Pluripotency and nuclear reprogramming

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Embryonic stem cells are promising donor cell sources for cell transplantation therapy, which may in the future be used to treat various diseases and injuries. However, as is the case for organ transplantation, immune rejection after transplantation is a potential problem with this type of therapy. Moreover, the use of human embryos presents serious ethical difficulties. These issues may be overcome if pluripotent stem cells are generated from patients’ somatic cells. Here, we review the molecular mechanisms underlying pluripotency and the currently known methods of inducing pluripotency in somatic cells.

Keywords: embryonic stem cell; induced pluripotent stem cell; transcription factor; retrovirus

1. INTRODUCTION

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst stage embryos and have the unique capacity to proliferate extensively while maintaining pluripotency. The isolation of the first ES cells from mouse embryos (Evans & Kaufman 1981; Martin 1981) led to the development of the revolutionary ‘knockout mouse’ technology (Doetschman et al. 1987; Hooper et al. 1987), which is widely used today. Since ES cells theoretically have the capacity to develop into any cell type, the generation of ES cell lines from human blastocyst embryos (Thomson et al. 1998) has offered the possibility of using these cells as a donor source for cell transplantation therapies. Potential clinical applications include treatment of degenerative diseases such as juvenile diabetes, Parkinson’s disease and heart failure as well as spinal cord injury and burns. However, as is the case for organ transplantation, tissue rejection is a concern for ES cell transplantation. One possible means to avoid immune rejection is the reprogramming of the nuclei of differentiated cells to an ES cell-like, pluripotent state, and using these cells to generate appropriate donor cells for transplantation. Here, we will review the following three methods of inducing nuclear reprogramming and pluripotency in somatic cells: nuclear transfer, fusion with ES cells and reprogramming using defined factors (figure 1).

2. REPROGRAMMING BY NUCLEAR TRANSFER

Much progress has been made in the last 50 years in understanding how differentiation and pluripotency are controlled. First, in order for the somatic cells to regain pluripotency, it is essential that they maintain complete genomes. While it is now widely accepted that all cells in an organism possess the same genome, historically this has not always been the case. For instance, in his 1893 book ‘The germ-plasma: a theory of heredity’, Weismann proposed that somatic cells lose or irreversibly inactivate unnecessary genes in the course of differentiation. It took more than 50 years to disprove this theory (Gurdon & Byrne 2003).

In 1952, Briggs & King showed that nuclear transfer was possible in frogs by transplanting nuclei from blastula stage embryos into enucleated Rana pipiens eggs. The resulting embryos developed into normal hatched tadpoles (Briggs & King 1952). However, their later work showed that transplantation of nuclei from tail-bud stage somatic cells into enucleated eggs resulted in abnormal embryonic development, suggesting that differences in nuclear state affect the capacity of somatic cells to be reprogrammed (King & Briggs 1955, 1956). Gurdon and colleagues then succeeded in producing adult frogs by transferring tadpole intestine cell nuclei into enucleated Xenopus laevis eggs (Gurdon 1962; Gurdon & Uehlinger 1966), showing that the genes required for normal development can be activated even in differentiated cells. However, when they transferred nuclei from adult somatic cells, animals developed to tadpole, but not to adult stage (Laskey & Gurdon 1970; Gurdon et al. 1975; DiBerardino & Hoffner 1983). This raised the possibility that terminally differentiated cells are unable to activate genes required for full development to adulthood.

Nuclear transfer in mammals is more difficult than in frogs due to the small size of eggs. Nonetheless, in 1975, Bromhall reported development to the morula stage following nuclear transfer of rabbit morula cell nuclei into enucleated rabbit eggs, albeit with low efficiency (Bromhall 1975). In 1983, McGrath & Solter obtained live mice by transferring a zygotic donor nucleus into an enucleated zygote (McGrath & Solter 1983). In 1986, Willadsen fused cells of 8- or 16-cell embryos with enucleated sheep eggs and was able to obtain healthy cloned animals (Willadsen 1986). Successful nuclear transfer of embryonic donor cell nuclei was subsequently reported in rabbits (Stice & Robl 1988),...
cells of patients suffering from spinal cord injury and juvenile diabetes (Hwang et al. 2004, 2005). However, in a much-publicized fraud scandal, their data were shown to be a fabrication; indeed, they were unable to generate a single cloned ES cell line from more than 2000 human eggs.

3. REPROGRAMMING BY FUSION WITH ES CELLS

Another strategy for reprogramming the somatic cell nuclei is fusion with ES, embryonic germ (EG) or embryonic carcinoma (EC) cells. In 1965, Harris showed that fusion of HeLa cells with Sendai virus was able to activate erythrocytes (Harris 1965; Harris & Watkins 1965; Harris et al. 1965). Their work facilitated studies in cell fate conversion by fusion with other cell types. In 1976, Miller & Ruddle demonstrated that thymocytes acquired pluripotency upon fusion with EC cells (Miller & Ruddle 1976), and similar results were later obtained by electrofusion with EG cells (Tada et al. 1997) and mouse ES cells (Tada et al. 2001). Reprogramming by spontaneous fusion with mouse ES cells was also demonstrated (Terada et al. 2002; Ying et al. 2002). In tetraploid cells generated by fusion of somatic cells with ES or EG cells, ES cell marker genes such as Oct3/4 acquire an ES cell-like epigenetic state, adopting the DNA methylation and histone modification patterns normally seen in ES cells (Kimura et al. 2004). The transplantation of these cells into nude mice results in formation of teratomas consisting of various tissues from all three germ layers, confirming the pluripotency of these cells. Finally, reprogramming by fusion with human ES cells was reported in 2005 (Cowan et al. 2005; Yu et al. 2005).

Thus, it appears that ES cells contain factors that induce pluripotency in somatic cells, though it is still controversial whether these factors reside in the nucleus (Do & Scholer 2004) or cytoplasm (Strelchenko et al. 2006) of ES cells. However, pluripotent cells generated by fusion contain both somatic cell and ES cell-derived chromosomes. As a result, rejection upon implantation remains an issue. In an effort to circumvent this problem, Tada and colleagues recently developed a system to remove whole chromosomes from tetraploid cells (Matsumura et al. 2007). In addition, other groups have been attempting to reprogramme the somatic cells with ES cell extracts (Taranger et al. 2005).

4. REPROGRAMMING WITH DEFINED FACTORS

The fact that somatic cell nuclei can be reprogrammed by transfer into oocytes or fusion with ES cells indicates that oocytes and ES cells contain reprogramming factors. It is probable that reprogramming factors largely overlap with those maintaining pluripotency in ES cells. Some of the candidate factors are described below.

(a) Oct3/4 (POU5F1)

POU5F1 is a POU family transcription factor specifically expressed in ES cells, early embryos and germ cells, and was originally designated as Oct3 (Okamoto et al. 1990) or Oct4 (Scholer et al. 1989). Oct3/4-null embryos die in utero at peri-implantation stages of

Figure 1. Three methods of inducing pluripotency in somatic cells.
development (Nichols et al. 1998). Although these embryos are able to reach blastocyst stage, in vitro culture of the inner cell mass of homozygous mutant blastocyst produces only trophoblast lineages, and Oct3/4 mutant ES cells differentiate only into trophoblast cells, while cells expressing a single copy of Oct3/4 maintain their pluripotential ES cell state (Niwa et al. 2000). Likewise, knock-down of Oct3/4 by siRNA in human ES cells caused these cells to differentiate into trophectoderm lineages (Zaehres et al. 2005). By contrast, twofold overexpression of Oct3/4 in ES cells causes differentiation into primitive endoderm and mesoderm (Niwa et al. 2000). Hence, the level of Oct3/4 expression is an important determinant of cell fate in mouse ES cells.

(b) Sox2
Sox2 is a Sox (SRY-related HMG-box) family transcription factor expressed in ES cells, early embryos, germ cells and neural stem cells (Koopman et al. 2004). The Sox2-null embryos die around implantation due to a failure in epiblast (primitive ectoderm) development (Avilion et al. 2003). Homozygous mutant blastocysts appear morphologically normal, but undifferentiated cells fail to proliferate when blastocysts are cultured in vitro, and only trophectoderm and primitive endoderm-like cells are produced. Disruption of Sox2 in mouse ES cells by either conditional knockout or RNAi resulted in rapid differentiation (Ivanova et al. 2006; Masui et al. 2007). These data demonstrate that Sox2 is indispensable for maintaining pluripotency in both early embryos and ES cells. Another Sox family protein, Sox15, is also highly expressed in ES cells, but its role remains unclear (Maruyama et al. 2005).

(c) Nanog
Nanog is a homeobox protein specifically expressed in pluripotent cells and the inner cell mass of the blastocyst stage embryo (Chambers et al. 2003; Mitsui et al. 2003). Nanog-null embryos show disorganization of extraembryonic tissues at E5.5, with no discernible epiblast or primitive ectoderm (Mitsui et al. 2003). Nanog-deficient blastocysts appear to be morphologically normal, but the inner cell mass produces only parietal endoderm-like cells and not epiblast-derived cells when blastocysts are cultured in vitro. ES cells lacking Nanog can be derived, but they tend to differentiate into extraembryonic endoderm lineages even in the presence of LIF. Another group reported that even heterozygous Nanog mutant ES cells were unstable and susceptible to spontaneous differentiation (Hatano et al. 2005). RNAi-mediated knockdown of Nanog led to differentiation in both the mouse (Hough et al. 2006; Ivanova et al. 2006) and the human (Hyslop et al. 2005; Zaehres et al. 2005) ES cells. Importantly, overexpression of Nanog in mouse ES cells permits cells to self-renew in the absence of LIF. Likewise, overexpression of Nanog in human ES cells enabled growth without feeder cells (Darr et al. 2006). In addition, Nanog-overexpressing ES cells showed markedly increased reprogramming activity after fusion with somatic cells (Silva et al. 2006). Nanog expression is upregulated by Oct3/4 and Sox2 (Kuroda et al. 2005; Rodda et al. 2005; Wu da & Yao 2005) and suppressed by p53 (Lin et al. 2005), GCNF (Gu et al. 2005) and TCF3 (Pereira et al. 2006). Genome-wide chromatin immunoprecipitation analyses demonstrated that Oct3/4, Sox2 and Nanog share many target genes in both the mouse and the human ES cells (Boyer et al. 2005; Loh et al. 2006).

(d) STAT3
Both the mouse and the human ES cells are usually derived and maintained on a feeder layer of mouse embryonic fibroblasts (MEFs) in order to promote self-renewal. Conditioned media from MEFs can also support self-renewal of mouse ES cells, indicating that MEFs secrete soluble factor(s) that inhibit differentiation (Smith & Hooper 1987). Indeed, MEFs are known to inhibit ES cell differentiation via production of the IL-6 family cytokine, leukaemia inhibitory factor (LIF; Smith et al. 1988; Williams et al. 1988). With the addition of recombinant LIF protein into the culture medium, mouse ES cells can be cultured without MEF feeder cells.

The LIF receptor is a heteromeric complex consisting of gp130 and the LIF receptor (LIFR; Ernst & Jenkins 2004). The tyrosine kinase Janus kinase (JAK) binds constitutively to the intracellular domain of this receptor complex in its inactive form. Upon LIF binding, JAK kinase phosphorylates tyrosine residues on both gp130 and LIFR. Phosphorylation of Y765/812/904/914 of the intracellular domain of gp130 and Y976/996/1023 of LIFR recruits signal transducers and activators of transcription (STAT) 1 and STAT3 through their SH2 domains (Stahl et al. 1995). The STAT proteins are then activated by JAK-mediated tyrosine phosphorylation to form homodimers and/or heterodimers and translocate into the nucleus, where they function as transcription factors (Auernhammer & Melmed 2000).

Among the many STAT proteins, STAT3 has been shown to be essential for the maintenance of pluripotency in mouse ES cells (Boeuf et al. 1997; Niwa et al. 1998; Matsuda et al. 1999; Raz et al. 1999). Using a tamoxifen-inducible form of STAT3 generated by fusion with the ligand-binding domain of the oestrogen receptor, it was shown that STAT3 activation is sufficient for self-renewal in the presence of foetal bovine serum (Matsuda et al. 1999). STAT3 also cooperates with Smad and Id proteins to support clonal expansion of mouse ES cells in the absence of serum (Ying et al. 2003).

The role of LIF appears to be different among different species. For example, LIF cannot promote self-renewal of human or monkey ES cells (Humphrey et al. 2004; Sumi et al. 2004). Human ES cells express relatively low levels of LIF signalling components (LIFR, JAK and STAT3), and high levels of suppressor of cytokine signalling (SOCS), which negatively regulates LIF signalling (Wei et al. 2005a). In monkey ES cells, suppression of LIF signalling by a dominant negative form of STAT3 does not cause them to differentiate (Sumi et al. 2004). Thus, human and monkey ES cells appear to maintain pluripotency using a LIF/STAT3-independent mechanism. Since contamination from feeder cells represents a potential problem for transplantation therapy, it will be useful...
to determine the mechanisms by which feeder cells maintain pluripotency of human ES cells.

(e) APC/β-catenin

β-catenin is a dual-function protein that participates in cell–cell adhesion by linking cadherins to the actin cytoskeleton and also acts as an intracellular signalling molecule of the canonical Wnt signalling pathway (Reya & Clevers 2005). In the absence of Wnt activation, β-catenin is phosphorylated by a complex consisting of adenomatous polyposis coli gene (APC), Axin and glycogen synthase kinase (GSK) β. Phosphorylated β-catenin is degraded by the ubiquitin–proteasome system, thereby keeping the level of cytoplasmic β-catenin low. Upon binding of Wnt to its receptors, Frizzled and LRP5/6, GSK3β is inactivated through a poorly understood mechanism involving the direct interaction of Axin with LRP5/6, and/or the action of an Axin-binding molecule, dishevelled. As a result, β-catenin accumulates in the cytoplasm and travels to the nucleus, where it regulates transcription through association with lymphoid enhancer factor (LEF)/T-cell factor (TCF) transcription factors.

Neural differentiation of mouse ES cells was attenuated by the activation of Wnt signalling, either by overexpression of Wnt1 or treatment with lithium chloride, an inhibitor of GSK3β (Aubert et al. 2002). Moreover, Wnt3a mutant mice display ectopic neural tube formation at gastrulation stage (Yoshikawa et al. 1997). In addition, ES cells with a mutant form of APC were impaired in their ability to differentiate into the three germ layers (Kielman et al. 2002). Wnt also accelerates the proliferation of stem cells in the intestinal, epidermal and haematopoietic systems, and may be a common factor controlling stem cell acceleration of proliferation of stem cells in the intestinal tract, testis and skin, we found that Klf4 is expressed in squamous cell carcinoma and breast cancers (Foster et al. 1999, 2000). Moreover, induction of KLF4 in basal keratinocytes blocks the prolifer–differentiation switch and initiates squamous epithelial dysplasia (Foster et al. 2005). Thus, Klf4 is associated with both tumour suppression and oncogenesis. Ectopic expression of Klf4 suppresses cell proliferation, but ablation of only one of its target genes, p21, is sufficient to neutralize the cytostatic effect of Klf4 (Rowland et al. 2005). In p21-null cells, Klf4 promotes cell proliferation by downregulating p53 (Rowland et al. 2005). This may in part account for the dual function of Klf4 in cancers.

In addition to its well-known expression in gastrointestinal tract, testis and skin, we found that Klf4 is highly expressed in undifferentiated mouse ES cells (Tokuzawa et al. 2004, unpublished data). We also found that inactivation of STAT3 in mouse ES cells dramatically decreases Klf4 expression and that forced expression of Klf4 enables LIF-independent self-renewal (Tokuzawa et al. 2004, unpublished data). Another group also reported a positive effect of Klf4 in self-renewal of mouse ES cells (Li et al. 2005), and Klf4 is known to cooperate with Oct3/4 and Sox2 to activate the Lefty1 core promoter (Nakatake et al. 2006).

(f) c-Myc

c-Myc is a helix-loop-helix/leucine zipper transcription factor that associates with its partner protein, Max (Adhikary & Eilers 2005). c-Myc is regulated by STAT3 (Kiuchi et al. 1999) and plays important roles in self-renewal and maintenance of pluripotency in mouse ES cells (Cartwright et al. 2005): forced expression of stable c-Myc causes mouse ES cells to self-renew without LIF, whereas the dominant negative form of c-Myc induces differentiation even in the presence of LIF. GSK3β negatively regulates c-Myc activity by phosphorylation–dependent degradation by the proteasome (Sears et al. 2000). Thus, c-Myc is a common target for both the LIF and Wnt signalling pathways. c-Myc has a large number of binding sites in the genome (Fernandez et al. 2003; Li et al. 2003; Caswley et al. 2004) and is thought to modify chromatin structure (Knoepfler et al. 2006) and activate expression of some miRNAs (O’Donnell et al. 2005).

(g) Klf4

Klf4 is a Kruppel-like transcription factor (also known as gut-enriched Kruppel-like factor, GKLK) (Rowland et al. 2005) originally identified as a tumour suppressor that is deleted in gastrointestinal cancers (Zhao et al. 2004; Wei et al. 2005b). A conditional knockout mouse model supports a role for Klf4 in gastrointestinal cancers (Katz et al. 2005). Klf4, however, is overexpressed in squamous cell carcinoma and breast cancers (Foster et al. 1999, 2000). Moreover, induction of KLF4 in basal keratinocytes blocks the proliferation–differentiation switch and initiates squamous epithelial dysplasia (Foster et al. 2005). Thus, Klf4 is associated with both tumour suppression and oncogenesis. Ectopic expression of Klf4 suppresses cell proliferation, but ablation of only one of its target genes, p21, is sufficient to neutralize the cytostatic effect of Klf4 (Rowland et al. 2005). In p21-null cells, Klf4 promotes cell proliferation by downregulating p53 (Rowland et al. 2005). This may in part account for the dual function of Klf4 in cancers.

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(h) Induction of pluripotent stem cells by four factors

To test these candidates for their pluripotency-inducing activity, we have developed a system in which induction of pluripotency can be detected as
marker gene expression. Fbx15 is specifically expressed in ES cells and early embryos, but is dispensable for self-renewal of ES cells and development (Tokuzawa et al. 2003). We inserted a β-geo cassette (a fusion of β-galactosidase and the neomycin-resistant gene) into the mouse Fbx15 locus by homologous recombination. ES cells homozygous for the β-geo knock-in (Fbx15βgeo/βgeo) were resistant to an extremely high concentration of G418 (up to 12 mg ml⁻¹), whereas somatic cells derived from Fbx15βgeo/βgeo mice were sensitive to the selection. Thus, it is probable that even partial induction of pluripotency would render somatic cells resistant to G418 at normal concentrations (0.3 mg ml⁻¹).

We introduced candidate genes into Fbx15βgeo/βgeo MEFs by retrovirus-mediated transfection and cultured them in ES cell medium containing G418. With any single factor, we did not obtain G418-resistant colonies. However, by combining four factors (Oct3/4, Sox2, c-Myc and Klf4), we obtained multiple G418-resistant colonies. These cells resembled ES cells in both their morphology and proliferation capacity. Furthermore, when transplanted into nude mice, these induced pluripotent stem (iPS) cells produced teratomas containing tissues of all three germ layers. Thus, pluripotent cells can be generated from fibroblasts by introducing just a few defined factors (Takahashi & Yamanaka 2006).

Fbx15-selected iPS cells do not contribute adult or germ line chimeras, indicating that their reprogramming is partial. Recently, we and two other groups reported significant improvement in quality of iPS cells (Maherali et al. 2007; Okita et al. 2007; Wernig et al. 2007). The three groups used the same four factors, but used Nanog as a selection marker. Nanog-selected iPS cells showed more similar gene expression pattern to ES cells than did Fbx15-selected ones. These improved iPS cells also contributed to adult and germline chimeras. These data showed that the combination of the four factors and proper selection can generate pluripotent cells that are indistinguishable from ES cells.

How the four factors induce pluripotency remains elusive. Our model is that c-Myc plays crucial roles. As a potent proto-oncogene, c-Myc should contribute to rapid proliferation of iPS cells. In addition, by binding numerous positions of genome and recruiting histone acetylase complexes, c-Myc should open up chromatin (Adhikary & Eilers 2005). However, overexpression of c-Myc alone in fibroblasts would induce p53-dependent apoptosis or senescence. We postulate that it is Klf4 that neutralizes the adverse effect of c-Myc by suppressing p53. Oct-3/4 and Sox2 then bind to their target sites and induce pluripotency by enhancing stemness genes and suppressing differentiation-associated genes. Niwa and associates recently showed that Klf4 also functions as a cofactor of Oct-3/4 and Sox2 (Nakatake et al. 2006).

At present, however, we do not know whether the same four factors can induce iPS cells from human somatic cells. It is possible that additional factors are required to induce human iPS cells, but more work is necessary to determine whether or not this is the case. In addition, the use of retroviral vectors and the proto-oncogene c-Myc poses serious safety concerns that must be resolved prior to application of human iPS cells in regenerative medicine. In fact, we showed that approximately 20% of mice derived from Nanog-selected iPS cells developed tumours (Okita et al. 2007). Thus, the retroviral introduction of c-Myc should be avoided prior to clinical application. Once established, human iPS cells should also be useful in the fields of drug discovery and toxicology.

### 5. CONCLUSION

In this review, an overview of the three currently proposed methods of inducing pluripotency in somatic cells is given. These technologies each have their own pros and cons for future clinical applications (table 1). However, rather than discussing which approach is best at present, basic research should push ahead on all fronts so that the best possible technologies may be developed in the future (Yamanaka 2007).

### 6. NOTE ADDED IN PROOF

After we submitted this manuscript, we and others succeeded in producing iPS cells from human fibroblasts with the same four factors (Takahashi et al. 2007; Yu et al. 2007; Park et al. 2008). In addition, the c-Myc retrovirus was shown to be dispensable for iPS cell generation (Nakagawa et al. 2008). Furthermore, another group reported generation of human iPS cells with a partially different combination consisting of Oct3/4, Sox2, Nanog and Lin28 (Yu et al. 2007). These data collectively showed that nuclear reprogramming in human cells can be achieved by combinations of small numbers of factors.

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