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An Insight into DNA-free Reprogramming Approaches to Generate Integration-free Induced Pluripotent Stem Cells for Prospective Biomedical Applications

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Abstract

More than a decade ago, a pioneering study reported generation of induced Pluripotent Stem Cells (iPSCs) by ectopic expression of a cocktail of reprogramming factors in fibroblasts. This study has revolutionized stem cell research and has garnered immense interest from the scientific community globally. iPSCs hold tremendous potential for understanding human developmental biology, disease modeling, drug screening and discovery, and personalized cell-based therapeutic applications. The seminal study identified Oct4, Sox2, Klf4 and c-Myc as a potent combination of genes to induce reprogramming. Subsequently, various reprogramming factors were identified by numerous groups. Most of these studies have used integrating viral vectors to overexpress reprogramming factors in somatic cells to derive iPSCs. However, these techniques restrict the clinical applicability of these cells as they may alter the genome due to random viral integration resulting in insertional mutagenesis and tumorigenicity. To circumvent this issue, alternative integration-free reprogramming approaches are continuously developed that eliminate the risk of genomic modifications and improve the prospects of iPSCs from lab to clinic. These methods establish that integration of transgenes into the genome is not essential to induce pluripotency in somatic cells. This review provides a comprehensive overview of the most promising DNA-free reprogramming techniques that have the potential to derive integration-free iPSCs without genomic manipulation, such as sendai virus, recombinant proteins, microRNAs, synthetic messenger RNA and small molecules. The understanding of these approaches shall pave a way for the generation of clinical-grade iPSCs. Subsequently, these iPSCs can be differentiated into desired cell type(s) for various biomedical applications.

Keywords Induced pluripotent stem cells · Cell reprogramming · Reprogramming factors · Non-integrative approaches · Transgene-free · Clinical-grade

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Introduction

John Gurdon's discovery demonstrated that nucleus of a differentiated somatic cell may reach a pluripotent state when placed in the cytoplasmic milieu of an egg cell [1]. Later, Davis and colleagues reported that overexpression of the transcription factor MyoD in fibroblasts converted them into myoblasts to show lineage conversion of mammalian cells [2]. The first successful mammalian cloning of Dolly via somatic cell nuclear transfer demonstrated that an adult somatic cell was capable of reverting back to an early embryonic totipotent state and further develop into a whole animal [3]. These remarkable studies demonstrated that restoration of pluripotency in an adult somatic cell, although thought to be an irreversible state, could be a possibility. However, *in vitro* settings for the successful derivation of pluripotent stem cells from terminally differentiated somatic adult cells were unknown. In the year 2006, two Japanese scientists made a

breakthrough and identified the right combination of reprogramming factors (Oct4, Sox2, Klf4 and c-Myc (OSKM); also popularly known as Yamanaka factors) to restore pluripotency in mouse embryonic fibroblasts (MEF) cells [4]. This study demonstrated that the differentiated state in somatic cells can be unlocked and reprogrammed back to an early embryonic-like state, namely induced Pluripotent Stem Cells (iPSCs). iPSCs generated by various studies [4–6] are similar to Embryonic Stem Cells (ESCs) isolated from the inner cell mass of a blastocyst [7, 8], and overcome the immunological and ethical concerns associated with ESCs. In addition, iPSCs are superior to adult stem cells, since the former have an unlimited self-renewal and differentiation potential. Therefore, iPSCs can be utilized as an excellent model system for the better understanding of developmental biology, disease modeling, drug discovery and toxicity testing, and autologous cell-based therapeutic applications [9–12].

Reprogramming techniques to generate iPSCs from adult somatic cells are constantly evolving due to technological advances in biology. To date, various integrative and non-integrative approaches are reported to derive iPSCs from different tissue-derived somatic cells [13, 14]. The classical integrative methods using γ-retro- and lenti-viral vectors are robust and efficient but result in permanent genomic modifications due to viral integration into the host genome. The clinical applicability of derived iPSCs using these techniques carry the risk of insertional mutagenesis and tumor formation [15, 16]. Moreover, silencing and activation of transgenes are not predictable and this ultimately affects the differentiation potential of these iPSCs [17, 18]. To circumvent these issues, non-integrative strategies of reprogramming are developed with minimal or no genetic modifications [13, 14]. This review primarily focuses on DNA-free reprogramming approaches (sendai virus, recombinant proteins, microRNAs, synthetic messenger RNA and small molecules) that are highly promising to generate integration-free, clinical-grade iPSCs for prospective biomedical applications.

Sendai virus

Sendai virus (SeV) is a non-pathogenic, enveloped virus having a non-segmented, negative-sense, single-stranded RNA genome. SeV belong to the Paramyxoviridae family, and their complete replicative cycle occurs in the cytoplasm (Fig. 1; [19]). The viral RNA genome encodes for six essential and two accessory proteins. The six essential proteins are nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the large protein (L) (Fig. 2; [20]). The accessory proteins are short protein V and a nested group of carboxy-coterminal proteins collectively termed as C [21, 22]. Thus, the viral RNA genome has six independent cistrons NP, P/C/V, M, F, HN and L that require only a gene end signal (3′–AUUCUUUUU), gene start signal (3′–UCCCNNUUUC) and an intervening conserved intergenic region (3’–GAA) to perform transcription [23, 24]. The catalytic subunit of RNA-dependent RNA polymerase is formed by the L protein that sustains viral replication and transcription [20]. The NP, P and L are bound to the viral RNA genome and forms the ribonucleoprotein complex, the latter is responsible for viral replication and transcription [25].

The first report to use SeV vectors demonstrated successful reprogramming of adult fibroblasts to derive integration-free iPSCs [26]. This group utilized F-deficient SeV vectors that offer a high-level expression of transgenes to introduce the reprogramming factors. Using a different approach, another study employed a temperature-sensitive mutated SeV vector to reprogram T cells without any genomic manipulation [27]. This approach generated a weaker transgene expression and this form of SeV vector cannot replicate at standard cell culture conditions. Various studies have successfully used SeV vectors to derive iPSCs and are summarized in Table 1. This vector has also been used to reprogram various other cell types: peripheral blood mononuclear cells [28], iris pigment epithelial cells [29], dental mesenchymal stem cells [30], renal epithelial cells [31] and hair follicle keratinocytes [32]. However, the residual presence of viral particles is always a concern for its therapeutic use, therefore, different strategies were designed to obtain SeV-free iPSCs. The first strategy is to use antibodies specific to the spike surface protein HN expressed on the surface of virus containing reprogrammed cells [26]. This approach will effectively allow us to screen and obtain transgene-free iPSCs. The second strategy is to use siRNAs that specifically target the viral replication machinery to remove viral genome rapidly from iPSCs [23]. Alternatively, temperature-sensitive mutant SeV vectors can be generated by introducing point mutations in structural genes to eliminate SeV viral residues from the reprogrammed cells by a short temperature shift [33]. Recently, a replication-deficient auto-erasable SeV vector was designed that responds to the microRNA-302 (miR-302) [34]. This miR-302 is highly expressed in iPSCs and absent in somatic cells [35–37]. Expression of this miR-302 during reprogramming suppressed the viral RNA-dependent RNA polymerase vital for the replication of SeV genome and inhibited the continuous expression of transgenes, resulting in automatic removal from the reprogrammed cells and derivation of integration-free, SeV-free iPSCs.

SeV proffers many advantages which have rendered it ideal for cell reprogramming. First, it binds to ubiquitous sialic acid receptors to enter a cell and thus has a broad tropism (Fig. 1). Second, the possibility of genome modifications or gene silencing by epigenetic modifications is eliminated due to the lack of a DNA phase [38]. Third, SeV has a high and rapid expression of proteins within twenty-four hours of transduction [39]. Fourth, the transduction efficiency of SeV is high for

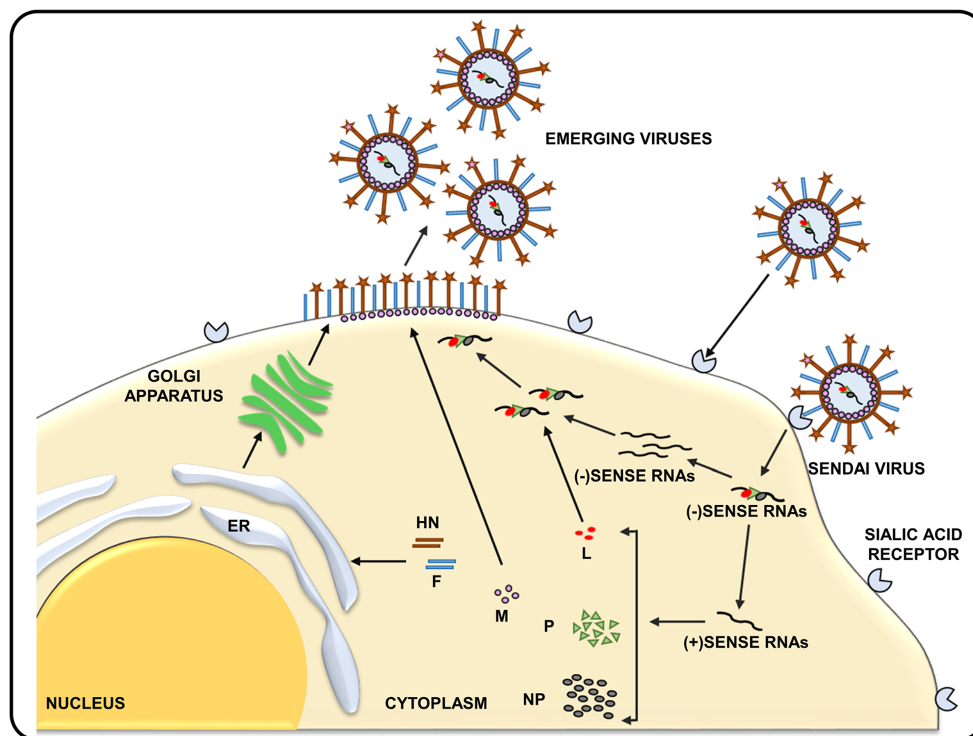

Fig. 1 The life cycle of Sendai virus. The HN proteins bind to the sialic acid receptors present on host cell membrane. The virus entry is achieved as the viral envelope fuses with the host envelope by F-mediated membrane fusion and release of nucleocapsid into the cytosol. Inside the cell, the negative sense RNA acts as a template for two distinct processes, transcription and replication. Both these processes occur entirely in the cytoplasm and do not require any host nuclear components. The SeV RNA-dependent RNA polymerase complex is comprised of L and P proteins, where L is responsible for RNA synthesis, cap formation and methylation of the cap, and P is an essential cofactor. To initiate transcription, the polymerase recognizes the gene start signals and the gene end signals, thus producing subgenomic capped RNAs with poly(A) tail due to stuttering of the polymerase at gene end signal. The proteins are expressed in a gradient as an expression of NP is the highest and decreases progressively towards the L gene. The newly synthesized NP, P and L proteins directly bind to the genomic RNA to form the nucleocapsid and this is tightly linked to RNA synthesis. M protein migrates towards the cell membrane. The F and HN proteins are glycosylated envelope proteins and hence they are processed by the endoplasmic reticulum (ER) and the Golgi bodies. Replication is initiated when adequate numbers of NP protein is produced as this stabilizes the polymerase-template RNA-nascent anti-genomic RNA complex. The nucleocapsid buds out at sites containing the M and the envelope proteins. NP: nucleoprotein; P: phosphoprotein; M: matrix protein; F: fusion protein; HN: haemagglutinin-neuraminidase protein; L: large protein

a broad range of tissues and cell types, and cellular uptake requires only a brief contact time [38]. Next, all the viral particles are eventually diluted and lost after approximately ten passages [40, 41], or can be removed promptly by alternative strategies [23, 26, 33, 34] . Importantly, the possibility of altering the stoichiometry of transgenes enable optimal expression of transgenes and achieve the highest reprogramming efficiency [23, 26]. Lastly, the SeV has been reported to derive high-quality iPSCs with low aneuploidy rates free from any viral contamination [42].

However, there are few shortcomings to this very appealing vector delivery system. First, the viral RNA-dependent RNA polymerase is extremely sensitive to the transgenic sequences [41]. Secondly, although the new generation SeV vectors are less cytotoxic/cytopathic, this concern is still associated with SeV delivery system [43]. Thirdly, it is highly laborious to prepare the SeV viruses and difficult to work with compared to retro- and lenti-viruses [40]. A commercial SeV-based reprogramming kit is available which minimizes the task of production of viruses, but this kit is very expensive. Lastly, SeV is fusogenic and immunogenic, although these concerns are reduced in SeV/ΔFΔMΔHN vector. Moreover, being a viral delivery system, thorough screening for integration and viral genes is necessary before the therapeutic applications of derived iPSCs. Despite these concerns, this vector is presently the most versatile and popular system used for cell reprogramming.

Recombinant Proteins

Pure bioactive form of reprogramming proteins can be produced in large quantities using prokaryotic or eukaryotic systems and delivery of these functional proteins represents a transgene-free approach to derive safe iPSCs (Fig. 3). Since proteins have limited ability to cross cell membrane, these reprogramming proteins can be fused with cell-penetrating peptides (CPPs) or also called protein transduction domains (PTDs) that can overcome cell membrane barrier and facilitate their intracellular delivery [44–47].

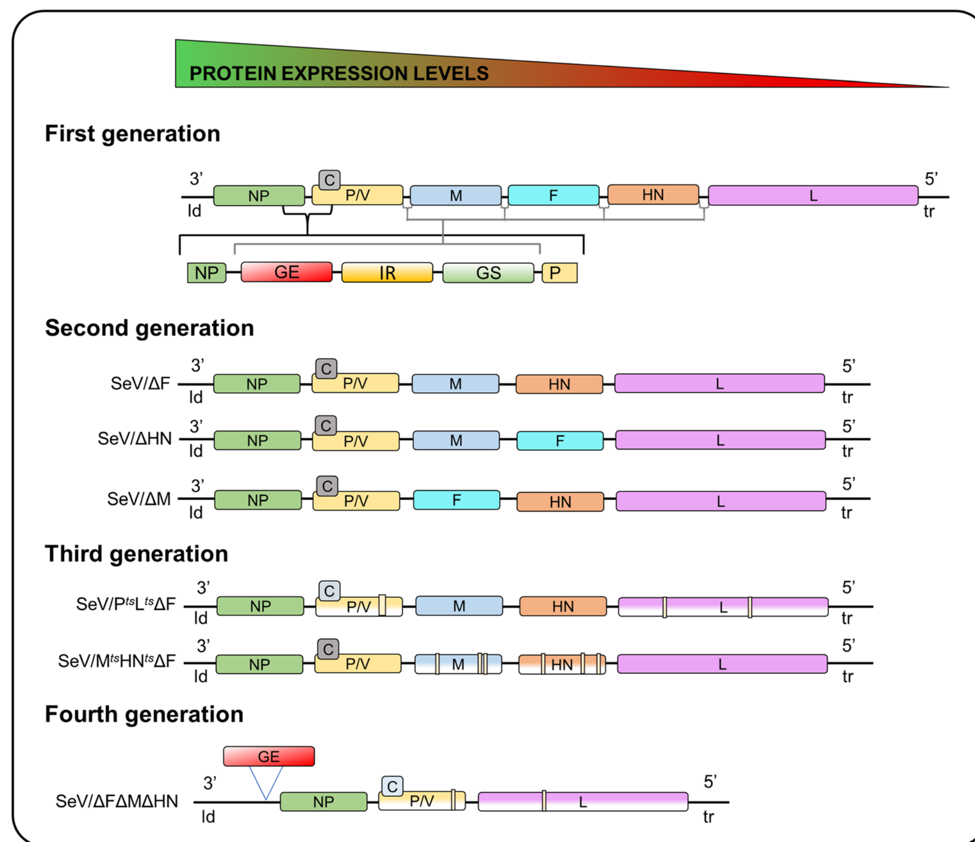

Fig. 2 Sendai virus vectors. The first generation SeV vector comprised of the complete viral genome consisting of six essential and two accessory proteins and therefore gave rise to infectious viral particles [164]. The leader sequence (ld) at the 3′-end is followed by these six viral genes with a small trailer (tr) sequence at the 5′-end. The protein expression levels decrease in a gradient with NP protein expressed the most and L protein expressed the least. A gene of interest can be inserted between the gene start (GS) signal and gene end (GE) signal in the sendai virus genome. The next generation of SeV vectors developed had a deletion/defect in one of the structural genes; either F [23, 165], HN [23] or M [23, 166]. These vectors were less immunogenic and had a diminished ability to self-replicate. However, the self-replicative ability was not completely abolished and could produce infectious virus-like particles, which can raise several regulatory and safety concerns [23]. The third generation vector was a temperature-sensitive mutant having three amino acid substitutions introduced in both the M and HN genes in the SeVV/ΔF vector that produced less infectious virus-like particles [25] . Alternatively, temperature-sensitive mutants in SeVV/ΔF vector were attained by point mutations in the viral P and L genes to eliminate viral-vector related genes from the derived iPSCs [33]. Further, a triple deficient vector lacking all the three structural genes (SeV/ΔFΔMΔHN) was developed [167] . The transgenic capacity of this fourth generation vector was higher due to the removal of all the structural genes. This new vector enabled the insertion of four genes into the vector cassette, and hence, a single vector was sufficient to carry all the four Yamanaka factors [23]. This single vector overcomes the homologous viral interference that occurs when multiple vectors carrying the different factors co-infect a cell. Moreover, the removal of F, M, and HN gene made the vector less cytopathic and less immunogenic. This vector was further improved by (a) incorporating gene end signal before NP gene and introducing specific missense mutations in the L gene which prevents Interferon beta (IFNβ) induction, and (b) missense mutations in P gene that enables stable transgene expression [23]. Viral replication is dependent on viral RNA-dependent RNA polymerase of which L is a crucial component. Therefore, viral replicons of SeV/ΔFΔMΔHN vector is eliminated using siRNA against L gene when expression of transgenes is no longer desired [23]. NP: nucleoprotein; P: phosphoprotein; M: matrix protein; F: fusion protein; HN: haemagglutinin-neuraminidase protein; L: large protein

Various studies have been reported employing recombinant protein-based approach to derive transgene-free iPSCs (Table 2). In 2009, Zhou and co-workers first reported the derivation of stable iPSCs from OG2/Oct4-GFP reporter MEF cells by transducing the four Yamanaka factors in the form of recombinant proteins [48]. To aid intracellular delivery the authors fused poly-arginine PTD (11-R) to the C-terminus of these four proteins and expressed them in *E. coli*. In addition, the authors had supplemented the culture media with 1 mM Valproic Acid (VPA), a histone deacetylase inhibitor, to enhance the reprogramming efficiency [48]. However, the efficiency and kinetics of reprogramming were extremely poor compared to the classical integrative techniques, as only three colonies emerged from 5×10^4 cells plated after 30-35 days. Likewise, in absence of VPA, the study failed to obtain stable GFP^+ colonies when transduced with three or four reprogramming factors as recombinant proteins [48]. The delivery of transduced proteins solely was inadequate to push the cells to an ultimate pluripotent state due to endosomal entrapment of transduced proteins. Concurrently,

Table 1 Summary of Sendai virus-based iPSC generation studies using Yamanaka factors

SeV used	Cell type used	Combination with	Reprogramming efficiency	Reprogramming kinetics (days)	Reference
*ts*SeV/ΔF	HDF, HBJ	–	1%	NR	[26]
*ts*SeV/ΔF	hTDCTC	–	0.1%	~25	[27]
*ts*SeV/ΔF	HBJ, HDF, CB CD34+ cells	–	>0.1%	~28	[33]
Cytotune kit	HNF	–	0.01–0.04%	17-21 (feeder-free) 21-25 (xeno-free)	[172]
Cytotune kit	HNF	–	0.01-0.1%	~21	[173]
Cytotune kit	PB from CMD patients	–	NR	14-30	[174]
Cytotune kit	Human Skeletal Myoblasts	NaB, SB431542	0.75%	18-24	[175]
Cytotune kit SeVdp	PBMCs	NaB	0.005% 0.014%	21 16	[176]
Cytotune kit	Finger-tip capillary blood, PBMCs	–	0.008–0.014%	20	[177]
Cytotune kit	AF-MSCs	–	0.01–0.05%	28	[178]
Cytotune kit	Dermal fibroblasts	–	–	>21	[179]
*ts*SeV/L(302L)ΔFΔHNΔM	HEF	–	–	~25	[34]

ts temperature-sensitive, *HDF* human dermal fibroblasts, *HBJ* human fibroblasts BJ, *hTDCTC* human terminally differentiated circulating T cells, *CB* cord blood, *HNF* human neonatal fibroblasts, *PB* peripheral blood, *CMD* craniometaphyseal dysplasia, *NaB* sodium butyrate, *PBMCs* peripheral blood mononuclear cells, *AF-MSCs* amniotic fluid mesenchymal stromal cells, *HEF* human embryonic fibroblasts, *NR* not reported

Kim and colleagues succeeded in generating iPSCs from human newborn fibroblasts by the direct delivery of the four Yamanaka factors in a recombinant form [48]. Unlike Zhou and co-workers, the authors fused the four reprogramming factors to a poly-arginine PTD (9R) and expressed in human embryonic kidney 293 cells, a mammalian expression system [48]. However, the authors did not use the reprogramming factors in pure form, instead applied extracts of human embryonic kidney 293 cells overexpressing Yamanaka factors to reprogram human newborn fibroblasts cells. The colonies emerged after six rounds of transductions i.e. after 8 weeks; albeit with a very low reprogramming efficiency [49]. Interestingly, the subsequent study successfully reprogrammed mouse cardiac fibroblasts to iPSCs by streptolysin O-mediated reverse permeabilization of ESC-derived extract proteins, with improved reprogramming efficiency and kinetics [50]. This agent is a cholesterol-binding bacterial exotoxin that promotes pore formation in the cell membrane of target cells up to the size of 35 nm that is adequate for recombinant proteins to translocate and small to prevent the escape of cell organelles [51]. The authors observed approximately 5-10 colonies (out of $1x10^6$ mouse cardiac fibroblasts initially seeded) between 4th -7th day and numerous secondary colonies between 20-25 days after induction [50]. Nonetheless, this study failed to reprogram adult fibroblasts with proteins extracted from ESCs of 129 background mice strain [50]. An important implication of this study was that iPSC colonies emerged only when mouse cardiac fibroblasts cells were incubated with both cytoplasmic and nuclear extract proteins, but not with any one of them. A significant increase in reprogramming efficiency was reported when a fifth transcription factor 'Nanog' was incorporated in the presence of VPA to reprogram human foreskin fibroblasts [52]. Apart from that, the authors fused the Yamanaka factors to Trans-Activator of Transcription (TAT) protein transduction domain, a special 10-20 amino acids sequence, derived from HIV-TAT protein to enhance cell membrane penetration [52]. They also showed that transcriptionally TAT-fused reprogramming factors are, in general, more efficient than their counterpart 11R-fused reprogramming factors [52]. Recently, a novel CPP of 10 amino acids in length was identified with nuclear trafficking activity which was fused with artificial transcription factors to induce pluripotency in MEF cells [53]. This CPP is reported to be more potent than commonly used CPPs, TAT and 11R.

It is noteworthy that none of the protein-based studies discussed above were able to increase reprogramming efficiency above 0.05% since no effort was made to improve nuclear localization. As a result, a majority of the transduced reprogramming proteins were trapped in endosomes. Therefore, Nemes and co-workers fused a Nuclear Localization Signal/Sequence (NLS) to aid nuclear localization of the Yamanaka factors, along with the CPP TAT [54]. Interestingly, the authors discovered that the genetic status of cells used also plays a significant role during the reprogramming process [54]. This was based on the fact that iPSC colonies only emerged from the outbred MEFs (ICR) but not from the inbred background (C57BL6) [54]. Instead of using CPP-based approach, a bolaamphiphile based study demonstrated much faster kinetics sharing similarity with those of RNA-based methods, and an efficiency that resembles with the efficiency of conventional virus-based

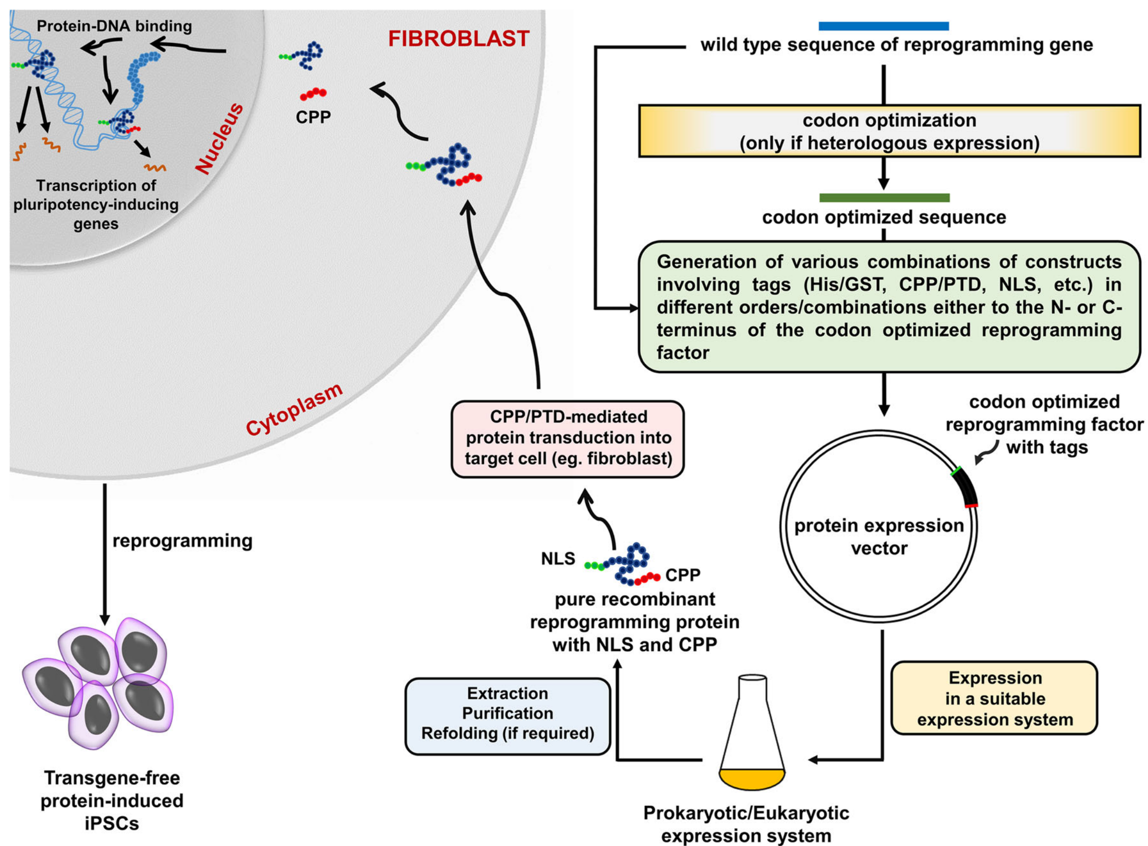

Fig. 3 A schematic diagram illustrating a recombinant protein-based approach to derive clinically potent iPSCs from mature somatic cells. Reprogramming factors can be produced in the form of pure recombinant proteins using prokaryotic or eukaryotic expression systems with retained biological activity. These reprogramming proteins can directly be delivered into a large number of cell types to generate exogene-free iPSCs. In case of heterologous recombinant protein expression, codon optimization of the wild-type genes of reprogramming factors is an important step to bypass rare codons of host species and maximize the protein yield. Likewise, designing of gene constructs either by N- or C-terminal fusion of a variety of tags, such as Histidine/Glutathione S-transferase (His/GST), etc. to aid purification, Cell Penetrating Peptide/Protein Transduction Domain (CPP/PTD) to enable cell membrane penetration, Nuclear Localization Signal (NLS) to facilitate nuclear translocation, etc., enhances cellular as well as nuclear delivery of pure recombinant proteins. These gene constructs can be overexpressed in suitable expression systems, isolated, purified and refolded (in case of proteins expressed in the form of inclusion bodies) to yield pure bioactive recombinant reprogramming factors. Upon transduction, these proteins permeate the cell membrane with the help of the fused CPP/PTD. In case of a certain reprogramming factor, the presence of CPP/PTD may adversely affect its biological activity, in such cases, intracellular cleavage of CPPs/PTDs from the reprogramming proteins after delivery into the cytoplasm at a specific time point is highly desirable. Presence of an NLS in the constructs directs the recombinant proteins towards the nucleus reducing time spent in the cytoplasm and thereby minimizes the risks of endosomal entrapment of these proteins. Upon reaching the nucleus, these reprogramming proteins bind onto the target sites of DNA and activate transcription machinery of various pluripotency inducing genes. Finally, activation of endogenous pluripotency networks leads to the transition of a somatic cell from a unipotent to a pluripotent state without genetic manipulation. Thus, this strategy offers great potential for clinical applicability of the generated iPSCs

approaches [55]. Bolaamphiphiles are molecules that contain a hydrophobic skeleton and two hydrophilic groups at both ends [56]. Khan et al. employed a 1,12-diaminododecane based bolaamphiphile to facilitate the intracellular delivery of the reprogramming proteins; where they substituted Yamanaka factor, OCT4 with NR5A2 [55]. The iPSCs thus obtained were positive for both *in vitro* and *in vivo* assays.

Protein-based reprogramming is promising but is less efficient compared to most of the non-integrative methods. To address this major issue, an interesting elegant study reported Toll-like receptor 3 (TLR3) signaling facilitates efficient pluripotency induction owing to the innate immune response to modified mRNA or viral-based approaches [57]. However, this essential signaling remains inactivated in CPP-mediated protein transduction resulting in low reprogramming efficiency. The inclusion of TLR3 agonist (Polyinosinicpolycytidylic acid (Poly I:C)), along with 11R CPP-fused Yamanaka factors, lead to an activation of this signaling pathway. This resulted in down-regulation of many HDACs (HDAC 1,2,5,7), which gave rise to iPSCs from human fibroblasts with faster kinetics and high reprogramming efficiency. Therefore, TLR3 stimulation by agonists is essential for an efficient protein-based reprogramming and will improve the robustness of this technology.

Table 2 Summary of recombinant protein-based cell reprogramming approaches to generate iPSCs

Recombinant proteins	Expression system	Cell type reprogrammed	Method of delivery	Small molecules used	*in vitro* differentiation assay	*in vivo* pluripotency assay	Efficiency	Reference
OSKM	*E. coli*	OG2-MEF	11R-PTD	VPA	Positive	Positive	0.006%	[48]
OSKM	*HEK293*	HNF	9R-PTD	None	Positive	Positive	0.001%	[49]
C57-ESC extract proteins	------	McFB/MsFB	Streptolysin O	None	Positive	Positive	0.0005–0.001 %	[50]
OSKMN	*E. coli*	HFF	TAT-PTD	VPA	Not carried out	Not carried out	0.01 %	[52]
OSKM	*E. coli*	HFF	11R-PTD	Poly I:C	Positive	Positive	0.04–0.08%	[57]
SKNNr5a2	*E. coli (SK) Wheat Germ (NNr5a2)*	HFF	Cationic bolaamphiphile	VPA	Positive	Positive	0.05 %	[55]
OSKM	*E. coli*	MEF	TAT-PTD + NLS	None	Positive	Positive	Not reported	[54]
ATF	*E. coli*	MEF	NTP	None	Not carried out	Positive	0.5–1.0%	[53]

OSKM Oct4, Sox2, Klf4, c-Myc, *N* Nanog, *L* Lin28a, *ATF* artificial transcription factor (composed of a transcription activator-like effector and transcription domain (VP64), designed to efficiently induce endogenous RNA expression at the proximal promoter region of the microRNA-302/367 cluster at an extremely low concentration), *E. coli Escherichia coli*, *ESC* embryonic stem cell, *HEK293* human embryonic kidney 293, *OG2* Oct4 promoter driven GFP reporter, *MEF* mouse embryonic fibroblasts; *HNF* human newborn fibroblasts, *McFB* mouse cardiac fibroblasts, *MsFB* mouse skin fibroblasts, *HFF* human foreskin fibroblasts, *11R* 11 arginine, *9R* 9 arginine, *TAT* transactivator of transcription, *PTD* protein transduction domain, *NTP* nuclear trafficking peptide (comprised of 10 amino acids (RIFIHFRIGC) of C45D18), *VPA* valproic acid, *Poly I:C* polyinosinic-polycytidylic acid

Delivery of reprogramming proteins in pure form is a safer alternative to generate exogene-free iPSCs. However, the recombinant proteins used in the aforementioned studies are usually challenging to reproducibly purify in the desired amounts, making them difficult to use routinely for reprogramming experiments. Moreover, poor *in vitro* solubility and stability of these recombinant proteins are the major obstacles hampering the regular use of this technology. This can be overcome by optimizing media conditions with certain components that maintain solubility and improve stability [58, 59]. In addition, non-toxic and non-viral CPPs such as 30Kc19 conjugated to reprogramming factors displayed enhanced solubility and stability *in vitro* [60]. An additional serious issue is that majority of the transduced reprogramming proteins are sequestered by endosomal compartments [46]. To facilitate the release of the reprogramming proteins from endosomes, use of lysomotropic agents such as chloroquine, sucrose, ammonium chloride and so forth, should be employed. Moreover, it has been reported that fusion of CPP/PTD with a protein of interest compromises its function whereas intracellular excision of the fused CPP/PTD using a specific protease restores the function of reprogramming proteins [61]. Therefore, these parameters should be accounted for, while designing a protein-based reprogramming approach. Upon overcoming these bottlenecks, recombinant protein-based reprogramming is a method of great interest as it provides precise control over dosage and time of application. In addition, it gives complete flexibility to try and test various recipes of reprogramming factors to investigate their role during different phases (early, intermediate or late) of the reprogramming process.

mRNA Transfection

Transfection of messenger RNA (mRNA) encoding reprogramming factors is a desirable, straightforward strategy to derive patient-specific clinical-grade iPSCs from somatic cells without compromising genomic integrity [62]. The original study to generate iPSCs using *in vitro* synthesized non-modified mRNAs of reprogramming factors was reported in 2010, when Yakubov and co-workers transfected mRNAs of reprogramming factors into human foreskin fibroblast cells [63]. iPSCs on further propagation were positive for alkaline phosphatase staining and the critical pluripotency markers. However, whether these cells are functionally pluripotent is still an open question, as the iPSCs generated were not further evaluated to demonstrate pluripotency using *in vitro* and *in vivo* assays. A similar conclusion can be drawn from another study carried out at the same time using non-modified RNA to reprogram human fibroblasts [64]. In addition, non-modified RNAs used in this study can generate an immune response and stimulate cellular defense mechanisms, which can result in cytotoxicity and deter mRNA translation [65].

Therefore, suppression of innate immune response is a must to enable multiple transfections of RNA encoding reprogramming proteins [66].

Taking this into account, an exhaustive elegant study was reported in the same year to derive human iPSCs from fibroblasts and keratinocytes in two weeks by repeated administration of modified mRNA cocktail of Yamanaka factors [67]. The reprogramming efficiency obtained was 1.4% compared to 0.04% obtained for retroviral-based approach performed in parallel. In addition, the reprogramming kinetics of mRNA-based approach was also much faster (13–15 days vs. 25–29 days) compared to the retroviral-based approach. Subsequently, the study showed that inclusion of a fifth factor LIN28A to this modified mRNA cocktail regime had a higher reprogramming efficiency (i.e. more than 2.5%) in both ambient (20%) and low oxygen (5%) conditions [67]. The high reprogramming efficiency and improved kinetics are mainly due to important aspects taken into consideration while generating the synthetic mRNA (Fig. 4). The artificially synthesized mRNAs of the five factors had slightly altered sequences, where 5-methylcytidine (5mC) was substituted for cytidine and pseudouridine (psi) for uridine. This was done to reduce innate immune response and increase mRNA stability and translational efficiency after transfection. The authors further incorporated an anti-reverse di-guanosine cap analog to the mRNAs to facilitate efficient translation and boost RNA half-life in the cytoplasm, followed by removal of residual 5ʹ-triphosphate moieties through phosphatase treatment to avoid innate immune response. Further, the authors supplemented the media with recombinant B18R protein, an inhibitor of interferon type I, which mitigates innate immune response and thereby enhances cell viability as well as mRNA translation profile [67]. However, repeated daily transfections for longer duration can exert substantial stress on the cells. A more recent study claimed derivation of high-quality iPSCs from human fibroblasts using a six-factor mRNA cocktail after 9 days of transfection [68]. The authors used commercially available synthetically modified (as described in Warren et al. [67]) mRNA premix of the reprogramming factors; at initially low concentration followed by increasing concentrations from 2nd day onwards till the 8th day.

Use of non-modified or modified *in vitro* transcribed RNA raises few concerns. The short half-life of *in vitro* transcribed RNA in cell culture conditions demands repeated transfections which increase cellular stress. Moreover, the effect of a transfection reagent on the health of cells is also a concern. Considering this, Rohani et al. generated human iPSCs using non-synthetic mRNA [69]. The authors replaced the cap analog system used in Warren et al. [67] by 2′-O-methyltransferase enzyme derived from V. virus to ensure 100% proper cap orientation during *in vitro* transcription of mRNA. This enzyme produces a cap1 structure observed in higher eukaryotes and thereby has a high translation efficiency. Moreover, this study used 200-300 adenosine residues long poly(A) tail to provide higher stability to the *in vitro* transcribed RNA transcripts, compared to shorter poly(A) tails (120 residues) used earlier [67]. Even removal of residual 5′-triphosphates from non-capped RNA transcripts (due to 100% capping) and supplementation with B18R was not essential using this system [69]. In addition, the study used polyethylenimine, a polymeric transfection reagent, which showed enhanced transfection efficiency and cell viability [69]. In general, this system showed reduced immunogenicity and cytotoxicity requiring reduced transfections to derive iPSCs in a short time [69]. Concurrently, Choi and co-workers demonstrated prevention of mRNA degradation and delivery of this mRNA encoding reprogramming factors using graphene oxide-polyethylenimine complexes to derive footprint-free iPSCs without the need for repetitive daily transfections [70].

To further reduce the number of transfections, Tavernier and colleagues reported establishment of pluripotency in MEFs with just three mRNA transfections [71]. This study used cationic lipid mediators to transfect MEFs with mRNAs of the four Yamanaka factors. The cell-clusters emerged expressed alkaline phosphatase and early pluripotency markers, SSEA-1 and Nanog [71]. Similar to the previously reported studies [63, 64], the further establishment of pluripotency was not carried out. With just five mRNA transfections of specially synthesized mouse-specific mRNAs of the four Yamanaka factors, El-Sayed and co-workers were able to restore pluripotency in MEFs in twelve days [72]. However, the success rate in terms of reprogramming efficiency was very moderate i.e. 0.1-0.13%. An interesting study generated human iPSCs using a combination of non-modified reprogramming mRNAs with immune suppressive factors (E3, K3, and B18R) to reprogram fibroblasts within 11 days and blood-derived endothelial progenitor cells within 10 days with four and eight transfections, respectively [73]. To avoid multiple transfections, an elegant study demonstrated generation of human iPSCs from newborn or adult fibroblasts by just one transfection employing a single, synthetic polycistronic self-replicative RNA replicon that encodes four reprogramming factors in the presence of B18R [74]. All these major studies using mRNA-based approach to derive iPSCs are summarized in Table 3.

Derivation of integration-free iPSCs using mRNA-based approach has become popular and used by several labs now [62, 75–77]. Human iPSCs derived using this highly efficient approach are of high-quality with low aneuploidy rates and no major alterations in the copy number variations in the established lines [42]. Human iPSCs were even derived from cells isolated from patients and/or healthy subjects above 40 years of age [77–79]. However, a major limitation is the need for feeder cells and the cell culture media containing animal-derived components, which carries an additional risk of transmitting overlooked human pathogens. To obviate this, several

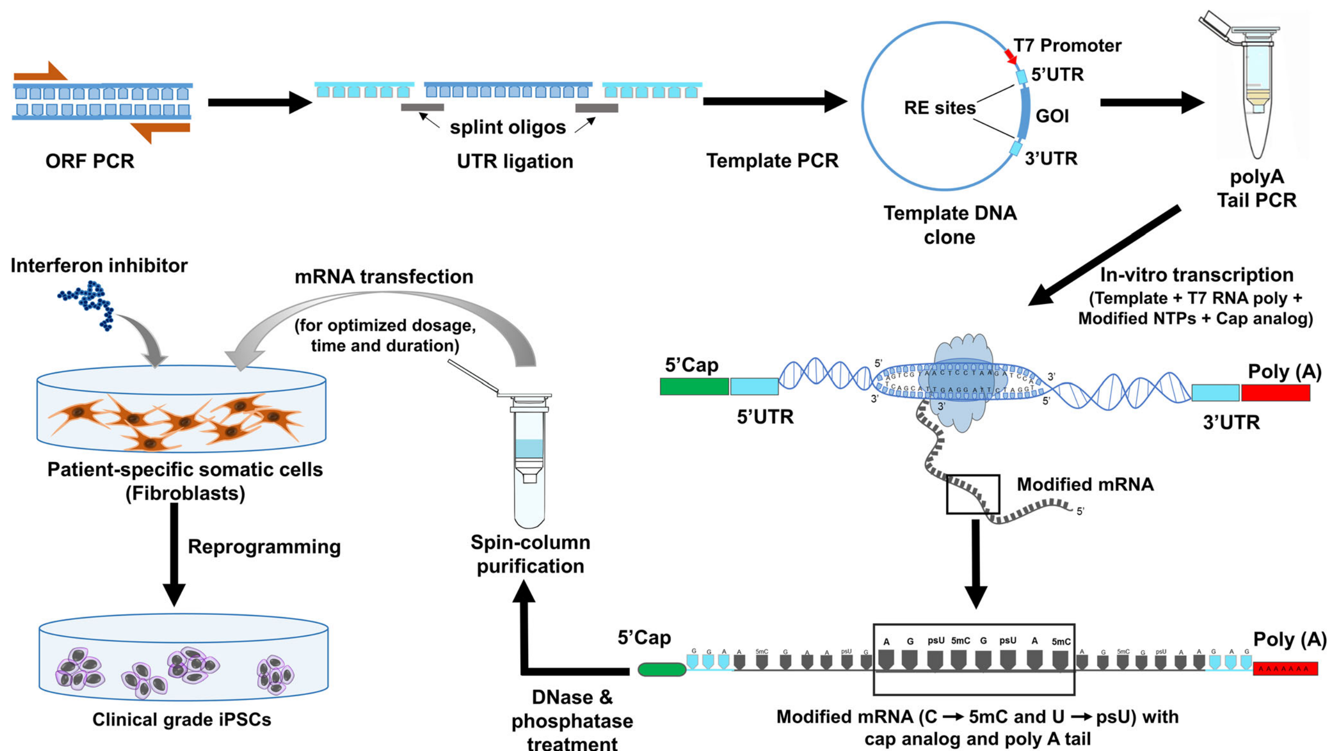

Fig. 4 A schematic diagram depicting *in vitro* production of synthetic messenger RNAs (mRNAs) of reprogramming factors and their transfection into somatic cells to derive transgene-free iPSCs. First, the open reading frame (ORF) of a reprogramming factor is amplified by polymerase chain reaction (PCR) from a cDNA to construct the template for *in vitro* transcription reactions. 5' and 3' UTRs in the form of long oligonucleotides sequences are ligated to the sense strand of ORF amplicons using a thermostable DNA ligase and the ligation of the desired single-stranded DNA (ssDNA) ends are mediated by splint oligos. Using generic primers, the ssDNA product is PCR amplified after incorporating a T7 promoter at 5' UTR sequence and TA cloning is carried out avoiding the use of restriction enzymes. Likewise, employing a T_{120}-heeled reverse primer, a polyA tail is added at end of 3' UTR fragments. The resulting amplicons are used as a template for IVT reactions using unmodified and modified (where 5-methylcytidine (5mC) was substituted for cytidine and pseudouridine (psi) for uridine) nucleobases. Modification of nucleobases is done to reduce innate immune response, increase mRNA stability and translational efficiency after transfection. Further, in the IVT reaction, an anti-reverse di-guanosine cap analog is incorporated using at a concentration four-fold higher than guanosine triphosphate to facilitate efficient translation and enhance RNA half-life in the cytoplasm. The IVT product so obtained is spin column purified followed by DNase and phosphatase treatment to remove the template DNA and the residual 5' triphosphate moieties to avoid innate immune response. Additionally, the media is supplemented with an inhibitor of interferon type I (recombinant B18R protein) which further suppresses innate immune response and thereby enhances cell viability as well as the efficiency of mRNA translation. The modified mRNAs after purification can be used to transfect a variety of cell types employing a suitable transfection agent to derive exogene-free iPSCs (production of modified mRNA protocol from Warren et al., 2010 [67]). *GOI* gene of interest, *UTR* untranslated region

groups have derived iPSCs under feeder-free and/or xeno-free conditions with high reprogramming efficiency employing mRNA-based reprogramming [79–85], that can be applicable for the clinical translation of iPSCs. Taking all together, mRNA-based reprogramming strategy holds great promise for potentiating clinical efficacy and safety profile of the derived iPSCs and the cell types obtained after differentiating them.

microRNAs

MicroRNAs (miRNAs) are naturally occurring, small (around ~22 nucleotides in length), non-protein-coding RNAs that play a decisive role in post-transcriptional regulation of gene expression [86]. miRNAs are transcribed in the nucleus and processed by Drosha/DGCR8. Subsequently, the processed miRNA is transported by Exportin-5 protein to the cytoplasm, where Dicer processes it to produce mature miRNAs along with other major players to be able to bind and destabilize the target mRNA(s) (Fig. 5). Recently, many studies have identified various miRNAs that are essential for vital functions in controlling the metabolism, cell cycle, pluripotency, and differentiation of pluripotent stem cells [87–90]. In addition, miRNAs are also reported to play a crucial role in cell reprogramming to derive iPSCs. Suppressing crucial miRNA processing machinery such as Dicer, Drosha, and Ago2 resulted in a substantial decrease in reprogramming efficiency [91]. Corroborating this, two studies showed that Dicer-deficient MEFs were deficient in functional miRNAs, and therefore, failed to reprogram efficiently when cell reprogramming factors were introduced in these cells for iPSC generation [92, 93]. Interestingly, the

Table 3 Summary of major studies using mRNA-based reprogramming approach to derive iPSCs

mRNA	Modification/Capping	Cell type used	Supplements	Transfection reagent	*in vitro* differentiation assay	*in vivo* pluripotency assay	Efficiency	Reference
OSML	None	HFF	None	Lipofectamine	Not carried out	Not carried out	0.05 %	[63]
OSKM; OSKML	5mC for cytidine Psi for uridine ARCA	HEF	B18R	RNAiMAX	Positive	Positive	1.4 %; >2.5%	[67]
OSKM	None	MEF	None	Lipofectamine or RNAiMAX	Positive	Not carried out	Not reported	[71]
OSKM	None	MEF	None	TransIT	Positive	Positive	0.1-0.13 %	[72]
OKSMNL	5mC for cytidine Psi for uridine ARCA	HFF	B18R	Stemfect	Positive	Not carried out	Not reported	[68]
OSKM/OSKG	None	HFF	B18R	Lipofectamine 2000	Not carried out	Positive	0.012-0.18%	[74]
OSKMN	None/2-O-Methyltransferase	HFF	None	Polyethylenimine (JetPei)	Positive	Positive	Not reported	[69]

OSKMNL Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28, *G* Glis1, *5mC* 5-methyl cytidine, *Psi* pseudouridine, *ARCA* anti reverse di-guanosine cap analog, *HFF* human foreskin fibroblasts, *HEF* human embryonic fibroblasts, *MEF* mouse embryonic fibroblasts

expression of Dicer in these cells reestablished their reprogramming potential [92].

Two independent studies by the Morrisey and Mori groups reported that mouse and human somatic cells can be reprogrammed to iPSCs by expressing specific microRNAs [37, 94], eliminating the requirement for ectopic expression of reprogramming factors. The study by Morrisey group demonstrated that lentiviral expression of the ESC-specific miRNAs, mir-302(b,c,a,d)-mir-367 cluster, in fibroblasts is sufficient for the derivation of iPSCs with faster kinetics and higher reprogramming efficiency [37]. Surprisingly, few subsequent studies were unable to obtain the same result [95–97]. This discrepancy might be due to the different delivery approaches employed to deliver and express miRNAs [37, 95] or different starting cell type used for reprogramming [37, 96, 97]. Interestingly, various other studies have also reported that this cluster or a member of this cluster when used in combination with reprogramming factors resulted in enhanced reprogramming efficiency and kinetics due to the promotion of mesenchymal to epithelial transition (MET) [85, 95–101]. This cluster is a direct target of core reprogramming factors, OCT4 and SOX2 [102]. Notably, deletion of this entire cluster completely blocked the formation of human iPSCs [103], indicating that this cluster is vital for efficient cell reprogramming. The Mori group delivered a combination of seven mature double-stranded miRNAs belonging to mir-200, mir-302 and mir-369 families by repeated transfections in multipotent human and mouse adipose stromal cells to reprogram them to iPSCs. This study did not utilize any integrating vectors for delivery, instead used direct transfection of mature miRNAs to derive integration-free iPSCs. However, the reprogramming efficiency in this study was lower compared to the study by Morissey group [37], probably due to the insufficient number of transfections (four times within the first 8 days) during the course of reprogramming. Notably, a number of miRNAs have been reported to induce or enhance (mostly pluripotent stem cell-specific miRNAs) or act as a barrier (mostly expressed in starting cell type to be reprogrammed) to cellular reprogramming and are listed in Fig. 6 and summarized in Table 4 (act as inducers/enhancers) and Table 5 (act as barriers). Since miRNAs are small, stable, easy to synthesize and transfect, they could represent an ideal approach for cell reprogramming to obtain transgene-free iPSCs [104].

Small molecules

Generating iPSCs exclusively by small molecules would be the most convenient and practical approach since they are small, non-immunogenic, stable, inexpensive, and easy to synthesize and deliver into cells. This approach is tunable, spatially and temporally controllable, and reversible, therefore, these characteristics provide a pliable modulation of

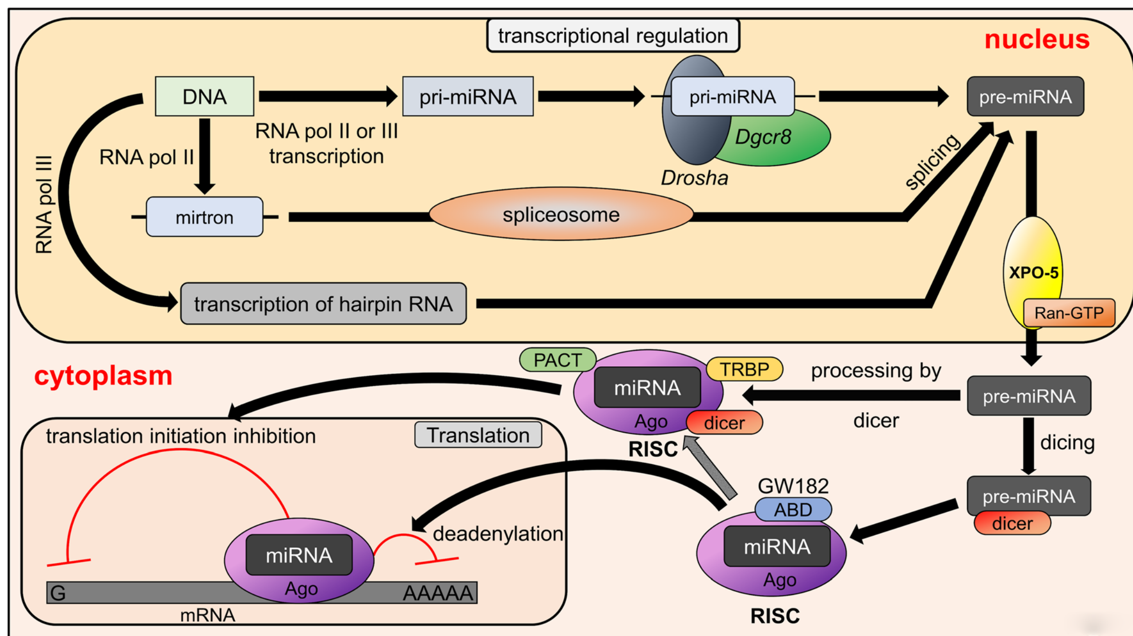

Fig. 5 miRNA biogenesis and gene expression control in human cells. miRNAs generated in the nucleus by either RNA polymerase II (RNA pol II) or RNA polymerase III (RNA pol III) are long, primary transcripts (pri-miRNA) [168, 169]. Numerous miRNAs transcribed by RNA pol II have a high expression level and usually their expression is cell type-specific [168]. Mostly, human miRNAs acquire a hairpin structure that is characteristic of miRNA genes, and cleaved by a microprocessor complex comprised of ribonuclease enzyme Drosha (an RNase III enzyme) and RNA-binding protein Dgcr8 (a cofactor of Drosha) to produce pre-miRNA hairpin in the cell nucleus [170]. Alternatively, pre-miRNAs formation also occurs by transcription of hairpin RNAs by RNA pol III [169] and a splicing-mediated hairpin formation mechanism, known as the mirtron pathway [171]. Pre-miRNAs produced by any of these pathways is subsequently exported to the cytoplasm by a nuclear RNA-export factor, Exportin-5 (XPO5), when the latter is bound by Ran-GTP. Once the pre-miRNA is exported out of the nucleus into the cytoplasm, it is cleaved and processed by a second RNAse III enzyme, Dicer, to produce symmetric RNA duplexes of a specified length [170]. The processed RNAs are then loaded onto RNA-induced silencing complex (RISC) and subsequently repress complementary target mRNAs via translational initiation inhibition, deadenylation, and mRNA degradation [170]

chromatin modifications and signaling pathways increasing its importance in the field of cell reprogramming [105]. Numerous small molecules have been identified: (i) small molecules that can enhance the reprogramming efficiency and kinetics (Table 6), or (ii) substitute the vital reprogramming factors (Table 7), or (iii) small molecule cocktails that are sufficient to induce pluripotency in absence of exogenous reprogramming factors (Table 7) [106]. These molecules either modulate chromatin modifications or target specific signaling pathways to regulate various cellular processes to favor cell reprogramming.

The first study to report the application of small molecules involved in chromatin modifications identified the HDAC inhibitor VPA and the DNA methyltransferase inhibitor (DNMT) 5-Azacitidine (5-AZA) to enhance the reprogramming efficiency by more than a hundred-fold and ten-fold, respectively [107]. In addition, the same study also identified two other HDAC inhibitors, Suberoylanilide hydroxamic acid (SAHA) and Trichostatin A (TSA), to promote reprogramming efficiency [107]. Increase in reprogramming efficiency by VPA was most likely due to alteration in the chromatin structure [107, 108] and suppression of reprogramming-induced senescence stress [109]. 5-AZA was also reported to enhance reprogramming efficiency in MEFs up to four-fold by accelerating the transition of partially reprogrammed cells to attain full pluripotency [110]. Additionally, butyrate, a histone deacetylase inhibitor and natural small fatty acid, was reported to significantly increase the reprogramming efficiency of mouse [111] and human [112] cells, by enhancing the expression of pluripotency-associated genes and epigenetic remodeling [112]. Further, Pasha and co-workers converted mouse skeletal myoblasts transduced with a single factor Oct4 and treated with a DNMT inhibitor RG108 to derive iPSCs having similar molecular and differentiation characteristics to ESCs [113]. RG108 does not incorporate into DNA, therefore, its cytotoxicity is less than 5-AZA. These studies demonstrated that chromatin modification is a key step in attaining the pluripotent state.

Numerous other groups were also investigating and identifying novel small molecules that could enhance reprogramming efficiency (Table 6). A study reported that Vitamin C, a water-soluble antioxidant, also significantly enhanced the reprogramming of mouse and human somatic cells [114], to some extent by alleviating cellular senescence [114] and possibly by inducing DNA demethylation [115, 116].

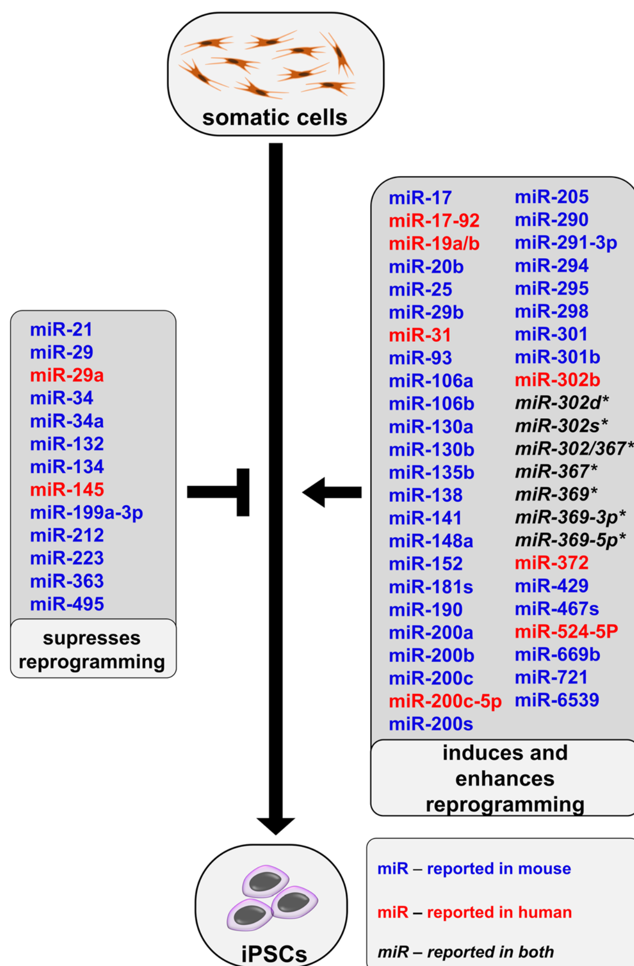

Fig. 6 The role of miRNAs in the generation of iPSCs. Numerous studies have reported the role of miRNAs that induce/promote or act as a barrier to iPSC formation. miRNAs highlighted in blue and red are reported in mouse and human, respectively. miRNA highlighted in black having a * at the end are reported in both mouse and human

This natural compound promotes gene expression changes and accelerates the conversion of partially reprogrammed colonies to a completely reprogrammed state, resulting in improved efficiency and kinetics of reprogramming. Lithium, an anti-psychotic drug, was also reported to significantly enhance iPSC generation of both mouse and human cells with one (Oct4) or two factors (Oct4-Sox2 or Oct4-Klf4) [117]. Lithium increased the transcriptional activity and expression of NANOG and facilitated epigenetic alterations via suppression of an H3K4-specific histone demethylase, lysine-specific histone demethylase 1. To further identify small molecules that increase reprogramming efficiency, a high-throughput screening of chemical libraries was performed [118]. This study identified Oct4-activating compounds that enhanced both reprogramming efficiency and kinetics and showed that one of these compounds increased transcription of the core pluripotent stem cell regulators Oct4-Sox2-Nanog, and Tet1, a gene involved in DNA demethylation.

Small molecules, apart from working as reprogramming enhancers, can also act as promising replacers for one or more crucial reprogramming transcription factors (Table 7). The small molecule, BIX-01294, a G9a histone methyltransferase inhibitor, has been reported to replace cMyc and greatly enhance the reprogramming efficiency of neural progenitor cells (that endogenously express Sox2) transduced with only Oct4 and Klf4 factors [119]. G9a histone methyltransferase was reported to irreversibly inactivate Oct4 during early embryogenesis resulting in de novo DNA methylation at the promoter region by the enzymes Dnmt3a/3b, thereby preventing reprogramming [120]. In addition, BIX-01294, along with BayK8644, an L-type calcium channel agonist, enabled reprogramming of MEF cells transduced with Oct4 and Klf4 [121, 122]. BayK targets upstream in the cell signaling pathways without having any effect on the epigenome. Therefore, this molecule is of particular interest and will be investigated further to identify its specific role in cell reprogramming. Furthermore, VPA was also shown to replace the oncogenes Klf4 and c-Myc in reprogramming primary human neonatal fibroblasts to derive iPSCs with a similar global gene expression profile and epigenetic state to ESCs [108]. Jaenisch group performed a high-throughput screen to identify a small molecule Kenpaullone that could substitute Klf4, however, the former was less efficient than the latter to derive mouse iPSCs [123]. CHIR99021, a glycogen synthase kinase 3 (GSK-3) inhibitor, was reported to induce reprogramming in MEF cells by only two factors, Oct4 and Klf4 [124]. Combination of Parnate (Tranylcypromine) along with CHIR99021 can reprogram human primary keratinocytes transduced with Oct4 and Klf4 without exogenous Sox2 expression [124]. Further, Sox2 was shown to be replaced by Repsox and SB431542 [125], LY-364947 [126], and Pan-Src family kinase inhibitors [126] to generate mouse iPSCs. Furthermore, a small-molecule EPZ004777, a DOT1L inhibitor, enhances the reprogramming efficiency of both mouse and human reprogramming three-fold and three-six-fold, respectively. This small molecule was reported to replace Klf4 and c-Myc by repressing the catalytic activity of DOT1L, a H3K79 methyltransferase, and by upregulation of Lin28 and Nanog [127].

To further reduce the number of reprogramming factors, Zhu and co-workers generated human iPSCs by reprogramming neonatal human epidermal keratinocytes with single reprogramming factor, Oct4, and a cocktail of small molecules including HDAC inhibitor sodium butyrate, transforming growth factor β (Tgf-β) receptor inhibitor A-83-01, 3'-phosphoinositide-dependent kinase-1 activator PS48 and extracellular signal-regulated kinase (ERK) inhibitor PD0325901 [128]. This study also reported that sodium butyrate was more efficient than the previously reported VPA when primary human cells were transduced with *OCT4* and *KLF4* reprogramming factors. Subsequently, iPSCs were derived by reprogramming mouse embryonic and adult fibroblasts using a

Table 4 Various miRNAs that act as inducers or enhancers for iPSC generation

miRNAs	Role	Starting cell type	Reprogramming factors/molecules used	References
hsa-miR-302	Suppressed developmental genes involved in initiation or facilitation of lineage-specific differentiation	Human cancer cell lines	None	[180]
hsa-miR-302	Inhibited four epigenetic regulators, namely AOF2 (also known as KDM1 or LSD1), AOF1, MECP1-p66 and MECP2), which resulted in enhanced global demethylation and facilitated cell reprogramming	Human hair follicle cells	None	[181]
mmu-miR-291-3p or mmu-miR-294 or mmu-miR-295 or mmu-miR-302d	Enhanced reprogramming efficiency, but only with OSK	MEF	OSK or OSKM	[98]
mmu-miR-205 and mmu-miR-200 family	Promoted MET and synergized with OSKM to accelerate progression through the initiation phase of cell reprogramming	MEF	OSKM	[139]
mmu-miR-302-367 or mmu-miR-106a or mmu-miR-20b	miR-302-367 accelerated MET and increased reprogramming efficiency in combination with OSK; miR-302b/c/a/d combination was equally potent as the intact miR 302–367; miR-106a or miR-20b of the cluster miR 106a-363 had a strongest positive effect in reprogramming efficiency, albeit not as strong as miR 302b/c/a/d combination; miR-302-367 also targeted Tgf-β receptor 2 to promote MET	MEF	OSK or OSKM (+ Vitamin C)	[99]
mmu-miR-93 or mmu-miR-106b or mmu-miR-106a or mmu-miR-17	Involved in acceleration of MET and directly targeted Tgf-β receptor 2 and p21, to greatly enhance reprogramming efficiency	MEF	OSK or OSKM	[91]
hsa-miR-302b or hsa-miR-372 or mmu-miR-294	Inhibition of Tgf-β–induced EMT and promotion of MET during reprogramming; downregulation of genes involved in EMT, cell cycle, vesicular transport and epigenetic regulation to promote cell reprogramming	Human foreskin and lung fibroblasts	OSK or OSKM	[100]
mmu-miR-130b or mmu-miR-301b or mmu-miR-721 or mmu-miR-106a or mmu-miR-106b or mmu-miR-20b or mmu-miR-93 or mmu-miR-130a or mmu-miR-301 or mmu-miR-148a or mmu-miR-152 or mmu-miR-190 or mmu-miR-19b or mmu-miR-669b	14 microRNAs were reported to promote reprogramming; miR-130/301/721 family targeted the transcription factor Meox2 and strongly promoted iPSC generation	MEF	OSK	[182]
mmu-miR-302/367	Enhanced reprogramming by two orders of magnitude more efficient compared to using standard OSKM reprogramming factors; inhibition of HDAC2 was required	MEF	VPA	[37]
mmu-miR-200c + mmu-miR-302s + mmu-miR-369-3p, -5p	Faithful reprogramming	Mouse adipose stromal cells	None	[94]
mmu-miR-302/367	Highly expressed in pluripotent stem cells [36]; Enhanced reprogramming efficiency	Human foreskin and dermal fibroblasts	None	[37]
mmu-miR-200c + mmu-miR-302s + mmu-miR-369-3p, -5p	Faithful reprogramming	Human adipose stromal cells and dermal fibroblasts	None	[94]
mmu-miR-25 or mmu-miR-302/367 or mmu-miR-290 or mmu-miR-298	Promoted reprogramming efficiency; miR-25 regulated ubiquitin ligases Wwp2 and Fbxw7 that are reported to regulate Oct4, c-Myc and Klf5 genes	MEF	OSKM	[95]
mmu-miR-138		MEF	OSK or OSKM	[183]

Table 4 (continued)

miRNAs	Role	Starting cell type	Reprogramming factors/molecules used	References
	Significantly improved iPSC generation with a threefold higher efficiency by targeting 3'-UTR of p53 and decreasing the expression of p53 and its downstream target genes			
mmu-miR-200a or mmu-miR-200b or mmu-miR-200c or mmu-miR-141 or mmu-miR-429	Promoted MET process and facilitated iPSC generation; repressed ZEB2 expression by directly targeting its 3′ UTR to promote iPSCs	MEF	OSKM	[184]
miR-302/367 cluster	Highly expressed in pluripotent stem cells [36]; Vital for faithful reprogramming	Human foreskin fibroblasts	OSKM	[103]
miR-302 cluster (without miR-367)	Essential for faithful reprogramming; suppressed MBD2 expression that resulted in an increase in Nanog expression and converted partially reprogrammed cells to completely reprogrammed iPSCs	Human $CD34^+$ cord blood cells	OSKM	[96]
miR-302 cluster (without miR-367)	Enhanced reprogramming efficiency by suppressing the expression of transcription factor NR2F2	Human adipose stem cells	OSKM	[97]
mmu-miR-29b	Expression of miR-29b is induced by Sox2; miR-29b enhanced iPSC formation by specifically repressing Dnmt3a and Dnmt3b expression; miR-29b-Dnmt signaling promoted early stage of MET and transcriptional silencing of the imprinted Dlk1-Dio3 region	MEF	OSK or OSKM	[185]
mmu-miR-181 family or mmu-miR-302 family or mmu-miR-467 family or mmu-miR-294 family	Promoted the initiation phase of reprogramming	MEF	OSK/Wnt3a+TGFβR inhibitor + AMPK inhibitor + Bestatin	[101]
mmu-miR-135b	Two-fold enhancement in iPSC formation by targeting Tgfbr2 and extracellular matrix genes Wisp1 and Igfbp5 that act as a barrier to reprogramming	MEF	OSKM	[186]
hsa-miR-17-92 cluster	Highly expressed in pluripotent stem cells [36, 187]; enhanced cell reprogramming specifically during the early stage of reprogramming; miR-19a/b of this miR-17-92 cluster exhibited the most potent reprogramming effect on stimulating human fibroblasts to iPSCs by targeted inhibition of PTEN, a tumor suppressor gene	Human fibroblasts	OSK or OSKM	[187]
mmu-miR-369	Stimulated Pkm2 splicing and promoted induction of cell reprogramming	Mouse adipose-derived mesenchymal stem cells	OSKM	[188]
hsa-miR-31	Enhanced reprogramming efficiency by suppressing SDHA expression and oxygen consumption rates in partially reprogrammed iPSCs; overexpression of miR-31 repressed mitochondrial complex II activity via downregulation of SDHA transcription	Human $CD34^+$ cord blood cells and fibroblasts	OSKM	[189]
hsa-miR-524-5p	Two-fold enhancement in somatic cell reprogramming by inhibiting TP53INP1 expression; promoted MET essential for initiating cell reprogramming by downregulating EMT-related genes, namely ZEB2 and SMAD4.	Human foreskin fibroblasts	OSKM	[190]
mmu-miR-6539	Overexpression of miR-6539 enhanced reprogramming efficiency by inhibiting translation of Dnmt3b via the target site in the coding sequence; facilitated DNA demethylation during the initial stage of reprogramming and increased iPSC formation	MEF	OSKM	[191]
hsa-miR-200c-5p		Human BJ dermal fibroblasts	OSKM	[192]

Table 4 (continued)

miRNAs	Role	Starting cell type	Reprogramming factors/molecules used	References
	Specifically targeted SIRT2 and suppressed SIRT2 expression via binding sites in the sirtuin gene coding sequence; enhances metabolic reprogramming via SIRT2 suppression; downregulation of SIRT2 significantly enhances iPSC formation			
hsa-miR-302-367	Facilitated induction of reprogramming; enhanced reprogramming efficiency as well as stability of derived iPSCs	Human primary neonatal fibroblasts	OSKMLN	[85]

OSK Oct4, Sox2, Klf4, *OSKM* Oct4, Sox2, Klf4, c-Myc, *OSKMNL* Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28, *MEF* mouse embryonic fibroblasts, *AOF* amine oxidase (Flavin containing) domain, *KDM* lysine demethylase, *LSD* lysine-specific histone demethylase, *MECP* methyl-CpG binding protein, *MET* mesenchymal to epithelial transition, *Tgf* Transforming growth factor, *EMT* epithelial to mesenchymal transition, *Meox2* mesenchyme homeo box 2, *HDAC* histone deacetylase, *VPA* valproic acid, *Wwp2* WW domain containing E3 ubiquitin protein ligase 2, *Fbxw7* F-Box and WD repeat domain containing 7, *Klf* kruppel-like factor, *UTR* untranslated region, *ZEB* zinc finger E-box binding homeobox, *MBD* methyl-CpG binding domain protein, *NR2F2* nuclear receptor subfamily 2, group F, member two, *Dnmt* DNA methyltransferase, *Dlk1-Dio3* delta-like homolog 1 gene and the type III iodothyronine deiodinase gene, *Wnt3a* Wnt family member 3A, *AMPK* AMP-activated protein kinase, *Wisp1* WNT1 inducible signaling pathway protein 1, *Igfbp5* insulin like growth factor binding protein 5, *PTEN* phosphatase and tensin homolog, *Pkm2* pyruvate kinase 2, *SDHA* succinate dehydrogenase complex subunit A, *TP53INP1* tumor protein P53 inducible nuclear protein 1, *SMAD4* SMAD family member 4, *SIRT* sirtuin, *let-7* lethal 7

single gene, Oct4, and a cocktail of small molecules designated as 'VC6T' (VPA, CHIR99021, 616452 and tranylcypromine), possibly by downregulating the two major reprogramming barriers: epigenetic (histone deacetylation and H3K4 demethylation) and signaling barriers (Tgf-β and GSK3) [129]. All the studies involving small molecules demonstrated that the role of OCT4 was indispensable and irreplaceable.

Remarkably, Deng and co-workers obtained iPSCs from MEF cells at an efficiency of 0.2% using a cocktail of seven small molecules [130], eliminating the need for exogenous expression of reprogramming factors, including the indispensable Oct4 factor. This was the first study to demonstrate reprogramming of mouse somatic cells to pluripotency without the use of genetic manipulation using small molecules. Later, Long and colleagues supplemented the reprogramming small molecule cocktail identified by Deng and co-workers [130] with a thymidine analog bromodeoxyuridine (BrdU) and showed enhanced OSKM-mediated reprogramming [131]. This molecule played a vital role during the early phase of reprogramming (day 3 to day 7) and was reported to substitute the core reprogramming factor Oct4 to derive mouse iPSCs. Notably, inclusion of BrdU reduces the number of small molecules required for acquisition of pluripotency with the minimal cocktail comprising of BrdU, RepSox, CHIR99021 and forskolin, albeit with an extremely low reprogramming efficiency. Concurrently Deng and colleagues elucidated the molecular mechanisms involved in chemical reprogramming to enhance the reprogramming kinetics and efficiency [132]. In this study, the authors included new small molecules in the reprogramming protocol at three different stages, altered concentration of CHIR990021 and components of the 2i medium, and optimization of the seeding of the cells and the time of the application of small molecules. Furthermore, the investigators used a DOT1L inhibitor SGC0946 and its application in stage 2 resulted in improved reprogramming efficiency. Application of SGC0946 during stage 1 resulted in decreased cell viability and therefore was not able to substitute EPZ004777, but its application in stage 2 resulted in increased expression of pluripotency-associated genes, such as Oct4 and Dppa family genes. This protocol yielded an increase by up to 1000-fold reprogramming efficiency in comparison to previous protocols. Subsequently, this group generated integration-free iPSCs from specialized cell types derived from different origins by chemical induction using the protocol reported earlier [132], with minor modifications in the concentration and time of application to reprogram these cell types [133]. Importantly, the same reprogramming chemical cocktail can be used across different cell types, but fine-tuning of the concentration and time of application of small molecules is essential to attain full pluripotency [133]. Therefore, small molecules can not only aid as reprogramming enhancers but also can substitute one or all the crucial reprogramming factors.

Table 5 Various miRNAs that act as a barrier to iPSC generation

miRNAs	Role	Starting cell type	Reprogramming factors/ molecules used	References
mmu-miR-21 and mmu-miR-29	Enriched in MEF cells and absent in pluripotent cells; regulate p53 and ERK1/2 pathways; depletion results in two- to three-fold enhancement in reprogramming efficiency; repressed by c-Myc to promote reprogramming by increasing p85alpha and CDC42, thereby suppressing the p53 pathway	MEF	OSK or OSKM	[193]
mmu-miR-34	Depletion resulted in significant increase in reprogramming kinetics and efficiency, in part, by posttranscriptional derepression of pluripotency genes; exhibited p53-dependent induction during reprogramming and cooperated with p21 to repress generation of iPSCs	MEF	OSK or OSKM	[194]
mmu-miR-199a-3p or mmu-miR-363	Enriched in MEF cells and their inhibition enhances reprogramming efficiency; miR-199a-3p was reported to be a p53 target and was upregulated by p53 at the posttranscriptional level; miR-199a-3p promotes G1 cell cycle arrest via the upregulation of p21 gene	MEF	OSK or OSKM	[195]
mmu-miR-34a	Regulate direct downstream target SIRT1, a member of the sirtuin family of NAD^+-dependent protein deacetylases; inhibition of miR-34a enhanced iPSC formation	MEF	OSKM	[196]
mmu-miR-223 or mmu-miR-495	Inhibited iPSC formation	MEF	OSKM	[186]
hsa-miR-145	Highly expressed in dermal fibroblasts and low in pluripotent cells [197, 198]; inhibited ESC self-renewal, repressed 3'-UTR of pluripotency-associated genes Oct4, Sox2 and Klf4 and induced lineage-specific differentiation[197]; downregulated during reprogramming of somatic cells into iPSCs; inhibition of miR-145 downregulated mesenchymal markers and let-7b, upregulated epithelial markers and expression of pluripotency-associated genes, and thereby facilitated and increased cell reprogramming	Human dermal fibroblasts	OSKM	[198]
mmu-miR-134	Inhibition of miR-134 promoted cell reprogramming and maturation of iPSCs whereas overexpression of miR-134 suppressed iPSCs induction and formation; miR-134 directly targeted the 3' UTR of the pluripotency related factor Mdb3 to down-regulate its expression; miR-134-Mbd3 axis regulate induction of cell reprogramming and pluripotency of iPSCs	Mouse neural progenitor cells	OSKM	[199]
hsa-miR-29a	Highly expressed in fibroblasts; miR-29a depletion resulted in global DNA demethylation due to upregulation of TETs and TDGs, eventually leading to enhanced reprogramming efficiency	Human fibroblasts	OSKM	[200]
mmu-miR-212 or mmu-miR-132	Depletion of miR-212 or miR-132 resulted in significantly increased reprogramming efficiency; targeted two crucial epigenetic regulators, namely histone acetyl transferase p300 and H3K4 demethylase Jaird1a, during reprogramming	MEF	OSK +VPA	[201]
let-7	Stimulated the expression of pro-differentiation genes; suppression of the let-7 family facilitated de-differentiation of somatic cells to iPSCs; depletion of let-7 family enhanced reprogramming in absence of c-Myc	MEF [202]; Human fibroblasts [203]	OSK or OSKM	[202, 203]

OSK Oct4, Sox2, Klf4, *OSKM* Oct4, Sox2, Klf4, c-Myc, *MEF* mouse embryonic fibroblasts, *VPA* valproic acid, *ERK* extracellular signal-regulated kinase, *CDC42* cell division cycle 42, *UTR* Untranslated region, *MBD* methyl-CpG binding domain protein, *Wnt3a* Wnt family member 3A, *SMAD4* SMAD family member 4, *SIRT* sirtuin, *let-7* lethal 7, *TETs* ten-eleven translocation proteins, *TDG* thymine-DNA glycosylase, *H3K4* histone 3 lysine 4; *let-7* lethal-7

Table 6 A comprehensive list of small molecules that enhances reprogramming to generate mouse and human iPSCs

Small molecules	Functions	Factors	Host cells	Concentration	Efficiency	References
VPA	Inhibits HDAC	OSK	Mouse	2mM	>100-fold	[107]
5-AZA	Inhibits DNMT	OSKM	Mouse	2μM	~10-fold	[107]
5-AZA + Dexamethasone	Inhibits DNMT Synthetic glucocorticoid	OSKM	Mouse	2μM 1μM	2.6-fold	[107]
SAHA	Inhibits HDAC	OSKM	Mouse	5μM	–	[107]
TSA	Inhibits HDAC	OSKM	Mouse	20nM	–	[107]
5-AZA	Inhibits DNMT	OSKM	Mouse	0.5mM	4-fold	[110]
Thiazovivin + SB431542 + PD0325901	Inhibits ROCK Inhibits ALK4, ALK5, and ALK7 Inhibits MEK/ERK	OSKM	Human	0.5μM 2μM 0.5μM	~200-fold	[138]
Kenpaullone	Inhibits GSK3-β; Activates Nanog expression	OSKM	Mouse	5μM	~2-fold	[123]
NaB	Inhibits HDAC	OSKM	Mouse	1mM	7-fold,	[111]
NaB	Enhance Histone H3 Acetylase	OSKM	Human	0.5mM	>100-fold	[112]
A83-01+ PD0325901	Inhibits TGF-β Inhibits MEK-ERK	OSKM or OSK	Human	0.5μM 0.5μM	–	[128]
PS48	Activates PDK1	OSKM	Human	5μM	15-fold	[128]
PS48+ NaB	Activates PDK1 Inhibits HDAC	OSKM	Human	5μM 0.25mM	25-fold	[128]
Fructose 2,6-bisphosphate	Modulate glycolytic metabolism	OSKM	Human	10mM	–	[128]
Fructose 6- phosphate	Modulate glycolytic metabolism	OSKM	Human	10mM	–	[128]
2,4-Dinitrophenol	Modulate mitochondrial oxidation	OSKM	Human	1μM	–	[128]
N-Oxaloylglycine	Promotes glycolytic metabolism and activates HIF pathway	OSKM	Human	1μM	–	[128]
Quercetin	Activates HIF pathway	OSKM	Human	1μM	–	[128]
Vit. C	Inhibits cell senescence	OSKM or OSK	Mouse	10μg/ml	2.5-3 fold	[114]
Vit. C + VPA	Decreases p53 Inhibits HDAC	OSKM	Human	10μg/ml 1mM	–	[114]
Rapamycin	Inhibits mTOR	OSKM	Mouse	0.3nM	4.8-fold	[204]
PP242	Inhibits mTOR	OSKM	Mouse	0.1nM	5-fold	[204]
PQ401	Inhibits IGF1 receptor	OSKM	Mouse	1μM	4-fold	[204]
LY294002	Inhibits PI3K	OSKM	Mouse	0.3μM	–	[204]
Resveratrol or Fisetin	Activates sirtuin	OSKM	Mouse	3μM	–	[204]
Spermidine	Induces autophagy	OSKM	Mouse	3nM	–	[204]
Curcumin	Antioxidant	OSKM	Mouse	3μM	–	[204]
NaB + SB431542 + PD0325901	Inhibits HDAC Inhibits TGF-β Inhibits MEK/ERK	OSKM	Human	0.5mM 2μM 0.5μM	–	[205]
PD0325901 + A83-01 + CHIR99021 + HA-100	Inhibits MEK/ERK Inhibits TGF-β Inhibits GSK3-β Inhibits ROCK	OSKMNL and SV40LT	Human	0.5μM 0.5μM 3μM 10μM	6-fold	[146]
8-Br-cAMP	Activates cAMP	OSKM	Human	0.1mM	2-fold	[206]
8-Br-cAMP + VPA	Activates cAMP Inhibits HDAC	OSKM	Human	0.1mM 0.5mM	6.5-fold	[206]
LiCl	Inhibits GSK3-β Inhibits LSD1	OSKM	Mouse	5-10mM	–	[117]
RSC133	Inhibits DMNT and histone deacetylase	OSKM	Human	10μM	3-4 fold	[207]
BIM020717	Inhibits P38 kinase	OSKM	Mouse	1μM	–	[142]
BIM0207164	Inhibits IP3K	OSKM	Mouse	1μM	–	[142]

Table 6 (continued)

Small molecules	Functions	Factors	Host cells	Concentration	Efficiency	References
BIM0086660	Inhibits Aurora K Kinase	OSKM	Mouse	1μM	–	[142]
SB431542 or	Inhibits TGF-β	OSKM	Mouse	5μM	–	[208]
PD0325901 +	Inhibits MEK/ERK			1μM		
CHIR99021 +	Inhibits GSK3-β			3μM		
VPA	Inhibits HDAC			0.5mM		
Vit. C + CHIR99021	Antioxidant Inhibits GSK3-β	OSKM	Mouse	50ug/ml 3μM	–	[149]
CYT296	Inhibits H3K9Me / DNMT1 / DNMT3a / DNMT3b	OSKM	Mouse	250nM	10-fold	[148]
Cyclic pifithrin + A83-01 +	Inhibits p53 Inhibits TGF-β	pEP4EO2SET2K	Human	0.3μM 0.5μM	170-fold	[150]
CHIR99021 +	Inhibits GSK3-β			3μM		
Thiazovivin +	Inhibits ROCK			0.5μM		
Sodium Butyrate +	Inhibits HDAC			250μM		
PD0325901	Inhibits MEK/ERK			0.5μM		

O Oct4, *S* Sox2, *K* Klf4, *M* c-Myc, *N* Nanog, *L* Lin28, *VPA* valproic acid, *HDAC* histone deacetylase, *AZA* azacitidine, *DNMT* DNA methyltransferase, *SAHA* suberoylanilide hydroxamic acid, *TSA* trichostatin A, *ALK* activin receptor-like kinase, *ERK* extracellular signal-regulated kinase, *GSK* glycogen synthase kinase, *NaB* Sodium Butyrate, *TGF-β* transforming growth factor β, *PDK* phosphoinositide-dependent kinase, *HIF* hypoxia-inducible factor, *Vit. C* vitamin C, *mTOR* mammalian target of rapamycin, *IGF* insulin-like growth factor, *PI3K* phosphoinositide 3-kinase, *ROCK* rho-associated protein kinase, *cAMP* cyclic adenosine monophosphate, *LiCl* lithium chloride, *H3K9ME* histone 3 lysine 9 methyltransferase

Signaling pathways play a crucial role in the induction and maintenance of pluripotency. Wnt signaling stimulated by soluble Wnt3a molecule was reported to promote nuclear reprogramming of mouse cells [134, 135], even in the absence of c-Myc by activating pluripotency-associated genes giving rise to homogeneous iPS clones [135]. Activation of sonic hedgehog family by sonic hedgehog, oxysterol or purmopharmine in combination with Oct4 was sufficient to reprogram mouse embryonic and adult fibroblasts in the absence of Sox2, Klf4, and c-Myc [136]. Inhibition of Tgf-β signaling by small molecules targeting this specific pathway has been reported to promote reprogramming efficiency [125, 137, 138], and also substitute for Sox2 [125, 137], or c-Myc [137], by inducing Nanog expression [125] and perhaps by promoting MET [129, 138, 139]. Further, inhibition of Tgf-β signaling by Tgf-β inhibitor A-83-01 together with a protein arginine methyltransferase inhibitor AMI-5 facilitated reprogramming of MEF cells transduced with Oct4 alone [140]. Application of BMP7 improved the efficiency of reprogramming by increasing the number of reprogrammed colonies after MEFs were transduced with Yamanaka factors [139]. Silva and coworkers reported that neural stem cells could be rapidly reprogrammed to iPSCs by using only two factors, Oct4 and Klf4, and further treatment of the generated pre-iPSCs with GSK3 inhibitor CHIR99021 and ERK inhibitor PD0325901 aided them to enter a more genuine naive pluripotent state [141]. Li and Rana identified kinases such as Tgf-β (specifically activin receptor-like kinase 4, 5), inositol triphosphate 3-kinase, Aurora A kinase and p38 mitogen-activated protein kinase that acted as a barrier to cell reprogramming, and demonstrated that inhibition of these kinases by specific small molecules resulted in enhanced iPSC formation [142]. Inhibition of other kinases such as GSK3 [123, 124, 141, 143], MEK/ERK [121, 138, 141, 143], Src family tyrosine kinase [126] and rho-associated protein kinase [138, 144] have also been reported to enhance iPSC generation. In addition, small molecules that inhibits cellular senescence (cyclic Pifithrin-α, Vitamin C) and promote glycolytic metabolism (fructose 2, 6-bisphosphate, PS48, N-Oxaloylglycine, quercetin, etc.) and autophagy metoabolism (Rapamycin, PP242, etc.) also play a crucial role in reprogramming (Table 6). Unlike mouse somatic cells, human somatic cells are more complex making it difficult to reprogram them by using only small molecules. To the best of our knowledge, there is no study reported that demonstrates the derivation of human iPSCs by using exclusively small molecules. Importantly, the extra-cellular signaling pathways crucial for self-renewal and maintenance of pluripotency of mouse and human pluripotent stem cells are different, therefore, small molecules identified in mouse reprogramming studies may not possibly work for human studies. Hence, the effect of small molecules belonging to a specific class may need to be pursued individually for both mouse and human reprogramming studies.

Similarly, other studies have also reported the importance of various small molecule combinations to reprogram mouse and human iPSCs [133, 145–152]. However, amidst all the advantages, the major issues related to small molecules

Table 7 A comprehensive list of small molecules that can substitute one or more Yamanaka factors to derive mouse and human iPSCs

Small molecule(s)	Function	Host cells	Compensates for YF	Concentration used	Efficiency	References
VPA	Inhibits HDAC	Mouse	M	2mM	50-fold	[107]
VPA	Inhibits HDAC	Human	KM	0.5-1mM	10-20 fold	[108]
BIX-01294	Inhibits G9a HMTase	Mouse	SM	0.5-1μM	–	[119]
BIX-01294	Inhibits G9a HMTase	Mouse	O	0.5-1μM	–	[119]
BIX-01294	Inhibits G9a HMTase	Mouse	SM	1μM	–	[121]
BIX-01294 +	Inhibits G9a HMTase	Mouse	SM	1μM	–	[121]
BayK8644	L-type Ca+2 Channel agonist			2μM		
BIX-01294 +	Inhibits G9a HMTase	Mouse	SM	1μM	–	[121]
RG108	Inhibits DMNT			0.5-2μM		
616452	Inhibits ALK5	Mouse	S/M	1μM	2.5-fold	[137]
SB431542	Inhibits ALK4/5/7	Mouse	S/M	2μM	–	[137]
Kenpaullone	Activates Nanog expression	Mouse	K	5μM	10-fold lower	[123]
CHIR99021	Inhibits GSK3-β	Mouse	SM	10μM	–	[124]
CHIR99021 + Tranylcypromine	Inhibits GSK3-β Inhibits Lysine-specific demthylase	Human	SM	10μM 2μM	–	[124]
CHIR99021 + Tranylcypromine +	Inhibits GSK3-β Inhibits H3K4 demthylase	Human	SM	10μM 2μM	–	[124]
PD0325901 +	Inhibits MEK-ERK			0.5μM		
SB431542	Inhibits ALK4/5/7			2μM		
Repsox (E-616452)	Inhibits TGF-β type 1 receptor	Mouse	S	25μM	–	[125]
SB431542	Inhibits ALK4/5/7	Mouse	S	25μM	–	[125]
NaB+ PS48+	Inhibits HDAC Activates PDK1	Human	SKM	0.25mM 5μM	–	[128]
A83-01+	Inhibits TGF-β			0.5μM		
PD0325901	Inhibits MEK-ERK			0.5μM		
Tranylcypromine+ CHIR99021	Inhibits H3K4 demethylase Inhibits GSK3-β	Human	SKM	2μM 3μM	–	[128]
LiCl +	Inhibits GSK3-β,	Mouse	SKM	5mM	–	[117]
	Inhibits LSD1					
VPA +	Inhibits HDAC			5mM		
Repsox	Inhibits TGF-β type 1 receptor			1μM		
LiCl +	Inhibits GSK3-β, Inhibits LSD1	Human	SKM	5mM	–	[117]
PS48 +	Activates PDK1			5μM		
NaB+	Inhibits HDAC			0.25mM		
A83-01 +	Inhibits TGF-β			0.5μM		
CHIR99021	Inhibits GSK3-β			3μM		
A83-01 +	Inhibits TGF-β	Human	SKM	1μM	~0.02%	[140]
AMI-5	Inhibits protein arginine methyl transferase			5μM		
VPA +	Inhibits HDAC	Mouse	SKM	0.5mM	–	[129]
CHIR99021 +	Inhibits GSK3-β			3μM		
616452 +	Inhibits TGF-β			1μM		
Tranylcypromine	Inhibits H3K4 demthylase			5μM		
iPYrazine	Inhibits Src family tyrosine kinase	Mouse	S	10μM	–	[126]
Dasatinib	Inhibits Src family tyrosine kinase	Mouse	S	0.5μM	–	[126]
PP1	Inhibits Src family tyrosine kinase	Mouse	S	10μM	–	[126]
LY-364947	Inhibits TGF-β	Mouse	S	1μM	–	[126]
Sonic hedgehog	Activates Sonic hedgehog signaling	Mouse	SKM	500ng/ml	–	[136]

Table 7 (continued)

Small molecule(s)	Function	Host cells	Compensates for YF	Concentration used	Efficiency	References
Oxysterol	Activates Sonic hedgehog signaling	Mouse	SKM	0.5-1μM	–	[136]
Purmorphamine	Activates Sonic hedgehog signaling	Mouse	SKM	0.5-1μM	–	[136]
Forskolin +	cAMP agonist	Mouse	O	10-50μM	–	[130]
2-Me-5HT +	5-HT3 agonist			5μM		
D4476	Inhibits CK1			5μM		
VPA +	Inhibits HDAC	Mouse	OSKM	500μM	0.2%	[130]
CHIR99021 +	Inhibits GSK3-β			10μM		
616452 +	Inhibits TGF-β			5-10μM		
Tranylcypromine +	Inhibits H3K4 demethylase			5-10μM		
Forskolin + PD0325901 +	cAMP agonist Inhibits MEK/ERK			10-50μM 1μM		
DZNep	Inhibits S-adenosyl-homocystein hydrolase			0.05-0.1μM		
SB431542	Inhibits TGF-β	Mouse	O	5μM	–	[208]
BrdU	A synthetic nucleoside thymidine analog	Mouse	O	2.5-10μM	–	[131]
BrdU +	A synthetic nucleoside thymidine analog	Mouse	OSKM	0-10μM	–	[131]
CHIR99021 +	Inhibits GSK3-β			10μM		
RepSox +	Inhibits TGF-β type 1 receptor			10μM		
Forskolin +	cAMP agonist			50μM		
BrdU +	A synthetic nucleoside thymidine analog	Mouse	OSKM	0-10μM	–	[131]
CHIR99021 +	Inhibits GSK3-β			10μM		
RepSox +	Inhibits TGF-β type 1 receptor			10μM		
Forskolin +	cAMP agonist			50μM		
DZNep	Inhibits S-adenosyl-homocystein hydrolase			50nM		
BrdU +	A synthetic nucleoside thymidine analog	Mouse	OSKM	0-10μM	–	[131]
VPA +	Inhibits HDAC			0.5mM		
CHIR99021 +	Inhibits GSK3-β			10μM		
RepSox +	Inhibits TGF-β			10μM		
Tranylcypromine +	Inhibits H3K4 demethylase			5μM		
Forskolin +	cAMP agonist			50μM		
TTNPB +	Retinoic acid analog			1μM		
DZNep	Inhibits S-adenosyl-homocystein hydrolase			50nM		
VPA +	Inhibits HDAC	Mouse	OSKM	0.5μM	5-fold	[132]
CHIR99021 +	Inhibits GSK3-β			20μM		
616452 +	Inhibits TGF-β			10μM		
Tranylcypromine +	Inhibits H3K4 demethylase			5μM		
Forskolin +	cAMP agonist			10μM		
DZNep +	Inhibits S-adenosyl-homocystein hydrolase			0.05μM		
PD0325901 +	Inhibits MEK/ERK			1μM		
EPZ004777/SGC0946 +	Inhibits DOT1L			5μM		
AM580 +	Activates retinoic acid receptor			0.5μM		
5-AZA	Inhibits DNMT			0.5μM		
VPA +	Inhibits HDAC	Mouse	OSKM	0.5mM	–	[133]
CHIR99021 +	Inhibits GSK3-β			10μM		
616452 +	Inhibits TGF-β			10μM		
Tranylcypromine +	Inhibits H3K4 demethylase			10μM		

Table 7 (continued)

Small molecule(s)	Function	Host cells	Compensates for YF	Concentration used	Efficiency	References
Forskolin +	cAMP agonist			10μM		
AM580 +	Activates retinoic acid receptor			0.05μM		
DZNep	Inhibits S-adenosyl-homocystein hydrolase			0.05μM		
VPA +	Inhibits HDAC	Mouse	OSKM	0.5mM	–	[133]
CHIR99021 +	Inhibits GSK3-β			15μM		
616452 +	Inhibits TGF-β			2 or 5μM		
Tranylcypromine +	Inhibits H3K4 demethylase			10μM		
Forskolin +	cAMP agonist			20μM		
Ch 55 +	Retinoic acid receptor agonist			1μM		
EPZ004777 +	Inhibits DOT1L			5μM		
DZNep	Inhibits S-adenosyl-homocystein hydrolase			0.05μM		
VPA +	Inhibits HDAC	Mouse	OSKM	0.5mM	–	[133]
CHIR99021 +	Inhibits GSK3-β			10μM		
616452 +	Inhibits TGF-β			10 or 20μM		
Tranylcypromine +	Inhibits H3K4 demethylase			10μM		
Forskolin +	cAMP agonist			10μM		
AM-580 +	Retinoic acid receptor agonist			0.05μM		
DZNep	Inhibits S-adenosyl-homocystein hydrolase			0.05μM		

YF Yamanaka factor, *O* Oct4, *S* Sox2, *K* KIf4, *M* c-Myc, *VPA* valproic acid, *HDAC* histone deacetylase, *HTMase* histone methyltransferase, *DNMT* DNA methyltransferase, *ALK* activin receptor-like kinase, *GSK* glycogen synthase kinase, *ERK* extracellular signal-regulated kinase, *TGF-β* transforming growth factor β, *NaB* Sodium Butyrate, *LiCl* lithium chloride, *PDK* phosphoinositide-dependent kinase, *H3K4* histone 3 lysine 4, *cAMP* cyclic adenosine monophosphate, *2-Me-5HT* 2-methyl-5-hydroxytryptamine, *BrdU* Bromodeoxyuridine, *CK1* casein kinase 1, *AZA* azacitidine, *DZNep* 3-deazaneplanocin A, *DOT1L* Disruptor of telomeric silencing 1-like (histone H3K79 methyltransferase)

curbing their widespread usability is their pleiotropic effects, unexpected toxicity and side effects [152, 153]. For example, 5-AZA is a cytosine analog, incorporated into DNA during S-phase, where it covalently binds to DNMTs. It is also a potent chemotherapeutic drug but is reported to be fatal for renal patients [154]. It is also speculated to induce mutations [155], causing tumor formation subjecting patients at high risk [156]. Another analog, BrdU is a synthetic thymidine analog that is incorporated into DNA during cell division, and such DNA modifications may induce mutations if used at high concentrations and result in genomic instability [131]. Another example is VPA, which is an anti-epileptic and mood-stabilizing drug. It has been shown that VPA downregulated expression of proteins essential for maintenance of chromatin [157]. In addition, VPA also showed various organ level side effects such as dermatologic effect, abnormalities in fetus development and so forth [158]. Importantly, some of these small molecules identified are potent modifiers of DNA and chromatin, and may possibly introduce unwanted epigenetic modifications resulting in a global dysregulation of gene expression in the iPSCs generated [159]. Therefore, iPSCs derived using such molecules require thorough screening at the genomic level before its therapeutic use. However, molecules like RG108 of non-nucleoside analog inhibitor family are specific and require no incorporation into DNA, hence, are less cytotoxic compared to nucleoside analog inhibitor family compounds like 5-AZA. Additionally, small molecules that promote cell cycle or target specific signaling pathways are of particular interest compared to those which alter the epigenetic landscape. Likewise, the current list of small molecules used for the derivation of iPSCs needs a thorough understanding of the chemical nature. Hence, more research is needed to elucidate promising combination of small molecules, their dosage and time of administration to bring forth the full potential of iPSCs in patient-specific treatments in the near future. In conclusion, small molecules represent a powerful complementary alternative and establishment of small molecules that are highly specific without modifying the genome is desirable to produce clinical-grade iPSCs for biomedical applications [160].

Conclusion

The discovery of iPSCs brought with it a wave of new opportunities, and represent a non-controversial, unlimited source of pluripotent stem cells. These cells can be differentiated into any desired cell type(s), and therefore has a tremendous potential for personalized human medicine and to understand developmental biology. Most of the reprogramming studies that have generated iPSCs from a variety of cell types involved an ectopic expression of reprogramming factors using commonly used integrating viral vectors. However, the risk of transgene integration and reactivation, insertional mutagenesis and induction of malignant transformation has made these approaches unsuitable for the generation of transgene-free iPSCs. This has prompted scientists to explore and develop novel methods of iPSC derivation that do not involve permanent genomic alterations.

Taking this into consideration, various alternative techniques are explored with minimal or no genetic modifications of cells such as adenoviral vectors, adeno-associated viral vectors, Sendai virus vectors, plasmid transfection, minicircle vectors, transposon vectors, episomal vectors, liposomal magnetofection, recombinant proteins, microRNAs, synthetic messenger RNA and small molecules [13, 14]. These methods circumvent the possibilities of transgene reactivation and insertional mutagenesis [161–163]. In this review, we have provided a comprehensive overview of the most promising DNA-free reprogramming approaches that involve no genomic integration. Non-integrating Sendai virus-based approach avoids the risks posed by integrating viral vectors and have shown commendable potential in ectopically expressing reprogramming factors and generating footprint-free iPSCs with high reprogramming efficiency from both mouse and human cells. Development of novel chimeric viral vectors is in progress combining special features to generate clinical-grade iPSCs. To date, the recombinant protein-based reprogramming method is slow and inefficient, and therefore it is less popular among the stem cell researchers. But this approach is the safest among all the reprogramming techniques employed [161–163], and therefore further improvements addressing various bottlenecks associated with it should be pursued vigorously. Significant improvements are made in the mRNA-based approach to reduce immunogenicity and the requirement of the number of transfections to derive iPSCs with high efficiency and faster kinetics. Several miRNAs and small molecules have been identified that can either enhance reprogramming efficiency or replace one or more of the essential reprogramming factors to derive transgene-free iPSCs. However, all these reprogramming methods still have a host of challenges to overcome for its efficient use and have been reported to reprogram only limited cell types such as fibroblasts. Further improvements to these approaches are in pursuit to make them more promising to derive iPSCs from different cell sources. Numerous recent studies have demonstrated improved efficiency and/or better kinetics when more than one of these techniques are used in a combination. Therefore, the current advancements are directed towards establishing simple, efficient and rationally designed standardized integration-free techniques that can reprogram easily accessible cell source obtained non-invasively from patients, particularly under feeder-free and xeno-free conditions. This permits us to achieve non-immunogenic, clinical-grade, patient-specific iPSCs with higher reprogramming efficiency and faster kinetics. In the near future, these cells can then be differentiated into desired cell type(s) using robust differentiation protocols for personalized regenerative medicine.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interests.

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