

Reprogramming with defined factors: from induced pluripotency to induced transdifferentiation

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ABSTRACT: Ever since work on pluripotency induction was originally published, reporting the reprogramming of somatic cells to induced pluripotent stem cells (iPS cells) by the ectopic expression of the four transcription factors Oct4, Sox2, Klf4 and c-Myc, high expectations regarding their potential use for regenerative medicine have emerged. Very recently, the direct conversion of fibroblasts into functional neurons with no prior pluripotent stage has been described. Interconversion between adult cells from ontogenically different lineages by an induced transdifferentiation process based on the overexpression of a cocktail of transcription factors, while avoiding transition through an embryonic stem cell-like state, provides a new impetus in the field of regenerative medicine. Here, we review the induced reprogramming of somatic cells with defined factors and analyze their potential clinical use. Beginning with induced pluripotency, we summarize the initial objections including their extremely low efficiency and the risk of tumor generation. We also review recent reports describing iPS cells' capacity to generate viable offspring through tetraploid complementation, the most restrictive pluripotency criterion. Finally, we explore the available evidence for 'induced transdifferentiated cells' as a novel tool for adult cell fate modification.

Key words: reprogramming / iPS cells / induced pluripotency / transdifferentiation / induced cell fate change

Introduction

Research with human embryonic stem cells (ESCs) has been controversial due to the political, religious and ethical implications about the use of human embryos. In an attempt to circumvent these issues, researchers have focused on somatic (adult) stem cells (ASCs) as a source of pluripotent cells. However, they face the following main hurdles: (i) ASCs are relatively rare undifferentiated cells found in many organs and differentiated tissues, (ii) their isolation into pure populations is not always possible and (iii) they have a limited capacity for both self-renewal (in the laboratory) and differentiation with their varying differentiation potential linked to the lineage from which they originated, what it means that ASCs are multipotent instead pluripotent cells. Moreover, ASCs causes immunological rejection in allotransplantation (<http://stemcells.nih.gov>).

The idea of generating pluripotent cells derived directly from the patient's own somatic cells, having the capacity to replace tissue and, thus, avoiding allotransplantation problems, is very attractive for researchers, pharmaceutical companies and clinicians. More than a

decade ago, *Wilmot et al.* (1997) showed that adult somatic cells could be reprogrammed back into an undifferentiated embryonic state using somatic cell nuclear transfer (SCNT). However, since then, attempts to generate patient-specific cells using SCNT have proven unsuccessful (*French et al.*, 2008; *Chung et al.*, 2009; *Kim et al.*, 2009a). In 2006, groundbreaking work by Takahashi and Yamanaka from the University of Kyoto brought about a turning point in the field of stem cells as they were able to reprogram somatic cells to pluripotent cells by the viral expression of four transcription factors: Oct4, Sox2, Klf4 and c-Myc. They termed these cells induced pluripotent stem (iPS) cells (*Takahashi and Yamanaka*, 2006) (Fig. 1).

iPS cells share many features with ESCs, such as morphology, immortal proliferation and pluripotency, as defined by their ability to generate teratomas and differentiate into all the lineages of the three germ layers, including germ cells that can ultimately give rise to offspring (*Takahashi and Yamanaka*, 2006; *Maherali et al.*, 2007; *Okita et al.*, 2007; *Wernig et al.*, 2007). In 2009, two research groups, headed by Gao and Zhou, simultaneously published online

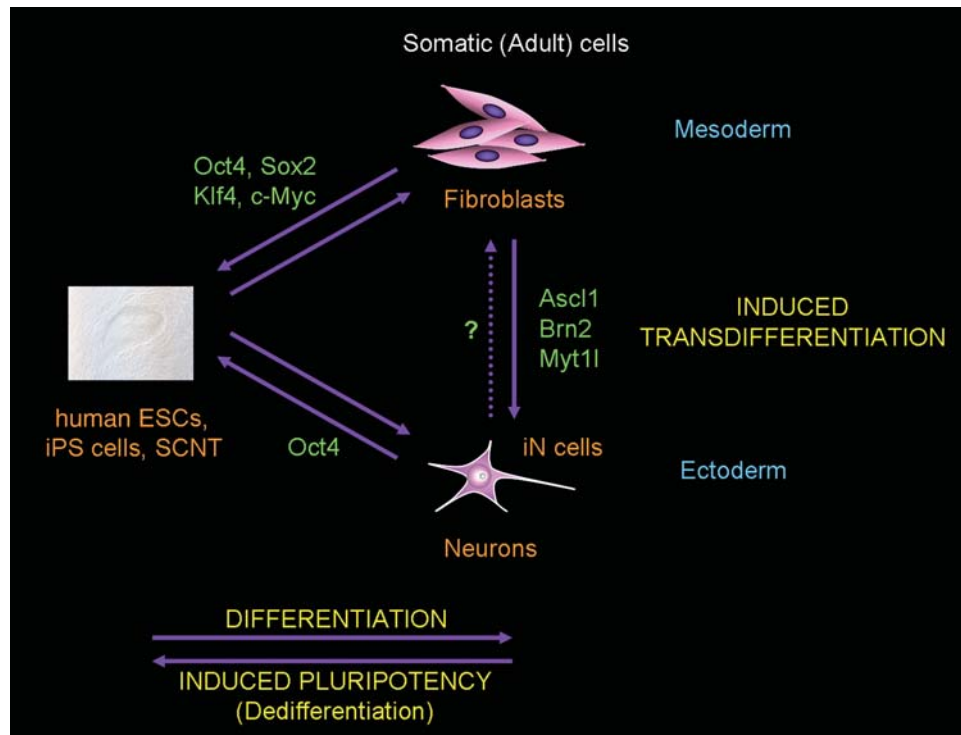


Figure 1 Differentiation and induced cell fate change. Differentiation process drives from left to right side in the schema. De-differentiation or induced pluripotency can be achieved by ectopic expression of four transcription factors in fibroblasts or just one in neurons (see text for more details regarding endogenous levels of transcription factors). In the schema, induced pluripotency drives reversal of differentiation. Induced transdifferentiation can be achieved from fibroblasts (mesoderm) to induced neuronal (iN) cells (ectoderm) by ectopic expression of three transcription factors as described in text. The reversal conversion has not been described yet.

that iPS can generate viable, fertile live-born mice by a tetraploid blastocyst complementation assay (Kang *et al.*, 2009; Zhao *et al.*, 2009). One week later, Baldwin and co-workers published additional data reporting the generation of fertile adult mice derived entirely from iPS cells (Boland *et al.*, 2009). The tetraploid complementation assay consists of fusing two blastomeres, which are then cultured to produce a tetraploid morula or blastocyst without an inner cell mass. This blastocyst is then injected with iPS cells and implanted into a rodent uterus where the cells from the tetraploid blastocyst form the trophoblast and ultimately the placenta, whereas the developing fetus is derived from the iPS cells (summarized in Lo *et al.*, 2010).

Although iPS cells, and ESCs are very similar, they are not identical as they show differences in gene expression signatures (Chin *et al.*, 2009), DNA methylation patterns (Deng *et al.*, 2009) and the efficiency to differentiate to specific lineages which has been reported to be superior in ESCs compared with iPS cells (Feng *et al.*, 2010).

Induced pluripotency by defined factors

iPS cells were initially generated by an ectopic expression of four transcription factors: Oct4, Sox2, Klf4 and c-Myc (the so-called Yamanaka factors) in mouse embryonic and adult fibroblasts (Takahashi and

Yamanaka, 2006). In 2007, two articles were published on the same day in *Cell* and *Science* (Takahashi *et al.*, 2007a; Yu *et al.*, 2007), respectively, and were the first evidence of induced pluripotency in adult human fibroblasts with two different cocktails of factors. Yamanaka and co-workers (Takahashi *et al.*, 2007a, b) overexpressed the transcription factors Oct4, Sox2, Klf4 and c-Myc by retroviral transduction of adult human dermal fibroblasts from the facial dermis of a 36-year-old Caucasian female, while Thomson and co-workers (Yu *et al.*, 2007) replaced Klf4 and c-Myc with Nanog and Lin28, and transduced the IMR90 fetal fibroblast line, as well as post-natal fibroblasts from human newborn foreskin, which are both available in the American Type Culture Collection. Both groups obtained comparable results with similar but not identical methods and validated the reproducibility of obtaining human iPS cells from fibroblasts. In 2008, Park *et al.* (2008a, b) derived iPS cells from fetal, neonatal and adult human fibroblasts. Since these initial reports, iPS cells have also been generated from fibroblasts of different species: canine (Shimada *et al.*, 2010), pig (Esteban *et al.*, 2009; Ezashi *et al.*, 2009; Wu *et al.*, 2009), rat (Liao *et al.*, 2009), non-human primates (Liu *et al.*, 2008; Wu *et al.*, 2010) and humans (Takahashi *et al.*, 2007a, b; Yu *et al.*, 2007; Lowry *et al.*, 2008; Park *et al.*, 2008a, b). iPS cells have also been generated from several mouse cell types: stomach cells (Aoi *et al.*, 2008), liver cells (Aoi *et al.*, 2008; Stadtfeld *et al.*, 2008b), pancreatic β -cells (Stadtfeld *et al.*, 2008a), lymphocytes (Hanna *et al.*, 2008), meningiocytes (Qin *et al.*, 2008), neural

progenitor/stem cells (Eminli et al., 2008; Kim et al., 2008, 2009b; Silva et al., 2008; Tat et al., 2010), melanocytes and melanoma cells (Utikal et al., 2009b), as well as adipose tissue-derived cells (Tat et al., 2010). iPS cells have also been produced from rat fibroblasts and bone marrow cells (Liao et al., 2009) and rat liver progenitor cells (Li et al., 2009a, b, c). In humans, many different cell types have been used for reprogramming, such as keratinocytes (Aasen et al., 2008; Aasen and Belmonte, 2010), CD34+ hematopoietic stem cells (Loh et al., 2009), cord blood-derived CD133+ stem cells (Giorgetti et al., 2009), cord blood-derived endothelial cells (Haase et al., 2009), melanocytes (Utikal et al., 2009b), neural stem cells (NSCs) (Kim et al., 2009c), amniotic fluid-derived cells (Li et al., 2009a, b, c), CD34+ peripheral blood cells from patients with myeloproliferative disorders (Ye et al., 2009), adult human adipose stem cells from lipoaspirate (Sun et al., 2009), human mesenchymal-like stem/progenitor cells of dental tissue origin (Yan et al., 2010) and mesenchymal stem cells from umbilical cord matrix and amniotic membrane (Cai et al., 2010). During 2010, many other cell sources have been used for inducing pluripotency (Table 1).

The original work of Takahashi and Yamanaka in 2006 established the possibility of generating iPS cell colonies by the co-transduction of an initial 24 factors which were narrowed down during screening to just four: Oct3/4, Sox2, Klf4 and c-Myc. Apparently, the most important factor is Oct3/4 since its expression is highly specific for pluripotent stem cells and cannot be replaced by other members of the Oct family (Nakagawa et al., 2008). Researchers in Schöler's team have generated iPS cells from human NSCs derived from human fetal brain tissue by the ectopic expression of Oct3/4 alone (Fig. 1). They established two clones from one-factor iPS cell colonies which yielded teratomas and adult chimeric mice. The Oct4-GFP expression was detected in the gonads of adult chimeras, thus demonstrating germ-line contribution (Kim et al., 2009b, c). These studies revealed the importance of Oct4 in inducing pluripotency in NSCs, although it must be emphasized that these cells endogenously express the rest of the Yamanaka factors, as well as several intermediate reprogramming markers, which possibly facilitates reprogramming in the absence of these factors added exogenously. Other studies have demonstrated that it is also possible to generate iPS cells by retroviral transduction of different combinations of factors, e.g. by combining Oct4 and Sox2 with Lin28 and Nanog (Yu et al., 2007; Haase et al., 2009), by expressing exogenous Oct4 together with either Klf4 or c-Myc (Kim et al., 2008), by transducing three factors Oct4, Sox2, Nanog (Zhao et al., 2010) or just two factors Oct4 and Sox2 (Huangfu et al., 2008b; Giorgetti et al., 2009, 2010), or Oct4 and Klf4 (Tsai et al., 2010), and by expressing Sox2, c-Myc and Tcf-1A (Picanço-Castro et al., 2010). Sox2 has been reported to be dispensable for reprogramming neural progenitor cells (Eminli et al., 2008), and also melanocytes and melanoma cells (Utikal et al., 2009b). Klf4 can be replaced with Esrrb, an orphan nuclear receptor, in reprogramming mouse embryonic fibroblasts (MEFs) (Feng et al., 2009). Interestingly it has been shown impossible to replace Oct4 with its closely related family members Oct1 and Oct6 (Nakagawa et al., 2008) to date. However a recent report describes how the nuclear receptor Nr5a2 can replace exogenous Oct4 in the reprogramming of murine somatic cells to iPS cells (Heng et al., 2010). In summary, these studies reveal that the differences needed in the cocktail of factors for

reprogramming specific cell types are directly related to the endogenous levels of these factors in the target cell(s).

In general, the efficiency of this process is poor when any of the indicated approaches are used (Yamanaka, 2007; Nakagawa et al., 2008; Wernig et al., 2008a, also commented in Hong et al., 2009). In order to improve the efficiency of inducing pluripotency, the stimulation of Wnt signaling can be used in combination with nuclear factors Oct4, Sox2 and Klf4 (Marson et al., 2008). Moreover, inhibition of transforming growth factor- β signaling co-operates in the reprogramming of murine fibroblasts by enabling a faster and more efficient generation of iPS cells (Maherali and Hochedlinger, 2009). A high-efficiency system for the generation of iPS cells has been developed on an inducible lentiviral system to generate 'secondary' pluripotent cells in which human iPS cell clones are differentiated *in vitro* to yield fibroblast-like cells. These cells harbor the inducible viral transgenes required for reprogramming (Maherali et al., 2008). Nevertheless, such systems could arouse concern in terms of safety, as reviewed below. At the end of 2008, a p53 (also known as TP53 in humans and Trp53 in mice) short-interfering RNA (siRNA) and undifferentiated embryonic cell transcription factor 1 (UTF1) were described to enhance the efficiency of iPS cell generation by up to 100-fold, even when oncogene c-Myc had been removed from the reprogramming gene combinations (Zhao et al., 2008). Activation of p53, the 'guardian of the genome' (Lane, 1992), leads to cell cycle arrest, a program that induces cell senescence/cellular apoptosis in response to a variety of stress signals, including overexpressed oncogenes such as c-Myc (Vogelstein et al., 2000; Jin and Levine, 2001). Klf4 can either activate or antagonize p53 depending on the cell cycle target and the level of expression (Rowland et al., 2005) (Fig. 2). Therefore, overexpression of c-Myc and Klf4 oncogenes seems to activate the p53 pathway, leading to cell cycle arrest and/or to apoptosis, and ultimately to reduced reprogramming efficiency. In 2009, five reports were published simultaneously and described how the suppression or alteration of the p53 pathway enhances the efficiency of human iPS cell generation (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a, b, c; Marión et al., 2009; Utikal et al., 2009a). These works are extremely interesting as they have established remarkable similarities between the reprogramming process and oncogenic transformation, which may provide insights into new approaches to cancer therapy (Krizhanovsky and Lowe, 2009). However, inactivation or suppression of the p53 pathway to enhance reprogramming efficiency does not seem to be recommendable, since p53 inactivation can contribute to tumorigenesis by propagation of genomic instability. Thus, inhibition or alteration of p53 pathway could increase the reprogramming efficiency in global terms, regarding number of cells reprogrammed, but not in terms of safety, as an altered p53 pathway could render iPS cells with genomic instability and tumorigenesis, clearly, not desirable for clinical use.

Limitations for iPS clinical applicability

iPS cells offer enormous clinical potential, although their future is limited by the possible risks of tumor formation. It has been demonstrated in the mouse system that iPS cells-derived chimeras frequently develop tumors resulting from the reactivation of the oncogenes c-Myc and Klf4 (Okita et al., 2007; Kim et al., 2009b; Markoulaki et al., 2009). To avoid these problems, iPS cells have been generated from mouse

Table 1 iPS cells generation: somatic cell sources, reprogramming factors used and reprogramming efficiencies.

Species	Somatic cell source	Reprogramming factors	Reprogramming efficiency	References
Canine	Embryonic fibroblasts (CEFs)	Oct4, Sox2, Klf4, c-Myc	n.d.	Shimada <i>et al.</i> (2010)
Human	Fibroblasts	Oct3/4, Sox2, Klf4, c-Myc	~0.02%	Takahashi <i>et al.</i> (2007a, b)
		Oct4, Sox2, Nanog and Lin28	~0.15%	Yu <i>et al.</i> (2007)
		Oct4, Sox2, Klf4, c-Myc + hTERT + SV40 LT	~0.21% in neonatal foreskin fibroblasts BJ1	Park <i>et al.</i> (2008a, b)
		Oct4, Sox2, Klf4, c-Myc, Nanog	n.d.	Lowry <i>et al.</i> (2008)
		Sox2, c-Myc, Tcl-1A	From 0.045 to 0.0575% (n = 2)	Picanço-Castro <i>et al.</i> (2010)
	Amniotic fluid-derived cells (hADFCs) 'precursor state'	Oct4, Sox2, Klf4, c-Myc	From 0.059 to 1.525%	Li <i>et al.</i> (2009a, b, c)
	Terminally differentiated amniotic fluid cells	Oct4, Sox2, Klf4, c-Myc	n.d.	Galende <i>et al.</i> (2010)
	Amnion-derived cells	Oct4, Sox2, Nanog	~0.1%	Zhao <i>et al.</i> (2010)
	Aortic vascular smooth muscle cells (HASMCs)	Oct4, Sox2, Klf4, Nanog, Lin28	0.002%	Lee <i>et al.</i> (2010)
	Bone marrow mononuclear cells (BM-MNCs)	Oct4, Sox2, Klf4, c-Myc	n.d.	Kunisato <i>et al.</i> (2010)
	CD34+ hematopoietic stem cells	Oct4, Sox2, Klf4, c-Myc	~0.01–0.02% (n = 3)	Loh <i>et al.</i> (2009)
	CD34+ peripheral blood cells from patients with myeloproliferative disorders (KAK2-V617F heterozygous genotype)	Oct4, Sox2, Klf4, c-Myc	n.d.	Ye <i>et al.</i> (2009)
	Cord blood-derived endothelial cells	Oct4, Sox2, Nanog and Lin28	From 0.0001 to 0.03%	Haase <i>et al.</i> (2009)
	Cord blood-derived CD133+ stem cells	Oct4, Sox2, Klf4	0.45 ± 0.27%, (n = 5)	Giorgetti <i>et al.</i> (2009)
		Oct4, Sox2	~0.00625%	Giorgetti <i>et al.</i> (2010)
	Dental tissue (dental pulp stem cells)	Oct4, Sox2, Klf4, c-Myc	~0.1% (n = 2)	Yan <i>et al.</i> (2010)
		Oct4, Sox2, Nanog and Lin28	From 0.002 to 0.1% (n = 4)	
	Dermal papilla cells (specialized skin fibroblasts)	Oct4, Klf4	0.024%	Tsai <i>et al.</i> (2010)
		Oct4, Sox2, Klf4, c-Myc	1.38%	
	Gastrointestinal cancer cells	Oct4, Sox2, Klf4, c-Myc	~0.1% GFP-expressing sphere formations	Miyoshi <i>et al.</i> (2010)
Hepatocytes	Oct4, Sox2, Klf4, c-Myc	~0.1–0.2% (n = 3) (Tra-1-60-positive colonies)	Liu <i>et al.</i> (2010)	
Keratinocytes	Oct4, Sox2, Klf4, c-Myc	~1.0%	Aasen <i>et al.</i> (2008, 2010)	
Lipoaspirate adipose stem cells	Oct4, Sox2, Klf4, c-Myc	~0.2%	Sun <i>et al.</i> (2009)	
Malignant cells (chronic myeloid leukaemia cells)	Oct4, Sox2, Klf4, c-Myc	n.d.	Carette <i>et al.</i> (2010)	
Melanocytes	Oct4, Sox2, Klf4, c-Myc	~0.05%	Utikal <i>et al.</i> (2009b)	
	Oct4, Sox2, Klf4	~0.01%		
	Oct4, Klf4, c-Myc	~0.01%		
Neural stem cells	Oct4	0.004%	Kim <i>et al.</i> (2009c)	
	Oct4, Klf4	0.006%		
Peripheral blood mononuclear cells (PB-MNCs)	Oct4, Sox2, Klf4, c-Myc + hTERT + SV40 LT	~0.0006% (three colonies from 5 × 10 ⁵ PB-MNCs cultured with ST3FP6)	Kunisato <i>et al.</i> (2010)	
Umbilical cord matrix and amniotic membrane mesenchymal stem cells	Oct4, Sox2, Klf4, c-Myc + Vitamin C and valproic acid	Up to 0.4 and up to 0.1%, respectively	Cai <i>et al.</i> (2010)	
Umbilical vein endothelial cells	Oct4, Sox2, Klf4, c-Myc	0.03%	Lagarkova <i>et al.</i> (2010)	

Continued

Table 1 Continued

Species	Somatic cell source	Reprogramming factors	Reprogramming efficiency	References	
Mouse	Adipose tissue-derived cells (ADCs) Fibroblasts (embryonic, MEFs, and adult)	Oct4, Sox2, Klf4, c-Myc	0.14 ± 0.77%	Tat et al. (2010)	
		Oct4, Sox2, Klf4, c-Myc (+ selection for Fbx15)	~0.0125% (MEFs)	Takahashi and Yamanaka (2006)	
	Liver cells	Oct4, Sox2, Klf4, c-Myc (+ selection for Fbx15)	n.d.	Aoi et al. (2008)	
		Oct4, Sox2, Klf4, c-Myc	~0.0006% (adult liver cells) ~0.0018% (fetal liver cells)	Stadtfield et al. (2008b)	
	B lymphocytes	Oct4, Sox2, Klf4, c-Myc + C/EBPα or shPax5 (mature B cells)	1/30	Hanna et al. (2008)	
	Melanocytes	Oct4, Sox2, Klf4, c-Myc	~0.19%	Utikal et al. (2009b)	
		Oct4, Sox2, Klf4	~0.02%		
		Oct4, Klf4, c-Myc	~0.03%		
	Melanoma cells	Oct4, Klf4, c-Myc	n.d.		
	Meningiocytes	Oct4, Sox2, Klf4, c-Myc	~0.8%	Qin et al. (2008)	
	Neural progenitor cells	Oct4, Sox2, Klf4, c-Myc	Oct4, Klf4, c-Myc	From 0.0002 to 0.0008%	Eminli et al. (2008)
			Oct4, Klf4, c-Myc	From 0.001 to 0.002%	
		Oct4, Sox2, Klf4, c-Myc	3.6 ± 0.5%	Kim et al. (2008)	
		Oct4, Klf4	0.11 ± 0.02%		
		Oct4 (adult mouse neural stem cells)	0.014%	Kim et al. (2009b)	
Oct4, Sox2, Klf4, c-MycT58+Mek/Erk and GSK3 inhibitors + LIF		(4F) 0.11%			
Oct4, Klf4, c-MycT58+Mek/Erk and GSK3 inhibitors + LIF		(3F) 0.34%	Silva et al. (2008)		
Oct4, Klf4+Mek/Erk and GSK3 inhibitors + LIF		(2F) ~0.0042% around one-third of the colonies stably activated Oct 4-GFP			
Neural stem cells from sub ventricular zone (NSCs-SVZ)		Oct4, Sox2, Klf4, c-Myc	0.13 ± 0.06%	Tat et al. (2010)	
Pancreatic β-cells		Oct4, Sox2, Klf4, c-Myc	0.16%	Stadtfield et al. (2008a)	
Stomach cells	Oct4, Sox2, Klf4, c-Myc (+ selection for Fbx15)	n.d.	Aoi et al. (2008)		
Porcine	Embryonic fibroblasts from Tibetan miniature pig	Oct4, Sox2, Klf4, c-Myc	From ~0.07 to 0.11%	Esteban et al. (2009)	
	Fetal fibroblasts	Oct4, Sox2, Klf4, c-Myc	0.1%	Ezashi et al. (2009)	
	Primary ear fibroblasts (PEFs) or primary bone marrow cells (BMCs)	Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28	~0.18%	Wu et al. (2009)	
	Bone marrow mesenchymal stem cells	Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28	n.d.	West et al. (2010)	
Rat	Bone marrow cells	Oct4, Sox2, Klf4, c-Myc	~0.024%	Liao et al. (2009)	
	Fibroblasts (adult)	Oct4, Sox2, Klf4, c-Myc	~0.054%		
	Liver progenitor cells	Oct4, Sox2, Klf4 in mESC standard medium containing MEK, GSK3β and ALK5 inhibitors + LIF	~0.4%		
Non-human primates	Marmoset skin fibroblasts	Oct4, Sox2, Klf4, c-Myc	~0.025%	Wu et al. (2010)	
	Rhesus monkey fibroblasts	Oct4, Sox2, Klf4, c-Myc	~0.033%	Liu et al. (2008)	

n.d., not determined.

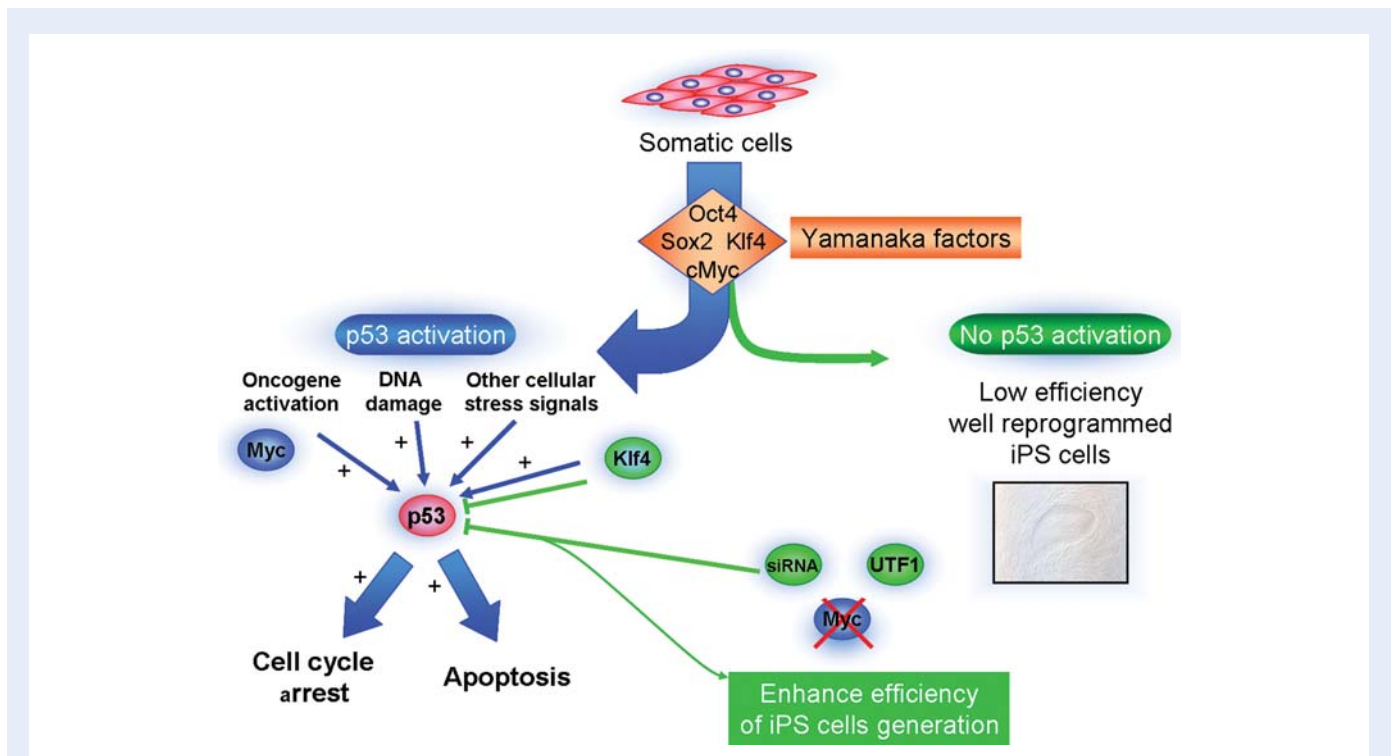


Figure 2 iPS generation and the p53 pathway. Reprogramming with Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc) drives most cells through the blue route to cell cycle arrest/apoptosis as the p53 pathway is activated by DNA damage and/or other cellular stress signals. This way does not produce iPS cells. However, the few cells with no activated p53 pathway develop to full, well-reprogrammed iPS cells through the green route. The induced pluripotency process is globally considered poorly efficient, as p53 ensures that only cells with no DNA damage progress to full, well-reprogrammed iPS cells. The role of Klf4, siRNA and UTF1 is described in the text. Partial or transitory inhibition of the p53 pathway could render the process of iPS cell generation more efficient in terms of the number of cells reprogrammed, although it would be much more inefficient in safety terms, as inhibition of the p53 pathway enhances tumorigenicity.

and human fibroblasts without oncogenes c-Myc and Klf4 (Aoi *et al.*, 2008; Huangfu *et al.*, 2008b; Nakagawa *et al.*, 2008; Wernig *et al.*, 2008a), although with viral integration.

The use of genome integrative methods, such as retroviral/lentiviral vectors, may also cause, by itself, tumor formation. Although the expression of encoded genes is silenced in fully reprogrammed iPS cell lines with retroviral integrative methods (Hotta and Ellis, 2008), and nearly complete silencing of lentiviral transgenes has been observed in the context of induced pluripotency (Yu *et al.*, 2007; Ebert *et al.*, 2009), the integrated foreign DNA remains in the genome and could disrupt/alter the host genome expression, causing tumor formation (Hochedlinger *et al.*, 2005). Furthermore, it has been proposed that residual transgene expression may explain some of the observed differences between ESCs and iPS cells, such as the altered differentiation into functional cell types (Yu *et al.*, 2007; Soldner *et al.*, 2009).

Generation of iPS cells without viral integration has proved possible in mouse hepatocytes using adenovirus (Stadtfeld *et al.*, 2008b); however, the frequency of reprogramming was extremely low and a high percentage of clones were tetraploid. iPS cells have been generated in MEFs by using a serial transient expression of two plasmids (Okita *et al.*, 2008, 2010). iPS cells have also been generated by nucleofection of a polycistronic construct co-expressing Oct4, Sox2, Klf4 and c-Myc in MEF with no evidence of integration in the host

genome (González *et al.*, 2009). iPS cells have also been derived from the genomic integration of the four reprogramming factors using plasmids (Kaji *et al.*, 2009), lentiviruses (Soldner *et al.*, 2009) or transposons (Woltjen *et al.*, 2009) followed by transgene removal with Cre-mediated excision or the re-expression of transposase (Yamanaka, 2009). One step forward, involving iPS cells of human origin, has been generated by transfection with non-integrating episomal vectors (Yu *et al.*, 2009). The use of chemicals or small molecules has proved a safer strategy for generating iPS cells (Huangfu *et al.*, 2008a, b; Shi *et al.*, 2008; Ichida *et al.*, 2009). Generation of human iPS cells by direct delivery of reprogramming proteins (p-hiPSCs), using peptides that are capable of overcoming the cell membrane barrier, anchored to reprogramming proteins, is very slow, inefficient and requires further optimization (Kim *et al.*, 2009a). Similarly, reprogramming with recombinant proteins combined with chemical compounds (e.g. valproic acid), as reported by the Ding team (Zhou *et al.*, 2009), has not proven completely satisfactory since the use of refolded proteins after expression in *Escherichia coli* by genetic manipulation and chemicals may induce DNA alterations and not fully functional proteins. Another step forward, this year has been the induction of pluripotency in human fibroblasts using the mRNA of the four Yamanaka factors (Yakubov *et al.*, 2010). Also the reprogramming of human fibroblasts to iPS cells under strict xeno-free conditions, avoiding the use of products of animal origin, as well as

the use of autologous feeder cells devoid of xenobiotics (Rodríguez-Pizà et al., 2010), implies an important advance for iPS cell-based therapies. More recently, it has been reported that it is possible to reprogram up to a pluripotent-like state, without the forced expression of ectopic transgenes, by a transfer of cellular extracts from ESCs into adult mouse fibroblasts (Cho et al., 2010). This study is based on previous ones which have shown a modest effect on reprogramming into specific lineages (Håkelién et al., 2002; Qin et al., 2005) or reprogramming that did not reach the pluripotent state (Taranager et al., 2005; Rajasingh et al., 2008).

In summary, safer transient and/or non-integrative methods should be used for generating iPS cells. Moreover, before iPS cells prove suitable for application in regenerative medicine, the differentiation efficiency of iPS cells into required functional cells should be enhanced by improving differentiation protocols as well as defining the best chemical cocktails of differentiation factors. Another important aspect to consider is the choice of the reprogramming target cell population. Utilization of somatic cells from easily accessible sources which do not imply invasive methods is desirable, along with cells that do not produce an immune rejection after transplantation due to the immunological incompatibility between patient and donor cells. Thus, the use of human keratinocytes from the patient's own plucked hair (Aasen and Belmonte, 2010) could be a potential source of autologous iPS cell generation as both requisites are met with this type of cell. Given all aforementioned aspects, the most important point for the clinical applicability of these cells is the generation of safer iPS cells in terms of non-tumorigenicity. It has been recently demonstrated that the teratoma-forming propensities of secondary neurospheres vary significantly depending on the iPS cells' tissue of origin (Miura et al., 2009). Thus, teratoma formation by derivatives of iPS cells appears to be affected by reprogramming and differentiation methods, and also by the transplantation site, among several other factors. For these reasons, Yamanaka recommended whole-genome sequencing of iPS cells to identify retroviral integration sites or other alterations such as integration of small plasmid fragments or chemically induced mutations prior to any possible application in human therapy (Yamanaka, 2009). Finally, before using iPS cells for regenerative medicine in humans, iPS cells should be generated under Good Manufacturing Practice standards. However, there are opinions which consider the generation of iPS cells from each patient unfeasible in economic terms. Nevertheless, private companies could obtain considerable incomes by performing these procedures once the absolute safety of iPS cells for regenerative medicine has been demonstrated, without forgetting ethical principles that ensure responsible use for the benefit and progress of humanity (Lanza, 2007). Until such a time, iPS cells from patients (disease-specific iPS cells) offer an unprecedented opportunity and enable investigation and drug development *in vitro* for specific diseases. In 2007, Hanna et al., by using a humanized sickle cell anemia mouse model, showed that mice can be rescued after transplantation with hematopoietic progenitors obtained *in vitro* from autologous iPS cells. This was achieved after the correction of the human sickle haemoglobin allele by gene-specific targeting (Hanna et al., 2007). In 2008, Park et al., generated iPS cells from patients with a variety of genetic diseases with either Mendelian or complex inheritance; these diseases include adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman–

Bodian–Diamond syndrome, Gaucher disease type III, Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy, Parkinson's disease, Huntington's disease, juvenile-onset, type I diabetes mellitus, Down syndrome/trisomy 21 and the carrier state of Lesch–Nyhan syndrome (Park et al., 2008c). Also, in 2008, Wernig et al. reported that Neurons derived from reprogrammed fibroblasts could functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease (Wernig et al., 2008b). Ebert et al. (2009) generated iPS cells from fibroblasts of a spinal muscular atrophy patient, being the first study to show that human iPS cells can be used to model the specific pathology seen in a genetically inherited disease. Using Cre-recombinase excisable viruses, Soldner et al. generated iPS cells from Parkinson's disease patient fibroblasts, free of ectopic reprogramming factors. These factor-free human iPS cells were pluripotent, and as judged by molecular criteria, were more similar to embryo-derived human ESCs than to the conventional vector-carrying parental human iPS cells (Soldner et al., 2009). In 2009, Raya et al. demonstrated that on correction of the genetic defect, somatic cells from Fanconi anemia patients can be reprogrammed to pluripotency to generate patient-specific iPS cells (Raya et al., 2009, 2010). iPS cells generated with Oct4, Sox2 and Klf4 from the DMD mouse model and fibroblasts of a DMD patient, respectively, combined with the use of a human artificial chromosome (HAC), with a complete genomic dystrophin sequence (DYS-HAC), have allowed the complete correction of this genetic deficiency in both the mouse model and the patient-derived iPS cells (Kazuki et al., 2010). Recently, reprogrammed iPS cells have been generated from patients with different diseases such as fragile-X syndrome (Urbach et al., 2010) and LEOPARD syndrome (an acronym formed from its main features, that is, lentigenes, electrocardiographic abnormalities, ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, retardation of growth and deafness) (Carvajal-Vergara et al., 2010). These data offer proof-of-concept that iPS cell technology can be used for the generation of disease-corrected, patient-specific cells with potential for cell therapy applications (Raya et al., 2009, 2010).

Generation of germ-line-competent iPS cells

Original iPS cells (termed Fbx15iPS cells) failed to generate adult chimaeras and differ from ESCs in terms of gene expression and DNA methylation (Takahashi and Yamanaka, 2006). As another progressive step forward, in 2007, this group reported that the ectopic retroviral expression of Oct3/4, Sox2, Klf4 and c-Myc (OSKM), followed by selection for Nanog expression, resulted in iPS cells with an increased ES cell-like gene expression and DNA methylation patterns. These iPS cells functionally differentiate into all cell lineages in chimeric mice, including germ cells which can ultimately give rise to offspring (Okita et al., 2007). The authors injected eight different clones of Nanog iPS cells into blastocysts, which were then transferred into the uteri of pseudo-pregnant mice. They obtained adult chimaeras in seven of the eight cases assayed and reported that most of the Nanog-positive iPS clones were competent to generate adult chimeric mice. These Nanog iPS cells contributed to various organs in the chimeras, with a highly variable percentage ranging

from 10 to 90%. Moreover, the chimeras from one of these eight assayed clones showed the highest iPS cell contribution in testes. When three of these chimeric mice were crossed with wild-type females, the resulting F1 generation had transgenes originating from the chimeric parent, indicating germ-line transmission to the F1, and also to the F2 generations when intercrosses of F1 mice were performed. These data confirm the germ-line transmission of Nanog iPS cells in mice, although around 50% of the F1 mice (aged 8–41 weeks) derived from one of the two viable clones either died or were sacrificed because of severe illness (Yamanaka, 2009). Neck and other tumors were observed in all the necropsied mice, and reactivation of the retroviral expression of c-Myc, but not of Oct3/4, Sox2 or Klf4, was detected in these tumors. These data indicate that tumor formation is attributable to the reactivation of the c-Myc retrovirus (Okita *et al.*, 2007; Yamanaka, 2009).

Tbx3, a transcription factor directly related to Nanog and Tcf3, has been recently identified to greatly improve the germ-line competency of iPS cells (Han *et al.*, 2010). Cells generated by the retroviral infection of MEFs, bearing the Oct4-GFP transgene with the classic cocktail (Oct4, Sox2 and Klf4) combined with Tbx3 (OSKT cells), accelerate the reprogramming process by diminishing the number of days required to obtain iPS cell colonies if compared with infection with three factors (OSK) and the classical four factors (OSKM). Furthermore, OSKT cells were more efficient in colonizing the germ tissues than OSK cells, thereby more efficiently generating viable mice composed entirely of cells engineered through tetraploid complementation. Moreover, these authors proved that iPS cells generated by combining Oct4 with Sox2 and the orphan nuclear receptor Esrrb,

also called OSE iPS cells (Feng *et al.*, 2009) tended to contribute poorly to chimaerism. In summary, the iPS cells generated with OSK and Tbx3 are superior in both germ-cell contribution to gonads and germ-line transmission frequency, suggesting that exogenous Tbx3 favors the induction of pluripotency (Han *et al.*, 2010) (Fig. 3).

Transdifferentiation induced by defined factors

Reprogramming somatic cells to an undifferentiated ES cell-like state to further differentiate them into cell types of interest has raised the issue of whether reprogramming could be successfully achieved by directly converting one differentiated cell type into another (Figs 1 and 4). This so-called transdifferentiation process has received significant attention since it could have considerable applications for cellular therapy.

Several studies have described how the ectopic overexpression of isolated factors can induce transdifferentiation from one differentiated cell type into another, generally within the same lineage: in 1987, Davis *et al.* were able to convert mouse fibroblast-like 10T1/2 cells into stable myoblasts by transfecting a single myoblast-specific cDNA (myoD). However, the overexpression of this gene did not induce muscle-specific properties when introduced into hepatocytes (Schäfer *et al.*, 1990). Other examples of transdifferentiation by defined factors can be found in the literature (reviewed in Vierbuchen *et al.*, 2010). Thus, the overexpression of interleukin-2 receptor and granulocyte-macrophage colony-stimulating factor receptor induced

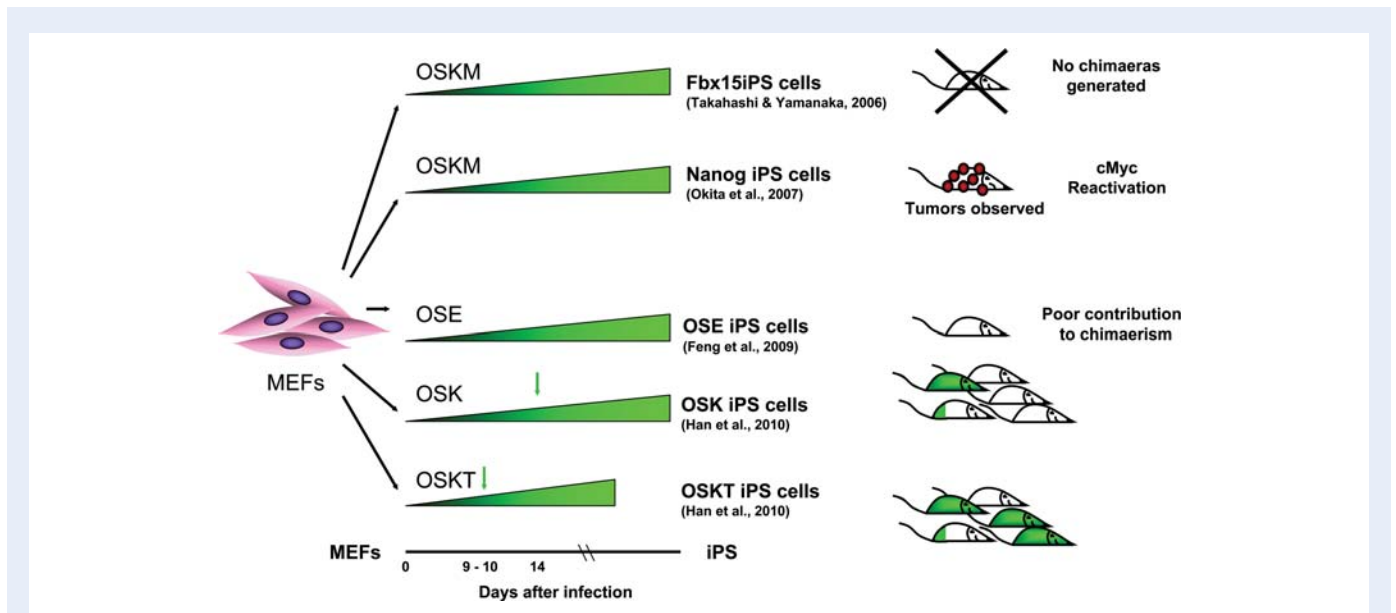


Figure 3 Reprogramming factors and germ-line competency. The reprogramming of MEFs to iPS cells can be achieved with different combinations of factors: oct4 (O); Sox2 (S); Klf4 (K); c-Myc (M); orphan nuclear receptor Esrrb (E); Tbx3 (T). Fbx15iPS cells (Takahashi and Yamanaka, 2006) failed to generate adult chimaeras. Nanog iPS cells (Okita *et al.*, 2007) generated adult chimaeras, but tumors were detected due to the reactivation of oncogene c-Myc. Different combinations were assayed by Han *et al.* 'Although activation of Oct4 typically required 14 days after infection with OSK and OSKM, the use of OSKT took 9-10 days' (sic) (Han *et al.*, 2010). Green arrows indicate activation of the Oct4-GFP transgene. Green wedges indicate time needed for the generation of iPS cells. By the most stringent pluripotency criterion of tetraploid blastocyst complementation, OSE iPS cells produced adult mice, but tended to contribute poorly to chimaerism, whereas OSK and OSKT iPS cells were more efficient for germ-line transmission and the production of viable F2 offspring, where OSKT iPS cells were the most efficient.

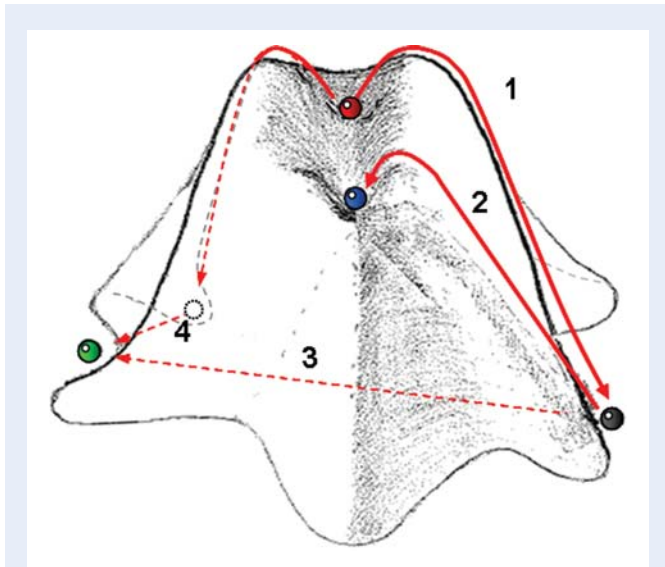


Figure 4 Differentiation and reprogramming. Schematic representation of the processes followed during the differentiation and reprogramming by a cell (a rolling ball in the schema). In energetic terms, as in chemical reactions, each event in nature tends to drive to lower energy levels by overcoming the initial resistance to the process. In this schema, undifferentiated cells (red ball) lie in a crater, at the top of a hill. (1) The differentiation process has to initially overcome slight resistance to differentiate into an adult cell type. This event is represented in the schema by the ball climbing the hill, and then rolling down to the bottom of the hill where it differentiates into adult cell types (black ball and hidden ball, back left). (2) These differentiated cells can transform into an undifferentiated-like state (SCNT or iPS cells), which is different to the original undifferentiated state. These differences between them reside in genetic (Chin et al., 2009) and, probably and additionally, epigenetic differences (Deng et al., 2009). This process is represented in the schema by the black ball rolling from the bottom to a different upper level (blue ball) to the original one (red ball). Differentiated cells (black ball and hidden ball, back left) can also be reprogrammed to another differentiated cell by following a direct way (3) (black ball to green ball), or (4) (hidden ball to green ball). In these particular cases it is interesting to note that the factor or cocktail of factors, which is (are) adequate for transdifferentiate one cell into another (v.g. black ball to green one, route 3), not necessarily have to induce transdifferentiation of another different cell type into the same final one (v.g. hidden ball to green one, route 4), as described by (Davis et al., 1987 and Schäfer et al., 1990). For this reason, in the schema are represented two different routes with two possible different factors or cocktail of factors, to induce transdifferentiation in cells from two different origins, which give rise to the same final cell type. Schema adapted from Waddington (1957).

a myeloid conversion of committed lymphoid progenitor cells (Kondo et al., 2000). The expression of C/EBP alpha and beta in B cells (Xie et al., 2004) induces macrophage differentiation, as well as the overexpression of PU.1 (also called Sfp1), and transcription factors C/EBP alpha and beta, in fibroblasts. (Feng et al., 2008; Bussmann et al., 2009; Graf and Enver, 2009). Moreover, deletion of Pax5 can induce B cells to de-differentiate toward a common lymphoid progenitor (Cobaleda et al., 2007).

Accumulated evidence suggests that a specific combination of multiple factors, rather than a single one, might be the most effective tool to transdifferentiate adult cells. Melton and co-workers described the transdifferentiation process in adult mouse pancreas from exocrine pancreatic cells to cells that closely resembled β -cells by adenoviral infection of three transcription factors (Zhou et al., 2008). These factors were narrowed down from nine genes exhibiting β -cell developmental phenotypes when mutated (Murtaugh and Melton, 2003; Jensen, 2004). After a screening process, the authors were able to directly reprogram fully differentiated exocrine cells to cells that produce insulin and closely resemble β -cells by using a combination of Ngn3 (also known as Neurog3), Pdx1 and Mafa, which is important in the embryonic development of the pancreas and β -cells. Reprogramming exocrine cells to β -cells occurred relatively quickly, with the first insulin-positive cells appearing on Day 3 and an efficiency of up to 20%, which may be due to the fact that pancreatic exocrine and β -cells are closely related cell types and share many of their epigenomic features (Zhou et al., 2008). In 2009, Takeuchi and Bruneau described the direct transdifferentiation of mouse mesoderm to heart tissue by transient transfection of Gata4, Tbx5 and Baf60c in cultured mouse embryos (Takeuchi and Bruneau, 2009). The authors observed that when Baf60c together with the cardiogenic transcription factor Gata4 is injected outside the heart fields, these tissues express heart-specific genes, and remarkably, addition of another transcription factor, Tbx5, transforms non-cardiac embryonic cells into beating heart muscle (reviewed in Liang and Crabtree, 2009).

More recently, Wernig and co-workers described the direct conversion of fibroblasts into functional neurons by defined factors (Vierbuchen et al., 2010) (Figure 1). Initially, 19 genes specifically expressed in neural tissues or implicated in neural development were screened. After the screening process was determined that just three factors (Ascl1, Brn2 and Myt1l) sufficed to convert MEFs, carrying a green fluorescent Tau protein (Tau-GFP) reporter, into functional neurons. Despite the relevance of this study, several obstacles must be overcome to ensure the success of future experiments (Nicholas and Kriegstein, 2010). Thus, it must be shown that induced transdifferentiated (iT) cells can be established and maintained after silencing the expression of the introduced transcription factors to confirm that an intrinsic and stable conversion of cell fate actually occurs. Additionally, safe methods with transient and/or non-integrative methods should be used for generating iT cells, as tested with iPS cells (Kim et al., 2009a). Likewise, human fibroblasts and other sources of human cells should be tested, and *in vivo* assays should be performed, to determine the suitability of iT cells for transplantation and applicability in regenerative medicine (Nicholas and Kriegstein, 2010). Therefore, iT cells represent potential new cell sources for regenerative medicine.

Conclusions

New discoveries of somatic cell reprogramming have opened up the possibility of creating iPS cells using innovative strategies. The aim of these studies is to generate pluripotent cells from any cell that can be isolated from a patient (both mature differentiated cells and tissue-specific stem cells), partially erase the somatic epigenetic program and reactivate the machinery involved in pluripotency. These iPS cells, often referred to as 'patient-specific cells', eliminate the immune

rejection problems in allotransplantation and avoid the ethical concerns of using ESCs. Future therapeutic application of iPS cells in humans requires overcoming several obstacles: (i) bypassing the use of harmful oncogenes as part of the reprogramming factors (Okita *et al.*, 2007), (ii) avoiding the use for gene delivery of integrative vectors that carry the risk of insertional mutagenesis and (iii) developing robust and reliable differentiation protocols for human iPS cells (Hanna *et al.*, 2007). Nevertheless, the fact that iPS cells are derived from cells of patients with a variety of diseases makes them valuable tools to study different disease mechanisms in the laboratory, perform customized toxicology tests and create patient-specific stem cell lines that might lead to the discovery of drugs to treat patients (Belmonte *et al.*, 2009). Cell transdifferentiation methods by directly reprogramming through the ectopic expression of defined factors are also an alternative strategy for cellular therapy.

In the near future, iPS cells and/or iT cells could be used as disease models to help elucidate disease mechanisms, study metabolic pathways and/or screen new drugs, as well as models for normal development, oncology and differentiation processes in humans. Reprogrammed cells have certainly opened a new era in regenerative medicine research with tremendous potential for new discoveries which may help establish therapies for current and new diseases.

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