Finding Expandable Induced Cardiovascular Progenitor Cells

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Expandable Cardiovascular Progenitor Cells Reprogrammed From Fibroblasts
Zhang et al
Cell Stem Cell. 2016;18:368–381.

Lineage Reprogramming of Fibroblasts Into Proliferative Induced Cardiac Progenitor Cells by Defined Factors
Lalit et al
Cell Stem Cell. 2016;18:354–367.

Cardiovascular progenitor cells (CPCs) are a promising cell source for cardiac regenerative therapy because of their ability for self-renewal and differentiation into various cardiovascular cell types beneficial for myocardial repair: cardiomyocytes, smooth muscle cells, and endothelial cells. Previous evaluations of exogenously derived CPCs have focused mainly on their capacity for trilineage differentiation rather than self-renewal because of the lack of an effective protocol to maintain and expand CPCs long-term in culture. In a recent issue of *Cell Stem Cell*, 2 groups of investigators independently reported their success in isolating, maintaining, and expanding mouse CPCs in culture for >18 to 20 passages (10^{10}- to 10^{15}-fold expansion), with faithful preservation of progenitor phenotype and ability for trilineage-restricted differentiation in both cell culture and mouse models of myocardial infarction (MI). This commentary will discuss the unique findings of these 2 studies, highlight the strengths and weaknesses of each CPC derivation/expansion technique, and propose additional steps necessary to accelerate their clinical translation.

Why CPCs?

The past 2 decades have witnessed some of the most dramatic developments in the field of cardiac regeneration. Central to numerous controversies is the debate on whether certain stem cell types can differentiate into functional cardiomyocytes at a clinically relevant efficiency after implantation for repair of MI. Bone marrow–derived hematopoietic stem cells[1,2] and c-kit$^+$ resident cardiac stem cells[3] are 2 notable cell types at the center of contention. Although many adult stem cells can still confer modest therapeutic benefits via paracrine effects, the search for a bona fide progenitor cell type that can generate a significant amount of cardiomyocytes in situ for contractile support remains a priority for regenerative therapy.

CPCs are increasingly recognized as a promising source for myocardial regeneration because of their potential for proliferation and differentiation into cardiovascular cell types that can assist with myocardial repair: cardiomyocytes, endothelial cells (ECs), and smooth muscle cells (SMCs).[4] The discovery of endogenous CPCs in the adult heart has spurred numerous studies, demonstrating their expandability in culture and ability to augment cardiac function post MI in both rodents and humans.[5,6] However, the developmental origins of these cells are debatable, and their cardiac differentiation potentials, especially for c-kit$^+$ CPCs, lately have been challenged.[7] Although extensive evaluation of endogenous CPCs continues, increasing efforts are being directed toward generating CPCs in cell culture via either differentiation of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) or direct reprogramming/transdifferentiation from somatic cell sources. These CPCs are safer for implantation than undifferentiated ESCs/iPSCs, which even at low levels (hundreds of cells) can cause teratoma.[8] Compared with cardiomyocytes alone, CPCs offer additional therapeutic benefits by serving as in vivo sources of ECs and SMCs, thus contributing to neovascularization.

Expandable Induced CPCs in Focus

To date, most attempts at capturing CPCs from ESCs/iPSCs using various markers (eg, Flk-1, Isl1, or Nkx2-5) have focused on demonstrating tripotency, if not bipotency, with ECs not always generated.[4] However, the prolonged expansion of CPCs in culture has remained challenging.[4] In a recent issue of *Cell Stem Cell*, 2 groups of investigators addressed this shortcoming by taking 2 distinct approaches to deriving expandable CPCs (Figure). In 1 approach, Zhang et al[9] used cell activation and signal-derived lineage conversion to isolate CPCs by transiently reprogramming mouse embryonic and tail tip fibroblasts via overexpression of reprogramming factor genes (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) under doxycycline regulation followed by further cardiac specification via glycogen synthase kinase 3 inhibition and concurrent suppression of pluripotency via Janus kinase (JAK) inhibition. These so-called induced CPCs (iCPCs), obtained via identification of double Flk-1$^+$/PdgfR-α$^+$ positivity, were further maintained in culture using BACS, a cocktail of BMP4, activin A, CHIR99021 (GSK-3 inhibitor), and SU5402 (inhibitor of fibroblast growth factor, vascular endothelial growth factor, and platelet-derived growth factor signaling). Impressively, these iCPCs were propagated for >18 passages (10^{10}-fold expansion)

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(*Circ Res*. 2016;119:16-20.
DOI: 10.1161/CIRCRESAHA.116.308679.)

***Circulation Research* is available at http://circres.ahajournals.org**
DOI: 10.1161/CIRCRESAHA.116.308679

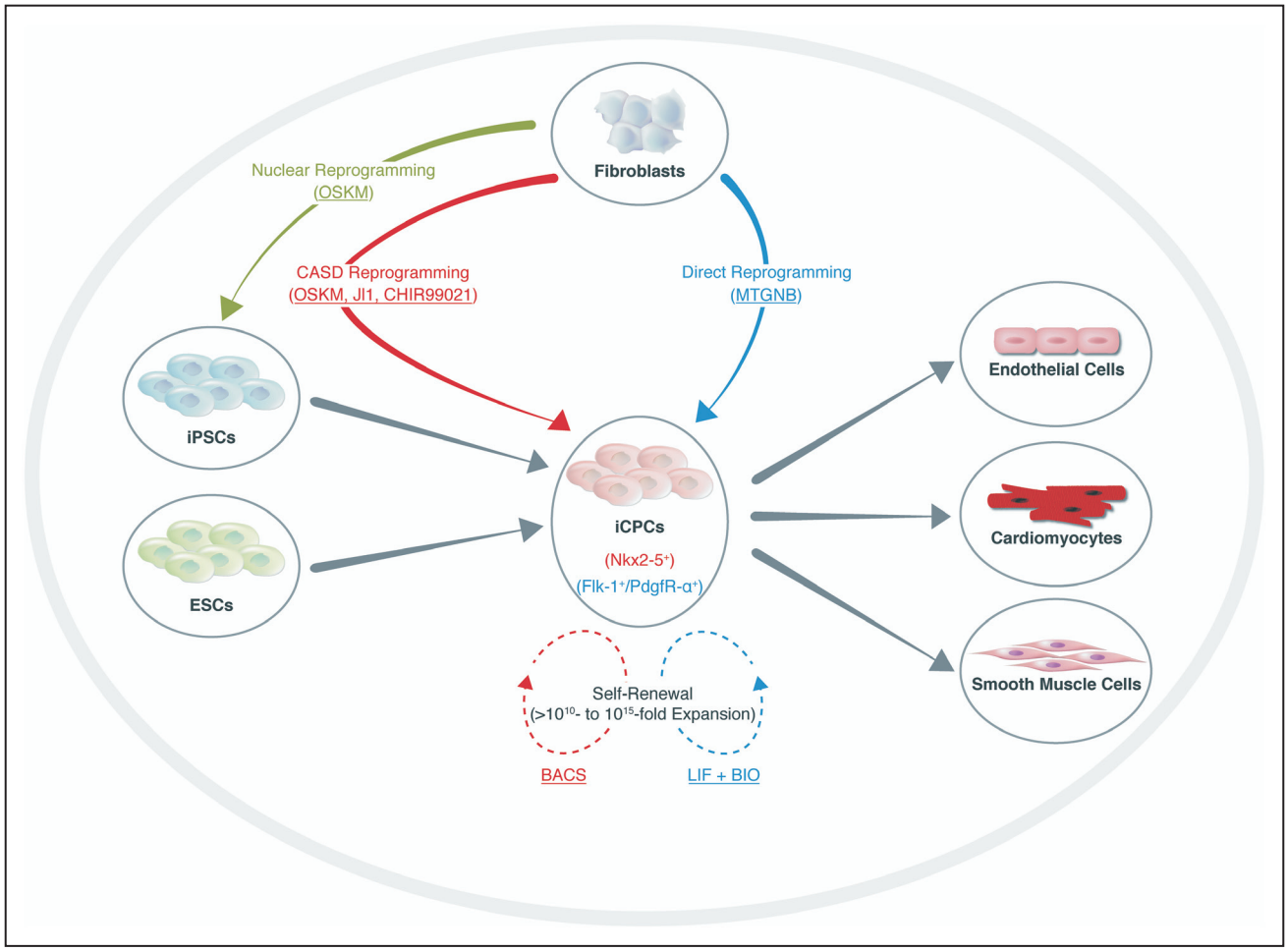

Figure. Generation of expandable mouse induced cardiovascular progenitor cells. Two different approaches for isolating expandable induced cardiovascular progenitor cells (iCPCs) are presented. In cell activation and signal-derived (CASD) reprogramming (red solid arrow), somatic cells (eg, fibroblasts) are transiently exposed to Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc [OSKM]) followed by cardiac specification toward iCPCs using Janus kinase (JAK) inhibitor (JI1) and glycogen synthase kinase 3 inhibitor (CHIR99021). Unlike conventional nuclear reprogramming for generation of induced pluripotent stem cells (iPSCs; green arrow), the derivation of iCPCs via CASD reprogramming does not involve a transition through the pluripotent state, which is prevented by JAK inhibition. The iCPCs generated, identified by Nkx2-5$^+$ positivity, can be expanded in culture using a cocktail of small molecules termed BACS (BMP4, activin A, CHIR99021, and SU5402; red dotted arrow). In another approach, iCPCs can be derived by direct reprogramming using overexpression of 5 transcriptional factors, including Mesp1, Tbx5, Gata4, Nkx2-5, and Baf60c (MTGNB; blue solid arrow). The iCPCs generated, identified by Flk-1$^+$/PdgfR-α^+ positivity, can be expanded using a combination of LIF (JAK/STAT activator) and BIO (Wnt activator; blue dotted arrow). Note that iCPCs also form as intermediates during the differentiation of iPSCs/embryonic stem cells (ESCs) toward distinct cardiovascular cell types (paths indicated by gray arrows): cardiomyocytes, endothelial cells, and smooth muscle cells.

under BACS conditions with preservation of cell morphology, growth rate, Flk-1$^+$/PdgfR-α^+ positivity, transcriptome, and trilineage potency either in culture or after implantation into mouse MI models, the latter being associated with improved cardiac function at 12 weeks post MI. Importantly, BACS also enabled the derivation of expandable CPCs from mouse ESCs, thereby providing additional avenues for the clinical translation of CPCs using existing patient-specific iPSC lines as sources.

Using a different approach, Lalit et al[10] conducted an extensive search for CPC-inducing factors, starting with 22 candidate factors that included cardiac transcriptional, cardiac chromatin remodeling, and iPSC reprogramming factors. Lentiviral vector–mediated delivery and overexpression of genes that encode only 5 of these factors (*Mesp1*, *Tbx5*, *Gata4*, *Nkx2-5*, and *Baf60c*) in mouse adult cardiac fibroblasts were sufficient to induce Nkx2-5$^+$ CPCs, which were maintained and expanded in culture for at least 20 passages on further exposure to a combination of LIF (a JAK/STAT activator) and BIO (a canonical Wnt activator). With passaging, these iCPCs retained the expression of expected cardiac progenitor genes but not markers associated with either pluripotent stem cells or differentiated cell types of any tissue origin. Clonal analysis of iCPCs revealed cells with mostly tripotency and some bipotency. These results were recapitulated for a total of 40 iCPC lines derived from various fibroblast sources (heart, lung, and tail tip), lending support to the generalizability of this iCPC derivation protocol. Interestingly, the iCPC-derived cardiomyocytes failed to beat spontaneously; a small fraction (5%–10%) of which started to contract only after coculture with mouse ESC-derived cardiomyocytes. Limited karyotype analysis of 3 iCPC lines derived from lung fibroblasts revealed

that 1 line was nearly tetraploid. Nevertheless, on implantation into the cardiac crescent of mouse embryos, the iCPCs derived from adult cardiac fibroblasts differentiated into contracting cardiomyocytes that integrated with the heart tube. As early as 4 days post injection into the peri-infarct regions of the mouse hearts, these iCPCs differentiated into all 3 cardiovascular lineages, leading to improved animal survival at 4 weeks compared with phosphate-buffered saline–injected controls. Importantly, no tumor formation was evident after iCPC implantation, further confirming the safety benefits of iCPCs over undifferentiated ESCs/iPSCs.

Pushing the Limits of Expansion at Limited Costs

The feasibility of expanding exogenously derived CPCs in culture has been previously established. Moretti et al[11] first demonstrated that Isl1$^+$ CPCs derived from mouse ESCs could be propagated on a feeder layer of cardiac mesenchymal cells for 1 week. This accomplishment was later attributed to the feeder layer's induction of Wnt/β-catenin signaling in the CPCs.[12] Likewise, Christoforou et al[13] showed that mouse ESC-derived Nkx2-5$^+$ CPCs cultured on mitotically inactive mouse embryonic fibroblasts were able to expand and passage for >100 doublings in high serum culture medium. Cao et al[14] subsequently generated MESP1/2$^+$/SSEA1$^+$/Isl1$^+$ CPCs from human ESCs and iPSCs in feeder-free and serum-free conditions using a combination of BMP4, CHIR99021 (glycogen synthase kinase 3 inhibitor), and ascorbic acid and succeeded in expanding these cells for >15 passages (10^7-fold expansion). However, there were concerns as to whether MESP1/2$^+$ CPCs could also differentiate into noncardiovascular cell types. More recently, Birket et al[15] succeeded in arresting and expanding CPCs for >40 population doublings at a pre-NKX2-5 stage by overexpressing *c-Myc* and modulating the insulin growth factor-1 and hedgehog pathways in these cells.

The 2 studies recently published in *Cell Stem Cell* further pushed the limits of mouse CPC expansion in culture to consistently >18 to 20 passages (10^{10}- to 10^{15}-fold expansion) under feeder-free conditions using 2 distinct combinations of small molecules (ie, BACS versus LIF/BIO combination), without any forced transgene expression, such as *c-Myc*. Worth applauding were the efforts in both studies to comprehensively confirm similar CPC characteristics and differentiation potentials at both low and high passages, which were not always thoroughly evaluated in previous studies. Particularly impressive was the generalizability of the LIF/BIO formulation for reproducibly expanding a total of 40 Nkx2-5$^+$ iCPC lines derived from mouse fibroblasts of different tissue origins. Notably, a near-tetraploid karyotype was observed in 1 of the 3 iCPC lines derived from lung fibroblasts subjected to chromosomal analysis, which is consistent with previous studies showing abnormal karyotypes in ≈30% of cardiac stem cells expanded in prolonged culture.[16] Given the increasing attention to genomic stability validation of patient-specific iPSCs before clinical usage, further investigation of the potential sources of these karyotype abnormalities during iCPC derivation or expansion is warranted.

In both studies, the iCPCs obtained demonstrated tripotency in cell culture by clonal analysis. However, in the study by Lalit et al[10], the cardiac differentiation efficiency notably varied depending on the tissue source of the fibroblasts used and not all Nkx2-5$^+$ iCPCs differentiated into 1 of the 3 cardiovascular cell types. This degree of variability, attributable, in part, to epigenetic memory of the source fibroblasts, could profoundly alter the in vivo differentiation capacity and therapeutic efficacy of the derived iCPCs. With regard to in vivo differentiation, the iCPCs derived in both studies were able to differentiate into all 3 cardiovascular cell types after transplantation into the peri-infarct mouse myocardium. However, the amount of each cell type engrafted was only quantified in the study by Zhang et al[9], which revealed predominantly SMCs (59.2%) rather than cardiomyocytes (30.8%) or ECs (6.8%) at 2 weeks post transplantation. This finding was consistent with the observation that these Flk-1$^+$/PdgfR-α$^+$ iCPCs also more readily differentiated into SMCs (92.1%) than cardiomyocytes (33.2%) in culture. Although the in vivo differentiation efficiency of these iCPCs could not be fully assessed because the survival kinetics of each iCPC derivative was not determined, the fewer number of cardiomyocytes engrafted compared with SMCs after iCPC implantation still resulted in better cardiac function and less myocardial fibrosis at 3 months compared with control fibroblast implantation. The mechanisms underlying these favorable outcomes were presumed to be related to de novo cardiomyocyte generation leading to restoration of contractility, engraftment of newly generated ECs and SMCs leading to enhanced neovascularization, as well as paracrine effects. Considering that almost twice as many SMCs as cardiomyocytes actually engrafted in this particular study, it would be interesting to know whether this ratio of SMCs to cardiomyocytes favored neovascularization. Nevertheless, the possibility of augmenting CPC therapy (ie, improving cardiac function or neovascularization) by manipulating the relative ratios of cardiomyocytes, ECs, and SMCs differentiated in vivo could be further explored, particularly in light of a recent study showing limited neovascularization after human ESC-derived CPC implantation into infarcted rat myocardium.[17] It is worth noting that the iCPC-derived cardiomyocytes derived in the study by Lalit et al[10] did not spontaneously contract and could only do so after coculture with mouse ESC-derived cardiomyocytes. This phenomenon has been reported for both mouse-induced cardiomyocytes (iCMs) generated via overexpression of GMT (Gata4, Mef2c, and Tbx5) transcriptional factors,[18] as well as human iCMs produced via overexpression of GMT plus Mesp1 and Myocd.[19] The lack of spontaneous contractions in these iCPC-derived cardiomyocytes was attributed to either immature cardiomyocyte phenotypes or incomplete reprogramming. This functional deficit seems to be more commonly seen with GMT-based transdifferentiation protocols than with either cell activation and signal-derived reprogramming or cardiac differentiation from ESCs/iPSCs. Interestingly, it did not significantly affect the performance of iCPCs in vivo as the mouse myocardial environment, similar to mouse ESC-derived cardiomyocyte coculture, was thought to facilitate maturation and rapid differentiation into functional cardiomyocytes. Nevertheless, a head-to-head comparison of these iCPCs with those derived from either ESC/iPSCs or somatic cells via cell activation and signal-derived reprogramming is needed to further evaluate the broader implication of this functional impairment.

Challenges and Strategies for Clinical Translation

Expandable iCPCs are a particularly attractive cell source for myocardial regenerative therapy because of their ability to provide millions to billions of cells in situ required for effective myocardial repair. The successful clinical translation of these cells rests on safe, efficient, and reliable protocols to expand them in culture, as well as mechanisms to augment their functions in vivo. Further optimization of the techniques discussed herein for manipulating expandable iCPCs will be crucial for accelerating their clinical translation: first, the aforementioned iCPC expansion techniques will need to be further validated using human somatic cells because the generation of human iCPCs, compared with mouse iCPCs, may require additional transcriptional factors. This has been the case when using GMT-based transdifferentiation techniques to generate human iCMs compared with their mouse counterparts.[19] Second, the use of lentiviral vectors in parts of both studies to deliver transcriptional factors could be replaced by nonintegrating vectors to minimize the risk of insertional mutagenesis. In this regard, cell activation and signal-derived reprogramming has the advantage in that only transient upregulation of reprogramming factors is needed for cell activation. Agents, such as synthetic mRNAs, are particularly suitable for this purpose. Third, the use of reporters (eg, Nkx2-5-YFP) for purifying iCPCs requires a priori genetic manipulation of the source cells and therefore are less convenient and less clinically ideal than isolating iCPCs based on cell surface markers. However, although preferred, the latter approach may be limited to only certain iCPC populations because of the dearth of distinct cell surface markers for iCPCs thus far. Fourth, although the survival of implanted iCPCs was not specifically addressed in the 2 studies presented, several previous studies using noninvasive longitudinal molecular imaging approaches have shown that the majority of cells (ie, CPCs, bone marrow mononuclear cells, mesenchymal stem cells, skeletal myoblasts, ESC-derived ECs, and ESC-derived cardiomyocytes) that were transplanted into the heart died rapidly.[20–24] Therefore, it would be worthwhile to explore the possibility of adapting the iCPC expansion techniques presented herein to promote iCPC proliferation in vivo, which may counteract the drastic acute donor cell death that has hampered nearly all forms of cardiac regenerative therapy. Fifth, many studies, including the study by Zhang et al,[9] demonstrated a low potential for the iCPCs to differentiate into ECs that engraft long-term in vivo.[17] As such, the advantage of implanting ESC-derived CPCs versus ESC-derived cardiomyocytes, in terms of neovascularization, has recently been challenged.[17] Therefore, effective strategies to modulate the relative yields of the 3 cardiovascular cell types in vivo may improve the overall therapeutic efficacy of iCPCs. Finally, in contrast to cardiac regeneration iPSC-derived cardiomyocytes, which take at least a couple of weeks for proper cardiac differentiation from existing iPSCs, iCPCs generated a priori could differentiate into functional cardiovascular cell types within a few days after implantation. This opens the possibility of banking stably reprogrammed iCPCs and thawing them in a subacute scenario for rapid expansion and implantation afterward. Thus, efficient biostorage and rapid functional recovery techniques for iCPCs could enable a more versatile clinical use of iCPCs for myocardial regeneration. As there are already many trials ongoing for endogenous CPC-based therapy, the expandable iCPCs may play a useful role in clinical trials once these aforementioned issues are addressed.

Acknowledgments

We thank Amy Thomas for her assistance with the figure. This publication was supported, in part, by research grants from the National Institutes of Health (NIH) 2T32EB009035 (I.Y. Chen), AHA 13EIA14420025, NIH HL130020, NIH R01 HL128170, and NIH R01 HL126527 (J.C. Wu).

Disclosures

None.

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Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. 2016;119:16-20
doi: 10.1161/CIRCRESAHA.116.308679
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571