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Limitations and challenges of direct cell reprogramming *in vitro* and *in vivo*

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Summary. Direct reprogramming, whether *in vitro* or *in vivo*, has attracted great attention because of its advantages of convenience, short-term conversion, direct targets, no immune rejection, and potential clinical applications. In addition, due to its independence from the pluripotent state, direct programming minimizes some safety concerns associated with the use of human pluripotent stem cells. However, the significant limitations of reprogrammed cells, such as poor proliferative ability, low efficiency, and immature function, need to be addressed before the clinical application potential can be expanded. Here, we review the recent achievements of direct reprogramming in 2D and 3D systems *in vitro* and *in vivo*, covering cells derived from the three germ layers from stem/progenitor cells to terminal cells, such as hepatocytes, pancreatic β cells, cardiomyocytes, endothelial cells, osteoblasts, chondrocytes, neurons, and melanocytes. Combining our lab experiences with current work, we summarize the practical and potential issues that need to be solved and the prospects of strategies for addressing the current dilemmas. Through comprehensive analyses, it is concluded that the directions for dealing with efficiency and functionality issues could be the optimization of transcription factors, the upgradation for delivery systems, the regulation of epigenetic factors and pathways, and the improvement of cellular maintenance conditions. Besides, converting cells into the progenitor state firstly and then differentiating them into the desired cell types with chemical compounds may provide an approach to obtaining functional and safe converted cells in batches with a better proliferative ability. With the emergence of more and more direct reprogramming techniques and approaches with both safety and effectiveness, it is bound to bring a new dawn for mechanism research and therapeutic applications for relevant diseases in the future.

Key words: Direct reprogramming, 3D, *in vivo*, Efficiency, Functionality, Proliferation

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Introduction

Producing the desired cells with the method of regenerative medicine, which refers to using biological and engineering techniques to create lost or functionally impaired cells or tissues, has been widely implemented in recent years. How to obtain specific cell types in a functional and effective way has attracted widespread attention from scholars, and the strategies of cell reprogramming and direct reprogramming are popular nowadays. Direct reprogramming (also called transdifferentiation) is a regeneration method that can directly convert one cell lineage into another cell lineage (Bajohr and Faiz, 2020). Compared with cellular reprogramming (converting initial cells into human pluripotent stem cells) and then inducing differentiation, direct reprogramming does not require the intermediate pluripotent stage (Chambers and Studer, 2011; Cherry and Daley, 2012). Moreover, direct reprogramming also has the advantages of convenience, rapidity, and low immunogenicity and tumorigenic risk, yielding new opportunities for cell therapy (Xu et al., 2015).

The work in related fields of direct reprogramming has made rapid progress, and the situation in direct reprogramming research needs to be urgently updated and summarized. Also, according to our practical experience, many weaknesses and key scientific problems are waiting to be solved. The induced cells obtained by direct reprogramming have low efficiency, show generally poor proliferation, and lack comprehensive functionality, thus causing serious difficulties for the development of follow-up work. This article reviews the recent progress in direct reprogramming, including the transition of different germ layer cell lineages with 2D and 3D systems *in vitro* and *in vivo* (Fig. 1), clarifies the above limitations, and discusses a series of attempts and efforts made by scholars to overcome these difficulties. Limitations are often accompanied by challenges and opportunities, so we hope to provide an overview for colleagues who are engaged in or about to carry out this work.

In vitro direct reprogramming

The transformation between cell lineages *in vitro* can not only build disease models for disease simulating and drug screening but also provide a new way of regenerating some rare cells. In recent years, direct reprogramming *in vitro* has evolved rapidly, and we focus on reviewing studies of major cell types in different germ layers (Table 1).

2D culture systems

Endoderm-related cells

Hepatocytes. The treatment of liver diseases, such as acute liver injury and liver failure, is always urgent, so the generation of functional hepatocytes is a popular option for direct reprogramming. A wide range of transcription factors (TFs) is available for generating hepatocytes by direct reprogramming, now that it has been demonstrated that the transduction of Gata4, Hnf1α, and Foxa3 (Huang et al., 2011) or TFs Hnf4α plus Foxa1/Foxa2/Foxa3 can directly convert mouse fibroblasts into induced hepatocytes (iHeps) (Sekiya and Suzuki, 2011). Huang et al. (2014) reported that human induced hepatocytes (hiHeps) were generated from fibroblasts by lentiviral expression of FOXA3, HNF1A, and HNF4A, and follow-up studies came to the same

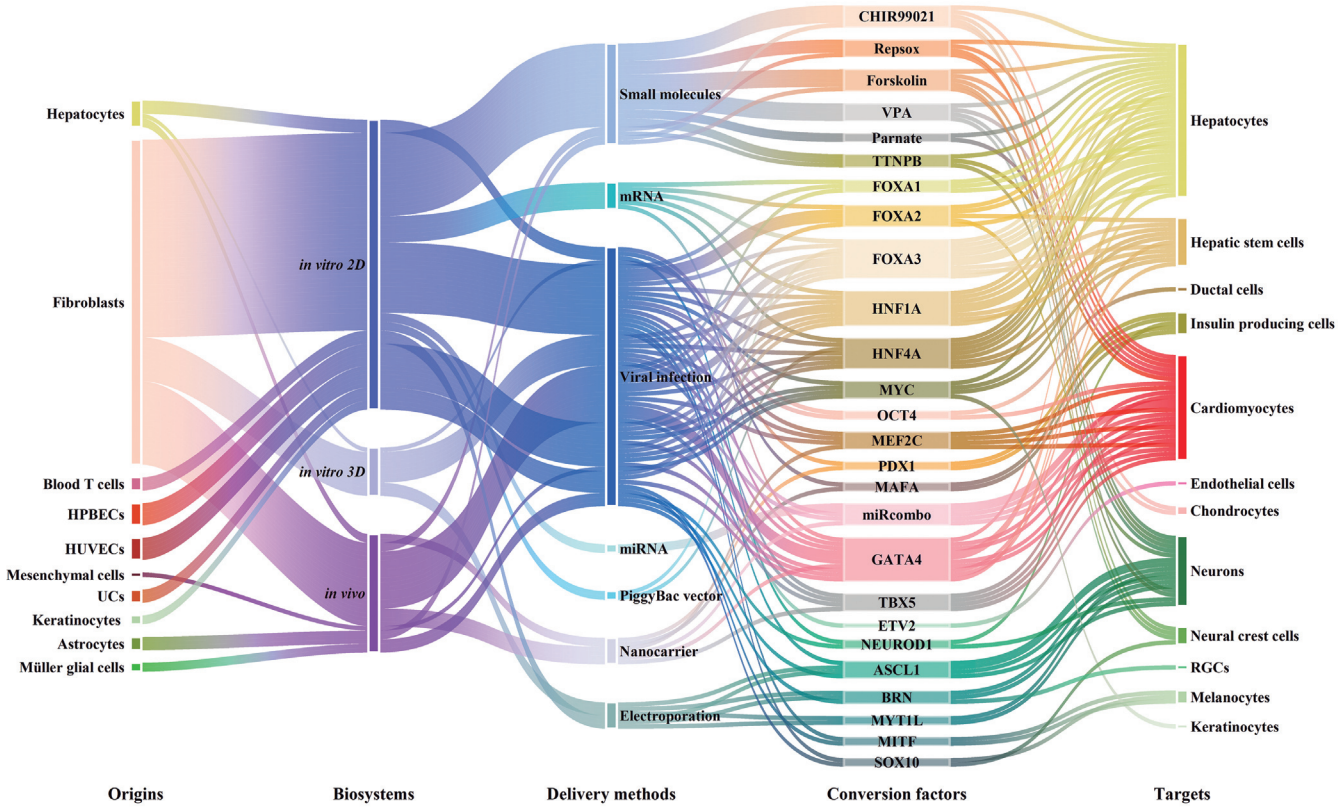

Fig. 1. Representative ingredients of direct reprogramming *in vitro* and *in vivo*. The alluvial map of frequently used origins, delivery methods, conversion factors and their main targets, as well as their biosystems for direct reprogramming *in vitro* and *in vivo* (yellow palette: endoderm-related cells; red palette: mesoderm-related cells; green palette: ectoderm-related cells; UCs: urine-derived epithelial-like cells; HPBECs: human peripheral blood-derived endothelial cells; HUVECs: human umbilical vein endothelial cells; miRcombo: microRNA1, 133, 208, and 499).

Table 1. Updates of *in vitro* direct reprogramming and their limitations.

	Targets	Origins/Species	Biosystems	Delivery Methods	Conversion Factors	Limitations	References
Endoderm	Hepatocytes	Fibroblasts/m	2D	Viral infection, small molecules	Foxa1/Foxa2/Foxa3, chemical cocktail CRFVPTD	Immature function	Guo et al., 2017
	Hepatocytes	Fibroblasts/m	2D	PiggyBac vector	Hnf4α, Foxa3	Immature function	Katayama et al., 2017
	Hepatocytes	Fibroblasts/h	2D	Viral infection	FOXA3, HNF1A, HNF4A	Low conversion efficiency	Huang et al., 2014; Ni et al., 2016; Sun et al., 2019
	Hepatocytes	Fibroblasts/h	2D	Viral infection	CTNNB1, TERT, MYC	Tumorigenicity	Sahu et al., 2021
	Hepatocytes	Fibroblasts/h	2D	mRNA	FOXA2, GATA4, FOXA1, FOXA3, HNF4A, HNF1A	Immature function	Simeonov and Uppal, 2014
	Hepatocytes	UCs/h	2D	Viral infection	FOXA3, HNF1A, GATA4	Lack of functional validation *in vivo*	Wu et al., 2020
	Hepatic progenitor cells	Fibroblasts/h	2D	Viral infection	HNF4A, HNF6A, GATA4, FOXA2, HHEX, p53 siRNA, c-MYC	Complicated steps, low efficiency	Xie et al., 2019
	Hepatic progenitor cells	HUVECs/h, HPBECs/h	2D, 3D	Viral infection	FOXA3, HNF1A, HNF6	Undetermined biosafety	Inada et al., 2020
	Insulin-producing cells	Hepatocytes/h	2D	Viral infection	PDX1, MAFA, NEUROD1	Poor proliferation	Cohen et al., 2018; Meivar-Levy et al., 2019
	Intestinal progenitor cells, spherical organoids, budding organoids	Fibroblasts/m, HUVECs/h	3D	Viral infection	Hnf4α, Foxa3, Gata6, Cdx2	Low conversion efficiency	Miura and Suzuki, 2017
	Hepatocytes, liver organoids	Fibroblasts/h	3D	Viral infection	FOXA3, HNF1A, HNF4A	Undetermined biosafety	Sun et al., 2019
Mesoderm	Cardiomyocytes	Fibroblasts/m	2D	Small molecules	Chemical cocktail CRFVPTD	Low efficiency, immature function	Fu et al., 2015
	Cardiomyocytes	Fibroblasts/m	2D	Transient transfection	miRcombo	Immature function	Kang et al., 2020; Yang et al., 2021
	Cardiomyocytes	Fibroblasts/h	2D	Viral infection	GMT	Immature function	Mohamed et al., 2017
	Cardiomyocytes	Fibroblasts/h	2D	Transient transfection	miRcombo	Immature function	Paoletti et al., 2020
	Endothelial cells	Fibroblasts/h	2D	Viral infection	ETV2	Immature function	Morita et al., 2015
	Endothelial cells	Fibroblasts/h	2D	Small molecule	Poly I:C	Low conversion efficiency	Sayed et al., 2015
	Hematopoietic progenitors	Fibroblasts/m	2D	Viral infection	Erg, Gata2, Lmo2, Runx1c, Scl	Immature function	Batta et al., 2014
	Hematopoietic progenitors	Fibroblasts/h	2D	Electromagnetic field exposure	-	Immature function	Habibi et al., 2020
	Osteoblasts	Fibroblasts/h	2D	Viral infection	OCT4, RUNX2, OSX, MYC	Tumorigenicity	Yamamoto et al., 2015
	Chondrogenic cells	Fibroblasts/h	2D	Electrical stimulation	-	Immature function	Lee et al., 2019
	Osteo-chondro-progenitors	Fibroblasts/m	2D	Viral infection	c-Myc, Klf4, Sox9	Immature function	Wang et al., 2017
	Germline stem cells, ovarian organoids	Spermatogonial stem cells/m	3D	Viral infection	H1, Stella, Zfp5, inactivation of Plzf	Undetermined biosafety	Luo et al., 2021
Ectoderm	Neurons	Fibroblasts/m	2D	Small molecules	Chemical cocktail FICB	No specific neuronal subtypeNo specific neuronal subtype	Li et al., 2015
	Neurons	Fibroblasts/m	3D	Electroporation	Brn2, Ascl1, Myt1	No specific neuronal subtype	Jin et al., 2018
	Neurons	Fibroblasts/h	2D	Small molecules	Chemical cocktail VCRFSGY	Immature function, no specific neuronal subtype	Hu et al., 2015
	Neurons	Blood T cells/h	2D	Electroporation	BRN2, ASCL1, MYT1L, NGN2	No specific neuronal subtype	Tanabe et al., 2018
	Serotonergic (5HT) neurons	Fibroblasts/h	2D	Viral infection	ASCL1, FOXA2, LMX1B, FEV	Poor proliferation	Xu et al., 2016
	Motor neurons	Fibroblasts/h	2D	Viral infection	NGN2, SOX11, ISL1, LHX3	Poor proliferation	Liu et al., 2016; Tang et al., 2017
	GABAergic neurons	Fibroblasts/h	2D	Viral infection	ASCL1, DLX5, LHX6, SOX2, FOXG1, shRNA REST	Undetermined biosafety	Bruzelius et al., 2021
	Neural crest cells	Keratinocytes/m	2D	Viral infection	Sox10, Snail1, Snail2, Twist1, Tcfap2a	Complicated steps, viral toxicity	Motohashi et al., 2020
	Neural stem cells	Fibroblasts/m	2D	Small molecules	Chemical cocktail ATPV	Poor proliferation	Zheng et al., 2016
	Neural crest cells	Fibroblasts/m	2D	Small molecules	Chemical cocktail VCSFEA	Low conversion efficiency	Pan et al., 2021
	Melanocytes	Fibroblasts/h, m	2D	Viral infection	Mitf, Sox10, Pax3	Low conversion efficiency	Yang et al., 2014; Zhang et al., 2021
	Melanocytes	Keratinocytic lineage/h	2D	Viral infection	MITF, PAX3, SOX2, SOX9; MITF, LEF1, SOX9, SOX10	Immature function	Fehrenbach et al., 2016
	Sweat gland cells, sweat gland organoids	Keratinocytes/h	3D	Viral infection	Ectodysplasin-A	Undetermined biosafety	Sun et al., 2021

UCs: urine-derived epithelial-like cells; HUVECs: human umbilical vein endothelial cells; HPBECs: peripheral blood-derived endothelial cells; GMT(G: Gata4; M: Mef2c; T: Tbx5); miRcombo: microRNA1, 133, 208, and 499; CRFVPTD (C: CHIR99021; R: RepSox; F: Forskolin; V: VPA; P: Parnate; T: TTNPB; D: Dznep); VCRFSGY (V: VPA; C: CHIR99021; R: Repsox; F: Forskolin; S: SP600625; G: GO6983; Y: Y-27632); FICB (F: Forskolin; I: ISX9; C: CHIR99021; B: I-BET151); ATPV (A: A-83-01; T: Thiazovivin; P: Purmorphamine; V: VPA); VCSFEA (V: VPA; C: CHIR99021; S: SB431542; F: Forskolin; E: EPZ004777; A: 5-Aza); shRNA REST: short hairpin RNA of RE1 silencing transcription factor.

conclusion (Ni et al., 2016; Sun et al., 2019). Although Huang et al. (2014) found that the removal of C/EBPβ and GATA4 was able to enhance hepatic gene expression, the whole process with a low conversion efficiency of less than 1% still took at least 2 to 3 weeks. After summarizing the literature, we found that TFs FOXA3, HNF1A, and HNF4A can successfully reprogram hepatocytes directly in both humans and murines, but whether GATA4 can promote the process remains controversial.

To reduce the viral toxicity, one TF (Foxa1/Foxa2/Foxa3) plus the chemical cocktail CRFVPTD (CHIR99021, RepSox, Forskolin, VPA, Parnate, TTNPB, Dznep) can transdifferentiate mouse fibroblasts into iHeps (Guo et al., 2017), which expanded for more than 30 passages. With the support of a combination of small molecules (A-83-01, BMP4, and CHIR99021), a single TF (HNF1A) was also sufficient to obtain proliferative hiHeps effectively (Lim et al., 2016; Park et al., 2019), but some gaps remained compared with primary hepatocytes (PHHs). We found that the addition of chemical cocktails can promote the proliferation of iHeps to a certain extent, but their functionality cannot be guaranteed. On the other hand, mouse fibroblasts can be converted into iHeps with HNF4A and FOXA3 via a non-viral PiggyBac vector (Katayama et al., 2017), and human embryonic fibroblasts can also be converted into hiHeps with FOXA2, GATA4, FOXA1, FOXA3, HNF4A, and HNF1A via synthetic modified mRNA (Simeonov and Uppal, 2014). Safer methods should be developed to facilitate clinical applications of cell therapy in iHeps.

Hepatic stem/progenitor cells. Besides hepatocytes, hepatic stem/progenitor cells can also be generated efficiently by direct reprogramming. The important TFs Hnf1α or Hnf1β combined with Foxa3 in hepatic organogenesis were sufficient to convert mouse embryonic fibroblasts (MEFs) into induced hepatic stem cells (iHepSCs). iHepSCs have self-renewal ability both *in vitro* and *in vivo* and the potential for bidirectional differentiation into hepatocyte and cholangiocyte lineages (Yu et al., 2013; Lim et al., 2018). Human fibroblasts can also be converted into expandable hepatic progenitor-like cells (hHPLCs) by HNF4A, HNF6A, GATA4, FOXA2, HHEX, c-MYC, and p53 siRNA, although cell proliferation has been increased by improving the hepatic expansion medium, the steps are more complicated (Xie et al., 2019). In contrast, functional hHPLCs can be obtained in human endothelial cells using only three TFs (FOXA3, HNF1A, and HNF6) (Inada et al., 2020). In the direct reprogramming of the hepatic progenitor lineage, the TFs used in humans and murines are quite different, but the HNF family certainly plays an indispensable role in the process.

Pancreatic β cells. Since the embryonic origin of hepatocytes and insulin-producing cells (IPCs) is very similar and the intercellular plasticity is relatively large, using hepatocytes as initial cells to produce IPCs has become a valuable method. Unlike the diversity of TF combinations when converting generating hepatocytes, the combination for the direct reprogramming of IPCs from hepatocytes mostly consists of PDX1, MAFA, and NGN3 (PMN) (Ham et al., 2013; Luo et al., 2014; Donelan et al., 2015). NGN3 in the PMN combination was occasionally replaced by other TFs, such as NEUROD1 (Cohen et al., 2018; Meivar-Levy et al., 2019) or PAX4 (Berneman-Zeitouni et al., 2014). The transformation of fibroblasts into IPCs is much easier and does not even require genetic manipulation. Mouse fibroblasts can obtain some properties of pancreatic β cells in conditioned media that contain dexamethasone and pancreatic extract (Usman et al., 2016). MEFs can also become functional islet-like cells after being cultured in a serum-free medium containing insulin, transferrin, and selenium for a while (Chandravanshi and Bhonde, 2018). The generation of IPCs from non-beta cells has emerged as a potential treatment for type 1 diabetes.

Mesoderm-related cells

Cardiomyocytes. Massive autologous functional induced cardiomyocytes (iCMs) obtained by direct reprogramming have become a new promising hope for heart regeneration because these cells have potentially fewer clinical problems. The three classical TFs GATA4, MEF2C, and TBX5 (GMT) can be used to transform mouse fibroblasts into iCMs (Ieda et al., 2010), whereas it is required to add some TFs in the basic GMT for human induced cardiomyocytes (hiCMs), such as MESP1 and MYOCD (Wada et al., 2013), or to use other combinations with GATA4, TBX5, HAND2, and MYOCD combined with miR-1 and miR-133 (Nam et al., 2013). The combination of GMT is not sufficient for direct reprogramming of human cardiomyocytes, although it is enough in mice, suggesting that cardiac regeneration in humans requires more transcriptional activation than in mice to activate the underlying pathways.

In addition to TF transduction, another usual method for direct reprogramming into cardiomyocytes is microRNA (miRNA). The minimal miRNA combination miRcombo (miR-1, miR-133, miR-208, and miR-499) was found to convert mouse (Jayawardena et al., 2012) and human cardiac fibroblasts (Paoletti et al., 2020) into cardiomyocytes. To increase the possibility of clinical application, in recent years, cardiac conversion has gradually transitioned from genetic manipulations to GMT supplemented with small molecules such as SB431542 and XAV939, which also yield hiCMs with relatively high proliferation (Mohamed et al., 2017). There are even completely chemical small molecules (CRFVPT) (Fu et al., 2015) to transdifferentiate iCMs successfully. However, although these cells' proliferation was acceptable, the function and efficiency were

relatively low.

Endothelial cells. Research on the direct lineage transformation of endothelial cells (ECs) for the treatment of severe myocardial ischemia and peripheral vascular diseases is on the rise. Transient expression of ETV2, co-expression with FLI1 and ERG1, and TGFβ-inhibition can generate mature induced endothelial cells (iECs) in human amniotic cells (ACs). These iECs can form stable vasculature *in vitro* and *in vivo* (Ginsberg et al., 2012). When fibroblasts were used as initial cells, it was feasible to use the five TFs ETV2, Foxo1, KLF2, Tal1, and Lmo2 for direct reprogramming of ECs in mice, but the efficiency was only about 4% (Han et al., 2014). The TFs ETV2 and FOXC2 transformed human fibroblasts into proliferative functional hiECs. However, although hiECs significantly improved blood flow recovery in hindlimb ischemia mice, the expression level of some endothelium-specific genes that are not related to the Fox:ETS motif, such as NOS3, was relatively low (Morita et al., 2015). In conclusion, the TF ETV2 always plays an important role in the transformation of ECs, regardless of the type of initial cells or species, but more strategies should be developed to promote cellular maturity or procedural efficiency.

Hematopoietic stem cells. There is a large clinical demand for blood cells for blood transfusions, so hematopoietic stem cells (HSCs) obtained by direct reprogramming could become an important choice for clinical blood supply. The TFs Erg, Gata2, Lmo2, Runx1c, and Scl can convert mouse fibroblasts into hematopoietic progenitors with multilineage hematopoietic potential, including lineages of myeloid, megakaryocytic, erythroid, and lymphoid cells (Batta et al., 2014). In order to remove the application limitations caused by viral transduction, human fibroblasts were exposed to electromagnetic fields and cultured in the HSC expansion medium, which can increase the expression of CD34 and CD38 genes without other functional verifications (Habibi et al., 2020). Although non-viral methods avoid viral toxicity, they come with low functionality and efficiency, and the reprogramming method needs to match the purpose.

Osteogenic and chondrogenic cells. Due to the limited number of cells and the difficulty of maintaining functionality in *in vitro* cultures, the regeneration of osteoblasts and chondrocytes is of great significance for the repair of bone tissue damage. The TFs OCT4, RUNX2, OSX, and L-MYC convert 80% of human fibroblasts into osteocalcin-producing cells (osteoblasts), which contributed to bone repair *in vivo* (Yamamoto et al., 2015). The same team subsequently developed a non-viral method, using an inhibitor of ALK5 in combination with vitamin D3, to induce functional osteoblast-like cells (Yamamoto et al., 2018). Without exogenous gene transduction or the addition of other factors, electrical stimulation (ES) with suitable intensity can drive the conversion of human fibroblasts into hyaline chondrocytes (Lee et al., 2019). On the other hand, bipotential osteo-chondro-progenitor nodules were generated from mouse fibroblasts through the overexpression of Klf4 and c-Myc for 6 days followed by the addition of Sox9. However, although the induced cells possessed osteo-chondro-progenitor lineage characteristics *in vitro*, they tended to differentiate into the osteogenic lineage only *in vivo* (Wang et al., 2017). The validation of the induced cells needs to be thorough, and the conclusion may differ *in vivo* and *in vitro*.

Ectoderm-related cells

Neurons. The cell regeneration ability of the nervous system is limited. Functional human induced neurons (iNs) can be obtained by the classic neural factors BRN2, ASCL1, and MYT1 (BAM), from either fibroblasts (Pang et al., 2011) or peripheral blood cells (Tanabe et al., 2018). Furthermore, chemical iNs (ciNs) can be obtained by seven compounds (VPA, CHIR99021, Repsox, Forskolin, SP600625, GO6983, Y-27632) from the fibroblasts of normal and familial Alzheimer's disease patients (Hu et al., 2015). However, specific neuronal subtypes were not clearly distinguished in these studies.

The direct induction of human fetal lung fibroblasts into dopaminergic (DA) neuron-like cells can be conducted based on functional small molecules and protein factors (Qin et al., 2020). A combination of (GABA) fate-determining TFs (ASCL1, DLX5, LHX6, SOX2, and FOXG1) together with RE1 silencing transcription factor (REST) inhibition in human fibroblasts can generate GABAergic neurons (Bruzelius et al., 2021). Overexpression of ASCL1, FOXA2, LMX1B, and FEV directly converted human fibroblasts into induced serotonergic (i5HT) neurons, which exhibited active serotonergic synaptic transmission within 12 days (Xu et al., 2016). The combination of the TFs NGN2, SOX11, ISL1, and LHX3 was identified to produce human induced motor neurons (iMNs) from fibroblasts of adult human and amyotrophic lateral sclerosis (ALS) patient (Liu et al., 2016; Tang et al., 2017). In these studies, ASCL1 acted as the pioneer "neuron-target" factor, supplemented by other "subtype-target" factors, which can induce various subtypes of neurons.

Neural progenitor cells. The direct transformation of neural progenitor cells from somatic cells has been proposed to enhance the cellular lineage differentiation potential. Restriction of Oct4 activity in the early stages while inducing continuous expression of Sox2, Klf4, and c-Myc yielded induced neural stem cells (iNSCs), which could be differentiated into multiple lineages, including neurons, astrocytes, and oligodendrocytes (Thier et al., 2012). Thereafter, chemical iNSCs derived from MEFs by A-83-01, thiazovivin, purmorphamine, and VPA were able to differentiate these cells into DA, GABAergic,

and cholinergic neurons, as well as astrocytes and oligodendrocytes, *in vitro* (Zheng et al., 2016).

Isolation of the human neural crest (NC) is difficult, limiting our ability to understand the mechanism of NC-related cellular development. Overexpression of a single TF (SOX10) combined with the activation of WNTs can directly transform human induced NC (iNC) cells, which can differentiate into four major NC lineages, including neurons, Schwann cells, melanocytes, and mesenchymal cells (Kim et al., 2014). In the keratinocyte lineage, TFs (Snail1, Snail2, Twist1, and Tcfap2a) related to the early development of NC are required to cooperate with Sox10 to simulate the sequence of NC occurrence (Motohashi et al., 2020). In addition, a complete chemical cocktail consisting of VPA, CHIR99021, SB431542, Forskolin, 5-Aza, and EPZ004777 can directly reprogram mouse fibroblasts into ciNC cells with an efficiency of about 4% (Pan et al., 2021). We found that SOX10 plays an absolute leading role in the development of NC cells, but although appropriate chemical molecules can also work, the conversion efficiency is low.

Melanocytes. The direct reprogramming of melanocytes has offered a new potential therapeutic approach for melanocyte-related diseases such as vitiligo or melanoma. (Yang et al., 2014) directly reprogrammed mouse and human fibroblasts into functional induced melanocytes (iMels) using the three TFs SOX10, MITF, and PAX3, providing the possibility of melanocyte regeneration for vitiligo. Overexpression of the TF combination MITF, PAX3, SOX2, and SOX9 can convert non-tumorigenic keratinocyte (HaCaT) cells into melanocyte-like cells, while MITF, LEF1, SOX9, and SOX10 induced melanocyte marker expression and melanosome formation in squamous cell carcinoma (MET-4) cells, which altered the tumorigenic potential (Fehrenbach et al., 2016).

Recently, we transformed MEFs into melanocytes with six transcription factors related to the development of the melanocytic lineage. Then, we further screened these TFs and arrived at the same conclusion as before: the three TFs Sox10, Mitf and Pax3 play an important role in melanocytic conversion (Zhang et al., 2021). We confirmed that Sox10 first causes cellular change toward the neural crest direction and then the fate determining TFs, Mitf and Pax3 direct further cellular transformation to melanocytes, which reveals the important role of key TFs in transforming melanocytes.

3D culture systems

Lineage reprogramming in a three-dimensional (3D) culture system is more suitable and convenient for studying the mechanisms of cellular phenotypic plasticity. Using a 3D culture system as a model for studying the mechanism of conversion from hepatocytes to duct-like biliary cells, it was found that the program was mediated by specific tyrosine phosphorylation pathways (Nishikawa et al., 2005), and Oct4 was gradually induced during the process (Doffou et al., 2018), and these may be new targets for the treatment of chronic biliary diseases. (Sun et al., 2019) constructed 3D liver organoids using hiHeps and genetically engineered these organoids to mimic hepato-carcinogenesis. It was found that c-Myc was a promoter for hepatocellular carcinomas (HCCs) due to mitochondria-endoplasmic reticulum hypercoupling and that activated RAS could induce the occurrence of intrahepatic cholangiocarcinomas (ICCs). The direct reprogramming of somatic cells into organoids might provide a model for mimicking disease development.

The factors H1, Stella, and Zfp5, combined with the inactivation of Plzf, were screened by 3D ovarian organoids for the transformation of mouse spermatogonial stem cells (SSCs) into induced germline stem cells (iGSCs), with the emergence of extensive chromatin reorganization; the subsequent 3D culture of follicles resulted in the development of mature oocytes. 3D systems facilitate genetic and epigenetic reprogramming and biotechnology discoveries (Luo et al., 2021).

Regeneration of the skin appendage sweat gland (SwG) is difficult. Researchers directly converted human keratinocytes into induced SwG cells (iSwGCs) and then constructed iSWG organoids (iSwGOs) with complete structural and biological characteristics through a 3D culture system. These iSwGOs were able to develop into SwGs in skin-damaged mice, which has important implications for functional skin regeneration in patients with extensive burns (Sun et al., 2021). In intestinal regeneration, spherical organoids and budding organoids derived from directly induced intestine progenitor cells in a 3D system were able to generate colonic and intestinal epithelium, respectively. The transplantation of these epithelia offers new opportunities for the development of intestinal diseases therapies (Miura and Suzuki, 2017).

Direct reprogramming combined with 3D culture systems and even the development of organoids can further help to explore the mechanisms of cellular phenotypic changes, simulate disease development, identify potential preventive therapies, and provide patients with transplantable organoids in a cost-effective manner, which has great significance for individualized organ regeneration.

***In vivo* direct reprogramming**

The basic body plan of the mammalian embryo is established through gastrulation, a pivotal early postimplantation event during which the three major germ layers (endoderm, ectoderm, and mesoderm) are specified with cellular and spatial diversity (Zhai et al., 2022). Direct reprogramming *in vivo* can not only induce conversion of homoderm cells but also enable cell fate switching between different germ layers (Wang et al., 2021a) (Table 2).

Endoderm

Because the *in vitro* expansion of endodermal homolog cells of the liver, stomach, intestine, colon, pancreas, thyroid, and other organs is often very difficult, *in vivo* reprogramming has brought back the possibility of generating endodermal cells as well as restoring organ function (Fang and Li, 2022).

To rescue liver failure, Rezvani et al. (2016) converted myofibroblasts into iHeps *in vivo* by expression of the liver TF genes Foxa1, Foxa2, Foxa3, Gata4, Hnf1a, and Hnf4a by established AAV vectors. Researchers have also tentatively realized the possibility of transforming the spleen into a liver-like organ. Liu et al. (2022) innovatively proposed and validated the concept of a mouse transsplenial regenerative liver. They transformed the fibroblasts *in vivo* by silicon dioxide particles (SiO_2) with lentiviral transfection (Foxa3, Gata4, and Hnf1a) into hepatocytes with essential functions such as glycogen storage, lipid accumulation, and drug metabolism, increasing the survival rate to 90% of hepatectomized mice, representing a new strategy for liver function reconstruction. As in *in vitro* conversion, the TF families of FOX and HNF are popular, and researchers have highlighted their interest in applying these families when it comes to *in vivo* conversion.

Coming from the same lineage during embryonic development, researchers have wondered whether hepatocytes can be reprogrammed *in vivo* into pancreatic-like cells. Therefore, Banga et al. (2012) forced the expression of Pdx1, Ngn3, and MafA with a polycistronic vector in the livers of NOD-SCID mice modeling diabetes and observed relieved diabetes. Subsequently, Zhang et al. (2016a) used genealogical tracing with genetic markers of insulin- or Sox9-expressing cells. Their results suggested that moderately high glucose combined with chronic administration of low doses of gastrin and epidermal growth factor (GE) is a way to induce *in vivo* differentiation of Sox9+ conduit cells into β-cells in adult mice. Taking biosafety into account, Yang et al. (2020) adopted a system of ultrasound targeted microbubble destruction (UTMD) to preprogram hepatocytes into IPCs by simultaneously delivering Pdx1, Ngn3, and MafA, highlighting the

Table 2. Updates of *in vivo* direct reprogramming and their limitations.

	Targets	Origins	Delivery Methods	Conversion Factors	Superiority	Limitations	References
Endoderm	Hepatocytes	Hepatic myofibroblasts	Viral infection	Foxa1, Foxa2, Foxa3, Gata4, Hnf1a, Hnf4a	Potential clinical translation therapy for liver fibrosis	Complicated steps, low efficiency	Rezvani et al., 2016
	Hepatocytes	Hepatic myofibroblasts	Viral infection	Foxa3, Gata4, Hnf1a, Hnf4a	Attenuated fibrosis	Undetermined biosafety	Song et al., 2016
	Hepatocytes	Fibroblasts	Viral infection	Foxa3, Gata4, Hnf1a	Promoted proliferation	Undetermined biosafety	Liu et al., 2022
	Insulin-producing cells	Ductal cells	Small molecules	GE, medium hyperglycemia	Novel nontoxic induction	Low conversion efficiency	Zhang et al., 2016a
	Insulin-producing cells	Hepatocytes	UTMD	Pdx1, Ngn3, MafA	Non-invasiveness, safety, versatility	Doubtful human suitability	Yang et al., 2020
Mesoderm	Cardiomyocytes	Cardiac fibroblasts	Viral infection	miRcombo	Complete maturity, normal function	Undetermined biosafety	Jayawardena et al., 2015
	Cardiomyocytes	Cardiac fibroblasts	Viral infection	GMT	Attenuated fibrosis	Complicated steps, undetermined biosafety	Miyamoto et al., 2018
	Cardiomyocytes	Fibroblasts	AuNPs	GMT	Improved function, low toxicity, non-DNA integration	Strict inducing environment	Chang et al., 2019
	Cardiomyocytes	Fibroblasts	NPs	miRcombo	Improved regeneration, non-DNA integration	Doubtful human suitability	Wang et al., 2021a
	Chondrocytes	Fibroblasts	Small molecules	VCRTc	Non-tumorigenicity, non-immunogenic repair	Doubtful human suitability	Chen et al., 2020a
Ectoderm	Neurons	Astrocytes	Viral infection	NeuroD1	Improved function	Low efficiency, undetermined biosafety	Chen et al., 2020b
	Neurons	Astrocytes	Small molecules	FICBY	Non-DNA integration	Low efficiency	Ma et al., 2021
	Nigral neurons	Astrocytes	Viral infection	PTBP1	Brief steps	Incomplete conversion, immature function	Qian et al., 2020
	Retinal neurons	Müller glia cells	Viral infection, small molecules	Ascl1, histone deacetylase inhibitor	Improved function	Doubtful pharmacological value	Jorstad et al., 2017
	RGCs	Müller glia cells	Viral infection	Math5, Brn3b	Untriggered proliferation, high efficiency	Long duration	Xiao et al., 2021
	Keratinocytes	Mesenchymal cells	Viral infection	DGTM	Skin epithelial regeneration	Undetermined biosafety	Kurita et al., 2018

GE: gastrin and epidermal growth factor; RGCs: retinal ganglion cells; UTMD: ultrasound targeted microbubble destruction; AuNPs: cationic gold nanoparticles; NPs: non-viral nanoparticles; miRcombo: microRNA1, 133, 208, and 499; GMT (G: Gata4; M: Mef2c; T: Tbx5); VCRTc (V: valproic acid; C: CHIR98014; R: Repsox; T: TTNPB; c: celecoxib); FICBY (F: Forskolin; I: ISX9; C: CHIR99021; B: IBET151; Y: Y-27632); DGTM (D: DNP63A; G: GRHL2; T: TFAP2A; M: MYC).

efficacy and safety of the method. It was reported that antral stomach cells can be used as initial cells for the conversion into functional pancreatic β cells (McCauley and Wells, 2016). *In vivo* conversion into pancreatic cells shares common TFs (Pdx1, Ngn3, and MafA) with *in vitro* conversion, and researchers devote themselves to exploring more possible initial cell types with a brief process and determined biosafety.

Mesoderm

In the embryos of triploblastic animals, the mesoderm germ layer develops into the circulatory system, cartilage, adipose tissue, dermis, etc. During gastrulation, some of the cells that migrate inward contribute to the mesoderm, an additional layer between the endoderm and the ectoderm (Brand, 2003).

Consistent with *in vitro* conversion, GMT is also a classical TF combination for cardio-conversion *in vivo* through viral infection (Miyamoto et al., 2018), whose biosafety can be improved by alternative delivery methods, such as cationic gold nanoparticles (AuNPs) (Chang et al., 2019). Optimizing the conventional TFs, Jayawardena et al. (2015) reported that miRcombo in a viral vector could induce *in vivo* reprogramming of non-cardiomyocytes in the heart into cardiomyocyte-like cells with morphological and functional properties of mature adult ventricular cardiomyocytes. To avoid viral toxicity, Wang et al. (2021a) fabricated a non-viral nanoparticle (NP) that could precisely deliver miRcombo to fibroblasts in the injured heart, thereby effectively enabling direct *in vivo* reprogramming through systemic administration. Cardio-conversion *in vivo* has been increasingly refined with brief processes and biosafety.

Chondrocytes have a poor capacity for self-renewal, yet there is increasing damage and degeneration of articular cartilage. Chen et al. (2020a) used a 3D induction system and a chemical cocktail, VCRTc (valproic acid, CHIR98014, Repsox, TTNPB, and celecoxib), to directly reprogram mouse fibroblasts into articular chondrocytes *in vivo*, thereby enhancing the mechanical and histological repair of the knee surface, providing a viable option for salvaging joint damage. Researchers have managed to achieve functional regeneration with less organic harm and higher delivery efficiency.

Ectoderm

The ectoderm generates the outer layer of the embryo, and it forms from the embryo's epiblast. The surface ectoderm develops into the epidermis, hair, nails, lens of the eye, sebaceous glands, etc (Brand, 2003).

As the skin is the largest and most external defense organ of the human body, its regeneration *in situ* is of great significance. The conversion of wound-resident mesenchymal cells into keratinocytes recovered all regions of the wound. Although biosafety is undetermined, this non-surgical technology brings hope for patients with cutaneous lesions, especially those who are in a pathological situation (Kurita et al., 2018).

In mammals, retinal cells are not regenerative, and the hope for vision restoration lies in the direct conversion of Müller glia (MG) into retinal cells. Pollak et al. (2013) identified Ascl1 as a possible TF that restores the neurogenic potential of the retina. After overexpressing Ascl1 in isolated mouse MG cultures, they found an upregulation of genes specific to retinal progenitors and a downregulation of glial genes. Then, it was found that Ascl1-expressing neuroglia triggered a response in the early stages of retinal regeneration only when the retina was damaged (Ueki et al., 2015), and more effective retinal neurons can be obtained from MG by TF Ascl1 combined with an inhibitor of histone deacetylase *in vivo* (Jorstad et al., 2017). Ascl1, as the main inducer of neurons, plays an important role not only *in vitro* but also *in vivo*. Xiao et al. (2021) reported that the TFs Math5 and Brn3b were able to reprogram mature MGs into retinal ganglion cells (RGCs), restoring vision in mice with glaucoma and other optic neuropathies. Researchers always strive for the complete recovery of function in retinal tissue damage.

The delivery of transforming factors directly to the brain to transform endogenous glial cells is being developed as an alternative to *in situ* generation of therapeutic neurons (Vignoles et al., 2019). Astrocytes represent the greatest potential for salvaging irreversible neurodegeneration in terms of the choice of initial cells for neuron conversion (Liddelow and Barres, 2017). Chen et al. (2020b) demonstrated that NeuroD1-mediated conversion of astrocytes into neurons *in situ* can regenerate large numbers of functional new neurons after ischaemic injury, while Qian et al. (2020) simplified the conversion with one efficient step by PTBP1. Ma et al. (2021) successfully transformed endogenous astrocytes into neurons in the adult mouse brain using an optimized cocktail, FICBY (Forskolin, ISX9, CHIR99021, I-BET151, and Y-27632), which resemble endogenous neurons in terms of neuron-specific marker expression, electrophysiological properties, and synaptic connectivity. Focusing on rescuing irreversible damage, neuron conversion *in vivo* has great potential to achieve safe and effective brain regeneration with higher efficiency and simpler methods in the near future.

Limitations and challenges

Based on this review of studies, the current limitations of direct reprogramming techniques lie in low efficiency and unstable functionality. Cells obtained by direct reprogramming still differ in important ways from the true target cells. Efficiency and functionality often complement each other, and we have identified the following four aspects that can improve the efficiency and functionality of direct reprogramming: (1) modifying the factors involved in direct reprogramming,

(2) upgrading the delivery systems, (3) regulating the epigenetic factors and pathways, and (4) improving cellular maintenance.

On the other hand, the challenge we face is poor cellular proliferation. TF transduction is still the main method for direct reprogramming at this stage which impairs the cellular viability absolutely. Attempts by researchers to solve this problem by converting cells into progenitors are summarized below.

Efficiency and functionality improvement

Deep screening and optimizing TFs

Lim et al. (2016) found that the addition of the TFs c-Myc and Klf4 (CK) in combination with small molecule compounds (A-83-01, BMP4, and CHIR99021) increased the iHep conversion efficiency 30 times. However, the boundary between proliferation and tumorigenicity needs to be controlled, otherwise, the cells would turn into tumorigenic hiHeps induced by the TFs MYC, CTNNB1, and TERT (Sahu et al., 2021). The conversion efficiency of iHepSCs with Hnf1α and Foxa3 increased 3.2 to 6.8 times compared with that of the combination of Hnf1β and Foxa3 (Lim et al., 2018). Compared with the classical TFs GMT, a TF combination of Hand2, NKX2.5, Gata4, MEF2C, and Tbx5 (HNGMT) resulted in a more than 50-fold increase in the conversion efficiency of cardiomyocytes, a higher expression of cardiac-related genes, and a higher percentage of cells exhibiting calcium transients and spontaneous pulses (Addis et al., 2013). For the direct conversion of induced oligodendrocyte progenitor cells, the combination of Sox10, Olig2, and Nkx6.2 was more potent (~80%) in mouse fibroblasts, while the combination of Sox10, Olig2, and Zfp536 was more potent (~90%) in rat fibroblasts; the conversion efficiency of different cell types will vary depending on the chosen TFs (Lee and Park, 2017). Sequential expression of TFs (overexpression of Pdx1 on the first day followed by Pax4 on the second day and Mafa on the third day) can improve the yield and maturity of directly reprogrammed insulin-producing cells (Berneman-Zeitouni et al., 2014), which is also more in line with the opinion that maturation occurs in a sequential manner. According to our experience, the selection, expression level, and sequence of TFs need to be continuously explored and optimized in the process of direct reprogramming to achieve the desired results.

Upgrading the delivery systems

Appropriate factor delivery systems are crucial for improving the efficiency of direct reprogramming. A polycistronic system was developed in which all four miRNAs of miRcombo were at an equivalent expression level from one single construct, and the cardiac direct reprogramming efficiency was increased in this system (Kang et al., 2020). A polycistronic GMT reprogramming cocktail with a microRNA (miR-133) was able to generate cardiac troponin T-positive (cTnT+) hiCMs with 40-60% efficiency within two weeks, approximately twice the efficiency of previous schemes in which GMT was transduced one by one (Garbutt et al., 2020). It has been shown that nanocarriers have low cytotoxicity and long-term expression of microRNAs, so they can carry miRcombo for cardiac reprogramming in a non-genomically integrated manner (Wang et al., 2021b; Yang et al., 2021). Long-term safety should be regarded as at challenge for clinical application.

Regulating the epigenetic factors and pathways

Inhibition of the p53 pathway was able to increase the direct reprogramming efficiency and functionality of the hepatic lineage (Du et al., 2014), hematopoietic progenitors (Batta et al., 2014), and functional DA neurons (Liu et al., 2014; Jiang et al., 2015). Knockdown of p53 and a hypoxic environment also significantly enhanced the transformation efficiency of serotonergic neurons (Xu et al., 2016). BECN1, an autophagy-related factor, was found to inhibit the induction of iCMs in an autophagy-independent manner. Depletion of *BECN1* resulted in an about 3- to 4-fold increase in the direct reprogramming efficiency of iCMs both in mice and humans, as well as a 3-fold increase in the number of spontaneously beating cells with periodic calcium oscillations and an elevated frequency of field potential spikes compared with the control group (Wang et al., 2020). Overexpression of the hepatocytic TFs HNF1A, FOXA3, and GATA4 was sufficient to convert hUCs into hiHeps, while knocking down the genes *p62* and *BECN1* in hUCs using CRISPR/Cas9 technology nearly doubled the direct reprogramming efficiency compared with that in controls, which indicated an essential role of autophagy in the direct reprogramming of hiHeps (Wu et al., 2020). The addition of the demethylase KDM2B was able to improve the efficiency of HNF4A and FOXA3 in the conversion of mouse fibroblasts into iHeps (Zakikhan et al., 2016). The TFs ER71, KLF2, and TAL1 were able to convert fibroblasts into functional endothelial cells in humans, but the efficiency was only about 5%. Through whole-transcriptome analysis, it was found that the gene *TWIST1* was able to block the conversion, and the *TWIST1*-knockdown combined with rosiglitazone (an inhibitor of epithelial-mesenchymal transition, EMT) can enhance the conversion efficiency up to ~20% (Han et al., 2021). Rational epigenetic adjustment of cells not only improves the efficiency of direct reprogramming but may also uncover unexpected mechanisms of tissue development and disease occurrence.

The Wnt/β-catenin pathway improved the efficiency (from less than 15% to more than 60%) and the predisposition of the livers to pancreas reprogramming (Cohen et al., 2018). In addition, SB431542 (transforming growth factor-β inhibitor) and XAV939 (WNT inhibitor) were also able to enhance the efficiency

(≥50%), speed (beating cells appeared as early as 1 week, which is much earlier than the 6 to 8 weeks using GMT alone), and quality (gene expression profiles closer to those of cardiomyocytes) of the GMT-based direct reprogramming of cardiomyocytes (Mohamed et al., 2017). In our own research, the addition of the WNT pathway activator CHIR99021 promoted the transformation of fibroblasts into melanocytes by enhancing the expression of TF Mitf (Zhang et al., 2021). Correctly mimicking the activation and inhibition of pathways during development is indispensable for regenerating target cells or tissues.

Improving cellular maintenance

The addition of some specific nutrient growth factors can further enhance direct reprogramming. Treatment with platelet-derived growth factor (PDGF) promoted Pdx1- and Ngn3-induced reprogramming of hepatocytes into functional pancreatic β cells, improving the ability of induced cells to respond to glucose stimuli (Chang et al., 2016). A combination with vitamin D3 also obtained more induced osteoblast-like cells (Yamamoto et al., 2018) than only inhibiting ALK5, and the conversion efficiency reached about 90%. A co-culture of endothelial colony-forming cells (ECFCs) and human bone marrow mesenchymal stem cells (MSCs) promoted IPC generation from human hepatocytes, which resulted in induced IPCs with a significantly increased expression of pancreas-specific genes and an increased glucose-regulated insulin secretion, implying a promoting role of the vascular niche in the conversion of liver cells into pancreatic cells (Meivar-Levy et al., 2019). One of the key points for the success of direct reprogramming is to create an appropriate cell survival environment to promote effective communication between cells and transduction factors.

Multicellular microenvironments in 3D systems may mimic the real tissue developmental environment, thereby improving the unstable functionality of induced cells obtained by direct reprogramming. In endodermal research, our team has cultured hiHeps in a 3D environment with hMSCs and human umbilical vein endothelial cells (HUVECs) to establish hepatic organoids. Compared with the expression in cultures on a flat surface, the expression of hepatocytic genes and proteins such as ALB, AAT, and HNF4A, as well as liver metabolism enzyme genes of liver organoids, was upregulated, which demonstrated that hiHeps are more usable and functional in a 3D multicellular micro-environment (Wang et al., 2018). Compared to conventional cultures in dishes, the 3D hydrogel environment enhanced miRcombo mediated cardiac direct reprogramming in terms of gene profile, protein level, and the expression of early cardiac transcription factors and resulted in a ~5-fold increased efficiency by MMP-dependent mechanisms (Li et al., 2016). Furthermore, a novel Matrigel-based hydrogel culture system (soft matrix) was developed to mimic myocardial tissue stiffness (10 kPa) *in vitro*. Compared with common 2D culture systems, the hydrogel system not only increased the number of functional iCMs 3-fold but also inhibited the fibroblastic program by inhibiting YAP/TAZ signaling to promote the cardiac reprogramming efficiency up to 15% (Kurotsu et al., 2020). After mesodermal chemically induced chondroid-like cells were transplanted into the defective joint cartilage, a 63.4% mechanical function repair rate was achieved. The researchers believed that these results are due to the fact that they used a 3D organoid culture system, resulting in their cells maintaining a more chondrocyte-like phenotype (Chen et al., 2020a). For ectodermal conversion, 3D scaffolds with a brain extracellular matrix (BEM), which was decellularized from human brain tissue, significantly increased the generation efficiency of plasma-transfection-based (Brn2, Ascl1, and Myt1) iNs nearly 1-fold (~18-25%) (Jin et al., 2018). The 3D environment can provide a richer microenvironment for cell or tissue generation, which is undoubtedly one of the main directions for improving direct reprogramming in the future.

Enhancing proliferation

Cells transformed to the progenitor state and then induced with chemical compounds have both a stronger proliferative ability and a better functionality, which greatly compensates for the limited proliferative ability of cells obtained by conventional direct reprogramming (Ge et al., 2019). Fibroblasts were first directly converted into human hepatic progenitor-like cells, which were able to substantially expand *in vitro* for more than 40 passages in a hepatic expansion medium containing the small molecules CHIR99021, LPA, SB431542, S1P, and EGF. Further, a large number of functionally competent hiHeps were generated in a hepatic maturation medium (Xie et al., 2019). Endoderm-like cells were generated with pluripotent TFs in mouse fibroblasts firstly and then expandable pancreatic lineages were obtained with combinations of small molecules, of which the TGF-β receptor inhibitor, the p38 MAPK inhibitor, and 2-phospho-L-ascorbic acid played important roles (Li et al., 2014). To acquire expandable cells for transplantation, mouse fibroblasts were also directly reprogrammed into proliferative induced cardiac progenitor cells (iCPCs), and these iCPCs were able to differentiate into numerous cardiomyocytes, smooth muscle cells, and endothelial cells both *in vitro* and *in vivo* (Lalit et al., 2016; Zhang et al., 2016b). A similar approach was used to directly reprogram nephron progenitor-like cells (Gao et al., 2021) and neural stem cells (Zheng et al., 2016) for subsequent differentiation and the regenerating of cells in batches.

In direct reprogramming *in vivo*, SiO_2 stimulation tripled the number of activated splenic fibroblasts, and in combination with protein of TNF-α, overexpression of EGF and HGF resulted in a large number of functional

iHeps (Liu et al., 2022). In addition, according to our own experience with converting fibroblasts into melanocytes, after adding hormone agents such as insulin and hydrocortisone combined with adenine (a substance that affects cell metabolism), the proliferation of iMels was significantly improved (Zhang et al., 2021). The selection of appropriate cell energy promoters may have unexpected benefits in inducing cell proliferation.

Conclusion and future perspectives

Direct reprogramming technology has been developed and improved over the years. The main ingredients of direct reprogramming *in vitro* and *in vivo* are shown in an alluvial map (Fig. 1). The conversion efficiency is low, the cellular proliferation is poor, and the functionality needs to be enhanced. It is still a great challenge to induce completely functional target cells. Improvements in the efficiency and functionality of direct reprogramming can be achieved by choosing appropriate TFs and culture conditions, and the 3D culture system has been proved to be one of the means to enhance efficiency. Epigenetic modification of cells with CRISPR-Cas9 might also be a solution to the current dilemmas. The delivery of TFs, miRNAs, and synthetic proteins in a controlled manner provides a potential method for translational applications. The cells reprogrammed by a chemical cocktail have no risk of mutations caused by genetic manipulation and have a relatively high proliferative capacity, but also poor functionality and efficiency. Conversion into the intermediates of expandable stem or progenitor cells, followed by chemical cocktails for lineage induction differentiation, provides a unique way to solve the above problems. In conclusion, in recent years, researchers have been committed to improving efficiency, ensuring cellular quality, and exploring more suitable maintenance conditions for direct reprogramming. In the future, safer and more efficient conversion systems are bound to be developed, opening up new avenues for disease modeling, drug screening, organ transplantation, and clinical regenerative therapy.

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