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REVIEW

Chemical-only reprogramming to pluripotency

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Abstract **Direct reprogramming technology has emerged as an outstanding technique for the generation of induced pluripotent stem cells (iPSCs) and various specialized cells directly from somatic cells of different species. Reprogramming techniques conventionally use viral vectors encoding transcription factors to induce fate conversion. However, the introduction of transgenes limits the therapeutic applications of the reprogrammed cells. To overcome safety-related concerns, small molecules offer some advantages over the existing methods for the control of gene expression and induction of cell fate conversion. Technical advances in optimizing concentrations, durations, structures, and combinations of small molecules make chemical reprogramming a safe and feasible method. This review provides a concise overview of cutting-edge findings regarding chemical-only reprogramming as one of the integration-free approaches to iPSC generation.**

Keywords cellular reprogramming, small molecule, chemical reprogramming, induced pluripotency, regenerative medicine

Introduction

To date, thousands of papers have cited the first report of induced pluripotent stem cell (iPSC) generation (Takahashi and Yamanaka, 2006), demonstrating the importance of pluripotency in the study of early developmental stages, epigenetics, disease modeling, drug screening, cell therapy, and regenerative medicine. Current reprogramming methods for the generation of iPSCs and therapeutic somatic cell types mostly use genetic material, including viral vectors. Direct reprogramming using viral vectors has been associated with safety concerns such as mutation, genomic alteration and dysregulation in reprogrammed cells, defects in differentiation potential, and the risk of tumorigenicity and induction of dysplasia caused by the insertion of exogenous DNA into the host genome (Foster et al., 2005; Hochedlinger et al., 2005; Okita et al., 2007; Koyanagi-Aoi et al., 2013; Lee et al., 2013; Lu and Zhao, 2013; Okano et al., 2013; Ma et al., 2014; Ohnishi et al., 2014; Sugiura et al., 2014). Interestingly, iPSCs have been generated by different integration-free methods. In general, integration-free methods include DNA-free approaches and those that use DNA (Silva et al., 2015). DNA-based methods make use of non-integrative vectors including adenoviruses (Stadtfeld et al., 2008) and episomes [e.g., expression plasmids (Okita et al., 2008), EBNA1/oriP expression plasmids (Chou et al., 2011), oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-based episomal vectors (Chou et al., 2015; Yu et al., 2009), EBNA1-based episomal plasmid vectors (Okita et al., 2013), episomal vectors (Piao et al., 2014), small-molecule-aided episomal vectors (Yu et al., 2011), and episomal vectors in multistage culture system (Valamehr et al., 2014)]. DNA-free approaches include RNA-based methods [e.g. Sendai virus (Fusaki et al., 2009; Ban et al., 2011), synthetic modified mRNAs (Warren et al., 2010; Lee et al., 2012;Durruthy-Durruthy et al., 2014), and miRNAs (Anokye-Danso et al., 2011)], protein-based methods (Kim et al., 2009; Zhou et al., 2009; Lee et al., 2012), and small molecule-based methods (Hou et al., 2013; Long et al., 2015; Zhao et al., 2015; Ye et al., 2016). Integrating but excisable vectors also have been used for the generation of transgene-free iPSCs (Kaji et al., 2009; Soldner et al., 2009; Woltjen et al., 2009). Such non-integrating methods have been reviewed elsewhere (Higuchi et al., 2015) and (González et al., 2011; Goh et al., 2013; Zhou and Zeng, 2013; Schlaeger et al., 2015; Silva et al., 2015). Non-integrating vectors do not insert genetic material into the host genome and therefore avoid the risk of

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insertional mutagenesis and can overcome potential safety-related concerns (Okita and Yamanaka, 2011; Silva et al., 2015).

Small molecules (chemicals) have been used to improve the efficiency and kinetics of traditional reprogramming methods and can substitute for some of the reprogramming factors (Su et al., 2013; Davies et al., 2015; Jung et al., 2014; Lin and Wu, 2015). More importantly, it has been recently demonstrated that small molecules can induce pluripotent reprogramming by replacing all reprogramming transgenes. Indeed, the chemical-only reprogramming approach can generate iPSCs without the use of reprogramming genes (Hou et al., 2013; Long et al., 2015; Zhao et al., 2015; Ye et al., 2016). The current review describes recent findings in chemical-only reprogramming to pluripotency as one of the integration-free techniques.

Although chemical reprogramming offers several advantages, the process still suffers from low efficiency and slow kinetics. In this regard, it has been hypothesized here that utilizing the methods that enhance reprogramming efficiency and kinetics (Ebrahimi, 2015a) in chemical-only reprogramming approaches can provide a blueprint toward a safe, reliable, and efficient method of pluripotent reprogramming. Suggestively, highly efficient chemical reprogramming approaches offer promises for patient-specific iPSC disease modeling, drug screening, and clinical applications. Notably, chemical-only iPSC generation is a new reprogramming paradigm and the current study reviews single pioneer reports. Thus, the reproducibility of the protocols remains to be unanimously validated in different settings.

Why do small molecules matter?

Numerous small molecules, which are capable of governing the self-renewal, reprogramming, transdifferentiation, and regeneration processes, have been introduced in the field of stem cell biology. These chemical compounds are versatile tools for cell fate conversion toward the desired outcomes (Li et al., 2012a, 2013a, 2013b). Small molecules have special advantages; for example, they are cost-effective, have a long half-life, have diverse structures and functions, and allow temporal and flexible regulation of signaling pathways. Moreover, chemical approaches have superiority over arduous traditional genetic techniques in several aspects, including their rapid, transient, and reversible effects on activation and inhibition of functions of specific proteins (Li et al., 2012a, 2013a). Additionally, their effects could be adjusted by optimizing concentrations and combinations of different small molecules (Li et al., 2012a, 2013a). Therefore, chemicals are powerful tools for induction of cell fate conversion and study of stem cell and chemical biology *in vitro* and *in vivo* (Li et al., 2012a, 2013a).

Small molecules as enhancers of transcription factor-mediated reprogramming to pluripotency

To enhance reprogramming efficiency using small molecules, it has been shown that chemical inhibitors of the MEK (PD0325901) and TGFβ (SB431542) pathways facilitate the mesenchymal to epithelial transition (MET). These chemicals improve the efficiency and kinetics of human OSKM (Oct4, Sox2, Klf4, and Myc) reprogramming up to 100-fold and further up to 200-fold in the presence of small-molecule thiazovivin (a ROCK inhibitor) (Lin et al., 2009). Furthermore, it has been demonstrated that ascorbic acid participates in suppression of the somatic/native program, and can improve the quality and efficiency of reprogramming (Esteban et al., 2010; Wang et al., 2011; Stadtfeld et al., 2012; Chen et al., 2013; Vidal et al., 2014).

In keeping with the use of chemicals to reduce the number of transcription factors in pluripotent reprogramming, it has been revealed that a combination of two small molecules, BIX-01294 and BayK8644, can substitute c-Myc and Sox2 (Shi et al., 2008). Additionally, small-molecule EPZ004777 increases the efficiency of mouse and human reprogramming three- to 4-fold. This chemical can also substitute c-Myc and Klf4 by inhibiting the catalytic activity of DOT1L, a H3K79 methyltransferase, and by upregulation of *Nanog* and *Lin28* (Onder et al., 2012). Compound δ is another novel synthetic small-molecule that rapidly induces several pluripotency genes in mouse embryonic fibroblasts (MEFs). This compound significantly induces expression of endogenous *Oct4* and *Nanog* during mouse cellular reprogramming (Pandian et al., 2012). Moreover, Ichida and colleagues showed that small-molecule RepSox (E-616452) can replace Sox2 (Ichida et al., 2009). In addition, Zhu and colleagues demonstrated that ectopic expression of Oct4 alone with a defined small-molecule cocktail substituting for three reprogramming factors (Sox2, Klf4, and c-Myc) induces reprogramming of several human primary somatic cell types into iPSCs (Zhu et al., 2010). However, this iPSC reprogramming procedure using small molecules and forced expression of Oct4 alone takes at least 5 weeks (Zhu et al., 2010) and represents the slow kinetics of this approach. Likewise, Deng and colleagues reported the induction of pluripotency in mouse fibroblasts using forced expression of *Oct4* and small molecules substituting for Sox2, Klf4, and c-Myc (Li et al., 2011). In addition to the aforementioned compounds, the small molecules OAC1/2/3 (Oct4-activating compound 1/2/3) can activate the *Oct4* and *Nanog* promoters, enhance reprogramming efficiency and dynamics, and reduce the time required (Li et al., 2012b). Together, chemical compounds can be convenient substitutes for iPSC transcription factors. Notably, most compounds that can substitute reprogramming transcription factors are also capable of improving the efficiency and kinetics of conventional reprogramming

methods. The roles of small molecules as enhancers of pluripotent reprogramming and as substitutes for iPSC transcription factors have been reviewed elsewhere (Su et al., 2013; Jung et al., 2014; Lin and Wu, 2015).

Chemical-only induction of pluripotency

In line with the use of small molecules as substitutes for reprogramming factors, Deng and colleagues have demonstrated that the forced expression of pluripotency master genes is dispensable for mouse iPSC reprogramming (Hou et al., 2013). In a stepwise procedure, mouse somatic cells were reprogrammed into pluripotent cells using a combination of seven small molecules (Fig. 1). In the first (induction) step, VC6PFZ (CHIR99021 (C), 616452 (6, or RepSox), forskolin (F), DZNep (Z), Parnate (P, or tranylcypromine), and valproic acid (V)) were used with TTNPB as an enhancer. In the next (maturation) step, the dual inhibition (2i) of glycogen synthase kinase-3 (GSK3) and mitogen-activated protein kinase (ERK) was achieved with CHIR99021 and PD0325901, respectively. Then, iPSC colonies were obtained with a frequency of up to 0.2% after about 40 days (Table 1 and Table 2). The produced cells were named chemically induced pluripotent stem cells (CiPSCs) (Hou et al., 2013). However, the main disadvantage of this chemical reprogramming method is that it takes about 40 days in mouse, and may require an even longer period for the generation of human CiPSCs (Hou et al., 2013; Masuda et al., 2013). To date, there are few reports of chemical-only reprogramming to pluripotency (Hou et al., 2013; Long et al., 2015; Zhao et al., 2015; Ye et al., 2016) and it is a new and growing field of study. Notably, all the reports of chemical-only reprogramming have been accomplished in mouse cells; however, for regenerative medicine purposes, development of protocols for chemical-only production of human iPSCs is in demand.

Although the 2013 chemical approach of Deng and colleagues was a step toward a safe reprogramming method, its efficiency was very low with delayed kinetics. However, this finding demonstrates that somatic cells can be reprogrammed into pluripotent cells by using small molecules alone without the need for ectopic expression of exogenous master genes.

Two years after the first report of chemical-only induction of pluripotency (Hou et al., 2013), Long and colleagues (Long et al., 2015) repeated the CiPSC protocol and improved it with the addition of bromodeoxyuridine (BrdU) to the previous chemical cocktail of Deng and colleagues (Fig. 1). It was discovered that BrdU, a synthetic analog of thymidine, is able to facilitate OSKM-mediated reprogramming (Table 2) (Long et al., 2015). Interestingly, BrdU has been found to be capable of replacing Oct4, the most critical factor in iPSC production, and induction of pluripotent reprogramming using SKM (Long et al., 2015). Furthermore, it was indicated that BrdU enhances the efficiency of VC6PFTZ-mediated CiPSC production by about threefold (Long et al., 2015). Remarkably, BrdU can reduce the number of small molecules needed for induction of chemical reprogramming with the minimal recipe consisting of BrdU, CHIR99021, RepSox, and forskolin, although with an extremely low efficiency (Table 1) (Long et al., 2015).

The exact mechanism by which BrdU improves the transcription factor- and chemical-induced reprogramming is yet to be explored. Results indicated that BrdU at the concentrations needed to induce reprogramming does not induce genotoxicity in reprogrammed cells (Long et al., 2015). Regarding the use of BrdU in humans (Eriksson et al., 1998), development of a chemical-only protocol for induction of human iPSCs in the future can provide a safer strategy for the generation of clinical-grade iPSCs.

More recently, the molecular mechanisms of chemical reprogramming have been dissected by Deng and colleagues (see subsequent section), which successfully improved the efficiency and kinetics of previous chemical protocols in three steps (Fig. 1) (Zhao et al., 2015). The chemical reprogramming protocol was improved by the addition of new chemicals to the cocktails at each stage, modification of the concentration of CHIR99021, alteration of the components of the 2i-medium, optimization of the cell density and the duration of small-molecule treatment (Table1). Moreover, modifying the structure of EPZ004777 to produce SGC0946 and its administration in stage 2 significantly increased the reprogramming efficiency. In contrast to the old protocol, applying these modifications enhanced the total yield of CiPSC colony formation by up to 1000-fold (Zhao et al., 2015).

It has been found that different cell types have different requirements for their efficient reprogramming (Vidal et al., 2014; Ebrahimi, 2015a). In the case of chemical reprogramming, different small molecules, at different concentrations and durations are required to reprogram different cell types into pluripotent cells. Thus, protocols for induction of chemical reprogramming should be designed and optimized according to the requirements of each cell type (Ye et al., 2016).

Concurrent with the report of their modified chemical reprogramming protocol (Zhao et al., 2015), Deng and colleagues demonstrated that their strategy for chemical induction of pluripotency is reproducible in different donor cell types (Table 1). Donor cells were taken from different mouse tissues, including fibroblasts, neural stem cells (NSCs), and small intestinal epithelial cells (IECs), which are derived from mesoderm, ectoderm, and endoderm, respectively (Ye et al., 2016). Although the core components of the chemical cocktails (VC6PF) are the same, optimizing the concentrations and durations of the small-molecule treatments is necessary for the initiation of reprogramming in different cell types. For example, reprogramming of IECs needs up to 20 μM of RepSox (616452) during stage 1, whereas a reduced concentration of RepSox is favorable for

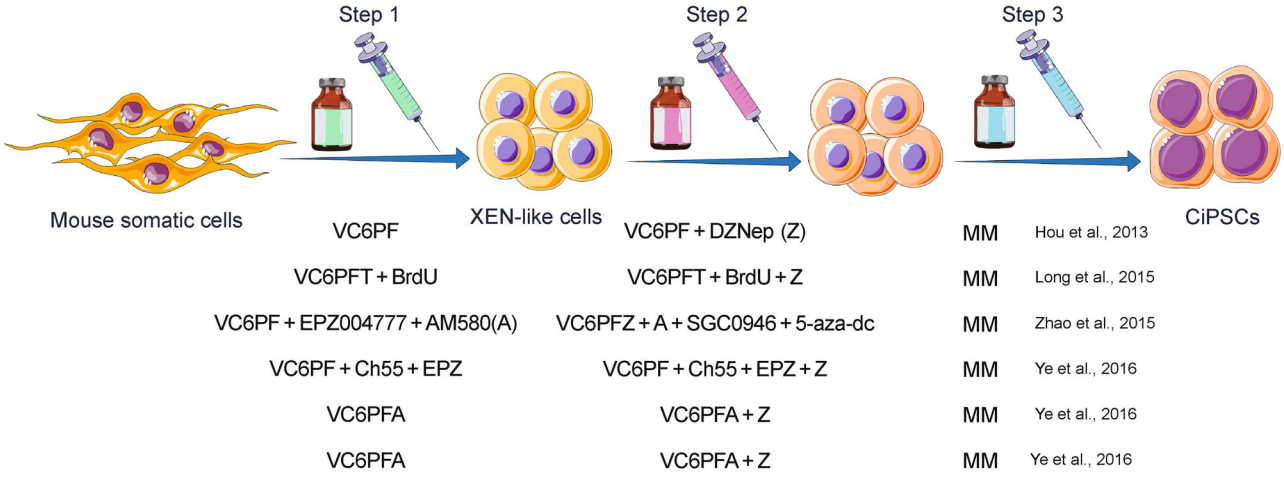

Figure 1 Pluripotent reprogramming of somatic cells using small molecules alone. Generally, three steps have been exploited by different authors for chemical-only reprogramming to pluripotency. Abbreviations are explained in Table 1 and Table 2. MM indicates maturation medium.

reprogramming of NSCs. Therefore, the primary chemical protocol for pluripotent reprogramming appears to be cell type-independent, representing a general mechanism underlying chemical reprogramming (Ye et al., 2016).

According to these discussions, it could be suggested that the efficiency and kinetics of this chemical approach might be further improved by inhibition of reprogramming barriers and enhancing methods (e.g., inhibition of Mbd3, p57, $p16^{Ink4a}$/$p19^{Arf}$, $p21^{Cip1}$, and p53) (Ebrahimi, 2015a, 2016). This could lead to the development of a rapid and efficient CiPSC production strategy. Moreover, an enhanced chemical reprogramming method may allow for highly efficient reprogramming of adult/stem progenitor cells, which are amenable to the acquisition of pluripotency (Ebrahimi, 2015b).

Collectively, the efficiency of chemical reprogramming can be significantly improved by precise optimization of the reprogramming conditions and the addition of small-molecule enhancers in each step during the chemical reprogramming process (Zhao et al., 2015; Ye et al., 2016). Ultimately, application of chemical modulators could be regarded as a promising strategy for the development of new drugs intended for targeting endogenous (stem) cells and induced regeneration (Li et al., 2012a, 2013b).

Molecular mechanisms underlying chemical reprogramming to pluripotency

The identification of the main molecular events and intermediate cell states during the trajectory of chemical reprogramming can assist in improving the efficiency of reprogramming by optimizing the small molecules and culture conditions required for each stage of reprogramming. Recently, Deng and colleagues endeavored to reveal cell fate dynamics and molecular events underlying chemical reprogramming to pluripotency (Zhao et al., 2015). Surprisingly, compared to the transgenic strategy, which transiently establishes a primitive streak-like state during OSKM-induced reprogramming, chemical reprogramming establishes an extra-embryonic endoderm (XEN)-like state, indicating different routes underlying chemical reprogramming and OSKM-induced reprogramming (Takahashi et al., 2014; Zhao et al., 2015; Ye et al., 2016). Interestingly, in addition to mouse fibroblasts, the XEN-like state is also essential in the small-molecule-mediated pluripotent reprogramming of mouse NSCs and IECs (Zhao et al., 2015; Ye et al., 2016). Indeed, dissection of the cellular and molecular mechanisms underlying chemical reprogramming allowed for the efficient reprogramming to pluripotency through the XEN-like state and by the step-wise administration of appropriate small molecules in each step (Fig. 1) (Zhao et al., 2015). Therefore, identification of the intermediate state of chemical reprogramming gives an opportunity for enhancing the efficiency of the process.

The XEN-like cells express some of the pluripotency genes, (e.g. *sall4* and *lin28a*) and some genes (i.e. *gata4* and *gata6*) which can substitute for Oct4 in OSKM-mediated reprogramming (Shu et al., 2013). Accordingly, the XEN-like cells are in a unique intermediate state, which is primed for reprogramming toward pluripotency (Zhao et al., 2015). Suggestively, XEN-like cells may also be amenable to differentiation into different lineages. Thus, the identification of the XEN-like state as a unique route toward pluripotency may allow for cell fate transitions toward different lineages and clinically relevant cell types.

Collectively, these findings suggest that small molecules induce pluripotent reprogramming in different cell types by a almost similar molecular mechanism, including the activation of *sall4*, *gata4* and *sox17* genes and through an intermediate XEN-like state (Zhao et al., 2015; Ye et al., 2016).

Table 1 Different protocols that have been used for chemical-only induction of pluripotency

	Starting cells	Induction stage (chemical agents)	Maturation	Product cells	References
1	Mouse fibroblasts	Stage 1: a cocktail of five small molecules, "VC6PF" (VPA, CHIR99021, 616452 (RepSox), Parnate (or tranylcypromine), and forskolin), was used for 16–20 days. Stage 2: small-molecule, DZNep (Z), was added to the VC6PF for the next 20–24 days. TTNPB (T) was used as an enhancer. [C6FZ essential compounds; VT optional compounds]	Reprogramming medium was replaced with 2i-medium with dual inhibition (2i) of glycogen synthase kinase-3 (GSK3) and mitogen-activated protein kinase (ERK) with CHIR99021 and PD0325901, respectively for the last 12–16 days.	Chemically-induced pluripotent stem cells (CiPSCs)	Hou et al., 2013
2	Mouse fibroblasts	Chemical cocktail consists of VC6PFT plus BrdU were added from day 0 to day 32, and Z (DZNep) was added from day 16 to day 32. The minimal influential set was BrdU, CHIR99021, RepSox (6) and forskolin.	After day 32, medium containing chemicals was replaced with the 2i-medium.	Chemically induced pluripotent stem cells (CiPSCs)	Long et al., 2015
3	Mouse fibroblasts	Stage 1: VC6PF + EPZ004777 + AM580 (A) for 16 days Stage 2: VC6PFZ + A + SGC0946 + 5-aza-dc for 12 days	N2B27/ 2i + LIF for 12 days	Chemically induced pluripotent stem cells (CiPSCs)	Zhao et al., 2015
4	Mouse neural stem cells (NSCs) and	Stage 1: 0.5 mM VPA, 15 μM CHIR, 2 μM 616452, 10 μM Parnate, 20 μM forskolin, 1 μM Ch 55, 5 μM EPZ. Stage 2: From day 20, 0.05 μM DZNep was added into the stage 1 chemical reprogramming medium.	2i-medium (with DMEM/F-12 containing N2 and B27 supplements) from day 40-44. (2i-medium: Knockout DMEM containing 10% KSR, 10% FBS + 2 mM GlutaMAX, 1% NEAA, 55 μM β-mercaptoethanol + 3 μM CHIR99021 + 1 μM PD0325901 + 10 ng/ml mouse LIF)	Chemically induced pluripotent stem cells (CiPSCs)	Ye et al., 2016
5	Mouse fibroblasts	Stage 1: 0.5 mM VPA, 10 μM CHIR, 10 μM 616452, 10 μM Parnate, 10 μM forskolin, 0.05 μM AM580. Stage 2: from day 20, 0.05 μM DZNep was added into the stage 1 chemical reprogramming medium.	2i-medium (with DMEM/F-12 containing N2 and B27 supplements) from day 40.	Chemically induced pluripotent stem cells (CiPSCs)	Ye et al., 2016
6	Mouse small intestinal epithelial cells (IECs)	Stage 1: 0.5 mM VPA, 10 μM CHIR, 20 μM 616452, 10 μM Parnate, 10 μM forskolin, 0.05 μM AM 580). Stage 2: From day 16, 0.05 μM DZNep was added into the stage 1 chemical reprogramming medium, and AM580 was withdrawn.	2i-medium (with DMEM/F-12 containing N2 and B27 supplements) from day 40.	Chemically induced pluripotent stem cells (CiPSCs)	Ye et al., 2016

Note: NEAA (Non-essential amino acids), KSR (knockout serum replacement), FBS (fetal bovine serum).

Furthermore, these findings indicate that chemical reprogramming and transcription factor-induced reprogramming go through different mechanisms (Takahashi et al., 2014; Zhao et al., 2015; Ye et al., 2016).

Discussion

Reprogramming methods predominantly utilize the overexpression of transcription factors, mostly by using viral vectors. However, the genetic manipulation of reprogrammed cells limits their use for regenerative purposes (Xu et al., 2015). Surprisingly, recent findings have shown that exogenous master genes are dispensable for pluripotent reprogramming, and pure small-molecule cocktails can drive direct reprogramming without genetic manipulation (Hou et al., 2013; Long et al., 2015;Zhao et al., 2015; Ye et al., 2016). Up to the present time, small molecules have been used to (1) improve the efficiency and kinetics of OSKM-reprogramming (Lin et al., 2009; Esteban et al., 2010;

Table 2 Different small molecules and factors that have been used in chemical-only reprogramming to pluripotency

Small-molecule	Effect	Role in generation of	Necessity	reference
Forskolin (F)	Activator of adenylate cyclase	Mouse CiPSC	Essential inducer	Hou et al., 2013; Long et al., 2015; Zhao et al., 2015;Ye et al., 2016
TTNPB (T)	A synthetic retinoic acid receptor (RAR) ligand; activates retinoic acid receptors	Mouse CiPSC	Enhancer	Hou et al., 2013; Long et al., 2015
Parnate (P, Tranylcypromine)	An epigenetic modifier that inhibits lysine-specific demethylase 1 (LSD1)	Mouse CiPSC	Essential inducer	Hou et al., 2013; Long et al., 2015; Zhao et al., 2015;Ye et al., 2016
5-aza-dc (5-Aza-2′-deoxycytidine)	Inhibitor of DNA methyltransferase	Mouse CiPSC	Enhancer	Zhao et al., 2015
VPA (V, valproic acid)	an inhibitor of histone deacetylase (HDACs)	Mouse CiPSC	Essential inducer	Hou et al., 2013; Long et al., 2015; Zhao et al., 2015;Ye et al., 2016
CHIR99021	An inhibitor of GSK-3 kinases	Mouse CiPSC	Essential inducer	Hou et al., 2013; Long et al., 2015; Zhao et al., 2015;Ye et al., 2016
RepSox (6, or 616452)	An inhibitor of TGF-β pathways	Mouse CiPSC	Essential inducer	Hou et al., 2013; Long et al., 2015; Zhao et al., 2015;Ye et al., 2016
DZNep (Z, 3-Deazaneplanocin A)	An S-adenosylhomocysteine synthesis inhibitor and a histone methyltransferase EZH2 inhibitor	Mouse CiPSC	Essential inducer (Hou et al., 2013) and Enhancer (Long et al., 2015) (Ye et al., 2016)	Hou et al., 2013; Long et al., 2015; Zhao et al., 2015;Ye et al., 2016
PD0325901 (P)	An inhibitor of the MEK/ERK pathway	Mouse CiPSC	A component of maturation medium	Ye et al., 2016
BrdU (B, Bromodeoxyuridine)	A synthetic analog of the nucleoside thymidine	Mouse CiPSC	Essential inducer	Long et al., 2015
EPZ004777 (E)	An inhibitor DOT1L methyltransferase	Mouse CiPSC	Essential inducer	Zhao et al., 2015
AM580 (A)	An agonist of retinoic acid receptor α (RARα)	Mouse CiPSC	Enhancer	Zhao et al., 2015
SGC0946 (S)	An inhibitor DOT1L methyltransferase	Mouse CiPSC	Enhancer	Zhao et al., 2015
LIF (Leukemia inhibitory factor)	An interleukin 6 class cytokine that inhibits differentiation of mouse embryonic stem cells and iPSCs	Mouse CiPSC	A component of maturation medium	Zhao et al., 2015; Ye et al., 2016

Wang et al., 2011; Stadtfeld et al., 2012; Chen et al., 2013; Vidal et al., 2014), (2) to replace some of the reprogramming factors (Shi et al., 2008; Ichida et al., 2009; Zhu et al., 2010;Li et al., 2011; Li et al., 2012b; Onder et al., 2012) and (3) to replace all transgenes in pluripotent reprogramming (Hou et al., 2013; Long et al., 2015; Zhao et al., 2015; Ye et al., 2016) and transdifferentiation (Pennarossa et al., 2013; Cheng et al., 2014; Cheng et al., 2015; Fu et al., 2015; He et al., 2015; Hu et al., 2015; Li et al., 2015; Sayed et al., 2015; Zhang et al., 2015).

Nowadays, chemical compounds are considered convenient tools for the regulation of protein functions, epigenetics, and signaling pathways, which can influence the quantity and quality of reprogramming products. Moreover, new findings have shown that chemical-mediated reprogramming could be a safe, reliable, feasible, standardized, and cost-effective strategy for translation of reprogramming technology into clinical applications (Li et al., 2012a, 2013a, Yu et al., 2014). As the optimization of current chemical protocols concerns the induction of reprogramming in mouse cells, further prospective investigations are needed to optimize protocols for chemical reprogramming of human cells.

In 2013, Hou *et al*. showed that a set of small molecules can induce pluripotent reprogramming in mouse fibroblasts, demonstrating the feasibility of fate conversion of mouse somatic cells toward pluripotency using only chemicals (Hou et al., 2013). In 2015, while attempting to dissect the molecular mechanisms of chemical reprogramming, the process was improved in a stepwise manner according to a molecular trajectory specific to chemical reprogramming (Zhao et al., 2015). In this new study, Zhao et al. showed that chemical reprogramming process passes through the XEN-like state. They identified small-molecule enhancers, which were used in appropriate time windows, to establish a robust chemical reprogramming method with a yield of up to 1000-fold greater than that of their previous protocol (Hou et al., 2013; Zhao et al., 2015). Surprisingly, Long et al. found that BrdU (a synthetic analog of thymidine and a commonly used

biologic reagent for tracing DNA replication) is able to improve the basic chemical reprogramming protocol by approximately threefold (Long et al., 2015).

Interestingly, passing through the XEN-like state is a unique route in the chemical reprogramming of somatic cells toward pluripotency that differs from the primitive streak-like state in OSKM-induced reprogramming (Takahashi et al., 2014; Zhao et al., 2015; Ye et al., 2016). In another recent study, Deng and colleagues showed that their chemical approach is generalizable and reproducible for pluripotent reprogramming of different mouse cell types from mesodermal, endodermal, and ectodermal lineages. Requiring the same cocktail of small molecules for induction of reprogramming in different cell types and activation of the same genes in the early stage of chemical reprogramming suggests that a conserved molecular roadmap underlies chemical reprogramming to pluripotency (Ye et al., 2016).

Reprogramming to pluripotency is an inefficient process due to the functions of various genetic and epigenetic barriers in donor cells that impede reprogramming (Ebrahimi, 2015a, 2016). For enhancing reprogramming efficiency and kinetics, a convenient approach is the inhibition of barriers. Thus, it could be assumed that the removal of barriers (Ebrahimi, 2015a) and transient disruption of the somatic gene regulatory networks (GRNs) (Cahan et al., 2014; Morris et al., 2014; Tomaru et al., 2014; Ebrahimi, 2016) can significantly enhance the efficiency and kinetics of chemical reprogramming strategies. Therefore, the inhibition of roadblocks in a chemical reprogramming paradigm would be a safe, reliable, and effective approach in pluripotent reprogramming.

To achieve regulatory approval and platform standardization, small molecules seem to be the best candidates among integration-free approaches (Silva et al., 2015). Collectively, expanding our knowledge of chemical reprogramming and improving chemical recipes for human reprogramming will lead to the generation of patient-specific cell lines and significant progress in disease modeling and regenerative medicine in the future (Babos and Ichida, 2015).

Conclusion

iPSC technologies have profoundly changed the fields of stem cell and developmental biology. In addition, the discovery of iPSCs has sparked new hopes for treating genetic and degenerative diseases. These technologies have experienced dramatic progress and have reached the stage of clinical application, albeit to a limited degree (Cyranoski, 2014). Recent advancements in the identification of different barriers and enhancers of reprogramming (Ebrahimi, 2015a) and chemical substitutes for reprogramming factors allow for safer and more highly efficient generation of iPSCs. These advancements have shed light on both personalized and regenerative medicine purposes. Collectively, the evidence addressed here increases the opportunity to gain a greater understanding of the chemical reprogramming, modeling diseases, and regeneration by using specific chemicals or drugs, instead of genetically manufactured products for the generation of medically relevant cell types.

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Compliance with ethics guidelines

The author declares that there are no competing interests.

This manuscript is a review article and does not contain any studies with human or animal subjects.

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