Hindawi Publishing Corporation
New Journal of Science
Volume 2014, Article ID 756240, 22 pages
http://dx.doi.org/10.1155/2014/756240

Review Article

Myocardial Reprogramming Medicine: The Development, Application, and Challenge of Induced Pluripotent Stem Cells

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Received 16 January 2014; Revised 1 April 2014; Accepted 2 April 2014; Published 16 June 2014

Academic Editor: Stefan Liebau

Induced pluripotent stem cells (iPSCs) can be generated by reprogramming of adult/somatic cells. The somatic cell reprogramming technology offers a promising strategy for patient-specific cardiac regenerative medicine, disease modeling, and drug discovery. iPSCs are an ideal potential option for an autologous cell source, as compared to other stem/progenitor cells, because they can be propagated indefinitely and are able to generate a large number of functional cardiovascular cells. However, there are concerns about the specificity, efficiency, immunogenicity, and safety of iPSCs which are major challenges in current translational studies. In order to bring iPSC technology closer to clinical use, fundamental changes in this technique are required to ensure that therapeutic progenies are functional and nontumorigenic. It is therefore critical to understand and investigate the biology, genetic, and epigenetic mechanisms of iPSCs generation and differentiation. In this spotlight paper the discovery, history, and relative mechanisms of iPSC generation are summarized. The current technological improvements and potential applications are highlighted along with the important challenges and perspectives. Finally, emerging technologies are presented in which improvements to iPSC generation and differentiation approaches might warrant further investigation, such as integration-free approaches, direct reprogramming, and the development of iPSC banking.

1. Introduction

Myocardial infarction (MI) is an important manifestation of coronary artery disease (CAD) and major cause of death and disability worldwide. MI occurs when prolonged ischemia irreversibly destroys distal blood vessels and myocardium, causing apoptosis or cell death, eventually triggering cardiac remodeling or sudden death [1, 2]. Recurrent MI leads to chronic postinfarct heart failure in patients with a longer life span. Currently, traditional therapeutic approaches focus on limitation of the initial injury and secondary maladaptive complications in order to prevent the death of existing myocardium. Despite significant advances in medical treatments over the past decades, chronic heart failure remains as a leading cause of death [3, 4]. Indeed, heart transplantation is the only available viable therapeutic option for end-stage heart failure, but this option is limited by the paucity of matched donor tissue specimens and by the requirement for life-long treatment with immunosuppresive agents.

Regenerative therapies offer great promise for patients with heart disease by using angiomyogenesis to create a source of replacement for lost or damaged cardiac tissues associated with MI [3, 4]. Therefore, a pivotal current task is to obtain ideal pluripotent stem/progenitor cells with cardiovascular potential. These characteristics include but are not limited to safety, sufficient quantity and quality for clinical applications, stable capacity of self-renew and differentiation, low immunogenicity, and a minimization of any ethical issues.

First seen in studies of experimental embryology, mouse or human embryonic stem cells (ESCs) were discovered as a kind of pluripotent stem cells derived from the inner cell mass of a developing blastocyst. A great milestone in stem cell biology occurred when it was found that these cells could be propagated in culture in an undifferentiated state [5–7]. Multiple studies have since demonstrated that ESCs can differentiate into all the cell lineages of the embryo, fetus, and adult under defined conditions [8–10]. ESCs can be

propagated indefinitely and serve as a potentially inexhaustible supply of functional cardiomyocytes under defined differentiation conditions. Although ethical issues and teratoma formation potentially hinder the clinical application of ESCs, they are important and useful tools for cell-line models of pluripotency (stemness) in both basic research and clinical medicine [11]. For decades, there has been tremendous enthusiasm in the quest to identify the master regulators of pluripotency (or totipotency) from investigating ESCs [12, 13]. Various signaling pathways were found to modulate the pluripotency and function of ESCs both *in vivo* and *in vitro*, as summarized in other reviews [11, 14]. Large scale gene expression profiling was performed to identify the regulators controlling pluripotency and lineage specificity in ESCs [13, 15]. Notably, it was found that the pluripotency and self-renewal of ESCs are maintained by a network of transcription factors (TFs), such as octamer-binding transcription factor-4 (Oct4), signal transducer and activator of transcription 3 (Stat3), and homeobox transcription factor Nanog [16, 17].

The technique of somatic cell nuclear transfer (SCNT) was established during the 1950s to probe the developmental potential of nuclei by transplanting them into enucleated oocytes [18, 19]. SCNT studies demonstrated that differentiated cells or somatic cells retain all of the genetic information in nuclei as early embryonic cells, which are required for the entire organism development. In these trials, the nucleus of oocyte was exchanged with the genome of a somatic cell using SCNT technology to generate pluripotent stem cells for regenerative applications [20]. Stem cell lines derived from these artificial blastocysts were capable of differentiation into cell types of all three germ layers. However, this reprogramming approach requires private egg donation from women and the support of ethical policies [21]. Thus, this approach is technically challenging and ethically questionable and therefore not suitable for translational research [22, 23]. A hypothesis arose that a combination of master regulators or factors isolated from nuclei can reprogram somatic cells (nonpluripotent cells) back into the embryonic state of pluripotency. This procedure of changing cell phenotypes is referred to as "reprogramming". Thus, the transfer of TFs which are the dominating active proteins in nuclei could be an alternative reprogramming strategy.

Initially, Yamanaka showed that stem cells with properties similar to ESCs could be generated from mouse or human fibroblasts by simultaneously introducing four TFs [24, 25]. Thomson subsequently reported the generation of novel human stem cells using a different combination of factors [26]. These novel stem cells were designated as induced pluripotent stem cells (iPSCs). The iPSC technology using adult somatic cells avoids the ethical issues raised in ESCs. In addition, the differentiation of iPSCs into functional cells is beneficial for cell based therapy and also plays an important role in the establishment of patient-specific disease models for drug discovery and development. The simplicity and reproducibility of iPSC technology have increased interest for their usage in regenerative medicine. In this spotlight article, I summarize the generation and potential application of iPSCs, as well as the major challenges, highlights, progress, and future directions.

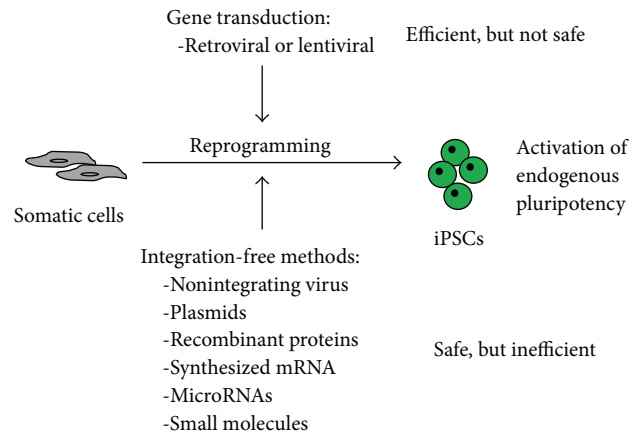

Figure 1: The current approaches of iPSC generation. There are a number of different approaches for iPSC generation from somatic cell reprogramming, including gene transduction and integration-free methods. Retroviral or lentiviral gene transduction is the most efficient and widely used method, but the integration of TFs into a host genome increases the concerns of oncogenicity and mutagenesis. The integration-free methods include using nonintegrating virus, plasmids, recombinant proteins, synthesized mRNA, microRNAs, and small molecules. Most of these approaches are safe, but reprogramming efficiency is lower than that of gene integration. The activation of endogenous pluripotency is considered an important criterion of high-quality fully reprogrammed iPSCs.

2. Generation of iPSCs

In order to efficiently produce iPSCs as research tools, and ultimately translate laboratory results into clinical applications, a number of different somatic cell reprogramming approaches have been developed, including gene transduction and integration-free methods (Figure 1).

2.1. Gene Transduction

2.1.1. Cell Transfection. Gene transduction can be used to generate iPSCs using retroviral or lentiviral approaches, which are currently the most efficient and widely used methods, although progress is rapidly growing in the use of other gene delivery methods. Twenty four pluripotency-associated candidate genes were initially evaluated in an assay system by retroviral transduction in order to identify the effective reprogramming factors. After successive elimination of individual factors, the minimal four genes comprising Oct4, Klf4, Sox2, and c-Myc (OSKM) were determined to induce reprogramming of mouse embryonic fibroblasts (MEFs) [25]. This method of iPSC production has many advantages, not only because the biological properties are similar to ESCs, but also because there are no ethical and legal issues and they are technically more feasible. Several laboratories began to use, reproduce, and improve the procedure of modifying the combination of different reprogramming factors or expanding the selection of target adult cells [22, 27]. In addition to OSKM genes, other core pluripotency genes such as Nanog and Lin28 were also determined as efficient

reprogrammers of human fibroblasts into pluripotent cells [26, 28].

As the reprogramming approaches rise in number, it is important to generate optimal reprogramming factor cocktails for various adult cells. Each protocol must take into consideration the reprogramming efficiency because currently it is at a very low level in the majority of current studies (typically much less than 1% [22]). This means that the reprogramming process of most transfected cells is never completed. Moreover, there is great variability in the quality of iPSC clones, even when using the same protocol. This variability is probably attributable to a multitude of complicated events, including gene delivery methods, growth conditions, culture timing, target cell selection, and stochastic genetic integration. Hematopoietic stem and progenitor cells were reported as an attractive cell resource to generate iPSCs with efficiency of up to 28%, as compared to terminally differentiated B and T cells [29]. The modification of cell culture conditions and the addition of molecular compounds also proved to enhance the reprogramming efficiency [30, 31].

Of great concern in these protocols is safety, as many reprogramming factors can be linked with the pathophysiology of cancer, such as c-Myc which is a very strong proto-oncogene [32, 33]. To decrease the risk of teratoma formation in the future applications, often genes can be replaced by other members of the same family. For instance, L-Myc or N-Myc can substitute for c-Myc [34]. This simple elimination of c-Myc in iPSCs eliminated the development of tumors during the study period, albeit with a decreasing reprogramming efficiency [35]. This low-efficiency issue, however, was minimized by using a modified low-serum culture protocol to obtain high quality iPSCs [36]. Poly (ADP-ribose) polymerase-1 was also found to act as a replacement for c-Myc or Klf4 and enhanced the efficiency of iPSC generation [37]. Nuclear reprogramming independent of c-Myc (using Sox2, Oc4, and Klf4) enhanced the innate cardiogenic potential of pluripotent stem cells, with the observation of consistent beating activity, sarcomere maturation, and rhythmical intracellular calcium dynamics [38].

In a different example, a detailed protocol was developed to derivate iPSCs from cord blood stem cells using retroviral transduction with only two factors (Oct4 and Sox2) that are a prerequisite in the majority of present studies [39]. However, the latest study shows that Oct4 and Sox2 can be replaced by some identified factors completely (Gata3 for Oct4; ZNF521, OTX2, and PAX6 for Sox2) to induce the reprogramming of human fibroblasts [40]. It has also been found that Oct4 and its substitutes (Gata3, HNF4a, and GRB2, etc.) are involved in mesendodermal specification, while Sox2 and its substitutes (Sox1, RCOR2, and GMNN, etc.) are involved in ectodermal specification regulating the balance of pluripotency and differentiation [41]. DNA hydroxylase Tet1 can replace Oct4 by promoting Oct4 demethylation and reactivation to initiate somatic cell reprogramming [42]. These studies indicate that all reprogramming factors are potentially interchangeable, which increases the feasibility and flexibility in the manipulation and improvement of iPSCs generation.

2.1.2. Inducible Transduction. As discussed above, the low efficiency of iPSC generation is a great challenge for the study of the molecular processes or mechanisms during the early phase of reprogramming. The use of an inducible transduction system, which can be flexibly controlled by the inert drug tetracycline (Tet) or doxycycline (Dox), allows for increasing efficiency and the selection of fully reprogrammed iPSCs [43]. This approach relies on the addition of Dox and is more convenient than the conventional methods which require *de novo* delivery of reprogramming genes into target cells. Lentiviral vectors can be used to generate iPSCs by encoding the four reprogramming factors and have high infection efficiency [44, 45]. Lentiviral vectors can also be modified to establish an inducible transgene expression system for iPSC generation. Briefly, the system is established in target cells containing two main components, including expression of reverse-tetracycline-dependent transactivator (rtTA) and the corresponding inducible promoter controlling the expression of reprogramming genes (Figure 2(a)). The specific promoter lacks binding sites for endogenous TFs, so it is virtually silent in the absence of induction. In the presence of Tet or Dox, the transactivator binds tightly and specifically to the promoter and activates transcription of the downstream genes of interest [46].

To generate genetically homogeneous cell populations, primary somatic cells (such as hepatic cells, hematopoietic cells, or fibroblasts) can be infected with Dox-inducible lentivirus encoding OSKM to generate the "primary" iPS cell lines with Dox in culture medium [43, 47]. These can then be injected into blastocysts to create chimeras for the generation of "secondary" somatic cells that carry the reprogramming factors. These can then be isolated and generate new iPSCs by Dox induction (Figure 2(b)). This method of generating iPSCs was indirectly derived from gene transduction and is termed as "secondary" iPSCs (or secondary reprogramming systems) [48]. Reprogramming efficiency was radically improved by 25- to 50-fold greater efficiency than using "primary" iPSC generation techniques [43]. However, the production of "secondary" somatic cells from chimeras is tedious and expensive.

Current studies are seeking an even more ideal resource of "secondary" somatic cells. Using "primary" iPSCs generated from primary human fibroblasts and keratinocytes with the Dox-inducible system, cells can be differentiated into fibroblast-like cells as the "secondary" somatic cells (Figure 2(b)). These can finally be used to produce "secondary" iPSCs using Dox treatment, resulting in an even higher efficiency (100-fold greater than the initial conversion) [49]. It was also reported that up to 2% of the "secondary" human fibroblasts derived from primary iPSCs were reprogrammed to "secondary" iPSCs using this system [50]. This provides a powerful and efficient platform for studying the underlying molecular mechanisms during reprogramming as well as for screening chemical and genetic factors that enhance or block reprogramming.

Unfortunately retroviral transgenes are epigenetically silenced towards the end of reprogramming [51, 52]. Consequently, the partially reprogrammed cells are dependent on exogenous factors and can fail to activate the corresponding

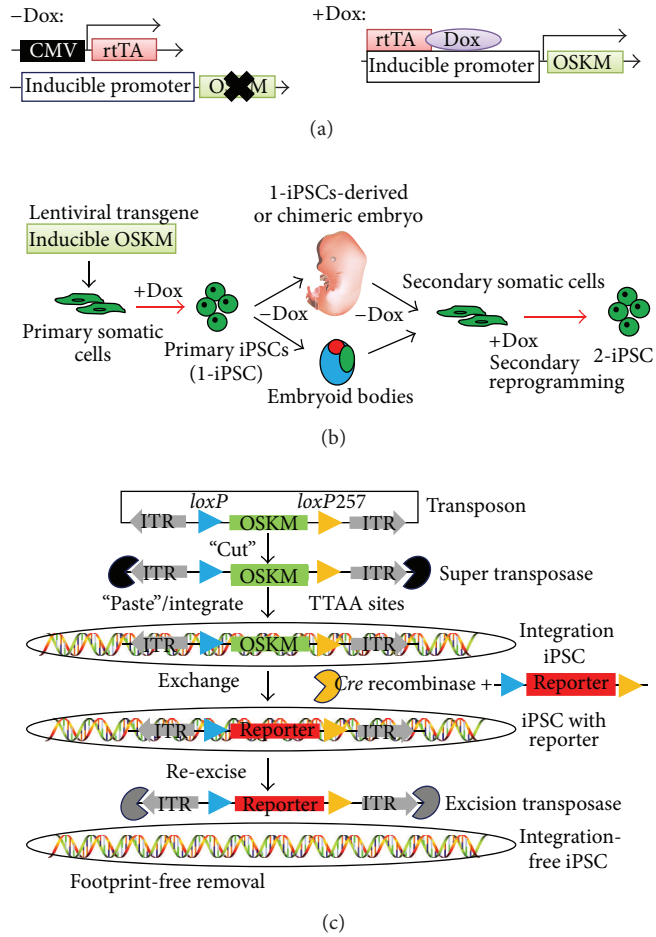

FIGURE 2: The inducible transgene and excisable system for iPSC generation. (a) The inducible transgene system conta ins two main components, reverse-tetracycline-dependent transactivator (rtTA) and the inducible promoter controlling the expression of reprogramming genes (OSKM). In the absence of doxycycline (Dox) induction, the specific promoter lacks binding sites for endogenous TFs, so it is virtually silent. In the presence Dox, the transactivator binds tightly and specifically to the promoter and activates transcription of OSKM genes. (b) The primary somatic cells (such as fibroblasts) were infected with inducible lentivirus encoding OSKM to generate "primary" iPSCs (1-iPSCs) with Dox. These were then injected into blastocysts to create chimeras or formed into embryoid bodies for the generation of "secondary" somatic cells. These then carry the inducible reprogramming factors, which were isolated and used to generate secondary iPSCs (2-iPSCs) using Dox induction. (c) The schematic of integration-free iPSCs generated by the transposon and *Cre-loxP* excisable system. The OSKM cassette was flanked by compatible loxP-loxP257 sites and inserted between the specific inverted terminal repeat (ITR) sequences of transposon vector. The insertion was excised ("cut") by hyperactive transposase from ITR to integrate ("paste") into the TTAA sites of chromosomal DNA in transfected somatic cells. After integration-iPSC generation, the reporter tracking gene (such as RFP) can be introduced by Cre recombinase which can specifically recognize loxP sites, resulting in the removal of OSKM. Finally, the integrated fragment can be re-excised by excision transponsase to generate integration-free iPSCs.

endogenous genes [22, 53]. This issue becomes exacerbated when lentiviral vectors are used and subjected to variable levels of silencing causing a differentiation block [54, 55]. Although viral transgene approaches were adequate for initial studies, current research requires more precise tools to explore deeper into the events and mechanisms underlying reprogramming, in particular for *in vivo* study [56].

Recently, double transgenic reprogrammable mouse systems have been developed as the most advanced techniques in mouse inducible iPSC generation. After a generation of "primary" iPS cells are generated using the inducible system, cells were used to breed chimeric mice whose somatic cells could be isolated and reprogrammed *in vitro* by addition of Dox [57]. This study also compared the reprogramming efficiencies among MEF lines containing different combinations of a defined set of Dox-inducible proviral genomes, providing a unique platform for identifying components that can substitute for a given reprogramming factor. However, it was difficult for the random segregation of multiple transgenes to maintain all factors in one mouse and identification of the genotype was needed for offspring in each generation. This limitation was alleviated by using inducible collagen type I (Col1a1) locus-targeted transgenic mouse models as described in two back-to-back publications [58, 59]. Different

strategies were used to link the four reprogramming genes (OSKM) in one vector under the control of the Dox-inducible promoter. The various somatic cell types from their offspring (any embryonic, fetal, or adult tissue) allowed iPSC generation with Dox treatment. Breeding is effective, but most importantly no tumors or other health problems were detected in these offspring. A line of conditional, inducible, and exchangeable "reprogrammable mice" was established for studying cellular (de)differentiation [60]. This mouse model can realize tissue or cell-specific expression and is also a means of selecting OSKM-expressing cells, allowing for the exchange of the reprogramming factors by other genes at a defined locus. These reprogrammable mice have emerged as an excellent *in vivo* tool for studying both cellular reprogramming and lineage-directed differentiation factors, particularly for the identification and functional characterization of the genetic/epigenetic determinants [60, 61].

2.2. Integration-Free Method. Current retroviral or lentiviral reprogramming strategies are utilizing the integration of TFs into host genome, increasing concerns of oncogenicity and mutagenesis [62, 63]. These viral transgene approaches are unlikely to be suitable for future clinical applications but are still important tools for basic studies. The transduction of exogenous TFs aims to activate the expression of endogenous pluripotent genes as a means of triggering the reprogramming process. This presents a need for an alternative way to accomplish this purpose. To solve this technical limitation of iPSC generation, reprogramming processes could potentially be induced using nonintegrating strategies. Recently, integration-free approaches are being developed as a promising technique for iPSC generation.

2.2.1. DNA Based Approaches. Using replication-defective adenoviral vectors, the first integration-free iPSCs were generated from fibroblasts and liver cells using adenoviruses transiently expressing OSKM [64]. A second group concurrently generated the virus-free iPSCs from mouse MEFs by repeated transfection of two expression plasmids containing OSKM [65]. The integration-free iPSCs were produced by nonintegrating gene delivery strategies, including *Sendai* virus [66, 67], episome [68, 69], minicircle vector [70], and polycistronic plasmids [71, 72]. These studies provided safe approaches with extremely low risk of tumorigenicity, although their reprogramming efficiencies were lower when compared to the transgene integration strategy. These inefficient approaches are poor for generation of patient-specific iPSC lines, particularly from their primary biopsies.

In order to eliminate the risk of oncogenicity in the integration protocols, several laboratories have refined the process by using "excisable" methods, such as transposon systems and *Cre-loxP* systems, to remove the chromosomally integrated genes (Figure 2(c)). Transposons with mobile genetic elements are semiparasitic DNA sequences which can replicate and spread through the host's genome, and thus can be introduced as discrete pieces of DNA using plasmid transfection protocols [73, 74]. Recently, the novel *piggyBac* (PB) and *Sleeping Beauty* (SB) transposon systems have emerged as promising approaches for *ex vivo* gene therapy [75]. PB transposons are host-factor independent and integrate stably into the target genome, which can be seamlessly removed by re-expression of PB transposase [74]. Therefore, transposons can be modified as a novel and viable vectors for the delivery of reprogramming factors into somatic cells, providing a safer alternative to viral methods [73]. In PB protocols, a PB transposon vector containing reprogramming factors is transfected into MEFs and then removed without trace using PB transposase to generate stable integration- and mutation-free iPSCs with equivalent efficiencies to retroviral transduction [76, 77]. Compared to the PB transposon system, SB transposon demonstrated several advantages, including higher transfer/integration efficiency and safety [78]. SB transposon containing OSKM can be cotransfected with hyperactive SB transposase to generate iPSC lines and then the integrations completely removed by transient transfection of excision SB transposase [79, 80]. The exogenous genes integrated by other methods such as retroviral or lentivial vectors can also be removed using a *Cre-loxP* system to decrease the risk of potential insertional mutagenesis [81–83]. Interestingly, SB transposons combined with the *Cre-loxP* system can also be utilized to create integration-free iPSC lines [78]. In this protocol, the OSKM cassette is flanked by compatible *loxP* recombination sites in a SB transposon vector and then excised using *Cre* recombinase after iPSC generation (Figure 2(c)). The reprogramming efficiency of these "excisable" methods was reported up to 1–3%. Despite their success in iPSC generation without a genetic footprint, the use of DNA based approaches unfortunately cannot eliminate the possibility of genomic integration of an exogenous sequence [84].

A more attractive approach allowed for iPSC generation using transfection of mRNAs coding for the reprogramming factors into human primary somatic cells [85]. Although this difficult and uneconomical approach is challenging in practical applications, its reprogramming efficiency is higher than that of conventional approaches and the authors have already made technical improvements [86]. The cDNA sequences of OSKM were amplified from individual vectors and then modified and switched into mRNA using an *in vitro* transcription kit. Finally, the synthesized mRNA can be directly transfected to generate transgene-free iPSCs, typically yielding several hundred colonies.

2.2.2. DNA-Free Methods. The above studies support the theory that transient expression of the exogenous reprogramming factors is sufficient to induce pluripotency in somatic cells. They also do not require genomic integration and can be eliminated after transfection, giving them an independence that is considered an important criterion of high-quality fully reprogrammed iPSCs. Recently, other DNA-free methods with more convenient manipulations are in development to activate the endogenous pluripotent genes for reprogramming such as microRNAs, recombinant protein, and small molecules.

It is first required to understand the molecular changes underlying iPSC derivation for the development of alternative and safer strategies for reprogramming. Gene expression profiling studies in fibroblasts have revealed three phases

of reprogramming termed initiation, maturation, and stabilization [87]. During the early phase of reprogramming, exogenous OSKMs bind the enhancers and promoters of four different classes of genes. This increases proliferation of reprogrammable cells, changes histone modifications of somatic genes, initiates mesenchymal-to-epithelial transition (MET), and activates DNA repair and RNA processing. OSKMs thus act as a "pioneer" factor for remodeling the epigenome before cells enter their maturation phase. During maturation phase, stochastic activation of pluripotency markers occurs in addition to transient activation of developmental regulators and glycolysis. In the last phase, the cells eventually stabilize into the pluripotent state with silenced exogenous transgenes, remodeled ESC-like cytoskeleton, reset epigenome, and activated core pluripotency circuitry. The early epigenetic priming events in the process may be critical for pluripotency induction [88, 89]. Thus, substitutions for OSKM can achieve the reprogramming of somatic cells by activating the epigenetic remodeling of pluripotency and MET genes.

2.2.3. Recombinant Protein Delivery. Recombinant protein delivery represents a straightforward reprogramming strategy. The first protein-induced pluripotent stem cells (piPSCs) generated using these methods were reported by two independent research groups [90, 91]. Unfortunately, the poor ability of macromolecules such as protein to cross the cellular membrane often hinders their intracellular delivery. Several groups have attempted to design a protein transduction domain to assist transmembrane transport of the four reprogramming factors (OSKM) [92, 93]. After repeated transfer of these recombinant proteins, results of only three iPSC colonies per 5×10^4 treated cells were achieved [90]. This is an efficiency of iPSC generation of about 0.001% of input cells, which was significantly lower than that of virus-based protocols [91]. Moreover, a single transfer of ESC-derived extract proteins into adult somatic cells achieved full reprogramming up to the pluripotent state, avoiding repeated transfer or prolonged exposure to materials [94]. Mechanistically, the cell-permeant Oct4 and Sox2 proteins were shown to specifically bind to the promoter of pluripotent gene, modulating the transcriptional machinery while maintaining pluripotency [95, 96]. Due to the extremely low efficiency and delayed reprogramming, it is essential to develop a robust and efficient protocol to generate recombinant reprogramming proteins modified for high stability and cell permeability.

2.2.4. Small Molecules. In order to improve the efficiency of nonintegrating reprogramming approaches, several screens for chemical compounds have been performed. A number of small molecules have been identified since, that modulate the induction of reprogramming while significantly improving the efficiency and quality of iPSC generation and functionally replacing exogenous OSKM. A single chemical compound is not able to entirely replace the function of a reprogramming factor in current studies but it can work with other modulators. Named on the basis of their reprogramming mechanisms, they can be classified into epigenetic modifiers, signaling pathway modulators, cell senescence alleviators, and metabolism regulators [84].

Epigenetic modifiers are used most commonly in various iPSC generation protocols because the epigenetic changes (e.g., DNA demethylation and histone acetylation) of pluripotency genes are the key feature of reprogramming. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, was initially reported to effectively reprogram primary human fibroblasts without introduction of the oncogene c-Myc [97, 98]. The addition of VPA can also improve the reprogramming efficiency of viral OSKM transduction [99, 100] or recombinant protein delivery [90] directly regulating Oct4 promoter activity to ensure the acquirement and maintenance of pluripotency [101]. Moreover, other HDAC inhibitors and combinations of inhibitors were found to significantly improve the overall efficiency of the reprogramming process in the context of TFs including trichostatin A [98], suberoylanilide hydroxamic acid [102] and sodium butyrate [103, 104], DNA methyltransferase inhibitors (5-azacytidine [98, 105] and RG108 [106–108]), and histone methyltransferase inhibitors (BIX01294 [106, 107, 109]).

In addition to epigenome remodeling, signal transduction pathways mediated by extrinsic factors and intrinsic transcriptional networks cooperate to reprogram somatic cells into iPSCs [87]. Several small molecules have been identified and employed to generate iPSCs by targeting the signaling pathways relevant to reprogramming. For example, CHIR99021 was reported to activate wingless/INT (Wnt) signaling, which is important in the maintaining and the pluripotency and self-renewal of adult stem cells [110] and significantly improved the reprogramming efficiency in MEFs [111, 112]. The inhibition of epithelial phenotype in MET mechanisms significantly enhanced reprogramming of human somatic cells [113–115]. Molecules affecting other signaling pathways can also facilitate iPSC reprogramming, such as cAMP analog and inhibitors of Src family kinases [116, 117].

Organismal aging or senescence was identified as another one of the barriers for iPSC reprogramming, resulting in slow kinetics and low efficiency of processes [118, 119]. Telomere shortening in cell senescence led to a dramatic decrease in reprogramming efficiency, which was restored by telomerase reintroduction [120]. Longevity-promoting (anti-aging) compounds were also reported to promote somatic cell reprogramming [121]. In one example study, vitamin C, an antioxidant agent, was shown to enhance mouse iPSC generation by alleviating cell senescence [122].

iPSC generation requires energy transition from mitochondrial oxidative phosphorylation to perform glycolytic metabolism [123, 124]. As a result, several metabolism regulators have been identified to upregulate expression of several key glycolytic genes and facilitate iPSC generation. Reprogramming efficiency was also significantly increased by other small molecules, as summarized in other articles [84, 125, 126].

Remarkably, after the screening of nearly 10,000 small molecules, seven molecule compounds (VPA, CHIR99021, Tranylcypromine, 616452, FSK, and DZNep) were found,

reprogramming mouse fibroblasts into iPSCs solely, obtaining dozens of iPSC colonies per 50,000 cells [127]. Although the reprogramming efficiency is lower than that of convention viral approaches, it provides an ideal chemical protocol which is more flexible and convenient than the biological protocols dependent on TFs. The usage of a cocktail of chemical compounds eliminates many drawbacks (e.g., tumorigenesis) of the other integration methods. Importantly, the chemical iPSCs can be easily manufactured under current good manufacture practices (cGMP) conditions, making it an economic, practical, and regulatory matter [128]. The chemical reprogramming approach also provides new insight into the minimal or alternate requirements for pluripotency induction.

2.2.5. MicroRNA Based Methods. In addition to pluripotent TFs, endogenous specific microRNAs (miRs) have been reported to be highly expressed in ESCs, termed ESC-specific miRNAs [129, 130]. They have been demonstrated to play a critical role in the control of pluripotency-related genes mediating self-renewal, differentiation, and dedifferentiation [131, 132]. miRs are small evolutionary conserved noncoding RNAs and usually act as endogenous repressors controlling gene posttranscription, either by inducing mRNA degradation or by blocking translation [133, 134]. Each miR can target and suppress hundreds of mRNAs, and one gene can be targeted by various miRs, dramatically changing the expression profile and identity of cell phenotype. Several studies have demonstrated that regulation networks are very important in governing cell differentiation, development, and disease [135, 136]. And as a result, researchers are attempting to revert somatic cells back to an ESC-like state using ectopic introduction of ESC-specific miRs. These in turn can be used to generate viral free iPSCs in the absence of any exogenous reprogramming TFs.

The transfection of miR-302-family, for instance, was reported to reprogram several human cancer cell lines into an ESC-like pluripotent state, with similar gene expression patterns and differentiation functions [137]. Several miRNA mimics have also been found to have similar effects. For instance, miR-291, miR-294, and miR-295 can increase the efficiency of reprogramming by OSK, without the requirement of c-Myc induction, and were identified as the downstream effecters of c-Myc and can substitute for its reprogramming effect [138]. Two independent *Cell Stem Cell* reports showed that human and mouse somatic cells can be reprogrammed to produce iPSCs by expressing microRNAs, completely eliminating the need for ectopic gene expression [139]. miR-based reprogramming approach was two orders of magnitude more efficient than the conventional OSKM methods [140]. Importantly, miR-200 family was showed as the unique mediators of the endogenous reprogramming factors Oct4/Sox2 [141]. Intriguingly, Sox2 directly regulated endogenous miR-29b expression which was required for OSKM- and OSK-mediated reprogramming [142]. Mechanistically, the ESC-specific miRs (such as miR-302 family) enhanced reprogramming of somatic cells by repressing multiple target genes [143, 144]. A new miRNA family (miR-130/301/721) was reported as an important regulator of iPSC induction by targeting transcription factor [145]. Several microRNAs, including miR-93 and miR-106b, have very similar seed sequence regions greatly enhancing iPSC induction by modulating identical target genes in the initiation stage of reprogramming [146]. Their target genes regulate various complicated cellular processes, including cell cycle, MET, epigenetic regulation, and vesicular transport, which are involved in the mechanisms of cell reprogramming [143, 147]. These genes could be the barrier of somatic cell reprogramming, which can be alleviated by the addition of miRs. At the last step, the core endogenous pluripotency networks were activated in iPSC colonies, similar to TF-mediated reprogramming.

The inhibition of microRNAs enriched in somatic cells may also facilitate the reprogramming procedure. For example, loss of miR-145 impaired differentiation and elevated its direct target genes, including Oct4, Sox2, and Klf4, but its increasing expression inhibited ESC self-renewal, repressed pluripotent genes, and induced lineage-restricted differentiation [148]. Another study showed that depletion of miR-21 and miR-29a enhanced reprogramming efficiency in MEFs by regulating p53 and ERK1/2 pathways [149]. As a p53 target, the miR-34 family can repress expression of pluripotent genes and its genetic ablation promoted iPSC generation without compromising self-renewal or differentiation [150]. Sirtuin1 was also reported to facilitate iPSC generation from mouse MEF through the inhibition of the miR-34a and p53 pathways [151]. A recent study showed that the let-7 family acts as an inhibitory influence on reprogramming through a prodifferentiation pathway (including EGR1) and the inhibition of let-7 in human cells promotes reprogramming due to an increasing level of its target LIN-41/TRIM71, which is important for overcoming the let-7 barrier to reprogramming [152]. Thus, the microRNA based strategies, including the increase of ESC-enriched miRs and the decrease of ESC-depleted miRs, contribute to the mechanism studies of cell reprogramming. Furthermore, the miR mimic or inhibitor induction approach needs to be optimized, since the reprogramming efficiencies dependent on various miR families may differ during the course of reprogramming and sequential delivery paradigms.

3. The Potential Application of Somatic Cell Programming Strategy

The somatic cell reprogramming and iPSC generation approach have only recently become a popular target for regenerative medicine research, but there has been tremendous enthusiasm in translational studies of iPSCs within every field of medicine. Specific differentiation into the 3 primary germ layers and all the cell types potentially provides many benefits for cell-based therapies, disease modeling, and drug development [153–155].

3.1. Differentiation Potentials for Cell Transplantation Therapy. Various cell delivery approaches have been developed to enhance stem/progenitor cell engraftment, survival, and integration to host tissues after MI and have been performed in multiple clinical trials of cardiac regenerative therapy, as summarized in our review [156]. However,

the most urgent task is to identify ideal seed cells with the highest differentiation potentials for the repair of injured heart. Most current clinical trial studies on MI are using endogenous stem/progenitor cells, including cardiac progenitor cells (CPCs), bone marrow mononuclear cells (BMMC), endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), and skeletal myoblasts (SMBs) due to the safety concerns [157, 158]. Resident CPCs (or cardiac stem cells, CSCs) have demonstrated positive therapeutic effects using cardiac differentiation, while other circulating progenitor cells have had mixed results with limited cardiac potential in clinical trials [159–161]. Unfortunately, the ability of CPCs to acquire a cardiomyogenic phenotype is subject to temporal limitations and display limited potential for self-renewal and differentiation [154, 162]. The therapeutic benefits of CPCs resulted mainly from release of paracrine factors and the isolation and identification of various populations still needs to be investigated [158, 163]. These limitations do not apply to ESCs or iPSCs which can be propagated indefinitely. Most laboratories can expand various iPSCs robustly using commercially available products, including feeder and feeder-free protocols [164, 165]. They are also able to generate large numbers of unambiguous cardiomyocytes with defined induction protocols. Although there may be some epigenetic differences between iPSCs and ESCs, they are remarkably similar and their derived cardiomyocytes have a very similar phenotype [166, 167]. Since the use of ESCs is limited due to ethical considerations, iPSCs are emerging as an ideal potential supply of human cardiomyocytes and offer a viable option for an autologous cell source for cardiac regenerative therapy.

3.1.1. Cardiovascular Differentiations. Patient specific or autologous iPSC-derived cells will play an increasingly important role for replacing lost or damaged tissues [168]. The correction approaches to autologous mutant iPSCs can be employed for repairing single-gene disorders, such as Duchenne muscular dystrophy and antitrypsin deficiency [169, 170]. Cardiovascular cells derived from iPSCs provide a promising seed cell resource for myocardial tissue engineering and have emerged as a novel therapeutic paradigm for MI treatment.

There are 3 major approaches for differentiation of human iPSCs to cardiomyocytes including embryoid body (EB) [167, 171, 172], monolayer culture [173], and inductive coculture [174]. These *in vitro* differentiation approaches were established mainly according to the sequential stages of embryonic cardiac development and modified by various signaling factors, such as bone morphogenetic proteins (BMPs), Wnts, and fibroblast growth factors (FGFs) [175–177]. The timing and relative expression of various growth factor combinations can induce the development of a cardiogenic mesoderm. Multiple complex interactions between these conserved signaling pathways control the initial differentiation, proliferation, and maturation of myocardium [178].

The aptitude of iPSCs for *de novo* cardiac differentiation has been demonstrated, as well their potential for MI therapy. Despite *in vitro* protocols recapitulating the cardiomyogenic phenotype after the discovery of iPSCs, the first *in vivo* study in MI treatment was reported in 2009 [179]. The intramyocardial delivery of iPSC colonies was first demonstrated to rescue postischemic myocardial structure and function, achieving in situ regeneration of cardiac, smooth muscle, and endothelial tissue [179]. Human iPSCs can also be established from dermal fibroblasts of patients with advanced heart failure and differentiated into functional cardiomyocytes and *in vivo* transplantation studies in the rat heart revealed structural integration with host cardiac tissue [180]. Because of the huge demands from basic research and clinical applications, there is great demand for large numbers of functional cardiovascular cells derived from iPSCs or ESCs. To this end, the iPSC- or ESC-derived contracting EBs which contained high percentages (up to 95%) of pure functional cardiomyocytes were generated by using an optimal culture protocol [181]. An efficient protocol using glycogen synthase kinase 3 inhibitor and other molecules was also reported to produce a high yield (0.8–1.3 million cells/cm^2) of pure (80–98%) functional cardiomyocytes from multiple cell lines without cell sorting or selection [182, 183].

The cardiomyocytes derived from iPSC or ESCs have been shown to improve cardiac functions after transplantation, but the functional integration of grafts into injured heart tissue has not been demonstrated. The transplantation of ESC-derived cardiomyocytes in uninjured heart had consistent 1 : 1 host-graft coupling, while the grafts in injured hearts were more heterogeneous and typically included both coupled and uncoupled regions [184]. Thus, the functional integration of implanted cardiomyocytes needs to be improved by optimized culture and induction systems which include morphological integration and electromechanical coupling. Intriguingly, the coculture of MSCs or MEFs was showed to improve electrical integration of iPSC-derived cardiomyocytes with ventricular tissues, indicating that soluble factors were involved in electrical coupling [185].

In addition to cardiomyocytes, other cardiovascular cell lineages, such as endothelial cells (ECs) and smooth muscle cells, play an important role in the functional restoration of MI [186, 187]. After injection into ischemic rat heart, the human iPSC-derived cardiac progenitor cells engrafted, differentiated into cardiomyocytes, endothelium and smooth muscle, and persisted for at least 10 weeks after infarction [188]. Compared to BMMC and HUVECs, the functional ECs generated from ESCs or iPSCs demonstrated better therapeutic efficacy for attenuation of ischemic tissues [189]. The purified CD31 and CD146 positive vascular progenitors from cord blood-derived iPSC were reported to home and engraft into ischemic tissues, with incorporation into damaged vessels [190]. Importantly, the refinement of EC differentiation methods enriched for subtype-specific iPSC-ECs, including arterial, venous, and lymphatic subtypes, with functional benefits of enhancing neovascularization [191].

It has become clear that cell therapies combined with tissue engineering techniques (such as myocardial cell sheets) can increase stem cell survival and retention, thereby enhancing therapeutic effects [192]. A cardiac tissue sheet reassembled with defined cardiovascular populations (including purified hiPSC-derived cardiomyocytes and

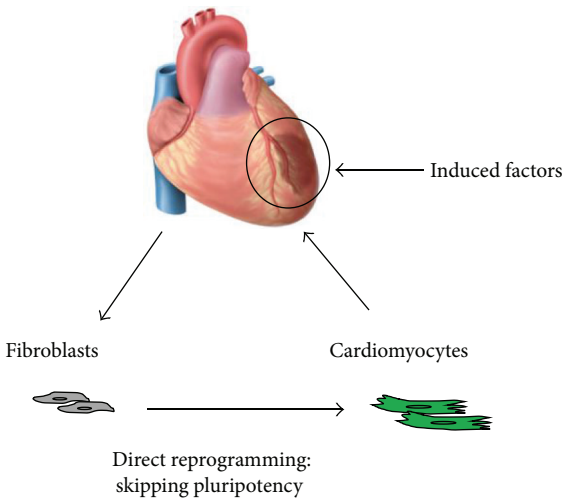

Figure 3: The model of direct reprogramming of fibroblasts into cardiomyocytes in infarct heart. The transient overexpression of cardiac specific TFs, GMT, is able to generate cardiac lineage-specific cells directly from somatic cells. This process "skipping pluripotency" does not require transition through a pluripotent intermediate, immensely eliminating the risk of tumorigenicity.

ECs) was implanted in an animal MI model which showed significant and sustained improvement of cardiac function accompanied by neovascularization [193, 194]. In our studies, a tricell omentum patch (seeded with iPSC-derived cardiomyocytes, ECs, and MEFs) was created and implanted into mice resulting in significantly higher cell engraftment accompanied by angiomyogenesis in the infarcted area and improvement in heart function [195, 196]. The combination of cell sheet and the omentum flap has emerged as a promising iPSC technique for the development of tissue-engineered vascular-rich new myocardium *in vivo* [197]. Notably, the long-term persistence of transplanted iPSC-derived cardiac cells demonstrated an electrical integration into host tissue with the presence of electrophysiological maturation and improved quality, supporting their potential for myocardial replacement therapy [198].

3.1.2. Direct Reprogramming for Cardiovascular Regeneration. Transdifferentiation is a new paradigm that has been devised to generate cardiovascular lineage-specific precursor cells directly from somatic cells (Figure 3), by combining transient overexpression of the cardiac specific TFs. At first, the transfections of specific TFs (Gata4, Mef2c, and Tbx5) transdifferentiated mouse postnatal cardiac or skin fibroblasts directly into cardiomyocyte-like cells, but with a very low efficiency [199]. Alternative *in vitro* methods demonstrated temporal overexpression of OSKM with the addition of signaling molecules that directly reprogrammed mouse fibroblasts into spontaneously contracting patches of cardiomyocytes over a period of 11-12 days, during which no pluripotent intermediates arose [200].

Direct myocardial reprogramming continues to be explored under *in vivo* circumstances using ischemic heart models. For instance, the injection of GMT retrovirus into mouse infarcted hearts induced infected cells to express cardiac-specific genes [201]. Furthermore, genetic lineage tracing showed that resident nonmyocytes can be reprogrammed into cardiomyocyte-like cells by the retroviral delivery of GMT in mouse infarcted heart [202, 203]. Their induced cardiomyocytes presented binucleate and assembled sarcomeres, as well as cardiomyocyte-like gene expression, and electrical coupling. They also found that the delivery of the proangiogenic and fibroblast-activating peptide thymosin beta4 along with GMT resulted in further improvements of cardiac function. Alternatively, four transcription factors, Gata4, Hand2, Mef2C, and Tbx5, can cooperatively reprogram adult mouse tail-tip and cardiac fibroblasts into cardiac-like myocytes *in vitro* and their overexpression improved cardiac function and reduced adverse ventricular remodelling following MI by *in vivo* direct reprogramming into functional cardiac-like myocytes [204].

However, the GMT overexpression in murine tail-tip fibroblasts and cardiac fibroblasts is an inefficient method to induce direct myocardial reprogramming, and lacks the molecular and electrophysiological phenotypes of mature cardiomyocytes [205]. The authors also found that transplantation of GMT infected cardiac fibroblasts into injured mouse hearts resulted in decreased cell survival with minimal induction of cardiomyocyte genes. Thus, there are significant challenges in the direct reprogramming of fibroblasts into cardiomyocyte-like cells and an increase in the translational potential of this strategy is required.

Interestingly, pretreatment of VEGF to infarcted myocardium was reported to enhance the efficacy of the GMT-mediated reprogramming strategy, improving myocardial function and reducing the extent of myocardial fibrosis [206]. Since the initial GMT approach was not sufficient for cardiac induction in human cardiac fibroblasts, a study screening for additional factors found that the transduction of Mesp1 and Myocd enhanced the direct reprogramming efficiency [207]. This strategy used a discrete set of factors but had critical epigenetic blocks in the process of *in vitro* cardiac reprogramming, which were attenuated in their native environment *in vivo* [199, 202, 204]. This phenomenon suggests the important role of epigenetic mechanisms in reprogramming process. DNA methylation and histone modifications are the major components of epigenetics in the regulation of gene expression [208]. The promoter regions of cardiac specific genes were comparatively demethylated in the induced cardiomyocytes [199]. With regards of the enrichment of histone modifications, trimethylated histone H3 of lysine 4 (H3K4me3, marks transcriptionally active chromatin) was increased on the promoter regions of cardiac specific genes, while trimethylated histone H3 of lysine 27 (H3K27me3, marks transcriptionally inactive chromatin) was significantly reduced upon reprogramming [199]. Therefore, the understanding of epigenetic mechanisms which were found to be involved in this process is important for enhancing the conversion efficiency [209, 210]. The inefficient generation of iPSCs reveals that the major epigenetic barriers in somatic cells cannot be easily overcome [89, 211]. Thus, it is required to continue the genome-wide epigenetic studies of cardiac

reprogramming and to develop alternative approaches (such as small molecules) targeting the stable epigenetic events. For example, epigenetically active small molecules were employed to change global histone modifications of human cardiac stromal cells, thereby driving them reprogrammed into functional cardiovascular precursors [212].

"Skipping pluripotency" is an alternative strategy for the production of functional endothelial cells directly induced from somatic cells. This reprogramming approach mediated by epigenetic mechanisms does not require transition through a pluripotent intermediate during the process of transdifferentiation, immensely eliminating the risk of tumorigenicity. This provides a new line of thought for the generation of lineage-specific cell types derived from somatic cells and may facilitate the potential applications of reprogramming in cardiac regenerative therapy. In one study, human fibroblasts were induced to generate partial-iPSC for 4 days by transfecting OSKM into the nuclei and clearly displayed the potential to differentiate into endothelial cells in response to defined media and culture conditions [213]. The human fibroblasts also can be transdifferentiated into functional endothelial cells by the lentiviral transduction of Oct4 and Klf4, in chemically defined medium with soluble factors such as BMP4, VEGF, and bFGF [214].

Alternatively, based on the potential of miRNAs regulating developmental and reprogramming processes, a combination of miRNAs was identified to induce direct reprogramming of fibroblasts to cardiomyocyte-like cells *in vitro* and *in vivo* [215]. The knowledge and the techniques gained from the development of iPSC reprogramming will be especially beneficial in designing new methods of transdifferentiation. The multipotent cardiovascular precursor cells derived from direct reprogramming of human somatic cells can be a versatile cell source for cell-based therapy in MI treatment. The low efficiency of direct reprogramming, however, will need to be improved.

3.2. Disease Modeling and Drug Screening. In addition to their regenerative capacity in cell therapy, iPSC-derived myocardium provides an important *in vitro* tool for modeling cardiac diseases, allowing us to study the molecular mechanisms involved in cardiac syndromes and test novel specific gene targets for drug discovery (Figure 4) [216, 217]. It is very convenient and feasible to generate iPSCs from somatic cells (such as skin fibroblasts and blood cells) of specific patients. The iPSCs and derivatives (all of adult cell types) containing specific genetic information of a patient can potentially contribute to development of personalized medicine for diagnostic testing and selecting appropriate therapies [218, 219].

3.2.1. Disease Modeling. The application of human iPSC-derived cell types can accurately recapitulate relevant human diseases from animal models. Stem cells are available in essentially unlimited quantities and exhibit a more stable phenotype in long-term culture, which is superior to primary cells. The human specific iPSCs also can also be differentiated into any human cell type and early efforts have focused on four main areas: neurons, hepatocytes, cardiomyocytes, and pancreatic beta islet cells, with widely known molecular and biochemical signals driving *in vivo* differentiation [220].

The first disease-specific iPSC line was generated from an 82-year-old woman diagnosed with a familial form of amyotrophic lateral sclerosis (ALS) and it was successfully differentiated into motor neurons which were destroyed in ALS [221]. Multiple iPSC lines were then generated from patients with a variety of genetic diseases with either Mendelian or complex inheritance, which can induce both normal and pathologic tissue formations *in vitro*, thereby enabling disease investigation and drug development [222]. The first time that the application of iPSCs modeling the specific pathology seen in a genetically inherited disease occurred when a child suffering from spinal muscular atrophy was used to produce motor neurons that showed selective deficits when compared to those derived from the child's unaffected mother [223].

Applications of iPSCs for modeling diseases *in vitro* are destined to expand to encompass numerous disease conditions, as discussed in other reviews [216, 217, 224].

In the study of cardiovascular diseases, human iPSC-derived cardiomyocytes can serve as a cardiac model to be used for diverse basic studies ranging from cellular electrophysiology to biochemistry [225]. For example, the iPSCs from patients with hypertrophic cardiomyopathy (who have a mutation in the PTPN11 gene) have been derived and *in vitro* differentiated cardiomyocytes presented a higher degree of sarcomeric organization and preferential localization of NFATC4 in the nucleus, the disease phenotype [226]. The patient-specific iPSCs from members of a family affected by long-QT syndrome type 1 (ventricular tachyarrhythmia due to mutations occur in the KCNQ1 gene), and the patient-derived "ventricular" and "atrial" cells, recapitulated markedly prolonged duration of action potential, the electrophysiological features of the disorder [227]. The development of an iPSC-derived long-QT syndrome type-2 disease model (which is due to the A614V missense mutation in the KCNH2 gene) was also reported to show significant prolongation of the action-potential duration [228]. Moreover, the cardiomyocytes derived from Timothy syndrome patient-specific iPSCs showed irregular contraction, excess Ca^{2+} influx, prolonged action potentials, irregular electrical activity, and abnormal calcium transients [229]. Finally, iPSC-derived myocardium can be used to reproduce the *in vitro* characteristics of familial hypertrophic cardiomyopathy [230, 231], right ventricular cardiomyopathy [232], and cardiac Na^{+} channel disease [233, 234]. These iPSC-derived cell models can provide a physiologically and functionally relevant human cellular context *in vitro* to reveal the pathogenic mechanism underlying the specific disease phenotype [235].

3.2.2. Drug Screening and Evaluation. In addition to the investigation of the mechanisms of human diseases, iPSC-derived cell models also play an important role in the evaluation of the potential therapeutic efficacy of drugs or small molecules. In this regard, access to abundant populations of human cardiomyocytes is of particular interest to the pharmaceutical industry as a tool to develop new cardioactive compounds for correcting cardiac disease phenotypes and for screening the potential cardiotoxicity of new compounds.

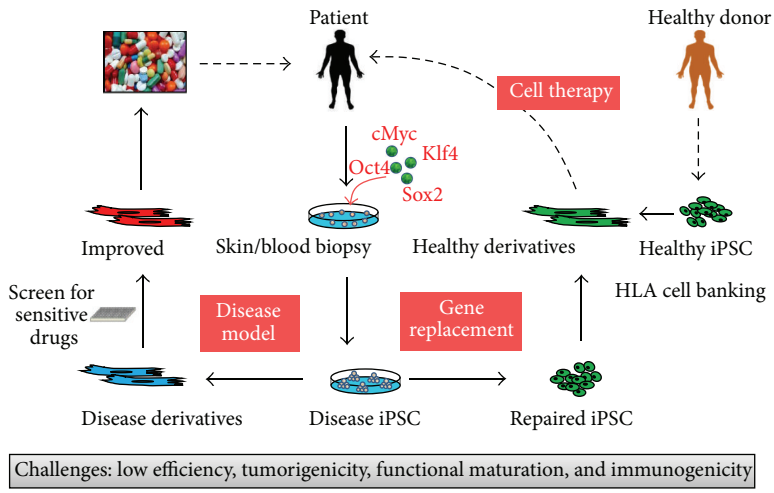

FIGURE 4: The potential applications and challenges of iPSCs in cardiac regenerative medicine. iPSCs are a cell therapy strategy for replacing lost or damaged cardiac tissues. They can be generated from patient specific somatic cells and are capable of generating large numbers of unambiguous cardiomyocytes using defined protocols. The major challenges in the current translational studies are concerns about the specificity of function maturation, low efficiency, immunogenicity, and safety of iPSCs. In addition to their applications in cell therapy, iPSC-derived cardiovascular cells provide an important *in vitro* tool for modeling cardiac diseases and drug screening. Clinical-grade HLA cell banking is one strategy being developed to minimize the risk of immunogenicity of iPSCs.

One such study employed the use of cardiomyocytes differentiated from patients with arrhythmogenic disorders. Patient-specific iPSCs were used as a guide for assessing drug therapeutic effects and tailoring medical treatment for patients [236]. This cardiac-tissue model was used to evaluate the potency of existing and novel pharmacological agents on cardiomyocytes derived from patients with long-QT syndrome and the disease phenotype was found to be impacted by the treatment with various β-adrenergic receptor blockers [227]. Dantrolene, for example, was identified to restore abnormal Ca^{2+} spark properties and rescued the arrhythmogenic phenotype of an iPSC-derived model of catecholaminergic polymorphic ventricular tachycardia [237].

Finally, iPSC can be used for the identification of novel target genes that play a potential role in the development of therapies for myocardial diseases. When molecular mechanisms of relevant diseases are recapitulated, the identification of gene candidates could be used for target-agnostic drug screening. RNA interference (RNAi) technology has been applied on a genome-wide scale for several biological processes to identify the potential new targets effectively [238]. Since gene therapy techniques are complicated and controversial, conventional chemical compounds are preferable in the discovery of new drugs by targeting the diseased genes. The combination of patient-derived iPSC disease models with RNAi technologies presents a great opportunity to not only identify disease-relevant biological signatures but to also discover novel drug targets [224]. With the expression profiling of healthy and diseased cells, systematic RNAi knockdown of gene candidates can identify potential therapeutic targets, if the specific gene knockdown can correct a disease phenotype. By screening the compound libraries based on the target genes, compounds could be used that mimic the effect of gene knockdown and then be tested for efficacy in the relevant iPSC disease models as new drugs [224].

Adverse reactions and drug toxicity represent major challenges for pharmaceutical industries, contributing to the high cost of drug development. Thus, it is important to develop the predictive human cellular systems for the complementation of current toxicity tests to establish routine screening for toxicity pathways [239]. Given that the human ESC-derived cell types are most susceptible to drug toxicity, the human iPSCs also can be used in the drug toxicity assays offering a great potential for assessing adverse drug effects [240]. Before the establishment of cell-based toxicity assays that accurately reflect mature tissue phenotypes as discussed above, it would be necessary to improve the hiPSC generation and differentiation protocols significantly [239]. Cardiomyocytes differentiated from human iPSC long-QT syndrome models have actually been used to reflect the tendency of drugs to exacerbate cardiac conduction defects, and thus potentially could be used to screen compounds for cardiotoxicity [241]. Disease-specific hiPSC-cardiomyocytes demonstrated increased susceptibility to known cardiotoxic drugs as measured by action potential duration and quantification of drug-induced arrhythmias, which recapitulated drug-induced cardiotoxicity profiles at the single cell level [242]. And they found that use of disease-specific hiPSC-derived cardiomyocytes may predict adverse drug reactions more accurately than the standard gene test or healthy controls. Notably, some kinase inhibitors used in cancer therapy have been associated with toxicities to the heart and vasculature and their cardiotoxicity can be predicted by hiPSC-derived preclinical models [243]. Thus, the iPSCs are employed in personalized drug development to characterize the protective effects or optimal safety doses of therapeutic agents. The current screening models have low-to-moderate

throughput, and high throughput drug screening models need to be developed for practical use in the pharmaceutical industry [244]. This will require the cell types for reproducible disease modeling with adequate quantity, purity, and quality. The bioreactor process development, cell banking, and automation protocols must also be devised [244]. They will prove to be a powerful approach to reduce drug toxicity, to stratify patient response, and to make less failure in the late-stage clinical trial.

4. Challenges and Perspectives of iPSCs

Although the discovery of iPSCs is encouraging and has brought forward numerous successful studies, it is just beginning to be used in the preclinical phase of human trials and the potential is still emerging [245, 246]. It will take a long time and great effort to overcome the major challenges of iPSCs present in cardiac regenerative medicine prior to the development of any clinical applications (Figure 4).

4.1. Low Efficiency. Given the successes of iPSC transplantation experiments in animal models, clinical trials will be taking place in the near future [245, 246]. Most iPSCs in current studies are produced with integrating DNA vectors which may not get silenced efficiently or potentially disrupt endogenous genes after *in vivo* transplantation and increasing the risk of oncogenicity or mutagenesis. One favorable factor is that iPSCs can be generated by using integration-free approaches, particularly the combination of small molecules or microRNAs. However, the biggest challenge for the transgene-free approaches is increasing efficiency.

Current progress has contributed to an in-depth investigation of pluripotency mechanisms that are part of the processes of ESC or iPSC generation. Pluripotency reporter models such as Oct4 promoter and GFP transgene mouse [98, 127], large scale screening in other DNA, small molecule, or microRNA libraries are all contributing to the discovery of novel pluripotent regulators or specifiers with higher induction efficiency. Ongoing improvement and optimization of current culture systems are also beneficial for enhancing reprogramming efficiency and the maintenance of iPSC self-renewal.

4.2. Safety Issues. One major pitfall is the tendency for iPSCs to form teratomas and current differentiation protocols cannot completely eliminate residual undifferentiated cells. This problem can be improved by morphological selection, label or staining strategies using fluorescence activated cell sorters (FACS), and drug selection approaches [247–249]. For instance, after defined differentiation induction of iPSCs, our previous studies used FACS to isolated CD31 positive derived ECs and purified NCX1 positive derived cardiomyocytes by puromycin resistance selection [195, 196]. NKX2-5 positive cardiomyocytes from iPSCs were purified by FACS and survived upon transplantation into the infarcted mouse heart without formation of teratomas [250]. Stage-specific embryonic antigen (SSEA) positive cardiovascular progenitors were purified using magnetic beads and were capable of generating cardiomyocytes, smooth muscle, and endothelial cells without the development of teratomas [251]. A novel selection system was reported to purify iPSC-derived cardiomyocytes using nanoscale probes with FACS and the transplantation improved cardiac function without forming tumors [252]. Remarkably, a nongenetic purified method based on the marked glucose/lactate metabolism characteristic of cardiomyocytes, generated abundant cardiomyocytes of up to 99% purity that did not form tumors after transplantation [253].

Although the purity of these isolation protocols was reported up to ~99%, it is still difficult to remove the undifferentiated cells and the differentiated noncardiac cells completely to obtain entirely purified derived cardiovascular cells. Even if very few native iPSCs (included differentiated) are left over, the pluripotent residual and unwanted populations can proliferate after implantation *in vivo* and bring many unpredicted consequences including benign teratoma formation and malignant transformation [254]. "Skipping pluripotency" has emerged as an alternative strategy for production of functional cell types directly reprogrammed from somatic cells, significantly eliminating the risk of tumorigenicity.

It is advantageous for iPSCs or ESCs to be propagated indefinitely *in vitro*, because current models of cell therapy can require hundreds of millions to billions of cells for each patient [255]. However, prolonged expansion of iPSCs or ESCs has been found to produce genomic abnormalities including chromosomal aneuploidy, translocations, mega-scale duplications and deletions, and point mutations [256]. The generation of iPSC is analogous to the inner cell mass during embryogenesis, which is accompanied with naturally prevalent chromosome instability [257]. Thus, in response to DNA damage and replicative stress such as ultraviolet exposure, a minority of pluripotent stem cells can still proliferate with deficient DNA damage repair and cell-cycle arrest, leading to point mutations [258]. The accumulation of such stochastic genetic lesions can cause the loss of tumor suppressor genes or gain of proliferation genes, increasing the tumorigenic potential of iPSCs or ESCs during long periods of culture and propagation. These findings highlight the need for stringent enforcement of *in vitro* expansion conditions and maintenance standards for iPSC lines to ensure the normal chromosomal integrity prior to clinical application.

4.3. Immature Differentiation. Although some laboratories describe successful variable cell differentiation approaches, the conversion of pluripotent cells to terminally differentiated cells (such as cardiomyocytes) is initially inefficient and not readily transferable across various cell lines. It is also very difficult to find the derivatives of many mature cell types. These limitations must be overcome in order to obtain high quality generation of *in vitro* mature myocardium for basic research and clinical applications. The cardiomyocytes derived from iPSCs or direct reprogramming of somatic cells is immature and lacks the consistent characteristics of

adult mature ventricular cardiomyocytes, so they are called cardiomyocyte-like cells. These cells are reflective of very early human cardiac development, limiting their utility in the generation of *in vitro* models of mature myocardium. Immature differentiated cardiomyocytes lack the consistent properties of adult mature ventricular cardiomyocytes such as gene expression profile, morphology, and electrophysiological function [158, 259, 260]. For instance, the majority of hiPSC-derived cardiomyocytes lack the action potential notch characteristic which is central to the disease phenotype of inherited cardiac arrhythmia syndromes [261]. The diversity of various induced cardiomyocyte phenotypes is dependent on their derivation, age, and culture conditions. However, when the immature cardiomyocytes are maintained in culture for a prolonged period, they progressively develop a more mature phenotype without signs of differentiation and acquired functionality of both gene expression and electrophysiology [262].

Scientists continue to refine current protocols and improve cardiac differentiation and maturation of iPSCs, combining optimal devices, culture conditions, materials, and timing [178]. Small molecules and microRNAs were also reported to improve cardiac differentiation and maturation of iPSCs and ESCs [263, 264]. The treatment of ascorbic acid, a small molecule, was also reported to enhance cardiac differentiation and maturation of iPSCs simply, universally, and efficiently [265]. This was demonstrated by dramatically augmenting yield, better sarcomeric organization, and improved electrophysiological functions. Interestingly, biowires is a platform that combines three-dimensional cell cultivation with electrical stimulation to mature hiPSC-derived cardiac tissue, increasing myofibril ultrastructural organization, elevating conduction velocity, and improving both electrophysiological and Ca^{2+} handling properties [266]. Cell surface markers (such as Flt1 and Flt4) can be used to identify and isolate the cardiac cardiovascular progenitor population derived from iPSCs, and their transplantation has demonstrated a robust ability for engraftment and differentiation into morphologically and electrophysiologically mature adult cardiomyocytes [267].

4.4. Immunogenicity. Before any clinical applications are feasible, the translational studies of stem/progenitor cell based therapy must be able to ensure that the progenies are stable (no differentiation), functional (therapeutic recovery), and safe (no tumorigenicity). Grafts should not elicit severe immune responses of any sort that could threaten the survival of donor cells after transplantation. The probability of immunogenicity associated with the transplantation of stem/progenitor cells and their derivatives has not been clearly addressed and remains one of the greatest obstacles to clinical applications [268]. Initially, teratomas formed by autologous iPSCs can induce T-cell-dependent immune responses in syngeneic recipients, caused by the abnormal expression of multiple genes [269]. Transplantation of allogenic iPSC-derived cardiomyocytes was observed an acute rejection from receipts, while transplantation of syngeneic iPSCs and derived cardiomyocytes was also observed progressive immune cell infiltration, as well the *in vivo* differentiation of syngeneic iPSCs [270]. Although these reports are challenging against such an optimistic view of iPSC studies, many unanswered questions are still left, such as their various derivatives and generation approaches [271]. It is encouraging that immune responses and abnormal gene expression were not observed in various tissues derived from the integration-free iPSC after transplantation [272] and there has been evidence supporting increased T cell proliferation and immune response in syngeneic iPSC-derived specific cells after transplantation [273]. These conflicting conclusions from the studies may be attributable to the different iPSC lines they used. It also may be attributed with partial reprogramming and genetic instabilities in iPSCs that then elicit immune responses. Alternatively, gene integration in transcriptionally active sites could occasionally cause continuous leakage of transgenes or activation of neighbor genes that may be correlated to aberrant expression of an immunogenic protein [274]. It is necessary to carefully screen the gene expression profiles in each cell type for potential immunogenicity for future clinical applications as well as the karyotype information of each iPSC clone.

The iPSC-based autologous methods can avoid the issues of immunogenicity or immunological rejection, while the allogeneic iPSCs can induce severe immune reactions. Autologous iPSCs generation is often associated with high medical costs and longer hospital duration using the current methods as discussed above. The rapidly effective treatment of some disorders, such as acute MI and spinal cord injury, cannot be achieved within the necessary time frame and it is unrealistic to generate autologous iPSCs from the patient's biopsy during the ongoing surgery. Although the healthy autologous iPSCs can be collected and stored in "private cell banking" and serve as primary sources for future use, it will be problematic to achieve this on a large scale due to relatively inefficient reprogramming techniques and high costs [275, 276]. Therefore, it is important to take the allogeneic iPSCs into consideration for regenerative medicine and tissue engineering. Yamanaka and other groups are currently developing clinical-grade "public cell banking" of primary and differentiated iPSCs from health and disease donors based on their types of human leukocyte antigen (HLA) [277, 278]. One important purpose of their projects is to clarify the immunological impact of HLA matching for allogeneic iPSC transplantation. According to their estimate, an iPSC bank from the selected homozygous HLA-typed volunteers could cover up to 90% of their ethnic groups [271, 278]. Based on experience in bone marrow transplantation, matching the three major types of HLA loci (including A, B, and DR) between the recipient and donor is expected to eliminate immune rejection after transplantation. In an HLA-matched but minor antigen mismatched allogeneic transplantation setting, it must be careful to select less immunogenic iPSC clones and differentiated cells, and also to monitor and minimize the risk of immunogenicity by using various strategies. A short-term immunosuppressive approach is capable of inducing engraftment of transplanted ESCs and iPSCs by blockade of leucocyte co-stimulatory pathways [279].

5. Summary

Contributions from multiple studies have guided iPSC research from their discovery to theory and methods of generation, as well as future directions in clinical use. These studies indicate that a network of TFs plays a central role in the maintenance of pluripotency and self-renewal of pluripotent stem cells. The reprogramming of somatic cells back into the embryonic state with pluripotency was realized by overexpressing four pluripotent factors. Most laboratories can now expand various iPSCs robustly using commercially available products according to established protocols. There has since been tremendous enthusiasm in studies of iPSCs within almost every field of medicine. However, there are many limitations for retroviral reprogramming approaches. Many nonintegrating strategies are being developed for the reprogramming process, and the chemical reprogramming protocols can be successful with a combination of different compounds, providing new insight into the minimal or alternate requirements for pluripotent TFs. Studies on the mechanisms of pluripotency induction indicate that all the reprogramming factors are interchangeable. This is a very important consideration in that the manipulation and improvement of iPSC generation is feasible and flexible with an ultimate goal of activation of endogenous pluripotent networks. All of these approaches need to be optimized including gene delivery methods, growth conditions, culture timing, and target cell selection in order to increasing the reprogramming efficiency.

Compared to other stem/progenitor cells, iPSCs can be propagated indefinitely and are able to generate large numbers of functional cardiomyocytes with defined induction protocols. However, prolonged expansion and culture should be avoided due to the increasing risk of chromosomal abnormalities. Derived cardiovascular cells are emerging as an ideal potential option for an autologous cell source for cardiac regenerative therapy. Importantly, a reprogramming strategy that "skips pluripotency" has been developed for the production of functional cell types directly induced from somatic cells under defined conditions. It does not require transition through a pluripotent intermediate and virtually eliminates the risk of tumorigenicity by mediating epigenetic mechanisms. This approach of *de novo* cell generation provides a new direction for the potential applications of cardiac regenerative therapy.

In addition to their applications in cell therapy, iPSC-derived cardiovascular cells provide an important *in vitro* tool for modeling cardiac diseases for defining the molecular mechanisms involved in cardiac syndromes. The contained specific genetic information is essential for personalized medicine. They also play an important role in the potential therapeutic efficacy or cardiotoxicity evaluation of new drugs. The application studies of iPSCs are just at the beginning of use at the preclinical phase and their various challenges still need be overcome before extensive clinical usage can occur. Ultimately iPSCs offer a safe and efficient approach for controlling cell fate toward specific cell types and will have a profound effect on bringing iPSC technology closer to clinical application.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by NIH Grants, HL089824, HL107957, and HL110740 (Y. Wang).

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