

# Impelling force and current challenges by chemicals in somatic cell reprogramming and expansion beyond hepatocytes

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### Abstract

In the field of regenerative medicine, generating numerous transplantable functional cells in the laboratory setting on a large scale is a major challenge. However, the *in vitro* maintenance and expansion of terminally differentiated cells are challenging because of the lack of specific environmental and intercellular signal stimulations, markedly hindering their therapeutic application. Remarkably, the generation of stem/progenitor cells or functional cells with effective proliferative potential is markedly in demand for disease modeling, cell-based transplantation, and drug discovery. Despite the potent genetic manipulation of transcription factors, integration-free chemically defined approaches for the conversion of somatic cell fate have garnered considerable attention in recent years. This review aims to summarize the progress thus far and discuss the advantages, limitations, and challenges of the impact of full chemicals on the stepwise reprogramming of pluripotency, direct lineage conversion, and direct lineage expansion on somatic cells. Owing to the current chemical-mediated induction, reprogrammed pluripotent stem cells with reproducibility difficulties, and direct lineage converted cells with marked functional deficiency, it is imperative to generate the desired cell types directly by chemically inducing their potent proliferation ability through a lineage-committed progenitor state, while upholding the maturation and engraftment capacity posttransplantation *in vivo*. Together with the comprehensive understanding of the mechanism of chemical drives, as well as the elucidation of

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specificity and commonalities, the precise manipulation of the expansion for diverse functional cell types could broaden the available cell sources and enhance the cellular function for clinical application in future.

**Key words:** Chemical induction; Pluripotent reprogramming; Direct lineage conversion; Direct lineage expansion; Hepatocyte expansion; Cell fate specificity; Transcriptional memory; *In vivo* induction

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**Core tip:** Chemical-mediated reprogramming is a promising strategy for generating desired cells. However, chemical-mediated pluripotent reprogramming has reproducibility difficulties, and direct lineage conversion shows significant deficiency in cell function maturation. On the other hand, direct lineage expansion from target cells not only bypasses pluripotency-related tumorigenesis but also has superior posttransplantation advantages in engraftment and functional maturation. Recent achievements in chemical expansion of human hepatocytes may help solve the cell source limitation in liver disease treatment.

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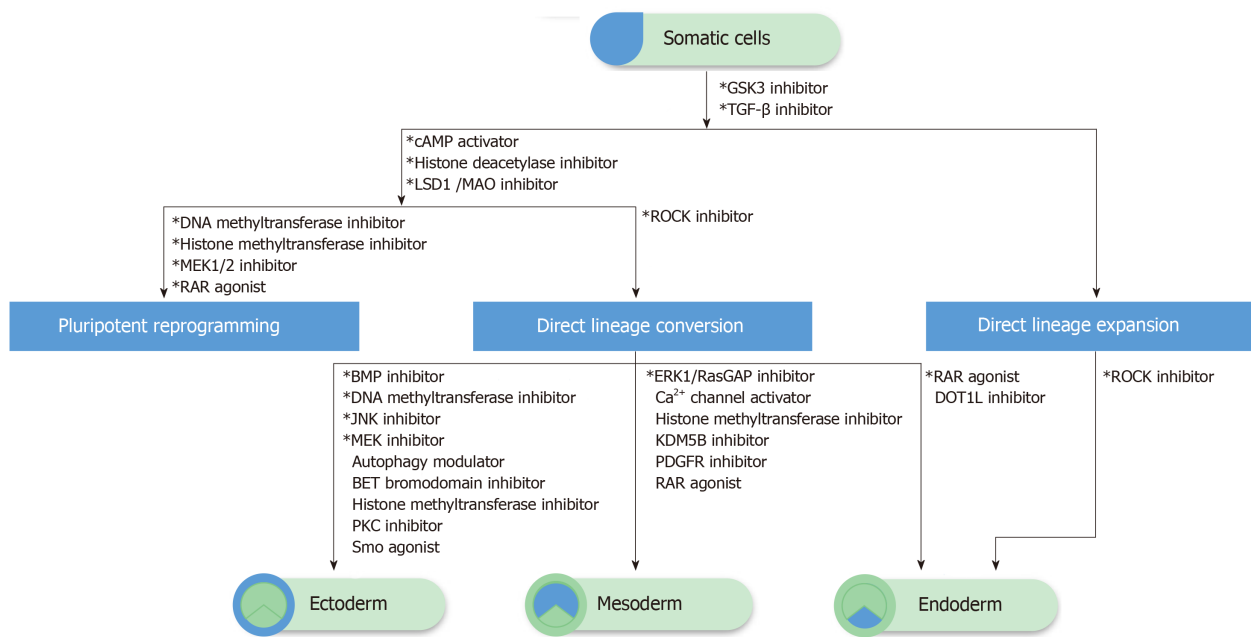
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## INTRODUCTION

The barriers to cell fate conversion between somatic cells and pluripotent cells had a breakthrough with the proposition of the induced pluripotent stem cell (iPSC) reprogramming strategy in 2006, when Takahashi *et al*<sup>[1,2]</sup> reported a significant discovery that the ectopic expression of four defined transcription factors (TFs; *Oct4*, *Sox2*, *Klf4a*, and *c-Myc*) could force the cell fate conversion. Remarkably, iPSCs can be converted into multiple functional cells under optimal differentiation strategies, not only contributing to the establishment of patient-specific disease models but also benefiting drug discovery and development<sup>[3-5]</sup>. Later, combinations of lineage-specific TFs were screened and applied to generate various desired cell types, known as direct lineage conversion, including neurons, cardiomyocytes, hepatocytes, and pancreatic  $\beta$ -cells<sup>[6-9]</sup>; this strategy bypasses the transition pluripotent stage, rendering the process faster and more effective and, meanwhile, evading the risk of pluripotency-related tumorigenesis, holding great promise for biomedical applications<sup>[10,11]</sup>.

However, to date, TF-based reprogramming approaches face numerous challenges in efficiency, especially in safety, regarding the use of oncogenes and likely genetic integration of exogenous factors. Researchers have made substantial efforts to optimize the reprogramming using nonviral and nonintegrating methods, including synthetic RNAs<sup>[12,13]</sup>, cell membrane-permeable proteins<sup>[14]</sup>, episomal plasmids<sup>[15]</sup>, and chemical compounds<sup>[16]</sup>. Comparatively, chemicals offer several unique advantages such as structural stability; spatiotemporal flexibility; easy for screening, application, and delivery; amenability to manufacturing and scale-up; and the possibility of fine-tuning their effects by altering their concentrations and combinations<sup>[17,18]</sup>. To date, several small molecules have been proved to facilitate the iPSCs reprogramming and lineage conversion, either by their substitute role for replacing certain TFs, or synergetic effect for augmenting the efficiency<sup>[19-22]</sup>. Additionally, the progressive attainment of full chemical-induced human pluripotent and lineage-committed cells merits great promise to resolve the cell source limitation for the therapeutic purpose (Figure 1), as well as offer an alternative option for human disease modeling and drug development.

Perhaps, establishing a stable and efficient chemical induction strategy could fundamentally alter the principal concept of the conventional cell reprogramming strategy. Nevertheless, to date, the specificity of chemical targets and the correlative gene network dynamics remain unclear<sup>[17,23]</sup>. Thus, elucidation of the individual or synergetic chemical force on the cell pathway signaling and epigenetic pattern



**Figure 1 The schematic of chemical-driven cell fate change and expansion.** The transforming growth factor (TGF)- $\beta$  and GSK3 pathway inhibitors are commonly required in pluripotency reprogramming, direct lineage conversion, and expansion. Additional epigenetic modulators (histone deacetylase inhibitors and/or deoxyribonucleic acid/histone methyltransferase inhibitors) are applied for pluripotency reprogramming, as well as direct lineage conversion, while different lineage commitments require specific signaling modulations. The combination of TGF- $\beta$ , GSK3, and ROCK pathway inhibitors could induce the direct lineage expansion of endoderm-committed cells such as hepatocytes. The direct expansion of ectoderm and mesoderm-committed cells remains unclear and could not be listed here. \*Necessary and/or commonly used compounds.

modulation would be vital to endorse the induction of desired cell types proficiently. Hence, this review aims to summarize the updated progress and discuss the advantages, limitations, and challenges of the impact of full chemicals on stepwise reprogramming of pluripotency, direct lineage conversion, and direct lineage expansion on somatic cells.

## PLURIPOTENT REPROGRAMMING

The first breakthrough for pluripotency reprogramming by full chemical treatment was attained in 2013<sup>[24]</sup>. By screening, Hou *et al.*<sup>[24]</sup> first successfully identified the chemical substitutes for Takahashi *et al.*<sup>[1,2]</sup>'s four TFs, including VPA, CHIR99021, 616452, tranylcypromine, Forskolin, and DZNep (VC6TFZ), which could convert mouse fibroblasts into a partially reprogrammed state with high Oct4 expression; embryonic stem cell-like chemically-iPSCs (CiPSCs) could be generated after switching to 2i (CHIR99021 and PD0325901) medium. Additionally, it was revealed that an endogenous pluripotency program could be established by chemically manipulating the cell signaling pathways. Although the routes and mechanisms underlying pluripotency reprogramming remain unclear to date, two primary hypotheses reveal how chemicals drive somatic cells stepwise toward pluripotency.

### Stepwise bridges

During the attainment of a pluripotent program, unique extraembryonic endoderm (XEN)-like state was first discovered linking fibroblasts to pluripotency, which markedly expressed XEN genes *Sall4*, *Gata4*, *Gata6*, *Sox17*, and *Sox7*<sup>[25]</sup>. Remarkably, the knockdown of XEN genes during the reprogramming markedly impairs the generation of CiPSCs, whereas their overexpression is adequate to replace essential chemicals (CHIR99021, 616452, and Forskolin) for the Oct4-positive XEN-like colony formation, illustrating that the XEN-like state is a vital intermediate state toward CiPSCs. Of note, despite different cell origins, the similar activation of the XEN-like program was reported during the early stage of reprogramming from neural stem cells (NSCs) and small intestinal epithelial cells (IECs)<sup>[26]</sup>, highlighting that the XEN-like state serves as a vital and exclusive bridge toward pluripotency. Remarkably, the reprogramming kinetics and frequency of XEN-like state were highly distinct between NSCs and IECs. Compared with NSCs, IECs exhibited much rapid and efficient XEN-like colony formation, which could be explained by the fact that the inherent

expression of *Gata4* and *Gata6* in IECs facilitated the conversion of XEN-like program at an early stage.

After 3 years, the same group reported that the 2C (two-cell stage)-like programs were key bridges linking the XEN-like state to pluripotency, and the expression level of the 2C-like program (*Zscan4c*, *Zscan4f*, *Tcstv1*, *Tcstv3*, *Lmx1a*, and *Sp110*) well correlated with the reprogramming potential<sup>[27]</sup>. The knockdown of 2C genes markedly impaired the reprogramming efficiency of CiPSCs. Additionally, extensive loss of deoxyribonucleic acid (DNA) methylation was detected in this stage, which corroborated the hallmark of global epigenetic reprogramming in early embryogenesis, elucidating further the stepwise establishment of “early embryonic-like programs” toward the complete pluripotency network. Notably, when the 2C-like program was enhanced by the optimized treatment of VPA (histone deacetylase inhibitor), the reprogramming efficiency was markedly augmented.

### **Chromatin accessibility dynamics**

BrdU, commonly used in tracing DNA replication, was occasionally reported to facilitate OSKM-induced reprogramming and demonstrated further that it could replace Oct4 in Yamanaka factors-mediated reprogramming and promote full chemical reprogramming<sup>[28]</sup>. Nevertheless, to date, the precise mechanisms by which BrdU promotes TFs and chemical-mediated reprogramming remain unclear. Based on the benefits of BrdU on closing or opening of chromatin loci, the dynamic chemical reprogramming process was revealed in the view of chromatin accessibility<sup>[29]</sup>. At stage 1 of chemical induction, the AP1 families-related chromatin loci in fibroblasts were closed gradually, whereas chromatin loci enriched with *GATAs*, *FOXs*, *KLFs*, and *SOXs* were opened, which highly corroborated the XEN-like intermediate state, as reported previously<sup>[25]</sup>; however, it markedly differed from TF-mediated reprogramming, which does not need to bypass through this particular state<sup>[30]</sup>, illustrating the unique epigenetic dynamics driven by chemicals. Following the treatment of 2iL at stage 2, *GATAs*- and *FOXs*-related loci turned to close gradually, along with the opening for OCT/SOX/KLF families. Notably, BrdU is essential to correctively open and close chromatin loci enriched with the XEN-like and fibroblast program at stage 1. Remarkably, in this study, the overexpression of master XEN genes could not replace BrdU for opening specific loci for the Oct4 expression, which contradicted a previous study<sup>[25]</sup>.

Despite published protocols, currently, the successful induction of CiPSCs is restricted in limited groups, and the core factors and induction efficiency remain debatable<sup>[25,27-29,31]</sup>, which could be partially explained by the nonspecificity of chemicals. Additionally, the targeted signaling pathway and/or epigenetic modulation could change much because of the tiny bias of chemical concentrations and combinations, making it difficult to establish stable intermediate states or correctly open or close the required chromatin loci. Generally, the current low reproducibility raises severe challenges for enhancing the stepwise protocol for generating CiPSCs.

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## **DIRECT LINEAGE CONVERSION**

Recently, the advancements of TF-mediated direct lineage conversion from somatic cells to other cell types have garnered considerable attention; this strategy bypasses the acquisition of pluripotent state and serves a promising approach for generating numerous types of functional cells. The impact of chemicals was first reported to facilitate the TF-mediated lineage conversion. Supplemented with specific chemicals, multiple cells from different germ layers have been successfully generated from fibroblasts or other somatic cells with minimal utilization of lineage-specific TFs, including neuronal cells<sup>[32-35]</sup>, cardiac cells<sup>[36,37]</sup>, hepatocytes<sup>[22,38]</sup>, and pancreatic cells<sup>[39,40]</sup>. Additionally, chemical cocktails could markedly augment the TF-mediated conversion with relatively high efficiency and purity<sup>[22,32]</sup>. However, from the perspective potential risks of genetic integration, direct lineage conversion by full chemicals merits much anticipation at present.

### **Ectoderm**

The first full chemical cocktail reported to generate neural progenitor cells (NPCs) directly from mouse and human somatic cells was a simple and effective VCR (VPA, CHIR99021, and RepSox) combination<sup>[41]</sup>. The chemical-induced NPCs (CiNPCs) exhibited similar transcription profiles as brain-derived NPCs and could self-renew and further differentiate into different neural lineage cells both *in vitro* and *in vivo*. Later, using alternative chemical cocktails, NSC-like cells were attained by other groups<sup>[42,43]</sup>. Notably, FGF and Shh signaling pathways recompiled the transcriptional

and epigenetic programs from fibroblast to neural lineage by modulating the binding and activation of immediate downstream TFs Elk1/Gli2 to master neural genes<sup>[43]</sup>.

Bypassing the NPC state, direct chemical conversion of mature neurons (CiNs) from mouse and human fibroblasts has been achieved<sup>[44-46]</sup>. Compared with fibroblasts, astrocytes are extensively considered as a preferable starting cell source for neurogenesis, as well as direct neuron conversion<sup>[47,48]</sup>. Under chemical-mediated epigenetic silencing of glial genes, as well as the transcriptional activation of crucial neural transcriptional factors (NEUROD1 and NEUROGENIN2), human astroglial cells were reportedly converted into functional neurons efficiently, with the ability to integrate into local circuits *in vivo*. Additionally, a recent preprint report of *in vivo* direct conversion garnered considerable attention. A study reported successfully converting resident astrocytes to functional neurons *in situ* in adult mouse brain<sup>[49]</sup>. Remarkably, such *in situ*-generated neurons acquired electrophysiological functions and could functionally interact with resident neurons in the brain.

### Mesoderm

Under the two-step chemical treatment based on CRFVPT (CHIR99021, RepSox, Forskolin, VPA, Parnate, and TTNPB), for the first time, mouse fibroblasts were successfully converted to spontaneously beating cardiomyocyte-like cells (CMs) through a *Scn-1*<sup>+</sup> cardiac precursor-like stage<sup>[50]</sup>. Later, another study reported the generation of mouse chemical-induced CMs (CiCMs) by replacing the core chemical RepSox with another TGF- $\beta$  inhibitor, A83-01<sup>[51]</sup>. Notably, CiCMs induced by either approach expressed cardiomyocyte-specific markers and displayed typical cardiac calcium flux and electrophysiological features, resembling primary cardiomyocytes. Remarkably, CiCMs could also be induced directly from resident cardiac fibroblasts *in vivo*. Despite the relatively low efficiency, the induced cardiomyocytes could markedly enhance cardiac functions in myocardial infarction mice<sup>[52]</sup>.

In particular, during the conversion of human CiCMs, platelet-derived growth factor receptor inhibitors reportedly facilitated the cardiac conversion by suppressing the fibroblast program<sup>[53]</sup>. Additionally, the synergistic effects of chemicals for epigenetic modulation were determined, enabling cells responsive to extrinsic cardiogenic signals. Furthermore, the chemicals promoted the chromatin accessibility of core cardiogenesis genes loci, enabling effective binding of cardiogenic signal effectors, such as  $\beta$ -catenin and Smad1, and ultimately facilitating the cell fate conversion.

### Endoderm

During the chemical induction of mouse CiPSCs, some studies reported a unique XEN-like intermediate state<sup>[25,26,29]</sup>; these extraembryonic endoderm-like cells shared similar global gene expression patterns and *in vivo* developmental potential to the embryo-derived XEN cells<sup>[25]</sup> and displayed high plasticity for directing endoderm and ectoderm lineage cells. Under favorable induction conditions, both hepatocytes and neurons could be generated<sup>[54]</sup>. Remarkably, when cultured in a lineage-favorable condition, the multipotential intermediate status appeared prone to incline to a specific direction. Combined with the hepatocyte culture medium and activin A, mouse endoderm progenitor cells (EPCs) were induced with the robust expression of endoderm markers *Sox17*, *Foxa2*, *Gata4/6*, and *Hnf4a*, while lacking ectoderm markers<sup>[55]</sup>. Additionally, chemically induced EPCs (CiEPCs) displayed marked foregut/liver differentiation potential regarding the markedly elevated expression of *Krt8*, *Krt18*, and *Krt19*. Furthermore, under specific differentiation conditions, both hepatocyte-like cells (HLCs) and pancreatic lineage cells could be generated.

To date, the induction of human EPCs from somatic cells was restricted to the same germ layer. Initiated from gastrointestinal epithelial cells, human CiEPCs were reportedly generated under the support of tissue-specific mesenchymal feeders<sup>[56]</sup>, endowed with bi-potential differentiation capacity toward hepatocytes, pancreatic endocrine cells, and IECs. Nevertheless, despite growing evidence demonstrating that chemicals could facilitate the TF-mediated induction of human endoderm lineage cells from fibroblasts or other distant lineages, such as hepatocytes<sup>[22,38]</sup> and pancreatic cells<sup>[39]</sup>, there was seldom achievement mediated by a full chemical strategy. Perhaps, gastrointestinal epithelial cells are more amenable for CiEPCs induction than fibroblasts because of their proximity in lineage distance. Regarding the difference in transcriptome and pathway profiles between humans and mice, further chemical cocktail screening is warranted to attain the direct endoderm conversion from other germ layers.

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## DIRECT LINEAGE EXPANSION IN HEPATOCYTES

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Although direct lineage conversion from fibroblasts or other somatic cells has established predominant advantages for regenerative medicine<sup>[41,43]</sup>, the relative deficient functional maturation and engraftment *in vivo* remain a major obstacle<sup>[57]</sup>. For decades, human hepatic cell source is in high demand for liver disease treatment because of the shortage of available liver organs<sup>[58,59]</sup>. The generation of a large number of functional and transplantable hepatic cells merits considerable clinical significance and has garnered substantial attention. In recent years, TF-mediated direct reprogramming of human-induced hepatocytes (hiHeps) has garnered more attention, overwhelming the conventional iPS-derived HLCs, in terms of markedly reduced risk of tumorigenesis. Despite the progressive enhancements in efficiency and purity of hiHeps, the extremely low *in vivo* repopulation capability, as well as deficient functions regarding metabolism, markedly hampered their transplantation applications<sup>[60,61]</sup>. Most recently, the successive achievements in the chemical induction of primary hepatocytes highlight the acquisition of highly expandable characteristics (Table 1), which could markedly promote the development of hepatic cell-based liver disease therapies.

### **Lineage-committed cells and progenitors**

To break the blockage for expansion, a simple and effective combination of three core chemicals YAC (Y-27632, A83-01, and CHIR99021) was first reported in 2017, enabling the conversion of terminally differentiated rat and mouse hepatocytes to highly expandable liver progenitor-like state, with bi-potential differentiation capacity toward mature hepatocytes and biliary epithelial cells<sup>[62]</sup>. Later, using the same core chemical combination, the induction of progenitor-like state was established further by another group with *in vivo* repopulation and maturation capability<sup>[63]</sup>.

However, the induction effect of YAC was only restricted to hepatocytes originated from rodents, until the discovery of HGF, which was highlighted to be essential for establishing a human hepatic progenitor-like state through the ERK-1/2 signaling activation<sup>[64]</sup>. Remarkably, during the induction of human hepatocytes by modified cocktail HAC (HGF, A83-01, and CHIR99021), not only were hepatic progenitor markers markedly elevated but also endoderm and pluripotency markers were detected<sup>[64]</sup>, suggesting the potential acquisition of multilineage differentiation capacity other than the hepatic fate. Additionally, nicotinamide, commonly used for hepatocyte culture<sup>[65,66]</sup>, inhibited the proliferation and even induced apoptosis through the inactivation of SIRT1, offering a clue for long-term culture optimization. Of note, under the three-dimensional differentiation condition, the expanded progenitor-like cells could regain the expression of hepatitis B virus (HBV) receptor sodium taurocholate cotransporting polypeptide, which could markedly support the HBV infection or reactivation modeling<sup>[67]</sup>.

Besides the markedly elevated expression of progenitor-associated markers, the HAC-based induction approach resulted in the suppressed expression of most mature hepatocyte markers<sup>[64,67]</sup>. However, when one of the core chemicals, CHIR99021, was replaced with Wnt3a, a unique proliferative state was established, which partly retained mature hepatocyte characteristics while exhibiting progenitor-associated identity<sup>[68]</sup>. Besides the GSK3-mediated Wnt pathway activation, special insight was provided into the crosstalk between Wnt3a and specific cell signaling regarding hepatic progenitor self-renew and proliferation such as the Hippo-YAP pathway<sup>[69]</sup>. Moreover, it implied that the CHIR99021-mediated pathway modulation could largely erase the initial memory of hepatocyte identity, while the Wnt3a-mediated modulation partially sustained these signatures. Remarkably, when induced cells were transplanted *in vivo* for over 4 mo, the human albumin secretion and repopulation efficiency could attain a comparable level to primary human hepatocytes, suggesting that these induced cells could serve as the most compatible hepatic cell source that had ever been reported beyond primary hepatocytes.

### **Organoid expansion**

Using a highly similar chemical and cytokine cocktail, both human and mouse primary hepatocytes have been established to form organoids (Hep-Orgs) in a 3D culture system and stably expand for a long term<sup>[70]</sup>. Hep-Orgs comprise noncycling mature hepatocytes and cycling hepatocyte progenitor cells, exhibiting comparable gene expression profiles and functions to primary hepatocytes. Remarkably, progenitor markers were not markedly elevated in Hep-Orgs, which was considerably different from that in conventional 2D culture<sup>[68]</sup>. Despite applying a different Wnt agonist, R-spondin, instead of Wnt3a, the major discrepancy could be contributed by the special circumstance in the organoid culture system, which seemingly recapitulated the regeneration microenvironment of the adult liver *in vivo*. In Hep-Orgs, cell cycle and ribosome synthesis-related gene expression was markedly enhanced, resembling the response of hepatocytes to acute liver damage such as

Table 1 Expandable hepatic cells induced from primary hepatocytes to date

Hepatocyte source(s)	Chemicals	Growth factor(s)	Medium and supplements	Expansion potential		Yr
				Passage count	Doubling time	
Mouse and Rat	A83-01, CHIR99021, Y-27632	EGF	DMEM/F12, HEPES, L-proline, ITS, dexamethasone, nicotinamide, ascorbic acid-2 phosphate, BSA, antibiotic/antimycotic	Rat: >10; Mouse: >20	Rat: 14.7 ± 1.1 h	2017 <sup>[62]</sup>
Mouse	A83-01, CHIR99021, Y-27632	EGF, HGF	DMEM/F12, N2 or ITS, S1P, LPA	>30	15-20 h	2017 <sup>[63]</sup>
Human (resected patient liver tissue, non-lesion)	A83-01, CHIR99021	EGF, HGF	DMEM/F-12 (high glucose), FBS, nicotinamide, dexamethasone, ITS, penicillin/streptomycin	>10	37.9-39.8 h	2018 <sup>[64]</sup>
Human (resected patient liver tissue, non-lesion)	A83-01, CHIR99021, Y-27632	EGF, HGF	Advanced DMEM/F-12, N2, B27, sodium pyruvate, ascorbic acid, S1P, LPA	>10	24.7 ± 1.4 h	2018 <sup>[67]</sup>
Human (normal, cryopreserved)	A83-01, Y-27632	EGF, FGF10, HGF, Wnt3a	Advanced DMEM/F-12, FBS, N2, B27 (minus vitamin A), N-acetylcysteine, nicotinamide, [Leu15]-gastrin I, penicillin/streptomycin,	4 (normoxia); 8 (hypoxia)		2018 <sup>[68]</sup>
Human (normal, freshly isolated and cryopreserved)	A83-01, CHIR99021, Y-27632	EGF, FGF7, FGF10, HGF, TGFa	Advanced DMEM/F-12, HEPES, B27 (minus vitamin A), R-spondin1 conditioned medium, N-acetylcysteine, nicotinamide, gastrin, GlutaMAX, penicillin/streptomycin,	Fetal hepatocytes: >16	5-7 d	2018 <sup>[70]</sup>

partial hepatectomy.

### Limitations

Notably, human hepatocyte-induced proliferative cell sources displayed large scale expandable potential, as well as superior compatibility and functional maturation, for transplantation compared with iPSCs-derived HLCs or hiHeps, exhibiting a remarkably higher repopulation rate and human albumin secretion<sup>[61,71]</sup>. As the maturation direction of human hepatocyte-induced proliferative cell sources is lineage-committed, they did not necessarily require an extra differentiation process, severing as ready-to-use transplantable sources, and might merit broader prospects for breaking through the obstacle for liver disease treatment. Nevertheless, currently, the expansion efficiency of human hepatocytes is remarkably lower than that of rodent hepatocytes. Compared with rodent hepatocytes, human hepatocytes required approximately a 10 h longer period for cell doubling, whereas the maximum passage counts for stable expansion declined approximately 10 times<sup>[63,67]</sup>. Regarding the proliferation kinetics, rodent hepatocytes essentially required three to five passages for establishing an accelerated proliferative state, while human hepatocytes exhibited a gradual loss of proliferation potential after three to four passages, implying that the building of a proliferative intermediate state for human hepatocytes might be relatively insufficient or unstable. Although a hypoxic expansion condition possibly inhibited the senescence and prolonged proliferation<sup>[68]</sup>, an understanding of the different transcriptome and pathway network profiles between humans and mice is required to reveal specific signaling for human hepatocyte expansion.



Telomere shortening during prolonged expansion is another limiting factor<sup>[70]</sup>. The unlimited self-renewal and genomic stability of pluripotent stem cells were facilitated by sufficiently long telomeres<sup>[72]</sup>. During pluripotent reprogramming, which was either TF- or chemical-mediated, short telomeres of somatic cells were significantly elongated in a process mediated by telomerase activity upregulation or by recombination-based ALT mechanisms<sup>[31,73]</sup>. Moreover, reprogramming triggers telomere elongation regardless of donor age status<sup>[74,75]</sup>. Thus, telomere erosion, characterized in aged and senescent cells, can be efficiently rejuvenated after pluripotent reprogramming. However, direct lineage conversion and expansion from somatic cells bypass the pluripotent intermediate status, thereby preserving age-associated features. Compared with the iPSC-derived counterpart, extensive DNA damage, loss of heterochromatin and nuclear organization, and increased SA- $\beta$ -Gal activity were observed in lineage-converted functional cells<sup>[76]</sup>. Therefore, even though the conversion is successfully completed, the converted cells seem to inherit aging signatures from parental cells, including short telomeres, and be prone to senescence after passages. During chemical-mediated expansion of primary human hepatocytes, the inherently short telomeres and insufficient telomerase activation may play a key role in eliciting senescence of induced proliferative cells after limited passages<sup>[68]</sup>, which remains a major obstacle for large-scale expansion *in vitro*. In contrast, we found that when human iPSC-derived HLCs are chemically induced for expansion, they exhibit a superior expansion advantage compared with primary hepatocytes. Induced cells can stably expand for extended passages while sustaining hepatic differentiation potency (unpublished data). The relatively high telomerase activity inherited from iPSCs potentially restores or elongates telomeres, facilitating long-term expansion. The ability to use chemicals to activate telomerase and elongate telomeres in human terminally differentiated cells such as hepatocytes would be of great importance for an extended expansion capability.

Moreover, the advantage of the organoid culture system has been proved to be supportive for the long-term expansion with enhanced functional maturation; however, the current expansion efficiency was much lower than the 2D system, and the culture condition optimization, such as the extracellular matrix, warrants further investigation.

Despite the achievements in hepatocytes, direct expansion of other lineage cells, such as neurons and cardiomyocytes, has been seldom reported. Undoubtedly, the direct conversion from fibroblasts has already proved a feasible option for generating these functional cells. It is expected that when the direct lineage expansion is applied, the *in vivo* repopulation and functional maturation could be enhanced further.

## CHALLENGES

The chemical-mediated direct lineage conversion and expansion, bypassing potential risks using transgenic methods, as well as pluripotency-related tumorigenesis, offer promising options for therapeutic purpose. However, several challenges remain regarding the unrevealed mechanism of chemical effects.

### **Cell fate specificity**

To date, several dozens of chemicals have been identified in various combinations for cell induction<sup>[11,16,18,77]</sup>. Although the precise mechanism underlying a particular conversion between different cell fates remains unclear, chemical inducers could be classified into two major groups – epigenetic modulators and signaling regulators. Epigenetic modulators, typically HDAC inhibitors and DNA/histone methyltransferase inhibitors, such as VPA and BIX01294, are commonly used for the induction of CiPSCs, CiNSCs, and CiCMs<sup>[27,41,53]</sup>. Conversely, signaling regulators specify the characteristics of the designated cell identity. Reportedly, ISX9 and AS8351 were specific for the CiNs induction<sup>[44,78]</sup>. Additionally, SC1 was specific for the CiCMs induction<sup>[51,53]</sup>. The 2i combination (CHIR99021 and PD0325901) was always required for the late stage of the CiPSCs induction<sup>[24,25]</sup>. However, RepSox, A83-01, CHIR99021, and Forskolin were extensively used as essential factors for inducing various cell types, including CiPSCs, CiNPCs, CiNs, CiCMs, and CiEPCs<sup>[24,43,53,55,78]</sup>. The nonspecific feature of a significant portion of the chemicals could be attributed to the fact that same signaling pathways are often shared by the multilineage development such as the Wnt and TGF- $\beta$  pathways<sup>[79]</sup>; it may not only extend the induction application for a broad range of cell types but also possibly lead to undesired cell fates. Using the same chemical cocktail for the CiPSCs induction, the generation of an unexpected cardiac fate was uncovered<sup>[50]</sup>. The possible explanation was that the chemicals might induce a nonspecific multipotential intermediate state, which was highly plastic and

unstable; or a heterogeneous mixture of multiple progenitor cell types. Under favorable conditions, the induction direction could incline further to cardiac or pluripotent fate.

To exclude the unanticipated induction fate, it would be challenging to elucidate further the individual and synergistic effect of chemical cocktails regarding cell signaling and epigenetic regulation, as well as the specificity and commonality for distinct lineages (Table 2), and then develop stepwise reliable chemical cocktails that could precisely direct the desired cell types.

### Cell identity memory

Each cell type has its unique transcriptome, which defines its cell identity. Reprogrammed cells more or less inherit the cell identity memory of their ancestor. An analysis of molecular traces during the induction of iPSC-HLCs and hiHeps identified original fibroblast identity efficiently but not completely erased in both cell types<sup>[61]</sup>. Additionally, iPSC-HLCs exhibited the expression of endoderm progenitor (*FOXA2* and *GATA6*) and hepatoblast (*AFP* and *EPCAM*) markers, suggesting that the memory of molecular traces during the differentiation path was also sustained in iPSC-HLCs; however, the hiHeps induction bypasses these cell identities transition, exhibiting no expression of these markers. Overall, the sustained memory during reprogramming and differentiation process could elucidate the fact that both c and hiHeps exhibited distinct characteristics from primary hepatocytes in gene expression and related functions<sup>[80-82]</sup>.

Additionally, chemical-mediated reprogramming was also challenged by the initial cell identity modulation. The investigation of the direct induction of neurons identified a bromodomain inhibitor, IBET151, as a core compound needed to erase the initial fibroblast transcriptional network program for cell identity rewriting<sup>[78]</sup>; the mechanism could be concerned with the blocking effect that IBET151 disrupted the accessibility of bromodomain proteins to acetylated histones related to fibroblast programs, resulting in transcriptional silencing<sup>[83]</sup>. Additionally, the bromodomain and extra-terminal domain inhibitors remarkably enhanced iPSC and NPC induction *via* switching off a large set of initial somatic transcriptional programs<sup>[84]</sup>. Besides, platelet-derived growth factor receptor (PDGFR) inhibitors, SU16F and JNJ10198409, reportedly accelerated the downregulation of fibroblast genes during the conversion of human cardiomyocytes, markedly enhancing the efficiency<sup>[53]</sup>. Perhaps, further investigation of chemicals with the erase effect on specific cell types would booster the efficiency of the cell fate conversion.

Conversely, the initial somatic transcriptional memory could also facilitate the transplantation. In a study, when direct lineage expansion was applied in primary hepatocytes, their partly sustained hepatocyte transcriptional memory could facilitate quick engraftment and functional maturation after transplantation *in vivo*<sup>[68]</sup>. Of note, based on our study, when cells were chemically induced from iPSC-HLCs, they exhibited relatively low *in vivo* repopulation capability, which corroborated that before induction (unpublished data). The possible explanation was that the induced cells inherited portions of transcriptional memory of iPSC-HLCs, including the deficiency in repopulation capability. This discrepancy not only highlights the hurdles for transplantation of iPSC-derived functional cells but also emphasizes the elucidation of the cell memory dynamics during induction.

### In vivo induction

Conventionally, long-term *in vitro* expansion is critical to obtain sufficient functional cells for transplantation, whereas the potential risk of functional alteration and genetic mutations from the *in vitro* microenvironment raises serious problems<sup>[57,85]</sup>. Theoretically, *in vivo* reprogramming could produce functional cells followed by inducing resident functional cells with bi-potency or proliferation capability, or directly convert the neighboring cell types proximal in lineage distance and, meanwhile, take advantage of the *in situ* niche to regenerate the damaged tissue or organ efficiently, which remains a major obstacle for *in vitro* circumstances.

In recent years, substantial TF-mediated *in vivo* reprogramming has demonstrated some exciting achievements, including neuroblasts or neurons induced from glial cells<sup>[86,87]</sup>, cardiomyocytes induced from cardiac fibroblasts<sup>[88-90]</sup>, hepatocytes induced from myofibroblasts<sup>[91,92]</sup>, and pancreatic  $\beta$ -like cells induced from exocrine cells<sup>[9,93]</sup>. Despite great promise for diseases treatment as demonstrated by previous studies, the TF-mediated strategy also poses risks of genome integration, tumorigenesis, as well as manufacturing and delivery problems. Comparatively, chemical-mediated strategy minimized the risk of genetic alteration and is easier for scalable manufacturing, stocking, and delivery<sup>[23]</sup>, eventually preferable for *in vivo* therapeutic applications. Recently, for the first time, *in vivo* chemical reprogramming was reported to successfully convert the resident astrocytes into functional neurons in the adult mouse

Table 2 The specificity and commonality of chemicals in direct lineage conversion

Compounds	Function	Target germ layer and cell types			Ref.
		Ectoderm: Neural stem/ progenitor cell, neuron	Mesoderm: Cardiomyocyte	Endoderm/ extraembryonic endoderm: Endoderm progenitor cell, XENs	
A83-01	TGF-βRI (ALK4/5/7) inhibitor	+	+		[42,43,51,53]
AM580	RAR agonist			+	[25,54]
AS8351	KDM5B inhibitor		+		[53]
Bay K 8644	Ca <sup>2+</sup> channel activator		+		[51]
BIX01294	Histone methyltransferase inhibitor	+	+		[42,53]
CHIR99021	GSK3 inhibitor	+	+	+	[25,41-46,50,51,53-55,78]
DMH1	BMP inhibitor	+			[46]
EPZ004777	DOT1L inhibitor			+	[25,54]
Forskolin	cAMP activator	+	+	+	[25,44-46,50,51,54,55,78]
GO6983	PKC inhibitor	+			[44]
Hh-Ag1.5	Smo agonist	+			[43]
I-BET151	BET bromodomain inhibitor	+			[78]
ISX9	Neurogenic agent	+			[78]
JNJ10198409	PDGF-RTK inhibitor		+		[53]
LDN193189	BMP type I receptor (ALK2/3) inhibitor	+			[43,45]
OAC2	Oct4 activator		+		[53]
Parnate (Tranylcypromine)	LSD1/MAO inhibitor	+	+	+	[25,43,50,54]
PD0325901	MEK inhibitor	+			[42,45]
RepSox (616452)	TGF-βRI (ALK5) inhibitor	+	+	+	[25,41,44,46,50,54,55]
RG108	DNA methyltransferase inhibitor	+			[42,43]
SB431542	TGF-βRI (ALK4/7) inhibitor	+			[45,78]
SC1	ERK1/RasGAP inhibitor		+		[51,53]
SMER28	Autophagy modulator	+			[43]
SP600125	JNK inhibitor	+			[44,46]
SU16F	PDGFRβ inhibitor		+		[53]
TTNPB	RAR agonist		+	+	[50,55]
VPA	Histone deacetylase inhibitor	+	+	+	[25,41,42,44,46,50,54]
Y27632	ROCK inhibitor	+	+	+	[44,46,53-55]

XENs: Extraembryonic endoderm-like cells.

brain, resembling endogenous neurons in both neuron-specific marker expression and electrophysiological properties<sup>[49]</sup>. Meanwhile, cardiomyocytes were induced from adult cardiac fibroblasts by direct full chemical administration *in vivo*, and although the reprogramming efficiency is relatively low, the chemical cocktail treatment markedly decreased the scar formation and enhanced cardiac functions in myocardial infarction mice<sup>[52]</sup>; these encouraging achievements not only provide a general strategy for *in vivo* reprogramming but also open a novel path to regenerate the diseased organs *in situ*.

However, the off-target or unspecific-target effects of chemicals pose challenges for the *in vivo* induction, as the chemical treatment could target undesired cell types *in situ* or nearby, potentially declining the intrinsic homeostasis of local cell populations. Moreover, exogenous chemicals could also break the homeostasis of local niche and

cause unintended tissue damage. Thus, it will be essential to evaluate the side effects *in vivo* in the long term, and the chemical combinations and concentration should be optimized to adapt physiological homeostasis synergistically. Finally, despite the high permeability advantage of chemicals in tissue, the arrival to unwanted location should be avoided. Advances in the delivery system are warranted to deliver chemicals to designated locations for an expected period precisely. Overall, enhanced targeting and shielding capabilities will be indispensable for attaining effective reprogramming *in vivo*.

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## PERSPECTIVES

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Generating substantial functional cells with tissue/organ regeneration capability is a major challenge for regenerative medicine. Despite the rapid progress of the conventional strategy regarding the differentiation from pluripotent stem cells<sup>[94,95]</sup>, the direct lineage conversion, bypassing the pluripotent stage, has highlighted a promising alternative strategy in recent years<sup>[57]</sup>. Additionally, chemical-mediated strategy, targeting the modulation of epigenetic status and signaling pathways without interfering the genome integration, exhibited the unique advantage over transgenic and other approaches for cell reprogramming, especially regarding the expansion potential<sup>[23,77]</sup>. Remarkably, chemically induced cells, including XEN-like state and lineage-specific stem/progenitors (CiNSCs, CiNPCs, and CiEPCs), exhibited highly expandable characteristics, which could markedly satisfy the predominant requirement for clinical use. Additionally, recently reported direct lineage expansion holds great promise for cell transplantation applications. As these induced cells not only exhibited the robust proliferation capability but also partly sustained the initial cell identity memory, it facilitated direct and rapid revert to mature state after *in vitro* differentiation or been transplanted *in vivo*. However, it remains a major obstacle for direct lineage conversion from fibroblasts or other somatic cells, which exhibited a significant deficiency in functional maturation. Although the achievement to date in direct lineage expansion of human functional cells remains highly limited, the success in human hepatocytes would undoubtedly offer a general idea for extending this strategy to other desired cell types. Moreover, the limited expansion capability of induced human hepatocytes also evokes the importance of exploring chemical candidates to activate telomerase and rejuvenate telomeres, which may potentially extend expansion of induced cells *in vitro* (Table 3).

Holding an excellent promise for direct lineage conversion and expansion, spatiotemporal flexibility and nonintegrative characteristics of chemical strategy could also be favorable for the *in vivo* induction approach, offering superior advantages for therapeutic potential<sup>[94]</sup>. As safety and efficiency might be the rate-limiting step for *in vivo* reprogramming<sup>[96]</sup>, assistant technology, such as nano-delivery system, might be required to deliver chemicals to specifically targeted sites in a controlled manner.

Along with the advances of chemical screening, discrete combinations of pathway-specific chemicals have progressively been identified to reprogram somatic cells into many lineages. Nevertheless, improved knowledge of the pathway networks, together with the epigenetic pattern that drives the cell fate conversion and proliferation, is warranted to intelligently enhance the induction efficiency and specificity *in vitro* and *in vivo*.

Table 3 Advantages and disadvantages of different strategies for functional cell induction

Strategy	Induction efficiency	Reproducibility/stability	Target specificity	Cellular function	In vivo engraftment	Safety	Scaling up	Cost	Ref.
TF-mediated pluripotent reprogramming followed by differentiation	Moderate	Highly reproducible/stable	High	Immature	Low	Genomic integration; tumorigenesis risk	Expandable before differentiation	Very high	[11,97-99]
TF-mediated direct lineage conversion	Fast and efficient	Reproducible/stable	High	Deficient	Low	Genomic integration	Expandable in progenitors	High	[100-103]
Chemical-mediated pluripotent reprogramming followed by differentiation	Controversial	Poorly reproducible/unstable	Low	Not clear	Not clear	Integration-free	Not clear	Low	[24,25,29,31]
Chemical-mediated direct lineage conversion	Low	Reproducible/unstable	Low	Deficient	Low	Integration-free	Expandable in progenitors	Very low	[18,77,104,105]
Chemical-mediated direct lineage expansion	Fast and efficient	Reproducible/unstable	Low	Close to primary	High	Integration free	Expandable in rodents/Limited in humans	Very low	[62,64,67,68]

TF: Transcriptional factor; XENs: Extraembryonic endoderm-like cells.

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