Adult c-kit<sup>pos</sup> Cardiac Stem Cells Fulfill Koch’s Postulates as Causal Agents for Cardiac Regeneration

To the Editor:

In a recent commentary, Molkentin and Houser<sup>1</sup> have focused on our article in *Cell* documenting the essential role of endogenous c-kit<sup>pos</sup> cardiac resident/stem/progenitor cells (eCSCs) in myocardial regeneration.<sup>2</sup> Their thorough review raised several issues that require our comment. We attempted to address these issues in the same format/space used by the authors but the Editors overruled us. We do not intend to defend our findings against the authors’ or others’ interpretation. Opinions and interpretations are free for everybody but facts are the basis of science and should be challenged with factual evidence. Our goal here is to clarify some overlooked/misinterpreted key factual aspects of our findings, which have significant relevance to the field of regenerative cardiovascular medicine.

By producing diffuse myocardial damage in several rodent and genetic fate-mapping murine models in combination with cell transplantation we have shown the following: (1) eCSCs regenerate the cardiomyocytes lost by the primary insult; (2) if the eCSCs are ablated, myocardial regeneration is stunted causing heart failure unless the eCSCs are replaced by exogenous CSCs; (3) Selective suicide of these exogenous replacement CSCs and their progeny abolishes regeneration and severely impairs ventricular performance; (4) the transplanted CSCs can be recovered from the recipient retaining their original characteristics and regenerative potential in vitro/in vivo. Therefore, these data fulfill all proverbial Koch’s postulates<sup>3</sup> for CSCs as the cell-type necessary and sufficient for myocardial repair and regeneration.

These conclusions rest mainly on 2 experimental facts specifically reviewed by Molkentin and Houser: the use of the isoproterenol (ISO) model of myocardial injury and the in situ labeling and genetic fate tracking of c-kit<sup>pos</sup>CSCs.

We respectfully disagree with the authors’ statement that ISO myocardial damage does not resemble any significant human cardiac disease. Human stress/apical ballooning cardiomyopathy (Takotsubo cardiomyopathy) accounts for up to ≈2% of all cases of suspected acute myocardial infarction.<sup>4</sup> This cardiomyopathy, in absence of coronary disease, is caused by severe emotional distress, which triggers catecholamine overload. The damage affects mainly the apical region and causes acute myocardial dysfunction that, similarly to our report in rodents, spontaneously resolves in ≈1 month. We think that it is difficult to find an animal model that better resembles a human disease than the ISO model does to Takotsubo cardiomyopathy. In our description of the ISO model, we acknowledged that mechanisms other than myocardial regeneration could also contribute to cardiac function recovery.<sup>5</sup> Nevertheless, participation of putative additional mechanisms does not challenge our conclusion about the pivotal role of eCSCs in the regeneration/repair observed. When eCSCs are eliminated, the damage becomes permanent and lethal.<sup>6</sup> After full eCSC deletion, if cloned CSCs are transplanted into the damaged myocardium, heart function and histology fully recover.<sup>2</sup>

Molkentin and Houser are also skeptical about the cardiac homing and functional engrafment of GFP-labeled CSCs when systemically injected in rats with ISO+5-fluorouracil cardiomyopathy. They point out that the rescuing CSCs were delivered 28 days after ISO, a time when, they argue, replacement fibrosis should have resulted in a lack of available niches. What they overlooked is that 5-fluorouracil during 28 days post-ISO killed not only the eCSCs, blunting myocyte regeneration, but also other proliferating cells including fibroblasts and inflammatory cells, preventing significant fibrosis. This latter effect reasonably explains why, even when injected after 5-fluorouracil treatment, exogenous CSCs home and engraft in the myocardium, replace the ablated eCSCs, and produce a functional myocyte progeny.<sup>7</sup>

As for the authors’ concerns about the validity of the lentiviral-based cre-lox strategy to tag the c-kit<sup>pos</sup>eCSCs and their progeny in vivo, a clarification is in order as we did not use the c-kit/enhanced green fluorescent protein construct 1 detailed in the original Ottolenghi’s study, which was shown to have some ectopic expression mainly in the central nervous system.<sup>8</sup> Instead, our c-kit/cre lentivirus was derived from the original construct 2, which, similarly to construct 3, is accurately expressed in c-kit<sup>pos</sup> cells and shows rare or absent ectopic expression (Figure A).<sup>6</sup> We ruled out c-kit reactivation in cardiomyocytes on ISO injury in transgenic c-kit/enhanced green fluorescent protein mice from construct 3.<sup>2</sup> These results show that (1) c-kit is not re-expressed either before or after ISO injury in adult cardiomyocytes, and (2) construct 2, when intramyocardially released is a valid tool to exclude c-kit reactivation in adult cardiomyocytes after myocardial damage.<sup>2</sup> This conclusion is further supported by unpublished tests showing that the c-kit/enhanced green fluorescent protein construct, used to obtain the lentiviral vector (Figure A), when injected into wild-type mice correctly labeled c-kit-positive cardiac cells (Figure B). However, not a single GFP positive mature myocyte was found in hearts either before or after ISO injury (Figure B). These data, together with the experimental evidence that no cardiomyocytes other than c-kit<sup>pos</sup> cardiac cells were reconstituted by the lentivirus c-kit/cre to express yellow fluorescent protein in R26-stop-EYFP transgenic mice,<sup>7</sup> should assuage Molkentin and Houser’s concerns that our experiments are not well controlled and are, therefore, questionable. Thus, our data provide the first genetic fate-mapping evidence that c-kit<sup>pos</sup>eCSCs differentiate into bona fide mature cardiomyocytes in vivo.

We do agree with Molkentin and Houser that a knockin c-kit/cre transgenic, if proven to work as desired, would be optimal to genetically fate map the contribution of c-kit<sup>pos</sup>eCSCs to heart generation and regeneration. Although we are currently working toward this goal, this strategy requires solving several problems. In the only available lac operon Z encoding β-galactosidase enzyme (LacZ)c-kit knockin (into the first exon of the mouse Kit gene), used to reveal/follow c-kit expression during development, the knockin null allele altered c-kit receptor expression.
and function. Moreover, even if the pattern of expression of the desired c-kit/cre knockin were correct, the data obtained would be partial and qualitative. Cre-recombination, when induced in the adult, has been repeatedly proven to label only a fraction of the target cells. Therefore, only a totally negative result, which convincingly rules out any new myocyte formation, would be unambiguous. The detection of any recombinant myocytes, because of the partial effectiveness of the cre/lox approach, will not rule out alternative sources of myocyte replacement. Additionally, such knockin would not allow tracking the fate of any particular c-kitpos cell (ie, cardiac or circulating) as the agent of myocardial regeneration after injury. Furthermore, when using an inducible Cre construct, fate mapping will have to deal with both the known acute toxic effects of the Cre construct and the reported epigenetic silencing of loxP-STOP loxP cassette in adult life. Thus, the only data now available tracking the fate of the eCSC cohort are those provided by our myocardial release of the lenti-c-kit/cre in R26-stop-EYFP transgenic mice, where the labeling is confined exclusively to cardiac c-kit expressing cells. Consistent with our results, an elegant study by Braun’s group, using a triple transgenic mouse, has lineage-traced cardiomyocyte formation in adult life to be at least partly the progeny of eCSCs expressing Sca-1 (a fraction of which express c-kit). In summary, applying Occam’s razor to the available data as interpreted by both sides of the ongoing controversy about the role of the eCSCs in myocardial cell homeostasis and repair, the simplest and most logical conclusion, which fits all available data, is that the eCSCs are the main cardiac regenerative agents in adult mammals.
Disclosures
None.

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