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Barriers in the generation of iPS cells

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Abstract. Since the discovery of induced pluripotent stem (iPS) cells by Takahasi and Tamanaka in 2006, a new era in regenerative medicine has began. Takahasi and Tamanaka were able to reprogram somatic cells back to pluripotent state by introducing retroviral the pluripotency genes Oct4, Sox2, Klf4 and c-Myc. After minor refinements in the initial reprogramming protocol, the iPS cells were shown to be germline-competent and capable of forming any cell type. This technology could deliver patient-specific derived iPS cells that can contribute to disease modeling, drug screening, toxicology tests and autologous cell-based therapies. However, there are still limits that indicate that iPS cell technology is still at its infancy. The reprogramming efficiency of iPS cells is very low and generated iPS cells are highly oncogenic. 4 years since the discovery of iPS cells, plenty of research has been done concerning iPS cells. New methods have been developed to refine and improve reprogramming and factors have been tested to improve reprogramming efficiency. This review will focus on why the generation of iPS cells is so inefficient and how the most recent developed methods can improve it. I will discuss the basic parameters of somatic cell reprogramming; why iPS cell generation is a long and inefficient process; newly developed methods to generate iPS cells; and factors that can improve reprogramming efficiency. With these topics I will try to answer the following question: what is the most efficient way to generate iPS cells?

Introduction

Since the discovery of embryonic stem cells (ESCs), a new promising standard has been found in cell-based regenerative medicine. ESCs are pluripotent, meaning that they have the ability to self-renew infinitely and are capable to differentiate into any existing cell type in the body [1]. These useful characteristics could have caused a medical revolution, because ESCs can be used to derive any kind of cell lines for patients. However, ES cell-based therapy has several limitations that have prevented its application in treating human disease so far. The derivation of autologous cell lines for patients is impossible, because ESCs can only be isolated from early-stage embryos, and transplantation of non-autologous cell lines would cause immune rejection. This immune rejection prevents the usage of ESCs for cell-therapy. Besides these technical limits of ESCs, there are also limits in researching ESCs because of ethical reasons.

A new technique called somatic cell nuclear transfer (SCNT) or nuclear cloning could have solved the technical problems surrounding ESCs. This technique involves the transfer of a nucleus taken from a somatic cell into an oocyte without a nucleus. Then, undefined factors reverse the epigenetic state of the somatic cell to that of a pluripotent embryonic cell. This cloned cell is then able to form a blastocyst stage with inner cell mass, able to act as autologous donor cells for cell-based therapy. In theory, this strategy is possible and its promising usage was proven in a mouse study [2]. However, SCNT has not yet been successfully performed in humans. But if perhaps SCNT is usable on humans in the future, ethics would still be a lasting issue concerning SCNT. Destruction of blastocyst and oocyte donation necessary to generate patient-specific pluripotent stem cell lines would certainly create moral and ethical dilemmas that would limit the usage of this therapy. So just like ESCs, SCNT has its limits, both technically and ethically. The technical problems could eventually be solved in the (near) future; however there will always be ethical problems limiting the use of these therapies. Now this ethical conundrum is about to be solved by a new upcoming technique that recently started a new era in regenerative medicine.

In 2006, Takahashi and Yamanaka [3] discovered that somatic cells that typically exhibit little or no capacity to differentiate, could be reprogrammed back to pluripotent stem cells. This reprogramming of somatic cells was achieved in mouse embryonic and adult fibroblasts by introducing the pluripotency genes Oct4, Sox2, Klf4 and C-myc by retroviral transduction. After minor refinements in the initial reprogramming protocol, the induced pluripotent stem (iPS) cells were shown to be germline-competent and capable of forming any cell type of the body. Not much later iPS cells were also generated from human fibroblasts [13]. This astonishing discovery truly represents a revolution in stem cell biology. For stem cell biologists, the study of iPS cells opens a new path to understanding the molecular mechanisms responsible for self-renewal and pluripotency. Not only could this discovery

provide us with new insights and knowledge, it could also help the medical community with developing new therapies for human diseases. iPS cells could offer patient-specific cells, which could demise the problem of immune rejection when transplanted, and can solve the ethical problems that surround the isolation of human embryonic stem cells from early embryos or the destruction of blastocyst and oocyte donation in SCNT. Also, iPS cell technology can contribute to disease modeling, drug screening and toxicology tests. The fact that there is no ethical pressure on iPS cells and pluripotent stem cells are now easier to gain, scientist are now stimulated to study pluripotent stem cells more than ever.
Although iPS cells sound very promising for the future, there are of course certain issues that need to be solved first if we want to use iPS for medical goals. For example, the first major problem is the oncogenicity of generated iPS cells. iPS cells have increased oncogenicy because of several reasons. iPS cells are generated by using retroviruses to integrate the pluripotency factors into the host genome. These retroviruses insert the pluripotency genes at random sites into the host genome. These random insertions of genes can cause insertional mutagenesis, which can eventually cause cancer. The genes used to induce pluripotency are oncogenes. Inappropriate expression or reactivation of these inserted transgenes can result in oncogency [13]. As long as iPS cells remain oncogenic, they cannot be used for clinical application.
The second major problem concerning iPS cells is the generation of it. So far the efficiency of generating iPS cells by reprogramming human adult fibroblasts and other easily accessible somatic cells remain at 0.01%-0.05% [4]. This low efficiency rate makes it difficult to derive a sufficient amount of autologous cell lines for a patient. Also, generation of human iPS cells is a very slow process [4]. Slow and inefficient generation of iPS cells is limiting iPS cell research and possible clinical application. Therefore, this review focuses on iPS cell generation efficiency.

Aim
To those who are interested in the latest developments concerning iPS cells, this review provides an overview of iPS cell generation and discusses a series of new developments in the research field. More specific, this review focuses on the reason why the generation of iPS cells is so inefficient and how the most recent discoveries can improve it. I will discuss the basic parameters of somatic cell reprogramming; why iPS cell generation is a long and inefficient process; different methods to generate iPS cells; and factors that could improve reprogramming efficiency. These topics will answer the following question: what is the most efficient way to generate iPS cells.

Generation and characterization of iPS cells
Molecular mechanism of reprogramming
In 2000 Takahashi and Yamanaka began testing the idea that factors that maintain pluripotency in ES cells might induce pluripotency in somatic cells. Twenty-four factors were selected as initial candidates for inducing pluripotency, on the basis of their important roles or specific expression in mouse ES cells. In 2006 Takahashi and Yamanaka demonstrated for the first time that it was possible to reprogram mouse somatic cells into pluripotent stem cells by retroviral delivery with a combination of just four genes. The genes they used for reprogramming were Oct4, Sox2, Klf4 and c-Myc. The reprogrammed cells were similar to ES cells in their morphology, expression of important ES cell marker genes, and their ability to form teratomas (tumours comprised of all three germlayers) when injected into mouse testes. After modifying the protocols, the generated iPS cells were also able to competent for adult chimaeric mice and germline cells (detailed characterization of iPS will be explained in ‘‘characterization of iPS cells’’). If reprogramming is done properly, reprogrammed somatic cells exhibit a wide range of characteristics (figure 1 [11].). Including: loss of somatic cell-specific markers, expression of embryonic antigens, telomerase activity, X chromosome reactivation (in female cells), reactivation of endogenous genes essential for pluripotency (e.g. Sox2, Oct4 and Nanog), self-renewal and silencing of exogenous factors used to initiate reprogramming.
All these cellular changes contribute to establishing pluripotency. An important step in reprogramming is reactivating the endogenous genes: Oct4, Sox2 and Nanog in somatic cells. These three genes are able

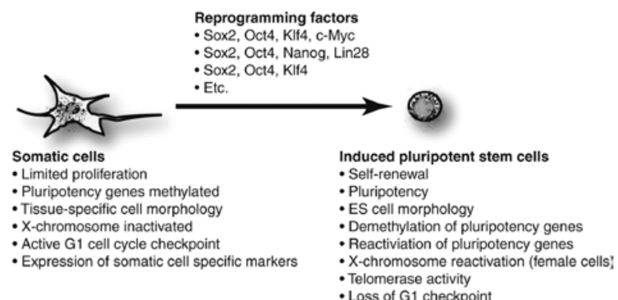

Figure 1. Reprogramming of somatic cells to induced pluripotent stem (iPS) cells. Examples of reprogramming factors are provided along with the characteristics of a typical starting somatic cell and those of an iPS cell [11].

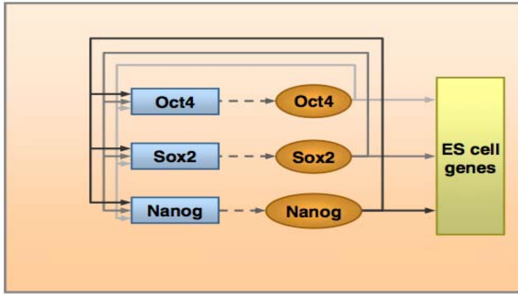

Figure 2. Oct4, Sox2 and Nanog form an interconnected autoregulatory circuit, by targeting both their own promoters and those of each other. In this way, the three key transcription factors are able to maintain their own expression, thereby contributing to the stable maintenance of pluripotency and activate ES cell genes. Proteins are depicted as circles, gene promotors as rectangles [21].

to promote the expression of their own genes and of each other [17,18], suggesting that Oct4, Sox2 and Nanog are able to form an interconnected auto regulatory loop to maintain their own expression (figure 2 [21]).
Auto regulatory circuits are believed to enhance the stability of gene expression and reduce response time to external stimuli [19,20]. These properties may provide valuable mechanisms through which pluripotency can be stably maintained while allowing stem cells to respond to developmental signals [18]. During this state, Oct4, Sox2 and Nanog can target up to several hundreds of other genes that contribute to cell pluripotency (figure 3 [21]).

Takahashi and Yamanaka used the genes Oct4, Sox2, Klf4 and c-Myc for reprogramming of somatic cells. Insertion of these genes into the host genome by retroviruses resulted in pluripotency, because this combination of genes is able to reactivate endogenous Oct4, Sox2 and Nanog. But what is more specifically the function of these genes? It is known that Oct4 acts as a key regulator of stem cell pluripotency [6]. Oct4 is expressed in the inner cell mass of developing mouse and human embryos and in differentiated somatic cells Oct4 is down regulated [6]. Oct4 also plays an essential role in normal mammalian development, because Oct4 knockout embryos fail to develop a pluripotent inner cell mass and die after blastocyst stage [46]. Additionally, pluripotent stem cells need to maintain fairly precise Oct4 protein levels to maintain their pluripotency. Slight changes in protein levels of Oct4 can lead to spontaneous differentiation of ES cells into trophoblast, endoderm or mesoderm lineages [7]. Sox2 is highly expressed in pluripotent lineage of the early embryo and just like Oct4, it plays an important role in maintaining pluripotency [8]. It is reported that Sox2 regulates expression of Oct4 itself. This indicates for a part, the importance of Sox2 in stem cell pluripotency [46]. Moreover, reduced levels of Sox2 in ES cells are associated with a loss of pluripotency and a tendency to differentiate [46]. Klf4 is involved in a wide range of cellular processes, including development,

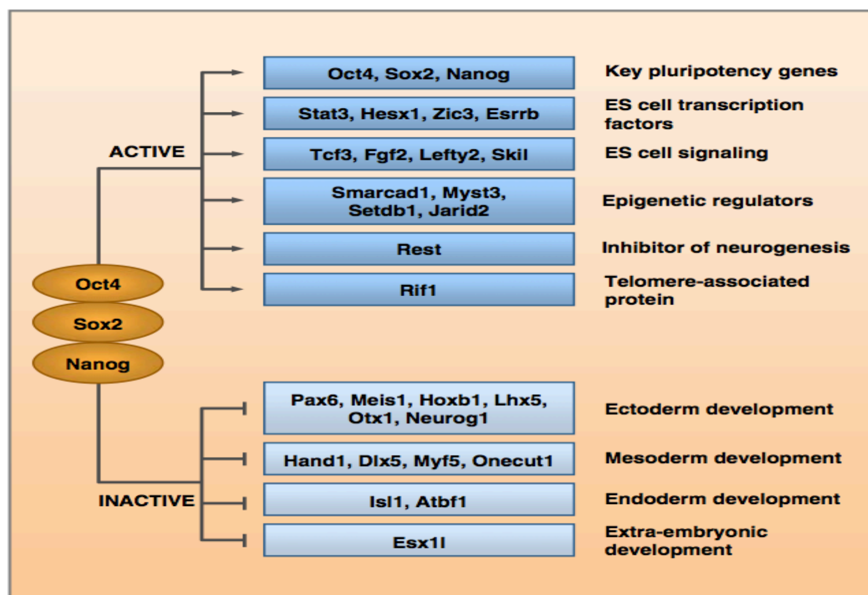

Figure 3. The Oct4, Sox2 and Nanog trio contribute to ES cell pluripotency by repressing genes linked to lineage commitment, and activating genes involved in pluripotency. Active genes include, besides Oct4, Sox2 and Nanog themselves. ES cell-specific transcription factors, components of ES cell signaling cascades and epigenetic enzymes. In contrast, repressed genes comprise regulators of differentiation into the three germ layers and extra-embryonic development. Proteins are depicted as circles, gene promotors as rectangles [21].

proliferation, differentiation and apoptosis [9]. Unlike Oct4 and Sox2, Klf4 has been identified as both an oncogene and a tumor suppressor gene [47]. So Klf4 is particularly involved in cancer formation. c-Myc is an oncogene as well and has important functions in cellular processes such as proliferation, differentiation, and cell growth [10]. In 70% of the human tumors, increased c-Myc activity is observed [10], indicating that c-Myc is one of the most frequently deregulated oncogenes in human cancer formation.

Variation in reprogramming factors

Since the first report of reprogramming, it has become evident that there are also variations possible in the usage of reprogramming factors to generate iPS cells. c-Myc is not strictly required for reprogramming of somatic cells. However, when c-Myc is removed from the protocol, the reprogramming time is increased dramatically and reprogramming efficiency drops significantly [12]. Other research has found that reprogramming of human fibroblasts is also possible by using the genes: Sox2, Oct4, Lin28 and Nanog [13].

Nanog, a recently discovered gene, plays a role in maintaining ES cell self-renewal in the absence of leukemia inhibitor factor (LIF) [16] and plays a critical role in maintaining pluripotency. Additionally, certain reprogramming factors can be replaced by transcription factors belonging to the same family. Sox1 or Sox3 can replace Sox2, L-myc or N-Myc can replace c-Myc, and Klf2 or Klf5 can replace Klf4 [14,15]. The best combination of reprogramming factors would depend on the cell type.

Characterization of iPS cells

There are several tests to check if somatic cells are successfully reprogrammed to iPS cells. Proper reprogrammed iPS cells should be able to differentiate into each of the three germ layers (ectoderm, mesoderm and endoderm) and should be germline-competent. A way to test this, is to check if iPS cells are able to from teratomas. Teratomas are actually tumors that contain derivatives of all three germ layers. iPS cells that are not able to form teratomas consisting of all three germlayers are not proper reprogrammed. These partially reprogrammed iPS cells do exhibit some characteristics of pluripotency, but are stuck in a so-called ''intermediate state'' and fail to become proper iPS cells.

An even more rigorous test to check the quality of reprogramming is by transplanting mouse iPS cells into tetraploid embryos [49]. In this test, the cells of tetraploid blastocysts are capable of contributing to extraembryonic tissues during development. Thus, for embryos to develop into viable adult mice, the inner cell mass must be reconstituted fully from the injected diploid pluripotent cells. It is difficult to generate iPS cells that are fully reprogrammed and checking the reprogramming status of human iPS cells. For ethical reasons, human iPS cells cannot be characterized on chimera formation, like mouse iPS cells.

Aditionally, human ES/iPS cells are not identical to mouse ES/iPS cells in terms of their developmental status. Mouse ES/iPS cells are more closely related to cells of the preimplantation epiblast. Human ES/iPS cells appear to be closely related to cells in the late epiblast. The mouse ES/iPS cells are in a so-called naïve pluripotent state, where as human ES/iPS cells are in a so-called primed pluripotent state [50]. Naïve pluripotent stem cells are able to contribute efficiently to chimeric embryos maintains both X chromosomes in an active state in female cells and are relatively stable in their potential to differentiate into primordial germ cells. Primed pluripotent stem cells, can give rise to differentiated teratomas, but are highly inefficient in repopulating the inner cell mass when injected in blastocysts, they have undergone X-chromosome inactivation and their differentiation potential has to be stabilized by primordial germ cells in vitro [50]. In short, there are several differences between human and mouse iPS/ES cells, despite the ability of both cell types to self-renew and contribute to all three germ layers during teratoma formation.

Generating iPS cells is an inefficient and long lasting process.

Generating iPS cells has been so far an inefficient process. Takahashi and Yamanaka had already observed in 2006 that only very few cells that were expressing all four transduced factors actually became iPS cells [1]. Other studies reported that reprogramming human adult fibroblasts and other easily accessible somatic cells had an efficiency of just 0,01%-0,05% [4]. This efficiency rate is even lower than the efficiency observed in cell reprogramming using SCNT [22]. Low efficiency hinders research on the molecular mechanism of reprogramming and limits the usage of iPS cells for possible clinical purposes. These observed low efficiency ratios are possibly caused by several reasons.

Stochastic process

Like told before in the molecular mechanism of reprogramming, slight changes in protein levels of Oct4 can lead to spontaneous differentiation of ES

cells [7]. This indicates that cells can be very sensitive to certain levels of specific proteins. Additionally, there are more proteins besides Oct4 that can have this kind of impact on ES cells. Now imagine that the generation of iPS cells may require specific, narrow-range expression levels of four transduced factors, and by chance, only small proportion of transfected cells may acquire these precise levels. Inappropriate balance of the four factors would result in improper reprogramming, senescence or apoptosis. This creates conditions that successful generation of iPS cells is quite rare.
The variation in expression of transduced factors depends on the random retroviral integration of these factors into unselected sites. Differences in the amount of copy numbers by viral insertions can also cause variations in expression levels [23]. So far generating iPS cells is just a random process of reprogramming factor insertion;, eventually a few cells will have the right proportion of transduced factors and will be successfully reprogrammed into iPS cells. What the best balance of these factors is to reprogram somatic cells, will be discussed in: ''factors that can improve reprogramming efficiency''

Epigenetic changes required for reprogramming
Even when the four factors are properly expressed for iPS cell generation, reprogramming can still be unsuccessful. An important requirement for correct functioning iPS cells is proper DNA methylation. In ES cells and iPS cells, the promotor regions of many pluripotency-associated genes are hypomethylated. In somatic cells like fibroblasts, the protomotor regions of these genes are heavily methylated [48]. DNA which is heavily methylated cannot be expressed. Therefore, in order to create proper functioning iPS cells, these promotor regions should be demethylated to activate them. The cell itself can only do demethylation of DNA, because the four inserted factors do not have intrinsic DNA demethylation activity. Correct demethylation will take several cell divisions to complete. This may be one reason why iPS cell generation is so slow and inefficient. Further in this review, I will discuss what effects demethylation-promoting agents such as 5-azacytidine, have on iPS cell generation in: ''factors that can improve reprogramming efficiency''.
Not only does the DNA methylation needs to be changed; histone formation needs to be adapted too in order to establish pluripotency. In both ES cells and iPS cells, histone H3 and H4 are hyperacetylated in the promotor regions of pluripotency-associated genes. Hyperacetylation enhances transcription by making RNA polymerases and transcription factors easier to access these promotor regions. In contrast, cells that are differentiated have hypoacetylated H4. Therefore, H4 of these regions should be acetylated during iPS cell generation. The cell itself can only do modifying of histones, because the inserted reprogramming factors do not have intrinsic histone modifying abilities. Histone deacetylase inhibitors, like valproic acid, can enhance modifying of these histones. I will discuss this late in: ''factors that can improve reprogramming efficiency''.
Another important step in reprogramming is silencing of the inserted genes. Transgene expression must be maintained during the first 10 to 14 days [31,32] in order to reactivate endogenous pluripotency genes (Oct4, Sox2 and Nanog). However, to achieve complete reprogramming, transgene expression should be silenced and then taken over by the endogenous genes. If cells fail to achieve such transgene silencing, it could result in partially reprogrammed cells.

Generation efficiency is cell-type dependent.
The efficiency of generating iPS cells could also depend on the type of cell that is used for reprogramming. Reprogramming of ectodermal keratinocytes has shown at least a 100-fold higher efficiency and twofold faster compared to fibroblasts [24]. In this study the same reprogramming factors (Oct4, Sox2, Klf4 and Nanog) and methods were used to induce pluripotency in both keratinocytes and fibroblasts. The expression levels of the four exogenous genes were similar in keratinocytes and fibroblasts. This suggests that differences in retroviral transduction efficiency were not a major parameter, indicating that the iPS cell generation efficiency depends on cell type. Li et al [53] extended this study by showing that mammary gland epithelial cells convert into iPS cells more readily than fibroblasts. An explanation for the differences in efficiency might be that, epithelial cells are more amenable to reprogramming, because unlike fibroblasts, they would not be required to undergo a mesenchymal-to epithelial transition (MET) to give rise to iPS cells (figure 4 [56]). Li et al [53] showed that E-Cadherin knockdown or Snail overexpression, both of which inhibit MET, substantially reduces the formation of iPS cells. A study by Samavarchi-Tehrani et al [54] also showed that MET formation in fibroblasts is a limiting step in the formation of iPS cells. They showed that treatment of reprogramming cultures with recombinant BMP-7 (MET promotor) significantly increased the number and accelerated the speed of iPS cell colony formation, whereas exposure to the BMP antagonist (Noggin) impaired colony formation. More evidence has been shown by Ichida et al [55]. They showed that treatment of fibroblasts with TGF- β inhibitors enchances iPS cell

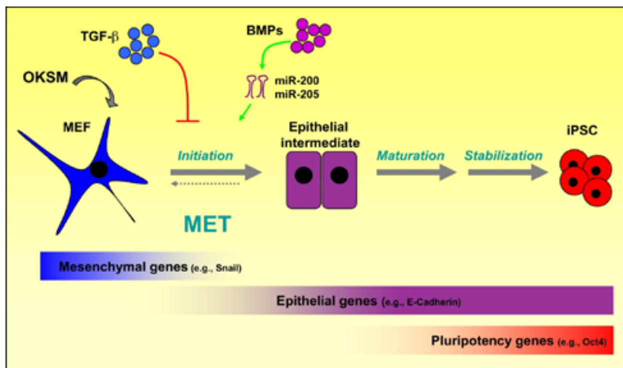

Figure 4. Fibroblast reprogramming into iPS cells entails a Mesenchymal-to-Epithelial transition. TGF-B inhibits MET. BMPs promotes MET [56].

formation. TGF- β is a well-known inducer of EMT, which, in light of current results, is expected to inhibit reprogramming of fibroblasts. These results give strong evidence that fibroblasts have an additional rate-limiting step to form pluripotent stem cells, compared to epidermal cells. Another explanation why some cells are more susceptible to reprogramming is because they possess a relatively more open chromatin organization that facilitates reprogramming.

A recent study by Loh et al [57] showed that peripheral blood (PB) CD34+ cells and peripheral blood mononuclear cells (PBMCs) can also be reprogrammed to iPS cells by infecting the cells with lentiviruses expressing Oct4, Sox2, Klf4 and c-Myc [57,58,59]. The observed reprogramming efficiency in PB CD34+ cells was 0.002% and for PBMCs the efficiency was 0.0008%-0.001%. From a scientific point of view, this achievement may relatively be a small step forward. However, practically and technically, their findings represent a huge and important progression towards clinical application of iPS cells. By being able to generate iPS cells from human PB, patients are not required to undergo an invasive skin biopsy to gather fibroblasts. This skin biopsy is not particularly free of risks, infections and other complications are possible. Instead, Just a small amount of blood (1ml) taken from the patient is enough for generating iPS cells.

Methods to generate iPS cells

The first iPS cell generated by Takahashi and Yamanaka was done by retroviral transduction of reprogramming factors. By using a retrovirus to insert reprogramming factors, cells risk insertional mutagenesis. Incurring mutations can cause cancer or improper functioning of these cells. Also, continued expression or later reactivation of the exogenously supplied reprogramming factors can cause the same serious problems. The efficiency of generating iPS cells by retroviral transduction is about 0.01%-0.05% [4] in human adult fibroblasts. Now 4 years further, scientists have developed new methods to generate iPS cells. These methods have brought a variety of possibilities to induce pluripotency in somatic cells. No longer is reprogramming dependent on retroviral transduction or nucleic acid-based reprogramming. Each of these new methods have positive and negative sides, which I will discuss.

Viral, non-integrating: Adenovirus

A way to reprogram somatic cells without integration of transgenes into the host genome is by using Adenoviruses. This non-integrating method prevents insertional mutagenesis in the host genome. Adenoviral particles containing the reprogramming factors are able to use the host cell's transcriptional and translational machinery to synthesize recombinant protein, without actually incorporating the host genome (figure 5 [51]). An important factor here is time of the viral infection. Somatic cells that are being infected with adenoviruses containing the reprogramming factors should be infected long enough to be able to trigger the endogenous loop of Oct4, Sox2 and Nanog. This is required to maintain active and stable pluripotency [17,18]. If cells aren't infected long enough, this endogenous loop cannot be activated and reprogramming fails.

Stadtfield et al [27] used this method to reprogram mouse fibroblasts and liver cells by using the traditional reprogramming factors Sox2, Oct4, Klf4 and c-Myc. They successfully reprogrammed the somatic cells and reported that no adenoviral sequences were integrated into the host genome. However, the efficiency of deriving iPS cells was extremely low, ranging from less than 0,0001% to 0,001%. This efficiency is remarkably lower than the retroviral integrating method. A reason for this low efficiency is probably that cells did not maintain viral transgene expression long enough. In summary, this method is able to induce pluripotency without viral integration into the host genome, but the remarkably low efficiency so far tells us that more research needs to be done in order to use this method for possible clinical applications in cell-based therapy.

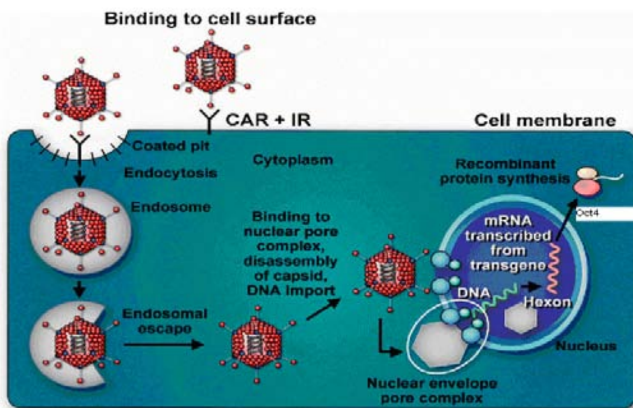

Figure 5. Adenoviral transduction. Recombinant adenovirus (containing the reprogramming factors) enters cells via CAR- and IR-mediated binding. Adenoviral particles are endocytosed, escape from the endosomes, and enter into the nucleus via nuclear envelope pore complex. Genetic material in the adenovirus is not incorporated into the host cell genome, but rather assumes an epichromosomal location, where it can still use the host cell's transcriptional and translational machinery to synthesize recombinant protein, in this case the reprogramming factor Oct4 [51].

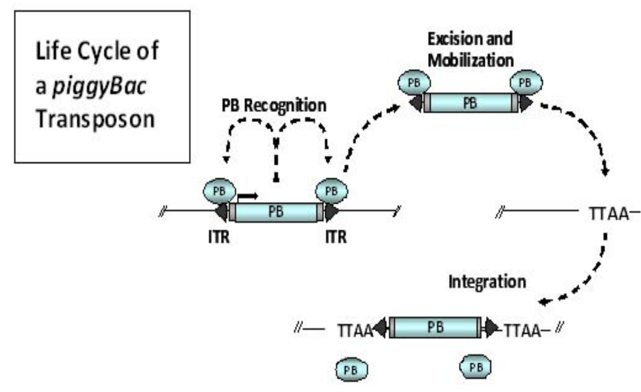

Figure 6. PiggyBac (PB) DNA transposons mobilize via a ''cut-and paste'' mechanism whereby a transposase enzyme, encoded by the transposon itself, excises and re-integrates the transposon at other sites within the genome. The amino acid PB transposase specifically recognizes PB inverted terminal repeats (ITRs) that flank the transposon; it binds to these sequences and catalyzes excision of the transposon. PB then integrates at TTAA sites throughout the genome, in a relatively random fashion [picture taken from transposagenbio.com]

Transgene free, Non-viral: PiggyBac transposon/transposase

PiggyBac is a refined system in which reprogramming factors can be delivered into the host genome and removed without any residual elements. Knut Woltjen et al [25] used this system to generate iPS cells for the first time. They used a polycistronic transgene, which includes all the 4 reprogramming factors Oct4, Sox2, Klf4 and C-myc. This polycistronic transgene is flanked by piggyBac terminal repeats, which allow the transgenes to insert into the host genome (figure 6). The PiggyBac transposon/transposease system does not require previously engineered sites into the starting cell population, because piggyBac transposon elements can integrate into sites already present in the mammalian genome. As soon the cells are in a pluripotent state and the transgenes are no longer required, the integrated piggyBac transposage elements will be expressed. The expressed piggyBac transposase removes the transgenes and also the piggyBac terminal repeats from the genome. By removing itself from the genome, no residual traces of genomic integration are left behind. This way the removed exogenous reprogramming factors cannot continue expressing themself or reactive in a later phase, which could cause serious problems. Reactivation of the transgene oncogen c-Myc can cause development of tumors [13]. Also, incomplete transgene silencing can affect differentiation of iPS cells negatively. Not only can PiggyBac improve the quality of the generated iPS cells, it can also simplify technical aspects and improve the accessibility of generating iPS cells. Specialized biohazard containment facilities or the production of high-titre, limited-lifetime viral stocks are no longer required. And susceptibility to viral infection is no longer a limiting factor for reprogramming cells.

All these positive factors mark an important advance towards achieving clinically acceptable methods of deriving reprogrammed cells. Also, it has been reported that these transposon/transposase-based systems were able to reprogram mouse embryonic fibroblasts at a rate significantly higher than most other protocols [25].

However, although piggyBac is removed from the genome after reaching pluripotency, critical genes required for proper cell functioning can become incorporated before removal.

Transgene free, non-viral: Cre-Loxp

Another method that integrates genetic material into the genome and removes it once pluripotency has been established, is described by Soldner et al [26]. Here they used a Cre-lox recombination protocol for reprogramming fibroblasts from Parkinson's patients. A lentivirus construct was used for inserting the reprogramming factors (Oct4, Sox2 and Klf4) and was flanked by loxP sites (figure 7.). When viral integration and reprogramming occurs, exogenous expression of Cre-recombinase will remove the reprogramming factors from the genome. Cre molecules will bind at both loxP sites flanking the reprogramming factors and will make an excision,

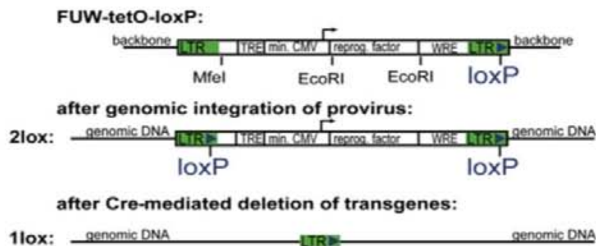

Figure 7. A schematic drawing of the DOX-inducible lentiviral construct FUW-tetO-loxP and the genomic locus after proviral integration (2lox) and Cre-recombinase-mediated excision (1lox). The FUW-TetO-loxP vector contains a tetracycline response element (TRE) located 5' of a minimal CMV promoter and a unique MfeI site used for diagnostic Souther blot digests. The reprogramming factors are flanked by EcoRI restriction sites. The 3' LTR of this lentiviral vector contains a single loxP site, which is duplicated during proviral replication into the 5' LTR. This duplication results in a transgene flanked by two loxP sites after genomic integration of the provirus (2lox). This allows the excision of the transgene in combination with the complete promotor sequences with the Cre-recombinase (1lox). (WRE, woodchuck response element.) [26]

thereby removing the reprogramming factors. However, one loxP site will be left behind in the genome of the reprogrammed cell. So basically, the cells are still at risk now for insertional mutagenesis, but reactivation of exogenous reprogramming factors will not occur. The efficiency observed here reached from 0.005% with three reprogramming factors to 0.01%with four reprogramming factors. Just like the piggyBac method, this method gives us an accessible and simplified way to generate iPS cells. But the fact that there still is a risk for insertional mutagenesis because the loxp sites will be left behind in the genome, makes this method less apropriate for generating iPS cells, compared to the piggyBac method.

Non-viral, non-integrating, non-genetic: direct protein delivery

Another way to avoid the insertion of exogenous genetic modifications into the host genome is by directly delivering the reprogramming proteins into the cells. Peptides that mediate protein transduction, such as HIV transactivator of transcription (HIV-TAT) and poly-arginine are able to transport a desired protein into a cell by conjugating them together. This way, relying on transcription of delivered genes is no longer needed. This method was demonstrated by Kim et al [28] where they fused the traditional reprogramming factors Oct4, Sox2, Klf 4 and C-myc to HIV-TAT and so enabling the factors to penetrate into the somatic cells. Induction of pluripotency went successfully, however the generation was very slow (twice as long as retroviral) and inefficient. 0,001% of the used cells were able to form iPS cells, compared to the retroviral method, which has 0,01% efficiency. This inefficient method has been improved by Zhou et al [29]. They used purified recombinant proteins (Oct4, Sox2, Klf4 and c-Myc) which were fused with their c-termus to a ploy-arginine protein transduction domain. The iPS cells generated from mouse embryonic fibroblasts were able to form chimeric embryos. The efficiency of iPS cell generation by treating cells with Oct4, Sox2, Klf4 and c-Myc and valproic acid (VPA) was 0,006% .
This method is the only method to generate iPS cells without the use of genetic material. By not being dependent on the usage of genetic material, any risks like insertional mutagenesis or reactivation of transgenes, by modifying the cell's genome is avoided. Direct protein delivery also provides a substantially simpler and faster approach then other DNA integrating methods, which require time-consuming sequential selection of the most potentially integration-free iPS cells. However, the low efficiency of 0,006% forms a large barrier in its potential for possible clinical applications.

Non-viral, non-integrating, non-genetic: direct mRNA delivery

The proteins used to generate iPS cells by the direct protein delivery method are produced in bacteria [29]. According to Yakubov et al [52], proper post translation modification of the proteins is better processed inside the somatic cell and not in bacteria. By directly delivering mRNA (encoding the reprogramming factors Oct4, Lin28, Sox2 and Nanog) into the cell, the translated protein will be proper modified. Yakubov et all [52] showed recently that the mRNA transfection resulted in protein expression level in less than eight hours in human fore-skin fibroblasts (hFF). In order to activate the endogenous loop of Oct4, Sox2 and Nanog and establish pluripotency, at least five consecutive transfections were needed. Colonies of iPS cells were observed between 1 and 2 weeks after the last transfection. However, the efficiency of generating iPS cells with this method was very low, only 0,00005% of the cells managed to reach pluripotency. Besides the low efficiency, the iPS cells were not even tested on the capability to form teratomas. This questions whether this method is reliable for generating iPS cells and indicates that improvements are needed.

Non-viral, Non-integrating: Plasmids

Using plasmids that express reprogramming factors is another way to avoid viral integration into the host genome. This is described by Okita et al [30]: They used repeated transfection of two expression plasmids, one containing the complementary cDNAs

of Oct3/4, Sox2 and Klf4 and the other containing c-Myc. This experiment was used on mouse embryonic fibroblasts and resulted in iPS cells, which were able to produce teratomas when transplanted into mice and were also able to contribute to adult chimeras. However, some iPS cells showed signs of plasmid incorporation into the host genome, which could cause insertional mutagenesis. The reprogramming efficiency was just like the use of adenoviruses remarkably low. An efficiency of $2.9x10^{-5}$ was reported, making this method very inefficient. Inducing pluripotency by plasmids requires repeated transfection of these plasmids into cells. This is required for cells to establish the endogenous loop of Oct4, Sox2 and Nanog so they can become pluripotent. This repeated transfection is quite an inaccessible and time-consuming way to generate iPS cells.

Factors that can improve iPS cell generation efficiency.

Reprogramming somatic cells back to pluripotent state is a process of breaking barriers on several levels that involve in proper reprogramming.

1. Reprogramming factors need to be expressed in an appropriate balance (depending on cell-type).
2. DNA of associated pluripotency genes needs to be proper demethylated and acetylated to become properly expressed.

These are the factors that should be intervened in order to improve reprogramming efficiency. Small molecules that are known to remodel the chromatin structure are actively investigated. They could contribute to proper demethylation and acetylation of associated pluripotency genes and so increase reprogramming efficiency.

DNA methyltransferase inhibitor5-aza-cytidine (AZA).

Several studies have noted that some cells may become trapped in a partially reprogrammed state [31,32]. They may inappropriately activate or fail to repress endogenous or exogenous transcription factors, and become ‘trapped’ in differentiated states. Eventually they may fail to reactivate hypermethylated pluripotency genes and so fail to become fully reprogrammed iPS cell. An explanation for this might be that cells induce anti-proliferative genes in response to proliferative stress. Mikkelsen et al [31] have shown that these partially reprogrammed iPS cells can become fully reprogrammed iPS cells by using the small molecule AZA. AZA is able to inhibit DNA methyltransferase and thereby preventing the methylation of promotors, essential for pluripotency like Oct4 [31]. Treating partially reprogrammed cell lines with AZA induced a rapid and stable transition to a fully reprogrammed iPS state. 7.5% of the cells treated with AZA became GFP-positive cells (indicating fully reprogrammed iPS state) compared to 0.25% in untreated cells. After five passages, GFP-positive cells comprised to 77.8% of the treated population, whereas the proportion in untreated remained stably low (0.41%). Clearly, AZA was able to increase the efficiency of generating iPS cells by promoting DNA demethylation.

Histone deacetylase inhibitor (HDAC): ValProic Acid (VPA)

Another chemical inhibitor besides AZA has also shown to have a positive effect on reprogramming efficiency. The Histone deacetylase inhibitor VPA enhanced reprogramming efficiency significantly, according to Huangfu et al [44]. By promoting histone acetylation on pluripotency associated genes, the cell is more susceptible to reprogramming. VPA treatment for a week improved the percentage of Oct4-GFP-positive cells by more than 100-fold when transfected with Oct4, Sox2 and Klf4. Cells that where transfected with Oct4, Sox2, Klf4 and c-Myc only showed a 50-fold increase when treated with VPA. In addition, AZA and VPA treatment also induced Oct4-GFP-positive colonies 2 days earlier than non-treated controls. Therefore, chemical treatment can be used as a strategy to improve both the kinetics and efficiency of reprogramming. Huangfu et al [44] reported that treatment AZA alone was not sufficient to reprogram cells. However, VPA treatment alone did have an effect on the cells. Remarkably, promoting histone acetylation caused a global transcriptional change in these uninfected cells. More specifically, ESC-specific genes were upregulated while MEFs-specific genes were downregulated. Acetylation of the genome induced by VPA and other histone deacetylase (HDAC) inhibitors could allow MEFs to adopt a relaxed chromatin structure that facilitates the binding of ectopically expressed transcription factors or downstream secondary factors.
Therefore, VPA may support a predisposition toward an ESC-like state.
By enhancing the reprogramming efficiency, VPA treatment may facilitate a reduction in the number of reprogramming factors required. Huangfu et all also showed that the addition of VPA enabled human fibroblasts to be reprogrammed with only two factors Oct4 and Sox2 at an efficiency similar three-factor Sox2,Oct4 and Klf4 reprogramming [45]. So Klf4 and

c-Myc are not required for reprogramming in the presence of VPA and so Oct4 and Sox2 are indispensable for the reprogramming of human fibroblasts.

Histone deacetylase inhibitor (HAT): Butyrate

Another investigated small molecule is Butyrate. Butyrate, a fatty acid, greatly enhances the efficiency of induced pluripotent stem cell derivation from human adult or fetal fibroblasts, according to Mali et al [39]. They reported an efficiency increase of 15-51 fold after transient butyrate treatment, using either retroviral or piggyBac transposon vectors expressing 4-5 reprogramming genes. Butyrate is a well know histone deacetylase inhibitor [40]. Butyrate treatment enhanced histone 3 acetylation, promotor DNA demethylation, and the expression of endogenous pluripotency-associated genes. By enhancing demethylation, there is a bigger chance that critical genes required for pluripotency will be re-activated. This would explain the improved reprogramming efficiency when cells are treated with butyrate. The iPS cells generated this way showed normal pluripotency and any possible harmful effects of butyrate are excluded.

Moreover, enhancing chromatin histone acetylation may not be the only mechanism that stimulates reprogramming. It could be possible that butyrate also stimulates reprogramming by modulating activities of non-histone key regulators, regardless it is HAT/HDAC dependent or not.

Stochasticy and reprogramming

In order to reprogram somatic cells back to pluripotent state, the introduction of reprogramming factors is required either retroviral or by other methods. These transgenefactors need to be expressed in a certain pattern to induce pluripotency. An inappropriate balans of the four reprogramming factors would result in improper reprogramming, senescence or apoptosis. Methods to introduce the reprogramming factors into the cell cannot precisely control the expression levels of these reprogramming factors, because insertion of reprogramming factors happens randomly. This stochastic process causes a different expression balance of reprogramming factors in every cell, but only the right balance of reprogramming factor expression can result in pluripotency. This is one of the reasons why reprogramming is an inefficient process, because only few cells will receive the right balance of reprogramming factors.

What exactly the right balance of reprogramming factor expression is unknown. But according to Papapetrou et al [5], stoichiometry of equal parts of all 4 reprogramming factors is highly effective. Most deviations from this stoichiometry have unfavorable effects to the efficiency of reprogramming however, they had seen a striking exception that a 3-fold increase of Oct4 only showed an increase in reprogramming efficiency (~2-fold) in human fetal fibroblasts.

It could be possible that every cell-type has different stoichiometric requirements. Because each cell-type has a unique methylation pattern and integration pattern for gene-delivery methods.

Disturbing the p53 pathway.

P53, a tumor suppressor, plays multiple roles in maintaining genomic stability in somatic cells. Whenever cells are in genotoxic or oncogenic stresses, p53 is activated and can induce cell cycle arrest, apoptosis or senescence. This way, accumulation of genetic mutations is prevented [38].

By silencing p53, Utikal [32] et al reported that somatic cells where able to form iPS cell colonies with up to threefold faster kinetics and a significantly higher efficiency than wildtype cells. Zhao et al reported that the disruption of the p53 pathway by siRNA and UTF1 expression enhances the efficiency of reprogramming by up to 100 fold [33]. Clearly, these results suggest that p53 not only plays a role in preventing accumulation of genetic mutations, but it might also play an important role in pluripotency activity. According to Lin et al [34] p53 is able to directly suppress the expression of several genes, including the pluripotency factor Nanog. As mentioned before, the expression of Nanog is required for maintaining an interconnected auto regulatory loop between Oct4, Sox2 and Nanog. Whenever this interconnected auto regulatory loop is established, genes that are required for pluripotency can be activated. By silencing p53, this auto regulatory loop between Oct4, Sox2 and Nanog can be maintained more stable. This could explain the higher efficiency of reprogramming somatic cells when the p53 pathway is disrupted.

According to Kanatsu-Shinohara et al, germ cells can be spontaneously be reprogrammed to pluripotent stem cells in the absence of p53 [35]. This finding suggests that p53 also plays a role in suppressing dedifferentiation of somatic cells. Reprogramming of somatic cells can be prevented this way. If somatic cells indeed must inactivate the p53 pathway to be successfully reprogrammed into iPS cells, it could explain the very low reprogramming efficiency often observed, because only a low percentage of cells could have spontaneously achieved the inactivation of p53 through insertional mutagenesis or direct gene mutation.

Inactivating p53 might increase efficiency of

generating iPS cells; it however, also might increase oncogenecy. In almost half of all human cancers, p53 is mutated [36]. Therefore, loss of p53 function is a cause for the development of human cancers. P53 is also important in maintaining genomic stability in somatic cells, permanent inactivation of p53 during reprogramming will lead to an instable genome with chromosomal alterations [37]. Taking these notions in consideration, iPS cell lines generated by depletion of p53 would have accumulated DNA damage and thus would not be appropriate for clinical use.

Using Hypoxic mediums to increase efficiency

A different approach to improve reprogramming efficiency was tested by Yoshida et al [41]. They tried to improve reprogramming efficiency of somatic cells by using hypoxic culture conditions. Observations of mammalian embryonic epiblasts that maintain in hypoxic conditions [42], gave rise to the hypothesis that hypoxic conditions might promote the reprogramming process and thus iPS cell generation. The results they found confirmed their expectations. Mouse and human somatic cells reprogramming was significantly increased when the cells were cultivated under 5% O2 (hypoxic) concentrations. More specifically, hypoxic treatment for 5 and 10 days increased the number of GFP-positive colonies with transient transfection of plasmid vectors. Using the piggyBac method combined with hypoxic treatment for 5 and 10 days increased the number of GFP-positive colonies with the piggyBac transposition system by 2.9-fold and 4.0-fold. They have also found that hypoxia showed a significant proliferative effect and increased the expression level of Oct3/4 and Nanog. More remarkable is that they also have found that exposure of mouse embryonic fibroblasts to hypoxic conditions shifted the overall gene expression pattern towards that of ESCs. These results suggest that hypoxic conditions may contribute to the reprogramming process itself, without even using reprogramming factors, because of the gene expression shift towards ESCs. Another factor that would contribute to reprogramming in hypoxic conditions is that the p53 is down regulated when conditioned in hypoxic cultures [43]. As told before the p53 pathway hinders somatic reprogramming. By down regulating this pathway, reprogramming will be more efficient. It should be noted that despite hypoxic conditions promote reprogramming, hypoxia also induces cytotoxicity. Susceptibility to this cytotoxicity depends on cell-type.

Concluding remarks

The main question of this review is: how can we improve the generation efficiency of iPS cells?
To answer this question, there are several other questions each of different perspectives that need to be answered first: 1. What is the best method for generating iPS cells so far (the most efficient and less oncogenic)? 2. Which reprogramming factors are the most suitable for reprogramming and at what balance? 3. Which cells are most suitable for reprogramming? 4. How can we make cells more susceptible for reprogramming? 5. What are the best conditions to generate iPS cells?

What is the best method for generation iPS cells?

Considering table 1, currently the best method to generate iPS cells is the piggyBac transposon method. The piggyBac method provides a relative efficient reprogramming efficiency, combined with a certainty that the generated iPS cells are free from permanent insertions in their genome. The piggyBac method integrates into the host genome and leaves the genome, as soon the transgenes are no longer required to induce pluripotency. This way, the created iPS cells do not have an increased chance of oncogenecy because of insertional mutagenesis or by reactivation of transgenes. Although direct protein delivery is a safer method to generate iPS, because it does not integrate into the host genome at all, the very low reprogramming efficiency makes this method far from usable. Other methods are less efficient or more oncogenic, making these methods inferior to the piggyBac method at the moment.

Which reprogramming factors are the most suitable for reprogramming?

The first reprogramming factors used to reprogram somatic cells into iPS cells were: Oct4, Sox2, Klf4 and c-Myc [3]. Klf4 and c-Myc are well known oncogenes [47,10]. iPS cells generated by inserting these genes permanently (retroviral) into the genome can become oncogenic if they are being overexpressed. By replacing these oncogenes for

Table 1. Summary of methods to generate iPS cells

Strategy	*Transduction method*	*Reprogramming factors used*	*Species*	*Cell type*	*Reprogramning efficiency*	*Safety advantage*	*Reference*
Lenti/retro viral	Integrating	OSKM	Hum	Fibroblasts	+++	+	[4]
Adeno virus	Non-integrating	OSKM	Mus	Fibroblasts	+	+++	[27]
PiggyBac transposon	Transgene free	OSKM	Mus	Embryonic fibroblasts	++	+++	[25]
Cre-Loxp	Transgene free	OSK	Hum	Fibroblasts	++	++	[26]
Direct protein delivery	Non-integrating, Non-genetic	OSKM	Mus	Embryonic fibroblasts	+	++++	[29]
Direct mRNA delivery	Non-integrating, Non-genetic	OSLN	Hum	Fore-skin fibroblasts	+	++++	[52]
Plasmids	Non-integrating	OSKM	Mus	Hepatocytes	+	++	[30]

O = Oct4, S = Sox2, K = Klf4, M = c-Myc, L = Lin28, N = Nanog
Hum = human, Mus = mouse
Reprogramming efficiency: + ≤ 0.001%, ++ 0.005% - 0.01%, +++ = 0.01% - 0.05%.

non-oncogenes Lin28 and Nanog [13], iPS cells have a smaller chance to develop a tumor. This way iPS cells would be more suitable for possible clinical applications.
It seemed that a 3-fold increase of the reprogramming factor Oct4 only has a positive effect on reprogramming efficiency of human fetal fibroblasts [5]. However, the possible methods used today to generate iPS cells are not able to control the ratio of inserting reprogramming factors into the cell's genome.

Which cells are most suitable for reprogramming?
Reprogramming of ectodermal keratinocytes has shown at least a 100-fold higher efficiency and was a twofold faster compared to fibroblasts [24]. When we want to reprogram somatic cells for a possible clinical purpose in the future, the choice of which cells we want to use depends on accessibility and efficiency of reprogramming. Ectodermal keratinocytes are less accessible than fibroblasts, however recent findings have shown that peripheral blood can also be reprogrammed back to pluripotent state [57]. Peripheral blood is a lot more accessible than fibroblasts, and collecting peropheral blood is less invasive, but the reprogramming efficiency of these cells is also less.

How can we make cells more susceptible for reprogramming?
VPA treatment combined with AZA in mouse embryonic fibroblasts resulted in improvement of Oct4-GFP-positive cells by more than a 100-fold when transfected with Oct4, Sox2 and Klf4 [44]. VPA and AZA treatment also induced Oct4-GFP-positive colonies 2 days earlier. By treating human fibroblasts with VPA, only 2 reprogramming factors (Oct4 and Sox2) are needed for reprogramming [45]. By removing oncogenes Klf4 and c-Myc, the created iPS cells will have a smaller to chance to develop tumors.
Butyrate treatment in human fetal and adult fibroblasts resulted in an efficiency increase of 15-51 [39]. Both VPA, AZA and butyrate have shown to increase reprogramming efficiency (table 2), however so far combined treatment of VPA and AZA have shown to highest increase in efficiency (100 fold).
Inactivating p53 would also result in a higher reprogramming efficiency, however it also creates an unstable genome. iPS cells generated this way have an increased oncogency and so are not suitable for clinical application.

Table 2. Summary of factors to improve iPS cells generation efficiency

Strategy	*Function*	*Reprogramming factors used*	*Method used*	*Species*	*Cell type*	*Effect*	*Reference*
AZA	DNA methyltransferase inhibitor5	OSKM	Lentiviral	Mus	Embryonic fibroblasts	7.25% increase in GFP-positive cells	[31]
VPA	Histone deacetylase inhibitor	OSKM	Retroviral	Mus	Embryonic fibroblasts	Improved reprogramming efficiency by 100-fold	[44]
VPA	Histone deacetylase inhibitor	OSK	Retroviral	Mus	Embryonic fibroblasts	Improved reprogramming efficiency by 50-fold	[44]
Butyrate	Histone deacetylase inhibitor	OSKM	Retroviral / PiggyBac	Hum	Adult and fetal fibroblasts	Improved reprogramming efficiency by 15-51-fold	[39]
P53	Inactivating p53	OSKM	Retroviral	Hum	Foreskin fibroblasts	Improved reprogramming efficiency by 100-fold	[33]
O = Oct4, S = Sox2, K = Klf4 M = c-Myc Mus = Mouse Hum = Human							

What is the best condition to generate iPS cells? So far, only 2 conditions have been tested for its effect on generation efficiency: regular conditions and hypoxic conditions. Generating iPS cells in hypoxic conditions when using the piggyBac method has showed a 2.9-4.0-fold increase of GFP-positive colonies compared to regular conditions. Also hypoxic conditions resulted in a shift of gene-expression pattern of somatic cells towards that of ESCs [41]. So using hypoxic conditions could facilitate reprogramming.

A strong demand for methods that can improve reprogramming efficiency and reduce oncogenecy of iPS cells, have driven researchers to develop new creative ways to develop or enhance iPS cell generation. 4 years since the discovery of iPS cells, progress has been made on different perspectives. PiggyBac has shown to be a reliable method to generate iPS cells with efficiency comparable to the retroviral method, but with reduced oncogenecy of iPS cells. The small molecules like VPA en AZA have shown to increase reprogramming efficiency, by adapting the chromatin structure. Ectodermal keratinocytes reprogramming showed a 100-fold higher efficiency in reprogramming and researchers now managed to reprogram peripheral blood into iPS cells. These discoveries enriched the iPS cell technology with new insights, that could combined together optimize reprogramming efficiency and minimize oncogenecy of iPS cells, bringing this promising technology another step closer towards clinical application.

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