Derivation of Human Induced Pluripotent Stem Cells for Cardiovascular Disease Modeling

Kamileh Narsinh,* Kazim H. Narsinh,* Joseph C. Wu

Abstract: The successful derivation of human induced pluripotent stem cells (hiPSCs) by dedifferentiation of somatic cells offers significant potential to overcome obstacles in the field of cardiovascular disease. hiPSC derivatives offer incredible potential for new disease models and regenerative medicine therapies. However, many questions remain regarding the optimal starting materials and methods to enable safe, efficient derivation of hiPSCs suitable for clinical applications. Initial reprogramming experiments were carried out using lentiviral or retroviral gene delivery methods. More recently, various nonviral methods that avoid permanent and random transgene insertion have emerged as alternatives. These include transient DNA transfection using plasmids or minicircles, protein transduction, or RNA transfection. In addition, several small molecules have been found to significantly augment hiPSC derivation efficiency, allowing the use of a fewer number of genes during pluripotency induction. We review these various methods for the derivation of hiPSCs, focusing on their ultimate clinical applicability, with an emphasis on their potential for use as cardiovascular therapies and disease-modeling platforms. (Circ Res. 2011;108:1146-1156.)

Key Words: induced pluripotent stem cells ■ somatic donor cells ■ derivation technique ■ cardiovascular disease ■ pluripotent stem cell-derived cardiomyocytes

Human embryonic stem cells (hESCs) initially generated much enthusiasm because of their self-renewing and pluripotent properties.1 hESCs theoretically can generate an unlimited number of any somatic cell, given the proper culture conditions. Directed differentiation can be promoted by varying the concentrations of various growth factors.2 Recently, the Food and Drug Administration approved 2 hESC-based clinical trials for treatment of acute spinal cord injury (Geron; http://www.geron.com) and Stargardt macular dystrophy (Advanced Cell Technology; http://www.advancedcell.com). Although hESCs have been established as a renewable source of definitive cardiac tissue cells, no clinical cardiovascular application of hESCs has yet been realized. hESC-derived cardiomyocytes (hESC-CMs) can be exploited as a human in vitro modeling system, in which the study of cardiogenesis, myocardial-related pathology, drug targets, drug screening, and tissue engineering can be easily conducted,3 paving the way for their future application in vivo as bona fide transplantation cells. Of note, the hESC-CMs derived to date are more akin to fetal/neonatal rather than adult cardiomyocytes,4,5 and enhancing their in vitro maturation using electric and mechanical cues is an ongoing area of investigation.6 Despite this, the advantages of using the human-based modeling system provided by hESCs remain appealing, given that nonhuman transgenic models may not accurately reflect all aspects of the human disease phenotype. However, in the United States, hESC research funding can be subject to the vagaries of the courts and Congress, as evidenced by the recently lifted ban on federal funding for hESC research.7 In addition, the issues of potential immunologic rejection8,9 and tumorigenicity10 continue to be challenges to clinical cell transplantation approaches. The recent discovery of human induced pluripotent stem cells (hiPSCs) has mitigated some of these concerns because hiPSCs can be generated autologously and do not require the destruction of ex utero embryos. hiPSCs are comparable to hESCs in morphology, feeder dependency, surface markers, gene expression, promoter methylation status, telomerase activities, in vitro differentiation potential, and in vivo teratoma-forming capacity.11 Hence, hiPSCs have great potential to replace hESCs as disease models or prospective treatment options. Although the results of these initial comparisons are promising, the functional equivalence and safety of hESCs and hiPSCs remain contested.12 For instance, hESCs display chromosomal instability with extended in vitro culture,13 and hiPSCs undergo dynamic changes in copy number during reprogramming and culture.14 Also, reactivation of repro-
programming transgenes in iPSCs may enable malignant transformation of the cells after transplantation.\textsuperscript{15} Most recently, variations in gene expression profiles between different hESC and hiPSC lines have been investigated, with evidence indicating that cell line–specific differences in epigenetic landscape underlie differences in differentiation propensity.\textsuperscript{12,16–21} In light of these variations in performance, it is becoming clear that choice of reprogramming technique plays a crucial role in the quality and ultimate utility of the resulting hiPSC lines.

Since the ability to directly reprogram somatic cells to pluripotency has been well established in adult mouse fibroblasts,\textsuperscript{22–27} attempts to generate hiPSCs have become more pronounced, and the logistics involved in appropriately generating and using hiPSCs is of significant current interest. Several techniques have evolved for derivation of hiPSCs suitable for cellular regenerative medicine and disease modeling. This review discusses the methods, benefits, and drawbacks of several techniques, with an emphasis on their potential for future cardiovascular clinical applications.

### Integrating Methods

Efforts to reprogram human somatic differentiated cell types to a state that resembles hESCs began with the pioneering work of Takahashi and Yamanaka.\textsuperscript{11} Their methods included retroviral integration of 4 vital reprogramming factors—\textit{OCT3/4}, \textit{SOX2}, \textit{KLF4}, and \textit{c-MYC}—into adult human dermal fibroblasts. These 4 transcription factors would later become known as the “Yamanaka factors,” and their roles in reprogramming are now known to be significant\textsuperscript{28} but not collectively necessary.\textsuperscript{29–36} Often the omission of 1 or more of these reprogramming genes was contingent on the endogenous network of the donor cell type. For example, one study found that hiPSC derivation from keratinocytes required only 10 days, whereas neonatal skin fibroblasts required \textasciitilde30 days.\textsuperscript{37} It was postulated that perhaps the keratinocytes’ higher endogenous expression levels of \textit{c-MYC} and \textit{KLF4} predispose them to quicker reprogramming.\textsuperscript{38} Starting cell type is thus an important consideration in the derivation process and a topic that is more thoroughly discussed elsewhere.\textsuperscript{29} Two other transcription factors, namely \textit{NANOG} and \textit{LIN28}, were initially shown to be able to substitute for \textit{c-MYC} and \textit{KLF4}, although a number of other different factor combinations have been subsequently demonstrated.\textsuperscript{26,29,36,40,41} In any event, several cocktails comprising any number of these 6 reprogramming factors, and in some cases, additional supplements such as small molecules and enzymes, have been shown to be capable of reprogramming cells to pluripotency. Some of the advances made in the generation of hiPSCs using such integrating methods are detailed in Table 1.

A chief aim of clinical hiPSC researchers is to achieve a high efficiency of derivation of hiPSCs, because current yields of bona fide hiPSCs can be as low as 0.001\% to 0.1\% of the starting cell population.\textsuperscript{42} Even in secondary reprogramming systems, using transgenic fibroblasts expressing all 4 transgenes simultaneously, the efficiency of pluripotency induction remains low, at 1\% to 5\%.\textsuperscript{37,43} Two mutually nonexclusive models have been proposed to explain the apparent resistance to pluripotency induction, termed the “elite” and “stochastic” models.\textsuperscript{44} The elite model proposes that only a small percentage of somatic cells, presumably resident tissue progenitor cells, are amenable to reprogramming. In support of this notion is evidence that hematopoietic stem cells undergo more efficient reprogramming than their differentiated progeny.\textsuperscript{45} However, reports of successful reprogramming of terminally differentiated cells such B-lymphocytes\textsuperscript{46} and pancreatic \textbeta-islets\textsuperscript{47} favor a stochastic model of reprogramming, in which successive cell divisions allow rare cells to acquire the stochastic changes necessary for conversion to full pluripotency.\textsuperscript{48} Perhaps these seemingly contradictory hypotheses can be reconciled by a model in which adult stem/progenitor cells require fewer stochastic changes to undergo reprogramming than more differentiated cells. Further investigation of the reprogramming process using single-cell resolution imaging and other techniques will undoubtedly help yield further insight into these reprogramming roadblocks.

Clearly, the choice of gene delivery vector can change reprogramming efficiency by directly affecting the degree of expression of the reprogramming genes. Retroviral/lentiviral infection provides the benefit of high transgene expression levels in primary cells as compared with nonviral methods of reprogramming. However, retroviral/lentiviral methods for hiPSC generation have come under scrutiny because of concerns regarding their ultimate clinical safety. In particular, the random integration of transgenes into the human genome can potentially cause insertion mutagenesis, leading to malignant transformation of a clonal cell population and disastrous consequences.\textsuperscript{49} Second, leaky expression caused by ineffective silencing of the transgenes may interfere with the physiological expression of the factors endogenously present within the cell, thereby potentially restricting differentiation propensity.\textsuperscript{50} This residual expression may hamper the validity of in vitro hiPSC uses, such as in disease modeling, drug screening, and toxicology tests. Third, reactivation of \textit{OCT4} and \textit{c-MYC} has been shown to promote tumor formation in chimeric mice,\textsuperscript{26,51,52} prompting legitimate concern over posttransplantation tumorigenic risk if such methods were used in human patients.
In one of the initial forays into generating safer hiPSCs, Maherali et al.37 created a doxycycline-inducible lentiviral system, attempting to maintain the silencing of transcription factors and thus reduce the tumorigenicity of the cells after differentiation. Although this system is a step toward safer hiPSCs, the leakiness of the doxycycline-inducible promoter and the permanent incorporation of oncogenes into the host genome still warrant concern.

Viral Integration Followed by Excision: Cre-loxP
Soldner et al.53 generated viable hiPSCs free of exogenous reprogramming factors using doxycycline-inducible lentiviral vectors that integrated into the host genome but were subsequently excised by Cre recombinase. Fibroblasts were obtained via skin biopsies from 5 patients exhibiting sporadic Parkinson disease and transduced using 3 or all of the Yamanaka factors. The reported reprogramming efficiency was 0.005% after transduction with the 3-factor combination and 0.01% with the 4-factor combination. Furthermore, the 3-factor transduced cells required 12 days of doxycycline exposure, as opposed to the 4-factor cells, which required only 8 days. Despite its lower reprogramming efficiency and temporal requirements, the 3-factor combination may be preferred over the 4-factor combination because the transduced cells are not overgrown by granulate colonies.11,31

Southern blot analysis demonstrated successful excision of the transgenes, and the resulting hiPSC lines maintained pluripotency independent of residual exogenous transcription factor expression.

In one of the initial forays into generating safer hiPSCs, Maherali et al.37 created a doxycycline-inducible lentiviral system, attempting to maintain the silencing of transcription factors and thus reduce the tumorigenicity of the cells after differentiation. Although this system is a step toward safer hiPSCs, the leakiness of the doxycycline-inducible promoter and the permanent incorporation of oncogenes into the host genome still warrant concern.

Viral Integration Followed by Excision: Cre-loxP
Soldner et al.53 generated viable hiPSCs free of exogenous reprogramming factors using doxycycline-inducible lentiviral vectors that integrated into the host genome but were subsequently excised by Cre recombinase. Fibroblasts were obtained via skin biopsies from 5 patients exhibiting sporadic Parkinson disease and transduced using 3 or all of the Yamanaka factors. The reported reprogramming efficiency was 0.005% after transduction with the 3-factor combination and 0.01% with the 4-factor combination. Furthermore, the 3-factor transduced cells required 12 days of doxycycline exposure, as opposed to the 4-factor cells, which required only 8 days. Despite its lower reprogramming efficiency and temporal requirements, the 3-factor combination may be preferred over the 4-factor combination because the transduced cells are not overgrown by granulate colonies.11,31

Southern blot analysis demonstrated successful excision of the transgenes, and the resulting hiPSC lines maintained pluripotency independent of residual exogenous transcription factor expression.

Because the isolated hiPSC lines are patient-specific, they provide a system for investigating the proposed molecular and cellular mechanisms of the disease. Soldner et al.53 demonstrated successful derivation of dopaminergic neurons from Parkinson disease patients’ cells, indicating that the

<table>
<thead>
<tr>
<th>Vector</th>
<th>Factors (+ Conditions)</th>
<th>Human Starting Cell Type</th>
<th>Approximate Reprogramming Efficiency</th>
<th>Approximate Speed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retroviral</td>
<td>OSKM</td>
<td>Fibroblasts</td>
<td>0.02%</td>
<td>25 d</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>OSK</td>
<td>Keratinocytes</td>
<td>&lt;0.01%</td>
<td>31 d</td>
<td>91</td>
</tr>
<tr>
<td>Lentiviral</td>
<td>OSKMN</td>
<td>Fibroblasts</td>
<td>0.001%</td>
<td>31 d</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>OSKM</td>
<td>Keratinocytes</td>
<td>0.002%</td>
<td>10 d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OSK</td>
<td>Adipose-derived</td>
<td>0.2%</td>
<td>18 d</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>OSNL</td>
<td>Hematopoietic ESC-derived</td>
<td>0.02%</td>
<td>20 d</td>
<td>41</td>
</tr>
<tr>
<td>OSN</td>
<td>Amnion-derived</td>
<td>Not shown</td>
<td>&gt;14 d</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Retroviral</td>
<td>OSK + VPA</td>
<td>Neonatal fibroblasts</td>
<td>1%</td>
<td>30 d</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>OS + VPA</td>
<td></td>
<td>0.002%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>OSKM + VPA + vitamin C</td>
<td>Adipose-derived</td>
<td>7.06%*</td>
<td>30–34 d</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibroblasts</td>
<td>3.7%*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Umbilical cord matrix-derived</td>
<td>0.4%</td>
<td>13–20 d</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placental amniotic membrane-derived</td>
<td>0.1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lentiviral</td>
<td>OSKM + butyrate</td>
<td>Fetal fibroblasts</td>
<td>16.3%</td>
<td>18 d</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>OSNL + hLIF + PD0325901 + A-83-01 + CHIR99021</td>
<td></td>
<td>0.0025%</td>
<td>28 d</td>
<td>95, 96</td>
</tr>
<tr>
<td></td>
<td>OK + Parnate + CHIR99021</td>
<td>Neonatal keratinocytes</td>
<td>0.002%</td>
<td>49 d</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>OK + Parnate + CHIR99021 + PD0325901 + SB431542</td>
<td></td>
<td>0.0075%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 + PD0325901 + butyrate + A-83-01 + PS48</td>
<td></td>
<td>0.00017%</td>
<td>56 d</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>0 + Parnate + CHIR99021 + PD0325901 + butyrate + A-83-01 + PS48</td>
<td>Keratinocytes</td>
<td>0.00025%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Umbilical vein endothelium-derived</td>
<td>0.01%</td>
<td>42 d</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amniotic fluid-derived</td>
<td>0.004%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Retroviral and lentiviral methods are shown with associated enhancements due to addition of small molecules to reprogramming cell culture media.

*The parameter used to define pluripotency in reference 76 only includes positive testing of alkaline phosphatase. Other parameters used to define bona fide pluripotent stem cells were not used.

O indicates OCT4; S, SOX2; K, KLF4; M, c-MYC; N, NANOG; L, LIN28; VPA, valproic acid; and hLIF, human leukemia inhibiting factor.
underlying age or disease of the donor most likely does not affect the ability of their cells to produce hiPSC-derived replacements ex vivo. Interestingly, in this study, factor-free hiPSCs were found to be more closely related to embryodervied hESCs than provirus-carrying parental hiPSCs, based on gene expression analysis. Hence, basal expression of proviruses carried in conventional hiPSCs can affect the molecular characteristics of the hiPSCs. Although transgenes are expected to be completely silenced in bona fide hiPSC lines, residual sequences and chromosomal disruptions during and after viral integration may still result in harmful alterations that could pose clinical risks. As previously mentioned, reactivation of reprogramming transgenens after transplant can result in malignant transformation of the cells and formation of a tumor.15 Such unpredictable effects of incomplete transgene silencing on downstream hiPSC phenotype further highlight the need for transgene-free hiPSC derivation methods. Some recent advances in a variety of such methods are detailed in Table 2.

Nonviral Integration Followed by Excision: PiggyBac Transposition

Although Cre recombinase–driven excision utilizes a highly efficient and widely used system, small residual vector backbone sequences remain at the site of integration and may engender unpredictable downstream effects. Wolftjen et al54 and Kaji et al55 demonstrated successful reprogramming of human embryonic fibroblasts using doxycycline-inducible reprogramming factors that were delivered as plasmids, stably integrated into the host genome, and subsequently excised using piggyBac transposition. Wolftjen et al noted that successful transposon-based nonviral reprogramming has several advantages over traditional lentiviral integration-based reprograming: (1) improved accessibility of reprogramming techniques through the use of plasmid DNA preparations and commercial transfection products, thereby eliminating the need for specialized biohazard containment facilities; (2) increased variety of reprogrammable donor cell types because susceptibility to viral infection is no longer an important consideration; (3) feasibility of xeno-free production of hiPSCs; and (4) most importantly, near complete elimination of the expression of reprogramming factors after establishment of hiPSC lines by piggyBac transposase–mediated excision. However, successful excision of the reprogramming cassette was only achieved in approximately 2% of the bona fide hiPSCs exposed to piggyBac transposase, limiting the amount of vector-free hiPSCs that could potentially be produced. Of note, Mali et al56 demonstrated the use of butyrate to enhance reprogramming efficiency 15- to 51-fold when used in conjunction with piggyBac transposase–driven integration and excision of the Yamanaka reprogramming factors. Genome-wide analysis of the effects of butyrate exposure at days 6 to 12 demonstrated significant changes in H3 acetylation and promoter methylation status in a variety of pluripotency-related genes, including DPPA2.

Nonviral Nonintegrating Methods

Despite the aforementioned progress toward safe and efficient reprogramming, the ideal method for hiPSC derivation would avoid temporary or permanent genomic modification. However, nonviral, nonintegrating reprogramming techniques typically have significantly lower reprogramming efficiencies than the aforementioned retroviral and lentiviral methods. Nonviral, nonintegrating methods for hiPSC generation include episomal plasmid DNA, minicircle DNA, protein, and synthetic RNA delivery. DNA- and RNA-based methods accomplish hiPSC induction by the transient expression of reprogramming factors, often through repetitive transfection protocols.

Episomal Plasmid

Yu et al57 derived hiPSCs from human foreskin fibroblasts using nonintegrating episomal plasmids vectors. Repeated transient transfection of 3 plasmids expressing 7 reprogramming factors resulted in hiPSCs that were completely

### Table 2. Summary of Different Nonviral, Nonintegrating Techniques for Generating hiPSCs

<table>
<thead>
<tr>
<th>Vector</th>
<th>Factors (+ Conditions)</th>
<th>Starting Cell Type</th>
<th>Approximate Reprogramming Efficiency</th>
<th>Approximate Speed</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excisional</td>
<td>Lentiviral</td>
<td>OSKM</td>
<td>Neonatal keratinocytes</td>
<td>0.00001%</td>
<td>22–35 d</td>
</tr>
<tr>
<td>PiggyBac transposition</td>
<td>OSKM</td>
<td>Embryonic fibroblasts</td>
<td>0.008%</td>
<td>14–28 d</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>OSKML + butyrate</td>
<td>Bone marrow–derived</td>
<td>0.008%</td>
<td>&gt;14 d</td>
<td>54</td>
</tr>
<tr>
<td>Episomal</td>
<td>Plasmid DNA</td>
<td>OSKM, SVLT, EBNA1</td>
<td>Fibroblasts</td>
<td>0.00045%</td>
<td>17 d</td>
</tr>
<tr>
<td></td>
<td>Minicircle DNA</td>
<td>OSNL</td>
<td>Adipose-derived</td>
<td>0.005%</td>
<td>26–28 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fetal fibroblasts</td>
<td>0.0005%</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Polyarginine tract tagged polypeptides</td>
<td>OSKM</td>
<td>Neonatal fibroblasts</td>
<td>0.001%</td>
<td>56 d</td>
</tr>
<tr>
<td>RNA</td>
<td>Modified synthetic mRNAs</td>
<td>OSKM</td>
<td>Fibroblasts</td>
<td>1.4%</td>
<td>17 d</td>
</tr>
</tbody>
</table>

Episomal, excisional, protein- and RNA-mediated methods are shown with associated enhancements due to addition of small molecules to reprogramming cell culture media.

O indicates OCT4; S, SOX2; K, KLF4; M, c-MYC; N, NANOG; L, LIN28; SVLT, SV40 large T gene; and EBNA1, Epstein-Barr nuclear antigen-1.
transgene-free (as confirmed by reverse-transcription polymerase chain reaction [RT-PCR]) but maintained the proliferative and developmental potential of hESCs. The 7 factors include OCT4, SOX2, c-MYC, KLF4, NANOG, LIN28, SV40 large T antigen (SVLT), and Epstein-Barr nuclear antigen-1 (EBNA1). Of note, stable extrachromosomal replication of these plasmid vectors is enabled by the presence of the cis-acting oriP sequence and the transacting EBNA1 protein.\(^{58–60}\) Interestingly, coexpression of the 6 reprogramming factors (OCT4, SOX2, NANOG, LIN28, KLF4, and c-MYC) in initial experiments resulted in substantial cell toxicity and produced no hiPSC colonies. Only the inclusion of the SVLT and modification of the reprogramming factor ratio allowed for isolation of bona fide hiPSCs.\(^{57}\) These results emphasize the importance of balanced absolute expression levels during the reprogramming process. Subsequently, the progressive loss of the episomal vectors enables isolation of hiPSCs devoid of vector or transgene sequences. However, reprogramming efficiency remained low, at approximately 3 to 6 colonies per \(^{10^6}\) input cells.

**Minicircle Vectors**

Jia et al\(^{61}\) constructed a minicircle vector for reprogramming consisting of a cassette of the reprogramming factors OCT4, SOX2, LIN28, and NANOG and a green fluorescent protein (GFP) reporter gene, each separated by sequences encoding the 2A ribosomal slippage site. Though minicircles are also supercoiled derivatives of plasmids, they are unique in that they primarily consist of eukaryotic expression cassettes, which lack both a bacterial origin of replication and antibiotic resistance gene. These vectors are preferred to standard plasmids because they have shown comparatively enhanced and more persistent transgene expression both in vivo and in vitro, perhaps on account of their smaller sizes and minimal expression cassettes.\(^{52–63}\) Indeed, Jia et al\(^{61}\) reported that a plasmid vector–based protocol failed to generate hiPSCs in their system. In their minicircle protocol, the vectors were introduced into human adipose stromal cells 3 times: first via nucleofection, a proprietary electroporation system of Lonza, then twice via Lipofectamine. On days 14 to 16, colonies that were morphologically similar to hESC colonies were produced from human adipose stem cells and neonatal fibroblasts, whose reprogramming efficiency was reported at \(~0.005\%\) and \(~0.0005\%\), respectively.\(^{64}\) Southern blot analysis confirmed the absence of genomic integration of the minicircle transgene in the subclones.

**Protein Delivery**

Direct delivery of reprogramming factor proteins into the cell is one way to entirely eliminate risks associated with viruses, genome manipulation, and DNA transfection. Kim et al\(^{65}\) reported successful generation of hiPSCs from human newborn fibroblasts by direct delivery of the Yamanaka factor proteins fused with a cell-penetrating polyarginine peptide. After 6 protein treatment cycles over 8 weeks, 5 alkaline phosphatase–positive hiPSC-like colonies were derived from a starting cell population of \(5 \times 10^5\) cells. Expansion of these hiPSC lines allowed confirmation of pluripotency by in vitro RT-PCR and in vivo teratoma formation assays. Despite the hampered reprogramming process created by the need for repeated treatments and relative subdued reprogramming efficiency (\(~0.001\%\)), reprogramming via protein transduction creates an attractive alternative by allowing generation of hiPSCs free of vectors or limitations caused by viral delivery. In conjunction with other methods such as use of purified recombinant mammalian proteins or concomitant use of small molecules, one could potentially further improve the reprogramming efficiency.

**RNA Delivery**

Nonintegrating DNA- and protein-based reprogramming strategies still have certain disadvantages. Even episomal DNA vectors used for hiPSC derivation entail a small risk of unintended genetic integration. Protein-based reprogramming requires relatively challenging production and purification of recombinant proteins. Recently, Warren et al\(^{66}\) demonstrated the successful production of hiPSCs via repeated transfection of modified synthetic mRNAs. These modified mRNAs potentially bypass any innate immune response to foreign DNA elements. Tra-I to 60’ hiPSCs were derived from BJ fibroblasts by repeated transfection of a combination of 4 modified synthetic mRNAs encoding the Yamanaka factors at an efficiency of 1.4%. Retrovirally mediated reprogramming experiments conducted in parallel achieved an efficiency of 0.04%. Therefore, RNA-mediated reprogramming represented a 36-fold improvement in reprogramming efficiency. The kinetics of hiPSC generation using modified mRNAs was also noted to be accelerated approximately 2-fold. Addition of LIN28 to the 4-factor cocktail and subjection of the cells to low oxygen culture conditions (5% \((\text{O}_2)\) generated a striking reprogramming efficiency of 4.4%. The significantly enhanced reprogramming efficiency of Warren et al may be attributable to several unique experimental advantages. First and foremost, transfected mRNA is translated into functional protein within several hours, whereas several days may elapse after lentiviral transduction before expression of functional protein. The ability to achieve stable intracellular stoichiometric concentrations of mRNA using transfection is presumably preferable to the somewhat variable expression patterns of transfected or transduced DNA constructs. Also, the immunogenicity of the foreign RNA was reduced by substitution of 5-methylcytidine and pseudouridine bases for cytidine and uridine, respectively, as well as media supplementation with the Vaccinia virus decoy protein B18R to allay any interferon-mediated immune response. These changes allowed for repetitive daily transfections for a period of \(~17\) days without significant cytotoxicity. Such results represent a significant advance in our ability to derive transgene-free hiPSCs. In agreement with previous reports, the authors observed that transgene-free hiPSCs derived by RNA transfection more faithfully recapitulated the global gene expression profile of hESCs than retrovirally derived hiPSCs.

**Small Molecules Augment Reprogramming Efficiency**

Small molecules may improve the reprogramming efficiency or completely replace 1 or more reprogramming factors...
during the hiPSC derivation process. Proponents of small molecules also assert that they are more timely in reaching their target, easier to control via concentration variations, simpler to use, and cheaper than the aforementioned reprogramming agents.\(^\text{67}\) Chemical approaches to screening combinatorial small-molecule libraries have successfully identified several molecules that can augment pluripotency induction. O’Malley et al\(^\text{68}\) separated these small molecules into 2 groups: (1) affectors of chromatin modification and (2) affectors of cell signaling pathways. This general classification system helps to organize and differentiate the targets of small molecules, allowing prediction of their effects and potential synergism. Detailed and comprehensive knowledge of the involved pathways is especially important because the effects of cell exposure to drugs may entail nonspecific and wide-ranging changes that unpredictably affect the safety and phenotype of the resulting cells. In addition, an understanding of the effects of small molecules on intracellular signaling mechanisms, developmental pathways, and cell fate may provide important insight into the molecular mechanisms of reprogramming.

Valproic Acid

Valproic acid (VPA) is a small-molecule histone deacetylase inhibitor that is already Food and Drug Administration–approved to treat epilepsy and bipolar disorder.\(^\text{69,70}\) VPA has been used to successfully reprogram neonatal foreskin fibroblasts when used in conjunction with OCT4 and SOX2. Thus, it can effectively replace the oncogenic c-MYC and KLF4 factors, albeit at the cost of a significantly reduced reprogramming efficiency (\(<0.005\%\)).\(^\text{32}\) When VPA was used with OCT4, SOX2, and KLF4, a reprogramming efficiency of 1\% was reported. Notably, reprogramming was accomplished in dermal fibroblasts, a differentiated cell type that does not endogenously express any of the reprogramming factors. Using VPA in conjunction with a cell type that endogenously expresses high levels of c-MYC or KLF4, such as keratinocytes or adipose stromal cells, might therefore significantly improve reprogramming efficiency.\(^\text{37,39}\)

SB431542, PD0325901, and Thiazovivin

Lin et al\(^\text{71}\) tested a combination of 3 molecules in an effort to precipitate speedier production of hiPSCs (created by retroviral transduction of fibroblasts [CRL2097 or BJ] with cDNAs encoding the Yamanaka factors). They concluded that dual inhibition of the transforming growth factor (TGF)-\(\beta\) and mitogen-activated protein kinase protein kinase pathways in a dose-dependent, temporal manner may guide and perhaps accelerate the kinetics of partially reprogrammed colonies to a fully pluripotent state. This is based on a \(\approx100\)-fold improvement in reprogramming efficiency that occurred when TGF-\(\beta\) inhibitor SB431542 and mitogen-activated protein kinase kinase inhibitor PD0325901 were added to the cultures, promoting improved induction at earlier stages than expected, and a decrease in the number of granulate noniPSC colonies produced. These results highlight the importance of signal transduction pathways in the reprogramming process.

For example, TGF-\(\beta\) signaling can be active in tumorigenic suppression and immune response and cell migration regulation because members of this family primarily promote cellular senescence, differentiation, and apoptosis.\(^\text{42}\) TGF-\(\beta\) antagonists probably benefit reprogramming in other ways as well, including inhibition of the aforementioned cellular processes and promotion of the mesenchymal-to-epithelial transition by upregulating genes such as E-cadherin,\(^\text{72}\) a regulator of \(\text{NANOG}\) expression.\(^\text{73}\)

An additional impediment to high reprogramming efficiency is the poor survival of hiPSCs after dissociation into single cells by trypsinization. Addition of thiazovivin to hiPSC reprogramming culture media has been found to improve cell survival during protocols involving splitting via trypsinization.\(^\text{71}\) Remarkably, by 30 days after transduction, \(\approx900\) hESC-like colonies were produced from 10 000 seeded cells after a single 1:4 split on day 14 by using thiazovivin in conjunction with SB431542 and PD0325901. Independent of its demonstrated ability to promote single-cell survival, thiazovivin appears able to enhance reprogramming efficiency by approximately 2-fold when used in conjunction with SB431542 and PD0325901.

**CHIR99021 and Parnate**

CHIR99021 and Parnate are 2 other small molecules that have been used to successfully optimize reprogramming efficiency. Li et al\(^\text{30}\) reported successful reprogramming of neonatal epidermal keratinocytes with only 2 transcription factors (OCT4 and KLF4) in conjunction with CHIR99021 and Parnate. On average, a reprogramming efficiency of 0.002\% was achieved. CHIR99021 is a glycogen synthase kinase-3 inhibitor that activates the Wnt signaling pathway, and Wnt3a-conditioned media is known to promote reprogramming in mouse embryonic fibroblasts.\(^\text{74}\) Parnate is a monoamine oxidase inhibitor used as a second-line treatment for depression. It is also classified as an epigenetic modifier because of its inhibition of lysine-specific demethylase 1.\(^\text{75}\) It is thereby thought to enhance reprogramming efficiency by inhibiting H3K4 demethylation. Interestingly, the use of Parnate and CHIR99021 in addition to SB431542 and PD0325901 did not result in any significant improvement in reprogramming efficiency because only 5 to 10 hiPSC colonies could be identified from \(10^3\) transduced cells. The pathways targeted by these additional molecules and their lack of synergy with other known small-molecule enhancers of reprogramming remain under investigation.

**Vitamin C**

Of particular interest is the effect of vitamin C on the reprogramming efficiency of hiPSCs. Esteban et al\(^\text{76}\) found that vitamin C and other antioxidants had no effect on reprogramming efficiency when added to cell culture media supplemented with knockout serum replacement. However, when added to cell culture media supplemented with Dulbecco modified fetal bovine serum, vitamin C used in conjunction with VPA produced roughly 3 times as many alkaline phosphatase positive colonies as VPA alone (7.06\% versus 2.10\% efficiency). Improved reprogramming efficiency (maximum efficiency of 0.40\%) using vitamin C was
also demonstrated by Cai et al.,77 using umbilical cord-derived cells. These cells were transduced with retroviruses using the Yamanaka factors and a chemical concoction of vitamin C, Dulbecco modified fetal bovine serum–based medium, and VPA. Given the known role of cellular senescence in impeding reprogramming, it is perhaps expected that vitamin C, a natural antioxidant, would enhance cellular reprogramming by reducing intracellular levels of reactive oxygen species.78 However, alternative mechanisms for vitamin C’s effects could include promotion of epigenetic modifications because vitamin C is a cofactor for many significant enzymes.

**Butyrate, A-83–01, and PS48**

Because keratinocytes express KLF4 and c-MYC endogenously, it was believed that full reprogramming was possible with the sole transduction of *OCT4* in the presence of small molecules.30 Only recently was this hypothesis definitively confirmed in a study by Zhu et al.,79 who used a mixture of sodium butyrate (sodium butyrate, a histone deacetylase inhibitor), PS48 (an inhibitor of phosphoinositide-dependent kinase-1), A-83–01 (TGF-β kinase activin receptor-like kinase inhibitor), PD0325901, CHIR99021, and Parnate to reprogram not only adult keratinocytes but umbilical vein endothelial and amniotic fluid derived cells at an average of 0.004% reprogramming efficiency. Interestingly, butyrate is believed to assist the reprogramming process epigenetically in mouse embryonic fibroblasts by modulating the flexibility of chromatin structure and repressing cancerous cell growth but only in the presence of c-MYC.80 PS48 was found to upregulate glycolytic gene expression,79 promoting a switch from mitochondrial oxidation, a metabolism used by somatic cells when they are no longer proliferating.81 The respective pathways affected by these molecules are critical in the reprogramming process, underscoring the need for further investigation into the mechanisms of dedifferentiation. Ideally, a chemical concoction without transcription factors would be sufficient to reprogram somatic cells to pluripotency, and progress toward this goal is readily apparent in recent work. Additional research into reprogramming enhancement using these and other small molecules is imperative, given the possibility that off-target effects of these small molecules may be detrimental to cell phenotype.

**Cardiovascular Applications of iPSC-Derived Cells**

Once derived, hiPSCs can serve as an inexhaustible source of more differentiated cardiovascular cells, including the cardiomyocyte, endothelial cell, and smooth muscle lineages. The adult heart has limited regenerative capacity, and pluripotent stem cell–derived lineages are ideal candidates for replacement therapies. Transplantation treatment of myocardial infarction may demand replacement of up to 1 billion damaged cardiomyocytes.82 Aside from the potential benefits that hiPSC-derived cardiomyocytes (hiPSC-CMs) can provide after myocardial infarction, endothelial cells generated from hiPSCs may help repair heart valves, vessels, and ischemic tissue,83 perhaps by preventing cell death and providing vascular support in grafts. Obstacles to overcome before applying hiPSC derivatives clinically include (1) standardization of lineage specification protocols to produce large quantities of pure, quality-controlled cells of the desired cell types, (2) methods to ensure their safe delivery, and (3) consideration of all possible adverse effects after treatment. As mentioned above, however, the hESC-CMs generated to date phenotypically resemble fetal/neonatal cardiomyocytes more closely than adult cardiomyocytes,4,5 and due care should be exercised in extrapolating results using hESC-CMs or hiPSC-CMs to the adult heart.

**Generation of Cardiovascular Cells**

Pioneering work by Gordon Keller’s group (Yang et al2) has definitively demonstrated the cardiovascular potential of a specific population of kinase insert domain receptor (KDR)low/C-KITneg cells derived from hESCs. Specifically, by mirroring developmental cues, they successfully induced generation of cardiovascular colony–forming cells from hESCs after temporal exposure to a combination of cytokines and signaling molecules, including bone-associated morphogenic protein 4, Activin A, basic fibroblast growth factor (bFGF), vascular endothelial growth factor, and Dickkopf-related protein 1. The KDRlow/C-KITneg cells were able to differentiate into all 3 cardiovascular lineages, cardiomyocytes, smooth muscle cells, and endothelial cells. Cardiovascular progenitors were identified by gene expression profiling showing upregulation of cardiac genes (including *NKX2.5, ISL1, TBX5*, and *TBX20*), expression of cardiac troponin T (CTNT), and contractile ability. The cells were also found to be clonal and therefore could be sources of cardiovascular regeneration. Chemical augmentation of the Wnt signaling pathway was explored because Dickkopf-related protein 1, a Wnt pathway inhibitor, increased production of CTNT+ cells, whereas Wnt3a suppressed development. The largest amount of CTNT+ cells (≈45% of the developing embryoid bodies [EBs]) was produced with a combination of bone-associated morphogenic protein 4, bFGF, and Activin A. KDRlow/C-KITneg/GFP cells transplanted into the hearts of nonobese diabetic/severe combined immunodeficient mice improved ejection fraction by 31%, and no further complications were reported. Other small-animal studies using transplanted hESC-CMs have revealed more modest short-term benefits,4,5 although grafted tissue is susceptible to acute donor cell death, tumorigenesis, and arrhythmogenesis.82 More recently, Zhang et al84 investigated the cardiovascular differentiation potential of hiPSC lines, potentially bypassing the aforementioned obstacles associated with hESC use. The cardiomyocytes derived from the EBs of hiPSCs lentivirally transduced with *OCT4, SOX2, NANOG,* and *LIN28* were comparable to cardiomyocytes derived from the EBs of hESCs. hiPSC-CMs and hESC-CMs both displayed relative downregulation of *OCT4* and *NANOG,* upregulation of cardiac genes, myofibronectin protein expression, and sarcomeric organization. hiPSC-CMs also proliferated robustly, generated atrial, nodal, and ventricular action potentials, and responded to electric and chemical stimulation of the β-adrenergic signaling pathway. There were somewhat fewer hiPSC-CMs that exhibited contractile ability than hESC-CMs, and ineffective silencing of the *OCT4* and *NANOG*
transgenes was demonstrated in RT-PCR analyses of the hiPSC-EBs. However, Zhang et al note that the differences between hiPSC-CMs and hESC-CMs are comparable to the differences already observed between hESC lines. Moreover, the aforementioned nonviral nonintegrating techniques to generate hiPSCs can bypass the problem presented by the transgene insertions in this study, thus showing promise for hiPSC lines as an alternative to hESC lines for a variety of applications.

Although they do not involve pluripotent stem cells, 2 recent studies warrant mention due to their novelty. Ieda et al demonstrated direct transdifferentiation of murine fibroblasts into cardiomyocytes, entirely bypassing the pluripotent stage, using a 3-factor combination of developmental transcription factors. In vitro, 30% of cells exhibited CTNT expression 1 week after viral transduction with the GATA4, MEF2C, and TBX5 factors. Immunocytochemistry confirmed the presence of sarcomeric α-actinin and atrial natriuretic factor in some of these cells. Additionally, transduction of these factors into murine hearts induced cardiomyocyte differentiation after a single day, demonstrating a potential proof-of-concept therapeutic application. Although the induced cardiomyocytes were found to be epigenetically and electrophysiologically similar to wild-type cardiomyocytes, the cardiomyocyte-specific genes ACTC1, MYH6, RYR2, and GJA1 were not detected.

Direct reprogramming of murine fibroblasts to cardiomyocytes was subsequently demonstrated using a slightly different transdifferentiation strategy. By briefly overexpressing the Yamanaka factors and carefully controlling cardiogenic media supplementation, Efe et al successfully reprogrammed up to 40% of mouse fibroblasts into functional CTNT+ cardiomyocytes within 18 days. Importantly, small-molecule signaling played a pivotal role in modulating cell fate, for example, by inhibiting JAK/STAT-driven pluripotency induction. These recently developed protocols provide an important alternate platform for the production of patient-specific cardiomyocytes while entirely avoiding the pluripotent state and its concomitant tumorigenic risk.

### Disease Modeling Using hiPSC-CMs

Several exciting demonstrations of the disease modeling capability of hiPSC-CMs have recently been published (Table 3). In all studies to date, the resulting patient-specific hiPSC-CMs have been found to at least partially exhibit the phenotype of the diseases under investigation: long-QT syndrome, Timothy syndrome, and LEAPORD syndrome.

A seminal study evaluated the use of hiPSCs in modeling LEOPARD syndrome, an autosomal-dominant developmental disorder of multiple organ systems resulting from a missense mutation in the PTPN11 gene. Compared with control hESC-CMs, diseased hiPSC-CMs were noted to have a higher mean cell surface area as well as nuclear translocation of the NFATC4 transcription factor, perhaps representing in vivo molecular surrogates of the disease’s cardiac hypertrophy phenotype. In addition, phosphoproteomic comparison of the diseased and wild-type hiPSCs revealed that diseased hiPSCs did not respond to attempted MAPK activation by bFGF. Although defects in MAPK activation are an expected result of mutation in the PTPN11 gene encoding SHP2 tyrosine phosphatase, it is worthwhile to note that the standard procedures for analysis of cardiomyocyte hypertrophy, such as protein synthesis rate and activation of the fetal gene program, could not be reliably assessed in the mixed population of cells resulting from attempted cardiomyocyte differentiation.

A subsequent study evaluated type 1 long-QT syndrome in hiPSCs by comparing wild-type cells with patient-specific cells containing a [569G→A] missense mutation in the KCNQ1 gene. Once the patient-specific hiPSCs had been directed to the cardiac lineage, the ventricular and atrial myocyte action potentials had significantly longer QT intervals and slower repolarization velocity as compared with wild-type cardiomyocytes. Type 1 long-QT syndrome is attributed to a reduction in the IKs (slow outward potassium current) responsible for mediating action potential repolarization, due to the defective KCNQ1 channel. In agreement with this, single-cell electrophysiological analysis on the ventricular patient-specific hiPSC-CMs revealed a reduction in this current, confirming that the KCNQ1 mutant interferes with the function of the wild-type subunit. Immunocytochemical tests of both populations of cardiomyocytes suggested that the phenotype is the result of a trafficking defect, in which the mutated KCNQ1 protein fails to achieve membrane targeting.

Last, stimulation of the ventricular patient-specific hiPSC-CMs using isoproterenol had little to no effect on the patient-specific hiPSC-CM repolarization and IKs currents, whereas a significant reduction of both was produced in the

### Table 3. Studies Using hiPSC-CMs for Cardiac Disease Modeling

<table>
<thead>
<tr>
<th>Disease</th>
<th>Reprogramming Method</th>
<th>Methods for Evaluation of Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEOPARD syndrome</td>
<td>Retroviral integration</td>
<td>Microscopic morphometry, Immunocytochemistry, Phosphoproteomic analysis using antibody arrays and Western blots</td>
<td>89</td>
</tr>
<tr>
<td>Type 1 long-QT syndrome</td>
<td>Retroviral integration</td>
<td>Single-cell patch clamping, Immunocytochemistry</td>
<td>87</td>
</tr>
<tr>
<td>Type 2 long-QT syndrome</td>
<td>Retroviral integration</td>
<td>Single-cell patch clamping, Microelectrode arrays, Pharmacological response</td>
<td>88</td>
</tr>
<tr>
<td>Timothy syndrome</td>
<td>Retroviral integration</td>
<td>Single-cell patch clamping, Calcium transient imaging, Response to roscovitine</td>
<td>90</td>
</tr>
</tbody>
</table>

hiPSC-CM indicates human induced pluripotent stem cell–derived cardiomyocyte.
wild-type cells. Additionally, the patient-specific hiPSC-CMs had reduced action potential duration:action potential interval ratios. These adrenergically stimulating tests cumulatively suggest that long-QT syndrome hiPSC-CMs are predisposed to arrhythmic events.

Another study has extended the above-mentioned findings by modeling type 2 long-QT syndrome using similar methods. These hiPSCs were derived from type 2 long-QT syndrome patients and contained a missense mutation in the KCNH2 gene. The resulting hiPSC-CMs exhibited the electrophysiological hallmarks of the disease, including prolonged action potential duration and early after depolarizations in patch-clamping studies, as well as prolonged field potential duration in microelectrode array studies. Diseased hiPSC-CMs displayed the expected defect in Ik, (delayed-rectifier potassium current) as well as increased susceptibility to pharmacologically induced arrhythmogenesis. The authors also demonstrate an important proof-of-concept drug screening experiment to evaluate the effects of nifedipine, pinacidil, and ranolazine on the electrophysiological properties of the diseased hiPSC-CMs.

Yazawa et al\textsuperscript{30} derived hiPSCs from patients with Timothy syndrome, a disorder in which patients have long-QT syndrome, autism, immune deficiency, and syndactyly caused by a mutation in the CACNA1C gene encoding the Ca,1.2 L-type channel. Beating hiPSC-derived EBs displayed irregular contraction rates, whereas single hiPSC-CMs displayed increased action potential duration as well as reduced voltage-dependent inactivation of the L-type calcium channel current. Interestingly, ventricular cardiomyocytes but not atrial cardiomyocytes displayed the prolonged action potential phenotype indicative of long-QT syndrome, in contrast to the results of Moretti et al.\textsuperscript{87}

Conclusion
The ability to safely and efficiently derive hiPSCs may be of decisive importance to the future of regenerative medicine, and it depends on researchers’ eventual ability to generate hiPSCs free from foreign chemical or genomic elements while maintaining a stable cell line. Many techniques for hiPSC derivation have been developed in recent years, utilizing different starting cell types, vector delivery systems, and culture conditions. A refined or perfected combination of these techniques might prove to be the key to generating clinically applicable hiPSCs. Although this review is by no means meant to be exhaustive, we have attempted to highlight the significant developments in clinical translation of hiPSCs and their relevance to cardiovascular disease. Among these developments, the most exciting techniques involve enhancement of reprogramming by small molecules (Table 1), non-viral nonintegrating methods for hiPSC derivation (Table 2), and their subsequent application to disease modeling platforms (Table 3). Although outside the scope of this review, it bears noting the critical need for standardization of the parameters that define a cell as an hiPSC.\textsuperscript{39} Without consensus on the criteria for identification of hiPSCs, comparison between the results of various investigators is difficult because the guidelines by which various investigators select their fully reprogrammed hiPSCs vary. Future endeavors to advance hiPSCs as clinically relevant cardiovascular therapies may use an exciting combination of the methods reviewed here, with additional improvements sure to follow.

Acknowledgments
Because of space limitations, we are unable to include all of the important studies relevant to induced pluripotent stem cell derivation and application; we apologize to those investigators whom we omitted here.

Sources of Funding
This study was supported by National Institutes of Health New Innovator Award DP2OD004437, HL091453, HL089027, and Burroughs Wellcome Foundation Career Award Medical Scientists (J.C.W.) and the Howard Hughes Medical Institute (K.H.N.).

Disclosures
None.

References
26:1269–1275.


Derivation of Human Induced Pluripotent Stem Cells for Cardiovascular Disease Modeling
Kamileh Narsinh, Kazim H. Narsinh and Joseph C. Wu

doi: 10.1161/CIRCRESAHA.111.240374

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/108/9/1146

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/