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Most of the dust has settled: cKit⁺ progenitor cells are an irrelevant source of cardiac myocytes in vivo

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Abstract

The concept that adult stem cells could be used to regenerate the infarction damaged heart to treat heart failure captured the imagination of both the public and scientific community. This concept dates back 14 years with the first description by Orlic and colleagues that cKit⁺ cells derived from bone marrow could abundantly transdifferentiate into new contracting myocardium when directly injected into the infarcted heart.¹ Two years later Beltrami and colleagues showed that cKit⁺ progenitors from the heart itself could be extracted, expanded ex vivo, and reinjected into the rodent heart to again abundantly transdifferentiate and create as much as 70% new contracting myocardium.² Fast forward to the present day and the current picture of myocardial regeneration from cKit⁺ progenitors or some other adult stem cell population seems rather unlikely. This current picture was made even more concrete with the recent publication of yet another definitive in vivo lineage tracing study for cKit⁺ cells in the heart, by Sultana and colleagues.³

Two sentence summary:

That cKit⁺ progenitor cells can transdifferentiate into new cardiac myocytes as a means of regenerating the heart after infarction injury has been hotly debated for nearly 15 years. A recent publication using exhaustive genetic reporters and lineage tracing shows definitively that endogenous cKit⁺ cells in the heart are in fact endothelial in nature and have an essentially meaningless capacity to make cardiomyocytes *in vivo*.

While cKit⁺ progenitor cells were originally reported to abundantly regenerate the heart after infarction injury, a number of subsequent papers in the ensuing years from independent groups were unable to repeat these results, despite considerable effort [reviewed in van Berlo and Molkentin].⁴ The controversy persisted with 2 opposing camps emerging, those that claimed cKit⁺ progenitors could meaningfully regenerate the heart, and those that observed no such effect.^{2, 5–10} In 2014, van Berlo and colleagues went one step further in attempting to resolve the ongoing debate by performing genetic lineage tracing for the very first time in the mouse heart, to specifically address if the heart uses cKit⁺ cells to make new myocytes *in*

vivo, during development, with aging or after injury.¹¹ The results of that study conclusively showed that cKit⁺ progenitors could make new cardiac myocytes in the heart during all three conditions, but at an extremely low level that was basically irrelevant from a physiologic perspective. The camp in support of cKit⁺ cells as master mediators of cardiac regeneration were quick to suggest potential limitations in the 2 primary genetic strategies used in the van Berlo study (Kit^{Cre} and tamoxifen regulated Kit^{MerCreMer} alleles), suggesting that another independent lineage tracing study with independently designed mice was needed. As requested, Sultana and colleagues recently published just such a paper in *Nature Communications*, which was entitled “*Resident c-kit⁺ cells in the heart are not cardiac stem cells*”.³

Sultana et al. generated 5 novel gene-targeted mouse models to exhaustively address the role of cKit⁺ cells in heart development or regeneration. This paper employed a series of highly definitive lineage systems with multiple genetic tracking alleles to directly quantify and characterize the nature of cKit⁺ cells in the heart and their ability to form new cardiomyocytes. The authors first generated a murine *Kit* allele (Kit^{H2B-tdTomato/+} mice) that allowed for in situ determination of current cKit expression using a fluorescent tdTomato-tagged histone 2B (H2B) protein. This allele identified many cKit expressing cells in the heart, even ones with very low cKit antibody reactivity, indicating a higher abundance of cKit⁺ cells than previously appreciated. The authors validated the sensitivity of this allele using in situ hybridization as well as immunohistochemistry for cKit expression. In development, as well as after birth, cKit⁺ cells identified with this active tdTomato-H2B allele were observed in the heart, but primarily in the endocardium with fewer cells in the myocardium. The authors identify the tdTomato-H2B expressing cKit⁺ cells as mainly endothelial in nature, both during development and after birth. The authors also applied another targeted allele in which eGFP was fused with H2B and placed in the Nkx2.5 genetic locus, which is a definitive marker of cardiac progenitors and the myocardial lineage. Upon cross-breeding Kit^{H2B-tdTomato/+} mice with Nkx2-5^{H2B-GFP/+} mice, virtually no double positive nuclei were identified in the heart. Only at E12.5 (but not at E9.5 or E15.5) did the authors detect a total of 11 double-positive nuclei. Also in adult hearts no double positive cells were found. This result is very conclusive as it shows direct labeling of essentially all cKit⁺ cells using a stable H2B nuclear marker, and their subsequent ability to take on the cardiomyogenic lineage when Nkx2.5 is expressed. But the authors did not stop there, as they also applied a separate knock-in line where eGFP tagged H2B was inserted into the cardiac troponin T locus (Tnnt2^{H2B-GFP/+}) and again they could only detect overlap with cKit expressed H2B-tdTomato at E12.5 in sparingly few cells. These findings indicate that cKit expressing cells in the heart are not predestined to become Nkx2-5 or Tnnt2 expressing cardiomyocytes.

Sultana et al. also generated a targeted *Kit* allele where a cassette containing a loxP flanked nuclear LacZ followed by H2B-eGFP was inserted in the translational start site of *Kit*. The LacZ pattern observed in these mice resembled the Tomato distribution in Kit^{H2B-tdTomato/+} mice. But more importantly, upon cross-breeding with endothelial specific Tie2-Cre mice, the results showed that virtually all cKit⁺ cells within the heart are endothelial, not cardiac myocytes. Sultana et al. also generated yet another targeted *Kit* allele, now containing a cDNA for the tamoxifen inducible MerCreMer protein, similar to that generated by van

Berlo and colleagues.¹¹ Similar to the results of van Berlo et al., Sultana and colleagues observed mainly endothelial cells upon cross-breeding to Rosa26-tdTomato reporter mice when tamoxifen was given, with very few detectable cardiomyocytes (see below).

To more carefully assess new cardiomyocyte formation with these 2 different Kit-Cre alleles, Sultana et al. applied a cardiomyocyte specific reporter allele by targeting the Tnnt2 locus with loxP flanked nuclear LacZ followed by GFP tagged H2B. Upon cross-breeding with the Kit^{MerCreMer/+} mice and addition of tamoxifen, they observed between 50 and 325 c-kit derived cardiomyocytes per heart (<0.04% of all cardiomyocytes). To further determine the potential regenerative capacity of endogenous cKit⁺ cells in terms of generating cardiomyocytes, Sultana et al. induced myocardial infarction in adult mice that were Kit^{H2B-tdTomato/+Nkx2-5H2B-GFP/+}. No double positive cells were identified up to 60 days after myocardial infarction, indicating that cKit⁺ cells do not become Nkx2-5⁺ cells after injury to the heart. To further corroborate the lack of expression of cKit in cardiomyocytes, Sultana et al. performed myocardial infarction in Tnnt2^{MerCreMer/+};Kit^{nLacZ-H2B-GFP/+}; Rosa26R^{tdTomato/+} mice. If cKit⁺ cells become cardiomyocytes and cKit expression is maintained, H2B-GFP positive cardiomyocytes would be expected, but none were identified in the infarct region. However, myocardial infarction in Kit^{MerCreMer/+} Tnnt2^{nLacZ-H2B-GFP/+} did show about 50 total H2B-GFP positive cardiomyocytes per heart. While the cellular origin of these low numbers of H2B-GFP⁺ cardiomyocytes is not known, Sultana et al. suggested that a very small fraction of cKit⁺ cells are in fact cardiomyocytes, despite the absence of a single Kit^{H2B-tdTomato/+Nkx2-5H2B-GFP/+} cell.

The latest findings of Sultana et al. are entirely consistent with the lineage tracing study by van Berlo et al. from last year that collectively suggest that cKit⁺ cells are not cardiomyocyte progenitor cells, and their ability to generate myocytes in the heart during development, with aging, or after injury is less than 0.05% and likely of little physiologic relevance.^{3, 11} A recent publication from Hare and colleagues that used a related Kit-CreERT2 gene-targeted mouse to perform lineage tracing in the heart also generated data that the authors state were consistent with van Berlo et al., meaning that very low levels of myocytes were traced in the heart.¹² One potential concern with the lineage tracing approach is that the *Kit* allele is heterozygous, an issue that was previously discussed in this journal and largely dismissed.^{13, 14} Moreover, Sultana et al showed abundant cKit progenitor cells in the hearts of their lineage traced mice, which were fully competent to generate new endothelial cells, again suggesting that heterozygosity of the *Kit* allele does not negatively impact cKit⁺ cells in the heart.

In conclusion, we believe that the hypothesis that cKit⁺ cells are a meaningful source for new cardiomyocytes in the heart has been essentially disproven. Hence, all previous literature suggesting that either bone marrow or endogenous heart-derived cKit⁺ cells can regenerate substantial new beating myocardium through transdifferentiation should be viewed as somehow incorrect. The lineage tracing data from multiple independent laboratories have definitely shown that cKit⁺ cells do not appreciably make cardiac myocytes *in vivo*, in their natural state. Although lineage tracing was performed in mice, there are no data to suggest that the role of cKit⁺ cells might be different in larger mammals. However, it remains possible that cell therapy approaches with cKit⁺ cells in humans after

myocardial infarction might still be beneficial, such as through a proposed paracrine effect of the transplanted cells, which is currently being tested in a randomized clinical trial supported by the Cardiovascular Cell Therapy Research Network (NTC02501811).¹⁴ Despite the potential for a therapeutic benefit, clearly cKit⁺ cells injected into the injured myocardium do not transdifferentiate, at a meaningful level, into new cardiac myocytes.

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