenous factor activation might be the (stochastic) epigenetic derepression of the endogenous *Nanog* locus by the reprogramming factors. *Nanog* and related pluripotency genes are activated late during reprogramming and may thus be limiting for efficient conversion of somatic cells into iPSCs. In agreement with this hypothesis, simultaneous overexpression of *Nanog* (Hanna et al. 2009b; Silva et al. 2009; Heng et al. 2010) as well as other pluripotency-associated transcriptional regulators—including *Tbx3* (Han et al. 2010) and *Sall4* (Tsubooka et al. 2009)—with *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, has been shown to enhance and/or accelerate reprogramming. It is interesting to note that *Nanog* expression also appears to be a limiting factor during fusion-mediated reprogramming (Silva et al. 2006), suggesting common mechanisms.

The promoters of pluripotency genes such as *nanog* and *Oct4* are stably silenced by DNA methylation in somatic cells (Gidekel and Bergman 2002), which likely interferes with transcription factor binding and gene activation during reprogramming. It is unclear how this roadblock is overcome during iPSC derivation, but mechanisms based on either passive or active DNA demethylation have been postulated (Hochedlinger and Plath 2009). In one scenario, an unknown reprogramming factor (or one of its targets) might antagonize the activity of the DNA methyltransferase *Dnmt1*, which maintains somatic DNA methylation patterns. This may lead to the progressive loss of DNA methylation with cell division. Since genome-wide demethylation is toxic for somatic cells (Li et al. 1992), this process would have to happen in a promoter-specific fashion, perhaps by the rapid binding of reprogramming factors to target sites on the newly synthesized and hence temporarily unmethylated DNA strand (Hochedlinger and Plath 2009). Otherwise, cell death pathways such as the *p53* pathway would have to be inactivated to ensure survival of cells (Jackson-Grusby et al. 2001). In support of this notion, fibroblasts undergoing reprogramming have been shown to inactivate the *ink4*–*arf* pathway (H Li et al. 2009; Utikal et al. 2009b).

In a second scenario, DNA is actively demethylated at key pluripotency promoters by an as-yet-uncharacterized enzymatic activity. Evidence for a role of active DNA demethylation during development comes from studies of germ cells (Hajkova et al. 2008, 2010) and zygotes (Mayer et al. 2000; Oswald et al. 2000) as well as some adult somatic cells, including neurons and T lymphocytes (Bruniquel and Schwartz 2003; Miller and Sweatt 2007). In the context of reprogramming, active DNA demethylation has been observed upon SCNT, although the molecules involved remain elusive (Simonsson and Gurdon 2004). The deaminase *Aid* has been proposed recently to play a role in the demethylation of the *NANOG* and *OCT4* promoters after cell fusion of mouse ESCs and human fibroblasts (Bhutani et al. 2010; Popp et al. 2010). Consistent with this finding, *Aid*, together

with *Gadd45* and *Mbd4*, has been shown to elicit DNA demethylation in zebrafish (Rai et al. 2008). Yet another class of enzymes potentially involved in active DNA demethylation is the Tet proteins, which catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (Tahiliani et al. 2009; Ito et al. 2010). This modification could be recognized by glycosylases, resulting in demethylation of DNA. However, a role for Tet proteins in iPSC formation has yet to be established.

The slow kinetics of somatic pluripotency gene activation after enforced factor expression in fibroblasts (~8–12 d) (Brambrink et al. 2008; Stadtfeld et al. 2008b) is in contrast to the rapid activation of these genes after SCNT (Egli et al. 2009) or cell fusion (~24–48 h) (Han et al. 2008). This observation might argue for a passive, replication-dependent mode of DNA demethylation during iPSC derivation. Alternatively, DNA demethylation might be involved, but key components of its machinery might be limiting until late during reprogramming.

In contrast to ESCs, which are immortal, fibroblasts and most other somatic cell types have a restricted proliferative potential and undergo apoptosis, growth arrest, or stress-induced senescence in culture (Collado et al. 2007). The acquisition of cellular immortality therefore appears to be yet another roadblock during iPSC cell generation. Increased proliferation and up-regulation of genes driving cell cycle progression are indeed early events seen during fibroblast reprogramming (Mikkelsen et al. 2008). However, the observed dependence of cells at intermediate stages of reprogramming on exogenous factor expression for continuous growth (Brambrink et al. 2008; Stadtfeld et al. 2008b) suggests that stable immortalization occurs late during the process (Fig. 1B). In agreement with the idea that the onset of cellular senescence is a barrier during fibroblast reprogramming is the finding that expression of the reprogramming factors in *p53* or *ink4a/arf*-deficient immortalized fibroblasts leads to dramatic increases in reprogramming efficiency and speed (Banito et al. 2009; Hong et al. 2009; Kawamura et al. 2009; H Li et al. 2009; Utikal et al. 2009b). In addition to its role in conferring immortality on fibroblasts, *p53* loss may also contribute to increased reprogramming by inhibiting DNA damage-induced apoptosis (Marion et al. 2009a). It is interesting to note, however, that loss of *p53* can elicit different responses in cells expressing the four factors. In fibroblasts, the main effect of *p53* loss appears to be inhibition of senescence and cell death, while, in blood cells expressing reprogramming factors, *p53* loss mainly contributes to reprogramming by accelerating cell cycle progression (Hanna et al. 2009b).

The analysis of partially reprogrammed cells has been informative in identifying additional roadblocks of reprogramming. For example, Mikkelsen et al. (2008) showed that the activity of lineage-associated transcription factors and DNA hypermethylation can be barriers for the transition of partially reprogrammed cells into a fully pluripotent state. It is unclear whether observations made in partially reprogrammed cells, which are immortal cell lines, are representative of events that take place in regular reprogramming intermediates, which are unstable and

transient cell populations. Nevertheless, support for an antagonizing role of somatic transcription factors during reprogramming comes from the observation that knock-down of the B-cell factor Pax5, which has been shown previously to elicit dedifferentiation of B cells into hematopoietic progenitors (Mikkola et al. 2002), significantly improves the reprogramming efficiency of mature B lymphocytes (Hanna et al. 2008).

A question that remains to be addressed in this context is whether reprogramming into iPSCs reverses normal development and thus entails dedifferentiation into a progenitor-like state before acquiring pluripotency. The appearance of partially reprogrammed cells, which exhibit similar phenotypes regardless of starting cell type (Mikkelsen et al. 2008), argues against dedifferentiation into lineage-specific progenitors. However, a definitive answer to this question will require the analysis of well-defined somatic cell populations, such as lymphocytes, and growth of cells in media that allow for the detection of potentially rare and transient progenitor cell populations. It has also been speculated that the enforced expression of transcription factors may generate artificial cell states that do not resemble any progenitor cell found in vivo (Nagy and Nagy 2010).

Role of reprogramming factors during epigenetic remodeling

How might individual reprogramming factors overcome the described roadblocks during iPSC derivation? To date, no studies have been performed that provide a comprehensive map of transcription factor binding, chromatin modifications, and gene expression in transient reprogramming intermediates to address this question. Nevertheless, extensive information is available on transcription factor binding in established human and mouse ESCs (Chen et al. 2008; J Kim et al. 2008; Kunarso et al. 2010) and iPSCs (Mikkelsen et al. 2008; Sridharan et al. 2009) that should be informative to build models on how they may act during reprogramming. These data suggest, for example, that Oct4, Nanog, Sox2, and Klf4 are part of a core pluripotency network that serves two main purposes: the repression of genes associated with differentiation, and the activation of ESC-specific targets (Boyer et al. 2005; Loh et al. 2006). Gene suppression by pluripotency factors in ESCs is associated with the recruitment of repressive chromatin remodeling complexes, such as NuRD (Kaji et al. 2006) and Polycomb (Boyer et al. 2006; Lee et al. 2006), to target promoters, leading to histone deacetylation and H3K27 trimethylation. A functional role for components of the Polycomb complex during epigenetic remodeling has been suggested by a recent report investigating fusion-mediated reprogramming between ESCs and lymphocytes (Pereira et al. 2010). ESCs lacking either PRC1 or PRC2 activity failed to remodel the genome of their somatic fusion partners, suggesting that these molecules might also be important during iPSC derivation.

Intriguingly, the mechanism by which pluripotency factors mediate transcriptional repression in pluripotent cells involves binding of single factors to target gene

promoters (Fig. 2; J Kim et al. 2008; Sridharan et al. 2009). In contrast, occupancy by multiple factors (J Kim et al. 2008) leads to recruitment of the basal transcription machinery and, consequently, strong gene activation. This dual control of target gene regulation might explain why somatic genes are usually silenced before pluripotency genes become activated: While repressive complexes can form immediately upon binding of individual exogenously expressed factors to cognate DNA sequences, key components of the more elaborate activating complexes, such as Nanog or Dax1 (Wang et al. 2006), may be limiting or absent at early stages of reprogramming and may become available only late, when their respective endogenous genomic loci have become activated (Fig. 2). The binding of pluripotency factors to their target genes might be facilitated by nucleosome remodelers such as Chd1 (Gaspar-Maia et al. 2009) and BAF (Singhal et al. 2010), both of which increase reprogramming efficiencies and kinetics when overexpressed. Once the majority of core pluripotency factors are expressed, they presumably engage in positive feedback and feed-forward loops of their own and other pluripotency promoters (Chen et al. 2008; J Kim et al. 2008) in order to sustain pluripotency in the absence of exogenous factor expression (Fig. 2). This model is supported by the observation that endogenous pluripotency genes, telomerase, and the silenced X chromosome in female cells become reactivated, while retroviruses are silenced, toward the end of the reprogramming process (Stadtfield et al. 2008b), although the precise order of these events has yet to be established.

Studies on bulk populations undergoing reprogramming as well as on partially reprogrammed cells suggest that incorrect binding of transcription factors to targets might be another reason for the inability of many cells to acquire pluripotency (Sridharan et al. 2009). This report confirms the previously noted cooperativity of Oct4, Sox2, and Klf4 in pluripotent cells. It further suggests that c-Myc plays an independent role early during reprogramming by inducing cellular proliferation and driving a concomitant switch toward an energy metabolism typical of cancer cells (Fig. 2C; Mikkelsen et al. 2008; Sridharan et al. 2009). Accordingly, for Myc to exert its enhancing effects, it has to be expressed only during the first few days of reprogramming (Sridharan et al. 2009). Consistent with this finding, premature expression of c-Myc and Klf4 in fibroblasts prior to activation of all four factors increases reprogramming efficiencies and speed, while early expression of Sox2 and Oct4 has no effect (Markoulaki et al. 2009). Thus, c-Myc might not be involved directly in inducing pluripotency, but rather in priming somatic cells for subsequent pluripotency factor expression. This could occur by facilitating the binding of Oct4 and Sox2 to target genes; for example, by establishing or maintaining activating histone methylation (CH Lin et al. 2009) and acetylation (Knoepfler 2008) marks. In addition, c-Myc might play a role later during reprogramming by solidifying pluripotency gene expression, as is suggested by its presence on promoters of many genes and microRNAs (miRNAs) highly expressed in ESCs (Chen et al. 2008; J Kim et al. 2008; Judson et al. 2009).

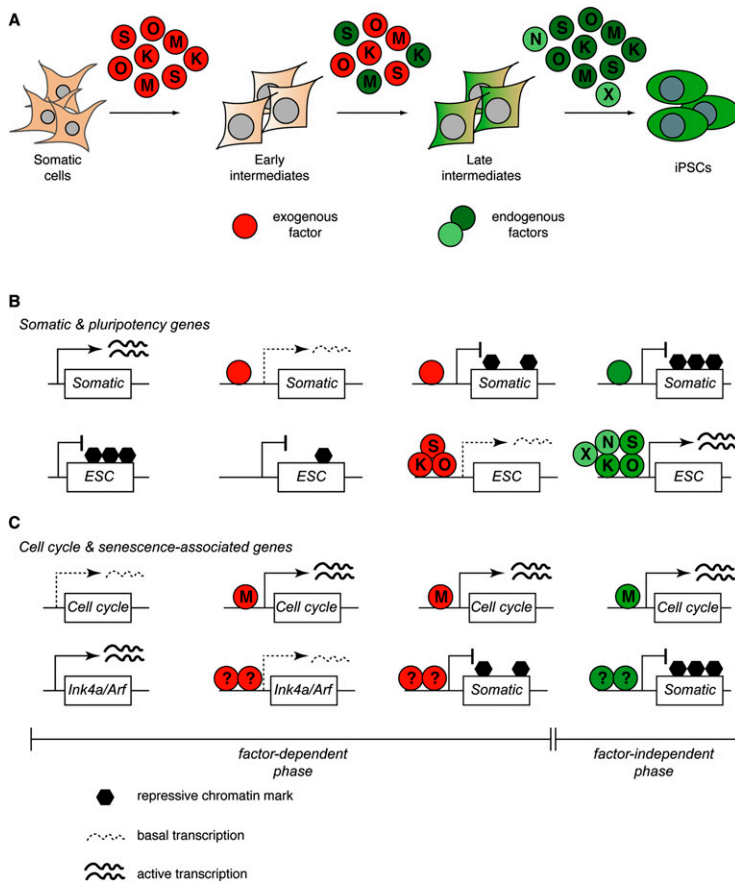


Figure 2. Putative role of reprogramming factors during iPSC formation. (A) Scheme depicting the expression of exogenous (red circles) and endogenous (dark-green circles) pluripotency factors at the protein level during different stages of reprogramming. The reprogramming process is initiated predominantly by the exogenous factors, which are gradually replaced by endogenous proteins as well as their targets, such as Nanog (N) or as-yet-undefined factors (X) (light-green circles). The endogenous loci of some reprogramming factors (such as *c-Myc*, *Klf4*, and *Sox2*) are expressed in some somatic cell types, and the corresponding endogenous proteins might thus become available before activation of the *Oct4* locus. (B) Scheme illustrating how the reprogramming factors may exert the rapid repression of somatic genes and the gradual activation of pluripotency (ESC) genes, two processes assumed to be mediated largely by *Klf4*, *Sox2*, and *Oct4*. Somatic gene silencing is associated by single-factor binding to promoter regions, while ESC gene activation involves the establishment of multiprotein complexes. The initial loss of repressive marks (such as DNA methylation and H3K27 histone trimethylation) at ESC promoters might be a passive process driven by multiple rounds of cell division. (C) Scheme showing activation of genes promoting cell division (such as cyclins) by *c-Myc* and repression of the *Ink4a/Arf* tumor suppressor locus conferring immortality by an as-yet-undefined combination of reprogramming factors.

Consistent with *c-Myc*'s role as a facilitator of early stages of reprogramming, exogenous *c-Myc* can be omitted entirely from the reprogramming cocktail (Nakagawa et al. 2008; Wernig et al. 2008b), albeit at the cost of reduced reprogramming efficiency and speed. Another reason why exogenous *c-Myc* expression is dispensable might be the observation that endogenous *c-Myc* is already expressed at low levels in many somatic cells, and may hence become readily activated by the remaining factors. Consistent with this notion, somatic cells that express other reprogramming factors endogenously do not require administration of the corresponding exogenous factor(s), as has been demonstrated for neural stem cells that can be converted into iPSCs by *Oct4* alone (Table 2; JB Kim et al. 2009a).

In this context, it is important to mention that *Oct4*, *Sox2*, *Klf4*, and *c-Myc* are not the only factor combination that can generate iPSCs. For example, human iPSCs have been derived by enforced expression of *Oct4*, *Sox2*, *Nanog*, and *Lin28* (Yu et al. 2007). This suggests that different routes may lead to a common pluripotent ground state, or, alternatively, that different transcription factors activate the same program by reinforcing each other's synthesis. Indeed, *lin28* represses *let-7* miRNAs (Viswanathan et al. 2008), which are negative regulators of *c-Myc* translation (Akao et al. 2006; HH Kim et al. 2009), thus establishing a link between the two reprogramming cocktails (*Oct4*, *Klf4*, *Sox2*, and *c-Myc*, and *Oct4*, *Sox2*, *Nanog*, and *Lin28*).

Similarly, *Sox2* and *Klf4* can be replaced by a number of closely related proteins, such as *Sox1* and *Klf2* (Nakagawa et al. 2008), respectively, suggesting that they recognize similar DNA-binding motifs. However, some of the classical reprogramming factors can be replaced by seemingly unrelated molecules—such as *Klf4* by *Esrrb* (Feng et al. 2009) and *Oct4* by the orphan nuclear receptor *Nr5a2* (Heng et al. 2010)—during mouse fibroblast reprogramming. The mechanisms by which these alternative factors function during reprogramming remain elusive.

Antagonists and cooperative factors

The finding that iPSC formation is slow in comparison with SCNT and cell fusion suggests that additional cooperative factors of *Oct4*, *Sox2*, *c-Myc*, and *Klf4* must exist and thus should be identifiable. Indeed, a number of modulators of the reprogramming process have been identified that can be divided into transcription factors, miRNAs, chromatin regulators, and growth factors, as well as small compounds targeting any of these molecules. An understanding of how these modulators interact with the reprogramming process has provided additional insight into the mechanisms of iPSC formation.

One group of cooperating factors appears to enhance reprogramming by impairing the activity or expression of components of the p53 pathway, further emphasizing the importance of overcoming cellular senescence and/or cell

death pathways during iPSC formation. For instance, overexpression of SV40 large T antigen (Mali et al. 2008; Park et al. 2008c) or Rem2 GTPase (Edel et al. 2010) has been shown to enhance reprogramming by inhibiting p53. The culturing of reprogramming cells in hypoxic conditions (Utikal et al. 2009b; Yoshida et al. 2009) or in the presence of ascorbic acid (Esteban et al. 2010) also seems to target the p53 pathway and thus may provide safer ways to boost iPSC generation. Interestingly, these culture conditions also bypass the requirement for c-Myc overexpression, thus indicating a possible functional redundancy between c-Myc overexpression and loss of p53.

Another class of modulators comprises components of signaling pathways. For example, addition of recombinant TGF β to fibroblast cultures almost completely abrogates iPSC formation, whereas inhibition of TGF β signaling by chemically antagonizing its receptor significantly increases reprogramming efficiency and kinetics. This was seen in both mouse (Ichida et al. 2009; Maherli and Hochedlinger 2009) and human fibroblasts (T Lin et al. 2009), with human cells requiring simultaneous inhibition of MAPK signaling. In mice, TGF β inhibition also abolishes the requirement for exogenous c-Myc and, to a lesser extent, Sox2 expression. While the effect of Tgf β signal inhibition might be partly explained by facilitating the activation of the *Nanog* locus (Ichida et al. 2009), another interpretation is that blocking TGF β signaling might be critical to initiate a MET that occurs early during reprogramming (Li et al. 2010; Samavarchi-Tehrani et al. 2010).

Similarly to Tgf β inhibition, activation of Wnt signaling increases the efficiency of fibroblast reprogramming in the absence of exogenous c-Myc (Marson et al. 2008). This is consistent with c-Myc being a downstream effector of Wnt signaling (He et al. 1998). Furthermore, chemical inhibition of GSK3, which is an inhibitor of the Wnt signaling pathway, allows for the reprogramming of human fibroblasts without exogenous Sox2 (W Li et al. 2009b) and, when combined with inhibition of MAPK signaling, enhances reprogramming of neural stem cells and triggers the conversion of partially reprogrammed cells into iPSCs (Silva et al. 2008). Of note, the combined inhibition of Gsk3 and MAPK signaling has been shown previously by the Smith laboratory (Ying et al. 2008) to be sufficient to maintain murine ESCs in a self-renewing, pluripotent state by suppressing their differentiation. Consistently, inhibition of MAPK/Erk signaling antagonizes differentiation of the ICM in the mouse blastocyst (Chazaud et al. 2006; Nichols et al. 2009b). The finding that inhibition of differentiation pathways enhances reprogramming is in further agreement with the observations that adult progenitor cells give rise to iPSCs more efficiently than their differentiated progeny (Eminli et al. 2009), and that inhibition of lineage-specific factors (Hanna et al. 2008; Mikkelsen et al. 2008) can facilitate the reprogramming process.

iPSC formation requires the extinction of a somatic epigenetic state and the establishment of a pluripotent epigenetic state, suggesting that the manipulation of epigenetic regulators or modifications should influence reprogramming as well. Indeed, a number of chromatin-

modifying agents have been identified that enhance the overall efficiency of reprogramming and also substitute for individual reprogramming factors or combinations of factors. For instance, inhibition of HDACs by VPA or butyrate allows for the efficient derivation of mouse iPSCs from fibroblasts in the absence of either c-Myc (Huangfu et al. 2008a) or Klf4 (Mali et al. 2010), and human iPSCs in the absence of both SOX2 and C-MYC (Huangfu et al. 2008b). Whether HDAC treatment enhances reprogramming by increasing the levels of endogenous c-Myc and Klf4 expression remains unclear. Likewise, inhibition of the histone methyltransferase G9a can replace either c-Myc or Sox2 during the reprogramming of mouse neural progenitor cells (NPCs) and fibroblasts, and it can substitute for Oct4 when administered to NPCs transduced with retroviruses expressing c-Myc, Klf4, and Sox2 (Shi et al. 2008). This effect is consistent with G9a's role in the post-implantation embryo, where it is involved in the stable epigenetic silencing of Oct4 (Feldman et al. 2006).

Lastly, a number of miRNAs have been identified that influence the reprogramming process. These include the previously discussed let-7 family, which blocks reprogramming by interfering with activation of the pluripotency factors c-Myc, lin28, and Sall4, while miR-294 activates these genes and thereby has a promoting effect on reprogramming (Melton et al. 2010). Both let-7 inhibition (Melton et al. 2010) and enforced miR-294 expression (Judson et al. 2009) have also been shown to functionally replace exogenous c-Myc expression during reprogramming. Recently, several miRNAs suppressing mesenchymal gene expression have been reported to enhance the reprogramming of fibroblasts, possibly by promoting a MET (Samavarchi-Tehrani et al. 2010).

Reprogramming factors and alternative pluripotent states

Pluripotent cell lines exist in two distinct states that are characterized by different growth factor requirements and developmental properties (Fig. 3). Murine ESCs established from the ICM of preimplantation blastocysts in the presence of LIF and BMP exist in a more primitive or "naïve" pluripotent state and fulfill all criteria of pluripotency (Table 1; Nichols and Smith 2009). In contrast, EpiSCs derived from post-implantation embryos in the presence of bFGF and activin represent a more advanced or "primed" pluripotent state that exhibits some pluripotency criteria, such as teratoma formation, but fails to contribute efficiently to tissues in mice (Brons et al. 2007; Tesar et al. 2007). ESCs exposed to bFGF readily give rise to EpiSCs (Guo et al. 2009), probably reflecting their normal differentiation path, whereas EpiSCs cultured in LIF and BMP reprogram into ESC-like cells at low frequency (Bao et al. 2009). Together, these results indicate that environmental changes are sufficient to interconvert these closely related cell types. Forced expression of Klf4 can promote the conversion of EpiSCs into ESC-like cells in the presence of LIF and BMP (Guo et al. 2009), presumably by reinforcing the LIF/Stat3 pathway (Hall et al. 2009).

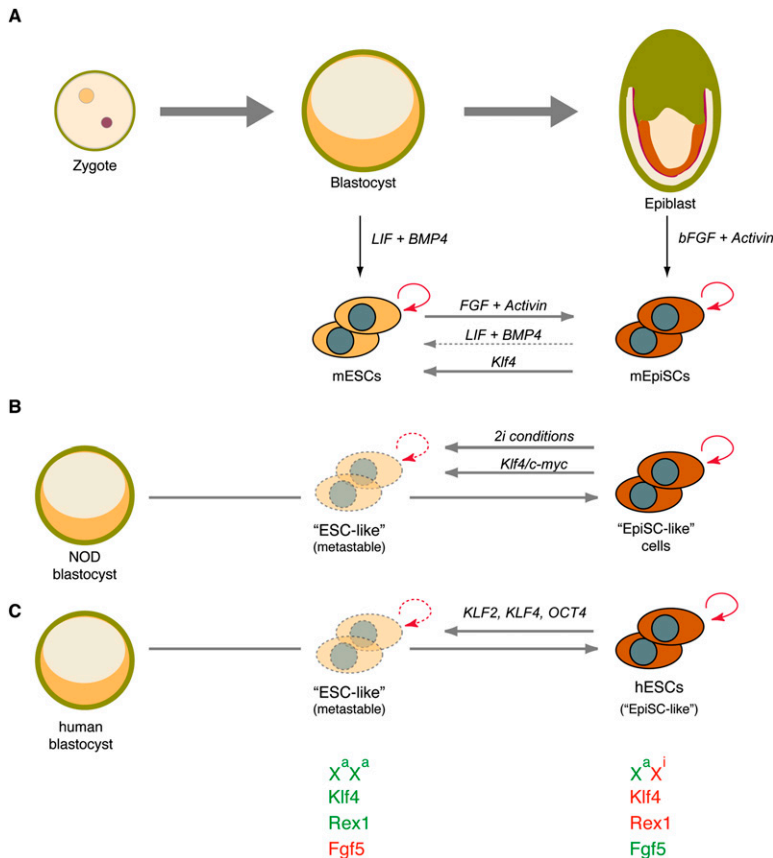


Figure 3. Transitions between alternative pluripotent states. (A) Model showing early developmental stages of the mouse embryo, from zygote to blastocyst and, subsequently, to post-implantation epiblast. ESCs are derived from the ICM (orange crescent) of the blastocyst and require LIF and BMP4 for indefinite self-renewal in vitro (indicated by the curved red arrow). EpiSCs are derived from epiblast stage embryos and require bFGF and activin for their propagation. ESCs readily differentiate into EpiSCs upon the switch to appropriate culture conditions, while the reverse transition is rare but can be enhanced significantly by enforced expression of *Klf4*. (B) Explant cultures of blastocysts from nonpermissive mouse strains (such as NOD) do not give rise to stable ESC lines in LIF and BMP4. However, a metastable ESC-like state can be attained by forced expression of *Klf4* and *c-Myc*, or by repression of both MAPK and GSK3 signaling ("2i condition"). (C) Human ESCs resemble mouse EpiSCs in their epigenetic configuration (one inactive X chromosome [Xi], and one active X chromosome [Xa]) and marker gene expression (such as *Fgf5*). A metastable murine ESC-like state can be induced in these cells by overexpression of *KLF2*, *KLF4*, and *OCT4*. Active genes are shown in green, and inactive genes are shown in red.

Enforced expression of combinations of the reprogramming factors have also been shown to facilitate the derivation of ESC-like cells from mouse strains that have thus far been considered refractory for ESC derivation, such as the nonobese diabetic (NOD) strain. While explanted blastocysts from NOD animals give rise to cells that are unstable in conventional ESC culture conditions, overexpression of either *Klf4* or *c-Myc* endows these cells with a murine ESC-like state (Hanna et al. 2009a). However, this state is "metastable," as it depends on the continuous expression of factors. Of note, the effect of *Klf4/c-Myc* overexpression can be mimicked by treatment of NOD stem cells with either drugs that induce *Klf4* expression (Hanna et al. 2009a) or, alternatively, compounds that inhibit MAPK and GSK3 (Nichols et al. 2009a).

Human ESCs and iPSCs also resemble EpiSCs more closely than ESCs, suggesting that previously used culture conditions capture a primed rather than a naïve pluripotent state. Recent data from the Jaenisch laboratory (Hanna et al. 2010) indicate that overexpression of *Oct4*, *Klf4*, and *Klf2*, together with LIF and inhibitors of MAPK and GSK3 signaling, induces a metastable naïve murine ESC-like state in human ESCs. In contrast to human ESCs grown in regular ESC conditions, treated cells showed responsiveness to LIF as well as reactivation of the silenced X chromosome, indicating the acquisition of murine ESC-like signaling and epigenetic patterns. Human ESCs with two active X chromosomes are also observed when cells

are derived in 5% oxygen (Lengner et al. 2010), suggesting that physiological oxygen levels help to maintain human ESCs in a developmentally more immature state. A metastable state that shares some morphological and molecular features with murine ESCs can also be directly induced in human somatic cells by overexpression of *Oct4*, *Sox2*, *Klf4*, *c-Myc*, and *Nanog*, and culture in the presence of LIF (Buecker et al. 2010).

Equivalency of iPSCs and ESCs?

The artificial nature of induced pluripotency raises the question of whether iPSCs and blastocyst-derived ESCs are molecularly and functionally equivalent. Analyses of genome-wide expression patterns and global histone modifications have shown a high degree of similarity between ESCs and iPSCs (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007; Mikkelsen et al. 2008). However, substantial differences between the two cell types have been reported as well. For example, a reduced and more variable neuronal potential has been described for a number of human iPSC lines, regardless of whether they carried reprogramming transgenes in their genome or not (Hu et al. 2010). Likewise, an increased propensity of iPSC-derived neural cells to form tumors after transplantation into the brains of immunocompromised mice has been observed (Miura et al. 2009). In addition, human iPSC-derived early blood progenitor cells appear to undergo premature senescence (Feng et al. 2010). At the

molecular level, gene-specific and global differences in DNA methylation (Deng et al. 2009; Doi et al. 2009; Pick et al. 2009) and in the expression of mRNAs and miRNAs (Chin et al. 2009; Wilson et al. 2009) have been reported between both mouse and human ESCs and iPSCs. Importantly, a contribution of genetic background (Brambrink et al. 2006) and residual transgene expression (Takahashi and Yamanaka 2006; Soldner et al. 2009), which can influence the properties of pluripotent cells, to the observed phenotypes could not be excluded in these studies.

A recent comparison of global mRNA and miRNA expression in genetically matched mouse ESCs and iPSCs revealed no consistent gene expression differences, with the exception of transcripts within the imprinted *Dlk1-Dio3* gene cluster (Stadtfeld et al. 2010b). This suggests that many of the previously observed transcriptional differences were due to experimental variables. Specifically, maternally expressed genes in this cluster were aberrantly silenced in the majority of iPSCs derived from fibroblasts, blood cells, and keratinocytes. In fact, the expression status of the *Dlk1-Dio3* locus served as a useful indicator for the quality of iPSC clones: While all iPSCs formed teratomas and contributed to low-grade chimeras, only cells with an ESC-equivalent expression pattern of *Dlk1-Dio3* had the capacity to contribute to high-grade chimeras and form viable “all-iPSC” mice after injection into tetraploid blastocysts (L Liu et al. 2010; Stadtfeld et al. 2010b). It is noteworthy that iPSCs with normal *Dlk1-Dio3* expression have so far been derived only from fibroblasts, which may be linked to high expression levels of this cluster in these cells. Importantly, the *Dlk1-Dio3* cluster is normally expressed in NT-ESCs derived from different cell types (Stadtfeld et al. 2010b), indicating that SCNT more frequently generates faithfully reprogrammed cells than factor-mediated reprogramming. Although the molecular reasons for the susceptibility of the *Dlk1-Dio3* cluster to acquire epigenetic abnormalities during iPSC formation are unclear, binding of reprogramming factors to specific sequences in this locus followed by the recruitment of repressive chromatin modifiers is likely involved. It remains an open question whether other epigenetic and/or genetic abnormalities, which are not detectable in the undifferentiated state or with current technology, are accrued during iPSC formation, especially in human cells. A recent study comparing human ESCs and iPSCs found no consistent differences in global gene expression and histone modification patterns (Guenther et al. 2010). However, these findings do not rule out that subtle differences exist, but they may have been masked by the use of stem cells that were not genetically matched.

A related issue is the question of whether iPSCs retain an “epigenetic memory” of their cell type of origin, which is defined here as remaining epigenetic marks originating from the starting cell that influence transcription in resultant iPSCs. Previous experiments using SCNT suggested that cloned embryos exhibit gene expression patterns reflective of their cell type of origin (Ng and Gurdon 2005, 2008). A recent study found gene expression differences indicative of a transcriptional memory

in human iPSCs derived from fibroblasts, adipose tissue, and keratinocytes (Marchetto et al. 2009). While the analyzed cell lines were derived in independent laboratories and with different technologies, which can confound gene expression analyses (Newman and Cooper 2010), another study confirmed and extended this finding by comparing genetically matched iPSCs derived from granulocytes, muscle progenitors, fibroblasts, and lymphocytes (Polo et al. 2010). iPSCs derived from these cell types exhibited discernible gene expression and DNA methylation patterns as well as differentiation biases into hematopoietic cells in vitro, some of which could be attributed to their cell type of origin. A parallel study corroborated these conclusions and further discovered that analysis of DNA methylation patterns in a given iPSC clone could predict the somatic cell from which it was derived (Kim et al. 2010). Notably, continuous passaging of iPSCs (Polo et al. 2010) or the treatment of cells with chromatin-modifying drugs (Kim et al. 2010) attenuated these differences. Together, these results suggest that low-passage iPSCs retain a transient epigenetic memory of their cell type of origin that can influence their differentiation potential.

To date, no comparison of genetically matched human ESCs and iPSCs has been described. Nevertheless, recent data suggest that female human iPSCs fail to reactivate the epigenetically silenced X chromosome (Tchieu et al. 2010). Female fibroblast populations are mosaic in that 50% of cells carry an active maternal X chromosome and an inactive paternal X chromosome, whereas 50% of cells carry an active paternal X and an inactive maternal X. iPSCs derived from female mouse fibroblasts faithfully reactivate the silenced X chromosome and undergo random X inactivation upon differentiation (Maherali et al. 2007). In contrast, human iPSCs derived from female dermal fibroblasts fail to reactivate the silenced X chromosome and keep the same X chromosome inactivated in differentiated cells (Tchieu et al. 2010). These results indicate differences in the reprogramming process between mouse and human cells. Moreover, the data show that female human iPSCs can also retain an epigenetic memory of their cell type of origin. In contrast to mouse iPSCs, however, this memory does not become attenuated with passage. This observation could be useful in disease modeling and potential cell therapy of X-linked disorders such as Rett syndrome or Duchenne’s muscular dystrophy, as it allows for the derivation of phenotypically wild-type and isogenic mutant cell lines from carriers harboring these mutations. At the same time, the possible clinical use of iPSCs derived from female carriers with unidentified X-linked mutations might be complicated by this finding.

Therapeutic potential of iPSCs

In addition to being an exciting research tool to probe mammalian development and epigenetic reprogramming, iPSCs have therapeutic potential for both custom-tailored cell therapy and so-called “disease modeling.” These two concepts are illustrated in Figure 4 and are discussed in the last section.

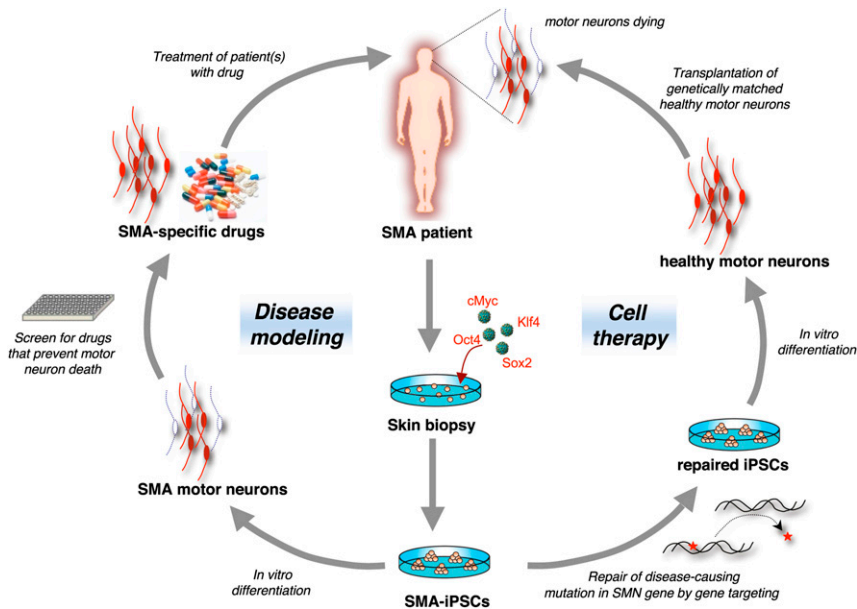


Figure 4. Potential applications of iPSCs. Shown are the potential applications of iPSC technology for cell therapy and disease modeling using SMA as an example. In SMA patients, motor neurons are afflicted and die, causing the devastating symptoms of the disease. SMA-specific iPSCs could be coaxed into motor neurons in vitro in order to establish a culture model of the disease that may lead to the identification of novel drugs that prevent the abnormal death of motor neurons in patients. Alternatively, if known, the disease-causing mutation could be repaired (in this case the *SMA* gene) in iPSCs by gene targeting prior to their differentiation into healthy motor neurons, followed by transplantation into the patient's brain.

iPSCs and cell therapy

Organ transplantation among nonrelated individuals is complicated by the limited availability of matched tissues and the requirement for life-long treatment with immunosuppressive drugs that can have serious side effects. iPSCs might potentially circumvent these problems, as they could be coaxed into the desired cell types that would already be genetically matched with the patient. Another key advantage of iPSCs over current transplantation approaches is the possibility of repairing disease-causing mutations by homologous recombination, a technology that has been used with limited success in adult stem cells because of notorious difficulties in growing them outside the body.

Promising experiments in mice indeed suggest that the treatment of genetic disorders with iPSCs is feasible. Specifically, Jaenisch and colleagues (Hanna et al. 2007) showed that iPSCs can be used to rescue the defects seen in an animal model of sickle cell anemia. Sickle cell anemia is the result of a single point mutation in the hemoglobin gene, rendering red blood cells nonfunctional. In this proof-of-concept study, skin cells from the mouse model, which recapitulates the human condition, were first reprogrammed into iPSCs. The disease-causing mutation was subsequently fixed in iPSCs by gene targeting, and the repaired cells were then coaxed into blood-forming progenitors. These now healthy progenitors were transplanted back into anemic mice, where they produced normal red blood cells and cured the disease (Hanna et al. 2007). In principle, this approach could be applied to any disease in humans for which the underlying mutation is known, and that can be treated by cell transplantation. This conclusion is further supported by the phenotypic correction of hemophilia A in mice transplanted with heterologous, iPSC-derived endothelial progenitor cells (Xu et al. 2009).

Drug development with iPSCs

The study and treatment of many degenerative diseases—such as type I diabetes, Alzheimer's disease, and Parkinson's disease—is limited by the accessibility of the affected tissues, as well as the inability to grow the relevant cell types in culture for extended periods of time. The idea behind so-called "disease modeling" is to derive iPSCs from patients' skin cells and then differentiate them in vitro into the affected cell types, thereby recapitulating the disease in a Petri dish (Fig. 4). The advantage of this approach over currently used strategies is that the very cell type that is compromised can be recreated in culture to be studied, even when the cell type is long gone from the patient. Moreover, because iPSCs grow indefinitely in culture, they provide an unlimited source for any desired specialized cells. Ultimately, the goal of this approach is to use these "Petri dish" models of disease to identify novel drugs to treat the disease; for example, drugs that prevent the pathological death of motor neurons in patients suffering from amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA), or the abnormal loss of insulin-producing β cells in diabetes patients.

Another problem associated with studying degenerative diseases that iPSCs may overcome is the fact that researchers are usually confined to cell material from patients whose disease has already progressed to advanced stages at the time of diagnosis. Since iPSCs likely need to pass through the same differentiation steps in vitro as patients' cells went through as they became sick, this technology may allow researchers to recapitulate and capture the very early stages of the disease. This may inform researchers when and why things went awry in the patient in the first place.

How far is research from identifying new drugs with iPSCs? In fact, several laboratories have already derived

iPSCs from patients suffering from Huntington's and Parkinson's disease, ALS, juvenile diabetes, muscular dystrophy, Fanconi anemia, Down syndrome, and others (Dimos et al. 2008; Park et al. 2008a; Raya et al. 2009; Soldner et al. 2009), which will facilitate these studies. Moreover, three promising reports showed that iPSCs derived from patients suffering from the devastating disorders SMA (Ebert et al. 2009), familial dysautonomia (FD) (Lee et al. 2009), and LEOPARD syndrome (Carvajal-Vergara et al. 2010) recapitulated the cell abnormalities in a Petri dish as they are seen in patients. Remarkably, when the cultured cells were exposed to experimental drugs for these diseases, the "symptoms" were partially alleviated in culture. This principle can now be applied to many other diseases and cell types for which we currently do not have treatments, and may result in the development of drugs from which not just one individual, as in cell therapy, but many patients may benefit.

Challenges for disease modeling and cell therapy

Despite successes in animal models, iPSC technology is not yet ready for transplanting cells into patients. The main issue is safety concerns; iPSCs, like ESCs, tend to form teratomas, and current differentiation protocols cannot efficiently eliminate residual undifferentiated cells (Wernig et al. 2008c). However, this problem should be surmountable by devising positive or negative selection strategies using FACS or drug selection approaches, as has been done before with cultured cells (Schuldiner et al. 2003). Most patient-specific iPSCs have been generated with integrating vectors, which may not get silenced efficiently or could disrupt endogenous genes, which also pose potential impediments for the use of human iPSCs in cell therapy. It will thus be critical to further improve the transgene-free approaches discussed previously to derive new patient-specific iPSC lines. For diseases that require gene targeting to repair mutant alleles, more efficient targeting strategies need to be developed. Regular targeting approaches in human ESCs are quite inefficient, and may result in karyotypic abnormalities due to extensive culturing (Draper et al. 2004). The use of zinc finger nucleases to target endogenous genes in human ESCs led to a significant increase in efficiency, and may hence become the method of choice for manipulating human ESCs (Hockemeyer et al. 2009; Zou et al. 2009). Another advance has been the recognition that human ESCs can be transiently converted into a murine ESC-like state that is more amenable to homologous recombination and single-cell subcloning by exposing cells to drugs or overexpressing certain reprogramming factors (Buecker et al. 2010; Hanna et al. 2010). Last, the observation that many mouse iPSCs harbor epigenetic abnormalities (Stadtfeld et al. 2010b) and retain a transient epigenetic memory of their donor cells (Kim et al. 2010; Polo et al. 2010) will require a careful molecular and functional evaluation of human iPSCs for similar potential aberrations, as well as for possible rare, nonrecurrent mutations.

Disease modeling faces some of the same challenges as cell therapy, but it also raises additional questions.

While promising preliminary data have been published on the modeling of monogenic diseases (see above), it remains unclear if multigenic diseases such as diabetes or Alzheimer's disease are equally amenable to in vitro modeling. Another question that needs to be addressed is whether late-onset diseases such as Alzheimer's and Parkinson's disease can be recapitulated in vitro within a few weeks or whether one needs to accelerate the phenotype by exposing cells to different types of environmental or genetic stress to unveil a phenotype. This challenge is underscored by the observation that neurons derived from "Parkinson iPSCs" did not exhibit obvious abnormalities compared with wild-type neurons (Soldner et al. 2009). Many diseases develop in a non-cell-autonomous manner and involve the interaction of multiple different cell types. Even though it should be possible, in principle, to derive all of the relevant cell types involved in disease from iPSCs, current differentiation strategies into functional cell types are inefficient and limited to a few tissues. Nevertheless, the idea of probing the effect of different cell types on the development of a disease has been demonstrated by different laboratories studying mouse and human models of ALS, another condition characterized by the loss of motor neurons (Di Giorgio et al. 2007, 2008; Marchetto et al. 2008). Specifically, Di Giorgio et al. (2007, 2008) and Marchetto et al. (2008) engineered ESCs to carry the ALS-specific SOD1 mutation and coaxed them in vitro into both motor neurons and astrocytes, which have been speculated to synergize during disease development in vivo. Coculture of both cell types indeed resulted in significantly more cell death of motor neurons, thus indicating that astrocytes, in addition to motor neurons themselves, contribute to the pathophysiology of ALS.

Concluding thoughts

The generation of iPSCs 5 years ago provided researchers with a unique platform to dissect the mechanisms of cellular reprogramming, which largely remained elusive for the past six decades. While many questions remain, interesting insights have been gained into the process of reprogramming, such as the finding that cells undergo defined sequential molecular events in an apparently stochastic manner, and these events are influenced by the choice and number of transcription factors as well as the starting cell type. The ease with which iPSCs can be generated with improved methodology has facilitated the development of chemical and siRNA screens as well as biochemical studies that should further unravel the mechanisms of this process.

The discovery of iPSCs has also influenced our view of normal development, as it demonstrated that activation of only a few transcription factors can potentially change cell fate and, hence, that mammalian cells must have developed epigenetic mechanisms to efficiently lock in a cell once it has differentiated. These mechanisms are often broken in cancer cells, which exhibit features of stem cells and signs of dedifferentiation. Notably, many signaling pathways mutated in cancer cells have been shown recently to affect the formation of iPSCs, indicating

remarkable similarities between tumorigenesis and cellular reprogramming.

The isolation of iPSCs has also sparked new interest in interconverting mature cell types directly into each other, which has already led to a number of remarkable examples for pancreatic, muscle, and neural cell types. It is likely that many other direct cell switches will be achieved in the near future. It remains to be tested, however, whether transdifferentiation works in the human system as well, and whether lineage-converted cells are functionally equivalent to their *in vivo* counterparts.

Despite numerous technical advances in the derivation of human iPSCs, relatively little is known about their molecular and functional equivalence to ESCs, which could affect their potential therapeutic utility. Addressing this question will require a careful analysis of the genomic and epigenomic integrity of human iPSCs, as well as the development of optimized differentiation protocols and reliable assays to evaluate the functionality of iPSC-derived specialized cells.

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