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Review Article

Direct reprogramming 101

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Direct reprogramming of somatic cells into a pluripotent state has been achieved with a set of just four transcription factors. Many scientists and medical doctors are trying to elucidate the causes of intractable diseases and discover new drugs using the newest types of technology. Various methods have been developed to produce clinical-grade fully reprogrammed cells for cell transplantation therapy. Augmenting agents, such as small-molecules, have been extensively screened to improve the reprogramming efficiency. The molecular mechanisms of reprogramming have been revealed by embryonic stem cell research. The accumulation of knowledge by the pioneers has driven the reprogramming field. In the present article, the contents of gift boxes from the studies of pluripotency to the nuclear reprogramming field are introduced.

Key words: iPS cells, pluripotecy, reprogramming.

Pluripotent stem cells

Cells derived from the inner cell masses (ICM) of blastocysts can grow in a manner well adapted for culture conditions, although the frequency is low, and continue expanding without losing their characteristics (Evans & Kaufman 1981; Martin 1981). They are broadly termed embryonic stem (ES) cells. Primordial germ cells have unipotency only for becoming gametes *in vivo*. However, if they are isolated prior to embryonic day 12.5 and transferred to a culture medium in dish with the addition of leukemia inhibitory factor (LIF) as well as stem cell factor, basic fibroblast growth factor (bFGF) and supported by feeder cells, they can convert into pluripotent cells, which have the potential to differentiate into all of the cell types in the body (Matsui *et al.* 1992; Labosky *et al.* 1994). These reprogrammed cells are called embryonic germ (EG) cells and they inherit unique DNA methylation patterns of imprinted gene loci similar to primordial germ cells. Interestingly, LIF, bFGF and stem cell factor are no longer required once EG cells are established. Germline stem (GS) cells established from mouse neonate testes can grow infinitely in an undifferentiated state although their differentiation potentials are restricted to sperm. GS cells can convert to ES-like cells when cultured in a medium suitable for ES cells, which contains serum, LIF and is supported by feeder cells (Kanatsu-Shinohara *et al.* 2004). However, the frequency is quite low. Despite their different origins, commonly these cells can be spontaneously reprogrammed to a pluripotent state in ES cell culture conditions (Hochedlinger & Jaenisch 2006). In contrast to their passive reprogramming, artificial methods have been developed for nearly 50 years.

Nuclear reprogramming

A landmark study performed by Dr John Gurdon demonstrated that the memory of somatic cells in tadpoles can be erased by injection into enucleated eggs (Gurdon *et al.* 1958). The birth of cloned frogs implied that some factors hidden in eggs could reprogram somatic nuclei to the embryonic state. The cloned sheep, Dolly, was proof that not only frogs but also mammals had reprogramming factors in their oocytes (Wilmut *et al.* 1997). These two reports strongly suggested that unfertilized eggs contain reprogramming factor(s).

The establishment of human ES cells was reported shortly after the success of nuclear transfer in mammals (Thomson *et al.* 1998). Human ES cells look closer to monkey ES cells, which were established a few years earlier rather than mouse ES cells. In addition, the culture conditions such as the cytokine

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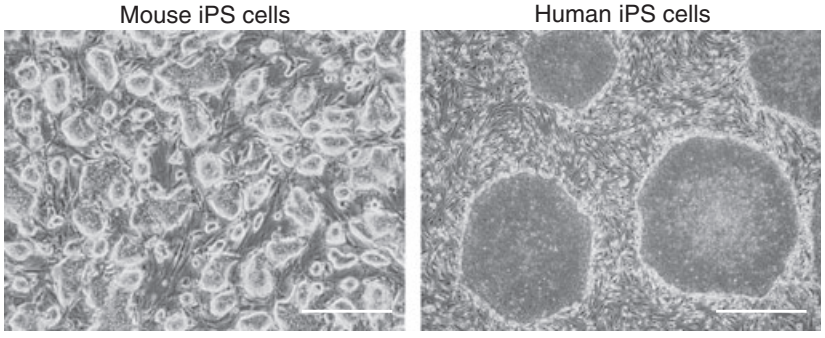

Fig. 1. Images of induced Pluripotent Stem (iPS) cells. Left, mouse iPS cells derived from adult tail-tip fibroblasts. Right, human iPS cells derived from adult dermal fibroblasts. Bars indicate 200 μm.

requirement differ between mice and primates (Boiani & Schöler 2005). The use of human ES cells is stringently regulated in some countries and regions, despite the great expectation for stem cell therapy (McLaren 2007). On the other hand, mouse ES cells are widely used in laboratories all over the world because of their easy handling and spreading transgenic and gene-targeting technologies.

The pioneer work of reprogramming using cell fusion was reported in 1983 (Takagi *et al.* 1983). It was shown that the X-chromosome of female somatic cells could be re-activated by fusion with murine teratocarcinoma-derived stem cells. Great advances, which were later announced at the beginning of the 21st Century, demonstrated that the nucleus of somatic cells such as T lymphocytes and fibroblasts could be reprogrammed by fusion with ES cells in mice and humans (Tada *et al.* 2001; Cowan *et al.* 2005). These findings suggested that the reprogramming factors exist not only in unfertilized eggs but also in ES cells. They were good news to stem cell scientists because embryos are difficult to use for large-scale analyses. The road to discovering the reprogramming factor was broadened at that moment although there was a paucity of data.

Learning by ES cells

What is reprogramming factor? Is it a transcription factor? A cytokine? Or an epigenetic modifier? How many reprogramming factors are there? Can a master gene change a somatic cell to be pluripotent? Although the possibility was daunting, more than a hundred factors were estimated to work together. However, one way to answer the questions was to understand the molecular mechanisms of pluripotency. Studies to understand the molecular mechanisms of pluripotency in mouse ES cells were energetically pursued in the last decade. At the same time, methods have also been developed to differentiate both mouse and primate ES cells into specific cell lineages such as dorpaminergic neurons, hematopoietic cells and vascular cells (Nakano *et al.* 1996; Kawasaki *et al.* 2000; Yamashita *et al.* 2000).

Some players with important roles in self-renewal of ES cells were identified at the end of the 20th Century. Oct3/4 (also known as POU domain, class 5, transcription factor 1) is expressed specifically in pluripotent cells and germ cells, rather than in somatic tissues. *In vivo* mutagenesis or conditional knockout experiments demonstrated that Oct3/4 is essential for maintenance of pluripotency in early embryos, primordial germ cells and ES cells (Nichols *et al.* 1998; Niwa *et al.* 2000; Kehler *et al.* 2004). Another player, SRY-box containing gene 2 (Sox2), is an inseparable partner of Oct3/4 in pluripotent stem cells. The expression of Sox2 is basically restricted in undifferentiated pluripotent cells, germ cells and nerve cells. The targeted deletion of Sox2 leads to embryonic lethality at the post-implantation stage (Avilion *et al.* 2003). On the other hand, Sox2 also plays important roles in neurogenesis in the brain and differentiation of retinal progenitors (Ferri *et al.* 2004; Taranova *et al.* 2006). Signal transducer and activator of transcription 3 (Stat3) is a well-analyzed molecule functioning in the self-renewal of mouse ES cells. LIF stimulates Stat3 via the LIF receptor-interleukin 6 signal transducer (gp130) complex, and Jak kinase. Activated Stat3 dimerizes and translocates into the nucleus, and then regulates the expression of downstream target genes as a transcription factor. Experiments using dominant active or negative mutant of Stat3 demonstrated that the LIF/Stat3 pathway is essential and sufficient for self-renewability of mouse ES cells (Niwa *et al.* 1998; Matsuda *et al.* 1999). Unfortunately, pluripotency and/or reprogramming were hardly explicable with the functions of these factors. Another key factor of pluripotency, Nanog, was discovered in 2003 (Chambers *et al.* 2003; Mitsui *et al.* 2003). The expression of Nanog is initiated at the morula stage, continues in the ICM of blastocysts, and then is promptly downregulated after implantation. The constitutive-expression of Nanog

allows LIF-independent self-renewal of mouse ES cells. The expression of endogenous *Nanog* gene remains static when LIF signals are removed in Nanog-transgenic ES cells (Chambers *et al.* 2003; Mitsui *et al.* 2003). These findings suggest that pluripotency of mouse ES cells depends on not only LIF/Stat3 signaling but also on Nanog.

The characteristics of ES cells go far beyond pluripotency. Another noteworthy characteristic of ES cells is immortality (Smith 2001). ES cells can carry on expansion semi-permanently in suitable culture conditions, maintaining their pluripotency without obvious chromosomal abnormalities. Such growth properties make them ideal cell sources for regenerative medicine. However, immortality of ES cells is inextricably linked to tumorigenicity. Indeed, ES cells form tumors called teratomas consisting of various tissue mixtures when they are injected into immune-deficient or isogenic animals. Residual undifferentiated cells can be a risk of tumor formation after *in vitro* differentiation of ES cells for therapeutic use. Therefore, although more than 10 years have passed since human ES cells were first established, clinical trials have never been conducted. On another front, these troubling aspects for clinical application provided more hints to researchers. Not only ES cell-specific molecules such as Oct3/4, Sox2 and Nanog, but also some genes known as oncogenes, play important roles in the circuitry of pluripotecy.

Issues of embryo-derived pluripotent stem cells

To convert somatic cells into pluripotent cells has profound social meaning. Most likely, reprogramming of patient's somatic cells can overcome the issues associated with ES cells. ES cells have two big issues to circumvent before they can be clinically used as cell therapies. One is the ethical issue that the destruction of embryos is unavoidable to establish ES cells. Although most human ES cells are derived from surplus embryos provided by fertility clinics, there are many opposing views on the use of embryos that have the possibility to eventually develop into a human being. Some attempts to establish pluripotent ES-like cells from one of the blastomeres have been successful (Chung *et al.* 2006, 2008; Klimanskaya *et al.* 2006). This scenario did not include the destruction of embryos because once they remove a blastomere from an 8-cell stage embryo for generation of ES-like cells, the remaining 7-cell embryo can develop normally after it is placed in a woman's uterus. Such single-cell biopsy has been used in pre-implantation genetic diagnosis. Realistically, however, only wealthy individuals may reap benefits from this technology.

Another issue associated with ES cells is the possibility of immunological rejection, because it is practically impossible to fit genetic types of eggs as origins of ES cells with those of patients. One of the possible ways to solve this diremma is to establish an ES cell bank. The Nakatsuji Group calculated that the proportion of patients who have at least one human leukocyte antigen (HLA) matched donors at three loci of HLA-A, -B, and –DR (Nakajima *et al.* 2007). They concluded that 170 randomly selected embryos can cover the needs of 80% of the patients. Moreover, 80% of patients are expected to meet a donor with complete matches at the three loci of HLA only if 55 independent ES cell lines carrying parthenogenetic homozygous are prepared.

One of the possible methods to avoid these problems is to use ES cells established by somatic cell nuclear transfer (SCNT). SCNT is the injection of a nucleus derived from a somatic cell such as fibroblast into an enucleated egg. SCNT-derived (nt) ES cells have the same genetic information as the donor except for mitochondrial DNA. On theoretical grounds, no immunological rejection is induced after transplantation of the cells derived from ntES cells to the donor. Actually, a therapeutic model with ntES cells to correct genetic defects was proposed (Rideout *et al.* 2002). ntES cells were established in mice and rhesus macaque, so far (Rideout *et al.* 2000; Byrne *et al.* 2007). Unfortunately, human ntES cell lines have never been established. Regardless, both the collection of HLA homozygous ES cell lines and making human ntES cells are ideal ways to solve the medical problems associated with stem cell therapy. However, these approaches commonly require many human eggs. Direct reprogramming of a patient's somatic cells into pluripotent stem cells can sweep away the dilemma of ES cells.

Reprogramming quartet

Functional screening for the reprogramming genes using primary *in silico* screening narrowed down the number of candidates. Mouse induced Pluripotent Stem (iPS) cells were first generated by introducing the combination of Oct3/4, Sox2, Krüppel-like factor 4 (Klf4) and myelocytomatosis oncogene (c-Myc) (Takahashi & Yamanaka 2006) (Fig. 1). With improved selection methods, these reprogrammed cells can contribute to germlines in chimeric mice (Maherali *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007). iPS cells derived from mouse embryonic fibroblasts (MEF) can produce cloned lived pups by tetraploid complementation, which is now regarded as one of the strictest hurdles of pluripotency (Boland *et al.* 2009; Kang

et al. 2009; Zhao *et al.* 2009). These data suggested that the differentiation potentials of iPS cells are already equivalent to those of ES cells at least in mice. Nakagawa *et al.* showed that Sox2 could be replaced with Sox1, Sox3, Sox7, Sox15, Sox17 or Sox18 (Nakagawa *et al.* 2008). They also demonstrated that Klf4 was able to be substituted by Klf2 or Klf5. Myc families such as N-Myc and L-myc mimic c-Myc function during direct reprogramming. However, Oct family genes such as Oct1 and Oct6 could not be used in the place of Oct3/4.

Oct3/4 is an essential factor in direct reprogramming. The Oct3/4 transgene alone can generate iPS cells in both mice and human neural stem cells, which express endogenous Sox2, although the efficiencies are extremely low (Kim *et al.* 2009b,c). There are only two reports of iPS cells that were established without the Oct3/4 transgene. Guo *et al*. demonstrated that mouse epiblast stem cells, which express endogenous Oct3/4 and Sox2, can be converted into iPS cells by introducing the Klf4 transgene only (Guo *et al.* 2009). Another report showed, surprisingly, that the transduction of Sox2, Klf4 and c-Myc into mouse neural progenitor cells in medium supplemented with BIX-01294, which selectively inhibits the enzymatic activity of histone methyltransferase (HMTase) G9a, produced fully reprogrammed iPS cells although the efficiency was as low as 0.001% (Shi *et al.* 2008a,b).

Sox2 is functionally redundant, at least in part with other Sox family proteins including Sox4, Sox11 and Sox15 (Maruyama *et al.* 2005; Masui *et al.* 2007). However, iPS cells cannot be generated with the normal strategy in the absence of Sox transgenes. Selectively inhibition of transforming growth factor β (TGFβ) signaling mimics the role of Sox2 in iPS cell generation from MEF (Maherali & Hochedlinger 2009). In addition, Sox2 is dispensable for the reprogramming of neural stem cells, melanocytes and melanoma cells into iPS cells in both mice and humans (Eminli *et al.* 2008; Kim *et al.* 2008; Hester *et al.* 2009; Utikal *et al.* 2009a). The transduction of Oct3/4 and Klf4 can reprogram MEF into iPS cells under hypoxic conditions (Yoshida *et al.* 2009). Recently, Ichida *et al*. showed that transduction of the *Nanog* gene could cover for the function of Sox2 (Ichida *et al.*, 2009). Hence, exogenous Sox2 is probably not essential. On the other hand, MEF cultures include a small population of cells expressing endogenous Sox2. There is no full evidence of Sox dispensability in reprogramming. Although the approaches are distinctly different, they are all developed and modified for one goal, in order to improve the reprogramming efficiency. A favorable environment may make Sox transgene dispensable for generation of iPS cells in some cases. Of course, iPS cells require Sox2 to maintain their pluripotency.

Klf4 was first noted as one of the downstream targets of LIF/Stat3 signaling in mouse ES cells (Li *et al.* 2005; Tokuzawa and Yamanaka, unpubl. data). Knockdown experiments with small interfering RNA (siRNA) revealed that the roles of Klf4 in mouse ES cells overlap with those of Klf2 and Klf5 (Jiang *et al.* 2008). Nakatake and colleagues reported that Klf4 functions with Oct3/4 and Sox2, and co-regulates with part of Oct/Sox target genes such as *Lefty 1* in mouse ES cells (Nakatake *et al.* 2006). Forced-expressed Klf4 can directly bind Oct3/4-Sox2 complex during direct reprogramming (Wei *et al.* 2009). Klf4 directly regulates the *Nanog* expression in human ES cells in association with homeobox protein, PBX1 (Chan *et al.* 2009). Treatment with valproic acid (VPA) which is a histone deacetylase (HDAC) inhibitor, can allow Klf4 transgene-free reprogramming in both mice and human (Huangfu *et al.* 2008a,b). In addition, exogenous Klf4 is dispensable for mouse iPS cell generation in the absence of tumor suppressor gene, *Trp53* (Kawamura *et al.* 2009). Klf4 directly responds to the promoter of *Trp53* gene and is then transcriptionally suppressed (Rowland *et al.* 2005). Feng and colleagues demonstrated that Klf4 can be substituted with estrogen-related receptor beta (Esrrb) or gamma (Esrrg) for the generation of mouse iPS cells derived from MEF even though the colony number decreased (Feng *et al.* 2009). Lyssiotis *et al.* screened chemical compound libraries in order to discover a substitute for Klf4 (Lyssiotis *et al.* 2009). They introduced candidate chemicals with Oct3/4, Sox2 and c-Myc to MEF carrying luciferase reporter gene driven by *Nanog* promoter. One of those hits that elevated the luciferase activity is kenpaullone, which is an inhibitor of glycogen synthase kinase 3 (GSK3) and cyclin dependent kinases (CDK). This molecule can not only mimic Klf4 but also improve the reprogramming efficiency even with a subset of reprogramming factors including Klf4. Interestingly, no similar effects were observed with other small-molecules inhibiting GSK3 or CDK, such as CHIR99021 and purvalanol. In addition, kenpaullone does not increase the Klf4 expression during the reprogramming process. These data suggest that the off-target effects of kenpaullone may be effective for the enhancement of reprogramming efficiency, and its unidentified target seems to be a downstream target of Klf4.

Exogenous Myc is dispensable for the generation of iPS cells although the efficiency is markedly decreased when used without Myc (Nakagawa *et al.* 2008; Wernig *et al.* 2008a). c-Myc is expressed in most cell types in both mice and human whereas the expression of N-Myc and L-Myc are limited. Therefore, the need for Myc in direct reprogramming remains unclear. The

activation of the Wnt pathway can compensate for the absence of exogenous Myc expression (Marson *et al.* 2008). The 58th threonine residue of c-Myc is a target of GSK3, and it results in c-Myc degradation by the proteosome pathway. Stimulation by the Wnt signal negatively regulates GSK3 activity by phosphlylation of its kinase core. GSK3 inhibitors such as CHIR99021 and 6-bromoindirubin-3′-oxime (BIO) enhance self-renewal of mouse ES and iPS cells (Sato *et al.* 2004; Silva *et al.* 2008; Ying *et al.* 2008). Bechard and Dalton showed that subcellular localization of GSK3 is associated with the self-renewal of mouse ES cells (Bechard & Dalton 2009). GSK3 is localized predominantly in the cytoplasm of undifferentiated mouse ES cells. GSK3 immediately accumulates in the nucleus after differentiation in response to LIF starvation. Moreover, the forced expression of c-Myc allows for the self-renewal of mouse ES cells in medium without additional LIF, whereas no such effects were observed in long-term culture (Cartwright *et al.* 2005). Inhibition of GSK3 and subsequent stabilization of Myc positively affect both the maintenance of pluripotency and progression of reprogramming. On the other hand, Wnt also stimulates T-cell factor 3 (Tcf3), which plays important roles in self-renewal of ES cells (Pereira *et al.* 2006; Tam *et al.* 2008; Yi *et al.* 2008). Tcf3 co-occupies the promoter regions that overlapped with Oct3/4 and Nanog throughout the genome (Cole *et al.* 2008). Therefore, the Wnt pathway has an important role for self-renewal of mouse ES cells through not only the stabilization of c-Myc but also transcriptional regulation by Tcf3.

Two independent groups reported the establishment of human iPS cells using different sets of reprogramming factors in 2007 (Takahashi *et al.* 2007; Yu *et al.* 2007) (Fig. 1). Yu *et al.* identified Oct3/4, Sox2, Nanog and Lin28 as factors to reprogram human fibroblasts to an ES-like state (Yu *et al.* 2007). They also showed that Lin28 was not essential for the generation of iPS cells, whereas it could elevate the efficiency of reprogramming. They described in the report that iPS cells were obtained using the subset of reprogramming factors lacking Nanog, albeit the number of colonies seemed to dramatically decrease.

Reprogramming boosters

c-Myc is the most highly powered booster of direct reprogramming, although its critical functions remain unknown (Singh & Dalton 2009). However, c-Myc should be precluded during the reprogramming of iPS cells for transplantation therapy because of its oncogenic nature. Finding enhancing molecules may therefore reveal novel mechanisms of reprogramming in the future.

Furthermore, the knowledge obtained from studies regarding the pluripotency of ES cells has greatly contributed to the technology of iPS cell generation. Needless to say, the conditions that iPS cells require are the same as those of ES cells. For example, both mouse and human iPS cells can be maintained when supported by feeder cells. The addition of LIF and bFGF into the medium are important for the self-renewal of iPS cells in mouse and human, respectively (Smith *et al.* 1988; Thomson *et al.* 1998; Maherali *et al.* 2007; Okita *et al.* 2007; Takahashi *et al.* 2007; Wernig *et al.* 2007; Yu *et al.* 2007). The combination of GSK3 and mitogen activated protein kinase kinase (MAPKK also known as MEK) inhibitors not only support self-renewal of iPS cells in the condition without serum and feeders but also improve the efficiency of reprogramming (Silva *et al.* 2008). The validity of these drugs in reprogramming was demonstrated in human cells (Lin *et al.* 2009a,b).

The transduction of Spalt-like 4 (Sall4) in reprogramming processes can increase the frequency of pluripotent stem cell induction not only in the generation of iPS cells but also in fusion with ES cells and somatic cells (Wong *et al.* 2008; Tsubooka *et al.* 2009). Sall4 is essential for embryogenesis, albeit not in mouse ES cells (Elling *et al.* 2006; Sakaki-Yumoto *et al.* 2006; Tsubooka *et al.* 2009). Sall1, another member of the Sall family, is also expressed predominantly in undifferentiated ES cells. Its expression functionally probably compensates for the deficiency of Sall4. On the other hand, Sall4 acts as a central tower of pluripotency in ES cells in association with Oct3/4, Sox2 and Nanog (Wu *et al.* 2006; Zhang *et al.* 2006; Zhou *et al.* 2007).

A third report about human iPS cell generation was published in 2008 (Park *et al.* 2008). These iPS cells were generated by using four orthodox factors with two additional factors, simian virus 40 large T antigen (SV40LT) and telomerase reverse transcriptase (TERT). Ectopic expression of the combination of SV40LT and TERT are sufficient to immortalize normal human diploid fibroblasts (Montalto *et al.* 1999). SV40LT contributes to immortalization by suppressing both p53 and retinoblastoma (RB) tumor suppressor protein (Stewart & Weinberg 2006). Generally, human cells other than stem cells, germ cells and transformed cells, do not express TERT. Constitutive expression of TERT allows human cells to avoid crisis. The transduction of SV40LT along with Oct3/4, Sox2, Klf4 and c-Myc greatly improved the reprogramming frequency of human fibroblasts (Mali *et al.* 2008). Immortalization without malignant transformation probably helps the progression of reprogramming because immortality is one of essential characteristics of pluripotent stem cells.

Epigenetic view in reprogramming

Epigenetics is the regulation of the gene expression by the acquired modification of chromatin and DNA, without changing the DNA sequence. ES cells and differentiated cells have significantly different epigenetic signatures (Bernstein *et al.* 2006, 2007). The epigenetic status of iPS cells and their origins are also markedly different, suggesting that the reprogramming process involves dynamic chromatin changes. Kimura *et al.* demonstrated with cell fusion experiments that the epigenetic status progresses from somatic types to pluripotent types during reprogramming (Kimura *et al.* 2004). Huangfu and colleagues reported that treatment with HDAC inhibitors such as Trichostatin A (TSA) and VPA can improve the frequency of iPS cell generation (Huangfu *et al.* 2008a,b). They also demonstrated that MEF treated with TSA showed upregulation of ES-specific genes and downregulation of MEF-specific genes without reprogramming factor transduction. In addition, treatment with TSA increases the success rate and prevents aberrations of DNA methylation patterns in SCNT (Kishigami *et al.* 2006; Rybouchkin *et al.* 2006). Scriptaid, another HDAC inhibitor, is much more effective than TSA for cloning efficiency via SCNT of inbred mice (Van Thuan *et al.* 2009). Therefore, histone acetylation plays important positive roles in reprogramming progression.

The ninth lysine residue of histone H3 (K9H3) is a target of G9a. Generally, methylated K9H3 are condensed in heterochromatin regions. Actually, mono- and di-methylation at K9H3 almost disappear in G9a-deficient ES cells (Tachibana *et al.* 2002). A G9a inhibitor, BIX-01294, increases the number of mouse iPS cell colonies (Shi *et al.* 2008b). In addition, RNA interference mediated knockdown of G9a promotes reprogramming by cell fusion of mouse ES cells and neural stem cells (Ma *et al.* 2008). The overexpression of Jhdm2a, which is a histone demethylase for K9H3, enhances reprogramming efficiency by cell fusion. Therefore, the methylation of K9H3 has negative effects on the somatic cell reprogramming to a pluripotent state.

The deletion of G9a also induces genome-wide DNA hypomethylation in mouse ES cells (Ikegami *et al.* 2007). Generally, DNA methylation reflects the region of gene silencing, and is also one of important epigenetic modifications (Li 2002). Cytosines of CpG dinucleotides in the promoter regions of silenced genes are frequently highly methylated. Methylated cytosines are highly condensed in the promoter region of pluripotent associated genes such as *Oct3/4* in somatic cells, whereas they are completely unmethylated in undifferentiated ES cells. *De novo* methylation of the *Oct3/4* locus is effectively prevented during differentiation of ES cells and early embryo formation in the absence of G9a (Feldman *et al.*, 2006; Epsztejn-Litman *et al.* 2008). G9a can interact with the *de novo* DNA methyltransferases, Dnmt3a and 3b, and guide them to the target sites (Epsztejn-Litman *et al.* 2008).

Mouse ES cells lacking Dnmt1, 3a and 3b can proliferate normally in the undifferentiated state despite the absence of CpG methylation (Tsumura *et al.* 2006). DNA methylation is important for normal differentiation in mouse ES cells rather than self-renewal. For example, mouse ES cells lacking both Dnmt3a and Dnmt3b show progressive global DNA hypomethylation and histone hyperacetylation, which eventually result in the loss of differentiation potentials (Jackson *et al.* 2004). Methyl-CpG binding domain protein 3 (Mbd3), a key component of the nucleosome remodeling and deacetylase (NuRD) complex, is also important for differentiation of mouse ES cells (Kaji *et al.* 2006). These findings suggest that DNA methylation and recruited molecules are important for normal differentiation rather than for self-renewal of ES cells. Moreover, treatment with a Dnmt inhibitor, 5-Aza-2′-deoxycytidine or RG108 improves the efficiency of iPS cell generation (Mikkelsen *et al.* 2008; Shi *et al.* 2008a). Taken together, inhibitors for Dnmts, HDACs and G9a possibly function in a similar manner during generation of iPS cells. These results strongly suggest that K9H3 methylation and DNA methylation are closely linked, and are important for normal differentiation, and are a negative blockade of reprogramming.

Bhutani *et al.* demonstrated by heterokaryoyic cell fusion of mouse ES cells and human fibroblasts, that cytosine deaminase Aid-mediated DNA demethylation is required for reprogramming (Bhutani *et al.* 2010). Aid is expressed predominantly in immune cells and pluripotent cells, but not in somatic cells (Morgan *et al.* 2004). Aid deaminates cytosine to uracil in DNA loci of immunoglobulin, and triggers either class switch recombination or somatic mutations via DNA repair machinery. Aid and its family enzyme Apobec1 can deaminate not only unmethylated cytosine but also methylated one (Morgan *et al.* 2004). A transition of cytosine to thymine by deaminases leads to the mismatch between thymine and guanine on paired strand. As a result, methylated cytosine can thus be replaced by unmethylated cytosine by DNA repair. In that sense, cytosines can be demethylated in pluripotent cells. Further investigations are expected to reveal whether aggressive demethylation can be induced, while also elucidating its importance during reprogramming.

Recent studies showed the association of reprogramming factors and chromatin modification. In mouse ES cells, Oct3/4 directly binds to the promoter

region of the *Eed* gene, which encodes a component of Polycomb repressive complex 2 (Ura *et al.* 2008). Eed enhances tri-methylation of K27H3 in the promoter region of differentiated associated genes such as *Pax3*, *Gata4* and *Cdx2*. Methylated K27H3 is located in the regions of silenced genes and recruits Polycomb complex, which is a regulator of gene expression through modulation of chromatin structures. In contrast, such modifications are dramatically decreased in Eed-deficient ES cells. The polycomb complex binds the promoter regions of developmental regulators in mouse and human ES cells, and represses their expression (Boyer *et al.* 2006; Lee *et al.* 2006). Therefore, Oct3/4 regulates pluripotency, at least in part, through chromatin remodeling to suppress the expression of differentiation-associated genes. Klf4 interacts with histone acetyltransferase p300, and positively regulates gene expression by modifying histone acetylation (Evans *et al.* 2007). In contrast, the co-expression of Klf4 and HDAC3 synergistically repressed target gene expression. These findings suggest that Klf4 can act as either an activator or a repressor depending on the interacting molecules. Lin and colleagues mapped c-Myc binding sites in mouse ES cells by chromatin immunoprecipitation combined with microarrays (Lin *et al.* 2009a). Their data suggest that c-Myc contributes to chromatin modeling in mouse ES cells. Therefore, epigenetic modifiers and reprogramming factors can interact at least in a pluripotent state (Fig. 2). Their relationships during the reprogramming process will be clarified by further studies.

Tumor suppressors as resistance forces

Inactivation of *Trp53* (*TP53* in human) gene which encodes p53 protein improves the efficiency of direct reprogramming comparably to c-Myc in both mice and human (Banito *et al.* 2009; Hong *et al.* 2009; Kawamura *et al.* 2009; Li *et al.* 2009b; Marión *et al.* 2009; Utikal *et al.* 2009b). p53 induces differentiation in mouse ES cells, by suppression of *Nanog* expression (Lin *et al.* 2005). Kanatsu-Shinohara and colleagues previously made reference to the relationship between reprogramming and p53 (Kanatsu-Shinohara *et al.* 2004). Although the conversion of mouse GS cells into a multipotent state is very rare, its efficiency can be dramatically increased in a p53-null background. Taken together, these data suggest that p53 blocks both active and incidental reprogramming. In contrast to p53, inactivation of RB1 in human fibroblasts does not increase the efficiency of iPS cell generation (Hong *et al.* 2009). One possibility is that two other RB-related proteins, p107 (also known as RBL1) and p130 (also known as RBL2), make up for the absence of RB1 (LeCouter *et al.* 1996; Robanus-Maandag *et al.* 1998). The targeted inactivation of all three RB-related genes in mouse ES cells, does not affect their proliferation but markedly reduces their differentiation potential in teratomas (Dannenberg *et al.* 2000; Sage *et al.* 2000). Generally, RB proteins regulate the procession in the G1 phase and subsequently accelerate entry into the S phase. However, these results suggest that the RB pathway does not regulate the cell cycle in mouse ES cells. In fact, most of the RB proteins are

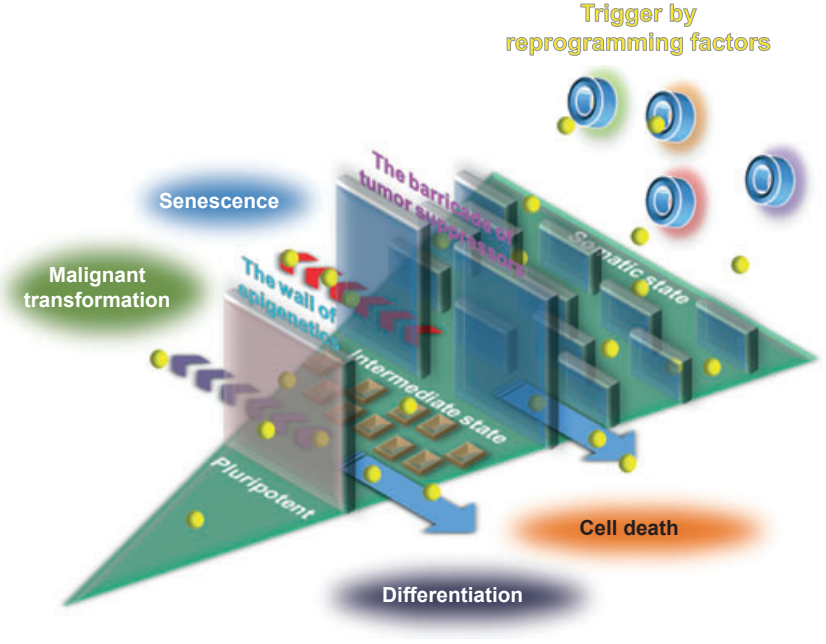

Fig. 2. A stochastic model of direct reprogramming. There are at least two blockades in the pathway to pluripotency. One is the barrier of tumor suppressor genes. Another is the wall of epigenetics. Some cells can stochastically remove such blockades.

hyperphosphorylated, implying their inactivation due to a lack of binding to E2F in mouse ES cells. Therefore, the dependency of RB in ES cells seems to be captured during ongoing differentiation. The resistibility for differentiation of ES cells was also observed in the case of p53 suppression both in mice and human (Sabapathy *et al.* 1997; Qin *et al.* 2007).

CDK inhibitor $p16^{Ink4a}$, encoded by the *Cdkn2a* gene, inhibits phosphorylation of RB by disrupting the CDK4-Cyclin D complex. Resistance to growth retardation by $p16^{Ink4a}$ is a common feature of RB-inactivated cancer cells (Lukas *et al.* 1995; Medema *et al.* 1995). Overexpression experiments show no growth inhibition induced by $p16^{Ink4a}$ in mouse ES cells (Savatier *et al.* 1996). Accumulation of hyperphosphorylated RB proteins via $p16^{Ink4a}$ and $p21^{Cip1}$ encoded by the *Cdkn1a* gene are highly related to replicative senescence (Brown *et al.* 1997). Therefore, MEF derived from the triple knockout of RB family genes are already immortalized in primary cultures, lack contact inhibition and escape from replicative senescence (Lukas *et al.* 1995; Medema *et al.* 1995). ES cells also carry on proliferation without crisis and senescence in the undifferentiated state. Taken together, these pathways show that tumor suppressor, and senescence-associated factors are important for differentiation of pluripotent stem cells. In direct reprogramming, inactivation or suppression of $p16^{Ink4a}$ and/or $p19^{Arf}$, which is an alternate reading frame products of the *Cdkn2a* loci, elevates the efficiency of iPS cell generation (Banito *et al.* 2009; Li *et al.* 2009b; Utikal *et al.* 2009b). These data indirectly suggest the blockade effects of p53 and RB in reprogramming (Fig. 2).

MicroRNA and pluripotency

MicroRNA (miRNA) are non-protein coding small RNAs that post-transcriptionally interfere with the expression of targets in a sequence-dependent manner. Tissue-specific miRNA often play important roles in organogenesis. For example, miRNA-1 (miR-1) is specifically expressed in skeletal muscles, and is directly regulated by muscle differentiation factors such as serum responsible factor, MyoD and Mef2 (Zhao *et al.* 2005). The targeted inactivation of miR-1 revealed that this gene promotes the expansion of ventricular cardiomyocytes during cardiogenesis by regulating the expression of transcription factor Hand2. miRNAs are active in ES cell differentiation in a variety of ways. Not only miR-1 but also miR-133 promotes differentiation of mouse ES cells into mesoderm while blocking entry to endoderm and neural lineages (Ivey *et al.* 2008). The core transcription factor circuitry of pluripotency, which consists of Oct3/4, Sox2 and Nanog, is modulated by miR-134, miR-296 and miR-470 that are induced by treatment with retinoic-acid in mouse ES cells (Tay *et al.* 2008). Xu and colleagues demonstrated that miR-145 controls the expression of Oct3/4, Sox2, and Klf4 and represses self-renewal of human ES cells (Xu *et al.* 2009).

Embryonic stem cells also express some specific miRNA (Houbaviy *et al.* 2003; Suh *et al.* 2004; Boyer *et al.* 2005; Marson *et al.* 2008). The members of the miR-290 family are specifically expressed in undifferentiated ES cells and promote rapid proliferation through regulation of G1-S transition (Wang *et al.* 2008). These small RNA can also enhance the reprogramming efficiency (Judson *et al.* 2009). The expression of mir-302s, which shares the same seed sequences with mouse miR-290, is directly regulated by Oct3/4 and Sox2 in human ES cells, and modulates Cyclin D1 expression (Card *et al.* 2008). On the other hand, mir-302s can also alleviate senescence by the transduction of Oct3/4, Sox2, Klf4 and c-Myc (Banito *et al.* 2009).

DiGeorge syndrome critical region gene 8 (Dgcr8), which expresses double-stranded RNA-binding protein, processes primary transcripts of miRNA (pri-miRNA) into pre-miRNA in the nucleus cooperating with RNase III enzyme Drosha (Gangaraju & Lin 2009). Mouse ES cells lacking the *Dgcr8* gene can differentiate but the expression of pluripotent stem cell marker genes are not fully silenced (Wang *et al.*, 2007). Dicer is an endoribonuclease that cleaves pre-miRNA into short double-stranded RNA fragments (Gangaraju & Lin 2009). Targeted disruption of the *Dicer* gene in mouse ES cells caused defects in differentiation (Kanellopoulou *et al.* 2005; Murchison *et al.* 2005).

The Argonaute subfamily is a subunit of RNA-induced silencing complex (RISC). Single strand RNA is loaded by Dicer into RISC, and then strikes the target mRNA and represses its translation. Both mouse and human genomes encode four closely related Argonaute subfamily proteins. Complete disruption of all four argonautes in mouse ES cells causes massive cell death due to apoptosis (Su *et al.* 2009). As expected, miRNA-mediated gene silencing was completely defective. These phenotypes can be rescued by re-introduction of human argonaute 2. In ES cells lacking all argonautes, the expression of an apoptosis facilitator Bim (also known as Bcl2-like 11), which is modulated by miRNA, is upregulated. Surprisingly, the forced expression of constitutive active mutant of Akt alone rescued their growth retardation by suppressing the Bim functions.

Lin28 is an RNA-binding protein and is related to processing miRNA such as let-7 (Viswanathan *et al.* 2008). Lin28 downregulates the process by blocking

pre-miRNA uridylation, resulting in degradation (Heo *et al.* 2008, 2009). The targets of let-7 include some ongogenic genes such as *c-Myc*, *K-Ras* and *Hmga2* (Kim *et al.* 2009a; Viswanathan *et al.* 2009). Lin28 can upregulate the efficiency of human iPS cell generation (Yu *et al.* 2007, 2009; Liao *et al.* 2008). Whether the positive effects of Lin28 on reprogramming are linked to miRNA machinery is still unclear. These observations indicate that, miRNA-mediated gene silencing plays critical roles in pluripotency, differentiation and reprogramming.

Conclusion

The acquisition and maintenance of pluripotency share many common mechanisms (Fig. 3). Interestingly, almost all reprogramming factors identified to date except for Oct3/4 and Sox2 are molecules that bypass the LIF signaling pathway in mouse ES cells, with the exception of Lin28 which has not yet been fully characterized (Chambers *et al.* 2003; Mitsui *et al.* 2003; Cartwright *et al.* 2005; Li *et al.* 2005; Ema *et al.* 2008; Zhang *et al.* 2008; Niwa *et al.* 2009). Self-renewal of human ES cells does not depend on the activity of LIF/Stat3 signaling (Sumi *et al.* 2004). Nevertheless, at least, some of the factors overlap in the maintenance of the undifferentiated state of both mouse and human ES cells. Like mouse ES cells, the forced expression of Nanog allows self-renewal of human ES cells in feeder-free differentiation conditions (Yasuda *et al.* 2006). In contrast, c-Myc induces differentiation and apoptosis of human ES cells (Sumi *et al.* 2007). However, c-Myc is quite effective to enhance the efficiency of human iPS cell generation (Nakagawa *et al.* 2008). This contradiction can be probably explained as that c-Myc is active in the early stage of reprogramming and will be silenced when cells achieve a pluripotent state (Sridharan *et al.* 2009).

The frequency of reprogramming somatic cells into iPS cells is generally around 1% (Yamanaka 2009). The balance among the individual reprogramming factors should be important (Papapetrou *et al.* 2009). However, even the ideal balanced expression of transgenes cannot accomplish 100% efficiency (Wernig *et al.* 2008b; Woltjen *et al.* 2009). Several reports claim that epithelial cells such as hepatocytes and keratinocytes could be reprogrammed more effectively than mesenchymal cells including fibroblasts (Aasen *et al.* 2008; Aoi *et al.* 2008). On the other hand, mouse neural stem cells are also a good source for reprogramming not only by gene transduction but also by fusion with ES cells (Silva *et al.* 2006, 2008). A sustainable effect of TGFβ inhibitors on iPS cell generation is possibly due to preventing epithelial-mesenchymal

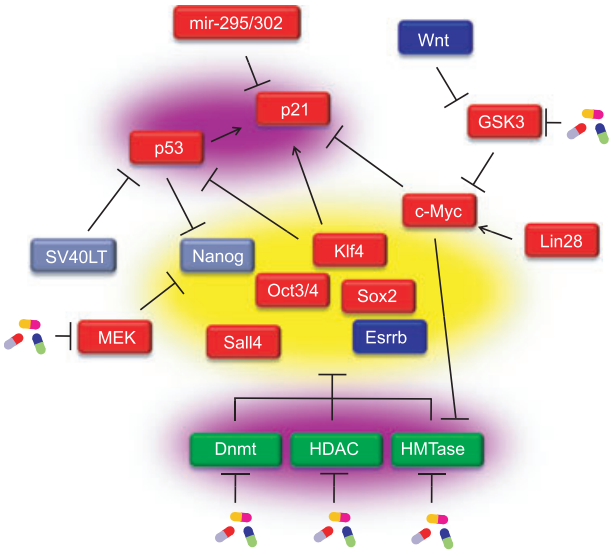

Fig. 3. Landscape of direct reprogramming. Red boxes, effects reported in both mouse and human cells; blue boxes, shown only in mouse cells; grey boxes, shown only in human cells; green boxes, epigenetic modifiers; capsules, small molecules whose effects were confirmed in direct reprogramming.

transition although the authors noted that they observed no such effects on human fibroblasts (Maherali & Hochedlinger 2009). Recent studies have revealed that cellular senescence could be one of the blockades of reprogramming. Actually, the efficiencies of mouse iPS cell generation are elevated to around 80% in a p53-null background by using a well balanced expression system (Utikal *et al.* 2009a,b). In addition, low efficiency is the common issue in all reprogramming technologies including SCNT and cell fusion. The reprogramming rate of MEF by fusion with ES cells is estimated to be <0.1%, and even NS cells can only convert with efficiency as high as 3–4% (Silva *et al.* 2006). For example, 100% of the cells will not become cancer cells even if oncogenic signals are activated in normal cells. Most of the cells escape from transformation by undergoing premature senescence or apoptosis. Similar barriers may therefore be faced after the introduction of reprogramming factors into normal cells. Moreover, all of the studies on iPS cells have limited the observation periods of the reprogramming processes within 2 months. Therefore, some cells that fail to be reprogrammed could probably become iPS cells after long term incubation (Hanna *et al.*, 2009). Not all of the cells derived from the ICM can commit to ES cells individually, thus changing cell fates including reprogramming always seems to be stochastic (Yamanaka 2009).

The concept of reprogramming was discovered more than 50 years ago. The recent rapid advances of

the field are closely associated with ES cell research. Direct reprogramming with defined factors can be used to decode the mechanism underlying pluripotency. iPS cell technologies are expected to expand our existing knowledge and contribute to a new view for the entire stem cell field.

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