Editorial

Stem Cell INVESTIGATION

Are we closer to cardiac regeneration?

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Received: 19 August 2016; Accepted: 21 September 2016; Published: 20 October 2016.
doi: 10.21037/sci.2016.09.16
View this article at: http://dx.doi.org/10.21037/sci.2016.09.16

Heart disease remains one of the leading causes of mortality. In contrast to adult teleost fish and amphibians, in adult mammals the resident cardiac cells are not able to regenerate heart tissue and restore efficiently the cardiac function after heart failure, being hypertrophy the most relevant compensation for the loss of cardiomyocytes. Since there are not enough heart donors available for transplantation, in recent years, new approaches have been explored, and a number of groups have been focused on the use of the stem cells. Although adipose tissue-derived cells or cardiac-derived stem cells are being explored (1,2), the largest clinical experience has been acquired with intracoronary delivery of bone marrow stem cells (BMSC) to treat acute myocardial infarction and chronic ischemic heart failure. However, the published data in the last 10 years do not show a substantial long-term benefit (3). The reasons that could explain BMSC inefficacy for cardiac repair could be: (I) only a very small fraction of the injected cells remain in the targeted area, and (II) BMSC are not able to differentiate into cardiac muscle. Actually, the current consensus is that any improvement in cardiac function after BMSC transplantation is likely to be the result of a paracrine action. Thus, success of cardiac cell therapy will be determined by the development of methods to improve engraftment and generation of cells capable to regenerate the damaged tissue (4).

Then, what is the optimal cell type for regeneration of the failing heart? Ideal candidate cell types, besides ensuring safety, should satisfy the following items: (I) be expandable or scalable; (II) be immunocompatible within the donor heart or at least immune-tolerant; (III) integrate and synchronize with the rest of host myocardium. Cardiovascular progenitor cells (CPC) have the potential to proliferate and differentiate into the main cardiovascular lineages (cardiomyocytes, smooth muscle and endothelial cells), thus constituting useful cellular models for studying cardiac development and potentially ideal cellular source for cardiac regenerative therapy.

Messina *et al.* first and other groups have demonstrated that CPC can be isolated from heart biopsies and enriched via cardiosphere culture, but their myogenic potential is limited (5,6). Alternatively, human CPC with tri-lineage cardiovascular differentiation potential can be isolated from differentiating human pluripotent stem cells *in vitro*, based on the expression of CPC-related transcription factors [Mesp1 (7), Isl1 (8), Nkx2.5 (9)], or surface markers [SSEA1$^+$ (10), KDR$^+$/PDGFR-α$^+$ (11), GFRA2$^+$ (12)]. Nevertheless, the study of CPC has been a real challenge since these cells undergo a rapid transition from multipotency to commitment. Wnt signaling plays essential roles during vertebrate heart development, and studies in embryos and pluripotent stem cells have shown that canonical Wnt pathway induces cardiac specification during early developmental/differentiation stages whereas inhibits it later (13). In this regard, several groups have used Wnt signaling activators (Wnt3a or GSK3 inhibitors) to promote pluripotent stem cell-derived CPC expansion (14-16).

Cell reprogramming has created great opportunities for studying cell specification and for modelling and treating certain diseases. Cell reprogramming methodologies, based on the forced expression of factors that determine the desired cell fate and/or alter the epigenome, potentially enable the obtainment of any cell type of the human body. Specifically, CPC, can be obtained using three different

reprogramming approaches:

(I) iPSC reprogramming. Yamanaka laboratory demonstrated in 2006 that somatic cells can be reprogrammed to induced pluripotent stem cells (iPSC) by ectopic expression of Oct4, Sox2, Klf4 and Myc transcription factors (17). iPSC have the capability to differentiate into any cell type, including CPC, and constitute an unlimited source of cells since these cells can be cultured and expanded in their undifferentiated cell state, and be cryopreserved. However, these reprogramming and differentiation procedures take months, and their tumorigenic potential limits their clinical application.

(II) "Plastic" reprogramming. A different reprogramming approach was described in 2011 by Ding laboratory by which the desired cell type, including CPC (18), could be produced in weeks. This reprogramming approach uses the 4 Yamanaka factors in combination with JAK inhibitor, to induce what it was called a "plastic" cell state, and the latter culture of these cells in defined conditions promoting the induction of the desired cell type. Initially, this "plastic" reprogramming approach was described as a direct cell fate conversion approach; however, two studies demonstrated that it generates a pluripotent intermediate state (19,20).

(III) Direct reprogramming. The direct reprogramming of human fibroblasts into KDR$^+$/Nkx2.5$^+$ induced CPC (iCPC) was achieved in 2012 by ectopic expression of ETS2 and MESP1 factors (21). iCPC were not extensively characterized and their function was not studied *in vivo* since these iCPC were spontaneously differentiated into immature cardiomyocytes.

A recent study by Lalit *et al*., demonstrated that at least 5 cardiac factors (Mesp1, Tbx5, Gata4, Nkx2.5 and Baf60c) in combination with the leukemia inhibitory factor (LIF) and the 6-bromoindirubin-3'-oxime (BIO), can reprogram adult fibroblasts into proliferative and multipotent iCPC (22). First, the authors selected 22 candidate genes: 18 cardiac genes (including transcription and chromatin remodeling factors) and the 4 Yamanaka factors. The coding sequence of each gene was individually cloned into a doxycycline inducible lentiviral vector. To identify easily reprogrammed iCPC and enable doxycycline inducible transgene expression, a Nkx2.5 cardiac reporter mouse model expressing enhanced yellow fluorescent protein (EYFP) crossed with a transgenic mouse expressing a reverse tetracycline transactivator (rtTA) was used.

In the first reprogramming assay, adult cardiac fibroblasts were infected with a mixture of lentivirus containing all 22 factors leading to a small number of EYFP$^+$ colonies after doxycycline treatment. The EYFP$^+$ colonies formation was not affected by the withdrawal of the 4 Yamanaka factors. Next, 11 factors were selected based on their early expression in cardiac development (Mesp1, Mesp2, Gata4, Gata6, Baf60c, SRF, Isl1, Nkx2.5, Irx4, Tbx5 and Tbx20). Single EYFP$^+$ cells were detected 6 days after the infection with the 11 factors, and by 3 weeks highly proliferative colonies of EYFP$^+$ cells were observed. Next, authors tried to expand these EYFP+ cells. The reprogramming medium (fibroblast medium supplemented with doxycycline) was not sufficient to maintain a proliferative state of the EYFP$^+$ cells and the cells senesced after few passages. Reasoning that Wnt and JAK-STAT signaling pathways play critical roles in proliferation of CPCs and cardiogenesis, respectively, the reprogramming medium was supplemented with BIO (a canonical Wnt signaling activator) and LIF which was called iCPC induction medium. Adult cardiac fibroblasts infected with 11 factors and cultured in iCPC induction medium produced EYFP$^+$ cells which could be robustly expanded for over 30 passages. EYFP$^+$ cells size reduction was observed during the first passages and the doubling time of EYFP$^+$ cells decreased when compared with their parental fibroblasts. Once again, in an attempt to reduce the number of reprogramming factors, reasoning that Mesp1, Tbx5 and Gata4 (MTG) are expressed earliest in cardiac development, this combination of factors was tested. Two of these three selected factors, Tbx5 and Gata4, were previously used together with Mef2c by Ieda *et al*. to reprogram mouse fibroblasts into induced cardiomyocytes (23). EYFP$^+$ colonies were not observed using MTG combination, however, MTG together with Nkx2.5 and Baf60c factors (MTGNB) produced proliferative and expandable EYFP$^+$ cells. Next, EYFP$^+$ cells were extensively characterized to demonstrate that they were truly iCPC. First, protein and RNA expression of CPC-related markers in EYFP$^+$ cells was verified by immunostaining and RNA sequencing analyses. Second, the ability of EYFP$^+$ cells to differentiate into cardiovascular lineages (cardiomyocytes, endothelial and smooth muscle cells) was confirmed *in vitro* and *in vivo*. EYFP$^+$ cells were induced to cardiac lineage using a protocol based on cell aggregate induction with BMP4, VEGF, bFGF and a canonical Wnt inhibitor (IWP4) for the first 4-6 days, and the posterior plating of

cell aggregates on gelatin-coated dishes and maintenance in fibroblast medium with 1% serum for 20 days. The *in vitro* differentiation potential of iCPC into cardiomyocytes (80% to 90%) smooth muscle (5% to 10%) and endothelial cells (1% to 5%) was demonstrated by immunostaining. However, the iCPC-derived cardiomyocytes, although manifested organized microfilaments, did not contract spontaneously, and only when cocultured with mESC-derived cardiomyocytes for two weeks, 5% to 10% of iCPC-derived cardiomyocytes started contracting. iCPC integrated with host cells within the heart tube and differentiated into cardiomyocytes when injected into cardiac crescent of E7.75 embryos, and the iCPC capability to differentiate into three cardiovascular lineages was shown when 1–1.5 million iCPC were injected in a mouse model of myocardial infarction. Interestingly, the survival of the animals that received iCPC was significantly improved when compared to animals that receive PBS (75% *vs.* 11%, respectively). Moreover, Lalit *et al.* showed the robustness and reproducibility of this procedure by the reprogramming of adult fibroblasts from different tissues (lung and tail-tip) into expandable iCPC.

The low reprogramming efficiency (0.008% or 0.014% with 11 or 5 factors, respectively) is offset by the fact that iCPC can be expanded in defined culture conditions generating billions of cells without losing their differentiation potential. However, a study led by Zhang *et al.* published simultaneously in the same scientific journal, reported the induction of expandable CPC using different culture conditions (18). In this study, iCPC were generated by the "plastic" reprogramming approach and were expanded with a cocktail called BACS (BMP4, Activin A, CHIR99021 and SU5402). The common signaling pathway activated in both expansion strategies, by BIO or CHIR GSK3 inhibitors, is the canonical Wnt signaling. Although both iCPC populations expressed CPC-related markers as Isl1 and Nkx2.5 and were tripotent *in vitro* and *in vivo*, these iCPC populations differed in the expression of surface CPC-related markers as PDGFR-α and Flk1. Presumably, the use of different markers to isolate iCPC may explain these molecular discrepancies and the different expansion requirements. In fact, the iCPC described by Lalit *et al.* using the Nkx2.5 reporter, showed very limited endothelial potential and heterogeneous cell surface and gene expression, so it is reasonable to think that these Nkx2.5^{+} iCPC may contain different subpopulations of CPC. The proportion of each cardiovascular lineage derived from iCPC may vary depending on the differentiation protocol used *in vitro*, but the utilization of other CPC markers expressed earlier in cardiac development might generate iCPC with increased potential, although a distinct combination of factors for their induction will probably be needed.

There are still pending issues to be solved. Although iCPC could be more malleable and adaptable to the recipient than their differentiated counterparts, it needs to be elucidated to what extent the microenvironmental cues, encountered following *in vivo* administration of iCPC, are enough to differentiate these cells into the cardiovascular lineages that are functionally required, and if the achieved stoichiometry is preserved in different subjects. On the other hand, a recent study demonstrated that the use of CPC-derived extracellular vesicles is sufficient for the functional recovery of mice with chronic heart failure (24), thus, another open question is if the survival of the animals receiving iCPC improved by their regenerative and/or paracrine action. Nonetheless, undoubtedly, the direct reprogramming of fibroblasts into expandable and multipotent iCPC constitutes a potent tool for cardiovascular research and opens up new therapeutic avenues. Next challenge should be obtaining proliferative and scalable human iCPC to determine their utility for disease modelling and drug discovery, and their capability to regenerate the injured heart.

Acknowledgements

Funding: This editorial is supported by the Spanish Ministry of Economy and Competitiveness (RYC-2012-10981 and SAF2013-46142-R to X Carvajal-Vergara), and the Spanish Cellular Therapy Network from Ministry of Health, Social Services and Equality-Institute of Health Carlos III (TERCEL ISCIII-RETIC RD12/0019/0031 to F Prósper).

Footnote

Provenance: This is a Guest Editorial commissioned by Editor-in-Chief Zhizhuang Joe Zhao (Pathology Graduate Program, University of Oklahoma Health Sciences Center, Oklahoma City, USA).
Conflicts of Interest: The authors have no conflicts of interest to declare.

Comment on: Lalit PA, Salick MR, Nelson DO, *et al.* Lineage Reprogramming of Fibroblasts into Proliferative Induced Cardiac Progenitor Cells by Defined Factors. Cell Stem Cell 2016;18:354-67.

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doi: 10.21037/sci.2016.09.16
Cite this article as: Carvajal-Vergara X, Prósper F. Are we closer to cardiac regeneration? Stem Cell Investig 2016;3:59.