Identity Crisis for Regenerative Cardiac cKit+ Cells

Onur Kanisicak, Ronald J. Vagnozzi, Jeffery D. Molkentin

The concept that cardiac-derived cKit+ cells can regenerate the injured adult heart by transdifferentiating into functioning cardiomyocytes was proposed 14 years ago although it remains controversial because of negative data from multiple independent laboratories. Irreproducibility of cardiac cKit+ cell studies was attributed to the differences in cell isolation, selection, and expansion before in vivo application. This Viewpoint will discuss recent results that again change the formula for how cardiac cKit+ cells must be isolated and processed to be cardiomyogenic, as well as discuss the uncertain in vivo relevance of cKit+ cells as putative cardiomyocyte-producing stem cells.

Introduction to the Cardiac Regeneration cKit+ Cell Controversy

The regenerative and myogenic potential of cKit+ cardiac-resident stem cells (CSCs) remains a dominant and contentious topic. In vitro expanded cKit+ CSCs were originally described 14 years ago as a source for robust new myocyte generation when injected directly into the adult rodent heart after myocardial infarction,[1] and although some laboratories have confirmed the basic principle, the magnitude of the effect remains unclear.[2] More recently, Ellison et al[3] showed that a clonal cKit+ cell line derived from a population of cardiac CD45− cKit+ cells was capable of widely regenerating isoproterenol-injured adult rodent hearts with abundant new cardiomyocytes, after simple tail vein infusion. However, many independent laboratories reported that adult cardiac-derived cKit+ cells did not generate appreciable new cardiomyocytes when directly injected back into the injured rat or mouse heart.[4–7]

In addition to controversies surrounding the regenerative potential of injected cKit+ cell formulations, until recently the field has not attempted to directly address if endogenous cardiac cKit+ cells are true cardiomyocyte producing stem cells of meaningful significance.[8] In an attempt to directly address this shortcoming, 4 independent laboratories separately generated and characterized mice with Cre recombinase (or H2B-tdTomato) targeted to the *Kit* gene (encodes cKit protein) to permit lineage tracing in vivo.[9–12] These studies uniformly showed that endogenous cardiac cKit+ cells did not generate appreciable new cardiomyocytes in the mouse heart. In response to these data and the ongoing controversy in the field, a collection of established cardiac-based researchers released a 2017 consensus statement in *Circulation* that provides guidance on cardiac regeneration. The general conclusion is that the adult mammalian heart only generates ≈1% new cardiomyocytes each year, which fate-mapping studies suggest is mediated primarily by cardiomyocyte proliferation.[13] However, this consensus statement did not dispute the likelihood that exogenously delivered cardiac cKit+ cells could have a beneficial effect when given to the injured heart, such as by enhancing vascular regrowth, extracellular matrix conditioning, and by paracrine-mediated induction of endogenous repair pathways.[14,15]

New Study From Vicinanza et al Changes Formula for Cardiogenic cKit+ Cells

A new study from Vicinanza et al[16] has reignited the debate over cardiomyogenesis from cKit+ CSCs as they now report that ≈99% of primary cKit+ cells isolated from the heart lack cardiomyogenic potential and have no rejuvenating benefit when injected into the injured hearts of mice or rats. Instead, only a rare subfraction of cardiac cKit+ cells defined by (1) expression of the marker cKit, (2) absence of the hematopoietic marker CD45, and (3) having been clonally derived and expanded in vitro are able to regenerate the heart with new cardiomyocytes. It is this fraction, representing only 1% of total cardiac cKit+ cells, that Vicinanza et al[16] redefine as the true cKit+ cardiac stem cell. Such a model, if correct, warrants re-examination of an entire body of past literature as it calls into question the identity and phenotype of isolated cKit+ CSCs that have already been used in numerous animal studies and in ongoing human clinical trials.[2,14]

Perhaps most striking, the new claims by Vicinanza et al[16] contradict the original characterization of cKit+ CSCs reported in *Cell* in 2003 (Beltrami et al[1]) by some of the same senior authors (Drs Nadal-Ginard and Torella). The 2003 study that began much of this field used total cKit+ cells to create 70% new functioning myocardium when directly injected into the infarcted rodent heart.[1] Cardiac cKit+ cells without additional isolation criteria were reported to be self-renewing, clonogenic, and multipotent, as well as widely expressing the cardiogenic transcription factor Nkx2.5.[1] Vicinanza et al[16] now contend that CD45-negative selection is required to designate a cardiac cKit+ cell as a CSC and that among all CD45− cKit+ cells, only a small portion (10%) have the potential to generate clones in culture with cardiomyogenic potential.[16] However, the histological end points for cardiac renewal and the physiological end points for functional improvement in Vicinanza et al[16] versus Beltrami et al[1] seem virtually identical, despite the fact that these new data

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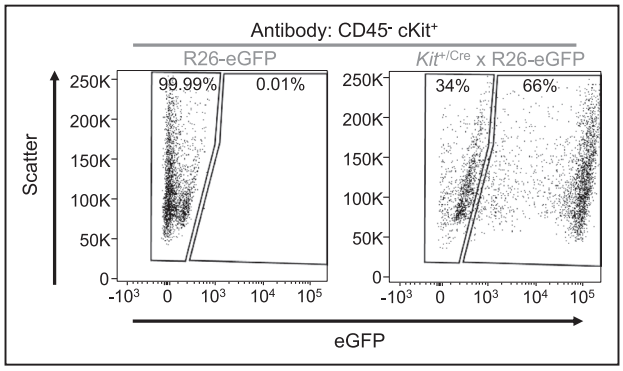

Figure. Kit-Cre lineage tracing of cardiac-resident stem cells. Flow cytometry plots of isolated cardiac interstitial cells pre-gated for the CD45⁻/cKit⁺ population from either *Rosa26*-loxP-eGFP (Cre-dependent reporter mouse line, R26-eGFP) or *Kit*$^{+/Cre}$×R26-eGFP mice. Approximately 66% of CD45⁻ cKit⁺ cells are eGFP positive and 34% did not show recombination of the R26-eGFP locus (Representative of cells analyzed from n=4 mice at 12 wk of age). These data indicate that the Kit-Cre allele effectively lineage traces cardiac cKit⁺ cells with the same antibody marker profile used in enriching for cardiomyogenic cells by Vicinanza et al.[16] eGFP indicates enhanced-green fluorescence protein.

contend that the population used in the original Beltrami et al[1] study was probably not cardiomyogenic. In addition, the new results by Vicinanza et al[16] are also at odds with their Ellison et al[3] study in which they stated that many of the c-kit^{pos} eCSCs expressed GATA4 and Nkx2.5, two early transcription factors of the cardiac lineage.

More perplexing, Vicinanza et al[16] reported that injection of broadly isolated cKit⁺ cells not negatively sorted for CD45 produced essentially no new cardiomyocytes (0.0001%) and no functional benefits in injured rodent hearts. Again, this result stands in contrast to their original observations from 14 years ago,[1] and it calls into question the newly emerging paracrine hypothesis[14] and the potential validity of ongoing clinical trials that also use broadly isolated cardiac cKit⁺ cells (NCT02501811). Recent studies have shown that the vast majority of cKit⁺ cells directly injected in the rodent heart simply die in a few days while the cells that persist lack appreciable cardiomyogenic potential.[7] The results of Vicinanza et al[16] are at odds with these newer concepts for how cardiac cKit⁺ cells might truly perform when injected back into the injured myocardium.

Understanding the Teleology of Endogenous cKit⁺ Cells

The potentially larger issue to consider is that Vicinanza et al[16] only showed that in vitro clonally derived cKit⁺ CD45⁻ cells could generate new cardiomyocytes when injected into the rat or mouse heart. This same basic experimental approach with clonally redrived cKit⁺ cells (in this case injected into the tail vein) was also used in Ellison et al[3] as the means of achieving meaningful regeneration of the mouse and rat heart.[3] We make no scientific argument against the likelihood that selected cardiac-resident mesenchymal cell types can be reprogrammed to adopt a more cardiomyogenic fate, whether by addition of cardiac lineage–specifying transcription factors, or perhaps by rigorous in vitro selection to identify a rare clonal cKit⁺ cell with myogenic characteristics. Our contention is related to interpretation because in vitro clonally selected cKit⁺ cells do not directly inform as to the true endogenous function of these cells within the heart.

To directly address the cardiomyogenic potential of endogenous cKit⁺ cells in vivo, we and others have taken a genetic approach by inserting Cre recombinase (or H2B-tdTomato) into the *Kit* gene. Our collective results showed that cardiac-resident cKit⁺ cells have extremely limited cardiomyogenic potential in vivo, either at baseline, with aging or after injury.[9,10,12] However, Vicinanza et al[16] claim that cKit⁺ cells are true cardiac stem cells albeit a rare population of them, by again attempting to discredit *Kit* allele knock-in lineage tracing approaches as flawed. As this argument was previously addressed[8] but still invoked by these authors, here we provide a simple figure of data definitively showing that lineage tracing with Kit-Cre knock-in mice identifies a majority of CD45⁻ cKit⁺ cells from the adult heart (Figure). This is the same starting population of cells that Vicinanza et al[16] claims have enhanced cardiomyogenic potential if clonally selected. Hence, disrupting one of the 2 *Kit* alleles in performing lineage tracing does not compromise identification of the newly proposed true cardiogenic cKit expressing subpopulation described by Vicinanza et al.[16]

Our previous lineage tracing results in the adult mouse heart showed detectable levels of new cardiomyocytes from cKit⁺ lineages[9] and while this rate was extremely low (1:20 000, when fusion was subtracted), it is consistent with the recent results of Vicinanza et al[16] whereby only 1% of the total cKit⁺ cells isolated from the heart can become cardiomyogenic when clonally selected in vitro. For example, when Vicinanza et al[16] inject total primary isolated cKit⁺ cells into the myocardial infarction–injured heart, which would contain these putative rare cardiomyogenic cKit⁺ cells, they report a rate of new cardiomyocyte production of 0.0001%. Hence, the results of Vicinanza et al[16] support the emerging consensus in the field that endogenous cardiac cKit⁺ cells are not true cardiomyocyte-producing stem cells of physiological significance. However, this does not discredit the observation that in vitro clonal selection of a subfraction of cardiac cKit⁺ cells can produce a cell line with enhanced cardiomyogenic potential, as appears to have been the case behind the Ellison et al[3] and Vicinanza et al[16] articles. Such highly selected cKit⁺ clonal cells could also be therapeutically relevant in treating patients, especially if they were autologously generated.

Implications of Vicinanza et al in Looking Forward

In moving forward, it will be important to better define the mechanisms of action underlying cardiac cell therapy while not forgetting the overarching concept that the adult mammalian heart is not an inherently regenerative organ and as such, it is unlikely to contain a physiologically relevant cardiomyocyte-producing stem cell, cKit expressing or otherwise. However, despite the ongoing controversy in the field, we should not be dissuaded from continuing our efforts to enhance the effectiveness of cell therapeutic approaches because the majority of animal studies continue to show a benefit of cKit⁺ cell administration when directly injected into the injured heart.

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Disclosures

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