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Targeting cell plasticity for regeneration: From *in vitro* to *in vivo* reprogramming

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ABSTRACT

The discovery of induced pluripotent stem cells (iPSCs), reprogrammed to pluripotency from somatic cells, has transformed the landscape of regenerative medicine, disease modelling and drug discovery pipelines. Since the first generation of iPSCs in 2006, there has been enormous effort to develop new methods that increase reprogramming efficiency, and obviate the need for viral vectors. In parallel to this, the promise of *in vivo* reprogramming to convert cells into a desired cell type to repair damage in the body, constitutes a new paradigm in approaches for tissue regeneration. This review article explores the current state of reprogramming techniques for iPSC generation with a specific focus on alternative methods that use biophysical and biochemical stimuli to reduce or eliminate exogenous factors, thereby overcoming the epigenetic barrier towards vector-free approaches with improved clinical viability. We then focus on application of iPSC for therapeutic approaches, by giving an overview of ongoing clinical trials using iPSCs for a variety of health conditions and discuss future scope for using materials and reagents to reprogram cells in the body.

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; AAT, α -1-antitrypsin; ALS, amyotrophic lateral sclerosis; CF, cystic fibrosis; CIRM, California Institute for Regenerative Medicine; CNV, copy number variation; DNA, deoxyribonucleic Acid; EBiSC, European Bank for induced pluripotent stem cells; ECM, extra cellular matrix; ESC, embryonic stem cells; GvHD, graft-vs-host disease; HLA, human leukocyte antigen; HSPC, hematopoietic stem and progenitor cell; hiPSCs, human induced pluripotent stem cells; iCeMS, Center for iPS cell research and application; iPSC, induced pluripotent stem cell; iPSC-CMs, iPSC derived cardiomyocytes; iPSCs-MSCs, iPSC derived mesenchymal stem cells; iPSC-NK, iPSC derived natural killer cells; LQTS, long QT syndrome; MEF, mouse embryonic fibroblasts; MET, mesenchymal to epithelial transition; MSC, mesenchymal stem/stromal cells; NP, nanoparticle; OKSM, Oct4, Klf4, Sox2, c-Myc (Yamanaka factors); PA, polyacrylamide; PDGF, platelet derived growth factor; PDMS, polydimethylsiloxane; PEG, poly(ethylene glycol); RECF, radio electric conveyed fields; RGD, arginine-glycine-aspartate; RGDSP, arginine-glycine-aspartate-serine-proline; RPE, retinal pigment epithelial cell; SeV, Sendai virus; TCP, tissue culture polystyrene/plastic; TGF, transforming Growth Factor; TTF, tail-tip fibroblasts.

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1. Introduction

iPSCs have proven to be an attractive cell source for various research areas since their successful derivation over a decade ago [1]. The use of somatic cells to produce a cell line which is genetically and functionally similar to embryonic stem cells [2], has revolutionized many fundamental and translational research ideas. Since the very first generated iPSCs from mouse embryonic fibroblasts (MEF) in 2006 [1] and in 2007 from human fibroblasts [3] by retroviral transduction of four transcription factors, various laboratories reported alternative methods for cell reprogramming. To date, the most commonly used method still remains the integration of reprogramming factors, including Oct4 (also known as Pou5f1), Sox2, Klf4, and c-Myc (known as Myc) into the genome by lentiviral or retroviral transduction [4,5]. These 2 methods have high reprogramming efficiency, defined as number of reprogrammed cells out of total initial cells, and have been proved efficacious in various disease animal models, such as Parkinson's disease [6] and sickle cell anaemia [7]. However, since viral DNA integrates in host cell's genome and can cause insertional mutagenesis and unpredictable genetic dysfunction, neither type of viral vector (retroviral and lentiviral) is ideal for clinical application. Moreover, reprogramming factors, in particular c-Myc have shown to increase tumour formation in iPSC-derived chimeric mice [8]. In fact, c-Myc functions as a "double-edged sword," promoting both iPSC generation and tumour formation [9]. c-Myc has been replaced with l-Myc, which has less of a propensity for tumorigenesis; however, cell reprogramming efficiency decreased [10,11]. Therefore, other methods for generating iPSCs are also currently under investigation, where the use of oncogenes can be avoided and render iPSC suitable for clinical application. In this respect, viral-free and transgene method have recently shown great interest from the scientific community.

In advance of the discovery of iPSCs, researchers had identified specific cytokines that were able to maintain embryonic stem cell pluripotency (bFGF, MEK/ERK and GSK3 inhibitors) and leveraged these compounds for somatic cell nuclear transplantation (SCNT), a process by which a somatic cell nucleus is fused with a mature enucleated oocyte, in order to generate an embryonic stem cell [12,13]. Much of these early studies set the groundwork for using soluble factors and design cell culture substrates to enhance reprogramming efficiency and reduce or eliminate viral vectors entirely.

Towards viral and transgene-free methods of reprogramming, in 2009 Kim et al. demonstrated human iPSCs (hiPSCs) by the integration of cell penetrating peptide (CPP) anchored reprogramming proteins [14]. However, the initial reprogramming efficiency was 0.001% compared to 0.01% using viral methods, and the procedure took twice as long as viral methods. CPPs contain a high proportion of charged amino acids, such as arginine or lysine, which facilitate cell penetration. To date over 100 different CPPs have been reported by a number of laboratories; however, challenges with delivering proteins through the cellular membrane remain. Pre-treatment with cell permeabilization agents for reversible permeabilization, that transiently open holes in cell membrane to allow proteins to penetrate provided modest improvements in efficacy, but these approaches are detrimental to cell survival. Moreover, unintended consequences of CPPs on the treated cells and neighbouring cells after transplantation remains a concern [15].

In 2010, Warren et al. advanced the field by reprogramming cells through transfection of synthetic modified mRNA, named mod-mRNA [16], thereby eliminating the need for viral transduction. Reprogramming efficiency on human fibroblast cell lines was among the highest achieved (4.4%), although at a high cost as the approach is relatively complicated and laborious, requiring daily transfection for 2 weeks. In addition, when applied to primary cells the same method resulted in inconsistent reprogramming efficiency [17]. To overcome the need of repeating the transfection step every day, there have been studies conducted with self-replicating mRNA, where a single long mRNA encoding for the Yamanaka factors, was prepared based on the non-infectious and self-replicating RNA replicon of Venezuelan Equine Encephalitis (VEE) virus [10,18,19]. Reprogramming efficiency was significantly improved compared to daily synthetic mRNA transfection method, whilst the process resulted to be 2 times longer than the original mRNA protocol. Alternatively, Miyoshi et al. used a combination of mature double stranded miRNA, mir-200c plus mir-302s and mir-369s family miRNAs to reprogram mouse and human somatic cells, including adipose stromal cells and dermal fibroblasts [20]. The synergistic effect of a combination of mod-mRNA cocktail of reprogramming factors, with embryonic stem cells (ESC)-specific miRNA-367/302 have also shown to enhance the generation of iPSCs from human fibroblasts [21].

Proteins and small molecules have emerged as a new approach for pluripotent reprogramming that obviates the need for viral factors and sensitive biomolecules. Moreover, key advantages reside also in their stability for long periods, and easy manipulation. The underlying concept of using soluble signals for reprogramming is to perturb pathways that prevent and/or promote the epigenetic reset underlying conversion to a pluripotent state [22,23]. Hou et al. used a combination of seven small-molecule compounds for mouse somatic cells and obtained iPSCs for the first time with a frequency up to 0.2% [24]. However, there remains some uncertainty surrounding the use of small molecules alone for generating human iPSC and the mechanism for induction of pluripotency has yet to be demonstrated [22]. Achievements obtained so far with the application of small molecules for cell reprogramming will be further discussed in Section 4 of this review.

Overall, at present iPSC generation faces problems related to reprogramming extent, epigenetic memory and immunogenicity [12]. In particular, high-throughput analysis has shown the presence of genetic variations in iPSCs, including genome instability, single nucleotide variations, copy number variation (CNV), and loss of heterozygosity. These mutations can be either inherited from their parental cells, or acquired during the reprogramming process, or even generated during prolonged culture *in vitro* [25]. A central tenet of modern reprogramming that may overcome these issues involves strategies that can increase efficiency and obviate the need for viral transduction, by lowering the epigenetic barrier to reprogramming. Towards this end, several approaches, such as small molecules or physical factors derived from designer cell culture materials, are currently under investigation from many researchers [5,26,27]. This review summarizes the current state of novel approaches for iPSC generation and for direct reprogramming. After giving an overview on criteria used to characterize iPSC and cell source types used for their generation, we will go through emerging alternative methods for iPSC generation, including physical and biochemical factors. We will further discuss ongoing efforts to leverage nuclear reprogramming as a therapeutic tool for

regenerative medicine, disease modelling and drug discovery. Taken together, these advances have the potential to pave the way to an optimal solution for regenerative medicine: *in vivo* reprogramming to combat injury and cure disease.

2. iPSC characterization

To ensure an accurate assessment of reprogramming, it is critical to have a baseline definition of what constitutes a pluripotent stem cell. Several criteria have been proposed, and according to the International Stem Cell Banking Initiative, there are specific criteria to meet for iPSC lines to be inserted in a biobank. Most biobanks share a common characterization protocol, which includes: (1) ESC like morphology, consisting in large nuclei and scant cytoplasm; (2) transgene silencing after reprogramming; (3) ESC markers, such as TRA-1-60, TRA-1-81, Nanog, Oct4; (4) differentiation potential both *in vitro*, by embryoid body formation, and *in vivo*, through teratoma formation; (5) karyotype analysis to indicate chromosomal abnormalities; (6) identity confirmation by DNA fingerprinting and short tandem repeat-PCR; and (7) microbiological assay to ensure that cells are not contaminated [28]. In addition to these criteria, other methods have been trialed including single cell assays and mass cytometry [29,30]. Kaji and colleagues were able to correlate cell-surface markers to different waves of pluripotency during the reprogramming process, using single-cell gene expression analysis and global RNA sequencing [31]. Mass cytometry, a technique where mass spectrometry and flow cytometry have been combined by using element isotope-conjugated antibodies, allows to screen more than 40 markers at once, giving the possibility to determine several intermediate cell groups during the reprogramming process and also characteristic marker expression profiles in hiPSCs [29]. Table 1 shows current available methods used for iPSC characterization.

3. iPSC cell source: impact on cell reprogramming and differentiation

After Yamanaka's pioneering discovery of iPSC generation from fibroblasts, extensive research has been conducted for the investigation of reprogramming different type of cells from fibroblasts. The need to reprogram other cells came from the realisation that using lineage-matched cells for iPSC derivation and subsequent differentiation could achieve a more efficient and robust lineage outcome, in part due to

some stability in lineage-specific epigenetics, towards targeting a particular tissue for treatment. Skin biopsy is an invasive and non-sterile procedure and requires 2 to 3 weeks to expand harvested cells before enough cells are available to accommodate the low efficiency of reprogramming. Moreover, skin cells are more susceptible to mutations due to environmental insults, such as UV irradiation, than cells from internal parts of the body [32].

There is now evidence that iPSC retain transcriptional, epigenetic and metabolic memory from their somatic cells of origin. iPSCs preserve some DNA methylation status similar to the cell of origin and thus being more prone to differentiate into a cell type rather than others [33]. Interestingly, this phenomenon gradually disappears with multiple passages [34]. Evans and colleagues have been working on a method to overcome the epigenetic memory and found that the introduction of an enzyme cytidine deaminase through retroviral vectors, can attenuate iPSC epigenetic memory [35,36]. Since then, chromatin-modifying drugs have been investigated for this purpose to modulate the epigenetic state during reprogramming [37].

iPSC derived from different cell source vary also in their propensity to form teratoma. In a study where MEF, tail-tip fibroblasts (TFF) and hepatocyte or gastric epithelial cells derived iPSC were induced to neural differentiation, it was observed that iPSC derived from either MEF or gastric epithelial cells had the lowest propensity to form teratomas, whereas TFF showed the highest. iPSC generated from hepatocyte epithelial cells showed an intermediate propensity. This result was correlated to the number of undifferentiated cells, defined by their Nanog expression, which was in fact highest in TFF [38]. Here we describe the most commonly used human cells to generate iPSCs. Table 2 includes examples of cell types for iPSC generation and their application.

3.1. Fibroblasts

Fibroblasts were the first cell type used to generate iPSC [3], and they are now considered a standard somatic cell type for reprogramming. The main advantage of using fibroblasts resides in the ease of harvesting and culturing *in vitro*, albeit they require uncomfortable biopsies. Most commonly used cells are in fact adult skin and foreskin human fibroblasts, as they can be obtained by skin biopsy samples from various parts of the body. Tissue specific cells, such as lung [39] and cardiac fibroblasts [40] have also been used for cell reprogramming. iPSC generated from fibroblasts have been then differentiated not

Table 1

iPSC characterization methods (hiPSC: human induced pluripotent stem cell; HDFs: human dermal fibroblasts; HLFs: human lung fibroblasts; 2D: two dimensional).

Characterization method	Cell source	Reprogramming method	Markers	Evaluation, efficiency	Limitations	Outcome	References
Cell morphology	dH1f fibroblast cell line	Retrovirus	Colony morphology	Low, invasive	Time consuming, qualitative, no precise lineage tracing	Lack of a single definitive assay for pluripotency	Chan et al. 2009 [1]
	Human fibroblast 253G1 and 201B7; human mammary epithelial cells (HMECs)	Sendai virus	Nuclear morphology	High, quantitative	Limited knowledge of iPSC nuclear morphology, system complexity; unable to identify single cell and heterogeneous colony	Can distinguish iPSCs effectively and objectively with proper machine learning algorithm	Tokunaga et al. 2014 [2]
	Human secondary fibroblast C1.2 and erythroid progenitor cells (EPCs)	H2B-mCherryplasmid	Cellular morphology and nuclear characteristics	Quantitative, single cell friendly	2D imaging, time consuming, dye compatibility with live-cell imaging	Non-invasive, identify intermediate cell state on computational model	Molugu et al. 2019 [3]
Immunofluorescence staining/reporter system	Human fibroblasts	Various method	Oct4, Sox2, and Nanog/ TRA-1-60, DNMT3B and REX1	Low	Require cell fixation or causing safety issue for downstream studies	Detect dynamic changes of pluripotency	Maherali and Hochedlinger 2009 [4]
Teratoma assay	Pre-established hiPSC cell lines (derived from HDFs, HLFs, etc)	Episomal plasmid, retrovirus, lentivirus, etc	Embryoid body/teratoma formation	Low	Time consuming, qualitative, cells cannot be used for downstream studies	Prudent approach to identify malignant potential	The International Stem Cell Initiative 2008 [5]

Table 2
Human cell source for iPSC generation (OKSM: Oct-4, Klf-4, Sox-2, c-Myc; EB: embryoid body; SeV: Sendai Virus; SFFV: spleen focus-forming virus; iNKT: invariant natural killer T; hDF: Human dermal fibroblasts; MSCs: Mesenchymal stem cells).

Tissue/group of cell source	Cell source	Method	Reprogramming efficiency	Purpose	Reference
Cord blood	Cord blood mononuclear cells (CBMCs)	SeV with OKSM	High	Skin EB for chondrogenesis	Kim et al. 2018 [6] Nam et al. 2017, Rim et al. 2016 [7,8]
		Combination of 19 episomal vectors	Lower than DF 100-fold higher than fibroblasts (352 iPSC lines per 10 ⁶ transfected cells)	Cartilage Hematopoietic progenitors	Rim et al. 2018 [9] Hu et al. 2011 [10]
Blood	Cord-blood-derived endothelial cells (CBECs) CD34 ⁺ cells	Lentiviral vectors for Oct4, Sox2, Nanog, and LIN28	Variables from sample to sample (directly dependent on proliferation activity)	3 germ layers (in particular cardiomyocytes) Efficiency: 12%	Haase et al. 2009 [11]
		Episomal vector with SFFV promoter containing Oct4 and Sox2	2%	3 germ layers	Meng et al. 2012 [12]
	CD133 ⁺ cord blood cells	SeV with OKSM	--	3 germ layers	Viera et al. 2019 [13]
	Peripheral blood cells (PBC)	Episomal vectors (pEV SFFV-OS (Oct4-2a- Sox2), pEV SFFV-MK (MYC-2a-KLF4), and pEV SFFV-B (BCL-XL)) Episomal vectors, CAG, EF1, or SFFV SeV with OKSM	-- Highest pluripotency in DF	MSCs, cartilage	Li et al. 2016 [14]
Fibroblasts	iNKT cells (obtained by healthy donors' PBC) Cardiac fibroblasts Dermal fibroblasts	Lentivirus with OKSM	--	Lung progenitor cells Cartilage Cardiomyocytes	Kamath et al. 2018 [15] Rim et al. 2018 [9] Riedelet al. 2014 [16]
		Retroviral pMXs-based vectors with OKSM	--	Cardiomyocytes	Sharp et al. 2015 [17]
		SeV with OKSM and d SV40 large T antigen with SeV18 +SV40LTA/TS15DF	High (quantification not shown)	iNKT	Kitayama et al. 2016 [18]
Cells from diseased patients	Dermal fibroblasts Lung fibroblasts with or without non-chronic obstructive pulmonary disease (COPD) osteoarthritis fibroblast-like synoviocytes (FLS)	pMXs retrovirus vectors with OKSM	--	3 germ layers	Linares et al. 2016 [19]
		pMXs plasmids with OCT3/4, SOX2 and KLF4	--	Epithelial cells	Yang et al. 2014 [20]
Mesenchymal stem cells	MSC, dermal fibroblasts, keratinocytes	SeV with OKSM	Highest pluripotency in DF	Cartilage Fibroblasts	Rim et al. 2016 [8] Basma et al. 2014 [21]
		Modified Lentivirus method with OKSM	--	MSCs	Kim et al. 2011 [22]
Hair	Keratinocytes	Retrovirus OKSM	--	MSCs	Kim et al. 2011 [22]
		Lentivirus (OCT4, SOX2, NANOG, and LIN28)	MSCs > fibroblasts > keratinocytes	Cardiomyocytes Efficiency: Lower than MSCs	Bömeke et al. 2013 [23]
Other tissues	Urine cells Hepatocytes	pMXs retroviral vector with Oct3/4, Sox2 and Klf4	30–100-fold higher efficiency than clonally expanded MSCs and hDF	3 germ layers	Oda et al. 2010 [24]
		SeV OKSM	--	MSCs (by ABB treatment) Efficiency: 19%	Nakayama et al. 2018 [25]
Other tissues	Neural stem cells Synovial cells	Retroviral plasmids with OKSM	--	3 germ layers	Zhou et al. 2011 [26]
		SeV with OKSM	--	Hepatocytes Efficiency: 75-85%	Takayama et al. 2014 [27]
		Retrovirus with OKSM	--	Drug screening,	Silva et al. 2008 [28]
		Lentivirus with OKSM	--	disease modelling	Rim et al. 2016 [29]

only into a variety of different tissue cells, but also into iPSC-derived fibroblasts (Table 1). Fibroblasts play an essential role in regulating normal tissue homeostasis and wound repair; however, it often remains difficult to acquire sufficient numbers of donor cells and because of their heterogeneity clinical outcomes can be unpredictable. For this reason Shamis et al. have investigated the possibility to generate iPSC-derived fibroblasts and demonstrated augmented production and assembly of extracellular matrix (ECM) proteins, showing an improved function of iPSC-derived fibroblasts compared to their parental fibroblasts [41].

3.2. Keratinocytes

Follicular keratinocytes can be isolated from different types of hair, and have proved very successful in reprogramming in short times with high efficiency [42]. When in culture, they need special low-calcium medium formulations to prevent the cells from becoming senescent. This is an advantage for when keratinocytes

are switched to a different medium for reprogramming which contains higher calcium levels. This process allows keratinocytes that have not been infected, to undergo senescence and increase reprogramming efficiency. Keratinocytes are generally reprogrammed within 1-2 weeks, whereas fibroblasts take 3-4 weeks. They have also shown a 100-fold higher reprogramming efficiency (1-2%) compared to fibroblasts [43].

3.3. Peripheral and cord blood cells

Peripheral blood cells (PBCs) are non-adherent cells present in cord blood, commonly utilized in clinical applications. PBCs are considered to be an ideal source of cells for reprogramming, due to their abundance and ease of isolation from patients [44,45]. As an example, PBCs have been reprogrammed with episomal vectors, CAG, EF1, or SFFV and used for the treatment of lung disease [45,46]. In addition, this cell type has been examined for their ability to repair cartilage [47,48],

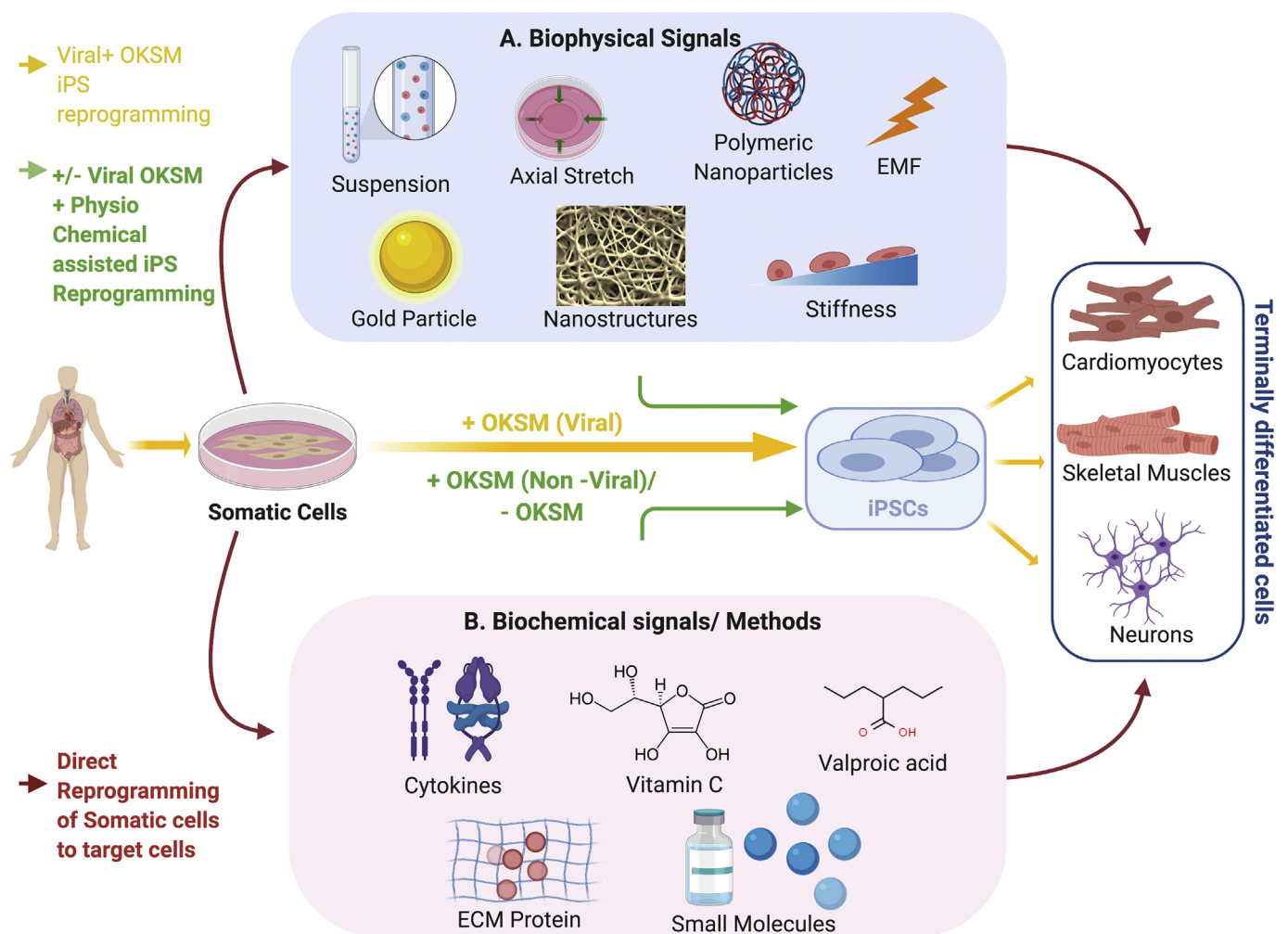


Fig. 1. Schematic representation of physical (A) and chemical (B) tools used for somatic cell reprogramming, either alone or in combination with viral or non-viral vectors coding for OKSM factors. OKSM = Oct4, Klf4, Sox2, c-Myc; iPSCs = induced pluripotent stem cells.

and to generate cardiomyocytes, for potential application as a therapeutic cell for cardiovascular disease [49,50].

Human cord blood has emerged as a potential cell source for regenerative medicine, since human leukocyte antigen (HLA) typing is mandatory during the cord blood cells banking process and thus gives easy access to these cells [51]. HLA is an important marker screened in every cell bank, as it is the most polymorphic gene in humans and is related to the major histocompatibility complex, which regulates immune responses and rejection of foreign organs. Moreover, cord blood cells are less immunogenic than other stem cells, because they are more immature [52].

Cord blood mononuclear cells (CBMCs) are abundant in the cord blood and have been tested for iPSC generation with different methods, including Sendai Virus [53] and other nonintegrating episomal vectors methods [54]. CBMC-iPSC have been reported to successfully generate skin [55] and cartilage [48]. When compared to dermal fibroblasts (DF), PBCs and osteoarthritis fibroblast-like synoviocytes (OAFSLs) for their chondrogenic potential, they showed highest level of chondrogenic markers among all groups, whereas PBCs had lower performance than standard DF [48].

In addition to CBMCs, cord blood comprehend other type of cells which unlike CBMCs they are adherent cells, and as such are considered a better source for generating iPSC compared to non-adherent ones [56]. Among them, cord blood endothelial cells (CBECs) and CD34⁺ cord

blood cells, corresponding to hematopoietic stem and progenitor cells (HSPC), have been reported as viable cell sources for iPSC generation [55,57,58].

CD34⁺ cord blood cells have been reprogrammed by transgene-free methods, using an episomal vector containing only 2 of the Yamanaka factors, namely Oct4 and Sox2 and showed a reprogramming efficiency of 2% [57]. CD133⁺ cells are another subset of adherent cord blood cells, which express pluripotency markers (Oct4, Sox2, Nanog and Cripto) and c-Myc and Klf4, and thus have an enhanced reprogramming efficiency than adult somatic cells [59].

In conclusion, both peripheral and cord blood cells represent an attractive cell source for iPSC generation. A study from Zhou et al. in 2015 showed the feasibility of reprogramming from a small amount of cryopreserved PBCs and CBMCs in a cost effective and scalable way. In this study, they also compare the two cell sources for their differentiation efficiency in either neural stem cells (NSCs), cardiomyocytes, or hepatocyte-like cells, concluding that no significant difference could be observed between PBCs and CBMCs; however, a variation was noted among different cell lines [60]. As a confirmation of the potential for these cells as an optimal iPSC source, iPSC cell banks derived from either peripheral or cord blood cells have recently started to appear [61]. Further details on iPSC banking will be provided in Section 6.

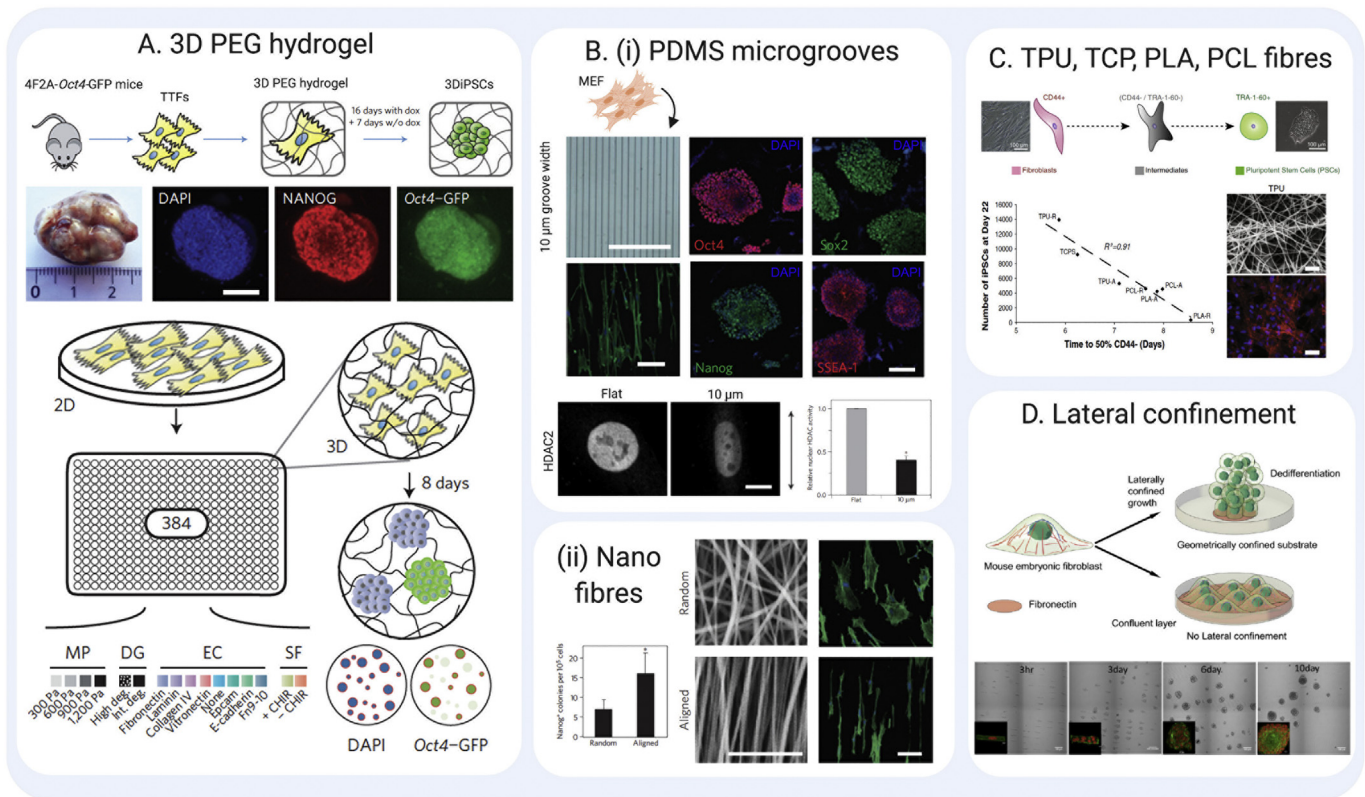


Fig. 2. Examples of mechanophysical stimuli used to reprogram somatic cells, including: (A) 3D PEG hydrogels Adapted with permission, copyright (2016) Springer Nature [119], (B) PDMS microgrooves (i) and nanofibers (ii), adapted with permission, copyright (2013) Springer Nature [122], (C) fibres of different synthetic materials (thermoplastic polyurethane (TPU), tissue culture polystyrene (TCP), polylactic acid (PLA), polycaprolactone (PCL), reproduced with permission, copyright (2014) Springer Nature [123], and (D) cell lateral confinement with micropatterns, adapted with permission, copyright (2018) [126].

3.4. Other cell types

To date, a variety of other cell types have been reprogrammed into iPSCs, such as hepatocytes [62], neural stem cells [63], pancreatic cells [64], different sources of adult stem cells and cancer cells [65]. The generation of iPSCs from primary hepatocytes has been shown to be faster compared to fibroblasts, blood cells and bone marrow cells, however, the reprogramming of this starting material shows an extremely low efficiency (0.0001%) [42]. Reprogrammed human hepatocytes showed 30% of colonies that could be picked up after only 6–9 days [66], whereas human fibroblasts typically take 30 days for first colony formation [67].

A potentially useful cell type for reprogramming are adult stem cells which are closer to the pluripotent state. Bone marrow MSCs (BM-MSCs) can be isolated fairly easily from the bone marrow, albeit a surgical procedure is still in need. When compared to fibroblasts and keratinocytes, BM-MSCs were more easily reprogrammed than keratinocytes and could differentiate into beating cardiomyocytes spontaneously and more efficiently than the other two cell types [68]. BM-MSC showed a reprogramming efficiency of 10 colonies from 10^4 starting cells, where fibroblasts had 5 and keratinocytes only about 2–3 colonies. iPSC derived from MSCs have also been shown to reliably re-differentiate to MSCs [69]. Cardiac progenitor stem cells (CPC) have also been used to generate iPSCs [70] (CPC-iPSCs), and when compared

Table 3
Direct reprogramming of cells with different biophysical stimuli (PA = Polyacrylamide; OKSM = Oct4, Klf4, Sox2, c-Myc).

Initial cells	Target cells	Biophysical stimuli	Reprogramming route	Reference
MEFs	Neurons	Microposts topography	Exogenous expression <i>Ascl1</i> , <i>Brn2</i> and <i>Myt1L</i>	Kulangara et al. 2014 [30]
MEFs	Dopaminergic neurons	Nanogrooves	Exogenous expression <i>Ascl1</i> , <i>Pitx3</i> , <i>Nurr1</i> and <i>Lmx1a</i>	Yoo et al. 2015 [31]
Mouse tail-tip mouse fibroblasts + MEFs	Cardiomyocyte-like cells	PEG hydrogel with functionalized proteins	OKSM + BMP4 based reprogramming protocols	Smith et al, 2013 [32]
Fibroblasts	Cardiomyocytes	nanopatterned substrates		Yoo et al. 2017 [33]
Neonatal murine cardiac fibroblasts	Cardiomyocytes	3D fibrin-based hydrogel	Micro RNA cocktails	Li et al. 2016 [34]
Human dermal fibroblasts	Hyaline chondrogenic cells	Electrical stimulation	Chemical chondrogenic differentiation	Lee, Kim, & Kwon 2019 [35]
Human dermal fibroblasts	Cardiac, neuronal, and skeletal muscle-like lineages	Radio electric conveyed fields	NA	Maioli et al. 2013 [36]
Cardiac progenitors	Cardiomyocyte-like cells	Parallel microgroove micropattern	Viral transfection of <i>Myocardin</i> , <i>Tbx5</i> , and <i>Mef2c</i>	Morez et al. 2015 [37]
Mouse tail tip fibroblasts	Cardiomyocyte-like cells	Mechanical stretch, stiffness, PA microgrooves	Retroviral transfection of <i>Gata4</i> , <i>Mef2c</i> , <i>Tbx5</i> and <i>Mkl1</i>	Sia, Yu, Srivastava, & Li 2016 [38]

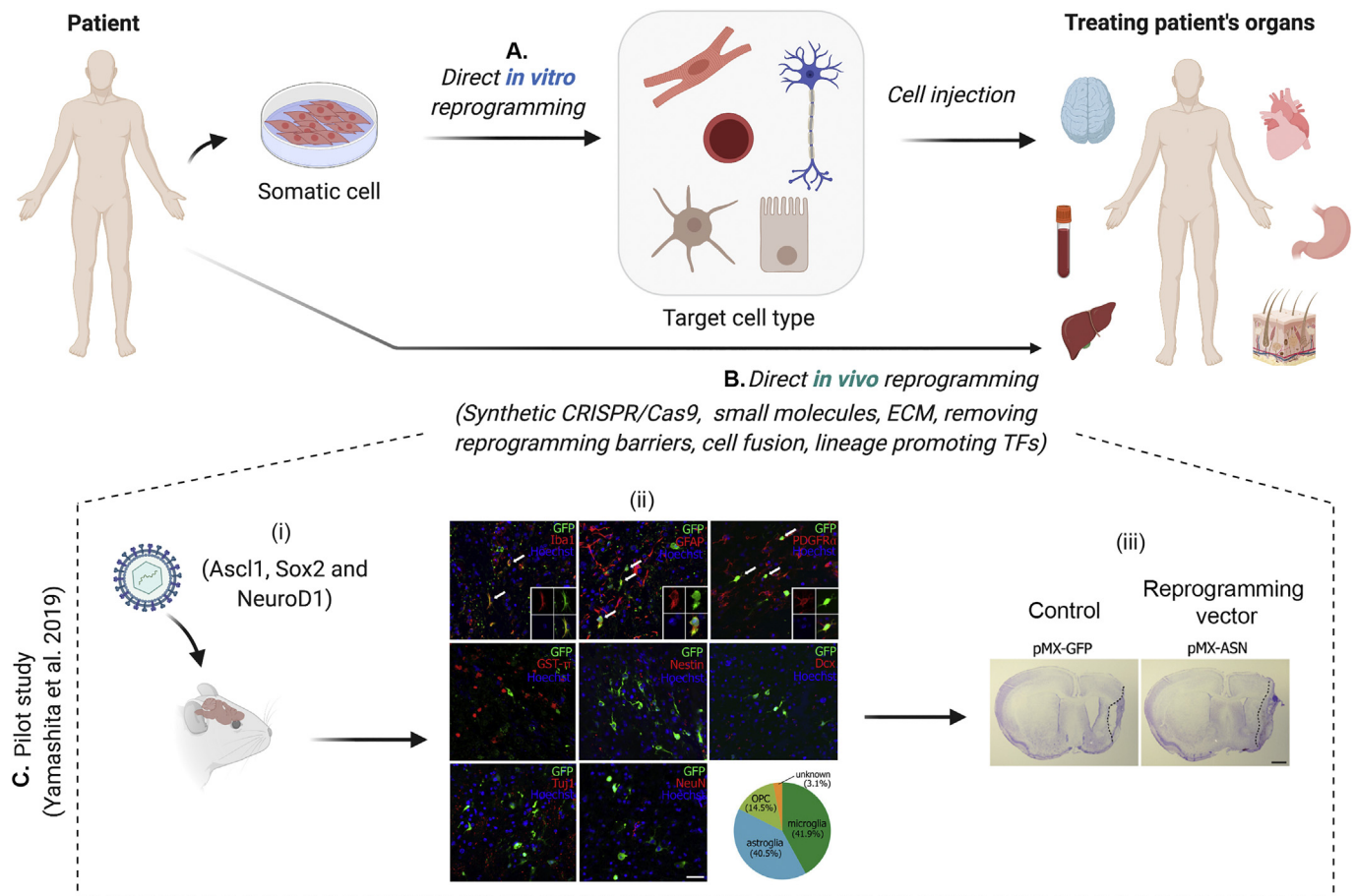


Fig. 3. Schematic of direct reprogramming *in vitro* (A) and potential application *in vivo* (B) with pilot study in mice shown in (C): (i) Yamashita et al. injected retroviral vector containing 2 reprogramming factors (Ascl1, Sox2 and NeuroD1) and obtained (ii) infected cells within brain including microglia, astroglia and oligoprogenitor cells (OPC), expressing related markers to these type of cells (Iba1, GFAP, PDGFR α), however, there was no evidence of oligodendrocyte, neural stem cell and neural markers; (iii) histological data confirmed although ectopic neurogenesis could be observed, the direct reprogramming method used did not therapeutically improve stroke animals.

to fibroblast derived iPSCs from the same donor for their ability to differentiate to cardiomyocytes, CPC-iPSCs showed significantly higher *in vitro* differentiation efficiency, measured by counting cells positive for cardiac troponin and number of beating cells. However, no significant functional differences were observed when cardiomyocytes derived from both cell types were implanted *in vivo* [70]. Human third molar mesenchymal stromal cells have also been employed for iPSC generation by retroviral transduction of 3 OKS factors, without c-Myc [71]. Because of their ease of access during wisdom teeth removal, they could be frozen and stored to be later used as a source for iPSC generation.

3.5. Cells from diseased patients

iPSC generated from patients with specific disease have been investigated with the intention of building an *in vitro* pathologic tissue model at first and subsequent potential treatment. The driving motivation for this approach came from the need for new model systems to analyze genetic diseases, in particular conditions arising from multiple genes, which cannot be applied with conventional disease modeling, including animal models or primary cells harvested from either healthy donors or patients with relevant genetic condition [72,73]. In addition, iPSCs have also been proposed as a tool for patient-specific drug screening. 95% of new drugs screened using artificially manipulated cells were withdrawn due to off-target effects [72], suggesting that a tissue model is needed instead of cell level assays.

Most of the investigations to date have centered around cardiovascular and neurological diseases, as they are major health threats in developed countries. iPSC derived from either cardiomyocytes (CMs) or neurons are in fact now used not only for drug screening and disease modeling but have also started to emerge as a source of cells for clinical applications [72]. Neurological diseases include stroke, neurodegenerative disorders, neurotrauma, multiple sclerosis (MS), and neurodevelopmental disorders. In order to analyze iPSC derived from patients with neurological diseases, Park et al. screened for the first time in 2008 iPSC generated from fibroblasts of patients affected by a variety of genetic diseases including adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophy, Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus, Down syndrome/trisomy 21, and the carrier state of Lesch-Nyhan syndrome [74].

In a study from Ochalek et al., PMBC were isolated from a patient with sporadic Alzheimer's disease, reprogrammed with Sendai virus delivery system of Yamanaka factors and have been proposed for drug testing and gene therapy studies [75]. iPSCs derived from patients with a variety of chronic neurological diseases could be successfully differentiated into neural cells, neuronal subtypes, or neural cell precursors *in vitro* [76–78]. They have been used for modeling neural diseases, including Alzheimer's disease [79], schizophrenia [80], Parkinson's disease [81,82], amyotrophic lateral sclerosis (ALS) [83], Rett Syndrome [84], Prader-Willi syndrome [85], spinal muscular atrophy (SMA) [86] and Huntington disease [77].

Cardiovascular diseases have also been explored by using iPSC derived from patient's cells [87,88]. iPSC colonies can be differentiated into functional CMs using a variety of methods, similar to traditionally employed protocols to generate CMs from hESCs. The most common method consists of a cocktail of pharmacological molecules, including GSK3 and Wnt inhibitors, to direct iPSCs to differentiate into the cardiac lineage [89]. Spontaneous embryoid body (EB) differentiation remains a viable strategy as well for cardiomyogenesis [90]. The first success in iPSC disease modeling in adult heart disease was achieved in 2010 from Carvajal-Vergara et al. for the Leopard syndrome [91]. Fibroblasts from two patients with Leopard syndrome were transduced with OCT4-, SOX2-, KLF4- and MYC-encoding VSV-pseudotyped Moloney-based retroviral vectors, to generate iPSCs [91]. iPSC derived CMs (iPSC-CMs) have been then used to model the long QT syndrome (LQTS), hypertrophic cardiomyopathy, dilated cardiomyopathy and cardiac rhythm disorders [92,93]. For modeling LQTS, iPSC were first generated from LQTS patient cells and subsequently differentiated into CMs (QTS human iPSC-derived CMs), which showed significant prolongation of the action-potential duration compared to cardiomyocytes of healthy donors and arrhythmogenicity. QTS human iPSC-derived CMs were used to make a cardiac-tissue model to test existing and novel drugs for their performance in changing the disease phenotype [94].

Nelson et al. demonstrated in 2009 that iPSCs delivered into infarcted hearts of mice could restore myocardial performance lost by the ischemic injury [95]. To date, iPSCs have been suggested for the treatment of cardiac diseases, including arrhythmias, cardiomyopathy, and regenerative medicine, and vascular diseases, such as hypertension, atherosclerosis and aortopathy [96]. iPSC technology has also been proposed for the development of cell-based therapies for biological pacing, which unlike electronic pacemakers can integrate with the host cardiac tissue and generate the electrical impulses required by the patient [87].

To give further examples of iPSC generated from diseased patients' cells, Basma et al. used lung fibroblasts from both patients having or not having chronic obstructive pulmonary disease, to reprogram and generate iPSCs. Although these two types of fibroblasts presented significant genetic and functional differences, when they were reprogrammed to iPSC and then re-differentiated into fibroblasts, these differences disappeared, because the differences present before could be related to epigenetics [39].

In regard to liver diseases, somatic cells from patient with rare hepatic disorders have been used to develop healthy and diseased liver tissue models. Although current hepatocyte like iPSC derived cells do not fully represent the mature form of hepatocytes, they perform better than many other liver cell models and they are sufficient to model most elements of liver disease. The rare diseases so far modelled through the iPSC technology account to around 30 and they have been reviewed in detail by Corbett and Duncan [97]. As an example, iPSC derived hepatocytes from patients with α -1-antitrypsin (AAT) deficiency were used to analyse accumulation of α -1-antitrypsin aggregates, in response to a drug library containing 3131 clinical compounds, and selected 5 of them that showed to consistently reduce AAT accumulation [98].

Finally, cells from patients affected from cancer have also been explored as a possible iPSC resource. Cancer is caused by a combination of environmental risk factors, genetic and epigenetic mutation, that lead to uncontrolled cell proliferation and tumor formation [99]. Understanding the reprogramming process of cancer cells could help defining the dynamic genetic and epigenetic changes of different state of these cells. Cells from human pancreatic ductal adenocarcinoma (PDAC) [100], triple negative breast cancer [101], leukemia [102] have been isolated to generate iPSC mainly for *in vitro* cancer model to understand mechanisms associated with cancer progression, and personal disease models for therapy and drug screening. The use of iPSC for cancer treatment remains questionable, as cancer cells seem to be often resistant to be reprogrammed, and when successful the entire process mechanism is still largely unknown. A study from Ko et al. compared human

fibroblasts, both human benign (MCF10A) and malignant (MCF7) breast cancer cell lines for their ability to become iPSCs with standard retroviral method, and showed that while fibroblasts could be successfully reprogrammed into iPSCs, both cancer cell lines were unable to achieve pluripotency [103]. Further work is necessary to optimize culture conditions—including the use of small molecule epigenetic modulators and advanced biomaterials—to guide pluripotency induction in cancer cells.

4. The influence of substrate and matrix properties on cellular reprogramming

The properties of the cell microenvironment plays an indispensable role in regulating cell identity through mechanotransduction cascades that ultimately affect nuclear shape and structure, and by extension the epigenetic state [104]. Various biomechanical factors in the microenvironment including local nanotopography, substrate stiffness and ligand presentation, as well as cell and tissue geometry, play a cumulative role in the final cell fate induction process [105]. Polo et al. demonstrated that genes responsible for cytoskeleton organization were activated during the first three days of reprogramming [106]. At the same time, major chromatin re-organization takes place [47] leading to complex and co-ordinated proteomic changes which facilitate the process [107]. Thus, cytoskeletal and epigenetic alterations are two critical events that mark the initiation phase of the reprogramming process. To assess how the cellular microenvironment affects these events, various synthetic and naturally occurring biomaterials have been used to alter the biomechanics of the cell niche [108], making the incorporation of biomaterials during nuclear reprogramming strategies a plausible approach to optimize and improve reprogramming efficiency [109–112] (Fig. 1A).

In this regard, several culture methods based on manipulation of the biophysical and biochemical microenvironment have been tested either alone or in combination with traditional viral transfection protocols, in order to enhance reprogramming efficiency and to improve scalability of iPSC generation methods. For instance, cell manipulation assisted by mechanical stretching has been applied in direct reprogramming of cells into various somatic cell types in 2D and 3D culture platforms [113]. Equiaxial mechanical stretching of the substrate to ~8% during the reprogramming of human dermal fibroblasts using the commercial Bioflex plates significantly improved reprogramming efficiency [110]. Physical properties of extracellular fluids like the viscosity [114] or fluid shear stress [115] acting upon cell aggregates during reprogramming in a stirred suspension bioreactor has been reported to accelerate reprogramming of mouse fibroblasts towards iPSCs, without the use of c-Myc. Non-ionizing radiation, like very low electro-magnetic fields, have been shown to affect cell behaviour in various biological systems, possibly by introducing chromosomal aberrations through increased free radicals in culture; however, the precise mechanism of these effects remains to be elucidated [115]. Extremely low-electromagnetic fields introduced to the somatic cell reprogramming process induced epigenetic modifications enough to activate histone lysine methyltransferase Mll2 and assist with the gain of pluripotency [116].

The contribution of matrix mechanics in coordinating cell behaviour has been reported in several studies towards enhancing cell reprogramming [117]. The reprogramming of mouse embryonic fibroblasts on a range of soft substrates (0.1–20 kPa) created using polyacrylamide hydrogels enhanced reprogramming efficiency by directly regulating mesenchymal-to-epithelial transition (MET) in fibroblasts cultured in 2D [118]. To assess reprogramming, PEG based hydrogels of 0.3–0.6 kPa functionalised either with fibronectin-derived adhesion peptide RGDSP (arginine- glycine-aspartate-serine-proline) or enriched with ECM proteins, such as laminin or epcam, resulted in marked improvement of iPSC generation, compared to 2D conditions [119] (Fig. 2A). Moreover, between a range of gel stiffness of 300 and 1200

Pa, 300–600 Pa appeared to be the ideal stiffness for improvement of iPSC generation in a 3D environment, in terms of acceleration of MET and expression of epithelial markers, concomitant to loss of the fibroblast phenotype. The pluripotent state was confirmed by the expression of Sox2, Nanog, Oct4 and SSEA-4, with a 2.5 fold higher reprogramming efficiency compared to cells cultured on 2D substrates [119]. Where 2D platforms provide a simpler, more straight forward approach to quantitatively analyse cellular behaviour during the reprogramming process, a 3D encapsulation which mimics the natural microenvironment can direct morphogenetic processes [120]. 3D biomaterials can be tuned to supply the cells with precise biophysical cues which help dictate cellular organization and gene regulation, thereby enhancing reprogramming events as compared to a 2D platform. Topographical cues have been demonstrated to control cellular signalling pathways via cytoskeletal control of nuclear shape and structure and chromatin dynamics [121]. For example, the reprogramming efficiency of mouse ear fibroblasts was enhanced when cultured on microgrooves of various widths through regulation of nuclear histone modifications, eliminating the need of exogenously adding epigenetic modifiers to assist the process [122] (Fig. 2B). Similar improvements in reprogramming was observed when fibroblasts were reprogrammed on randomly aligned nanofibrous substrates and graphene substrates as compared to TCP [123] (Fig. 2C). Similarly, Saha and colleagues demonstrated a micropatterning based method to dissect and understand the intermediate cell populations as well as the dynamics of the nuclear reprogramming process [124]. This study used micropatterns to modulate nuclear size and shape and established a set of nuclear characteristics to distinguish between fully and partially reprogrammed cells. Taken together, the control of microenvironmental cues during reprogramming can augment signalling pathways connected to reprogramming, and thereby increase efficiency and potentially reduce the need for viral factors.

Another aspect of using biomaterials for reprogramming, involves strategies for facilitating delivery of factors. To overcome the caveats associated with viral DNA, plasmid integration into the host genome, and increased possibility of mutations and tumorigenicity [8], biomaterials have been used to either deliver the Yamanaka factors – OKSM transiently to the MEF to initiate the reprogramming process, or biomaterials themselves were able to completely alleviate the need of OKSM induction to produce an iPSC like cell population [125,126]. The study from Wang et al. reported stable and efficient reprogramming of MEFs to iPSCs using an arginine-terminated gen4 polyamidoamine (G4Arg) nanoparticle as a non-viral and less cytotoxic OKSM carrier. The higher transfection efficiency in this case was owed to an optimal nanoparticle size and suitable zeta potential to assist with a greater endocytosis based cellular uptake and subsequent gene delivery [125]. Referring to the complete alleviation of the need for OKSM induction mentioned above, Shivashankar et al. reported that lateral confinement of MEFs on rectangular micropatterns showed ESC like characteristics without any induction but only due to nuclear deformation by the substrate topography [126]. These studies exemplify the use of biomaterials through various approaches to aid the iPSC reprogramming process.

Magnetic nanoparticles coated with biodegradable cationic polymers have been used for transient and controlled delivery of the Yamanaka factors to MEFs to alleviate the risk of genomic integration of viral DNA during traditional viral transduction methods [127]. As mentioned in the introduction, various combinations of CPP materials have been proposed as a safe way to induce cell reprogramming, albeit with low efficiency compared to other reprogramming methods. A major difficulty of using CPPs reside in the poor stability of recombinant proteins and the subsequent endocytic uptake [15]. Innovative nanocarriers, such as gold nanoparticles, in conjunction with a designer CPP – VG-21 (from the vesicular stomatitis virus (VSV) glycoprotein G (VSV-G), enhanced intracellular translocation through the combined effect of proteins or molecules to the cells due to their high tissue uptake capacity, low immune response and efficient clearance from the tissues

without having harmful cytotoxic effects on them, when being used for *in vivo* reprogramming trials [128]. Although it is clear that CPPs promote the cellular uptake of various anchored proteins in the simple culture system, the detailed mechanism of CPP internalization remains to be determined. Another issue that should be resolved is potential side effects of CPPs on the treated cells and neighbouring cells after transplantation. This issue is very important for the clinical application of cells generated by CPP-based technology.

Some biomaterials have been designed in conjunction with chemical signals to deliver the required small molecules, drugs, genes or microenvironment signals (e.g. adhesion ligands) to facilitate attachment, proliferation and reprogramming response. A common example for this is the use of nanoparticles (NPs) made of lipids or natural polymers to facilitate cargo delivery to the cells due to their small size, low risk of immunogenicity and preference over viral gene delivery methods [129], and were hence used for OKSM delivery to fibroblasts for a controlled iPSC induction [125,130]. These biomaterials-based delivery vehicles can either be used for surface adsorption or complete encapsulation of the OKSM factors [131], and their degradation kinetics be deployed as a tool for their strategic release of the cargo in a particular location [132] as the exposure times of the cells to these factors has been deemed important for efficient iPSC derivation [133].

Titanium oxide nanotubes have been used as a safer, non-cytotoxic tool for delivering reprogramming factors to fibroblasts; however, pluripotent colonies were produced after a slow and gradual process of 23 days and led to unstable iPSC like colonies [134].

More recently, the effects of physical constraints on cytoskeletal forces regulating nuclear deformation and downstream reprogramming related pathways have been under scrutiny [135–137]. For instance, we have demonstrated how microconfinement can trigger epigenetic reprogramming in cultured cancer cells, where the histone H3K4ac mark direct the activity of pluripotency regulator PRDM14 [138]. Shivashankar and colleagues demonstrated how lateral confinement of fibroblasts on micropatterned substrates was enough to trigger chromatin dynamics and induce iPSC-like cell populations, without the use of any exogenous factors [126] (Fig. 2D). This work presents evidence that fine-tuning the environment in which cells are cultured will direct epigenetic modifications to a broad extent, with scope for full epigenetic reset to pluripotency. Although considerable mechanistic insight remains to be gathered, the way in which cell culture materials can influence signal transduction cascades on the pathway to pluripotency, raises the provocative possibility of reprogramming through microenvironment engineering *in vitro* and *in vivo*.

5. Biochemical modification strategies: integration with biophysical regulation

Biochemical cues in the cellular microenvironment refer to a milieu of growth factors, small molecules, cytokines or even the components of the ECM which assist with stem cell behaviour [139]. In a cellular reprogramming context, small molecules as modulators of major signalling cascades may perturb the pathways underlying signals that promote or prevent reprogramming [140] (Fig. 1B). The efficiency of retroviral transfection-based reprogramming of MEFs using OKSM was tested after being aided with 5'-Azacytidine (a DNA methyltransferase inhibitor), or dexamethasone (a synthetic glucocorticoid) or several chromatin modifying small molecules, out of which valproic acid (a histone deacetylase inhibitor) proved to be the most potent [141]. Valproic acid also assisted with the successful use of recombinant protein CPP poly-arginine to deliver OKSM across somatic cell plasma membrane for non-viral induction and enhanced efficiency of pluripotency [142]. Supplementation of ascorbic acid (vitamin C) was reported to assuage cell senescence during the reprogramming process resulting in smoother and efficient transition [143,144]. With the identification of potent chemical inducers of pluripotency and a hope to alleviate the need for use of viral vectors from the process, there was an inflow of

studies reporting iPSC induction [145,146] by either using fewer transcription factors than traditional OKSM coupled with chemical induction [147] or by directly delivering OKSM to the cells using cell penetrating peptides [148]. These small molecules function in lieu of the exogenously expressed transcription factors by perturbing various signalling pathways and functionally replacing them for manipulating reprogramming kinetics [149]. This study reported that the regulation of factors which were known to directly promote MET, like TGF β or MEK-ERK pathway antagonists using small molecules (ALK5 inhibitor SB431542 and MEK inhibitor PD0325901) resulted in efficient and even alternative ways for reprogramming [149]. Inhibition of TGF β /Smad signalling using small molecules like E-616452 and E-616451 in the presence of HDAC inhibitor valproic acid (VPA) was able to alleviate the need of exogenous expression of Sox2 [150] or both Sox2 and c-Myc [151] during the reprogramming process.

Researchers recently reported a two-step method which was able to produce iPSC-like cells by reprogramming mouse astrocytes using small molecules and sonic hedgehog pathway activators, without the use of viral vectors or OKSM [152]. The small molecule approach has since assisted with direct reprogramming of somatic cells to target cells like cardiomyocytes [153], hepatocytes [154], neuronal cells [155,156], skeletal muscle cells and multipotent adipose derived stem cells [157].

Researchers have been using animal derived ECMs, such as laminins, Matrigel® and collagen for the adhesion and maintenance of iPSC in feeder free standard culture conditions. Surface functionalization of an otherwise inert PDMS surface using proteins like fibronectin, collagen or gelatin has proven useful for MSC [158] as well as ESC [159] adhesion and proliferation. In case of iPSCs, surfaces with modified and crosslinked hyaluronic acid with methacrylate functional groups were prepared and were then conjugated with a synthetic peptide from vitronectin (Ac-KGGPQVTRGDVFTMP), to maintain cell self-renewal, proliferation and pluripotency [160]. Various methods have been devised in order to chemically and physically functionalize biomaterials for desired outcomes using stem cells, as reviewed in detail [161]. As an alternative approach to induce fibroblasts to multipotent stem cells, recombinant human fibromodulin (FMOD) protein was used to generate FMOD reprogrammed (FR β P) cells which were iPS like in nature but did not form teratomas *in vivo* [162]. However, using similar approaches for assisting somatic cell reprogramming requires further exploration. Putnam and colleagues augmented OSKM reprogramming with supplements to direct cardiac differentiation [163] and demonstrated guiding roles for ECM composition and matrix mechanical properties. MEF were induced to dedifferentiation by combining lentiviral vectors containing OSKM and collagen I coated polyacrylamide substrates with four different stiffness values of 0.2, 2, 20, and 200 kPa. This study demonstrated that the optimum surface elasticity of 20 kPa along with a high ligand density of collagen I was optimum to promote higher reprogramming efficiency [164], hence confirming that physico-chemical properties regulate the dedifferentiation and cardiac differentiation phases of the reprogramming process.

6. Direct reprogramming: new approaches with potential for *in vivo* reprogramming

Over the past decade, the shift in focus has been from complete reprogramming to methods for manipulating cell plasticity to intermediate states; so-called direct reprogramming. With this approach, fibroblasts were directly converted to a variety of differentiated or progenitor cell types including neuronal cells [165,166], neural stem cells [167], functional cardiomyocytes [168,169], myoblasts [170], haematopoietic progenitors [171], angioblast-like progenitor cells [172], and melanocytes [173] using forced overexpression of a combination of selected transcription factors and/or defined culture conditions for each target cell type, such as feeder layer or growth factor use in culture media (Table 3). These transcription factors serve as initiators to bend the epigenetic silencing of the cell, shaping the chromatin

dynamics to enable specific regulatory sequences resulting in cell fate control [174]. Deploying these methods of cell fate alterations *in vivo*, could help circumvent the need for cell transplantation and/or the use of external (viral) vectors [175] (Fig. 3A, B). Reprogramming of endogenous cells is in fact a natural process that occurs within organs as part of tissue regeneration. Several studies have reported promising proof of concept results around this idea [176–178]. For instance, Yamashita et al. published a pilot study for a therapeutic application of *in vivo* neuronal reprogramming by intracerebral injection of retroviral solution of neuronal transcription factors *Ascl1/Sox2/NeuroD1* to produce ectopic neuronal cells in post-stroke mice [176] (Fig. 3C). Direct reprogramming results like these provide a possibility of tweaking the fate of endogenous cells to suit the microenvironment instead of iPSC transplantation, which carries a risk of teratoma formation *in vivo*.

Apart from injection of transcription factors carrying plasmids for direct *in vivo* reprogramming to a target cell type, several other strategies for *in vivo* reprogramming to target multiple genes have been proposed [179]. For example: CRISPR/Cas9 based manipulation of multiple disease genes by using lipid [180] or gold [181] nanoparticle as delivery systems have been reported, where one study also treated various human disease mouse models using this strategy [182]. Two recently published reviews provide details of various *in vivo* reprogramming strategies [179,183]. However, the direct delivery of genes and factors to remote tissue carries its own challenges in controlling the cell behaviour [175]. Direct *in vivo* reprogramming poses a risk of unintended reprogramming of tissue cells, which could also result in an unstable epigenetic state and initiation of stress related apoptotic signals in the cells. Hence, thorough knowledge of the cellular pathways leading to cell fate decisions is crucial to design safe direct *in vivo* cell manipulation trials.

Somatic cells have been directly converted to desired cell types using biophysical cues in conjunction with biochemical induction protocols guiding optimized stem cell behaviour [184]. For instance, neuronal induction of fibroblasts via overexpression of neuronal transcription factors experienced an increased efficiency on microgratings and microposts, highlighting the importance of topological cues in the process [185]. Similarly, enhanced conversion efficiency of fibroblasts to dopaminergic neurons on nanogrooved substrate, as compared to microgrooved and flat substrates and was attributed to nuclear shape and histone modification [186].

An improved protocol for conversion of mouse fibroblasts directly to cardiomyocyte-like cells using poly(ethylene glycol) (PEG) hydrogel substrate functionalized for MEF adhesion using laminin and RGD-binding integrins along with expression of Yamanaka factors was reported [187]. Fibroblasts have been reported to more efficiently convert to functional cardiomyocytes in response to nanotopographical cues in 2D [188], and to an increased expression of matrix metalloproteinases (MMPs) when confined in the fibrin based 3D hydrogel environment [189]. Exposure of dermal fibroblasts to electrical stimulation resulted in increased secretion of several growth factors including TGF- β 1, PDGF-AA, and IGFBP-2, 3, and facilitated its reprogramming to hyaline cartilaginous tissue [190] and their stimulation using radio electric conveyed fields (RECF) resulted in their conversion to several cell types including neural, cardiac and skeletal muscle like cells [191]. Direct conversion of somatic cells into target somatic tissues helps scrutinize the basis of cellular plasticity in a physiological context. Understanding the contribution of biophysical signals in the direct reprogramming process will provide important context to understand the myriad signals underlying reprogramming towards the use of reprogramming for *in vivo* therapies.

7. Therapeutic applications of iPSCs

Having the ability to derive into almost all mature cell types in human body and self-renew indefinitely, iPSCs have been in the centre of regenerative therapy and personalized medicine in the past decade.

The very first clinical iPSC trial was conducted in Japan in 2014 and consisted in treating age-related macular degeneration with an autologous iPSC-derived retinal pigment epithelial cell (RPE) sheet [192,193]. After 1 year, the first patient showed intact transplanted sheet, best corrected visual acuity neither worsened nor improved. However, since the authors found that the second patient iPSCs and iPSC-derived RPE reported three CNV, they decided to stop the second transplantation and end the clinical trial early, although iPSC-derived RPE passed the tumorigenicity test. While many preclinical studies and clinical trials have emerged in the recent years, the prevalent application of iPSCs has been in disease modelling and drug discovery.

7.1. iPSC for Disease modelling and drug discovery

Animal models such as rodents and primates are well-established and have served as the platform for disease modelling conventionally. However, it has always been hindered by significant interspecies differences, which limited the recapitulation of pathophysiology and aetiology of human diseases and frequently failed in clinic [194]. iPSCs have the potential to generate the patient specific disease model, and it is especially beneficial involving iPSC-derived cells and tissues that are otherwise hard to access, like neuronal cells and cardiomyocytes, for the discovery of reliable efficacy, pharmacokinetics and toxicity results [195]. Degenerative diseases including Alzheimer's disease and Parkinson's disease, and rare genetic disorders such as Cystic Fibrosis (CF) are life threatening diseases with practically no treatment available, due to limited understanding of the pathology and lack of adequate drug discovery platforms. Presently, iPSC-derived disease models have become the new 'gold standard' for phenotypic and target-based screening [196], by providing nearly unlimited cellular components specific to each patient in the form of organoid or mixed cell settings [197,198].

Significant numbers of observational clinical studies have been conducted on disease modelling and drug discovery with iPSCs, including amyotrophic lateral sclerosis (ALS) [199,200], Alzheimer's disease [201,202], Parkinson disease [203,204], long QT syndrome [94,205], and CF [206,207]. Whereas most of these clinical trials were largely observational, an important study was conducted by Sorscher et al

[208,209], where the efficacy of CF drug Trikafta was assessed by a Phase II study [210]. In this clinical trial, primary iPSCs derived airway epithelial tissue were evaluated in parallel with conventional human *in vivo* clinical response for the first time, to predict patient response towards therapeutic treatment. Using human iPSCs for drug screening avoids cross-species differences before they are taken to clinical trials. Further details on iPSC used for *in vitro* drug screening can be found in a review from Elitt et al [198].

7.2. iPSC biobanking

Another major development in iPSCs technology is the establishment of biobanks [28]. While autologous iPSCs programmed cellular components are idyllic for precise medicine, the process of reprogramming is particularly time consuming, cost sensitive and difficult to scale up by using current technology. On the other hand, allogeneic transplantation could solve the problem, but immunosuppression administration is required for allogeneic applications, which introduce long term complications. To overcome this problem, a genomic stable iPSC cell bank that originated from homozygous cell lines with human leukocyte antigen (HLA) matching could reduce immune rejection [211]. A clinical grade iPSC bank has been established in Japan [61,212], and a similar clinical trial was conducted in the USA, which has been suspended due to 'slow accrual' [213]. Alternatively, with the recent advancement of gene editing technology, CRISPR-Cas9 technologies [214] have prompted the development of 'universal non-immunogenic iPSCs' by overexpressing the transmembrane protein CD47 to inactivate major histocompatibility complex (MHC) to evade the host's immune response [215].

Most institutes offering iPSC generation, characterization and banking are non-profit and government funded organizations, focused on providing rare disease cell lines and improving stem cell research for both academic and industrial applications. Currently, the biggest cell banks are the European Bank for induced pluripotent stem cells (EBiSC), the Taiwan Human Disease iPSC Consortium, the California Institute for Regenerative Medicine (CIRM), Center for iPS cell research and application (iCeMS) in Kyoto University and the Korean Society for Society for Stem cell Biology (KSCB) [28].

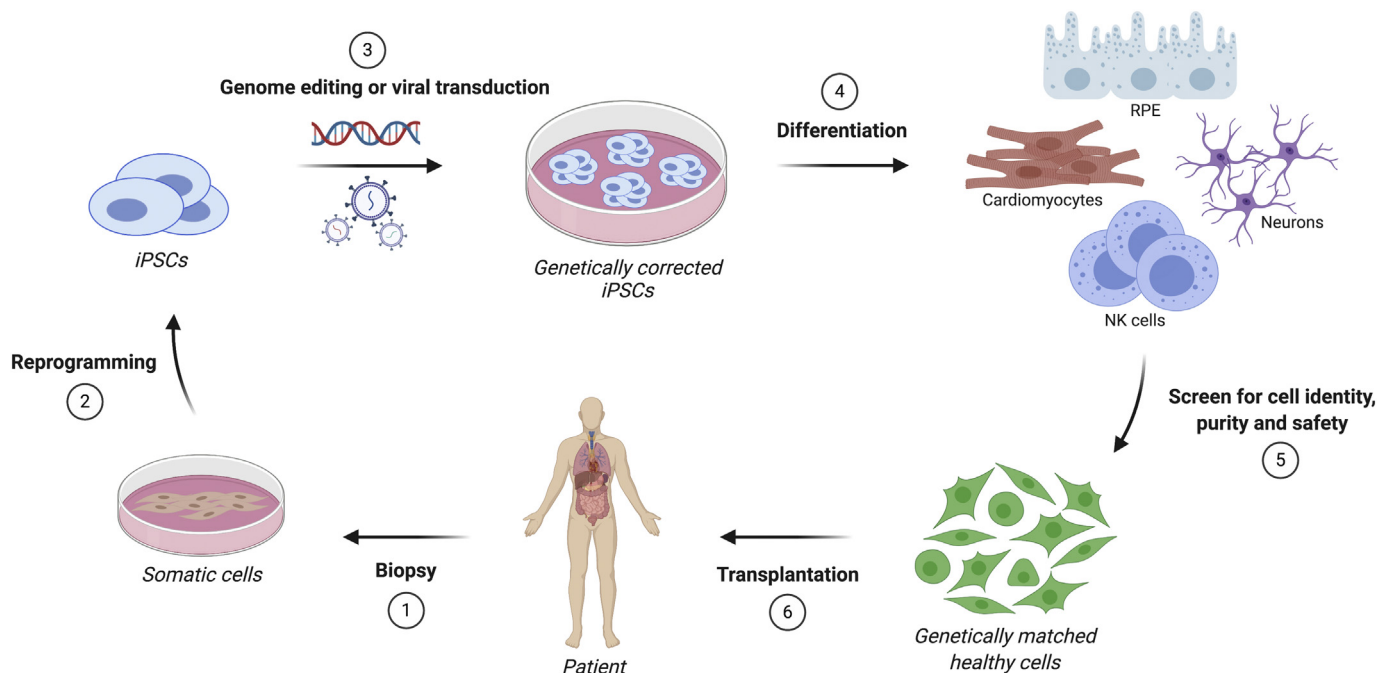


Fig. 4. A schematic illustration of human iPSCs-based regenerative medicine.

Table 4
Clinical trials of iPSC-based therapies in regenerative medicine.

Status	Conditions	Study title	Cell source	Enrolment	Sponsor	Location	Estimate study completion	Registration number
Phase I/II Completed	Exudative age-related macular degeneration (AMD)	A study of transplantation of autologous induced pluripotent stem cell (iPSC) derived retinal pigment epithelium (RPE) cell sheet in subjects with exudative age-related macular degeneration	autologous iPSC derived RPE cell sheet	2 (50+)	RIKEN, Foundation for Biomedical Research and Innovation Kobe City Medical Center General Hospital	Japan: Hyogo	February 28, 2019–October 2, 2013	UMIN000011929
Phase I/II Completed, follow up continuing	Neovascular AMD	A study of transplantation of allogenic induced pluripotent stem cell (iPSC) derived retinal pigment epithelium (RPE) cell suspension in subjects with neovascular age related macular degeneration	allogenic iPSC derived RPE cell suspension	5 (50-85)	Department of Ophthalmology, Kobe City Medical Center General Hospital	Japan: Hyogo	February 6, 2017 -	UMIN000026003
Phase I/II	limbal stem-cell deficiency	First-in-human clinical research of iPS derived corneal epithelial cell sheet transplantation for patients with limbal stem-cell deficiency	allogeneic iPSC derived corneal epithelial cell sheet (iCEPS)	4 (20+)	Department of Ophthalmology, Osaka University Graduate School of Medicine	Japan: Osaka	May 23, 2019–September 30, 2021	UMIN000036539
Phase I, II, III active, not recruiting	Parkinson's disease	Kyoto trial to evaluate the safety and efficacy of iPSC-derived dopaminergic progenitors in the treatment of Parkinson's disease	human iPSC-derived dopaminergic progenitors	7 (50-70)	Kyoto University Hospital, Japan Agency for Medical Research and Development	Japan: Kyoto	August 1, 2018–March 31, 2023	JMA-IIA00384, JMA-IIA00385, UMIN000033566, UMIN000033565, jRCTa050190117
Phase I recruiting	Aplastic anaemia with platelet transfusion refractoriness	Clinical study of autologous transfusion of iPS cell-derived platelets for thrombocytopenia	-	To be decided (20+)	Kyoto University Hospital	Japan: Kyoto	May 14, 2019 -	
Phase I/II pending	spinal cord injury	Regenerative medicine for spinal cord injury at subacute stage using human induced pluripotent stem cell-derived neural stem/progenitor cells	-	4 (18+)	Keio University School of Medicine	Japan: Tokyo	September 1, 2020 -	jRCTa031190228
Phase I/II pending	Damage of articular cartilage of the Knee	Development of treatment of knee articular cartilage damage with iPS-cell-derived cartilage	allogeneic iPS cell-derived cartilage	4 (20-70)	Asahi Kasei Corporation, Japan Agency for Medical Research and Development	Japan: Kyoto	January 6, 2020 -	jRCTa050190104
Phase I/II active, not recruiting	Ischemic cardiomyopathy	Clinical trial of human (allogeneic) induced pluripotent stem cell-derived cardiomyocyte sheet for severe cardiomyopathy	allogeneic iPS cell derived-cardiomyocyte sheet	3 (18-79)	Osaka University	Japan: Osaka	August 11, 2019–June 30, 2022	UMIN000032989
Phase I active, recruiting	Advanced Solid Tumors	FT500 as monotherapy and in combination with immune checkpoint inhibitors in subjects with advanced solid tumors	FT500 (allogeneic, iPSC-derived Natural Killer cell)	76 (18+)	Fate Therapeutics	US: California, Minnesota, Texas	February 15, 2019–June 2022	NCT03841110
Phase I active, not recruiting	Graft vs Host Disease	A study of CYP-001 for the treatment of steroid-resistant Acute graft versus host disease	Blood sample derived iPSC into MCAs and MSC	16 (18-70)	Cynata Therapeutics Limited	Australia: Sydney, Adelaide; UK: Bristol, Leeds	March 1, 2017–May 2020	NCT02923375
Phase I active, not recruiting	thoracic diseases COPD	A phase I Study of iPS cell generation from patients with COPD	Skin Biopsy derived iPSC	50 (18+)	Mayo Clinic	US: Minnesota	September 2009–January 2021	NCT01860898
Phase I/II recruiting	Ischemic Heart Failure	Treating heart failure with hPSC-CMs; The Study of human epicardial injection with allogenic induced pluripotent stem cell-derived cardiomyocytes in ischemic heart failure	Allogenic iPSC-derived Cardiomyocytes	5 (35-75)	Help Therapeutics The Affiliated Nanjing Drum Tower Hospital	China: Jiangsu	May 1, 2019–December 1, 2020	NCT03763136
Phase I/II pending	Beta-Thalassemia	Thalassemia treatment based on the stem cell technology	iPSC derived hematopoietic stem cells	2 (1-18)	The Third Affiliated Hospital of Guangzhou Medical University	China: Guangdong	January 1, 2015–June 14, 2016	NCT03222453
Preclinical Phase I/II	Advanced or recurrent gastrointestinal cancer	Basic research of immunotherapy using dendritic cells derived from induced pluripotent stem cells in healthy donors and cancer patients	Blood sample derived iPSC into dendritic cells	10 (20-75)	Second Department of Surgery, Wakayama Medical University, School of Medicine	Japan: Wakayama	March 1, 2016–March 1, 2021	UMIN000021105
Interventional: pre-clinical	Intellectual Deficiency with MYT1L Gene Mutations	Development of the tool " iPSC" for the functional study of mutations responsible for mental retardation	Cutaneous biopsy	4	University Hospital, Grenoble	France: La Tronche	September 2015–September 2017	NCT02980302

Interventional: pre-clinical	Myotonic dystrophy 1	Induction of pluripotent stem cells from human fibroblasts of DM1 patients	Skin biopsy, Blood and urine sampling	12 (age 20-50)	Institut de Myologie, France	France: Paris	November 2013–August 2014	NCT02084407
Observational pre-clinical, recruiting	Diabetes Complications, Diabetic Retinopathy	Human iPSC for repair of vasodegenerative vessels in Diabetic Retinopathy	Blood draw (peripheral blood cells)	20 (21-98)	University of Alabama at Birmingham	US: Alabama	January 11, 2018–January 31, 2021	NCT03403699
Observational, pre-clinical, Completed	Thrombocytopenia	iPSC-derived immortalized megakaryocyte cell lines (imMKCLs) from voluntary blood component donors with specific HLA haplotypes towards platelet products in transfusion therapy.	blood draw	3 (20+)	Center for iPSC Cell Research and Application (CiRA) Kyoto University, Kyoto University Hospital	Japan: Kyoto	November 1, 2014 -February 4, 2016	UMIN000015345

7.3. iPSC for regenerative medicine

Regenerative medicine, most prominently stem cell therapy, is a therapeutic approach that replaces damaged tissues or organs by stimulating self-repair or grafting *in vitro* generated components derived from patient or donor cells [194]. Previously, mesenchymal stem cells (MSCs) have been exploited in stem cell therapy for their availability, efficacy and safety in animal models among other endogenous stem cells. Yet, the clinical translation of MSCs has been restrained by the short life span that is endemic to adult stem cells, and recent reports of variable efficacy when trialled in humans [216,217].

Having the ability to differentiate into almost all mature cell types in the human body and self-renew indefinitely, iPSCs have garnered interest for treating degenerated organs suffering from ageing, genetic predisposition and trauma [218] (Fig. 4). iPSCs were used in stem cell therapy, at first as the source of growth factors [219] and dispersed target cells [220], however they showed limited therapeutic efficacy with these applications.

The initial attempt of iPSC-based therapeutic application was on degenerative ophthalmic diseases, namely age-related macular degeneration (AMD). Inspired by a successful study conducted on iPSC-derived retinal pigment epithelium (RPE) or iPSC-RPE in rats [221], a preclinical study was initiated at the Riken Centre (Japan) by Takahashi and colleagues to create a human iPSC-RPE cell sheets [222].

Later that year, the study reported promising results in a primate model, which paved the way to the world's first clinical trial of human iPSC products in 2014 [192,223]. An autologous iPSC-RPE cell sheet was grafted in the retina of a 77-year-old woman with no immunosuppression [193]. The trial reported positive results with no sign of tumorigenesis and immune rejection after one year follow-up, but it needed to halt due to genetic changes observed in autologous iPSCs. Since then, the group has shifted their focus onto HLA-matched allogeneic iPSCs with the aid of CiRA biobank (Kyoto Japan), and has a new ongoing clinical trial started in February 2017 [224].

There has been an emerging number of iPSC-based preclinical studies and clinical trials registered in the past 3 years and are under evaluation for both safety and efficacy. To date, there have been 14 clinical trials on iPSCs registered at the National Institutes of Health clinical trials website and Japan Primary Registries Network (JPRN), Table 4 has listed all registered clinical trials and selected preclinical studies that gave rise to clinical trials. One of the most ground-breaking results was received in September 2019, when the first limbal stem-cell deficiency patient experienced notable vision improvement one month after transplantation of a sheet containing allogeneic iPSC derived corneal epithelial cells [218,225].

No practical treatment is available for degenerative neurological disorders like Parkinson's disease, which encourages the development of novel treatments including iPSC-based regenerative medicine. Based on the safety and efficacy studies in rodent [6] and promising preclinical trial results in primates [226,227], millions of dopaminergic precursor cells that were derived from HLA homozygous allogeneic iPSC have been implanted in the patient's brain in Kyoto University [228,229]. With a sample number of 5, the clinical trial is expected to finish by early 2023. Another trial was approved on treating spinal cord injuries with iPSC derived neural progenitor cells from primate model studies [230,231], where remyelination and locomotor function recovery was noticed. The study was approved in 2018 and registered on February 2020 [232]. iPSC therapy is at the heart of promising treatments for ischemic cardiomyopathy, due to the limited regenerative ability and irreplaceability of cardiomyocytes. Remarkable cardiac function improvement was achieved in a preclinical study performed on porcine model with allogeneic iPSC-derived cardiomyocytes cell-sheet [233], and a clinical trial on 3 patients was initiated in August 2019 [234].

iPSC-derived cell-based products are an emerging trend for regenerative medicine. Presently, there are two major candidates, iPSC-derived natural killer cells (iPSC-NK) and iPSCs-MSCs. NK cells are cytotoxic

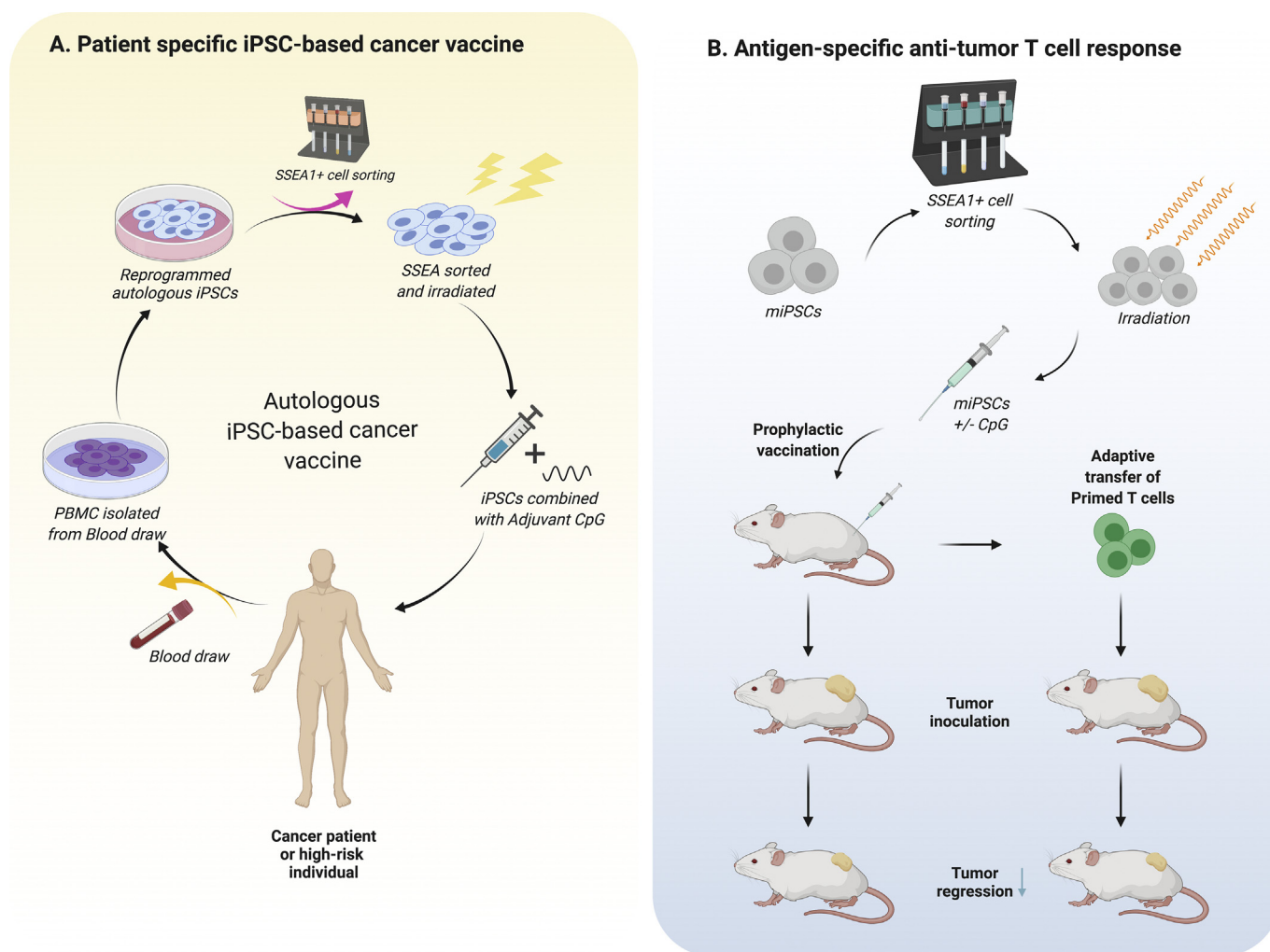


Fig. 5. Schematic illustrations of iPSCs application in cancer vaccines: (A) Autologous iPSC-based cancer vaccine can be generated by collecting peripheral blood mononuclear cells (PBMCs) isolated from patient's blood, and reprogrammed *in vitro*. The resulting iPSCs are then sorted for SSEA marker, irradiated and prepared in combination with CpG oligodeoxynucleotides and injected into the patient as an anti-cancer vaccine; (B) Schematic illustration of Kooreman et al. study: Murine iPSCs are at first sorted, then irradiated and combined with CpG solution and injected subcutaneously in mice. Kooreman et al. showed that autologous iPSC vaccines can not only prevent tumor in vaccinated mice, but also inhibit tumor growth in unvaccinated mice that have received T cells from vaccinated mice, indicating that iPSC vaccine can promote an antigen-specific anti-tumor T cell response.

lymphocytes that are genetically modified in graft-vs-host disease (GvHD) and cancer immunotherapy. iPSC-NK were first used in the treatment of ovarian cancer [235], and have proven their efficacy in treating other solid tumours that are otherwise hard to infiltrate by cytokine-activated NK cells [236]. A clinical trial with 64 candidates was recently initiated by Fate Therapeutics targeting different kinds of solid tumours [237]. An iPSC-MSCs named Cymerus was developed by Cynata Therapeutics, where a Phase I clinical trial targeting GvHD is underway [238]. With positive supporting feedback from Phase I study, it can now proceed to Phase II [239].

The majority of iPSCs clinical trials have originated from Japan. This is because of the vast funding provided by the government, and most prominently the implementation of Japan's regenerative medicine laws that came into effect on November 2014 [240], which enables fast tracking [241,242]. Although these clinical studies are still in their infancy, regenerative and cell therapies employing iPSCs may soon be more widely available. The United States and France have approved considerable numbers of iPSC applications, but almost all of them are observational studies with a focus of the basic biological and pathological mechanism or proof-of-concept preclinical interventional studies.

7.4. iPSC for cancer vaccines

iPSCs have also been suggested as vaccines for cancer treatment by inducing anti-tumor response. The first studies began over a decade ago; however, although iPSC induced IFN γ and IL-4-production against mouse colon cancer cells, no evidence of tumor rejection was observed [243]. In 2018, Kooreman et al. developed anti-tumor vaccines by using irradiated iPSCs expressing a range of tumor-associated antigens in combination with adjuvant CpG. When injected in mice, the iPSC vaccine was able to inhibit melanoma and breast cancer.

Moreover, when T cells produced from vaccinated mice were injected in unvaccinated mice, they were still able to inhibit tumor growth, providing a so called two-way immunity [244] (Fig. 5). Tumor immunity was related to shared epitopes between cancer cells and iPSCs, similarly to hESC. Moreover, there was a significant gene expression overlap among different cancer types and iPSCs [245]. Single-cell analysis demonstrated that iPSCs are more heterogeneous than hESCs and thus could provide a better source for anti-tumor immune response than hESCs by providing the host with a larger set of antigens [246]. Depending on treatment needs, anti-cancer vaccines can either function as prophylactic to delay cancer occurrence or as therapeutic agent to target

pre-existing tumors [247]. iPSC derived anti-cancer vaccines developed from Kooreman et al. can potentially act as an efficient whole-cell anti-cancer vaccine.

8. Conclusion and future directions

Somatic plasticity raises the exciting prospect of reprogramming a patient's cells to repair any tissue in the body. The advent of iPSC technology has already had huge impacts in fundamental studies, disease modelling and regenerative therapies. However, there are major hurdles to wide scale implementation in clinical settings including cost, standardisation, genetic stability, and concerns of immunogenicity. Nevertheless, with multivariate approaches involving small molecule epigenetic modulators and designer cell culture materials, there is tremendous promise in the development of reprogramming methodologies that increase efficiency, reprogramming time, and obviate the need for viral-based reagents.

Direct cell plasticity manipulation *in vivo* is an optimal approach to regenerative therapies. However, clinical implementation faces several challenges that need to be overcome in order to be viable as a therapeutic approach. Targeting the innate plasticity of a cell requires forced transcriptional and epigenetic manipulation thereby perturbing local homeostasis. Combining that with the *in vivo* heterogeneity of cells, full or partial change of cell identity requires them to go through several rigorous stages of cell fate and proliferation rates, while also altering their metabolic and environmental profiles [248]. Stable control of this process in a remote tissue environment poses considerable barriers. Another issue is the low and incomplete reprogramming efficiency of current methods, a lack of control over complex cellular subtypes and intrinsic heterogeneity, which have essential roles in forming an *in vivo* functional tissue.

While a new field with considerable challenges, our greater understanding of somatic cell plasticity coupled with the use of gene editing and advanced molecular delivery materials, provides a vision where *in vivo* cell engineering may someday become a reality.

Notes

Figures were created with [Biorender.com](https://biorender.com) and Adobe Photoshop, and have been exported under a paid subscription.

All information reported here was gathered from search engines Pubmed/MEDLINE, Google Scholar, and Scopus. Clinical trials information was obtained through NIH (<https://clinicaltrials.gov/ct2/home>), NIPH: Japan <https://rctportal.niph.go.jp/en/link> and WHO (<https://www.who.int/ictrp/search/en/>).

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