HHS Public Access

Author manuscript

Circ Res. Author manuscript; available in PMC 2018 January 20.

Published in final edited form as:

Circ Res. 2017 January 20; 120(2): 400–406. doi:10.1161/CIRCRESAHA.116.309710.

The Elusive Progenitor Cell in Cardiac Regeneration: Slip-Slidin' Away

Chen-Leng Cai and **Jeffery D Molkentin**

Department of Developmental and Regenerative Biology, and Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, New York, NY (C-L.C) and the Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical Center and Howard Hughes Medical Institute, Cincinnati, OH (J.D.M.)

Abstract

The adult human heart is unable to regenerate after various forms of injury, suggesting that this organ lacks a biologically meaningful endogenous stem cell pool. However, injecting the infarcted area of the adult mammalian heart with exogenously prepared progenitor cells of various types has been reported to create new myocardium by the direct conversion of these progenitor cells into cardiomyocytes. These reports remain controversial because follow-up studies from independent laboratories failed to observe such an effect. Also, the exact nature of various putative myocyte-producing progenitor cells remains elusive and undefined across laboratories. By comparison, the field has gradually worked towards a consensus viewpoint that proposes that the adult mammalian myocardium can undergo a low level of new cardiomyocyte renewal of approximately 1% per year, which is due primarily to proliferation of existing cardiomyocytes but not from the differentiation of putative progenitor cells. This review will weigh the emerging evidence suggesting that the adult mammalian heart lacks a definable myocyte-generating progenitor cell of biologic significance.

Keywords

Cardiac; regeneration; stem cells; proliferation; c-Kit

Introduction

The description of bone marrow derived c-Kit$^+$ mononuclear cells regenerating vast amounts infarct damaged myocardium in rodents, as described in Orlic et al in *Nature* in 2001, captured the imagination of the field and even the public.[1] However, these results were refuted by 2 reports in *Nature* 3 years later in which bone marrow derived c-Kit$^+$ cells were shown to be incapable of becoming cardiac myocytes under an array of conditions.[2,3] The

Correspondence to: Jeffery D. Molkentin, Howard Hughes Medical Institute, Cincinnati Children's Hospital Medical Center, 240 Albert Sabin Way, Cincinnati, OH 45229-3039, jeff.molkentin@cchmc.org, Or Chen-Leng Cai, Department of Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, New York, NY 10029, chenleng.cai@mssm.edu.

Disclosures

No financial or other conflicts of interest exist with any of the authors.

scenario with bone marrow c-Kit$^+$ cells is very much a primer for the greater field of cardiac regeneration and what has transpired over the past 15 years. Indeed, the subsequent assertion that the heart has an endogenous pool of stem cells, c-Kit$^+$ or otherwise, that can regenerate the heart by making new cardiomyocytes after injury or with aging[4,5] remains contentious and is the primary source of current ongoing controversy in the field.[6] Some of the underlying controversy surrounding these putative c-Kit$^+$ progenitor cells likely results from an array of experimental and theoretical considerations that are discussed in detail by Keith and Bolli in a recent review on this subject.[7] Moreover, despite the presence of c-Kit$^+$ cells and other proposed putative stem cells within the adult mammalian heart, this organ nonetheless remains poorly regenerative and not capable of producing more than 1% cardiomyocyte renewal per year, which primarily result from cardiomyocyte proliferation. There are also basic tenets of stem cell biology that the proposed c-Kit$^+$ cardiac progenitor cell hypothesis breaks (will be discussed below). These and other lines of evidence collectively suggest an emerging consensus hypothesis that the adult heart lacks a physiologically meaningful, cardiomyocyte producing stem cell.

Evidence that existing cardiomyocytes proliferate to create new myocardium

The early neonatal mouse heart was recently shown to fully regenerate after either a resection surgical procedure or after myocardial infarction (MI) injury due to the proliferation of myocytes surrounding the area of injury.[8] Moreover, when adults from lower vertebrates such as zebrafish or newt are subjected to an apical resection procedure they show complete regeneration with new myocardium due primarily to the proliferation of existing cardiomyocytes.[9–13] In fact, studies in zebrafish even showed that adult heart regeneration was not due to the action of a progenitor cells, c-Kit$^+$ or otherwise, indicating an evolutionary ground state for cardiac repair that does not require unrelated progenitor cells.[13] However, regeneration of the zebrafish heart is due to the activity of a subset of endogenous cardiomyocytes that contain a somewhat specialized transcriptional program that affords proliferative potential.[12]

The results discussed above suggest that the heart indeed has a program for regenerative activity through proliferation of existing cardiomyocytes, which in the neonatal mouse is because the fully differentiated state has yet to occur, while in zebrafish there is less need for highly differentiated cardiomyocytes throughout the heart given the reduced hemodynamic pressures of this species.[13] This general paradigm could extend to the adult mammalian heart, whereby very small numbers of endogenous cardiomyocytes retain some sort of molecular signature of a less differentiated state to account for a low level of new myocyte production that has been unanimously documented.[13] Recent studies have also identified molecular pathways that can be exploited to augment the inherent ability of some endogenous cardiomyocytes to re-enter the cell cycle.[14,15] The field is attempting to dissect the molecular circuitry and genes that underlie cell cycle control and the extent to which cardiomyocyte differentiation progresses or even how to coax a de-differentiated state of the cardiomyocyte in the hopes of attaining more substantial proliferation.[13,16,17] However, zebrafish and other lower organisms do not employ c-Kit$^+$ or other types of progenitor cells

in creating new cardiomyocytes in the heart, hence there is lack of evolutionary conservation for a proposed mechanism involving stem cells making new myocytes.

The adult mouse or human heart appears to have the ability to generate as much as 1–2% new cardiomyocytes each year.[6,17] Studies dating back nearly 20 years had already made a convincing technical argument that cardiomyocytes within the adult mouse heart can only undergo limited cell cycle activity of approximately 1% per year.[18] The most conclusive of these studies was from Field and colleagues in which they measured DNA synthesis in the mouse heart with [^{3}H]-thymidine in a transgenic model expressing β-galactosidase (also referred to as, LacZ) in only cardiomyocyte nuclei for unequivocal scoring.[19] More recently, *in vivo* cellular labeling in the mouse with [^{15}N]-thymidine multi-isotope imaging mass spectrometry showed a similar rate of 1% cardiomyocyte renewal per year, but from existing cardiomyocytes.[20] Finally, 2 studies in the human heart were conducted based on the post-mortem analysis of hearts for [^{14}C]-isotope labeling in individuals that were alive during the nuclear testing era when atmospheric levels of this carbon isotope were exceedingly high, as well as afterwards when atmospheric levels dropped to only trace levels.[21,22] In both a first study published in 2009 in *Science* and a subsequent study published in 2015 in *Cell*, the estimated rate of cardiomyocyte DNA synthesis was placed at 0.5–1.5% per year in adult humans.[21,22]

The conclusion that cardiomyocytes within the adult mammalian heart have a limited capacity for renewal of approximately 1% per year from existing myocytes would appear to be solid, as the data span at least 3 different approaches and some 20 years' time and emanate from independent laboratories lacking known association or co-publications. However, this potential conclusion apposed previous and ongoing claims from the laboratories that had reported cardiomyocyte renewal rates an order of magnitude higher, and that this renewal was due to the activity of progenitor cells.[4] The argument put forth by Drs Leri and Anversa in a recent review is based on assumptions of ongoing rates of cardiomyocyte apoptosis in the heart at baseline.[23] They suggest that the heart loses significant and progressively more numbers of cardiomyocytes each year due to ongoing apoptosis, so there must be a much higher level of new myocyte formation than 1% per year to maintain cellular homeostasis at baseline.[23] However, it is just as possible that rates of ongoing apoptosis in the human heart were previously overestimated by 5–10-fold, which would then bring the balance back into synchronization of approximately 1%. Indeed, Saraste et al estimated rates of ongoing cardiomyocyte apoptosis in control adult atrial and ventricular tissue of approximately 1.0–3.5% each year,[24] which is more similar to the accepted rates of yearly cardiomyocyte renewal that was discussed above. Hence, the assertion that the adult mammalian heart has a very limited endogenous capacity to generate new cardiomyocyte is likely consistent with the comparably low rates of cardiomyocyte loss each year.

Relevance of putative adult cardiac progenitor cell sources

We have attempted to focus our discussion on the natural ability of the heart to regenerate and produce new cardiomyocytes, either at baseline, with aging or after an injury event. This focus on endogenous repair seems important in defining a starting point in which we can all

agree, so that we can attempt to tackle more controversial areas while also holding to basic tenets of biology. For example, it is well established that the failing heart gradually loses cardiomyocytes over time and that this phenomenon underlies a significant aspect of heart failure progression towards death. We also observe clinically that areas of injured myocardium, whether scarred or not, do not appreciably resolve in patients.[25] Hence, the adult heart is arguably incapable of meaningful self-renewal, but the yearly 1% cardiomyocyte renewal rate discussed above could be physiologically meaningful when summated over several decades. Finally, this 1% cardiomyocyte renewal rate that is observed each year in the heart appears to be primarily due to the activity of existing cardiomyocytes, not the activity of an adult cardiac progenitor cell.

Some past studies employing progenitor cells collected from either bone marrow, the heart itself, or other selected tissues, have shown remarkable levels of new cardiomyocyte formation within the heart when injected, which also restored cardiac function after MI injury.[4,5] Indeed, as discussed in the opening paragraph, Orlic et al claimed that bone marrow c-Kit$^+$ Lin$^-$ fractionated cells could regenerate 68% of an infarcted region of the heart with new healthy myocardium by the direct re-commitment of these cells into cardiomyocytes and vascular cells.[1] Two years later Beltrami et al from the same laboratory claimed that c-Kit$^+$ cells isolated from the heart itself, expanded *ex vivo* and injected back into the infarction injured heart, could directly regenerate 70% of this region with new contractile tissue.[26] While these results are remarkable, they remain at odds with data from other laboratories and the known biology of c-Kit$^+$ cells in general, and the basic tenets of stem cell biology that were discussed so far and will be expanded upon in a sub-section at the end of this review.[6,27,28] It is also important to note that the approach by Beltrami et al,[26] in which they isolated and exogenously expanded c-Kit$^+$ cells for injection into the heart does not necessarily reflect the underlying natural endogenous repair capacity of the heart so that injections of such cells in a transplantation experiment might still provide some other benefit to the heart, such as a paracrine effect.[7,29]

If unique progenitor-like cells are someday reproducibly identified from the heart,[29] or properly re-engineered,[30] such cells could be therapeutically meaningful in generating new cardiomyocytes when injected *in vivo*. The reprogramming of cardiac fibroblasts with cardiac-inducing transcription factors was clearly shown to create new cardiomyocytes,[31] although endogenous fibroblasts are not cardiomyocyte progenitor cells. Another tenet to consider is that if cells were successfully re-engineered to make *bonafide* new cardiomyocytes *in vivo* at an appreciable level they would likely cause arrhythmia, as shown recently with human embryonic stem cells programmed to become new immature cardiomyocytes and injected into the non-human primate heart.[32] Such arrhythmias would develop because inductions of low-resistance pathways in the cardiac conduction syncytium promotes current re-entry leading to premature excitation. Hence, one could argue that perhaps the reason arrhythmias were not described in past animal studies or human clinical trials with c-Kit$^+$ derived cells, whether from bone marrow or expanded from the heart itself, is because new contracting cardiomyocytes were never generated. Otherwise it should have induced arrhythmias.

These caveats aside, an emerging consensus from animal studies and even human studies with cellular transplantation experiments is that the field has yet to identify, in an inter-laboratory reproducible manner, a putative progenitor cell population that can transdifferentiate into beating cardiomyocytes once injected *in vivo*.[29] Hence, past studies that reported the ability of injected c-Kit$^+$ progenitors to directly generate new cardiomyocytes in restoring heart tissue and function in experimental injury models need to be re-examined with exchanging of samples and personnel between independent laboratories. Indeed, such an exchange was indirectly accomplished when Bolli and colleagues published 2 recent papers whereby they isolated then injected cardiac c-Kit$^+$ cells into the injured rodent heart, showing that none of these cells become cardiac myocytes and that most simply perished and were no longer detectable within a few weeks.[33,34] Hence, even without a discussion of the most recent *Kit* allele lineage tracing results from the past 2 years, as will be discussed below, a consensus is already emerging that holds that c-Kit$^+$ cells are not physiologically relevant cardiomyocyte producing progenitors. However, while injection of c-Kit$^+$ cells into the injured rodent myocardium did not produce new cardiomyocytes, it nonetheless augmented cardiac function likely through a paracrine mechanism of action.[33,34]

Kit allele lineage tracing to examine endogenous cardiac regeneration

As discussed above, much of the data in support of an endogenous cardiac progenitor cell acting in the replacement or turnover of adult mammalian cardiomyocytes were from cellular transplantation experiments. To directly examine the potential of endogenous c-Kit$^+$ cells to have progenitor-like activity *in vivo*, van Berlo et al targeted the *Kit* genetic locus in the mouse with the Cre recombinase cDNA (*Kit^{Cre}*) for lineage tracing analysis of the cell-types that express c-Kit or are derived from them.[35] Mice containing the *Kit^{Cre}* allele were crossed with Cre-dependent reporter mice (*Rosa26-loxP-STOP-loxP-eGFP* (R-eGFP) in which eGFP (enhanced green fluorescent protein) is expressed after recombination in all c-Kit$^+$ cells and their progeny.[35] van Berlo et al showed that the *Kit^{Cre}* allele faithfully recapitulated endogenous c-Kit protein expression throughout all known regions in the mouse (bone marrow, intestine, testis, skeletal muscle, skin, lung), including endogenous c-Kit$^+$ mononuclear cells within the heart, both low and high expressing.[35] Remarkably, this lineage tracing strategy, at 4 weeks of age, showed only 0.027% cardiomyocyte labeling although endothelial cells were abundantly labeled.[35] Importantly, the *Kit^{Cre}* allele is expressed constitutively throughout all of development and adulthood, hence a rate of 0.027% cardiomyocyte labeling at 4 weeks of age indicated that c-Kit expressing progenitor cells are not appreciably part of the primary heart field during embryogenesis,[35] in contrast to previous reports suggesting otherwise.[7,36] These results also indicate that embryonic and adult cardiomyocytes do not express the *Kit* allele in its properly configured state. However, transgenic approaches that utilize fragments of the *Kit* promoter to drive expression will likely not reflect true endogenous expression, even producing ectopic expression in cardiomyocytes, as *Kit* allele promoter fragments (even very large ones) are unreliable *in vivo*.[28]

To isolate the formation of new cardiomyocytes in the adult mouse heart after injury, van Berlo et al also targeted the *Kit* allele with the tamoxifen-regulated MerCreMer cDNA

(*Kit*^{MerCreMer}).[35] Lineage tracing with the *Kit*^{MerCreMer} allele and the R-eGFP reporter allele identified extremely low rates of eGFP^{+} traced cardiomyocytes in the adult heart with various labeling times up through 6 months of age.[35] MI injury to the adult heart showed rates of new eGFP^{+} traced cardiomyocytes of only 0.016%, and only 0.007% after isoproterenol infusion-induced injury, indicating that endogenous c-Kit^{+} cells were not generating significant new cardiomyocytes *in vivo* after injury.[35] Importantly, these results were recently confirmed in a blinded manner by another independent laboratory.[37] More specifically, Houser and colleagues used the exact same mice as van Berlo et al and reported a nearly identical low rate of *Kit*-dependent lineage traced cardiomyocytes at baseline in the adult heart, as well as no appreciable increase after isoproterenol injury.[37]

While the results of van Berlo et al showed a very low rate of new cardiomyocyte generation in the heart from *Kit* lineage-traced cells, more careful investigation showed that approximately 85% of these labeled cardiomyocytes were simply due to fusion of c-Kit traced immune cells with cardiomyocytes.[35] Recall that all immune cells in the *Kit*^{Cre} lineage tracing mice will be eGFP positive, as they are descended from c-Kit^{+} hematopoietic progenitors. Hence, the true rate of c-Kit^{+} cells having cardiomyocyte progenitor capacity is even much lower, approximately 0.005% throughout development and 0.002% after injury to the heart.[35] This consideration notwithstanding, the results of van Berlo et al still demonstrate that c-Kit^{+} cells have some definable rate of generating *de novo* cardiomyocytes in the heart, even though it is highly unlikely to be physiologically meaningful. In contrast, c-Kit^{+} lineage traced cells did generate abundant CD31^{+} endothelial cells in the heart, indicating that these cells are endothelial progenitors that are likely to be important for vascular repair in the heart and of potential medicinal value. At the minimum, these results of widespread CD31^{+} cell production from a putative hemangioblast c-Kit^{+} progenitor cell demonstrates the validity of the *Kit* allele targeting, and that the heterozygosity of this locus does not disrupt the basic biology of c-Kit expressing cells.

The genetic lineage tracing data of van Berlo et al was immediately questioned,[38] but was quickly supported in the literature by another independent study from Sultana et al.[39] In this later study, Sultana et al created three knock-in mouse lines with insertion of H2B-tdTomato, nlacZ/H2B-GFP (dual reporter) or MerCreMer into *Kit* locus for lineage tracing (*Kit*$^{H2B\text{-}tdTomato}$, *Kit*$^{nlacZ/H2B\text{-}GFP}$ and *Kit*^{MerCreMer}). These targeted lines each mirrored endogenous c-Kit expression in the mouse in known regions such as heart, lung, liver, intestine, stomach, melanocytes, spleen and umbilical cord.[39] Using these genetic tools it was confirmed that c-Kit^{+} cells were indeed endothelial progenitors but that they did not generate appreciable cardiomyocytes.[39] More specifically, *Kit* allele traced cells did not express the myocardial-specific marker cardiac troponin T (cTnT) after birth or during development of the heart (<0.04% at embryonic day 13.5 to postnatal day 60; <0.007% after postnatal day 90). These results again support the conclusion that c-Kit^{+} cells are not myocardial progenitor cells, in contrast to previous suggestions.[7,36] Upon MI injury Sultana et al showed that the *Kit* allele lineage traced cells still maintained their endothelial cell propensity, with less than 0.002% of myocardial cells identified.[39] These observations support van Berlo and again suggest that c-Kit^{+} cells lack significant cardiomyocyte progenitor cell activity. Moreover, using another independent approach that would bypass concerns of cellular fusion, Sultana et al used mice containing a *Kit*^{MerCreMer} allele that

were crossed with mice containing a cardiac-specific reporter knock-in allele ($cTnT^{nlacZ-/H2B-GFP}$).[39] This elegant approach further confirmed that c-Kit$^+$ cells rarely generate *de novo* cardiomyocytes in the heart (~0.005% of total c-Kit$^+$ cells and ~0.002% of total myocardial cells in the heart).[39]

As even further evidence, a third independent research group also recently described the generation of *Kit* allele-dependent lineage tracing mice.[40] Liu et al targeted the *Kit* allele with the CreERT2 cDNA for tamoxifen inducible Cre recombinase activity, which they also used in conjunction with a *Rosa26* red fluorescent protein (RFP) reporter allele ($Kit^{CreERT2}$; $Rosa26^{RFP}$).[40] Here the authors examined *Kit* lineage cells in the heart at 48 hours after tamoxifen treatment, revealing a very low number of RFP$^+$ cardiomyocytes (0.035%). Importantly, these RFP$^+$ cardiomyocytes were unlikely to have been generated by differentiation of c-Kit$^+$ progenitors given such a short time window.[40] Indeed, Liu et al suggested that RFP$^+$ cardiomyocytes detected in the heart at baseline with injury were from cardiomyocytes that expressed the $Kit^{CreERT2}$ allele itself, rather than by differentiation of c-Kit$^+$ progenitors. This means that the *Kit* allele itself can be rarely and randomly activated in cardiomyocytes, further downplaying the cardiomyogenic potential of c-Kit$^+$ cells in the heart. They also showed that MI injury did not promote an increase in c-Kit$^+$ lineage traced cardiomyocytes (0.035% at baseline versus 0.034% after MI).[40] However, van Berlo generated data suggesting that c-Kit$^+$ cells can produce low levels of cardiomyocytes *in vivo*, and that it is not simply aberrant activation of Kit^{Cre} in rare cardiomyocytes.[35] van Berlo et al generated *Kit* null embryos and showed that they lacked this low rate of lineage traced cardiomyocytes in the developing heart.[35] Importantly, full deletion of the *Kit* gene (both alleles) essentially eliminates the progenitor activity of c-Kit$^+$ cells,[35] but it would have no effect on spontaneously activating the Kit^{Cre} allele in a cardiomyocyte, meaning that the loss of the low level of lineage traced cardiomyocytes in the embryonic heart with *Kit* deletion proves that c-Kit$^+$ cells have this very low level activity of *de novo* cardiomyocyte creation.

Finally, a fourth group also recently reported *Kit* allele-dependent lineage tracing in the heart. Saur and colleagues generated an independent version of $Kit^{CreERT2}$ knock-in mice, which were constructed with 2 independent ATG containing 1st exons.[41] Using these mice Hatzistergos et al traced the progeny of c-Kit$^+$ cells in the early embryonic and postnatal heart.[42,43] They used $Kit^{CreERT2}$ in conjunction with *Wnt1::Flpe* and dual-recombinase responsive indicators (*RC::Fela* and *RC::Frepe*)[44,45] and detected cardiac neural crest derived c-Kit traced cells in the early mouse embryo, although quantitation was not performed so that the potential significance of this finding is unclear.[43] However, Hatzistergos et al reported that the lineage traced cardiomyocytes in the early embryonic and postnatal c-Kit$^+$ cells were significantly lower than expected as well as lacking in labeled endothelial cells.[42,43] It should be noted that the $Kit^{CreERT2}$ allele used in this study has 2 engineered ATG start sites that appears to disrupt true *Kit* expression given that these mice show no labeling of bone marrow-derived c-Kit$^+$ cells, other than mast cells.[44,46] Nevertheless, all these studies with *Kit* allele-dependent lineage tracing do not exclude the ability of neonatal derived c-Kit$^+$ cells manipulated in culture to express select cardiomyocyte specific genes.[47,48] The results discussed in this section simply indicate that

endogenous c-Kit$^+$ cells lack full cardiomyogenic potential *in vivo*, whether during embryonic, postnatal development or in the adult heart under any conditions.

Concerns with *Kit* allele-based lineage tracing

The primary criticism of the lineage tracing approaches discussed above is that they produce a single *Kit* null allele based on the "knock-in" procedure itself (loss of function), meaning heterozygosity in c-Kit expression and a possible alteration in the activity of c-Kit expressing progenitor cells. We discussed this issue at length in a recent editorial and why this is unlikely to be a significant overall concern,[49] but will briefly discuss the evidence again here, as this issue continues to resurface.[50] First, single allele *Kit^{Cre}* mice (heterozygous) showed the same number of c-Kit$^+$ mononuclear cells within the heart and in bone marrow as wildtype controls, indicating no reduction in c-Kit$^+$ cellular content due to heterozygosity.[49] Moreover, isolation of these *Kit* allele heterozygous cells from the adult heart revealed the same potential as wildtype cells to upregulate aspects of cardiac gene expression under dexamethasone culture conditions, such as induction of Gata4 and troponin T expression (reference 35 [Extended Data Figure 9] and data not shown). Also, the recombination efficiency of the *Kit^{Cre}* allele and the lineage tracing approach is approximately 80%, generating the concern that the unlabeled, possibly lower expressing c-Kit$^+$ cells, might preferentially contribute to a more specialized pool of cells that more readily generates cardiomyocytes. However, van Berlo et al[35] have shown that recombined and nonrecombined c-Kit$^+$ progenitors (antibody sorted) from the heart equally induced Gata4 and cTnT expression under dexamethasone treatment, suggesting that the unlabeled c-Kit$^+$ cells are not more cardiomyogenic.[35] Finally, *Kit^{Cre}* heterozygous mice still showed abundant endothelial cell production in the heart, hence they maintain their true progenitor-like functionality in generating this cell-type, despite the loss of 1 *Kit* allele.[35,39]

Basic tenets of stem cell biology from known regenerative tissues

The proposition that cardiac c-Kit$^+$ cells function as endogenous cardiomyocyte producing "stem cells" breaks with the biology of how stem cells function and are defined in known regenerative tissues. For example, adult mammalian skeletal muscle is highly regenerative and within this tissue stem cells comprise as much as 5% of the total nuclei. More importantly, these stem cells are interspersed evenly throughout the tissue so as to respond to both small local areas of injury but also to generate widespread and temporally homogenous regeneration after larger injury events. Skeletal muscle stem cells (also referred to as satellite cells) are easily isolated and made to differentiate into myotubes in a highly reproducible manner across all laboratories that work in the field. Moreover, injecting skeletal muscle satellite cells into different tissues, such as the heart, directly generates only skeletal muscle cell containing grafts.[51] Indeed, even c-Kit$^+$ progenitor cells isolated from bone marrow, where such cells function as true hematopoietic progenitors, only generate immune cells when injected into the heart.[2,3] Finally, tissues that regenerate due to the activity of an endogenous stem cell show an evolutionary conservation in this mechanism at least down through zebrafish. These basic tenets of stem cell biology are all broken by the concept that the heart contains a c-Kit$^+$ progenitor cell predetermined to generate new cardiomyocytes. First, c-Kit$^+$ mononuclear cells in adult mouse heart are exceedingly rare with total levels

estimated at 1 to 5 cells per histological section of the entire heart.[35] This is clearly too sparse of a cellular concentration to coordinate meaningful renewal. Second, c-Kit$^+$ cells are not employed by zebrafish for heart regeneration, but instead existing cardiomyocytes are the underlying mechanism of regeneration. Finally, the field cannot reproducibly isolate a definable c-Kit$^+$ cell population from the adult heart that differentiates into beating cardiomyocytes in culture or when injected back into the heart.[33,34,47,48] Hence, the entire concept of a predestined cardiomyocyte producing c-Kit$^+$ stem cell being present within the heart breaks the known biologic principles of stem cell biology in regenerative tissues.

Conclusions and future directions

In this review we supported one view of a long-standing area of controversy in cardiac regeneration because we believe that the emerging body of data in the literature point only in this direction. More than this, basic tenets of biology support a broader conclusion that the adult mammalian heart is not an inherently regenerative organ when left to its own devices; hence it likely lacks a true physiologic stem cell pool as defined in other regenerative tissues. Second, the rate of cardiomyocyte renewal activity in the adult heart of 1% per year is astonishingly low, below the level that would be expected if there was a true endogenous stem cell present in the heart that was predetermined to make new cardiomyocytes. Not to mention that previous studies showed that such renewal was due primarily to proliferation of existing cardiomyocytes, which is a conserved mechanism in zebrafish. Third, genetic lineage tracing strategies in the mouse from multiple unlinked and independent laboratories do not support the concept that endogenous c-Kit$^+$ cells generate cardiomyocytes at physiologically meaningful levels, with estimates spanning from 1 in 3500 cells to well over 1 in 50,000, or even non-existent.

In addition to a lack of a c-Kit$^+$ cardiomyocyte producing stem cell, other genetic strategies have similarly down-played the likelihood that a non-c-Kit$^+$ expressing stem cell might exist in the heart contributing to meaningful renewal or repair (e.g., side population cells,[52] Sca-1 expressing cells,[53] cells derived from the adult epicardium[54]). Mesenchymal stromal cells were also previously proposed as having cardiomyogenic potential, but these cells are essentially fibroblast-like stromal cells from the bone marrow that recent studies have shown possess no ability to generate cardiomyocytes.[7] Finally, even studies with *ex vivo* expanded progenitor cells, or primary isolated bone marrow mononuclear cells, which were injected into the heart and claimed to generate abundant new cardiomyocytes, have been refuted due to lack of reproducibility and the inability to define these presumed stem cells consistently between laboratories.

While we highlighted an emerging consensus hypothesis here that the heart lacks a cardiomyocyte producing stem cell as classically defined, this new state of affairs does not refute in any way the greater concept of cellular therapy as a potential treatment for human heart disease. Indeed, cardiomyocytes derived from induced pluripotent stem cells, from embryonic stem cells,[32] and even from the transcriptional reprogramming of other cell-types,[31] have emerged as cellular approaches for attempting to regenerate the heart. In this context, perhaps reprogramming c-Kit$^+$ cells would be more efficient than attempting to reprogram fibroblasts with cardiogenic transcription factors as c-Kit$^+$ cells already have a

low predilection for inducing cardiomyocytes in vivo, or at least induction of cardiac differentiation-specific genes.[35] Lastly, cellular therapies have other potential benefits that requires more investigation, such as the paracrine hypothesis, which proposes that selected secreted factors produced by the injected cells within the damaged area of the heart can impart better cardiac function through multiple cellular rejuvenating mechanisms.[7,33,34] Thus, considering the data collected in animal models whereby injected progenitor cells from various sources all uniformly appear to benefit the heart though non-engraftment dependent mechanisms, more research is needed to understand the basic biology of how such cellular transplantation therapies provide benefit.

Acknowledgments

Funding Sources

J.D.M. and C-L C. were supported by grants from the National Institutes of Health and C-L.C. received funding from the American Heart Association. J.D.M was also supported by the Howard Hughes Medical Institute.

Non-standard Abbreviations and Acronyms

eGFP	enhanced green fluorescent protein
MI	myocardial infarction
RFP	red fluorescent protein
R-eGFP	Rosa26-loxP-STOP-loxP-eGFP
cTnT	cardiac troponin T

References

1. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature. 2001; 410:701–705. [PubMed: 11287958]
2. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature. 2004; 428:668–673. [PubMed: 15034594]
3. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004; 428:664–668. [PubMed: 15034593]
4. Anversa P, Kajstura J, Rota M, Leri A. Regenerating new heart with stem cells. J Clin Invest. 2013; 123:62–70. [PubMed: 23281411]
5. Nadal-Ginard B, Ellison GM, Torella D. The cardiac stem cell compartment is indispensable for myocardial cell homeostasis, repair and regeneration in the adult. Stem Cell Res. 2014; 13:615–630. [PubMed: 24838077]
6. van Berlo JH, Molkentin JD. An emerging consensus on cardiac regeneration. Nat Med. 2014; 20:1386–1393. [PubMed: 25473919]
7. Keith MC, Bolli R. "String theory" of c-kit(pos) cardiac cells: a new paradigm regarding the nature of these cells that may reconcile apparently discrepant results. Circ Res. 2015; 116:1216–1230. [PubMed: 25814683]

8. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. Science. 2011; 331:1078–1080. [PubMed: 21350179]
9. Oberpriller JO, Oberpriller JC. Response of the adult newt ventricle to injury. J Exp Zool. 1974; 187:249–253. [PubMed: 4813417]
10. Becker RO, Chapin S, Sherry R. Regeneration of the ventricular myocardium in amphibians. Nature. 1974; 248:145–147. [PubMed: 4818918]
11. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. Science. 2002; 298:2188–2190. [PubMed: 12481136]
12. Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss KD. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. Nature. 2010; 464:601–605. [PubMed: 20336144]
13. Foglia MJ, Poss KD. Building and re-building the heart by cardiomyocyte proliferation. Development. 2016; 143:729–740. [PubMed: 26932668]
14. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, Martin JF. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. Science. 2011; 332:458–461. [PubMed: 21512031]
15. Mahmoud AI, Kocabas F, Muralidhar SA, Kimura W, Koura AS, Thet S, Porrello ER, Sadek HA. Meis1 regulates postnatal cardiomyocyte cell cycle arrest. Nature. 2013; 497:249–253. [PubMed: 23594737]
16. Szibor M, Pöling J, Warnecke H, Kubin T, Braun T. Remodeling and dedifferentiation of adult cardiomyocytes during disease and regeneration. Cell Mol Life Sci. 2014; 71:1907–1916. [PubMed: 24322910]
17. Uygur A, Lee RT. Mechanisms of Cardiac Regeneration. Dev Cell. 2016; 36:362–374. [PubMed: 26906733]
18. Soonpaa MH, Field LJ. Survey of studies examining mammalian cardiomyocyte DNA synthesis. Circ Res. 1998; 83:15–26. [PubMed: 9670914]
19. Soonpaa MH, Field LJ. Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. Am J Physiol. 1997; 272:H220–H226. [PubMed: 9038941]
20. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP, Lee RT. Mammalian heart renewal by pre-existing cardiomyocytes. Nature. 2013; 493:433–436. [PubMed: 23222518]
21. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. Science. 2009; 324:98–102. [PubMed: 19342590]
22. Bergmann O, Zdunek S, Felker A, Salehpour M, Alkass K, Bernard S, Sjostrom SL, Szewczykowska M, Jackowska T, Dos Remedios C, Malm T, Andrä M, Jashari R, Nyengaard JR, Possnert G, Jovinge S, Druid H, Frisén J. Dynamics of Cell Generation and Turnover in the Human Heart. Cell. 2015; 161:1566–1575. [PubMed: 26073943]
23. Anversa P, Kajstura J, Rota M, Leri A. Regenerating new heart with stem cells. J Clin Invest. 2013; 123:62–70. [PubMed: 23281411]
24. Saraste A, Pulkki K, Kallajoki M, Heikkilä P, Laine P, Mattila S, Nieminen MS, Parvinen M, Voipio-Pulkki LM. Cardiomyocyte apoptosis and progression of heart failure to transplantation. Eur J Clin Invest. 1999; 29:380–386. [PubMed: 10354194]
25. Dawn B, Zuba-Surma EK, Abdel-Latif A, Tiwari S, Bolli R. Cardiac stem cell therapy for myocardial regeneration. A clinical perspective. Minerva Cardioangiol. 2005; 53:549–564. [PubMed: 16333238]
26. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell. 2003; 114:763–776. [PubMed: 14505575]
27. Hesse M, Fleischmann BK, Kotlikoff MI. Concise review: The role of C-kit expressing cells in heart repair at the neonatal and adult stage. Stem Cells. 2014; 32:1701–1712. [PubMed: 24585704]
28. Molkentin JD, Houser SR. Are resident c-Kit+ cardiac stem cells really all that are needed to mend a broken heart? Circ Res. 2013; 113:1037–1039. [PubMed: 24115067]

29. Quijada P, Sussman MA. Making it stick: chasing the optimal stem cells for cardiac regeneration. Expert Rev Cardiovasc Ther. 2014; 12:1275–1288. [PubMed: 25340282]
30. Nagalingam RS, Safi HA, Czubryt MP. Gaining myocytes or losing fibroblasts: Challenges in cardiac fibroblast reprogramming for infarct repair. J Mol Cell Cardiol. 2016; 93:108–114. [PubMed: 26640115]
31. Qian L, Srivastava D. Direct cardiac reprogramming: from developmental biology to cardiac regeneration. Circ Res. 2013; 113:915–921. [PubMed: 24030021]
32. Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Cook SM, Palpant NJ, Gantz JA, Fugate JA, Muskheli V, Gough GM, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem HP, Laflamme MA, Murry CE. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature. 2014; 510:273–277. [PubMed: 24776797]
33. Tang XL, Li Q, Rokosh G, Sanganalmath SK, Chen N, Ou Q, Stowers H, Hunt G, Bolli R. Long-Term Outcome of Administration of c-kit(POS) Cardiac Progenitor Cells After Acute Myocardial Infarction: Transplanted Cells Do not Become Cardiomyocytes, but Structural and Functional Improvement and Proliferation of Endogenous Cells Persist for at Least One Year. Circ Res. 2016; 118:1091–1105. [PubMed: 26838790]
34. Hong KU, Guo Y, Li QH, Cao P, Al-Maqtari T, Vajravelu BN, Du J, Book MJ, Zhu X, Nong Y, Bhatnagar A, Bolli R. c-kit+ Cardiac stem cells alleviate post-myocardial infarction left ventricular dysfunction despite poor engraftment and negligible retention in the recipient heart. PLoS One. 2014; 9:e96725. [PubMed: 24806457]
35. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marbán E, Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. Nature. 2014; 509:337–341. [PubMed: 24805242]
36. Wu SM, Fujiwara Y, Cibulsky SM, Clapham DE, Lien CL, Schultheiss TM, Orkin SH. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. Cell. 2006; 127:1137–1150. [PubMed: 17123591]
37. Wallner M, Duran JM, Mohsin S, Troupes CD, Vanhoutte D, Borghetti G, Vagnozzi RJ, Gross P, Yu D, Trappanese DM, Kubo H, Toib A, Sharp TE 3rd, Harper SC, Volkert MA, Starosta T, Feldsott EA, Berretta RM, Wang T, Barbe MF, Molkentin JD, Houser SR. Acute Catecholamine Exposure Causes Reversible Myocyte Injury Without Cardiac Regeneration. Circ Res. 2016; 119:865–879. [PubMed: 27461939]
38. Nadal-Ginard B, Ellison GM, Torella D. Absence of evidence is not evidence of absence: pitfalls of cre knock-ins in the c-Kit locus. Circ Res. 2014; 115:415–418. [PubMed: 24965482]
39. Sultana N, Zhang L, Yan J, Chen J, Cai W, Razzaque S, Jeong D, Sheng W, Bu L, Xu M, Huang GY, Hajjar RJ, Zhou B, Moon A, Cai CL. Resident c-kit(+) cells in the heart are not cardiac stem cells. Nat Commun. 2015; 6:8701. [PubMed: 26515110]
40. Liu Q, Yang R, Huang X, Zhang H, He L, Zhang L, Tian X, Nie Y, Hu S, Yan Y, Zhang L, Qiao Z, Wang QD, Lui KO, Zhou B. Genetic lineage tracing identifies in situ Kit-expressing cardiomyocytes. Cell Res. 2016; 26:119–130. [PubMed: 26634606]
41. Klein S, Seidler B, Kettenberger A, Sibaev A, Rohn M, Feil R, Allescher HD, Vanderwinden JM, Hofmann F, Schemann M, Rad R, Storr MA, Schmid RM, Schneider G, Saur D. Interstitial cells of Cajal integrate excitatory and inhibitory neurotransmission with intestinal slow-wave activity. Nat Commun. 2013; 4:1630. [PubMed: 23535651]
42. Hatzistergos KE, Saur D, Seidler B, Balkan W, Breton M, Valasaki K, Takeuchi LM, Landin AM, Khan A, Hare JM. Stimulatory Effects of Mesenchymal Stem Cells on cKit+ Cardiac Stem Cells Are Mediated by SDF1/CXCR4 and SCF/cKit Signaling Pathways. Circ Res. 2016; 119:921–930. [PubMed: 27481956]
43. Hatzistergos KE, Takeuchi LM, Saur D, Seidler B, Dymecki SM, Mai JJ, White IA, Balkan W, Kanashiro-Takeuchi RM, Schally AV, Hare JM. cKit+ cardiac progenitors of neural crest origin. Proc Natl Acad Sci USA. 2015; 112:13051–13056. [PubMed: 26438843]
44. Jensen P, Farago AF, Awatramani RB, Scott MM, Deneris ES, Dymecki SM. Redefining the serotonergic system by genetic lineage. Nat Neurosci. 2008; 11:417–419. [PubMed: 18344997]

45. Engleka KA, Manderfield LJ, Brust RD, Li L, Cohen A, Dymecki SM, Epstein JA. Islet1 derivatives in the heart are of both neural crest and second heart field origin. Circ Res. 2012; 110:922–926. [PubMed: 22394517]

46. Heger K, Seidler B, Vahl JC, Schwartz C, Kober M, Klein S, Voehringer D, Saur D, Schmidt-Supprian M. CreERT2) expression from within the c-Kit gene locus allows efficient inducible gene targeting in and ablation of mast cells. Eur J Immunol. 2014; 44:296–306. [PubMed: 24127407]

47. Zaruba MM, Soonpaa M, Reuter S, Field LJ. Cardiomyogenic potential of C-kit(+)-expressing cells derived from neonatal and adult mouse hearts. Circulation. 2010; 121:1992–2000. [PubMed: 20421520]

48. Jesty SA, Steffey MA, Lee FK, Breitbach M, Hesse M, Reining S, Lee JC, Doran RM, Nikitin AY, Fleischmann BK, Kotlikoff MI. c-kit+ precursors support postinfarction myogenesis in the neonatal, but not adult, heart. Proc Natl Acad Sci USA. 2012; 109:13380–13385. [PubMed: 22847442]

49. Molkentin JD. Letter by Molkentin regarding article, "The absence of evidence is not evidence of absence: the pitfalls of Cre Knock-Ins in the c-Kit Locus". Circ Res. 2014; 115:e21–e23. [PubMed: 25258403]

50. Leri A, Anversa P. Complexity of Tracking the Fate of Adult Progenitor Cells. Circ Res. 2016; 119:1067–1070. [PubMed: 27789586]

51. Menasché P. Skeletal myoblasts as a therapeutic agent. Prog Cardiovasc Dis. 2007; 50:7–17. [PubMed: 17631434]

52. Doyle MJ, Maher TJ, Li Q, Garry MG, Sorrentino BP, Martin CM. Abcg2-Labeled Cells Contribute to Different Cell Populations in the Embryonic and Adult Heart. Stem Cells Dev. 2016; 25:277–284. [PubMed: 26573225]

53. Noseda M, Harada M, McSweeney S, Leja T, Belian E, Stuckey DJ, Abreu Paiva MS, Habib J, Macaulay I, de Smith AJ, al-Beidh F, Sampson R, Lumbers RT, Rao P, Harding SE, Blakemore AI, Jacobsen SE, Barahona M, Schneider MD. PDGFRα demarcates the cardiogenic clonogenic Sca1+ stem/progenitor cell in adult murine myocardium. Nat Commun. 2015; 6:6930. [PubMed: 25980517]

54. Zhou B, Honor LB, Ma Q, Oh JH, Lin RZ, Melero-Martin JM, von Gise A, Zhou P, Hu T, He L, Wu KH, Zhang H, Zhang Y, Pu WT. Thymosin beta 4 treatment after myocardial infarction does not reprogram epicardial cells into cardiomyocytes. J Mol Cell Cardiol. 2012; 52:43–47. [PubMed: 21907210]