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Review

Induced pluripotent stem cells: opportunities and challenges

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Somatic cells have been reprogrammed into pluripotent stem cells by introducing a combination of several transcription factors, such as *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*. Induced pluripotent stem (iPS) cells from a patient's somatic cells could be a useful source for drug discovery and cell transplantation therapies. However, most human iPS cells are made by viral vectors, such as retrovirus and lentivirus, which integrate the reprogramming factors into the host genomes and may increase the risk of tumour formation. Several non-integration methods have been reported to overcome the safety concern associated with the generation of iPS cells, such as transient expression of the reprogramming factors using adenovirus vectors or plasmids, and direct delivery of reprogramming proteins. Although these transient expression methods could avoid genomic alteration of iPS cells, they are inefficient. Several studies of gene expression, epigenetic modification and differentiation revealed the insufficient reprogramming of iPS cells, thus suggesting the need for improvement of the reprogramming procedure not only in quantity but also in quality. This report will summarize the current knowledge of iPS generation and discuss future reprogramming methods for medical application.

Keywords: iPS cell; reprogramming; pluripotency

1. INTRODUCTION

Reprogramming has been studied extensively for decades. Nuclear transfer into an oocyte gives somatic cells pluripotency to produce cloned animals. For example, Dr J. Gurdon and his colleagues showed that frog somatic cell nuclei can be reprogrammed after transfer into enucleated oocytes, and they develop into feeding tadpoles [1]. Reprogramming in vertebrates was also proven by the creation of cloned animals from sheep [2] and mice [3]. In addition to oocytes, human [4] and mouse embryonic stem (ES) [5] cells also can reprogramme somatic cells into an ES cell-like state after cell fusion. These results demonstrate that terminally differentiated cells can revert to a state of pluripotency in response to external stimulation.

The accumulated understanding of the mechanisms underlying pluripotency in ES cells led to attempts to revert somatic cells into a pluripotent state using defined factors. Twenty-four candidate factors were transduced into mouse embryonic fibroblasts (MEFs) by retroviral delivery and this identified four factors that can convert fibroblasts into induced pluripotent stem (iPS) cells [6]. iPS cells have been generated from mouse [6], rat [7,8], monkey [9], pig [10], dog [11], rabbit [12] and human [13,14]. Most of the iPS cells are derived using the *Oct3/4*, *Sox2*, *Klf4* and *c-Myc* reprogramming factors. The original iPS cell induction system used retroviral vectors, which integrate transgenes into the host genome. The insertion of tumorigenic genes, like *c-Myc*, and activation of proto-oncogenes by LTR increase the risk of tumour formation [15,16].

Mouse iPS cells were generated using a plasmid vector in 2008, showing that iPS cells can be induced by the transient expression of reprogramming factors [17]. The goals of those experiments were to increase transfection efficiency in primary cells and to maintain transgene expression long enough (a few weeks) for iPS cell induction. Three essential reprogramming factors (*Oct3/4*, *Sox2* and *Klf4*) were connected in a single plasmid using the 2A sequence, which enables expression of multiple proteins from a single RNA transcript. The stoichiometric balance of these core transcription factors is thought to be important for iPS cell induction, and therefore all six possible orders of the factors in the retrovirus system were examined to determine the most effective arrangement. The three factors were then placed into a plasmid vector with a constitutively active CAG promoter, which yielded high expression [18]. This vector ensures co-expression of the three core factors in all of the transfected cells. In addition, another expression vector for *c-Myc* was constructed. The transfection of the plasmids into MEFs was repeated multiple times to achieve the sustained expression required for iPS cell generation. After four weeks we obtained iPS cell colonies, albeit at a very low frequency. As expected, iPS cell clones in which transgenes had been integrated into the host genome were frequently observed. However, no transgene

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Table 1. iPS induction methods in human fibroblasts.

type of vector	method	genomic integration	factors[d]	reprogramming efficiency in human fibroblasts [e]	reference
virus	retrovirus	+	OSKM	++++	[13]
	lentivirus	+	OSNL	+++	[14]
	adenovirus	−[a]	OSKM	+	[19]
	Sendai virus	−[b]	OSKM	++++	[20]
DNA	episomal plasmid	−[a]	OSKMNLT	+	[24]
	transposon	−[a,c]	OSKM	++	[21,22]
	minicircle	−[a]	OSNL	+	[23]
RNA	RNA	−	OSKM	+++	[25]
protein	cell transparent protein	−	OSKM	+	[26]

[a]Absence of genomic integration should be experimentally examined.
[b]Absence of virus RNA genome should be experimentally examined.
[c]Transposon vector is integrated into genome, but it can be removed.
[d]O, OCT3/4; S, SOX2; K, KLF4; M, C-MYC; N, NANOG; L, LIN28; T, SV40-large T antigen.
[e]+, <0.001%; ++, <0.01%; +++, <0.1%; ++++, >0.1%.

integration was detected in approximately one-third of the established mouse iPS cell clones. The integration-free iPS cell clones had the potential to differentiate into various cell types of the three germ layers. Furthermore, they were able to form chimeric mice when transplanted into blastocysts, which were competent for germline transmission.

The frequency of iPS cell generation by plasmids, however, was very inefficient. The estimated efficiency is less than 0.0002%, which is at least 1000-fold lower than that of viral induction. The fact that reprogramming efficiency of human fibroblasts with retrovirus is approximately 10-fold lower than that of mouse fibroblasts suggested that the generation of integration-free human iPS cells would be extremely inefficient using the same method. Subsequently, several methods for integration-free human iPS cell generation have been reported. The approach can be divided into four groups based on delivery methods of the reprogramming factors: (i) virus [13,19,20], (ii) DNA [21–24], (iii) RNA [25], and (iv) protein [26] (table 1). We calculated induction efficiency of the methods from the best result reported in each article. Because of the differences in their experimental settings, it is hard to compare their efficiency correctly. However, as predicted, non-integration methods are extremely inefficient in general. Notably, recent reports showed significant improvement of non-integration method. Sendai virus is a minus strand RNA virus. Fusaki *et al*. [20] infected Sendai virus vectors encoding reprogramming factor into human fibroblasts and obtained iPS cells. Because Sendai virus replicates its genome in the cytoplasm of infected cells, this vector system can stably express reprogramming factors and achieve high reprogramming efficiency. The established iPS cells, on the other hand, tended to carry the virus genome even after long-term culture. To obtain viral-free cells, an additional approach was needed such as the elimination of virus-containing cells through negative selection against virus antigen hemagglutinin–neuraminidase or using a temperature sensitive mutant. Direct delivery of synthetic mRNA also generated iPS cells at high efficiency [25]. The mRNA sustained high and relatively long expression of encoding reprogramming factors by using modified ribonucleotides. However, reprogramming via modified RNAs is technically difficult, sensitive to reagents and requires labour-intensive procedures. Therefore, further improvements in reprogramming methods are absolutely required for reproducible generation of integration-free human iPS cells. A summary of several topics associated with iPS cell generation and a discussion of the future in reprogramming methods for medical and other applications are herein provided.

2. Myc FAMILY

c-Myc is a potent inducer of reprogramming [27]. Its functions are not fully understood; however, one of the functions may be direct activation of pluripotent marker genes. Though c-Myc is ubiquitously expressed in several cell types, it has an essential function in maintenance of the pluripotent state in mouse ES cells. Mouse ES cells can be maintained by activation of the STAT3 signal through addition of its upstream cytokine, leukaemia inhibitory factor (LIF). Dalton and co-workers [28] showed that *c-Myc* is one of the STAT3 target genes and that forced expression of c-Myc alone can keep mouse ES cells in a pluripotent state. Both c-Myc and N-Myc inhibit differentiation of mouse ES cells into the primitive endoderm lineage, through suppression of Gata-6 expression, the master gene [29]. Interestingly, the incorporation of *c-Myc* in the reprogramming cocktail promoted the frequency of germline transmission from chimeric mice in comparison with iPS cells generated without the *Myc* transgenes [30]. Therefore, c-Myc affects the net reprogramming process. Another role of c-Myc is acceleration of the cell cycle, although it activates the p53 and p21 pathway. More than 4000 sites in the genome are reported as c-Myc binding regions [31]. Myc binding may loosen chromatin structure and facilitate the access of other reprogramming factors to their target sequences. c-Myc enhances iPS cell generation at least in part through these mechanisms; on the other hand, its tumorigenic properties could have an inhibitory effect on proper reprogramming

and increase the frequency of transformed cells during iPS generation. Myc also increases the risk of tumour formation when the transgene remains in established iPS cells [15].

The Myc family consists of three members: c-Myc, N-Myc and L-Myc. All three form heterodimers with Max protein and bind to target DNA. Their entire amino acid sequences are similar; however, L-Myc is shorter in the N-terminal region than the other two members. Interestingly, the N-terminal region contributes to transformation activity in cultured cells. Consistent with this, activation of L-Myc in human tumours is less frequently reported. Therefore, L-Myc was the candidate for a reprogramming enhancer without increasing the tumorigenic risk, and attempts were made to use L-Myc for iPS cell induction [30]. Using *L-Myc* for the induction of mouse iPS cells increases the number of iPS cell colonies in comparison with cells transduced with *Oct3/4*, *Sox2* and *Klf4*; however, the effect was weaker than for *c-Myc*. *L-Myc* showed less colony formation than *c-Myc*, which results in a higher proportion of iPS cell colonies among the total number of colonies. In contrast, *L-MYC* enhanced iPS induction more efficiently than *C-MYC* in human fibroblasts. *C-MYC* and *N-MYC* markedly increased the formation of non-iPS cell colonies, whereas *L-MYC* did not. Therefore, the proportion of human iPS colonies of the total colonies is significantly higher with *L-MYC* than with *C-MYC* or *N-MYC*. Therefore, different functional moieties of the *Myc* proto-oncogene products are involved in the transformation and promotion of directed reprogramming. A microarray analysis has shown the enhancing effect of *L-Myc* on reprogramming to be predominantly accomplished by the suppression of fibroblast-specific genes. In addition, *L-Myc* showed weaker activation of genes related to tumour formation than did *c-Myc*. These data demonstrated that *L-Myc* may therefore be more suitable for human iPS cell generation than *c-Myc*.

3. p53

The expression of pluripotent marker genes are often found in immature tumour cells. For example, breast cancer cells show elevated levels of OCT3/4 and SOX2 [32,33]. In addition, these cancer stem cells have a differentiation potential to transform into several cell types. Both immature tumour cells and iPS cells are derived from somatic cells and obtain differentiation potential. These similarities suggest an underlying common mechanism of reprogramming towards iPS cells and cancer cells and led to an examination of the roles of the tumour suppressor gene, *p53*, during iPS cell generation [34]. A relationship between the p53 pathway, c-Myc and Klf4 has also been reported. The transduction of p53-null MEFs with the four reprogramming factors revealed marked enhancement of iPS cell colony formation. They formed tightly packed compact colonies, similar to ES cells. They also express pluripotent marker genes including *Nanog* and *ECAT1*. The injection of these reprogrammed cells into immunodeficient mice results in the formation of teratomas containing various cell types of the three germ layers, such as neuronal tissue, cartilage, muscle and gut-like epithelium. Their pluripotency was further confirmed by chimeric mouse formation. Enhancement of reprogramming was also observed by induction of the dominant negative form of *p53* or its short hairpin RNA (shRNA) into wild-type human adult dermal fibroblasts. These data demonstrated that the loss of p53 markedly accelerates both mouse and human iPS cell generation.

p53 has many cellular functions, including regulation of apoptosis and senescence through p21. *c-Myc* stimulates p53 and then p21 during iPS cell generation, which both inhibit the reprogramming process. The forced expression of *MDM2* increases reprogramming efficiency through the inhibition of p53. On the other hand, p53 suppression may lead to genomic instability; thus continuous suppression should be avoided [35]. It is important to carefully examine the genomic mutations and tumorigenicity of established iPS cell clones.

The cell cycle seems to play important roles during generation of iPS cells. Some of the reprogramming steps are likely to depend on stochastic events, such as the initial cell condition, microenvironment, fluctuation of gene expression and epigenetic modification. Therefore, an increase in the cell numbers simply increases the chance of iPS cell induction. Hanna *et al.* [36] examined the relationship between cell number and reprogramming efficiency using the secondary induction system. They argued that reprogramming is a continuous stochastic process where almost all mouse donor cells eventually give rise to iPS cells after extended cultivation. Inhibition of the p53 and p21 pathway as well as the expression of Lin28 increases iPS cell generation predominantly by acceleration of the rate of cell division. On the other hand, an overexpression of Nanog seemed to enhance reprogramming in a proliferation independent manner.

4. LIN28

LIN28 is a negative regulator of the let7 microRNA (miRNA) family. Lin28 induces the uridylation of immature let7 RNA by a non-canonical poly (A) polymerase, TUTase4, which leads to degradation of the RNA [37]. Lin28 gradually decreases during ES cell differentiation, and mature let-7 family miRNAs becomes detectable with an inverse correlation. The targets of mature let7 include oncogenic genes, such as *K-Ras* and *c-Myc*. This is consistent with the analysis performed by Hanna *et al.* [36] which showed that Lin28 accelerates the efficiency of iPS cell generation in a cell cycle-dependent manner. However, Lin28 also facilitates the expression of Oct3/4 at the post-transcriptional level by direct binding to its mRNA [38]. In addition, let-7 family miRNAs promotes differentiation of breast cancer cells and inhibits their proliferation [39]. Therefore, Lin28 may facilitate iPS cell generation, not only in a cell cycle-dependent manner, but also by promoting Oct3/4 expression and suppressing the differentiation through the inhibition of let-7 family miRNA. Addition of Lin28 enhances the reprogramming efficiency from both human and

mouse fibroblasts. Further studies are required, however, to elucidate the precise mechanisms.

5. OESTROGEN RECEPTOR-RELATED BETA

Oestrogen receptor-related beta (Esrrb) is an orphan nuclear receptor which has a significant homology with oestrogen receptors. Esrrb regulates transcription constitutively through the oestrogen response elements or steroidogenic factor-1 response elements of target genes. The suppression of Esrrb in ES cells results in morphological changes and differentiation, thus suggesting an important role in self-renewal [40,41]. Esrrb positively regulates the expression of the key pluripotency gene *Nanog* [42], and the overexpression of Esrrb allows for short-term ES cell maintenance without the addition of exogenous LIF [43]. Esrrb is also capable of replacing *Klf4* in somatic cell reprogramming from MEFs, but to a lesser extent [44]. However, the roles of Esrrb in human iPS cell induction remain to be determined.

6. SALL4

Sall (Sal-like) 4 belongs to the Spalt (Sal) transcription factor family characterized by highly conservative C_2H_2 zinc-finger motifs. Knockdown of Sall4 in mouse ES cells results in the loss of the undifferentiated state and differentiation into trophectoderm-like cells, suggesting that Sall4 contributes to self-renewal of ES cells [45,46]. Importantly, many binding sites of Sall4 overlap with those of Oct3/4, Sox2 and Nanog in ES cells, as determined by chromatin immunoprecipitation (ChIP) on chip analyses [47]. These data suggest that Sall4 plays a pivotal role in pluripotency, hence the effect of Sall4 in iPS generation [48]. Addition of Sall4 to the reprogramming factors increases the iPS colony number from MEFs. However, the ectopic expression of *SALL4* showed variable effects on iPS generation from human dermal fibroblasts (HDF). Three out of seven lines of HDF showed more than twofold increment. On the other hand, no such effects were observed in the other four lines. A possible cause of the effects of SALL4 on different HDF lines is the expression levels of endogenous SALL4. A fibroblast line from a 36-year-old female, which showed the strongest effect of the *SALL4* transgene, had the lowest expression level of endogenous SALL4. Therefore, the addition of SALL4 could be beneficial when trying to achieve iPS cell generation from cells with a low endogenous SALL4 expression.

7. UNDIFFERENTIATED EMBRYONIC CELL TRANSCRIPTION FACTOR 1

The expression of UTF1 (undifferentiated embryonic cell transcription factor 1) is restricted in ES cells, embryonic carcinoma cells and primordial germ cells. UTF1 functions as a chromatin-associated transcriptional repressor with a dynamic behaviour similar to core histones [49]. Knockdown of *UTF1* in ES cells and embryonic carcinoma cells results in a substantial delay or block to differentiation [50]. UTF1 may also be important for proliferation. These data indicate a possible role for UTF1 in the maintenance of a specific epigenetic profile that is required for differentiation and proliferation of ES cells. Zhao *et al.* [51] screened several candidate factors along with conventional factors (*OCT3/4*, *SOX2*, *KLF4* and *C-MYC*) for their capacity to improve the reprogramming step, and found a dramatic increase by *UTF1* addition. They also found an inhibitory effect of p53 for reprogramming. However, none of other candidates including *NANOG*, *LIN28*, *DPPA4*, *DPPA5*, *ZIC3*, *BCL-2*, *h-RAS*, *TPT1*, *SALL2*, *NAC1*, *DAX1*, *TERT*, *ZNF206*, *FOXD3* and *REX1*, increased iPS cell colony formation dramatically in their culture conditions.

8. TBX3

Tbx3 is a transcription factor belonging to the T-box family. Tbx3 regulates the expression of Nanog and Sox2, and is involved in the transcriptional network for the maintenance of pluripotency in mouse ES cells [52]. The expression of Tbx3 is induced by the activation of the PI3K pathway, whereas it is inhibited by mitogen-activated protein kinase (MAPK) signalling. shRNA mediated loss-of-function assays indicated requirement of Tbx3 in ES cells [40]. Addition of Tbx3 in reprogramming factors seemed to improve the quality of iPS cells [53]. Mouse iPS cells induced by *Tbx3* together with *Oct3/4*, *Sox2* and *Klf4* do not show any significant difference in global gene expression profile in comparison with iPS cells without *Tbx3*. However, incorporation of *Tbx3* increases the frequency of germ-cell contribution and germline transmission when injected into early mouse embryos. ChIP-sequencing revealed that the direct regulatory targets of Tbx3 share a large number of common binding sites with Oct4, Sox2 and Nanog [53]. Tbx3 may increase the probability of fully reprogrammed cells in iPS cell population.

9. miRNA

miRNAs are small single-stranded RNAs (around 22 nt) that directly interact with target mRNAs through complementary base-pairing and inhibit translation of the target genes. miRNAs also modify gene expression at a transcriptional level. miRNAs are involved in many features of cell properties, such as proliferation, apoptosis and differentiation, by fine-tuning gene expression. miR-291-3p, miR-294 or miR-295 increase reprogramming efficiency from MEFs [54]. The three miRNAs share a conserved seed sequence, suggesting they work through common targets. They could be downstream targets of c-Myc, because the miRNAs did not enhance reprogramming efficiency in the presence of *c-Myc* transgene, and c-Myc binds the promoter region of the cluster. The miR-200 family also promotes iPS cell generation through enhancing mesenchymal-to-epithelial transition (MET; described below) [55].

10. GENE BALANCE

The low efficiency of iPS cell induction from somatic cells raises the possibility that the precise balance and/or amount of each transgene expression is important

for reprogramming. Retrovirally induced fibroblasts can integrate more than 10 copies of transgenes, and it is difficult to control the balance and their inserting position. Retroviral vectors preferentially integrate into the promoter regions of active loci, where the transgene expression level is affected by the flanking promoter and other elements. Dramatic epigenetic changes occur during the reprogramming process. This could influence endogenous promoter activities and retroviral expression. Therefore, the level and balance of retroviral/lentiviral transgene expression can change even after their integration. Papapetrou *et al.* [56] tried to monitor these expression levels through the process using lentivirus vectors encoding different fluorescent proteins connected to each reprogramming factor. They infected these vectors into human fibroblasts at various multiplicities of infection, and thus found the iPS cell induction rate to be highly sensitive to the OCT3/4 dosage. A threefold increase of OCT3/4 improved the reprogramming efficiency up to twofold, whereas threefold reduction severely decreased iPS generation. Interestingly, excess addition of OCT3/4 transgene spoiled the enhancement. On the other hand, a change of the other three factor dosages inhibited the reprogramming process. The overexpression of some reprogramming factors, such as Nanog, c-Myc and Klf4, can maintain a pluripotent state in mouse ES cells [57]. On the other hand, the forced overexpression of Oct3/4 or Sox2 results in the differentiation of mouse ES cells [58]. A small degree of imbalance can be compensated for in the mutual regulation networks because these reprogramming factors constitute a transcriptional circuit and maintain their expression level. However, an excess amount of imbalance would be harmful for iPS cell generation and maintenance.

11. CULTURE CONDITIONS

iPS cells have been established in the medium for ES cells. The external signals, LIF and basic fibroblast growth factor (bFGF) are important factors for mouse and human ES cell maintenance, respectively. Wnt signalling also supports the self-renewal of ES cells. The Wnt3a signal is mediated by glycogen synthase kinase (GSK) 3β. The absence of the Wnt signal causes GSK3β to inactivate targets, such as β-catenin and c-Myc by phosphorylation and proteasome-mediated degradation. Hence, the inhibition of GSK3β with a chemical drug, CHIR99021, results in activation of Wnt signalling [8,59]. The addition of Wnt3a or CHIR99021 enhances the reprogramming efficiency from both mouse and human cells [8,59]. Kenpaullone, an inhibitor whose targets are GSK3β as well as CDKs, can replace *Klf4* when MEFs are transduced with *Oct3/4*, *Sox2* and *c-Myc* [60]. Transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) signals regulate MET and could also play an important role in the reprogramming process (described below) [55,61].

O_2 tension is also an important aspect for stem cell maintenance and differentiation. For instance, low O_2 tension, hypoxia, promotes the survival of neural crest cells and haematopoietic stem cells, and prevents differentiation of human ES cells [62]. Therefore, iPS cell induction performed in hypoxic conditions (5% O_2) shows upto fourfold enhancement of the reprogramming efficiency in both mouse and human fibroblasts [63].

(a) *Epigenetic modifiers*

Epigenetic modifying drugs can also improve iPS generation. For instance, inhibitors of DNA methyltransferase (DNMT), such as 5′-azacytidine and RG108, increase reprogramming efficiency [64]. A putative mechanism of active DNA demethylation was recently reported in a cell-fusion based reprogramming system [65]. No consensus mammalian DNA demethylase has been identified. A complex consisting of a cytidine deaminase, AID, GADD45 and Mbd4 has been implicated in DNA demethylation [66]. These factors function as an active demethylation complex by a coupled mechanisms of AID-dependent deamination of 5-meC followed by thymine base excision by Mbd4. AID is primarily known for its role in B cell class switch recombination and hypermutation in mammals. Fibroblasts have methylated DNA at the *Oct3/4* promoter region. The activation and DNA demethylation of the *Oct3/4* promoter occurs within 24 h after cell fusion with ES cells. Bhutani *et al.* [65] found that the suppression of AID by shRNA inhibits this promoter activation and DNA demethylation status in fused cells. AID is also involved in genome-wide erasure of DNA methylation in primordial germ cells [67]. It is unclear whether AID also plays a role in the iPS cell induction process.

A global survey of DNA methylation in iPS cells and their parental origins (mostly fibroblasts) shows that iPS cells have rather higher DNA methylation than do their origins [68,69]. The upregulation of DNMT3b and DNMT3l occurs during the reprogramming process, and might be responsible for *de novo* methylation during iPS cell generation. The abnormal DNA methylation pattern, including hypermethylation and hypomethylation, can cause abnormal differentiation properties of iPS cells. Other epigenetic states, such as histone methylation and acetylation, probably participate in iPS cell reprogramming. Addition of a histone deacetylase (HDAC) inhibitor—valproic acid (VPA)—improves reprogramming efficiency in both mouse and human fibroblasts [70,71]. Other HDAC inhibitors, suberoylanilide hydroxamic acid and trichostatin A, also increase iPS cell generation from mouse fibroblasts [71].

12. ORIGIN

iPS cells can have some memories of their parental sources. iPS cells from peripheral blood differentiated into the haematopoietic lineage at a high efficiency [72]. They did show a differentiation potential into neuronal cells, but the efficiency was low. However, iPS cells from different origins show markedly similar gene expression patterns in the undifferentiated state. Their clonal diversity is not beyond that of ES cells. Therefore, the memory could be epigenetic status at loci that are not directly related to the pluripotent status. Several differentially methylated DNA regions (DMR) have been reported [68].

Although long-term passaging of iPS cells could largely attenuate these differences [73], studies of the relationship between DMR and differentiation potentials are needed to precisely evaluate iPS cells from different origins. The cell sources of iPS cells also influence the safety of the established iPS cells. The neural differentiation of mouse iPS cells derived from various tissues including MEFs, tail-tip fibroblasts (TTF), hepatocyte and stomach was performed to examine tumorigenicity [74]. Clones that originated from TTF showed many residual pluripotent cells after three weeks of *in vitro* differentiation into the neural sphere, which resulted in teratoma formation when transplanted into an immune-deficient mouse brain. The precise mechanism of this phenomenon is yet to be determined, but it may be attributable to incomplete reprogramming, epigenetic memory and/or genomic stability. A 'safe' iPS cell clone showed therapeutic effects in a mouse model of spinal cord injury [75]. The origin of iPS cells should also be important in humans. It is important to consider clinically available sources. Human iPS cells have been generated from keratinocytes [76], mesenchymal cells in fat [77], oral mucosa [78], dental pulp cells [79], peripheral blood [80] and cord blood, in addition to skin fibroblasts [81]. The properties and safety of these iPS cell clones should therefore be carefully examined.

13. CLONE DIFFERENCE

Each human ES clone shows a distinct differentiation potential [82]. Some tend towards the mesoderm lineage after embryoid body formation, but others prefer ectoderm differentiation. The characteristics of ES/iPS cells are also changed by culture conditions, such as growth medium, feeder cells, oxygen concentration and passage methods [83]. The culture time and passage number are particularly important with iPS cells, as reprogramming seems to continue even after establishment of iPS cell colonies. Chin *et al.* [84] reported that the continual cultivation of iPS cells yields a gene expression profile more similar to human ES cells than that of early passages. However, extended culture itself raises the risk of genomic instability. Some scientists believe that human ES/iPS cells in the naive state have less diversity than that of those in the primed state. However, it is important to note that even mouse ES cells demonstrate diversity in their differentiation potential.

14. NAIVE INDUCED PLURIPOTENT STEM CELLS

Pluripotent stem cells including iPS cells and ES cells are categorized into two groups by their morphology, gene expression profile and external signal dependence. Conventional mouse-type ES/iPS cells, which form compacted dome-shape colonies in culture, are called 'naive state' cells. They are largely dependent on external LIF signals and show a high proliferation rate. On the other hand, human-type ES/iPS cells, which show flat colonies, are predominantly dependent on the bFGF signal, and proliferate more slowly, are termed 'primed state'. Primed stem cells, called epiblast stem cells (EpiSCs), can be established from mouse epiblasts with the addition of bFGF in culture medium [85,86]. The EpiSCs form flat colonies, and can differentiate into various cell types; however, they fail to form chimeric mice when injected into blastocyst. It could be due to the limited differentiation potential of the primed stem cells or just show the difference of their developmental stage. The addition of several transgenes such as OCT3/4 and KLF4 into conventional human iPS cells could successfully convert their status like mouse ES cells [87]. These modified human iPS cells share several features with naive stem cells, including morphology, growth properties, an X-chromosome activation state, a gene expression profile and a signalling pathway dependence. However, the culture conditions of naive human pluripotent cells seem not to be optimized, because they easily revert to the primed state. Further study should be performed to identify and establish stable handling methods for naive human pluripotent stem cells.

15. MECHANISMS UNDERLYING THE REPROGRAMMING PROCESS

The reprogramming process has been gradually revealed by intense studies. Araki *et al.* [88] traced the conversion of MEFs to iPS cells via a live cell imaging system. They introduced the reprogramming factors and observed MEFs dividing several times in a morphologically symmetric manner, maintaining a fibroblastic shape. Thereafter, the ancestral cells gradually transformed their morphology into an ES-like shape. The transformation occurred within 48 h after retroviral induction of the four factors in most cases. Importantly, their time-lapse analysis uncovered frequent failure in reprogramming at late stages of iPS induction. One of descendant became an iPS cell after an asymmetric division of reprogramming cells. However, the other descendant with the same retroviral insertion underwent cell death. The process was largely dependent on the c-Myc transgene. They frequently found the transient expression of GFP driven by the Nanog promoter. This unstable expression may indicate spontaneous activation of the locus, and may reflect stochastic events during reprogramming, which can be a stressful time for cells.

MEFs start to lose their mesenchymal character after the induction of transgenes, and then transform their shape into epithelial cells, the phenomenon known as MET [55,61]. MET is a biological process that often occurs during organ development. The molecular analysis revealed that reprogramming factors cooperatively induce MET in MEFs. Oct3/4 and Sox2 suppress Snail, a key factor of the epithelial-to-mesenchymal transition (EMT), which is the opposite of the MET. TGF-β signals induce the EMT, at least in part, through the activation of Snail and thereby negatively regulate the MET. The c-Myc transgene enhances MET through the downregulation of TGF-β signals by suppression of TGF-β1 and TGF-β receptor 2 expression. In addition, Klf4 upregulates genes associated with epithelial cells, such as E-cadherin. A temporal gene expression analysis

revealed that the BMP signals promote the MET through the miR-200 family. The inhibition of MET by TGF-β signalling or by siRNA greatly reduced the reprogramming efficiency, suggesting that the MET is an important cellular event during reprogramming.

Chan *et al*. [89] observed human iPS cell induction by live cell imaging in detail and found that there are three types of human iPS cells based on the expression profiles of cell surface markers and retroviral silencing. The three types differed in the methylation status of the promoter region of *NANOG* and *OCT3/4* loci and their differentiation potential. The best reprogrammed type was positive for the pluripotency markers, SSEA-4 and TRA-1-60, and negative for the fibroblast marker, CD13, and showed inactivation of the retroviral promoter. Only this cell type could make teratomas containing tissues of all three germ layers. The discrimination of the high pluripotency cells from iPS cell induction cultures is necessary because the other two types of partially reprogrammed cells were morphologically similar to the correctly reprogrammed iPS cells.

16. CONCLUSION

Human iPS cells can be established with a variety of methods. There are several differences among (i) the cell source, (ii) induction method, (iii) reprogramming factors, (iv) culture conditions including small molecular supplements, and (v) type of stem cells (naive or primed). Retro/lentiviral induction is sufficient for *in vitro* use of iPS cells when the remaining reprogramming factors do not significantly interrupt designed assays. However, the transgene integration and alteration of the endogenous genomic organization could cause a negative safety issue when considering medical applications. Genomic integration sites of retro- and lentivirus in iPS cells range from 1 to 40, and PCR-based analysis can detect all the integration sites. Hence, it may be possible to estimate their risk beforehand. Non-integration methods of iPS cell generation have been reported, but their induction efficiencies are quite low and may give rise to insufficiently reprogrammed iPS cells. These integration-free methods could be improved by using better combinations of reprogramming factors, better parental cell sources, and better culture conditions. The establishment of methods of iPS cell generation for clinical applications is an ongoing process. More comprehensive knowledge of the reprogramming process is therefore crucial for future clinical applications of iPS cells.

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