Review of: "Setd4 controlled quiescent c-Kit+ cells contribute to cardiac neovascularization of capillaries beyond activation"

Marina Higuera[1], Antonio Bernad[1]

1 National Center for Biotechnology (CNB)

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Summary

For decades, the mammalian heart was believed to be a terminally differentiated organ, with no intrinsic capacity to regenerate––and adapting itself only to growth and changing physiological conditions. There is now compelling evidence that the mammalian heart is indeed in continuous albeit slow turnover (reviewed in [1][2]), which has sparked intense debate about the “source” of the turnover and the contradictory results regarding the generation of de novo cardiomyocytes. The controversy is far from being resolved, and the field needs new perspectives and approaches to address the old key question(s) that still remain.

Xing et al. have thrown their hat into the ring with a recent contribution[3] based on their expertise in the characterization of physiological quiescence, which is a critical determinant for the long-term maintenance of cellular pools, the preservation of proliferation capacity and––upon activation––tissue turnover[4][5]. In addition, quiescence seems also a key aspect in tumor biology[6]. Prior work by the Yang group studying the diapause embryos of Artemia[4] and human breast cancer stem cells[6] revealed an evolutionarily conserved mechanism for cellular quiescence that facilitated heterochromatin formation, with the methyltransferase Setd4 (SET Domain Containing 4) as the key determinant. In their recent article, Xing et al.[3] evaluated the potential involvement of such a mechanism for the regulation of a quiescent pool of cardiac cells expressing c-Kit (a receptor tyrosine kinase). We have endeavored to distill the main points of the Xing et al. article here, and we discuss the outstanding major challenges in the cardiovascular regenerative field.

Background on cardiac c-Kit+ cells

Before we review the Xing et al. study, it will be useful to appraise the existing literature on the c-Kit+ cardiac stem / progenitor cell field. Myocyte proliferation is a highly regulated process in human and pig hearts (reviewed in [7]) and has been demonstrated in young human individuals (<20 years old)[8], and is

potentiated in pathologic settings such as myocardial infarction (MI)[9]. This suggests the existence of an atypical resident progenitor/stem cell population(s) from which new myocytes can arise, as recently established in mammalian arteries for smooth muscle cells (SMC)[10]. An alternative proposal, however, is the de-differentiation/proliferation/re-differentiation of mature cardiomyocytes[11][12]. Both processes could also be acting coordinately.

Several markers that characterize stem cell populations in other tissues have been proposed for the isolation of cardiac resident stem/progenitor cells (multipotent cells) from the heart, including c-Kit, Sca1, Isl1, Abcg2 and Bmi1[13][14]. Among these, c-Kit+ cardiac cells were the first[15] and the most intensively studied population (reviewed in [16]) as a potential resource for cardiovascular therapy (reviewed in [17]), and were given the descriptive name cardiac stem cells (CSC). However, later findings from basic and preclinical research together with the failure of several clinical trial evaluations and compounded by historical and scandalous retractions of many seminal publications in the field[18] have fueled a long and bitter debate over the existence of c-Kit+ CSC that has led to a general skepticism in the field. While the polemic over the function of endogenous c-Kit+ cells is far from being resolved, several important clues have been uncovered during the last years, and the main technical problems at the origin of the conflict have been mostly clarified[19][20][21][22]. That being said, no study has thus far provided strong evidence for the precise mechanism of endogenous c-Kit+ cell action in the improvement of cardiac function after injury such as MI. A recent analysis on the possible mechanisms concluded that the evident functional benefits of cell therapy with exogenous c-Kit+ cells are likely due to acute inflammatory-based wound healing responses[23], involving a macrophage response that modulates the activity of cardiac fibroblasts, reducing the extracellular matrix content in the border zone and enhancing the mechanical properties of the injured area. Nonetheless, multiple questions remain unanswered that are critical to reconcile the published results.

Initial descriptions of cardiac c-Kit+ CSC stressed the relevance of c-Kit expression as the main criterion for their definition. Later, this was reinforced with the asseveration that “an adult cardiac c-Kit+ CD45- cell population is necessary and sufficient” for functional cardiac regeneration and repair, and that many of the endogenous c-Kit+ cells express Gata4 and Nkx2.5[24]. Challenging this notion, Molkentin et al. remarked in a letter to the Editor of Nature that they did not find, by lineage tracing, a significant contribution of c-Kit+ cells to de novo cardiomyocyte formation in the adult heart[25]. The Torella group later published that the number of cardiac c-Kit+ cells with cardiomyogenic-clone-producing potential is very low (~1% of total c-Kit+ cells) in the mouse adult heart[19] and that the c-Kit level of expression is much lower than other known c-Kit+ cell lineages[20]. This was the origin of a two-sided controversy based on cardiac c-Kit+ cells: the evidence of more than one c-Kit+ population in the heart, and the cardiac repair potential of these populations (based on de novo cardiomyocyte formation).

It is currently accepted that the adult heart contains other cell lineages that also express c-Kit. Indeed, the majority of c-Kit+ cells (≥90%) are associated with the blood cell lineage, expressing markers such as CD45 and CD31 (cardiac mast cells or endothelial progenitor cells, respectively), and only 10% of cardiac

c-Kit+ cells are enriched for CSC (c-Kit+lowCD45-CD31-) with significantly much lower c-Kit expression than found in embryonic stem cells, hematopoietic stem cells (HSCs) and bone marrow mast cells. To put it simply, among all the cardiac cells expressing c-Kit, only a small fraction (~10%) has the phenotype c-Kit+/lowCD45-CD31- and only about ~1% of clonogenic cells demonstrate the differentiation potential of true multipotent CSC[19].

Clearly, c-Kit does not now appear to be the ideal, practical, marker for the identification and isolation of CSC. After 20 years of research it would be reasonable to have more specific and robust marker(s) for CSC. It is almost explicit now that more than two good markers are needed to conclusively define (independently of the technique used) a cell population of interest, as it is almost assured that a potentially "unique" marker would also be expressed (lower levels, transiently, etc.) by other populations, which might result in interference and inaccurate conclusions. As stated by Cianflone et al.[26], the expression of c-Kit is necessary but not sufficient to identify CSC. Regarding this concept, these authors reviewed the phenotypic characterization of multipotent resident CSC based on the presence/absence of several surface markers. The isolation of c-Kit cardiac stem/progenitor cells (CSC/CPC) could be refined using this revised phenotype. In any case, the most recent publication on CSC[20] appears to support the main published features of the cells, although the authors maintain that cardiac c-Kit+lowCD45-CD31- cells include all the adult CSC pool. This is not completely compatible with a body of evidence based on other independent markers that were proposed to describe resident multipotent cardiac subpopulations (reviewed in [13][14][15]). The diversity of potential markers has hindered the unambiguous identification and molecular definition of endogenous CSC/CPC, and several lineage-tracing studies have yielded inconclusive results[27][28]. In 2017, a working group agreed on some relevant considerations regarding this conflictive issue[29]. In conclusion, there is currently no globally-accepted hallmark for CPC and whether they are supported by enduring multipotential cells (CSC?). It is evident that further collaborative-comparative research is needed to reach a final conclusion on this complex matter and, in this respect, Nadal-Ginard et al. have recently written an excellent overview looking to the future[30].

Regarding the second controversy, several groups have addressed the issue of the generation of de novo cardiomyocytes from c-Kit+ cardiac cells using genetic cell-fate approaches[5][31][32] that rely on the expression of the Cre recombinase (constitutive or conditionally-active) under c-Kit regulatory sequences. It is well known that the net activity of Cre recombinase (recombination of any target construct) is strongly dependent on the levels of Cre protein and on the complexity and accessibility of the target constructs[33]. In the analysis of cardiac c-Kit+ populations, most studies have used ad hoc mice carrying a modified knock-in c-Kit allele, where Cre or CreERT2 (KitCre alleles) are expressed under the control of a segment of the c-kit regulatory sequences, producing c-Kit haploinsufficient mice. Previous studies have demonstrated the complexity of the c-Kit regulatory domain to bona fide reproduce its physiological pattern of expression[34]. Results using these mouse lines (c-Kit-Cre/Rosa26-floxed-STOP-reporters)[31][32] or dual-recombinase approaches[5] concluded that cardiac c-Kit+ populations have a marginal cardiomyogenic capacity (0.01% of total cardiomyocytes).

The genetic tools were assumed to be cleaner and more powerful than classical methods of cell isolation, but mounting evidence has called attention to some pitfalls when they are used at the limit of their capability[35][36]. Regarding their use for the study of cardiac c-Kit+ cells, Torella and collaborators provided strong evidence that KitCre alleles––both inducible and constitutive––promote the inefficient recombination of the several reporter constructs evaluated in cardiac c-Kit+ cells[20]: a) ≤20% of all c-Kit+ cardiac cells were recombined but all of these were CD45+, CD31+, or both, representing cardiac mast cells or endothelial progenitor cells, respectively; b) c-Kit+lowCD45-CD31- cells demonstrated a very low level of recombination (≤1%). Compelling experiments concluded that c-kit haploinsufficiency in these genetic fate-mapping experiments is the most probable origin of the discrepancies, provoking growth and clonogenesis and cardiosphere-formation defects.

All of these limitations, including the cardiomyogenic differentiation capacity, were rescued by transfection of a BAC harboring the complete cloned c-Kit locus, in c-Kit+ cloned cells. Finally, Vicinanza et al. confirmed the described phenotypes in vivo using cloned cardiac c-Kit+lowCD45-CD31- cells[20]. However, this last feature has been a main argument by Molkentin et al. against some of the conclusions raised by Torella and collaborators, as it is not a given that cloned and expanded cells retain the full characteristics and reflect the physiological behavior of endogenous c-Kit+lowCD45-CD31- cells, especially in heart repair after severe damage[25]. Contrastingly, there is consensus that cardiac c-Kit+ cells have robust endothelial cell (EC) differentiation potential. Recently endogenous c-Kit+ CSC/CPC cells have been shown to participate in vascular turnover and repair in the aorta, differentiating to EC, which was confirmed by lineage-tracing and single-cell analyses[37]. Recent data with a new mouse strain incorporating the Cre cassette in the c-Kit UTR seemed to corroborate the overall hypothesis[38], but using two additional mouse lines, that avoid the haploinsufficiency in the c-Kit locus[39], Zhou and colleagues failed to confirm any cardiogenic potential in c-Kit CSC/CPC. Therefore this question remain open, although the recent characterization of human cardiac atrial myxomas as the first-described CSC (c-Kit+CD45-CD31-)-related human heart disease reinforces this line of research[40].

The methyltransferase Setd4 and quiescence regulation

Within this complex scenario, the study by Xing et al. first establishes that c-Kit+ populations in the adult and neonatal mouse heart include 75–80% of cells in G0/G1 phase(a), and concludes that adult primary cardiac c-Kit+ populations are predominantly in a quiescent state(b), in agreement with a previous description[24]. In addition, they analyzed 5-bromo-2′deoxyuridine (BrdU) retention in cardiac c-Kit+ populations, administrated at embryonic day 6 (E6), and found that almost all sorted cardiac c-Kit+ cells were BrdU+ in neonates, one day after birth, and approximately 30% of these cells were BrdU+ in young and adult animals. They concluded that a pool of cardiac c-Kit+ cells generated during development persists in the adult heart, also in quiescence. Second, Xing et al. convincingly demonstrate that quiescent c-Kit+ cells express higher levels of Setd4 than do actively proliferating c-Kit+ cells. The Setd4+c-Kit+

cells lacked expression of proliferation markers (Ki67 and amplification of H3pS19), suggesting that Setd4 could also be involved in the regulation of quiescence in cardiac c-Kit+ cells. In agreement with this notion, markers of heterochromatin––H4K20me3 and HP1α––were also preferentially observed in Setd4+c-Kit+ cells compared with Setd4 non-expressing (Setd4−) c-Kit+ cells, as previously described in other cell populations from pancreas, bone marrow or breast[41][42][6]. Based on the current vision of cardiac c-Kit+ cells, it seems that Xing et al. are evaluating quiescence in a mixed adult cardiac cell population, including mast cells and endothelial precursors (>90%) and progenitor/stem cells (<10%). We draw this conclusion because they do not deplete CD45+ cells (Methods. FACS sorting). It would be interesting to analyze whether the small (~1% of total cKit+) clonal cardiomyogenic population (c-Kit+/lowCD45-CD31-), which is also preferentially in quiescence (>90% BrdU- and Ki67-)[24], use the same described mechanism[6]. In addition, they compared the adult cardiac c-Kit+ population with that of the neonatal heart. In the context of the described controversy, the composition and relationship of both cardiac populations is not clear. It would be interesting to examine this in the context of quiescence and the putative CSC/CPC populations. In this sense, it would also be interesting to analyze the correlation of Setd4 expression with quiescence along heart development.

Epigenetic studies have shown that heterochromatin silences gene expression by virtue of its highly condensed structure, maintaining the reversibility of cellular quiescence. Heterochromatin is highly associated with the trimethylation of lysine 20 of histone 4 (H4K20me3) and HP1α protein, in contrast to euchromatin, which shows high levels of acetylated lysine 9 of histone 3 (H3K9ac) and trimethylation of lysine 4 of histone 3 (H3K4me3). In agreement with this, Xing et al. showed that the heterochromatin markers H4K20me3 and HP1α were also preferentially observed in Setd4+c-Kit+ cells over Setd4−c-Kit+ cells, but they did not observe any significant differences in H3K9ac between these cell types. Some studies reported that Setd4 could regulate H3K4 trimethylation (H3K4me3), a representative mark in quiescent muscle stem cells, suggesting a permissive chromatin state for transcription (reviewed in [43]).

We propose that it would be instructive to confirm whether the epigenetic regulation of chromatin by Setd4 methyltransferase activity in CSC is due solely to its silencing effect (heterochromatin formation) or also involves some activating effects (euchromatin formation). Furthermore, and in agreement with previous data in independent models[4][6], Xing et al. showed that adult cardiac c-Kit+ cells upon short in vitro culture show a correlation between reduction of Setd4 expression and increase in proliferations status (Ki67+ or Edu+). This was reinforced by the transduction of activated cardiac c-Kit+ cells with an adenoviral vector overexpressing Setd4/GFP, which halted proliferation in parallel with a sharp increase in the expression of heterochromatin markers. Taken together, Xing et al. conclude that Setd4 regulates cardiac c-Kit+ cell quiescence by facilitating heterochromatin formation via H4K20me3 catalysis.

Lineage tracing analysis of Setd4+ cells

To further study Setd4+ cells in the heart, Xing et al. generated a Setd4-Cre;Rosa26mT/mG line that

showed significant fluorescence (membrane-GFP; mG+) in the postnatal heart, indicating that Setd4 might contribute to heart development. Lineage tracing of Setd4+ cells in the heart of E6.5, E15.5 and postnatal (P14) mice demonstrated mG+ cells in over 75% cells in the primitive streak, by E6.5, with no or very low background. Moreover, mG+ cells comprised approximately 37% of cells in the E15 mouse heart and this level was maintained after birth (P14), contributing similarly (E15 vs. P14) to the most relevant cardiac cell lineages (including cardiomyocytes). It could be illuminating to elucidate the precise contribution of Setd4+ cells to each of the main cardiac lineages (endothelial, smooth muscle and cardiomyocyte) or even to the cardiomyocyte/non-cardiomyocyte fractions. Using this genetic tool, Xing et al. confirmed that, in neonates, Setd4 protein was highly preferentially expressed in cardiac c-Kit+ compared with c-Kit- populations. It would also provide clear insights to the field, given the controversy of cardiomyogenic potential of c-Kit+ cells, to corroborate that all the Setd4+ progeny cells are c-Kit+. Focusing on the putative CSC/CPC populations, it is assumed that the embryo population would be less quiescent that adult counterparts, and it would be interesting to know whether the role of Setd4 is equivalent in newborn and adult cells. Analysis of Setd4+ vs. c-Kit+/lowCD45-CD31- phenotypes complemented with G0/G1-S status analysis during embryonic development would be informative. The emergence of stem cell quiescence in several embryonic lineages varies significantly[44][45] and is dominated by the creation of the first functional niches[46].

Conditional knockout of Setd4 in c-Kit+ cells

Xing et al. also analyzed the functional implications of conditional elimination of Setd4 in adult c-Kit+ cells using a dedicated conditional mouse model (Setd4 knock-out; c-Kit-CreERT2$^{(c)}$ Setd4f/f Rosa26-stop-TdTomato). Four days after tamoxifen (Tx) administration the authors analyzed Ki67 in sorted TdT+ cells, finding a statistically significant increase (≈ 2-fold) compared with controls. This confirmed that knock-out of Setd4 leads to the significant activation of cardiac quiescent c-Kit+ population in vivo. The authors also defined some relevant clues for the quiescence regulation of c-Kit+ cells in adult heart: sorted TdT+ cells from Setd4 knock-out (48 h post-Tx) showed, by western blot analysis, a significant decrease in H4K20me3, HP1α and PTEN expression, and significantly increased PI3K, AKT, and mTOR phosphorylation. Thus, Setd4 controls c-Kit+ cell quiescence via the PI3K-AKT-mTOR signaling pathway and H4K20me3 catalysis.

As previously discussed, it would be interesting to refine the analysis with the best characterized putative CSC/CPC, c-Kit+/lowCD45-CD31-[19] or B-CPC (Bmi-1+ Cardiac Progenitors Cells)[14] cells, to evaluate the impact of Setd4 deletion in the putative more primitive cardiac population. It is only fair to note that this is not the first quiescence-controlling factor described in cardiac c-Kit+ populations. Dimova et al. demonstrated that the chemokine SDF1 (also known as CXCL12) facilitated c-Kit+ cell quiescence by blocking cell cycle progression at the G0 to G1 transition[47]. This mechanism was also described as being AKT-dependent, but in contrast to the Setd4 mechanism it was facilitated by β-catenin stabilization

through GSK3β activity[46]. Wnt, Notch and FGF pathways have also been implicated in quiescence regulation in stem cells (reviewed in [48]). All these data confirm that CSC/CPC could be actively regulated by different intrinsic mechanisms. Interestingly, the role of the main actors in both c-Kit+ mechanisms (Setd4 and SDF1) is related to inflammatory response, which supports the idea that quiescence is not merely a passive state, but rather that quiescent CSC/CPC have the ability to sense environmental changes––such as MI––and respond by re-entering efficiently a activate/proliferative state.

After four weeks of Tx-induction of adult Setd4 knock-out animals, TdT+ cells were increased (≈ 1.4-fold), involving an increment of CD31+TdT+ cells (EC) but not of TroponinT+ TdT+ (cardiomyocyte-like; CM) and PDGFRα+ TdT+ cells (fibroblasts); the nascent EC expressed the capillary marker FABP4 (Td+FABP4+) but minimally contributed to the inner layer of coronary arteries as marked by α-SMA. Comparable results were obtained using complementary markers and inducing Setd4 deletion in neonatal mice. Altogether, these results demonstrate that the deletion of Setd4 in c-Kit+ populations favors progeny generation but with a strong bias for capillaries and a loss in their capacity to generate new cardiomyocytes. This was in clear contrast to the multipotent capacity of embryo-born Setd4+ cells. As we previously discussed, the existence of mixed populations in the studied c-Kit+ cells could limit the interpretation of the results. Analysis of the differentiation capacity of each lineage (mainly the cardiomyogenic potential) of Setd4+c-Kit+ vs Setd4-c-Kit+ cells could be invaluable.

Response to acute myocardial infarction of conditional knockout of Setd4 in c-Kit+ cells

Finally, Xing et al. analyzed the consequences of Setd4 knock-out in c-Kit+ cells upon MI. c-Kit+-labeled (TdT+; Tx 2 d after MI) cells were significantly increased in number in all heart areas in Setd4 knock-out mice, 4 m post-MI, and the animals showed a reduced infarct size and a moderate but significant improvement in some functional cardiac parametersd. Again, Setd4 knock-out c-Kit+ cells mainly contributed to capillaries (TdT+FABP4+), with poor generation of SMC and only occasional generation of nascent cardiomyocytes. Moreover, at 2 w post-MI, Setd4 knock-out c-Kit+ cells showed reduced cardiomyocyte apoptosis, most likely by the concomitant neo-angiogenesis. Interestingly, the exact opposite response was described by Dai et al. in c-Kit+ cells with SDF1-CXCR4 signaling blockade––the previously described alternative mechanism that controls quiescence in c-Kit+ cells––after MI, where infarcted mice have expanded scarring and hypertrophic and dilated cardiac tissue. In this case, activation of c-Kit+ cells enhanced their proliferation but, ultimately, the heart showed left ventricle dysfunction, implying progression to heart failure[49]. In concluding, Xing et al. note that the activation of quiescent c-Kit+ cells by Setd4 deletion promotes the neovascularization of capillaries, inhibits cardiomyocyte apoptosis, and thus preserves cardiac function in response to MI-induced injury in the adult heart.

Global discussion on Setd4-associated mechanisms

Several studies by independent groups transplanting c-Kit+ cells in adult mice confirmed some functional improvements but failed to observe the occurrence of substantial new cardiomyocyte formation from the injected cells[50][51]. It would be interesting to confirm whether adult Setd4+ cells retain the multipotency described in P14 animals or whether they intrinsically lose the capacity to contribute to the cardiomyocyte and SMC lineages; it would be important to evaluate the phenotype in adult Setd4 knock-out animals four weeks after Tx-induction. Alternatively, this could be the result of a different composition of embryonic and adult c-Kit+ populations: embryonic cells could be enriched in multipotent progenitors whereas the adult population, although containing progenitors, are diluted (>90%) by other c-Kit-expressing cell types such as mast cells and endothelial progenitors. So, cardiomyocyte and SMC differentiation capacity is highly diminished within the adult c-Kit+ pool in vivo, and the net balance would be poor.

The authors discuss that previous work transplanting mesenchymal stem cells, endothelial precursors or CSC/CPC (including c-Kit+ cells) could lead to the activation of endogenous quiescent c-Kit+ cells, which coordinately promote angiogenesis and modulate the immune reaction response (immunoregulation) at the cardiac ischemic injury site. This process generates a substantial increase in capillaries that would facilitate the rescue of anoxic cardiomyocytes, explaining the inhibition of cardiomyocyte apoptosis that Xing et al. describe. However, as we have emphasized throughout this review, we need to consider that the activation of endogenous quiescent c-Kit cells could be co-regulated by different intrinsic mechanisms.

Regarding potential therapeutic implications of Setd4 deletion (or chemical inhibition) we cannot ignore a plausible global impact in other tissues. Similar to the Xing et al. publication, ablation of Setd4 in adult mice after irradiation damage improved survival and bone marrow failure, with recovery of long- and short-term HSCs and early progenitor cells, characterized by high c-Kit expression[42]. Moreover, genetic lineage tracing studies have shown that during pancreatic development Setd4+ cells contribute to each pancreatic lineage in homeostasis but, contrastingly, deletion of Setd4 in adult mice compromises regeneration of acinar cells, leading to a failure to repair cerulein-induced pancreatitis damage[41]. All of these recent reports highlight the relevant role of Setd4 both in development and regenerative activities during normal/pathological conditions, but clearly in an organ-dependent context.

Conclusion

The study by Xing et al. demonstrates that Setd4 epigenetically regulates the quiescence of cardiac c-Kit+ cells by H4K20me3 via the PI3K-AKT-mTOR signaling pathway. Setd4+ cells were demonstrated to have multipotential differentiation capacity for most cardiovascular lineages in the embryonic heart, but they only significantly contributed to the endothelial lineage in the adult context. Activation of endogenous

quiescent (Setd4+) c-Kit+ cells by conditional knock-out of the Setd4 gene in c-Kit+ cells induced an increase in vascular endothelial cells of capillaries during homeostasis and in response to ischemic injury, mitigating some aspects of MI. The association of Setd4 expression with the quiescent cardiac c-Kit+ subpopulations, and taking into consideration other cardiac progenitor pools, defined as independent of c-Kit expression, could be an invaluable criterion to obtain definitive conclusions on the true nature and origin of heart turnover. This new vision of heart homeostasis may also help to define more realistic targets for future clinical interventions and cellular therapies.

Footnotes

a) *There is an error in the figure key*

b) *Fig. S1. Negative controls are left; this is especially relevant in this complex field.*

c) *Xing et al. refer ([3]) that the c-Kit-CreERT2 transgenic mice were purchased from Shanghai Model Organisms Center, Inc. We could not confirm that the transgenic construct is the same as that used by Molkentin et al. ([5][31][32]). Due to the conflict in the field, this tempers our evaluation and becomes relevant for the whole comparison.*

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