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”

There has been great interest in the potential role of cKit+ cells as mediators of heart regeneration. To explore this issue, van Berlo et al1 recently used genetic lineage tracing in the mouse to mark cKit antibody reactivity by immunohistochemistry and nuclear plicite data in Figure 1E that directly demonstrates this issue. van Berlo et al1 used fluorescence-activated cell sorter imaging cytometry in light of a recent commentary published in Circulation Research by Dr Nadal-Ginard et al2 that question the conclusions and some theoretical aspects of the study by van Berlo et al.

The specific points in response to this commentary are as follows:

1. One of the Nadal-Ginard Commentary’s most important arguments is that the article by van Berlo et al1 did not verify expression of their Kit-Cre or Kit-MerCreMer approach in the cardiogenic progenitor cells (CPCs) of the heart, which they say is a major limitation. Their statement is “In fact, the recombination efficiency in the c-kit eCSCs is an indispensible piece of information and the main driver for the conclusions derived from the results reported but it is never specifically assessed in the paper itself or in the additional information.”

This is a surprising concern because van Berlo et al1 have explicit data in Figure 1E that directly demonstrates this issue. van Berlo et al1 used fluorescence-activated cell sorter imaging cytometry to show that ≈80% of the cardiac CPCs labeled with the cKit antibody are recombined by the Cre approach and express enhanced green fluorescent protein (eGFP) (and well >80% in bone marrow; Figure 1C and 1D).1 van Berlo et al1 even went 1 step further and showed the differentiation ability of these cells in extended data Figure 9.1 These differentiation data show that both the eGFP-positive cells (recombined by the Kit-Cre genetic system) and the less frequent population of eGFP-negative cells (unrecombined) could equally differentiate toward the myocyte lineage with dexamethasone stimulation, thereby expressing GATA4 and troponin T. Moreover, Figure 1F and extended Figure 1E from van Berlo et al1 show complete overlap between cKit antibody reactivity by immunohistochemistry and nuclear eGFP expression mediated by the Cre allele. Therefore, essentially all of the currently cKit expressing CPCs in the heart drive expression from the Kit-Cre knock-in allele. Given these results, the core concern of the authors as to why they wrote their commentary2 in the first place is perplexing because van Berlo et al1 plainly included the data that they claimed was required to validate the approach.

2. Another major criticism within the commentary of Nadal-Ginard et al2 is that only the lowest-c-kit expressing CPCs in the heart are the ones that generate cardiac myocytes, and because they are low expressing, the Kit-Cre recombination strategy probably fails to report them in van Berlo et al.1

As discussed above, van Berlo et al1 presented data directly demonstrating that recombined and nonrecombined CPCs from the heart equally differentiated toward the myocyte lineage. However, this criticism is surprising because it is inconsistent with ≥4 previous reports from the Anversa laboratory, all coauthored by Dr Nadal-Ginard. These previous studies showed robust and uniform cKit protein expression levels by immunohistochemistry in CPCs from both rodent3,4 and human hearts.5,6 In the latter report, the intensity of cKit staining in human CPCs also seemed equivalent to that in human bone marrow cells.7 So, although the publications by Dr Nadal-Ginard et al,2 up until this point, have suggested that expression levels of cKit in CPCs are similar to bone marrow cells, they now suggest (unpublished data) that the expression levels are actually different.2 This issue notwithstanding, there are no data to support the notion that cKit receptor density or expression levels per se affects CPC myogenic potential or does any of this affect the validity of data in van Berlo et al,1 which clearly show that CPCs in the heart are highly labeled with the used recombination system.

3. The authors also claim that it is known that cKit is expressed in the inner cell mass (ICM) and embryonic stem cells and that this means that the mice in van Berlo et al1 should have had complete recombination of the reporter in all cells of the mouse. They reference a article as a proof that cKit is expressed in the ICM.3

The reference cited to suggest that cKit is expressed in the ICM actually does not show this as claimed by Nadal-Ginard et al.2 The reference3 only shows that cultured embryonic stem cells can express cKit at some undefined relative level not the ICM. Although embryonic stem cells are generated from the ICM, their gene expression profiles are different for numerous reasons (culturing, etc).

4. Related to the above issue, the authors make the following inaccurate statement with inappropriate references: “In fact, all the c-kit transgenic or knock-ins produced to track c-kit expressing cells are far from closely recapitulating c-kit expression and function either in development or adult life.”8,9

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First, the statement is inexact because the 2 cited references8,9 are not a KIT allele knock-in approach, they both were transgene-based strategies, which indeed do not recapitulate endogenous expression. However, 3 separate GFP or LacZ knock-in mouse models for the Kit locus were previously generated and published, which the Nadal-Ginard et al.12 did not cite or discuss. Each of these 3 undisputed knock-in approaches showed overlap in GFP or LacZ expression from the Kit allele with known sites of KIT protein expression.10–12 Thus, the knock-in approach seems to be a valid means of performing lineage tracing from the Kit locus. The study of van Berlo et al.1 surveyed multiple tissues with known expression of KIT protein and found reliable lineage tracing, much in line with the GFP and LacZ knock-in models that were previously published.10–12

5. They also suggest that the KIT-Cre or KIT-MerCreMer alleles from van Berlo et al.1 should have been expressed in all the germ cells of the mouse and hence should have caused total recombination throughout all cells of the mouse.

This comment has no relevance to the study of van Berlo et al.1 or recombination detection in the heart, because only heterozygous mice were bred together, in which one mouse has the ROSA26 LoxP-dependent reporter and the other mouse has the KIT-Cre allele.1 Hence, these 2 alleles never go through the germ line together, and recombination as suggested is therefore impossible.

6. Another issue they raise is hemizygosity of KIT because of the Cre knock-in or the MerCreMer knock-in, which could affect the number, or activity of CSCs in the heart.

This is true and van Berlo et al.1 discussed this issue in the supplemental discussion at some length. However, there was no difference in the total number of KIT+ cells in the hearts of hemizygous mice versus wild-type mice, suggesting that the hemizygous state of the Kit allele was outwardly benign to CPC content in heart. Extended data Figure 9 also shows that hemizygous targeted CPCs are capable of differentiating toward the myocyte lineage, at least expressing GATA4 and troponin T.1

7. Nadal-Ginard et al.2 claim that KIT-Cre-ER2 mice from the Saur laboratory3 in Germany is a better strategy than the knock-in mice generated by van Berlo et al.1.4

It is not clear how this argument affects the validity of the results in van Berlo et al.1, even if the mice generated previously by the Saur laboratory were somehow superior. However, it actually seems to be the opposite of what was shown in the literature because the KIT-Cre-ER2 mice from the Saur laboratory showed only 2% Cre-mediated recombination in bone marrow cells, whereas the van Berlo et al.1 KIT-Cre and KIT-MerCreMer knock-in mice showed recombination in ≈80% of all known KIT expressing bone marrow cells. Hence, the van Berlo et al.1 knock-in approach clearly has more coverage with the known domains of KIT protein expression. The KIT-Cre-ER2 mice from the Saur laboratory were generated with 2 separate translational start sites and an IRES (internal ribosome entry site) insert that may have been responsible for the loss of appropriate allele expression in most of the bone marrow lineages (except for mast cells).13

8. Nadal-Ginard et al.2 claims that either the results of 0.03% reported by van Berlo et al.1 are correct or that 3% to 8% reported by their group earlier are correct.14 Both cannot be correct.

In my view, the results reported by Ellison et al.14 were not based on an approach that reliably assesses the level whereby endogenous cKIT+ CPCs might give rise to new cardiomyocytes in the heart; hence, their assertion that the data from van Berlo et al.1 are inconsistent with their past results cannot be inferred. Rather than rehash the technical reasons for this view point again, I would like to simply refer the reader to our past editorial comments on this issue and the article by Ellison et al.14–16

9. Nadal-Ginard et al.2 also raised concerns over Cre-dependent toxicity within the ckit+ cells from the knock-in allele. Cre can be toxic to cells in culture when expressed at high levels, such as by viral-mediated overexpression, thereby inducing nonspecific DNA rearrangements.15 We do not think that this is a concern with our knock-in allele because it is expressed at much lower levels; hence, we observed no difference in CPC numbers in the hearts of adult wild-type versus KIT-Cre mice, or in bone marrow–derived ckit expressing lineages. Moreover, we used a parallel strategy with Kit-MerCreMer allele, and expression of this protein causes no nonspecific recombination at base-line because it is essentially silent until tamoxifen is given. This means that expression of the MerCreMer protein in vivo could not cause genomic instability or otherwise affect the activity of ckit cells, yet we observe essentially the same data of low myocyte contribution from Kit locus-dependent lineage tracing between either the Cre or the MerCreMer alleles. Hence, Cre-mediated toxicity does not seem to be a significant concern with the observations reported by van Berlo et al.1.3

Conclusions

In summary, van Berlo et al.1 developed 2 unique cKit+ lineage tracing mouse models, performed initial experiments with these mice, and reported the most straightforward interpretation of the results. The hope was that these mice would help address the ongoing controversy in the cardiac stem cell arena from a new perspective. The data clearly showed that cKit-labeled progenitors did not prefer to generate cardiac myocytes although they do abundantly generate endothelial cells in the heart, as well as highly label the bone marrow and other sites of known ckit protein expression.1 Moving forward, it is our hope that these mice will stimulate new approaches that eventually lead to a better understanding of the cellular basis for cardiac regeneration, and possibly how to program CPCs toward the cardiomyocyte lineage more effectively. There is clearly an unmet clinical need to develop more potent cellular therapies for patients with heart failure.

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Disclosures

None.

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