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Mechanism of Induction: Induced Pluripotent Stem Cells (iPSCs)

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Abstract

Induced Pluripotent Stem Cells (iPSCs) are self renewable and can differentiate to different types of adult cells, which has shown great promises in the field of regenerative medicine. iPSCs are reprogrammed from human somatic cells through ectopic expression of various transcription factors viz. Oct4, Sox2, Klf4, and c-Myc (OSKM). This novel technology enables derivation of patient specific cells, which possess a potential cure for many diseases. During the last decade, significant progresses have been achieved in enhancing the reprogramming efficiency, safety of iPSCs derivation, development of different delivery techniques by various research groups. Nevertheless, it is important to resolve and define the mechanism underlying the pluripotent stem cells. Major bottleneck which arises during iPSCs generation is the availability of source material (cells/tissues), difficulty to deliver transcription factors with no aberrant genetic modifications and limited reprogramming efficiency. Reprogramming may be achieved by employing different cocktails with number of different transcription factors, application of miRNA and some small molecules such as (Valproic acid, CHIR99021, Sodium butyrate, Vitamin C, Parnate etc). Similarly, various starting source materials have been demonstrated for iPSC based therapies including fibroblasts, cord blood, peripheral blood, keratinocytes, urine, etc., with their specific uses and limitations. Moreover, with the advent of many new reprogramming techniques, various direct delivery methods have been introduced such as using synthetic mRNA expressing pluripotent gene network has been shown to be an appropriate technique to deliver transcription factors and a dozen of small molecules which can replace transcription factors or enhance reprogramming efficiency. This article addresses the iPSCs technology mechanisms, progresses and current perspectives in the field.

Keywords: iPSCs, Embryonic Stem Cells, Somatic Cell Reprogramming, Epigenetic reprogramming, pluripotency

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1. Introduction

Stem cell poses great promises in the field of regenerative medicine and is immensely useful for personalized medicine, curing a broad range of diseases. A large number of reports have established both scope and potential of Embryonic Stem Cells (ESCs) which are pluripotent cells, which can give rise to all three germ layers: ectoderm, mesoderm, and endoderm and can be used to regenerate the damaged tissues or specific cell lineage. However, due to many ethical issues such as mortality of an embryo that is considered to be a living thing, ESCs could not be a suitable source for tissue regeneration. Fortunately, programming somatic cell to develop self renewable pluripotent cells opened new paths and hence, iPSCs came into existence.

iPSCs are mature cells that have been genetically reprogrammed to an ESC-like state through ectopic expression of transcriptional factor genes critical for maintaining the properties of ESCs. This reversion of adult cells to a state resembling ESCs offers unprecedented potential to generate patient and disease specific tissues for targeted disease research, as well as drug screening methods for the development of new therapies. iPSCs are self renewable also. The possession of these two properties shows their importance in the field of regenerative medicine. iPSCs can be reprogrammed from somatic cells by implying different ways like Somatic Cell Nuclear Transfer (SCNT), Cell fusion, transduction of OSKM transcription factors and small molecules.

In 1938, Spemann first proposed nuclear transfer but experiment was unsuccessful [1] then, in 1952, Briggs and King studied the transplantation of the nuclei from blastula cells in to an enucleated egg in the frog and concluded that the cell differentiating genes are not silenced or lost [2] which gave rise to the idea of '*principle of reversion of somatic cells by reprogramming*' by specific factors present in oocytes. However, they were not able to reproduce the process with other specialized cells. In 1962, Gurdon studied the similar experiment on tadpole, but unfortunately did not succeed when used similar technique on other species [3]. The major milestone was achieved with demonstration of cloning technique for Dolly sheep which was developed with

an enucleated oocyte [4]. In 1997, Tada reprogrammed somatic cells to generate pluripotent hybrids by fusing the mice ESCs with female thymocytes and renewed tetraploid cell [5]. All these studies were valuable and helpful to reprogram the somatic cells. Then, another breakthrough came in 2006, when first iPSCs were derived from mouse fibroblasts with the use of retroviral transduction [6]. Subsequently, in 2007, Takahashi carried out similar experiment on human fibroblasts. They isolated human dermal fibroblasts from the facial dermis of Caucasian woman and transduced them with retroviral vector containing Oct4, Sox2, Klf4, and c-Myc genes [7]. Since then, newer approaches have been developed to reprogram a somatic cell to an iPSC, which includes exploring the four different factors, cell sources, delivery techniques for transcription factors, the cocktails to be used for reprogramming and the characterization of formed iPSCs, in order to achieve better reprogramming in terms of cost, ease and efficiency of reprogramming.

First aspect involves the source material, fibroblasts have been commonly used but using them for iPSCs generation is laborious and also results in lower efficiency. Overcoming the issue of sophisticated processing, Peripheral Blood (PB) and Cord Blood (CB) were reported as a source for iPSCs formation. CB possesses lower risks of mutation as compared to PB derived cells [8]. Many other sources have been reported in consequent years, likewise mesenchymal tissues result in lower efficiency, dental pulp results in better efficiency and urine was found to be more efficient and accessible source [9, 10]. Different sources have different limitations in their own processing and use. In 2012, exfoliated renal epithelial urine cells were reported to efficiently generate iPSCs and moreover, urine cells also possess advantages of accessibility and availability [11].

The second factor- the method of delivery of transcription factors plays an important role in iPSCs generation as different methods with different efficiencies have different requirements of lab setup and manual expertise. Retroviral delivery systems were the first used for introduction of transgenes into the host cell, but they lack the ability to introduce at specific locus region. This may cause insertional mutagenesis or teratoma formation. Lentiviral systems overcome the tumour formation and hence,

are used in reprogramming. For lowering the risk of chromosomal aberration in host cells, non integrating methods are used. Adenovirus, Sendai virus, polycistronic mini circle vectors and autonomous episomes have been studied but are reported to be less efficient. Dox lentiviral system and single polycistronic vectors reduce the chances of tumour formation [12]. Direct delivery methods have been defined to reduce the risk of genetic modification. Poly arginine protein tagged transduction domain has been used to generate iPSCs through direct delivery method, but this is a time taking process [13]. mRNA encoding transcription factors introduced into host cells are reported to have higher efficiency of reprogramming [14].

The third factor is the cocktail to be used for reprogramming. Yamanaka used 4 reprogramming factors- Oct4, Sox2, Klf4, and c-Myc, which came to be known as Yamanaka's cocktail. But, there have been studies where researchers have not used one or more factors from Yamanaka's cocktail or have used some other reprogramming factors/proteins and have achieved generation of iPSCs. Many combinations of transcription factors have been used experimentally for increasing efficiency and avoiding risk. Reprogramming with only O, OSK, OS etc, but their efficiency is very low. Recently, small molecules were used in reprogramming somatic cells to iPSCs. Some small molecules (VPA, CHIR, RG-108, Pannate, Sodium butyrate, etc) have been found to be able to replace some of the 4 basic transcription factors and also, to enhance the efficiency of reprogramming [15]. Small molecules are mostly involved in epigenetic regulation via inhibiting nucleosome remodelling complexes. Transcription factors and miRNA play an important role in reprogramming via epigenetic regulation of nucleosome remodelling complexes. Reprogramming mechanism involves expression of genes responsible for maintenance of pluripotent state and inhibition of genes involved in the differentiation of cells but still, the exact mechanism behind iPSCs generation is still unclear. Hence, it is required to study the role of pluripotency regulating factors.

The fourth factor- characterization of cells also plays a crucial role for the determination of whether the formation of iPSCs has occurred or not. Various markers have been used for the selection and

characterization of iPSCs colonies. The use of positive markers (alkaline phosphatase, SSEA-4, Tra1-81, Oct4 etc) in combination with negative marker CD44 can be used to select specific iPSCs colonies [16]. Generation of patient specific iPSCs revolutionized the field of clinical therapy. Applications of iPSCs technology are widely reported in modelling of many diseases, drug discovery and regenerative medicine but, there are some risks that are associated with the use of iPSCs. More independent knowledge about underlying mechanisms and various reprogramming networks importantly of OSKM would definitely help to enhance the therapeutic potential and technique. To understand the reprogramming mechanisms independent and integrated roles of Oct4, Sox2, Klf4, c-Myc and other binding proteins need to be studied. In this review, we will discuss the mechanisms behind iPSCs generation, how reprogramming factors are involved in pluripotency. This review also summarizes the different sources used for iPSCs generation and various techniques used for delivering reprogramming transcription factors.

2. Reprogramming at First Glance

At first glance, reprogramming means reversion of an adult cell to its initial state as ESCs which have the unique property of differentiation to all three germ layers- endoderm, ectoderm and mesoderm cell lineage. Cell Reprogramming in simpler way is to regulate an adult cell to repress the genes responsible for differentiation and activate the genes responsible for reprogramming. iPSCs generation protocol mainly involves - cellular sources, delivery methods, culturing media, and characterization methods. First, a suitable somatic cell source is required which is easily accessible, available and convenient in processing. Second, delivery method needs to be safe and reproducible, which can introduce the reprogramming factors efficiently without causing any genomic modification. The next consideration is the reprogramming factors to be induced. Reprogramming factors are needed to be safe, efficient, which can regulate the somatic cell epigenetically and more importantly avoid any risk of tumours. Another important aspect is the

characterization method which can analyze partially and fully reprogrammed iPSCs.

Mechanism for reprogramming refers to the remodelling of epigenetic markers, such as DNA methylation, acetylation etc during mammalian development. Reprogramming of somatic cells involves epigenetic changes in the pattern of histone modifications and DNA methylations. Since exact mechanism and the role of transcription factor is unclear, some valuable insights into molecular mechanisms are required, by which, cellular fate and pluripotency would boost iPSCs cell production

efficiency. Reprogramming efficiency refers to the ratio of number of somatic cells receiving the reprogramming cocktail to the number of resulting iPSCs colonies.

From the first published information about the fibroblast reprogramming via a retroviral method with OSKM factors for the iPSCs generation, a number of other cell sources, for example, cord blood, peripheral blood, urine, keratinocytes etc, delivery methods other than viral methods, non viral methods and direct delivery methods have been developed.

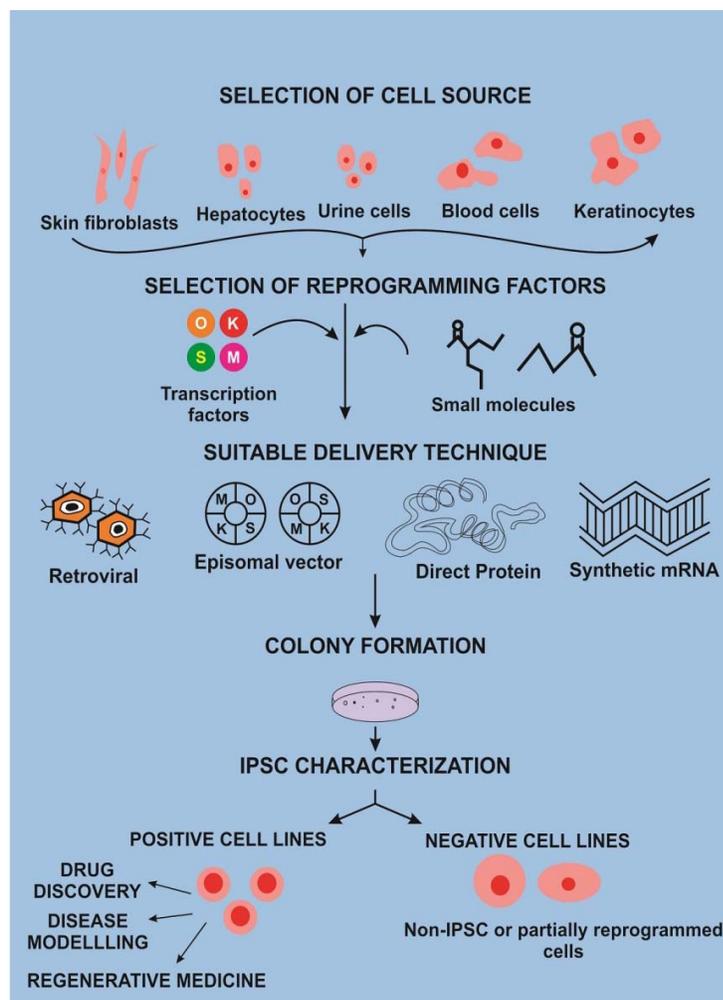


Figure 1. Reprogramming of somatic cells includes several steps- first selection of suitable cell sources and then selection of specific combination of pluripotent cocktail (OSKML) or small molecules with compatible delivery method (integrating or non-integrating). Induction of reprogramming factors suppresses the genes responsible for cell differentiation and activates pluripotency maintaining genes. Resulting iPSCs colonies are characterized by positive and negative cell makers and iPSCs are used for further application.

Advancements in consequent years, for characterization of iPSCs using the combination of positive and negative markers, which assess the reprogrammed iPSCs accurately at specific stages, have been defined. Alternatives of the transcription factors, other reprogramming cocktail combinations and small molecules have been found to reprogram efficiently with no tumourigenic risk.

3. Different Approaches to iPSCs Formation

There are various ways for achieving pluripotency: Somatic Cell Nuclear Transfer (SCNT), Cell fusion, Transduction of OSKM transcription factors (direct programming) and reprogramming with small molecules.

3.1. Nuclear Transfer

In SCNT technique, the nucleus of a somatic cell is introduced into an egg cell, by first removing the egg cell nucleus and then stimulated by a shock which forms blastocyst. Nuclear reprogramming was first achieved by SCNT in which cell nucleus from adult cells were transferred to an unfertilized enucleate oocyte. In 1996, first nuclear transfer cloned sheep was developed by transferring blastomere nucleus from sheep embryo to enucleated unfertilized egg [17]. Subsequent study reported production of successful animal clones using different somatic cell sources, including hepatocytes, leukocytes, neuronal cells, lymphocytes and germ cells [18]. In SCNT process, the embryo can be developed by electrical or chemical stimulation activating egg and ESCs can be removed from developing blastocyst. However, in 2007, only 2 out of 304 oocytes were reported to be successfully created as ES cells by the use of nuclear transfer, suggesting low induction efficiency.

3.2. Cell Fusion

Cell fusion is a natural process which is involved in the development and tissue regeneration. It is a process in which somatic cells fuse with pluripotent

cells to reprogram the somatic cell's genome. Fortunately, heterokaryon formed by cell fusion activates the silent differentiated genes. ESC nuclei are used to reprogram the somatic cells. Overexpression of embryonic transcription factors deletes the somatic cell properties and restores the pluripotency [19, 20]. Cell extracts from the undifferentiated human embryonic carcinoma NCCIT cells or mouse ESCs were reported to acquire pluripotency in the kidney epithelial cells HEK293 with increased expression levels of markers that signify non-differentiated state, including Oct4 [21], and the reprogrammed cells could be induced to differentiate into neurogenic, adipogenic, osteogenic and endothelial lineages. However, they could not be differentiated into three germ layers *in vivo*. Thus, this reprogramming method may be incomplete.

3.3. Transcription Factors

Direct introduction of reprogramming transcription factors to the somatic cells of interest is used to generate iPSCs. Yamanaka and the group generated iPSCs from mouse fibroblasts by inducing 24 transcription factors using the retroviral mediated delivery method. Subsequent studies showed that only four transcription factors Oct4, Sox2, Klf4 and c-Myc were together sufficient to regulate the reprogramming [6].

Direct induction of reprogramming factors has been reported to establish the adult cell as ESC-like cells. However, this reprogramming method is associated with certain issues such as lower reprogramming efficiency and teratoma formation. In the last decade, various research groups have worked on overcoming these issues. The researchers have used different combinations of transcription factors for reprogramming somatic cells like OSNL, OSML, OSK etc.

3.4. Small Molecules

Induction of the Yamanaka factors has been used for reprogramming somatic cells by various researchers, but mostly resulted in lower reprogramming efficiency and found to be associated with certain issues which eventually led to the

exploration of the small molecules which can mimic or replace the reprogramming factors. Small molecules mostly work by inhibiting the expression of genes involved in the cell differentiation and development.

Various small molecules defined were valproic acid, CHIR99021, sodium butyrate, vitamin C, parnate, 5-Azacytidine, RG108 etc, which can replace the factors from the core pluripotent network and importantly increase the efficiency of reprogramming.

4. iPSCs Sources

Generating the iPSCs involves an important aspect to consider, which is selection of a cell source. Desirable sources are required to be accessible, non-invasive and importantly, which do not cause the risk of immune rejection due to incompatibility during transplantation. Many cell types have been used for reprogramming into iPSCs. Yamanka used mouse fibroblasts as a source for iPSCs generation, after which various cell sources have been used. Among different sources, dermal fibroblasts are the most commonly used source because of their accessibility, but there is a problem associated as well. Skin biopsy for using dermal fibroblast is a laborious process and cell culturing is required prior the cells are used as a cell source which is time taking and UV exposure during skin biopsy might create chromosomal aberration [22].

Table 1. Different cell source types and reprogramming factors used for iPSCs generation (O- Oct4; S- Sox2; K- Klf4; M- c-Myc; N- Nanog; L- Lin28)

Cell types	Reprogramming factors	Reprogramming efficiency (approximate)	Reference
Fibroblasts	OSKM	0.02%	[7]
	OSLN	0.02%	[29]
Keratinocytes	OSKM	ND	[23]
Cord blood endothelial cells	OSLN	< 0.01%	[8]
Cord blood stem cells	OSKM	0.01%	[27]
Neural stem cells	O	< 0.004%	[30]
Melanocytes	OSKM	0.19%	[31]
Amniotic cells	OSKM	0.05-1.5%	[32]
Adipose derived stem cells	OSKM	0.50%	[33]
Hepatocytes	OSKM	0.10%	[34]
Circulating T cells	OSKM	0.10%	[35]
Astrocytes	OSKM	> 0.02%	[36]
Peripheral blood	OSKM	0.01%	[37]
Kidney mesangial cells	OSKM	ND	[38]
Urine cells	OS	4%	[11]

Some other sources have been used to derive iPSCs which have been mentioned in the Table 1. Mesenchymal stem cells of human hair follicles have

been reported to be used for iPSCs generation. The process of reprogramming involves lentivirus mediated transduction using OSKM factors. Colonies

observed resulted in very low efficiency of 0.001% which may be the reflection of low expression of endogenous Sox2 and Nanog genes in these cells [9].

Both fibroblast and mesenchymal cells take longer time to grow colonies and mesenchymal cells result in lower reprogramming efficiency. In 2008, Aasen demonstrated the use of keratinocytes with 2 times faster ability of colony formation than human fibroblasts [23]. Human immature dental pulp stem cells are undifferentiated cells hence, easily convertible to iPSCs than human fibroblast with better efficiency. Dental stem cells can be isolated from the pulp of exfoliated primary teeth stem cells from human exfoliated deciduous teeth (SHED), apical papilla, and tooth germs [10]. Comparatively, blood seems to be more appropriate source for iPSCs generation that does not require prior culturing. Laboratory venipuncture is an easier process than skin biopsy. Also, blood can be made available from donors or from blood banks [24]. Importantly, blood cells are used to generate iPSCs by the retrovirus mediated introduction of OSKM for reprogramming mouse B-lymphocytes and T- lymphocytes [25, 26]. Simultaneously, iPSCs from healthy donors and cord blood has also been reported. However, using mobilized peripheral blood for iPSCs generation is invasive and has potential risk for blood donor [27]. On the other hand, CB has youngest somatic cells and possesses lesser risk of somatic mutation [8]. iPSCs generated from newborn cord blood and adult peripheral blood using EBNA1/OriP plasmid takes lesser time of 14 days [28].

Recently, exfoliated renal epithelial cells present in urine were used for iPSCs formation. iPSCs derivation from urine is more efficient with about 4% more efficiency than the retrovirus mediated introduction of exogenous factors. Exfoliated renal epithelial cells are advantageous as they require lesser time of about 2 weeks for reprogramming and are easily available, accessible source which can be obtained from persons of any age [11]. iPSCs generated from the urine cells are widely used in various applications, likewise used in bone regeneration successfully. The choice of cell types is an important aspect which requires considering cell availability, accessibility, reprogramming efficiency with quality of resulting iPSCs colonies.

5. iPSCs Generation Mechanism

The mechanisms behind reprogramming of somatic cells are based on the core pluripotency transcription factors network, which involves the epigenetic regulation of the genes involved in the normal cell development. Reprogramming factors, miRNA, small molecules regulate the genes epigenetically for iPSCs generation. Epigenetic regulation includes DNA methylation, histone demethylation and chromatin remodelling. Determination of the epigenetic status in iPSCs would offer the meaningful approach to assess the completion of the reprogramming and the epigenetic modifications, which have the essential role in controlling the activity of genes and regulatory elements in the genome. One aspect of the epigenetic modifications is DNA methylation, which plays a crucial role in gene regulation. Promoters of pluripotent gene markers are demethylated in ESCs.

5.1. Transcription Factors Regulating Reprogramming

Core pluripotency network regulates via repression of genes associated with differentiation and reset of gene expression to an ESC-like profile and consequently achieve full reprogramming. Main factors responsible for maintenance of pluripotency of both hESC and mESC cells are: Oct4, Sox2, Klf4, c-Myc, and Nanog.

5.1.1. Interactions of Oct4, Sox2 & Nanog

Regulation of pluripotency is hypothesized to be mainly done by three core transcription factors, namely Oct4, Sox2, and Nanog. Most important factor Oct4, which is from a POU family of transcription factors, is mainly responsible for maintaining the pluripotency. It represses the expression of genes which are involved in the differentiation [39]. Oct4 locus sequencing revealed the extensive demethylation, an epigenetic state equivalent to human ESCs. Oct4 in cooperation to other genes like Sox2 is involved in somatic cell reprogramming. Sox2 is Sex determining region-Y box-2 related protein which is activated in response of Leukemia Inhibitory factor (LIF), which regulates the

JAK-STAT signalling pathway. Downstream expression of signalling pathway results in Sox2 activation [40]. Nanog arises through screening for transcription factors which maintains ESC in the presence of LIF [41]. These factors activate their own as well as other promoters' genes and form a network that is responsible for the regulation of pluripotency gene expression [42] and also, these factors are able to initiate cascades of many other genes which leads to suppression in development and hence differentiation [43]. The tryptophan repeat motif in Nanog is important for its interaction with tyrosine of triple repeat motif (SXT/SY) of Sox2. If the tyrosine of this motif in Sox2 gets mutated to alanine, the interaction between Sox2 and Nanog gets hampered and hence the ability of ES cell differentiation rescue gets reduced [44]. Johansson et al conducted in situ proximity ligation assay and showed that Oct4, Sox2 both individually interact with Nucleophosmin (Npm1) and translationally controlled tumour protein (tpt1) which have roles in maintaining ESCs phenotype, whereas Nanog only interacts with Npm1 [45]. Oct4, Sox2 and Nanog are important for the maintenance of Inner Cell Mass (ICM) and are also required for the self renewal of pluripotent cells [46]. Many genes are co-occupied by these three factors [47]. A complex called Stem Cell Coactivator Complex (SCC), which is a trimeric XPC- nucleotide excision repair complex, is important for the activation of Nanog by Oct4 and Sox2. The depletion of this complex results in decreased reprogramming of fibroblasts to iPSCs and compromised pluripotency in ESCs [48].

5.1.2. c-Myc and Klf4 Complementarity

Another important factor in reprogramming is c-Myc, which is an oncogene and acts as a catalyst in the reprogramming, increasing the efficiency of iPSCs formation [49]. The mutual co-operation of c-Myc & Klf4 is an essential thing in terms of the successful reprogramming. c-Myc increases the proliferation rate which is beneficial, but over-expression leads to an increased level of p53 protein, whereas Klf4 increases the levels of p21 protein that leads to decrease in proliferation rate and also, it decreases p53 levels which reduces the risk of apoptosis. The roles of these transcription factors are opposite to each other which

lead to the formation of an equilibrium that is required for the attainment of pluripotency [50].

In an experiment, it was found out that although Klf4 & c-Myc primarily mediate the repression of Thy1 which is required for the accomplishment of reprogramming and activation of pluripotency genes, timely coordination of Oct4 & Sox2 is also requisite for the efficient colony formation at the appropriate time in the later stages of reprogramming [51].

5.2. Epigenetic Regulation

During iPSCs formation, initial ectopic expression of reprogramming factors initiates the reprogramming process by epigenetically regulating the genes involved in the differentiation. iPSCs are required to be in ESCs like state with dispersed heterochromatin and abundant euchromatin modifications [52]. While reprogramming, pluripotency genes are associated with the recruitment of nucleosome remodelling complexes such as nucleosome remodelling and deacetylation complex (NuRD) and polycomb-group proteins (Polycomb). The combined action of the deacetylase activity of NuRD and the methyltransferase activity of Polycomb protein represses gene expression which provides the underlying mechanism for lineage commitment of pluripotent stem cells.

After reprogramming factors induction, histone modifications are an early event associated with initiation of reprogramming. The NuRD complex with its histone deacetylase activity is involved in chromatin remodelling. NuRD has been reported to repress the pluripotency factors and block the somatic cell reprogramming [53]. The NuRD complex consists of seven proteins, including histone deacetylase HDAC1, HDAC2 and the methyl-CpG-binding domain protein (MBD3). Histone deacetylases enzymes (HDAC1, HDAC2) regulate reprogramming negatively by inhibiting the histone acetylation, which is required to open a state of chromatin remodelling complexes [54]. MBD3 contains a histone deacetylase activity. MBD3 depletion is reported to improve reprogramming efficiency by facilitating the derivation of pluripotent cells that are capable of forming viable chimera mouse which suggests, MBD3 is an important

epigenetic regulator that restricts the expression of the pluripotency genes and down regulation of MBD3 may be a powerful means to improve reprogramming efficiency. However, in 2014, DOS Santos disagreed with the inhibitory role of MBD3. They reported that MBD3 silencing does not completely result in the reduction of reprogramming efficiency and plays an important role in the initiation of reprogramming of neural stem cells and dispensable in later stages. Further, understanding of MBD3/NuRD functioning during various stages of reprogramming is essential to define more elaborated mechanism for iPSCs generation [55].

In addition NuRD-mediated histone deacetylation also facilitates the recruitment of Polycomb Repressive Complex 2 (PRC2) to repress the gene expression [53]. Polycomb mediated gene silencing relies mostly on the regulation of chromatin structure by post-translational modifications of histones. Polycomb includes Polycomb Repressive Complexes PRC1 and PRC2. In ESCs, PRC2 represses developmental genes involved in cellular differentiation and development [56]. Deletion of PRC2 or its core components results in defects in ESCs differentiation in mouse. PRC2 occupancy and enzymatic activity at target genes in ESCs are coordinated by a JmjC-domain protein (Jarid 2) enriched in pluripotent cells. Jarid2 contains a DNA-binding ARID domain and is associated with DNA-binding proteins and is required for recruitment and efficient binding of PRC2 to the target cells [57]. PRC2 is composed of Histone-lysine N-methyltransferase- EZH2, which has catalytic subunit. The SET domain of EZH2 harbors histone lysine methyltransferase activity which is responsible for the formation of di- and tri-methylated lysine residue 27 within histone H3 (H3K27me_{2/3}). It suggests that there is interplay between PRC2 and Jarid 2 for fine-tunes deposition of the H3K27me₃ mark. Deposition of histone trimethylation characterizes the rearrangement of heterochromatin which indicates the derivation of iPSCs by inhibiting the MEK and glycogen synthase kinase 3 (GSK3) pathways [52].

H3K27me₂ may further demethylase Ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) which consequently regulates the efficient induction of pluripotency through the regulation of WNT signalling pathway. UTX

facilitates the formation of iPSCs using its histone demethylase activity by directly partnering with OSK reprogramming factors [58]. The depletion of UTX leads to change in the dynamics of H3K27me₃ repressive chromatin demethylation in somatic cells undergoing the re-establishment of pluripotency. H3K27me₃ may further lead to the recruitment of the PRC1 complex which consists of ubiquitin ligases Ring1A & Ring1B and monoubiquitinates Lys 119 of histone H2A (H2AK119ub) [59]. PRC1 ubiquitinates histone H2A, thereby represses the expression of the target genes. Some PRC1 complexes interact with nucleosomes and compact chromatin regardless of the presence of histone tails. Polycomb-mediated histone H3 lysine-27 trimethylation (H3K27me₃) has been proposed as marker for iPSCs.

Recently, Ten-Eleven Translocation methylcytosine dioxygenase 1 (TET1) proteins have been shown to regulate reprogramming epigenetically by inhibiting the DNA methyltransferase enzyme during cell cycle progression [60]. TET1 proteins are a class of enzymes involved in DNA demethylation, which catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (HMC) for its enrichment [61]. TET1 protein mediated oxidative demethylation epigenetic modification promotes gene activation, which appears to be functionally required for reprogramming [62]. TET1 mediated Oct4 demethylation and reactivation is essential for mesenchymal to epithelial transition responsible for reprogramming. Moreover, Tet1 can replace Oct4 and initiate somatic cell reprogramming in association with other reprogramming factors Sox2, Klf4, and c-Myc [63]. High expression of TET1 epigenetically regulates the reestablishment of ESCs like cells by LIF signalling which is known to promote self-renewal and pluripotency by opposing MAPK/ERK-mediated differentiation, whereas, the depletion of TET1 affects the stability of STAT3 to bind with its targets on chromatin and so, impairs the LIF-dependent Stat3-mediated gene activation. This will decrease the reprogramming efficiency [64, 65]. Another pathway found to be involved in reprogramming is TLR-3 receptor pathway. In 2012, Lee demonstrated important role of the viral delivery vector itself in promoting nuclear reprogramming. So far viral particles are reported to deliver the desired reprogramming molecules in any cells. But here it

was defined that the viral particles may elicit TLR-3 receptor pathway mediated innate immunity which may induce efficient nuclear reprogramming. This finding opened an interesting line of work where efficient reprogramming might be possible without even the need of essential reprogramming factors OSKM [66].

5.3. miRNA Regulation

MicroRNAs (miRNAs) are small non coding RNA molecules that bind to 3' untranslated region of target molecules and regulate gene expression post transcriptionally. Induction of pluripotent core network (OSKM) works by activating miRNAs which down regulate their target genes or by directly repressing the miRNAs which create barrier for reprogramming. Initially, c-Myc suppresses the miR-29 expression through binding to the promoter region and Oct4-Sox2 heterodimer activates the expression of clusters of the miR-200 family. miR-29a indirectly induces p53 protein levels by post-transcriptionally inhibiting CDC42 and p85 (the regulatory subunit of PI3 kinase) [67]. Human CDC42 protein plays an important role in the regulation of different signalling pathways controlling diverse cellular functions such as cell morphology, endocytosis, migration, and progression of the cell cycle. Furthermore, miR-29a can impair re-establishment of somatic cells by downstream targeting TET1 and thymine DNA glycosylase (TDG), hence miR-29a acts as a barrier for reprogramming of somatic cells [68].

Oct4 and Sox2 activated miR-200 expression significantly lowers the expression of zinc finger E-box binding homeobox-2 (ZEB2) through targeting its 3' untranslated region and directly targeting Cadherin11 and Neuropilin1 (proteins responsible for cell survival and growth). MiR-200/ZEB2 pathway helps fibroblasts to edge the mesenchymal to epithelial transition (MET) barrier [69, 70]. MET state is required for iPSCs generation as it suppresses the epithelial to mesenchymal transition (EMT) state signalling pathway responsible for cell differentiation and development [71].

Next step towards pluripotency involves repression of the miR-29 mediated upregulation of transformation-related protein 53 (p53). miR-34 is

downstream target of p53 and contributes significantly to p53-mediated cell cycle arrest and apoptosis. miR-34 family negatively regulates reprogramming through the repression of pluripotency genes such as LIN28a, Nanog, Sox2, N-Myc, and c-Myc. In murine, it has been found that miR-34 deficiency improves kinetics of reprogramming and the efficiency about 4 fold [72].

Another important miRNA molecule, miR302 known to reset the differentiated gene expression by binding to gene transcript of lysine specific histone demethylases protein (AOF1/2) and methyl CpG binding protein (MECP1/2) [73]. Silencing of MECP1/2 enhances the demethylation effect of AOF1/2. In addition, AOF2 silencing destabilizes DNA cytosine-5 methyltransferase (DNMT1) activity and prevents replication dependent DNA demethylation and further enhances global demethylation during somatic cell reprogramming. miR302 increases reprogramming by accelerating G1 to S phase transformation. It silences both cyclin-E CDK2 and cyclin D- CDK4/6 cell cycle pathway during G1 to S phase translation preventing iPSCs tumour formation [74]. With ongoing advances, several miRNAs have been shown to increase iPSCs reprogramming efficiency synergistically with combinations of the reprogramming factors (OSKM). In addition, miRNAs can be powerful tools for mediating iPSCs reprogramming without the need of pluripotency factors including the OSKM factors.

5.4. Small Molecules

Recently, small molecules and chemical compounds have been reported to improve the reprogramming efficiency. Small molecules inhibit the Histone deacetylase (HDAC) and DNA methylation transferase enzyme and hence, carry out epigenetic regulation. HDAC inhibitors reduce the cell differentiation capability and enhance the self renewable properties of ESCs [54, 75]. Histone acetylation open up the chromatin structure by binding to lysine residue of histone and inhibition of DNA methylation transferase enzyme facilitates demethylation of the somatic cells and activates the pluripotency genes [76].

Various small molecules found to improve the reprogramming and target the signalling pathways have been listed (Table 2). Valproic acid (VPA), histone deacetylase (HDAC) inhibitor, in conjugation with Oct4 and Sox2 alone used to successfully reprogram neonatal foreskin fibroblasts. Importantly, VPA can replace the proto oncogene c-Myc and overcome the risk of tumour formation [77]. Moreover, VPA in conjugation with Vitamin C produces about 3 times more colonies as compared to colonies generated with only VPA [78]. Vitamin C improves the reprogramming efficiency by inducing the DNA methylation, which indicates vitamins and other related proteins could be involved in reprogramming of somatic cells.

The combination of glycogen synthase kinase-3 inhibitor - CHIR and histone demethylase Inhibitor - Parnate with only Oct4 and Klf4 was found to reprogram neonatal epidermal keratinocytes with 0.02% reprogramming efficiency [79]. Parnate

inhibits the AOF1/2 protein and is considered to be an epigenetic modifier [80]. Another histone deacetylase inhibitor is sodium butyrate. It is thought to be involved in reprogramming by modulating flexibility of chromatin structure and reprogramming cell growth in presence of c-Myc in mouse fibroblast [81]. These molecules can be used in combination with or by replacing the transcription factors, to enhance the efficiency of reprogramming. Small molecules possess other advantages as they can pass through the cell membrane and can be removed after initiation of programming during reprogramming hence, minimizes the risk of mutation or any other side effects [15].

Despite the success of reprogramming of somatic cells small molecules substitution of reprogramming factors is associated with lower efficiency of induction. Finding more chemical alternatives for core pluripotency networks would enable derivation of safe iPSCs for clinical applications.

Table 2. Various small molecules in reprogramming the somatic cells and their specific roles in enhancing the reprogramming efficiency have been defined

Compound	Mechanism of action	Reference
Valproic acid	Inhibits of histone deactylase	[77]
CHiR99021	Inhibits of glycogen synthase kinase 3	[32]
Sodium butyrate	Inhibits of histone deacetylase	[81]
Vitamin C	Lowers reactive oxygen species	[78]
Parnate	Inhibits of histone demethylase	[79]
5-Azacytidine	Inhibits of DNA methyltransferase	[82]
RG108	Blocks DNA methyltransferase	[83]

6. Delivery Techniques

During reprogramming, mechanistically important consideration is the method for delivery of reprogramming factors into the cell source. For a different delivery method, a specific cell source attains different results and reprogramming efficiencies. Till date, after Yamanaka and the group first used retroviral vector for iPSCs study, a number of different approaches have been devised to introduce reprogramming factors into somatic cells.

Delivery techniques can be broadly categorized into two classes: integrating vectors and non integrating methods.

Integrating vector method includes retroviral and lentiviral vectors which integrate the host cell genome. Retroviral stably integrates with the transcription factors cocktail in the host genome. This method results in partial or incomplete reprogramming due to silencing of transcription factors at the end stage. Lower expression of transgenes in somatic cells arises due to activation of

histone methyltransferases [84]. Delivery of regulators in non dividing cells or slow dividing cells is resistant to transduction in a retroviral mediated method which results in random or improper integration of genes at different position [85], which may cause chromosomal aberration or teratoma formation. Efficiency of retroviral transduction varies from 0.1 to 1 %. The possible reason for the low efficiency of reprogramming is low infection efficiency of cells by viruses. The development of “secondary reprogramming systems” for the activation of reprogramming factors homogeneously has resulted in increase in the efficiency in 1-5 percent which indicates viral infection contributes to reprogramming efficiency. A second possible cause for the same might be the activation of endogenous genes by insertional mutagenesis. In addition to the expression of the four reprogramming factors, stochastic epigenetic events are equally important which affects the efficiency of reprogramming, for example, the down-regulation of cell cycle inhibitors p16/p19 inhibitors is crucial for the generation of pluripotent immortal cell lines [86, 87]. Hence, work is required to find better strategies with lesser risk and better efficiencies.

Lentiviral systems where transgene is less effective compensate the issues associated with the use of retroviral vectors and are used for a wide range of somatic cell reprogramming. Its expression is controlled by using doxycycline drug which specifically allows selection of reprogrammed cell lines [88]. Indisputable advantages of the application of lentiviral vector are: high-efficiency, long-term stable expression of a transgene, low immunogenicity and it does not express its own viral genes in host cells, however, limited insertion size, difficulties in the storage & quality control and a short half-life remain their serious drawbacks.

Integrating free technique is a second class of delivery techniques. Non integrating vector systems includes adeno virus, sendai vectors, polycistronic mini circle vectors, self replicating episomes and direct delivery methods. These methods reduce the risk of teratoma formation and are able to produce safe iPSC lines for further clinical applications. Reprogramming efficiency by plasmid transfection approach was reported to be 0.001 % and with adenoviral vector induction, efficiency was 0.0006 %

in contribution to multiple tissues in chimeric mice, which compared to reprogramming efficiency by retroviral and lentiviral approach was unfortunately very low. A possible reason behind the lower efficiency is less expression of transcription factors and partial epigenetic remodelling.

By using DOX-inducible lentiviral vector with loxP site patient specific iPSCs have been generated. loxP incorporated sites were excised from the host genome by Cre recombinase transient expression. LoxP site is inserted into the 3' LTR regions of lentiviral vectors. Removal of promoter and transgene in lentiviral vector reduces the risk of oncogenic transformation [89]. Single polycistronic DOX inducible lentiviral vector encoding for factors separated by three different two amino acid peptides was used to generate iPSCs. Combining reprogramming factors in the single polycistronic vector by inserting self cleaving short peptide or IRES sequence between two open reading frames decreases the risk of insertion mutagenesis [12]. A piggybac transposon system has been reported for iPSCs generation in human and mouse fibroblast. A piggybac transposon system by transient expression of transposase enzyme, inserts the reprogramming factors to the host [90]. PiggyBac transposition system exhibits many advantages including easy plasmid preparation and range of somatic cell types that can be reprogrammed which make it useful in iPSCs derivation. However, some issues like transposition into unknown sites in the genome of generated iPSCs and sometimes gene duplication are behind not so frequent use of this method.

Direct delivery methods avoid risk of genetic modification which are associated with viral methods and DNA transfections. Rather than relying on the delivery of vectors containing transcription factors, direct transfer of the reprogramming proteins to somatic cell seems to be convenient way. For direct delivery of reprogramming protein, there is a need of cell membrane penetrating mediator for protein transduction to the cell. Polyarginine conjugated protein and HIV transactivator of transcription has been demonstrated to transfer the reprogramming protein into the cell. Poly arginine tagged protein VPA and histone deacetylase inhibitor with repeated incubation to mouse fibroblast generates iPSCs [13]. However, certain issues associated with direct

delivery method are that it is a more time taking process (took 30 to 36 days), has extremely low reprogramming efficiency and recombinant protein generation/purification is another challenge.

More efficient way is RNA modification encoding for reprogramming. Synthetic RNA expressing pluripotent network with repeated transfections produces iPSCs. Modified mRNA bypasses any innate immune response to foreign DNA elements. Compared with Yamanka's method with RNA encoding OSKM, there was 36 fold increase in reprogramming efficiency. Addition of LIN28 to four

factors cocktail (OSKML) and subjection of cells to low O₂ culture conditions reprogram with higher efficiency of 4.4% [14]. It indicates that due to presence of O₂ an efficiency of 20% would be evaluated comparably less. Modified synthetic RNA has been described to be a safe and efficient way of transcription factor induction for iPSCs generation. Nonetheless, there are some drawbacks to mRNA-based reprogramming too. The expression of reprogramming factors is robust only for about 24 hours after mRNA is transfected.

Table 3. Different delivery methods for transfer of different combinations of transcription factors to cell sources with different reprogramming efficiency (O- Oct4; S- Sox2; K- Klf4; M- c-Myc)

		Reprogra m m g f a c t o r s	Cell sources t y p e	Reprogramming e f f i c i e n c y (A p p r o x i m a t e)	Pros	Cons	Reference
Integrating m e t h o d	Retroviral T r a n s d u c t i o n	OSKM	Mouse f i b r o b l a s t s	0.001-1%	Optimal efficiency, r e p r o d u c i b l e	Multiple i n t e g r a t i o n, r i s k o f g e n e t i c a b e r r a t i o n	[6]
	Lentiviral T r a n s d u c t i o n	OSKM	Human f i b r o b l a s t s	0.1-1%	Reasonable e f f i c i e n c y, c o m p a t i b l e w i t h n o n - d i v i d i n g c e l l	Multiple i n t e g r a t i o n, g e n e t i c a b e r r a t i o n	[29]
	Inducible l e n t i v i r a l	OSKM	Human f i b r o b l a s t s	0.1-2%	Appropriate e f f i c i e n c y, c o n t r o l l e d f a c t o r e x p r e s s i o n	Multiple i n t e g r a t i o n s i t e s, n e e d o f t r a n s a c t i v a t o r	[91]
Non I n t e g r a t i n g m e t h o d	Adenoviral t r a n s d u c t i o n	OSKM	Mouse f i b r o b l a s t s	0.001%	Low frequency of i n t e g r a t i o n	Lower r e p r o g r a m m i n g e f f i c i e n c y	[92]
	Sendai virus	OSKM	Human f i b r o b l a s t s	0.10%	Eliminates risk of g e n e t i c a b e r r a t i o n	Chances of i n t e g r a t e d v e c t o r f r a g m e n t s	[93]
	Plasmid DNA t r a n s f e r	OSK	Fibroblasts	0.001%	No insertion in g e n o m e, t a k e s l e s s t i m e t o r e p r o g r a m	Lower r e p r o g r a m m i n g e f f i c i e n c y	[94]
	PiggyBAC	OSKM	Fibroblasts	0.01%	Accurate deletion, N o n - i n t e g r a t i n g	Time taking p r o c e s s, L e s s e f f i c i e n t	[90]
	Protein m e d i a t e d (P o l y a r g i n e t a g g e d)	OSKM	Neonatal f i b r o b l a s t s	0.001%	No risk of genetic m o d i f i c a t i o n	Slow, L o w e r r e p r o g r a m m i n g e f f i c i e n c y	[95]
	Lox P l e n t i v i r a l	OSKM	Fibroblasts	0.1-1%	No genome insertion, a p p r o p r i a t e e f f i c i e n c y	Optimal r e p r o g r a m m i n g e f f i c i e n c y	[96]
	RNA m o d i f i e d s y n t h e t i c m R N A	OSKM	Human f i b r o b l a s t s	4.40%	Lox P sites remain i n t a c t	Various times t r a n s f e c t i o n s r e q u i r e d	[14]

It takes about two weeks of expression of factors to induce pluripotency in human cells and the time involved in performing the repeated transfections required for the generation of iPSCs. These iPSCs derivation techniques can be combined with advances in high-throughput propagation and quality of pluripotent clones, and large-scale autologous iPSCs lines can be generated for therapeutic uses.

7. iPSCs Colonies Characterization

During reprogramming somatic cells, it is very important to characterize cell lines and examine colonies for the fully reprogrammed iPSCs formation. iPSCs are morphologically examined to be round shape, having large nucleolus and scant cytoplasm as shown in figure 2. These cells are morphologically similar to ESCs [97]. Reprogrammed colonies are sharp edged, flat, tightly packed and mitotically very active due to their self renewable property. Human pluripotent stem cells also express the cell surfaces proteins such as alkaline phosphatase, SSEA-4, transcription factors Oct4, Nanog, Sox2, Tra1-81 etc which are used to characterize iPSCs [98]. In humans, different types of membrane enzymes are present but for pluripotency, specifically placental enzymes are used such as alkaline phosphatase which elicits the conformation changes of calorimetric reagents resulting in their precipitation. However, alkaline phosphatase is toxic to the cells and once cells are stained, they change cell morphology and cannot be propagated again. Overcoming the issue, live alkaline phosphatase stain have been developed which

specifically stains the pluripotent stem cell while preserving the cell integrity [99]. In addition, iPSCs colonies are selected by positive markers mainly Oct4, Sox2 and SSEA-4 and colonies are further analyzed using negative surface markers to identify non-pluripotent cells or partially reprogrammed cells by analyzing the expression of CD44 and SSEA-1 markers [100]. To characterize iPSCs, morphology and cell surface markers are commonly used to verify or isolate iPSC clones. However, it is not definitive since different subtypes of cells have been found to exhibit distinct DNA methylation patterns such as the subtypes of male and female iPSCs, or fetal and adult somatic cells [101]. For example, many cell lines derived from females still have one X chromosome inactivated, which is different from the ESCs where both Xs are actively transcribed. This suggests that ensuring the correct epigenetic features such as DNA methylation and other epigenetic regulations could be the key to define the iPSCs.

Using RT-PCR and immunofluorescence techniques, most groups studied the established iPSCs by expressing positive markers. Combination of positive and negative cell lineage selection can be used to determine specific reprogrammed colonies. *In vitro* iPSCs colonies can be analyzed by culturing colonies in suspension and determining embryoid formation by immunocytochemistry methods for pluripotent markers. *In vivo* iPSCs colonies are selected by teratoma formation through immunohistochemistry method for differentiation markers and used to assess the developmental potential of iPSCs [102].

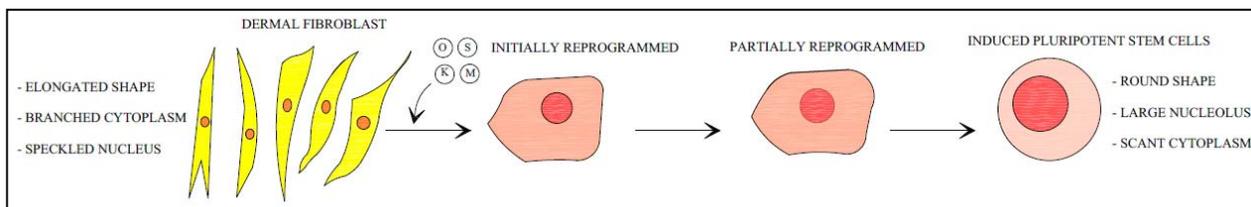


Figure 2. Morphological transformation of dermal fibroblast cells into iPSCs. Dermal fibroblasts have an elongated shaped, branched cytoplasm and speckled nucleus. Introduction of reprogramming factors is followed by the changes in the morphology of the fibroblasts during the generation of iPSCs which are round shaped, and possess large nucleolus and scant cytoplasm.

Recently, Fluorescence Activated Cell Sorting (FACS) has been used for the selection of iPSC lines

by the isolation of single cells expressing the cell surface marker signature CD13 negative, SSEA4

positive, and Tra-1-60 (positive) on day 7–10 after infection has been reported [103]. This technique is used for the isolation of fully reprogrammed iPSCs. It depletes both, the fibroblasts used as source and the fibroblasts which have been partially reprogrammed. This helps to reduce the time and reagents required for the generation of iPSCs lines without using different cocktails of small molecules. FACS derived iPSCs lines express common markers of pluripotency and possess spontaneous differentiation potential *in vitro* and *in vivo*. Hence, an important concern is the maintenance of undifferentiated state of iPSCs. The appropriate maintenance of cultures of stem cells depends on the play between the process of self-renewal and differentiation into different cell types. The optimization of the methods for maintenance is important for culture homogeneity, number of cells produced and the potential of differentiation. The maintenance of stem cell cultures requires regular replacement of the medium which greatly reduces but does not eliminate the process of differentiation. Studies have proved that the levels of growth factors are important for the maintenance of stem cells, for example, Fibroblast growth factor 2 (FGF2 or basic FGF) is used for maintenance of stem cells [104].

Conclusion

Breakthrough derivation of iPSCs overcomes ethical issues of mortality of embryos to be used for research or regenerative medicine. iPSCs technology provides a unique and promising future of regenerative and personalized medicine. Indeed, iPSCs have already been successfully used in many diseases. But there are some complications and risks which cannot be ignored. Major challenge is defining ideal source and transformation technique which would be inexpensive, use less man power, has less laborious work and take lesser time to derive iPSCs.

iPSCs have been generated from direct reprogramming with a pluripotent core network, which results in an epigenetic change in the cell lineage in terms of demethylation of nucleosome remodelling complexes and miRNA interfered repression of genes responsible for differentiation. Efficiency can be enhanced by replacing the transcription factors with small molecules.

Considering cell sources, peripheral blood cells with some other sources have shown better efficiency to generate iPSCs.

To reduce the risk of tumour formation, non-integrating delivery methods are used for iPSCs derivation. Among these methods, synthetic RNA has shown better efficiency as compared to Yamanaka iPSCs generation efficiency. For analyzing the partially reprogrammed or fully reprogrammed cell lines, characterization by combinatorial methods with both positive markers and negative marker selection is done and for characterization of iPSCs correct epigenetic features and regulation may be used as the key biomarkers. An essential consideration is to maintain the iPSCs culture in undifferentiated state which can be done by using growth factors (e.g. Fibroblast growth factor-2), however, a range of new methods need to be defined.

The application of small molecules in the generation of iPSCs requires detailed knowledge of reprogramming technique. Despite the advantages, other issues need to be resolved such as chromosomal aberration, immune rejection, higher efficiency, and teratoma formation. So, further study needs to address important issues and iPSCs mechanism, for fulfilling the promise of regenerative medicine. In future, iPSCs could be more efficiently generated using purified reprogramming proteins. High-efficiency and wide availability of recombinant protein production would contribute to broader and more economical application of this reprogramming method.

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Conflict of interest

Authors have no conflict of interest regarding the publication of the paper.

References

- [1] Speman H. Embryonic development and induction. New Haven : *Yale University press* 1938.
- [2] Briggs R, King TJ. Transplantation of living nuclei from blastula cells into enucleated frogs eggs. *Proc Natl Acad Sci USA* 1952; 38:455.
- [3] Gurdon JB. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *Journal of embryology and experimental morphology* 1962; 10: 622–640.
- [4] Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; 385: 810-813.
- [5] Tada M, Tada T, Lefebvre L, Barton SC and Surani MA. Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO J.* 1997; 16: 6510-6520.
- [6] Takahashi K and Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; 126 (4): 663–76.
- [7] Takahashi K, Okita K, Nakagawa M and Yamanaka S. Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc.* 2007; 2(12): 3081-9.
- [8] Haase A, Olmer R, Schwanke K, Wunderlich S, Merkert S, Hess C, Zweigerdt R, Gruh I, Meyer J, Wagner S, Maier LS, Han DW, Glage S, Miller K, Fischer P, Schöler HR, Martin U. Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell* 2009; 5(4): 434-41.
- [9] Wang Y, Liu J, Tan X, Li G, Gao Y, Liu X, Zhang L, Li Y. Induced pluripotent stem cells from human hair follicle mesenchymal stem cells. *Stem Cell Rev.* 2013; 9(4): 451-60.
- [10] Beltrão-Braga PC, Pignatari GC, Maiorka PC, Oliveira NA, Lizier NF, Wenceslau CV, Miglino MA, Muotri AR, Kerkis I. Feeder-free derivation of induced pluripotent stem cells from human immature dental pulp stem cells. *Cell Transplant.* 2011; 20(11-12): 1707-1719.
- [11] Zhou T, Benda C, Dunzinger S, Huang Y, Ho JC, Yang J, Wang Y, Zhang Y, Zhuang Q, Li Y, Bao X, Tse HF, Grillari J, Grillari-Voglauer R, Pei D, Esteban MA. Generation of human induced pluripotent stem cells from urine samples. *Nature Protocols* 2012; 7(12): 2080–2089.
- [12] Carey BW, Markoulaki S, Hanna J, Saha K, Gao Q, Mitalipova M, Jaenisch R. Reprogramming of murine and human somatic cells using a single polycistronic vector. *Proc Natl Acad Sci U S A.* 2009; 106(1): 157–162.
- [13] Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 2009; 4(5): 381-4.
- [14] Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ. Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Cell Stem Cell* 2010; 7(5): 618-630.
- [15] Federation AJ, Bradner JE and Meissner A. The use of small molecules in somatic-cell reprogramming. *Trends Cell Bio.* 2014; 24(3): 179-87.
- [16] Quintanilla RH, Asprer JST, Vaz C, Tanavde V and Lakshmi U. CD44 Is a Negative Cell Surface Marker for Pluripotent Stem Cell Identification during Human Fibroblast Reprogramming. *PLoS ONE.* 2014; 9(1): e85419.
- [17] Campbell KH, McWhir J, Ritchie WA and Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 1996; 380(6569): 64-6.
- [18] Hochedlinger K and Jaenisch R. Nuclear Transplantation, Embryonic Stem Cells, and the Potential for Cell Therapy. *N Engl J Med.* 2003; 349: 275-286.
- [19] Tada M, Takahama Y, Abe K, Nakatsuji N and Tada T. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr Biol.* 2001; 11(19): 1553-8.
- [20] Pralong D, Trounson AO and Verma PJ. Cell fusion for reprogramming pluripotency: toward elimination of the pluripotent genome. *Stem Cell Rev.* 2006; 2(4): 331-40.
- [21] Taranger CK, Noer A, Sørensen AL, Håkelién AM, Boquest AC, and Collas P. Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells. *Molecular Biology of the Cell* 2005; 16(12): 5719–5735.
- [22] Ikehata H, Masuda T, Sakata H and Ono T. Analysis of mutation spectra in UVB-exposed mouse skin epidermis and dermis: frequent occurrence of C->T transition at methylated CpG-associated dipyrimidine sites. *Environ Mol Mutagen* 2003; 41(4): 280-92.
- [23] Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bili J, Pekarik V, Tiscornia G, Edel M, Boué S, Belmonte JCI. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol.* 2008; 26(11): 1276-1284.
- [24] Zhao J, Jiang WJ, Sun C, Hou CZ, Yang XM and Gao JG. Induced pluripotent stem cells: origins, applications, and future perspectives. *J Zhejiang Univ Sci B.* 2013; 14(12): 1059-69.
- [25] Hanna J, Markoulaki S, Schorderet P, Carey BW, Beard C, Wernig M, Creighton MP, Steine EJ, Cassady JP, Foreman R, Lengner CJ, Dausman JA, Jaenisch R. Direct Reprogramming of Terminally Differentiated

- Mature B Lymphocytes to Pluripotency. *Cell* 2008; 133(2): 250–264.
- [26] Staerk J, Dawlaty MM, Gao Q, Maetzel D, Hanna J, Sommer CA, Mostoslavsky G, Jaenisch R. Reprogramming of peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell*. 2010; 7(1): 20–24.
- [27] Ye Z, Zhan H, Mali P, Dowey S, Williams DM, Jang YY, Dang CV, Spivak JL, Moliterno AR, Cheng L. Human-induced pluripotent stem cells from blood cells of healthy donors and patients with acquired blood disorders. *Blood* 2009; 114(27): 5473–5480.
- [28] Chou BK, Mali P, Huang X, Ye Z, Dowey SN, Resar LMS, Zou C, Zhang YA, Tong J, Linzhao Cheng L. Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Research* 2011; 21(3): 518–529.
- [29] Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; 318(5858): 1917–20.
- [30] Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS. Generation of Human Induced Pluripotent Stem Cells by Direct Delivery of Reprogramming Proteins. *Cell Stem Cell*. 2009; 4(6): 472–476.
- [31] Utikal J, Maherali N, Kulalert W and Hochedlinger K. Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *J Cell Sci*. 2009; 122(19): 3502–3510.
- [32] Li W, Zhou H, Abujarour R, Zhu S, Young Joo J, Lin T, Hao E, Schöler HR, Hayek A, Ding S. Generation of human-induced pluripotent stem cells in the absence of exogenous Sox2. *Stem Cells*. 2009; 27(12): 992–3000.
- [33] Sugii S, Kida Y, Kawamura T, Suzuki J, Vassena R, Yin YQ, Lutz MK, Berggren WT, Belmonte JCI, Evans RM. Human and mouse adipose-derived cells support feeder-independent induction of pluripotent stem cells. *Proc Natl Acad Sci U S A*. 2010; 107(8): 3558–63.
- [34] Liu H, Ye Z, Kim YH, Sharkis S and Jang YY. Generation of Endoderm derived Human iPS cells from Primary Hepatocytes. *Hepatology*. 2010; 1(5): 810–1819.
- [35] Seki T, Yuasa S, Oda M, Egashira T, Yae K, Kusumoto D, Nakata H, Tohyama S, Hashimoto H, Kodaira M, Okada Y, Seimiya H, Fusaki N, Hasegawa M, Fukuda K. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell*. 2010; 7(1): 11–4.
- [36] Ruiz S, Brennand K, Panopoulos AD, Herrerias A, Gage FH, Izpisua-Belmonte JC. High-Efficient Generation of Induced Pluripotent Stem Cells from Human Astrocytes. *PLoS ONE* 2010; 5(12): e15526.
- [37] Kunisato A, Wakatsuki M, Shinba H, Ota T, Ishida I, Nagao K. Direct generation of induced pluripotent stem cells from human nonmobilized blood. *Stem Cells Dev*. 2011; 20(1): 159–68.
- [38] Song B, Niclis JV, Alikhan MA, Sakkal S, Sylvain A, Kerr PG, Laslett AL, Bernard CA, Ricardo SD. Generation of Induced Pluripotent Stem Cells from Human Kidney Mesangial Cells. *J Am Soc Nephrol*. 2011; 22(7): 1213–1220.
- [39] Pardo M, Lang B, Yu L, Prosser H, Bradley A, Babu MM, Choudhary J. An Expanded Oct4 Interaction Network: Implications for Stem Cell Biology, Development, and Disease. *Cell Stem Cell*. 2010; 6(4): 382–395.
- [40] Niwa H, Ogawa K, Shimosato D and Adachi K. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature*. 2009; 460(7251): 118–22.
- [41] Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 2003; 113 (5): 643–55.
- [42] Rosenfeld N, Elowitz MB and Alon U. Negative autoregulation speeds the response times of transcription networks. *J Mol Biol*. 2002; 323(5): 785–93.
- [43] Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Nakatsuji N, Tada T. Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol Cell Biol*. 2005; 25(6): 2475–85.
- [44] Gagliardi A, Mullin NP, Tan ZY, Colby D, Kousa AI and Halbritter F. A direct physical interaction between Nanog and Sox2 regulates embryonic stem cell self-renewal. *The EMBO Journal*. 2013; 32: 2231–2247.
- [45] Johansson H and Simonsson S. Core transcription factors, Oct4, Sox2 and Nanog, individually form complexes with nucleophosmin (Npm1) to control embryonic stem (ES) cell fate determination. *AGING*. 2010; 2(11): 815–22.
- [46] Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CWH, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *NATURE GENETICS*. 2006; 38(4): 431–40.
- [47] Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. Core Transcriptional Regulatory Circuitry in Human Embryonic Stem Cells. *Cell*. 2005; 122(6): 947–956.
- [48] Fong YW, Inouye C, Yamaguchi T, Cattoglio C, Grubisic I, Tjian R. A DNA Repair Complex Functions

- as an Oct4/Sox2 Coactivator in Embryonic Stem Cells. *Cell*. 2011; 147(1):120–131.
- [49] Flinn EM, Wallberg AE, Hermann S, Grant PA, Workman JL and Wright AP. Recruitment of Gcn5-containing complexes during c-Myc-dependent gene activation. Structure and function aspects. *J Biol Chem*. 2002; 277(26): 23399-406.
- [50] Rowland BD, Bernards R and Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat Cell Biol*. 2005; 7(11): 1074-82.
- [51] Heffernan C, Sumer H, Malaver-Ortega LF and Verma PJ. Temporal Requirements of cMyc Protein for Reprogramming Mouse Fibroblasts. *Stem Cells International* 2012; vol.2012: article id 541014.
- [52] Fussner E, Djuric U, Strauss M, Hotta A, Perez-Iratxeta C, Lanner F, Dilworth FJ, Ellis J, Bazett-Jones DP. Constitutive heterochromatin reorganization during somatic cell reprogramming. *EMBO J* 2011; 30: 1778–1789.
- [53] Reynolds N, Latos P, Hynes-Allen A, Loos R, Leaford D, O'Shaughnessy A, Mosaku O, Signolet J, Brennecke P, Kalkan T, Costello I, Humphreys P, Mansfield W, Nakagawa K, Strouboulis J, Behrens A, Bertone P, Hendrich B. NuRD suppresses pluripotency gene expression to promote transcriptional heterogeneity and lineage commitment. *Cell Stem Cell* 2012; 10: 583–594.
- [54] Melcer S, Hezroni H, Rand E, Nissim-Rafinia M, Skoultschi A, Stewart CL, Bustin M, Meshorer E. Histone modifications and lamin A regulate chromatin protein dynamics in early embryonic stem cell differentiation. *Nat Commun* 2012; 3: 910.
- [55] Dos Santos RL, Tosti L, Radziszewska A, Caballero IM, Kaji K, Hendrich B, Silva JC. MBD3/NuRD Facilitates Induction of Pluripotency in a Context-Dependent Manner. *Cell Stem Cell*. 2014; 15(1): 102-10.
- [56] Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 2006; 441(7091): 349-353.
- [57] Pasini D, Cloos PA, Walfridsson J, Olsson L, Bukowski JP, Johansen JV, Tommerup N, Rappsilber J, Helin, K. JARID2 regulates binding of the polycomb repressive complex 2 to target genes in ES cells. *Nature* 2010; 464(7286): 306-10.
- [58] Wei Z, Yang Y, Zhang P, Andrianakos R, Hasegawa K, Lyu J, Chen X, Bai G, Liu C, Pera M, Lu W. Klf4 interacts directly with Oct4 and Sox2 to promote reprogramming. *Stem Cells* 2009; 27(12): 2969-78.
- [59] Christophersen NS and Helin K. Epigenetic control of embryonic stem cell fate. *J Exp Med*. 2010; 207(11): 2287-95.
- [60] Wu SC, Zhang Y. Active DNA demethylation: many roads lead to Rome. *Nat Rev Mol Cell Biol* 2010; 11: 607–620.
- [61] Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science* 2009; 324 (5929): 930–935.
- [62] Hu X, Zhang L, Mao SQ, Li Z, Chen J, Zhang RR, Wu HP, Gao J, Guo F, Liu W, Xu GF, Dai HQ, Shi YG, Li X, Hu B, Tang F, Pei D, Xu GL. Tet and TDG Mediate DNA Demethylation Essential for Mesenchymal-to-Epithelial Transition in Somatic Cell Reprogramming. *Cell Stem Cell* 2014; 14(4): 512–522.
- [63] Gao Y, Chen J, Li K, Wu T, Huang B, Liu W, Kou X, Zhang Y, Huang H, Jiang Y, Yao C, Liu X, Lu Z, Xu Z, Kang L, Chen J, Wang H, Cai T, Gao S. Replacement of Oct4 by Tet1 during iPSC Induction Reveals an Important Role of DNA Methylation and Hydroxymethylation in Reprogramming. *Cell Stem Cell* 2013; 12(4): 453–469.
- [64] Freudenberg JM, Ghosh S, Lackford BL, Yellaboina S, Zheng X, Li R, Cuddapah S, Wade PA, Hu G, Jothi R. Acute depletion of Tet1-dependent 5-hydroxymethylcytosine levels impairs LIF/Stat3 signaling and results in loss of embryonic stem cell identity. *Nucleic Acids Res*. 2012; 40(8): 3364-3377.
- [65] Costa Y, Ding J, Theunissen TW, Faiola F, Hore TA, Shliha PV, Fidalgo M, Saunders A, Lawrence M, Dietmann S, Das S, Levasseur DN, Li Z, Xu M, Reik W, Silva JC, Wang J. NANOG-dependent function of TET1 and TET2 in establishment of pluripotency. *Nature* 2013; 495(7441): 370-4.
- [66] Lee J, Sayed N, Hunter A, Au KF, Wong WH, MocarSKI ES, Pera RR, Yakubov E, Cooke JP. Activation of innate immunity is required for efficient nuclear reprogramming. *Cell*. 2012; 151(3): 547-58.
- [67] Yang CS, Li Z and Rana TM. microRNAs modulate iPSC cell generation. *RNA*. 2011; 17(8): 1451-60.
- [68] Morita S, Horii T, Kimura M, Ochiya T, Tajima S and Hatada I. miR-29 Represses the Activities of DNA Methyltransferases and DNA Demethylases. *Int J Mol Sci*. 2013; 14(7): 14647–14658.
- [69] Wang G, Guo X, Hong W, Liu Q, Wei T, Lu C, Gao L, Ye D, Zhou Y, Chen J, Wang J, Wu M, Liu H, Kang J. Critical regulation of miR-200/ZEB2 pathway in Oct4/Sox2-induced mesenchymal-to-epithelial transition and induced pluripotent stem cell generation. *Proc Natl Acad Sci U S A*. 2013; 110(8): 2858-63.
- [70] Mongroo PS and Rustgi AK. The role of the miR-200 family in epithelial-mesenchymal transition. *Cancer Biol Ther*. 2010; 10(3): 219–222.
- [71] Li R, Liang J, Ni S, Zhou T, Qing X, Li H, He W, Chen J, Li F, Zhuang Q, Qin B, Xu J, Li W, Yang J, Gan Y, Qin D, Feng S, Hong Song, Yang D, Zhang B, Zeng L, Lai L, Esteban MA, Pei D. A Mesenchymal-to-

- Epithelial Transition Initiates and Is Required for the Nuclear Reprogramming of Mouse Fibroblasts. *Cell Stem Cell* 2010; 7(1): 51–63.
- [72] Choi YJ, Lin CP, Ho JJ, He X, Okada N, Bu P, Zhong Y, Kim SY, Bennett MJ, Chen C, Ozturk A, Hicks GG, Hannon GJ, He L. miR-34 miRNAs provide a barrier for somatic cell reprogramming. *Nat Cell Biol.* 2011; 13(11): 1353-60.
- [73] Lin SL, Chang DC, Lin CH, Ying SY, Leu D, Wu DT. Regulation of somatic cell reprogramming through inducible mir-302 expression. *Nucleic Acids Res.* 2011; 39(3): 054-65.
- [74] Card DA, Hebbar PB, Li L, Trotter KW, Komatsu Y, Mishina Y, Archer TK. Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells. *Mol Cell Biol.* 2008; 28(20): 6426-38.
- [75] Ware CB, Wang L, Mecham BH, Shen L, Nelson AM, Bar M, Lamba DA, Dauphin DS, Buckingham B, Askari B, Lim R, Tewari M, Gartler SM, Issa JP, Pavlidis P, Duan Z, Blau CA. Histone deacetylase inhibition elicits an evolutionarily conserved self-renewal program in embryonic stem cells. *Cell Stem Cell* 2009; 4: 359–369.
- [76] Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM. Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature* 2010; 463: 1042–1047.
- [77] Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W, Melton DA. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nature Biotechnology* 2008; 26: 1269 – 1275.
- [78] Esteban MA, Wang T, Qin B, Yang J, Qin D, Cai J, Li W, Weng Z, Chen J, Ni S, Chen K, Li Y, Liu X, Xu J, Zhang S, Li F, He W, Labuda K, Song Y, Peterbauer A, Wolbank S, Redl H, Zhong M, Cai D, Zeng L, Pei D. Vitamin C Enhances the Generation of Mouse and Human Induced Pluripotent Stem Cells. *Cell Stem Cell* 2010; 6(1): 71–79.
- [79] Li H, Collado M, Villasante A, Strati K, Ortega S, Cañamero M, Blasco MA, Serrano M. The Ink4/Arf locus is a barrier for iPS cell reprogramming. *Nature.* 2009; 460(7259): 1136-9.
- [80] Mimasu S, Sengoku T, Fukuzawa S, Umehara T, Yokoyama S. Crystal structure of histone demethylase LSD1 and tranlycypromine at 2.25 Å. *Biochem Biophys Res Commun.* 2008; 366(1): 15-22.
- [81] Mali P, Chou BK, Yen J, Ye Z, Zou J, Dowe S, Brodsky RA, Ohm JE, Yu W, Baylin SB, Yusa K, Bradley A, Meyers DJ, Mukherjee C, Cole PA, Cheng L. Butyrate Greatly Enhances Derivation of Human Induced Pluripotent Stem Cells by Promoting Epigenetic Remodeling and the Expression of Pluripotency-Associated Genes. *Stem Cells.* 2010; 28(4): 713-720.
- [82] Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A. Dissecting direct reprogramming through integrative genomic analysis. *Nature.* 2008; 454(7200): 49-55.
- [83] Lin SW, Unno Y, Hou WS, Chang P, Adachi I, Aihara H et al. Difference in direct charge-parity violation between charged and neutral B meson decays. *Nature.* 2008; 452: 32-335.
- [84] Matsui T, Leung D, Miyashita H, Maksakova IA, Miyachi H, Kimura H, Tachibana M, Lorincz MC, Shinkai Y. Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 2010; 464(7290): 927-31.
- [85] Selvaraj V, Plane JM, Williams AJ, Deng W. Switching cell fate: the remarkable rise of induced pluripotent stem cells and lineage reprogramming technologies. *Trends in Biotechnology* 2010; 28 (4): 214–23.
- [86] Stadtfeld M and Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. *Genes & Dev.* 2010; 24: 2239-2263.
- [87] Marqués-Torrejón MÁ, Porlan E, Banito A, Gómez-Ibarlucea E, Lopez-Contreras AJ, Fernández-Capetillo O, Vidal A, Gil J, Torres J, Fariñas I. Cyclin-dependent kinase inhibitor p21 controls adult neural stem cell expansion by regulating Sox2 gene expression. *Cell Stem Cell.* 2013; 12(1): 88-100.
- [88] Kane NM, Nowrouzi A, Mukherjee S, Blundell MP, Greig JA, Lee WK, Houslay MD, Milligan G, Mountford JC, von Kalle C, Schmidt M, Thrasher AJ, Baker AH. Lentivirus-mediated Reprogramming of Somatic Cells in the Absence of Transgenic Transcription Factors. *Mol Ther.* 2010; 18(12): 2139–2145.
- [89] Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell.* 2009; 136(5): 964-77.
- [90] Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hämäläinen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 2009; 458(7239): 766-70.
- [91] Maherali N, Ahfeldt T, Rigamonti A, Utikal J, Cowan C, Hochedlinger K A. high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell.* 2008; 3(3): 340-5.
- [92] Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science* 2008; 322(5903): 945-9.
- [93] Fusaki N, Ban H, Nishiyama A, Saeki K and Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host

- genome. *Proc Jpn Acad Ser B Phys Biol Sci.* 2009; 85(8): 348–362.
- [94] Okita K, Nakagawa M, Hyenjong H, Ichisaka T and Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science.* 2008; 322(5903): 949-53.
- [95] Kim JB, Greber B, Araúzo-Bravo MJ, Meyer J, Park KI, Zaehres H, Schöler HR. Direct reprogramming of human neural stem cells by OCT4. *Nature.* 2009; 461(7264): 649-3.
- [96] Somers A, Jean JC, Sommer CA, Omari A, Ford CC, Mills JA, Ying L, Sommer AG, Jean JM, Smith BW, Lafyatis R, Demierre MF, Weiss DJ, French DL, Gadue P, Murphy GJ, Mostoslavsky G, Kotton DN. Generation of transgene-free lung disease-specific human induced pluripotent stem cells using a single excisable lentiviral stem cell cassette. *Stem Cells* 2010; 28(10): 1728-40.
- [97] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; 282(5391): 1145–1147.
- [98] Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol.* 2007; 25(7): 803–816.
- [99] Singh U, Quintanilla RH, Grecian S, Gee KR, Rao MS, Lakshmipathy U. Novel Live Alkaline Phosphatase Substrate for Identification of Pluripotent Stem Cells. *Stem Cell Rev.* 2012; 8(3): 1021–1029.
- [100] Quintanilla RH, Asprer JST, Vaz C, Tanavde V, Lakshmipathy U. CD44 Is a Negative Cell Surface Marker for Pluripotent Stem Cell Identification during Human Fibroblast Reprogramming. *PLoS ONE.* 2014; 9(1): e85419.
- [101] Nazor KL, Altun G, Lynch C, Tran H, Harness JV. Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. *Cell Stem Cell* 2012; 10: 620-634.
- [102] Keller GM. In vitro differentiation of embryonic stem cells. *Curr Opin Cell Biol.* 1995; 7(6):862–869.
- [103] Kahler DJ, Ahmad FS, Ritz A, Hua H, Moroziewicz DN, Sproul AA, Dusenberry CR, Shang L, Paull D, Zimmer M, Weiss KA, Egli D, Noggle SA. Improved methods for reprogramming human dermal fibroblasts using fluorescence activated cell sorting. *PLoS One.* 2013; 8(3): e59867.
- [104] Lotz S, Goderie S, Tokas N, Hirsch SE, Ahmad F, Corneo B, Le S, Banerjee A, Kane RS, Stern JH, Temple S, Fasano CA. Sustained levels of FGF2 maintain undifferentiated stem cell cultures with biweekly feeding. *PLoS One.* 2013; 8(2): e56289.

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