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Definitive lineage tracing of cKit+ cells in mice suggests they are not cardiac progenitor cells

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There has been great interest in the potential role of cKit$^+$ cells as mediators of heart regeneration. To explore this issue, we (van Berlo et al) recently used genetic lineage tracing in the mouse to mark cKit$^+$ cells.[1] When we looked at tissues from these mice, the lungs were green, the gastro-intestinal tract was green, the bone marrow was green, the testis were green, but the heart was not. This was a surprising finding, given the extensive literature implicating cKit$^+$ cells in regeneration of the heart. In depth microscopic analysis revealed that cardiac endothelial cells were abundantly labeled (as much as 50%), but only a few rare myocytes were GFP$^+$ (maximum of 0.03%). The report contained extensive control experiments to validate all observations and suggested the simplest conclusion; that there were very few adult cardiac myocytes derived from cKit$^+$ progeny. Below we highlight the key issues and attempt to bring clarity to this field in light of a recent Commentary published in *Circulation Research* by Dr. Nadil-Ginard and colleagues that questioned the conclusions and some theoretical aspects of the study by van Berlo et al.[2]

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

1) One of the Nadil-Ginard Commentary's most important arguments is that the paper by van Berlo et al did not verify expression of their Kit-Cre or Kit-MerCreMer approach in the CPCs of the heart, which they say is a major limitation. Their statement is exactly this: “In fact, the recombination efficiency in the c-kit+ eCSCs is an indispensable 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 al have explicit data in Figure 1e that directly demonstrates this very issue. van Berlo et al used FACS imaging cytometry to show that almost 80% of the cardiac CPCs labeled with the cKit antibody are recombined by the Cre approach and express eGFP (and well over 80% in bone marrow, Figure 1c and d).[1] van Berlo et al even went one 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 Kit-Cre genetic system) as well as the less frequent population of eGFP-negative cells (unrecombined) could equally differentiate towards the myocyte lineage with dexamethasone stimulation, thereby expressing GATA4 and troponin T. Moreover, Figure

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1f and Extended Figure 1e from van Berlo et al show complete overlap between cKit antibody reactivity by immunohistochemistry and nuclear e-GFP expression mediated by the Cre allele. Therefore, essentially all of the the currently cKit expressing CPCs in the heart drive expression from the Kit-Cre KI allele. Given these results, the core concern of the authors as to why they wrote their Commentary[2] in the first place is perplexing as van Berlo et al included the very data that they claimed was required to validate the approach.

2) Another major criticism within the Nadal-Ginard et al Commentary is that only the lowest-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.

As discussed above, van Berlo et al presented data directly demonstrating that recombined and non-recombined CPCs from the heart equally differentiated towards the myocyte lineage.[1] However, this criticism is surprising because it is inconsistent with at least 4 previous reports from the Anversa laboratory, all co-authored by Dr. Nadal-Ginard. These previous studies showed robust and uniform cKit protein expression levels by immunohistochemistry in CPCs from both rodent [3,4] and human hearts [5,6]. In the latter report, the intensity of cKit staining in human CPCs also appeared equivalent to that in human bone marrow cells.[6] So, although the publications by Dr. Nadal-Ginard, up until this point, have suggested 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, nor does any of this impact the validity of data in van Berlo *et al* which clearly show that CPCs in the heart are highly labeled with the employed recombination system.

3) The authors also claim that it is known that cKit is expressed in the inner cell mass (ICM) and ES cells and that this means that the mice in van Berlo et al should have had complete recombination of the reporter in all cells of the mouse. They reference a paper as proof that cKit is expressed in the ICM.[7]

The reference cited to suggest that cKit is expressed in the ICM actually does not show this as claimed by Nadal-Ginard and colleagues. The reference[7] only shows that cultured ES cells can express cKit at some undefined relative level, not the ICM. While ES 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]

First, the statement is inexact because the 2 cited references[8,9] are not a Kit allele knock-in (KI) approach, they both were transgene-based strategies, which indeed do not recapitulate endogenous expression. However, 3 separate GFP or LacZ KI mouse models for the *Kit* locus were previously generated and published, which the Nadal-Ginard et al did not cite or

discuss. Each of these 3 undiscussed KI approaches showed overlap in GFP or LacZ expression from the *Kit* allele with known sites of cKit protein expression.[10-12] Thus, the KI approach appears to be a valid means of performing lineage tracing from the *Kit* locus. The van Berlo study surveyed multiple tissues with known expression of cKit protein and found reliable lineage tracing, very 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 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 van Berlo et al study or recombination detection in the heart, as 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 cKit due to the Cre KI or the MerCreMer KI, which could impact the number, or activity of CSCs in the heart.

This is true and van Berlo et al discussed this issue in the supplemental discussion at some length. However, there was no difference in the total number of $cKit^+$ cells in the hearts of hemizygous mice versus WT 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 towards the myocyte lineage, at least expressing GATA4 and troponin T.[1]

7) Nadal-Ginard et al claim that Kit-Cre-ER^{T2} mice from the Saur laboratory[13] in Germany is a better strategy than the KI mice generated by van Berlo et al.

It is not clear how this argument impacts the validity of the results in van Berlo et al, even if the mice generated previously by the Saur laboratory were somehow superior. However, it actually appears to be the opposite of what was shown in the literature, as the Kit-Cre-ER^{T2} mice from the Saur laboratory showed only 2% Cre-mediated recombination in bone marrow cells,[13] while the van Berlo et al Kit-Cre and Kit-MerCreMer KI mice showed recombination in approximately 80% of all known cKit expressing bone marrow cells. Hence, the van Berlo et al KI approach clearly has more coverage with the known domains of cKit protein expression. The Kit-Cre-ER^{T2} mice from the Saur laboratory were generated with 2 separate translational start sites and an IRES 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 claims that either the results of 0.03% reported by van Berlo et al are correct, or that 3-8% 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 are inconsistent with their past results cannot be inferred. Rather than rehash the technical reasons for this viewpoint again, I would like to simply refer the reader to our past editorial comments on this issue and the Ellison et al paper.[15-16]

9) Nadal-Ginard et al also raised concerns over Cre-dependent toxicity within the cKit$^+$ cells from the KI allele.

Cre can be toxic to cells in culture when expressed at high levels, such as by viral-mediated overexpression, thereby inducing non-specific DNA rearrangements.[17] We do not believe this to be a concern with a KI allele because it is expressed at much lower levels; hence why we observed no difference in CPC numbers in the hearts of adult wildtype 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 recombination at baseline 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 very low myocyte contribution from Kit locus-dependent lineage tracing between either the Cre or MerCreMer alleles. Hence, Cre-mediated toxicity does not appear to be a significant concern with the observations reported by van Berlo et al.[1]

Conclusions

In summary, van Berlo et al 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 do 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 more effectively program CPCs towards the cardiomyocyte lineage. There is clearly an unmet clinical need to develop more potent cellular therapies for patients with heart failure.

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