I n 1998, Anversa et al.3 provided the first evidence that the mammalian heart is not a terminally differentiated organ, demonstrating the production of new cardiomyocytes in adulthood. Although intense debate still exists on the exact rate at which this occurs,7–11 this paradigm changing observation has been subsequently documented with sophisticated animal models8,12,13 and with14C-dating experiments in humans.8,9 After this initial experiment, evidence emerged that extracardiac cells, some of which express cKit, could contribute to cardiac renewal.13–17

In 2003, cKit+ cells in the heart were identified as resident cardiac stem cells, capable of generating new coronary vessels and cardiomyocytes in response to injury.18 These cells were described to reside in cardiac stem cell niches19; were capable of ex vivo propagation and clonal expansion; exhibited capacity for in vitro and in vivo differentiation into cardiomyocytes, endothelial cells, and smooth muscle cells20,21; participated in the in vitro formation of cardiospheres similar to neural stem cells22–24; and were negative for hematopoietic lineage markers.18,19 Several groups demonstrated ex vivo propagation of cardiac stem cells that were subsequently transplanted in injured hearts leading to enhanced regeneration via mechanisms involving their direct differentiation into new cardiomyocytes and vessels.18,21,22 These observations warranted the approval of clinical trials from the Food and Drug Administration to test the safety and efficacy of resident cardiac stem/progenitor cells for the treatment of human heart disease (NCT00893360).25,26

In 2014, a study by van Berlo et al2 reported that cKit cells in the heart have minimal cardiomyogenic ability; hence, their therapeutic importance may be marginal. Two mouse lines, carrying 2 different versions of Cre recombinase (a recombinase enzyme derived from the P1 bacteriophage; either a tamoxifen-inducible MerCreMer [a version of Cre, fused to 2 copies of a mutant murine estrogen receptor ligand–binding domain [amino acids 281–599] with a G525R substitution] or a noninducible Cre-IREs-eGFPnls [nls stands for nuclear localization signal]) under the control of the endogenous cKit promoter and Cre-reporter genes, were engineered to track the fate of cKit-expressing cells in the heart (Figure). Expression of reporters was analyzed either before or in response to myocardial infarction injury. In both cases, van Berlo et al.2 found that the vast majority of the cardiac cells that had undergone Cre-mediated recombination were endothelial cells and only rarely cardiomyocytes. This finding led the authors to conclude that cKit+ cardiac cells “may be dedicated vascular endothelial progenitor cells...[not] capable of regenerating the heart with new contracting myocardium to a physiologically meaningful extent.”11 However, because cKit expression occurs in several cardiac and extracardiac cell lineages,27 the role and identity of cardiac cKit+ cells remained elusive. Do the endothelial cells and cardiomyocytes arise from the same cKit-expressing lineage or are there multiple cKit+ lineages in the heart?27 Why do cKit+ cells differentiate into far fewer cardiomyocytes than endothelium? Are they bona fide cardiovascular progenitors or do they represent noncardiomyogenic cells that stochastically (trans)differentiate into myocardium?

Our group performed a high-resolution genetic lineage-tracing study using a different mouse line that expresses the CreER(T2 version (a version of Cre, fused to a mutant human estrogen receptor ligand–binding domain [G400V/M543A/L544A triple mutation]) of Cre recombinase from the endogenous cKit locus and a loxP-STOP-loxP–flanked Cre-reporter allele (Figure). In addition to addressing some of the issues raised by van Berlo et al.;2 we sought to not only genetically track the fate of cKit+ cells but also to identify their lineage and role in the heart.1 Importantly, we showed that cKit marks rare, bona fide cardiomyogenic progenitors in the neural crest...
lineage (as shown by intersectional genetic fate mapping with a *Wnt1*-reported mouse) with unequivocal capacity to produce new cardiomyocytes but not coronary endothelial cells. Furthermore, cardiomyocyte differentiation is governed by spatiotemporal changes in the activity of bone morphogenetic protein, a signaling pathway that subsides during migration of cKit+ neural crest cells to the heart, reducing an important activator of cardiomyocyte differentiation. Thus, the limited contribution of cKit+ cells in cardiomyocytes is not a function of deficient cardiomyogenic capacity but rather reflects a nonpermissive milieu.

The recent article by Sultana et al further clarifies the identity of the cKit+ cardiac lineage(s). The authors developed 3 new cKit knockout mouse lines to address the developmental origin of cardiac cKit+ cells, the identity of cKit+ cardiac cells, and whether cKit+ cells are useful for cardiomyocyte regeneration (Figure).}

**Overview of the cKit Knockin Alleles**

To monitor real-time expression of cKit, the authors developed 2 new reporter mouse lines (Figure). In the first mouse line, transcription of cKit results in nuclear expression of the red fluorescent protein tdTomato (tandem dimer tomato red fluorescent protein; *c-Kit*^H2B-Tomato/+). The second is a dual reporter mouse line, where transcription of cKit results in nuclear expression ofβ-galactosidase, which may be irreversibly substituted by nuclear expression of green fluorescent protein on Cre-mediated recombination (*c-Kit*^nLacZ-H2B-GFP/+). Importantly, once a cell no longer expresses cKit (ie, differentiates into a cKit-negative derivative), expression of the reporter genes (either TdTomato, GFP, or nLacZ [nuclearβ-galactosidase]) should no longer be detectable.

Both cKit reporter mouse lines exhibited broad reporter gene expression in the heart, during both development and adulthood. The degree of cKit reporter gene expression exceeds essentially all reports of cKit expression or fate-mapping studies in rodents, large mammals, and humans. For example, although van Berlo et al showed that cKit+ cardiomyogenic progenitors are not present before E12.5, and both of these studies document unequivocal evidence of cKit+/Nkx2.5+ cardiomyogenic progenitors after this time. The nature of these cells is further elucidated by experiments showing their generation in vitro from mouse induced pluripotent stem cell (iPSCs). An important limitation between all 3 studies is the utilization of knockin mouse models that render the gene-targeted cKit allele nonfunctional, which may cause abnormalities in cKit- cardiomyocytes in these mice (eg, proliferation, migration, and differentiation). BMP indicates bone morphogenetic protein; LV, left ventricle; and VS, ventricular septum.

**Figure.** The cKit receptor is expressed on Nkx2.5-negative vasculogenic cells and Nkx2.5+ cardiomyogenic lineage of neural crest origin. Three genetic lineage fate-mapping studies demonstrate cardiac cKit- cells forming cardiomyocytes in the mouse heart at a low level. The 3 different murine studies are not in full agreement on endothelial differentiation. Contrary to the study by van Berlo et al, Hatzistergos et al showed that cKit+ cardiac cells are not primed toward endothelial cell differentiation. Sultana et al also show that the coronary endothelium does not descend from cKit- cardiac progenitors, but as much as 43% of coronary endothelial cells express cKit (possibly at low levels). Both Hatzistergos et al and Sultana et al demonstrate that cKit+/Nkx2.5+ cardiomyogenic progenitors are not present before E12.5, and both of these studies document unequivocal evidence of cKit+/Nkx2.5+ cardiomyogenic progenitors after this time. The authors developed 3 new cKit knockout mouse lines to address the developmental origin of cardiac cKit+ cells, the identity of cKit+ cardiac cells, and whether cKit+ cells are useful for cardiomyocyte regeneration (Figure).
Identity of cKit+ Cell Lineage(s) in the Heart

A point of controversy between the studies by van Berlo et al11 and Hatzistergos et al1 is whether cKit+ cardiac cells are vascular endothelial progenitors. Although van Berlo et al2 described an extensive contribution of cKit+ cells to endothelial cells, Hatzistergos et al1 showed that coronary endothelial cells were not a product of cKit+ cardiac progenitor cell differentiation. This inconsistency has been shown in previous studies, which variably support18,21,29 or refute endothelial cell35 differentiation capacity of cKit cells. Similarly, most studies document that coronary endothelium—producing cells do not express cKit.37–39

Elucidating the developmental origin(s) and identity of the cKit+ cardiac cells contributes substantially to resolving this issue. Both Wu et al16 and Hatzistergos et al1 show that cardiomyogenic cKit+ progenitors express Nkx2.5 and do not contribute to coronary endothelium. Notably, although it was initially thought that the cKit+/Nkx2.5+ cardiac progenitors represent a subpopulation of Nkx2.5+ mesodermal progenitors,16,40–42 that are present in the developing mouse heart between approximately E7.5 and E9.0, Hatzistergos et al1 clarified a later appearance of these cells in development, supporting the idea that they are likely of cardiac neural crest and not mesodermal origin. This was further substantiated with an intersectional fate mapping approach in which the cKitcreERT2/+ mouse was crossed to mice carrying a Wnt1::Flpe and dual recombinase–responsive indicator alleles. Moreover, the majority of these cells were found within the interventricular septal wall1 and not the endocardium as was thought.40

After these findings, the question that remained unanswered was whether a relationship existed between the cKit+/Nkx2.5+ cells1,16 and the vasculogenic cKit+ cells described by van Berlo et al2 and others.18,21,29 Sultana et al3 now provide further evidence that the cKit+/Nkx2.5+ cardiomyogenic progenitors are distinct from the cKit+ vasculogenic cardiac cells.

Unlike the cKit+/Nkx2.5+ lineage, the cKit+ vasculogenic cells first appear in the heart during approximately E8.5 and do not express Nkx2.5.1 These Nkx2.5-negative cKit+ cells, which likely include the cKit+ endocardial cells,40 are actually Pecam1+ endothelial cells and not progenitors3 (Figure).

Compared with the study by van Berlo et al,2 Sultana et al2 show that the majority of cKit+ cells in the heart are mature endothelial cells of a Tie2 lineage (by crossing cKitcreERT2/H2B-GFP/+ to Tie2Cre mice) and not endothelial progenitors. In fact, they report that ≈43% of all endothelial cells in a 4-month-old adult mouse heart coexpress a cKitH2B-GFP/+ reporter gene, meaning that cardiac endothelial cells express cKit.3 This is a rather surprising finding, considering that with a similar approach, van Berlo et al2 did not find eGFPnls expression in either cardiomyocytes or endothelial cells of Kit+/Cre mice. Moreover, Fioret et al37 recently reported that virtually, all of the Tie1Cre–derived coronary endothelial cells do not express cKit. These discrepant findings likely represent differences in detection sensitivity between the Molkentin and Cai cKit reporter alleles.

Table. Comparison of Cardiomyocyte Regeneration From Pre-Existing Cardiomyocytes and cKit+ Cardiac Cells

<table>
<thead>
<tr>
<th>Study</th>
<th>Regenerative Cell Source Tested</th>
<th>Mouse Model</th>
<th>Mouse Model</th>
<th>Regenerated CMs Post Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Senyo et al2</td>
<td>Pre-existing CMs</td>
<td>MIMS in MerCreMer/ZEG (8-wk chasing)</td>
<td>Analyzed n=4 mouse hearts; found 11 regenerated CMs (MIMS+/GFP-, diploid) in total</td>
<td></td>
</tr>
<tr>
<td>Ali et al4</td>
<td>Pre-existing CMs</td>
<td>Myh6Creckl, MADMERT1Gfp/+ (4-wk chasing)</td>
<td>Analyzed n=5 mouse hearts; found 476 regenerated CMs in total</td>
<td></td>
</tr>
<tr>
<td>van Berlo et al</td>
<td>cKit+ cardiac cells</td>
<td>KitcreERT2/ and KitMCM/+ (4-wk chasing)</td>
<td>Analyzed n=2 mouse hearts; found 37 regenerated CMs in total</td>
<td></td>
</tr>
<tr>
<td>Sultana et al3</td>
<td>cKit+ cardiac cells</td>
<td>cKitcreOsCreERT2/Tnmt2ae2:Z/EGFP/+ (60-day</td>
<td>Analyzed n=3 mouse hearts; found 50 regenerated CMs per heart</td>
<td></td>
</tr>
</tbody>
</table>

CM indicates cardiomyocyte; GFP, green fluorescent protein; MADMERT1Gfp/, mice containing the Mosaic Analysis with double marker alleles; MIMS, multi-isotope imaging mass spectrometry; and ZEG, a Cre reporter mouse line expressing a LacZ/EGFP double reporter allele.
Another key example where a genetic fate map does not reflect therapeutic importance comes from studies in cardiomyocyte proliferation. Genetic lineage-tracing studies of proliferating cardiomyocytes illustrate that, similar to cKit+ cardiac progenitors, the degree of myocardial regeneration from pre-existing cardiomyocytes is functionally insignificant (Table). Intriguingly, here, the findings are not interpreted as therapeutically irrelevant, but as a justification for targeting cardiomyocyte replication as a meaningful therapeutic strategy for myocardial regeneration. We would use the same logic to continue to support the use of cKit+ cells in therapeutic trials.

The differentiation capacity of cKit+ cardiac progenitors is tested in the study by Hatzistergos et al through the combined use of genetic lineage tracing and induced pluripotent stem cell modeling. These experiments define that cKit+ cardiac progenitors are fully capable of generating therapeutically meaningful numbers of bona fide cardiomyocytes, but other factors (such as the activity of bone morphogenetic protein signaling pathway) limit their prevalence in in vivo cardiomyogenic fate-mapping studies.

Last, the findings by Sultana et al and a more recent (and fourth) report by Liu et al with a new Kit-CreER2 allele suggest that some of the resident cKit+ cells in the heart are differentiated cardiomyocytes. Again, this finding is in contrast with previous reports, including the reports by van Berlo and Hatzistergos et al both of which demonstrated that the original population of cells labeled with the Kit+/Cre, Kit+MCM, and cKit+/CreERT2+ alleles were not cardiomyocytes. However, because the Cai cKit reporter alleles seem to exhibit high sensitivity and label cells even with low cKit transcriptional activity, the possibility that some of the cardiomyocytes detected by Sultana et al are pre-existing cardiomyocytes expressing low levels of cKit, or perhaps cKit-derived cTriT+ cells at early stages of cardiogenic specification with residual cKit (or cTriT) expression, cannot be excluded.

Discussion

The identification of bona fide cardiac progenitors in the postnatal heart is a major milestone in the field of cardiovascular research that would signify the departure from the longstanding view of human heart disease as incurable. In our view, this milestone has now been reached through the development of 7 elegant research reagents, designed to address the cKit controversy.

Despite the heated debates and provocative headlines that are expected to characterize any type of research with such profound implications for human disease, all 4 independently conducted genetic lineage fate-mapping studies of cKit with 7 different knockin alleles unambiguously recorded the presence of cKit+ cardiomyogenic progenitors in the embryonic and postnatal mouse hearts. The physiological role of these cells in cardiomyogenesis may not be as extensive as was previously thought, but the fact that this unequivocal and evolutionarily conserved event is interpreted to mean that these cells are not cardiac progenitors is at odds with the current experimental data.

The intriguing similarities and differences on cardiac cKit expression that have been presented by these mouse lines (Figure) underline the vastness under which the cardiomyogenic and vasculogenic programs are operated in mammals. The development of these new reagents offers a unique opportunity to comprehensively study the role of cKit in the heart and identify possible pathways for deploying cKit+ cells for therapeutic myocardial regeneration. For example, although pharmacological or genetic modulation of the cardiac bone morphogenetic protein pathway may be required for therapeutically enhancing the cardiomyogenic activity of the cKit+/Nkx2.5+ progenitor lineage, whether bone morphogenetic protein pathway plays a role in vasculogenic cKit+ cells is unknown and needs to be explored because they are likely the most abundant cKit+ population in the heart. Similarly, we have previously shown that endogenous cKit+/Nkx2.5+ progenitors may be therapeutically stimulated with mesenchymal stem cell–based therapy. However, whether mesenchymal stem cells affect the cKit+ vasculogenic lineage is unknown. The combined use of >1 cKit knockin alleles may help address these questions and others.

On the other hand, these expression differences are also indicative of the inherent limitations of the Cre–loxP knock-in approach. The results from these studies may have been skewed by many possible factors, such as abnormal behavior of cKit+ cells because of inactivation of the targeted allele, limited detection sensitivity of cells with physiologically low cKit expression again because of allelic inactivation, improper expression of Cre from the cell of interest, improper expression of the reporter genes from the cell of interest, etc, all of which have been extensively discussed elsewhere. Thus, caution should always be exercised when interpreting the knockin genetic fate-mapping findings.

In summary, all independently conducted Cre–loxP knock-in genetic fate-mapping studies of cKit identify a rare, cKit+ bona fide cardiomyogenic cell lineage in the heart. These cells are likely of cardiac neural crest origin and are distinct from the mesodermal cKit+ vasculogenic lineage, which is abundantly present in the heart. More work is required to understand why these cells with clear-cut ability to generate cardiac myocytes enter the heart at a time when a required signal for their differentiation has waned.

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Disclosures

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References


Murine Models Demonstrate Distinct Vasculogenic and Cardiomyogenic cKit+ Lineages in the Heart

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