Inducing pluripotency *in vitro*: recent advances and highlights in induced pluripotent stem cells generation and pluripotency reprogramming

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Abstract

Induced pluripotent stem cells (iPSCs) are considered patient-specific counterparts of embryonic stem cells as they originate from *somatic cells* after forced expression of pluripotency reprogramming factors Oct4, Sox2, Klf4 and c-Myc. iPSCs offer unprecedented opportunity for personalized cell therapies in regenerative medicine. In recent years, iPSC technology has undergone substantial improvement to overcome slow and inefficient reprogramming protocols, and to ensure clinical-grade iPSCs and their functional derivatives. Recent developments in iPSC technology include better reprogramming methods employing novel delivery systems such as non-integrating viral and non-viral vectors, and characterization of alternative reprogramming factors. Concurrently, small chemical molecules (inhibitors of specific signalling or epigenetic regulators) have become crucial to iPSC reprogramming; they have the ability to replace putative reprogramming factors and boost reprogramming processes. Moreover, common dietary supplements, such as vitamin C and antioxidants, when introduced into reprogramming media, have been found to improve genomic and epigenomic profiles of iPSCs. In this article, we review the most recent advances in the iPSC field and potent application of iPSCs, in terms of cell therapy and tissue engineering.

Introduction

Pluripotency is the ability of cells to undergo indefinite self-renewal and differentiate into all specialized cell lineages (1). This developmental potential is a natural property of mammalian embryonic stem cells (ESCs) and enables their use in developmental studies and regenerative medicine (1). Clinical exploitation of this developmental plasticity, however, requires an alternative source of pluripotent cells to avoid ethical and mechanistic limitations inherent in consideration of the use of human embryonic stem cells (hESCs). Early cell reprogramming techniques, such as somatic cell nuclear transfer (SCNT) (2–4) and transdifferentiation (5) indicated that phenotype identity can be reprogrammed. Animal cells possess considerable plasticity which under certain *in vitro* conditions can switch their fate. This discovery paved the way for development of induced pluripotent stem cell lines (iPSC lines). In a revolutionary study, Takahashi and Yamanaka *et al.* demonstrated that forced expression of four ESC-specific transcription factors (TFs)/reprogramming factors – Oct4 (octamer-binding transcription factor 4), Sox2 (sex determining region Y box 2), Klf4 (Krueppel-like factor 4) and c-Myc (v-Myc myelocytomatosis avian viral oncogene homolog), the OSKM Yamanaka factors, reprogramme adult mouse fibroblasts to become mouse ESC-like cells, termed *induced pluripotent stem cells* (iPSCs) (6). In the following year, Takahashi *et al.* and Yu *et al.*, independently reported human induced pluripotent stem cells (hiPSCs) from human fibroblasts (Fig. 1) (7,8). A distinctive feature of OSKM-induced iPSCs (OSKM-iPSCs) is that they resemble ESCs. iPSCs conform to the major aspects of pluripotency, such as pluripotent state-specific marker gene expression, unlimited self-renewal, multilineage differentiation potential (assayed *via* embryoid body and teratoma formation techniques) and germline transmissibility (8–11). Mouse iPSCs are also used to produce viable all-iPSC mice by the tetraploid

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Figure 1. Reprogramming adult somatic cells into induced pluripotent stem cells (iPSCs) through ectopic expression of reprogramming factors. Forced expression of these pluripotency factors resets the epigenetic and transcriptional profile of the specialized cells and reverts them back to their embryonic state.

blastocyst complementation technique (12,13); a key assay for assessing true cell pluripotency, strictly ascribed to hiPSCs. The prospect of obtaining OSKM-iPSCs from somatic cell origins promises an authentic source of patient-specific pluripotent cells for clinical application. A plethora of studies published so far has reported obtaining authentic iPSCs from a large variety of mouse and human somatic cells, employing different strategies and combinations of reprogramming factors (see Table S1).

Early reprogramming endeavours relied on viral delivery systems such as by retrovirus or lentivirus (14–33), however, non-viral vectors, for example episomes, minicircle vectors, transposons, human artificial chromosome vectors and nanoparticle carriers, have subsequently emerged as alternatives to avoid complications of viral reprogramming (40–50) (Fig. 2). Analyses of the pluripotency gene regulatory network has helped distinguish alternative reprogramming factors (60–82) and small chemical inhibitors (87–107) to alleviate existing challenges to iPSC development, including poor reprogramming efficiency and conversion of partially reprogrammed cells into iPSCs. Recent studies also suggest that *in vitro* nutritional supplements such as vitamin C and antioxidants improve the quality of iPSCs (108–114). These advancements may enable clinical-grade

patient-specific iPSCs for therapeutic application. Hence in this review, we summarize the most recent advances and current status of iPSC technology.

Recent advances in pluripotency reprogramming

Delivery systems

Introducing reprogramming factors (RFs) to target cells is the first step in pluripotency induction. Several delivery systems have been developed for this task, including viral vectors (14–39), non-viral vectors (40–50) and the direct transfection approach (51–54) (see Fig. 2).

Viral vectors. Integrating viral vectors (IVVs) such as retroviruses (14–24) and lentiviruses (25–29,122) are the most common delivery system for cell reprogramming and iPSC generation (14–29). IVVs deliver and maintain persistent expression of RFs; however, this process results in integration of multiple proviral copies throughout host cell genomes – raising the possibility of genomic instability and chromosomal aberration (6,9,10). Polycistronic viral vectors (PVVs) can overcome problems associated with IVVs (30,31). PVVs are single viral constructs which carry all RFs that are separated by autonomous self-cleaving peptides such as 2As. In

Figure 2. Overview of the approaches available for generating induced pluripotent stem cells (iPSCs). Somatic cells can be reprogrammed into iPSCs using viral/non-viral delivery system or direct application of the reprogramming factors, their mRNAs or embryonic stem cell-specific miRNAs.

PVVs, RFs are expressed from a single promoter, producing a single transcript. Two existing lentivirus-based PVVs have shown adequate exogenous expression that only a single proviral copy is needed to reprogramme a somatic cell, with high fidelity (30,31), although leaky expression from these viral constructs may still occur. An alternative choice might be Lox-Cre-mediated excision of viral constructs from loxp-flanked genomic sites (32,33) which produces iPSCs free of exogenous elements. However, the likelihood of minute viral remnants is still a concern for clinical applicability of iPSCs (32). In this regard, non-IVVs that avoid genomic integration, such as Adenovirus and Sendai virus (SeV) systems, seem to be a reasonable choice for generating iPSCs (34–39). Adeno-iPSCs are devoid of viral integration (34,35), although minor incidence of tetraploid adeno-iPSC has also been reported (34). SeV vectors also generate authentic iPSCs without being incorporated into the host genome, while retaining high conversion

efficiency (36,37,119,120). SeV vectors gradually deplete from iPSC cytoplasm after several passages which makes subsequent iPSC generations exogenous element free (36,37,120). To ensure complete removal of viral constructs, a temperature-sensitive Sendai vector mutant may be used (38,39).

Non-viral vectors. Several non-viral vectors have been described for iPSC reprogramming which circumvent the problems of viral vectors. These include episomal vectors (40–42), minicircle (mc) vectors (43), synthetic RNA replicons (44), human artificial chromosomes (45), transposon systems (46,47) and nanoparticle carriers (48–50).

The Epstein–Barr virus-derived oriP/EBNA1 episomal vector has been extensively used to generate footprint free iPSCs. This vector is non-integrating, persists throughout reprogramming and subsequently diminishes in iPSCs (40,41). A further non-viral vector is p2phic31 plasmid-derived minicircle (mc). Its reprogramming

efficiency is greater than standard plasmid/episome vectors and generates true iPSCs (43). Self-replicating RNA vectors are another potential delivery method for footprint-free iPSCs (44). Based on non-infectious, self-replicating Venezuelan equine encephalitis (VEE) viral RNA, the positive-sense polycistronic VEE ssRNA construct (VEE-RF RNA replicon) can transduce RFs into host cells and maintain transgene expression for successful reprogramming (44). Interestingly, human artificial chromosome (HAC) vectors can also be effective in generating vector and transgene-free iPSCs (45).

In addition, transposon systems such as piggybac (PB) and sleeping beauty (SB) can be used to produce iPSCs. Successful excision of PB from iPSC-genomes has been reported (46), although SB systems require further optimization to ensure exogenous construct removal.

There is growing interest in nanoparticle and synthetic carriers as RF delivery systems for iPSC reprogramming. For example, using cationic Bolaamphiphile coupled with Klf4, Nanog, NR5A2 (orphan nuclear receptor) and Sox2, Khan *et al.* successfully reprogrammed human fibroblasts (48). Using a different cationic lipid carrier, Lipofectamine RNAiMAX (LF), fused with OSKM mRNAs, Tavernier *et al.* generated miPSCs from mouse fibroblasts (49). Sohn *et al.* utilized ESC-specific microRNAs (miR-302/367 cluster) fused with polyketal copolymer PK3 to generate miPSCs (50). While these synthetic vectors successfully generate transgene-free iPSCs, the labour-intensive method involved has largely limited their use.

Direct transfection. In addition to vector-based approaches, direct transfection methods have been adopted also. Warren *et al.* demonstrated that repeated administration of synthetic Oct4, Sox2, Klf4, c-Myc and Lin28 mRNAs generates authentic hiPSCs (51). Miyoshi *et al.* generated mouse and human iPSCs by direct delivery of ESC-specific miRNAs (mir-200c, mir-302s and mir-369) (52). Direct delivery of purified RFs also has been successful in producing iPSCs. Repeated administration of OSKM factors fused with cell-penetrating peptide 9-arginine (RFP-9R) can produce hiPSCs (53). Cho *et al.* showed that a single transfection of whole cell protein extract from mESCs can reprogramme adult mouse fibroblasts into iPSCs (54). In contrast, neither cytoplasmic nor nuclear fraction-derived protein extracts from mESCs was able to induce reprogramming alone (54). This suggests a combination of factors from different cell compartments acts together for pluripotency reprogramming.

Alternative reprogramming factors for iPSCs

While ESC-specific miRNAs can generate iPSCs (52,55), OSKM (Oct4, Sox2, Klf4 and c-Myc)-based

reprogramming remains the most convenient way of generating them (14–47,120,121); however, poor reprogramming efficiency of OSKM (usually less than 1%) is still a key concern (56). Lately, researchers have characterized a range of novel factors that may substitute OSKM and facilitate improved reprogramming with greater efficiency when inducing pluripotency (Table 1).

Alternative factors for c-Myc

c-Myc's role in maintenance and acquisition of pluripotency has been disputed. Despite its ability to boost reprogramming efficiency of a broad pool of somatic cells (10,56,57), forced expression of c-Myc can also cause tumourigenicity (6). In addition, a multitude of reports has demonstrated that deregulation of c-Myc can trigger genomic instability and induce a broad range of cancer progression (58,59). Thus, uncontrolled expression of c-Myc is a potential risk for iPSCs. As excluding c-Myc from the reprogramming cocktail reduces reprogramming efficiency (10,11), discovery of a potent alternative of this proto-oncoprotein needs to be prioritized. Recent studies suggest that previously reported factors may have a similar mode of action during pluripotency reprogramming. For example, substituting c-Myc with UTF1 (undifferentiated embryonic cell transcription factor 1) generates iPSCs with reprogramming efficiency of around 3×10^{-4} which is approximately 10 times higher than OSK transfection (60). Similarly, c-Myc's substitution with Wnt3a (a soluble Wnt signalling protein) can generate homogenous ESC-like colonies with higher efficiency – 20-fold more iPSCs from OSK-transfected mouse fibroblasts with Wnt3a versus OSK transfection only (61). A further alternative to c-Myc is GLIS1 (Glis family zinc finger protein 1), a maternally expressed transcription factor. GLIS1 increases numbers of fully-reprogrammed iPSCs in the absence of c-Myc (62). Kdm2b, a histone H3 Lys 36 dimethyl-specific demethylase, can also be used to substitute c-Myc and has resulted in improved reprogramming of OSK-transfected mEFs – 4 to 6 fold more iPSCs from OSK+Kdm2b than OSK only (63). Chiou *et al.* demonstrated that both Parp1 and Parp 2 proteins (poly ADP ribose polymerases) can replace c-Myc with increased reprogramming efficiency (64). Interestingly, Parp 1 together with OS can reprogramme mouse fibroblasts with higher efficiency – reprogramming efficiency close to 2.2% – suggesting Parp 1 to be a promising candidate for future pluripotency reprogramming. T-box transcription factor 3 (Tbx3) is a pluripotency-associated transcription factor which drastically improves iPSC quality when transduced with OSK (65). Compared to OSK-iPSCs, addition of Tbx3 accelerates the reprogramming

Table 1. Alternative reprogramming factors for induced pluripotency

Factors	Function	Accompanied factors	References
UTF1	Enhances reprogramming efficiency, improves pre-iPSCs to iPSCs transition and can replace c-Myc	OSKM or OSK	60
Wnt3a	Replaces c-Myc, endows higher reprogramming efficiency and homogenous iPSCs colonies	OSK	61
Esrrb	Replaces reprogramming factor Klf4	OSM or OS	71
Nr5a1/Nr5a2	Functionally substitutes Oct4, improves reprogramming efficiency and kinetics	SKM	81
Tbx3	Accelerates reprogramming, increases the number of fully reprogrammed cell, improves germline competency of iPSCs	OSK	65
BMP4	Can replace Sox2, Klf4 and c-Myc, facilitates mesenchymal-to-epithelial transition (MET)	SK	72
E-cadherin	Replaces Oct4 during reprogramming	SKM	80
GLIS1	Effectively replaces c-Myc	OSK	61
Bmi1	Can replace Sox2, Klf4 and c-Myc	Oct4	75
Oxysterol or purmorphamine	Can replace Sox2, Klf4 and c-Myc	Oct4	
Nanog	Can replace Oct4	Bmi1/oxysterol/purmorphamine	79
Parp1/Parp2	Significantly enhance reprogramming efficiency, able to replace Klf4 and c-Myc	OSKM or OSM	67
Kdm2b	Can replace c-Myc, improves reprogramming efficiency	OSK	63
Zic3	Promising candidate for c-Myc replacement, improves iPSCs yield	OSK	66
Zscan4	Replaces c-Myc, improves reprogramming efficiency, amend iPSC quality (complete developmental potential, enhanced telomere lengthening and stabilized genomic integrity)	OSK	68,69
RCOR2	Can substitutes Sox2	OKM	70
Id3	Alone can transdifferentiates mEFs into NSC/NPCs-like state, potential substitute for Sox2, Klf4 and c-Myc	Oct4	76
Tet1	Potent alternative of Oct4, improves reprogramming efficiency	SKM or SK	82
EpCAM	Improves iPSCs yield	OSKM and Cldn7	83
HMGA1	Enhances reprogramming efficiency up to 2-fold	OSKM	84
Zfp296	Increases number of iPSC colonies, accelerates reprogramming	OSKM	85

O, Oct4; S, Sox2; K, Klf4; C, c-Myc.

process and increases numbers of fully-reprogrammed cells – up to 34 more OSKT-iPSC colonies compared to OSK alone. OSKT-iPSCs also exhibit improved germ-

line transmissibility indicating closer resemblance to ESCs (65). Thus, Tbx3 action on hiPSC generation needs to be investigated.

Zic3 (zinc finger transcription factor in cerebellum-3) can also be used to replace c-Myc and results in improved iPSC yield from mouse embryonic fibroblasts (mEFs) with OSK. OSKZ-iPSCs remarkably resemble mESCs in aspects of morphology, epigenomic profile and developmental potential (66). However, it has been reported that OSKZ overexpression converts human fibroblasts into neuronal progenitor-like cells (NPCs) (67). Thus, further investigation needs to be performed to determine whether Zic3 primes cells into the neuronal fate only in human fibroblasts, or whether other human somatic cells are reprogrammable with Zic3.

Zscan4 is a promising candidate for c-Myc replacement too. Zscan4 is a zygote-specific factor, which in the absence of c-Myc, can reprogramme mouse and human somatic cells with markedly higher efficiency (up to 6.7%) in conjunction with OSK (68,69). Most importantly, Zscan4 substantially improves quality of resultant iPSCs by restoring complete developmental potential (all-iPSC mice), telomere elongation in OSK+Zscan4-iPSCs compared to putative OSKM-iPSCs, maintenance of genomic stability and reduction of DNA damage response underlying iPSC-based reprogramming (69).

Alternative factors for Oct4/Sox2/Klf4

Embryonic stem cells-specific co-repressor RCOR2 can be substituted for Sox2 in reprogramming mouse and human somatic cells, and generates iPSCs (70). In contrast, Esrrb (estrogen-related receptor beta) can replace Klf4, although reprogramming efficiency declines (71). Similarly, BMP4 (bone morphogenetic protein 4) can supplant Klf4 during mouse fibroblast reprogramming (72). Using modified culture conditions, BMP4 and Oct4 can reprogramme mouse fibroblasts with reasonable efficiency and kinetics, supporting BMP4 potential as a RF (72). However, previous reports have suggested that BMP4 supports mESCs maintenance (73) and triggers differentiation in hESCs (74). Thus, generating hiPSCs with BMP4 requires further investigation.

A further promising reprogramming factor is Bmi1 (polycomb complex protein BMI-1). Bmi1 can functionally replace Sox2, Klf4 and c-Myc, and along with Oct4 generate authentic iPSCs from mEFs with increased reprogramming efficiency of 0.17% (75). In addition, Bmi1-Oct4-iPSCs closely resemble mESCs and fulfil all criteria of pluripotency (75). Id3 (inhibitor of differentiation 3) seems to be another interesting candidate for replacing Sox2, Klf4 and c-Myc. Id3 overexpression transdifferentiates mEFs into NSC/NPC-like phenotypes which can be further reprogrammed into iPSCs with Oct4 alone (76). Both Bmi1 and Id3 should be further

investigated in human somatic cells, as they show promise as potential RFs.

Initially, researchers presumed that Oct4 presence was essentially indispensable for inducing pluripotency, however, recent reports have contradicted this notion. For example, Moon *et al.* (2013) reported that Nanog, the core guardian of ESC pluripotency (77,78), together with Bmi1 can reprogramme mouse fibroblasts into iPSCs, in the absence of Oct4 (79). Ectopic expression of E-cadherin can also compensate for exogenous Oct4 requirement and generates iPSCs in concert with Sox2, Klf4 and c-Myc (80). Although reprogramming efficiency is relatively low compared to the OSKM method, these Oct4-free ESKM-iPSCs display similarities in morphology, epigenetic and developmental potential, as mESCs (80). Similarly, orphan nuclear receptors (Nr5a2 and Nr5a1) can substitute Oct4 and generate iPSCs with Sox2, Klf4 and c-Myc (SKM) from mEFs (81). Tet1, a DNA demethylating factor in mammalian systems, can also substitute Oct4 and in conjunction with SKM or SK reprogrammes differentiated somatic cells into authentic iPSCs (82). Tet1 induction improves reprogramming efficiency, and TSKM-iPSCs resemble OSKM-iPSCs and ESCs. The Tet1 mode of action is by demethylating endogenous Oct4 loci in a 5mC-to-5hmC manner (82).

These studies indicate that the complexity of transcriptional regulation governing pluripotency involves crosstalk between several key factors and there are multiple counterparts of major pluripotency factors.

Chemical supplements for reprogramming: introducing small molecules

Even though methods employing miRNA, mRNA or direct delivery of RFs for inducing pluripotency are well established (see Table S1), development of novel reprogramming techniques involving fewer RFs while increasing iPSC yield are worth pursuing. This endeavour has ultimately prioritized small molecules/chemical supplements for cell reprogramming.

There is growing evidence to support the notion that small molecules (SMs) may revolutionize the iPSC field by replacing RFs and improving overall reprogramming efficiency-kinetics (Table 2). Many of these chemical supplements are inhibitors of major signalling pathways and epigenetic regulators (86). For instance, valproic acid (VPA) is a histone deacetylase (HDAC) inhibitor which ameliorates OSKM-based reprogramming efficiency (87). Similarly, 5'-azacytidine (5-azac), a DNA methyltransferase inhibitor, increases numbers of fully reprogrammed iPSCs from OSKM-transfected cells, in a dose-dependent manner (88). 5-azac + VPA treatment of

Table 2. Small molecules for induced pluripotent stem cells (iPSCs) generation

Compound	Function	Associated factors	References
Valproic acid (VPA)	A histone deacetylase (HDAC) inhibitor. Improves reprogramming efficiency, replaces c-Myc and Klf4 during reprogramming	OS	87
5'-azacytidine (5'-azac)	A DNA methyltransferase inhibitor. Amends reprogramming efficiency and number of reprogrammed cells in a dose-dependent manner	OSKM/OSK	88
BIX-01294 (BIX)	An inhibitor of the G9a histone methyltransferase. Functionally substitutes exogenous c-Myc and Sox2 during reprogramming, improves poor reprogramming efficiency	OK	93
PD0325901	MEK inhibitor. Promotes growth of fully reprogrammed iPSCs in later stage of reprogramming	OSKM	
Bayk	An L-calcium channel agonist. Together with BIX substitutes c-Myc and Sox2 and significantly increases number of reprogrammed cells	OK	100
CHIR99021	A GSK3b inhibitor. Together with PD0325901 and A-83-01 reprogrammes human somatic cells to ground state pluripotency,	OSNL	103
Repsox (E-616452)	Can replace Sox2 and c-Myc A transforming growth factor β receptor 1 (TGF β 1) kinase inhibitors. Substitutes exogenous Sox2 and c-Myc requirement during reprogramming. Assists partially reprogrammed cells to authentic pluripotent state	OSK or OK OK	95 99
SB431542	ALK5 inhibitor. Together with PD0325901 and thiazovivin drastically improves reprogramming efficiency and kinetics	OSKM	104
A-83-01	Potent TGF- β receptor inhibitor. Induces ground-state pluripotency in Oct4-transfected human somatic cell in conjugation with PS48, sodium butyrate and PD0325901	O	105
AMI-5	A protein arginine methyltransferase (PRMT) inhibitor. In concert with A-83-01 reprogrammes somatic cell with Oct4 alone	O	106

(continued)

Table 2 (continued)

Compound	Function	Associated factors	References
EPZ004777	Inhibitor of H3K79 histone methyltransferase Dot1L, yields OSKM-iPSCs from mouse and human fibroblasts up to 4-fold, can substitute Klf4 and c-Myc	OSKM	89
RSC133	An indoleacrylic acid analogue. Improves OSKM-hiPSCs yield up to 3.3-fold, accelerates reprogramming, improves cell proliferation potential	OSKM	90
CYT296	A chromatin de-condensing compound, improves reprogramming efficacy of OSKM-transfected cells up to 10-fold	OSKM	91
Inhibitors of inositol trisphosphate 3-kinase, Aurora kinase and P38 kinase	Improves reprogramming efficiency	OSKM	92
Dasatinib (PP1)	Inhibitors of pan-Src family kinase (SFK), can replace Sox2	OKM	96
ALK5 inhibitor	Inhibitor of Tgfb receptor I kinase/activin-like kinase 5, can replace Sox2, a potent alternative of c-Myc too	OKM or OK	97
Kenpaullone	Chemical substitute of Klf4	OSM	98
Parnate	An inhibitor of lysine-specific demethylase 1, in association with CHIR99021 replaces Sox2 and c-Myc	OK	95
OAC1	Chemical activator of Oct4, remarkably improves reprogramming efficiency (up to 20-fold) and accelerates the process	OSKM	101

O, Oct4; S, Sox2; K, Klf4; C, c-Myc; L, Lin28.

OSK-transfected cells accelerates the reprogramming process with high iPSC yield compared to OSKM transduction only (88). EPZ004777 is a potential chemical supplement which inhibits H3K79 histone methyltransferase Dot1L. Treatment with EPZ004777 yields a 3- to 4-fold increase in iPSC formation from human and mouse fibroblasts when used in conjunction with OSKM (89). Similarly, reprogramming stimulating compound RSC133, an indoleacrylic acid analogue, accelerates iPSC conversion and increases reprogramming efficiency up to 3.3-fold over OSKM-transfected human foreskin fibroblasts (90). RSC133 stimulatory effects stem from its ability to promote cell proliferation by down-regulating p53, p21 and p16/INK4A, and repressing epigenetic modulators such as Dnmt1 and HDAC1 (90). Wei *et al.* recently reported that CYT296, a chromatin de-condens-

ing compound, improves reprogramming efficiency of OSKM-transfected mouse somatic cells up to 10-fold (91). In addition, treatment with Aurora kinase, p38 kinase and inositol trisphosphate 3-kinase inhibitors result in increased OSKM-iPSCs yield (92). MEK inhibitor, PD0325901, acts in the later stages of somatic cell reprogramming during endogenous Oct4 expression, and inhibits non-iPSC colony formation while promoting authentic iPSC population growth (93). For successful reprogramming, these inhibitors can be useful, particularly for partially reprogrammed cells and cells resistant to reprogramming, as they represent a substantial portion of RF-transfected cell populations (94) (Fig. 3).

As mentioned earlier, some chemical inhibitors have the capacity to replace established RFs either individually or in concert. For instance, CHIR99021 [a glycogen

Figure 3. Small molecules improve pluripotency reprogramming efficiency. Administration of small molecules (chemical supplements) drastically improves the efficiency of pluripotency reprogramming and accelerates the process itself. Part of this amendment is facilitated by conversion of a large pool of partially reprogrammed pre-induced pluripotent stem cells (iPSCs) into fully reprogrammed iPSCs.

synthase kinase-3 beta (GSK3b) inhibitor] (95), Dasatinib/PP1 [inhibitors of pan-Src family kinase (SFK)] (96) and Tgfb receptor I kinase *aka* activin-like kinase 5 (ALK5) inhibitor (97) can functionally replace Sox2. ALK5 inhibitor is also an alternative for c-Myc and increases reprogramming efficiency up to 2.5-fold in comparison with OSKM transfection (97). Conversely, Kenpaullone is a chemical substitution for Klf4 that repeats Klf4 functions such as inducing endogenous *Nanog* expression (98).

There are SMs that can substitute multiple RFs, these include VPA (87), EPZ004777 (89), CHIR99021 (95), Parnate (95), Repsox (99) and BIX (93,100). Both VPA and EPZ004777 are capable of replacing Klf4 and c-Myc (87,89). CHIR99021 and Repsox (E-616452) are transforming growth factor β receptor 1 (TGF β r1) kinase inhibitors which can substitute Sox2 and c-Myc to generate iPSCs from mouse fibroblasts (95,99). Repsox treatment also assists partially reprogrammed somatic cells to transform into true pluripotent state. It has been suggested that Repsox replaces Sox2 by inducing *Nanog* expression and inhibiting TGF- β signalling (99). Parnate (tranylcypromine), which inhibits lysine-specific demethylase 1, in association with CHIR99021 generates authentic hiPSCs from primary human keratinocytes with Oct4 and Klf4, although this method is 100-fold less efficient than OSKM reprogramming (95). BIX (BIX-01294), an inhibitor of the G9a histone methyltransferase, can also substitute Sox2 and c-Myc, and produce iPSCs with Oct4 and Klf4 only (93,100).

Disputing the concept that exogenous Oct4 expression is fundamental to pluripotency reprogramming, BIX – serving as a substitute for Oct4 – generates iPSCs from mouse neural progenitor cells (NPCs) with Klf4, Sox2 and c-Myc only (93). Despite endogenous Sox2

expression in NPCs, exogenous Sox2 is required for NPC reprogramming, in the absence of Oct4. mEFs can be reprogrammed into iPSCs with Oct4, Klf4 (OK) and BIX, although with lower efficiency than with OSKM-based reprogramming or OK+BIX-based reprogramming of NPCs (100). Addition of Bayk, an L-calcium channel agonist, can further improve OK+BIX mEFs reprogramming (100). Also, *Nanog* in concert with small molecules (for example, oxysterol or purmorphamine) that activate sonic hedgehog (shh) signalling can reprogramme mouse somatic cells, in the absence of exogenous Oct4 (79). Thus, *Nanog* + small molecules might be a promising reprogramming approach. Li *et al.* reported a chemical activator of Oct4 termed Oct4-activating compound 1 (OAC1), which up-regulates Oct4, *Nanog* and Tet1 expression (101). OAC1-modified culture condition improves both efficiency and speed of reprogramming, with iPSCs emerging 4 days post-infection. Treatment of OSKM-induced cells with OAC1 has yielded more than 20-fold more iPSCs (101) suggesting OAC1 may be a suitable supplement for improving OSKM-based reprogramming.

Evidence suggests these SMs have greatest effect when combined. For instance, OSKM-transfected human myoblasts and fibroblasts had improved reprogramming with the HDAC inhibitor sodium butyrate (NaB) and ALK4/5/7 inhibitor SB431542 (102). Meanwhile, CHIR99021 and ALK5 inhibitor together have reprogrammed human fibroblasts transfected with Oct4, Sox2, *Nanog* and Lin28 into a pluripotent state that resembled mESCs (103). Using a cocktail of SB431542, PD0325901 and thiazovivin have improved reprogramming efficiency of OSKM-transfected human fibroblasts by up to 200-fold (104). Zhu *et al.* demonstrated that with Oct4, A-83-01, PS48 (3-phosphoinositide-depen-

dent kinase-1), Nab and PD0325901, neonatal human epidermal keratinocytes (nHEKs), human umbilical vein endothelial cells (hUVECs) and amniotic fluid-derived cells (hAFDCs) could be reprogrammed with significant efficiency (105). Authentic iPSCs from mEFs have been reported using Oct4 followed by treatment with AMI-5, a protein arginine methyltransferase inhibitor, and A-83-01 (106). A further potent SM cocktail consisting of VPA, CHIR99021 and 616452 has successfully reprogrammed mouse fibroblasts with Oct4 alone with greater efficiency than with OSKM and OSM (107). Adding tranylcypromine, an H3K4 demethylation inhibitor, to the existing cocktail can further enhance reprogramming efficiencies (107). Wei *et al.* also reprogrammed mEFs with Oct4 and a SM cocktail comprised of CYT296, VPA, CHIR99021, Parnate and RepSox which improved reprogramming efficiency (91).

Overall, these studies suggest that small molecules are an excellent resource for successful pluripotency reprogramming, and these chemically defined media may produce high-quality clinical-grade iPSCs with the least number of RFs and yet a satisfactory yield.

Nutritional supplements for pluripotency reprogramming

Some of the recent studies have sought to address nutritional requirements for pluripotency reprogramming and determined that some common dietary supplements have noteworthy potential for improving reprogramming efficiency and iPSC quality (108–114) (Table 3, Fig. 4). For example, butyrate, a natural nutritional

supplement and well-established HDAC inhibitor, improves reprogramming efficiency of OSKM-transfected human somatic cells such as foetal fibroblasts (108). Sodium butyrate (NaB) treatment has been reported to increase reprogramming efficiency of transfected fibroblasts up to 50-fold, with overall efficiency of 15–20% hiPSCs, and has greater efficacy than other HDAC inhibitors such as VPA and Trichostatin A (TSA). Even in the absence of *c-Myc* or *Klf4* transgenes (OSK or OSM), NaB stimulatory effects can increase iPSC yield (108). In addition to NaB HDAC inhibitory effect, NaB facilitates global promoter demethylation which accelerates endogenous pluripotency-associated gene expression (108). Zhang *et al.* have shown that NaB promoted iPSC reprogramming by augmenting Oct4-mediated transcriptional activation of pluripotency-associated miRNAs, for example, the miR-302/367 cluster (109,110). NaB not only promoted transcription and structural stability of the miR-302/367 cluster but also improved Oct4 transcriptional activity (110). Thus, NaB may be suitable for improving RF-based reprogramming methods.

Vitamin C (Vc) has also been demonstrated to improve reprogramming of mEFs, human skin fibroblasts and adipose stem cells transduced with OSKM or OSK (111). Its mode of action alleviates cell senescence by reducing p53 and p21 expression while maintaining minimum DNA repair machinery (111). Relatively higher numbers of authentic-iPSCs following Vc treatment may in part be due to Vc-mediated transformation of partially reprogrammed pre-iPSCs into fully reprogrammed iPSCs (111). Furthermore, Stadtfeld *et al.*

Table 3. Nutritional supplements for pluripotency reprogramming

Compound	Function	References
Sodium butyrate (NaB)	Acts as nutritional supplement and HDAC inhibitor. Accelerates endogenous pluripotency gene expression and enhances reprogramming efficiency	108
	Substantially up-regulates the miR-302/367 cluster expression through Oct4	109
	Enhances transactivity of the Oct4 transactivation domains	110
Vitamin C	Alleviates cell senescence by inhibiting p53 and p21 expression, enhances pre-iPSCs to iPSCs transition, increases reprogramming efficiency	111
	Endows full developmental potential to iPSCs by restoring active chromatin mark [H3 lysine 4 trimethylation (H3K4me3)] at Dlk1-Dio3 locus	112
N-acetyl-cysteine (NAC) – antioxidant	Reduces reactive oxygen species (ROS) build-up and copy number variations (CNV) in resultant iPSCs	113,114

Figure 4. Nutritional supplements improve the quality of induced pluripotent stem cells (iPSCs). Supplementing reprogramming media with nutritional supplements such as Butyrate, vitamin C and antioxidants substantially improves the quality of resultant iPSCs.

demonstrated that Vc treatment substantially improved the quality of iPSCs, reviving full developmental potential and unaltered genomic status (112). Generally, OSKM-transduced miPSCs bear altered epigenetic signatures at the imprinted *Dlk1-Dio3* gene locus which modulates their ability to generate iPSC-derived adult mice through tetraploid complementation. However, supplementing reprogramming media with Vc restores H3 lysine 4 trimethylation at *Dlk1-Dio3* loci of the OSKM-iPSCs, reviving full pluripotency (112).

Also, supplementing reprogramming media with antioxidants, such as N-acetyl-cysteine (NAC), can substantially reduce genomic instability of iPSCs, one serious side effect of pluripotency reprogramming (113,114). NAC tends to inhibit rapid build-up of reactive oxygen species and reduces *de novo* copy number variations in OSKM-iPSCs, due to metabolic shift during early phases of the reprogramming (113).

Thus, optimization of reprogramming culture media with appropriate nutritional supplements needs to be effectively prioritized to ensure clinical-grade iPSCs.

Chemical reprogramming: a novel approach for iPSCs

Application of small molecules (SMs) is the most recent development in somatic cell reprogramming (86). In continuation of prior studies (86), Hou *et al.* have reported that a combination of six SMs, *VC6TFZ* (VPA, *CHIR99021*, *616452*, *Tranylcypromine*, *Forskolin* and *dznep*) reprogrammes mouse embryonic fibroblasts (mEFs) into iPSCs with 2i media (115). These chemically induced pluripotent stem cells (ciPSCs) express typical pluripotency markers. In addition, their gene expression, methylation and histone profiles are similar to mESCs and OSKM-iPSCs. ciPSCs also exhibit multilineage

differentiation potential and germline transmissibility, supporting their complete reprogramming. Addition of TTNPB, a synthetic retinoic acid receptor ligand, significantly improves efficiency of VC6TFZ-based reprogramming (115). This finding paves the way toward chemically induced pluripotency which overcomes most of the prevailing complexities regarding RF-based reprogramming and may allow production of footprint-free clinical-grade iPSCs.

Potent cell sources for hiPSCs

Induced pluripotency provides an unprecedented opportunity for deriving patient-specific pluripotent cells from somatic cell lineages. Considering cell origin and epigenomic profile of somatic cells, determine RFs prerequisite and subsequent efficiency of iPSC reprogramming (56,57); based on previously published studies, we propose four promising candidates for generating patient-specific hiPSCs – keratinocytes, melanocytes, urine-derived renal epithelial cells and red blood cells (15,22,27,28,116–120).

Human keratinocytes are suitable as they are easily harvested from hair, contain high levels of endogenous Klf4 and c-Myc transcripts and are readily reprogrammed (116). Compared to fibroblasts, reprogramming of human keratinocytes is more than 100-fold more efficient with overall reprogramming efficiency close to 1% (15). Keratinocytes are also of epithelial origin and do not require mesenchymal-to-epithelial (MET) transition prior to pluripotency induction (15), making them an attractive candidate for clinical-grade iPSC production.

Human melanocytes are a further candidate, as in addition to OSKM-based reprogramming, primary melanocytes can be reprogrammed in the absence of exogenous Sox2 (27). This occurs as, similar to neuronal precursor cells (NPC), melanocytes are of neuroectodermal origin and express Sox2 endogenously.

Human urine-derived renal epithelial cells (hRECs) is our third candidate for clinical-grade hiPSCs (22,117). In addition to their availability and non-invasive collection method, hRECs are amenable to reprogramming with high conversion efficiency. OSKM-transfected hRECs can yield up to 4% urinary iPSCs (UiPSCs) within 5–6 weeks (22,117). Resultant UiPSCs have typical hESC morphology, expression and epigenetic profiles, and have multilineage differentiation potential. Thus, hRECs could conceivably be a suitable source for patient-specific hiPSCs.

Reprogramming blood cells – T lymphocytes, CD34 + blood cells, blood mononuclear cells – is potentially the safest and most efficient source of generating

patient-specific hiPSCs (28,118,119). Routine venipuncture can supply a sufficient quantity of PB34 (peripheral CD34⁺ blood cells) or PBMCs (peripheral blood mononuclear cells) with minimal risk to the donor (28,118). Human PB34 and PBMCs have previously been reprogrammed into hiPSCs with reasonable efficiency (28,118). With further optimization of reprogramming protocols and culture conditions, PB-iPSC yield can be improved. Tan *et al.* recently demonstrated that iPSC reprogramming using small volumes of finger-prick blood cells can generate authentic hiPSCs using OSKM-equipped Sendai viruses with reasonable efficiency (120). Convenience of collecting and preparing human finger-prick blood sample makes it a highly suitable source for generating patient-specific hiPSCs.

Potential application of hiPSCs

Induced pluripotency (iPSCs) continues to prevail as the best platform for disease modelling, drug screening and regenerative medicine. Increasingly successful derivation of a myriad of disease specific or functional cells from iPSCs and their clinical relevance suggests that a possible boon in stem cell therapy is on the horizon. In this section, we briefly discuss some recent reports concerning *in vitro* hiPSC differentiation, therapeutic potential and tissue engineering prospects (for focused reviews on iPSC-based disease modelling and drug screening studies, see refs 123–126).

Human induced pluripotent stem cells hold great promise for tailored cell-based therapies. Functional hiPSC derivatives such as astrocytes, dopaminergic neurons, retinal pigment epithelium cells, adipocyte progenitors, insulin-producing islet-like clusters, bone substitutes, hepatocytes, melanocytes, erythroblasts, cardiomyocytes and red blood cells have already been generated (127–137). Considering damaged tissues account for many chronic illnesses, such as neurodegenerative diseases, retinal degeneration, muscular dystrophy and myocardial infarction, patient-specific hiPSC derivatives can be a suitable therapeutic choice. These cells appear to exhibit remarkable resilience to *in vivo* physiological environments and readily acclimatise to cellular homeostasis. For example, hiPSC-derived neuronal progenitor cells (NPCs) successfully grafted into ALS (amyotrophic lateral sclerosis) rat model spinal cords, survived over an extended period following differentiation into mature neurons (138). Similarly, transplantation of hiPSCs-derived neuroepithelial-like stem cells (It-NES) into striatum or cortex of stroke-induced mice and rats have markedly recovered motor neuron function (139). Grafted It-NES cells acclimatise in host brains without tumourigenesis, survive for prolonged periods,

differentiate into mature neurons with electrophysiological properties and develop synaptic connections with host neurons (139). In a study by Lu *et al.*, hiPSCs-derived multipotent cardiovascular progenitor (MCPs) grafts repopulated decellularized mouse hearts by efficiently differentiating into cardiomyocytes, smooth muscle cells and endothelial cells (140). MCP-engineered heart tissue has been shown to exhibit normal electrophysiological function such as contraction and can respond to chemical treatments (140).

Human induced pluripotent stem cells-based organ fabrication is a further promising area of regenerative medicine. With successive improvements of artificial scaffolds and 3D culture systems, laboratory-grown organs may not be far off – at least, studies employing mouse progenitor cells suggest this to be so. For example, Greggio *et al.* developed a matrigel-based 3D culture system that promotes expansion of mouse embryonic pancreatic progenitors (mPPs) and pancreatic organoid formation *in vitro* (141). Resultant pancreatic organoids mimicked *in vivo* pancreas development and differentiated into endocrine-producing cells. Similarly, Eiraku *et al.* generated self-organized optic cup or retinal primordium-like structures from mouse ESCs, in a modified 3D culture system (142). As cell–cell interaction and niche dependency of progenitor cells greatly influence tissue architecture or morphogenesis, these artificial niche-3D culture systems may assist with developing personalized organ substitutes from hiPSCs or their functional derivatives.

3D printing technology, a recent addition to the tissue engineering toolkit, has become popular in fabricating complex tissue constructs (143). This bioprinting technique has enabled researchers to craft intricate tissue structures such as vascular channels (144), neocartilage constructs (145) and skin substitutes (146). Biophysiological activity of these 3D-printed tissues in an *in vivo* setting, harbingers the prospect of iPSC-driven organs in the near future.

Conclusions

Induced pluripotent stem cells are an invaluable resource for cell therapy and regenerative medicine. While generating authentic-iPSCs using a variety of methods is becoming routine, many of the current techniques have not yet been rigorously investigated in convenient human somatic cells. However, growing concern with regard to genomic and epigenomic instability, somatic memory or immunogenicity of iPSCs demands further investigation. We believe optimization of reprogramming protocols and potent cell sorting might enable existing hurdles to be overcome to exploit clinical-grade iPSCs

for upcoming stem cell therapies and regenerative medicine.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to formulate the concept for this review. IKR wrote the manuscript. MEI, MMB and KDI revised the initial manuscript. IKR revised and updated the manuscript according to editorial and reviewers suggestion. AB and JAB read and edited the updated manuscript and approved the final manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Generation of iPSCs; from different cell types, utilizing different approaches