c-kit+ cells minimally contribute cardiomyocytes to the heart

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If and how the heart regenerates after an injury event is highly debated. c-kit–expressing cardiac progenitor cells have been reported as the primary source for generation of new myocardium after injury. Here we generated two genetic approaches in mice to examine whether endogenous c-kit+ cells contribute differentiated cardiomyocytes to the heart during development, with ageing or after injury in adulthood. A complementary DNA encoding either Cre recombinase or a tamoxifen–inducible MerCreMer chimaeric protein was targeted to the Kit locus in mice and then bred with reporter lines to permanently mark cell lineage. Endogenous c-kit+ cells did produce new cardiomyocytes within the heart, although at a percentage of approximately 0.03 or less, and if a preponderance towards cellular fusion is considered, the percentage falls to below approximately 0.008. By contrast, c-kit+ cells amply generated cardiac endothelial cells. Thus, endogenous c-kit+ cells can generate cardiomyocytes within the heart, although probably at a functionally insignificant level.

The adult mammalian heart was originally proposed to be essentially incapable of renewal after injury or with ageing; although some recent studies have shown that the heart is capable of new cardiomyocyte formation with varying degrees of regenerative potential1. The concept that stem cells are the source for cardiomyocyte regeneration arose from initial observations in which bone-marrow-derived c-kit+ haematopoietic stem cells (HSCs) showed restoration of the myocardium after infarction injury when given exogenously2. However, subsequent studies demonstrated that HSCs possessed essentially no ability to make cardiomyocytes, calling into question these earlier reports3,4, at which time the field shifted to a focus on endogenous c-kit+ cardiac progenitor cells (CPCs) residing within the myocardium5. Such cells isolated from the rat heart were reported to differentiate into cardiomyocytes, smooth muscle cells and endothelial cells, even after clonal derivation, and when injected into the infarct region they produced substantial new myocardium6. Mouse and human c-kit+ CPCs were also isolated and marked, and after injection into an infarcted mouse heart, were shown to generate substantial levels of labelled cardiomyocytes, capillaries and fibroblasts5. More recently, resident c-kit+ CPCs were reported to be both necessary and sufficient for complete repair and functional restoration of the myocardium after isoproterenol-induced cardiomyocyte killing, whereas bone-marrow-derived c-kit+ cells had no regenerative effect6. However, other studies with adult cardiac resident c-kit+ cells have reported the opposite: that these cells do not possess the ability to generate cardiomyocytes in vivo7,8,9,10. To address ongoing controversy, we generated mice in which the Kit locus was used for lineage tracing analysis to examine if and how frequently c-kit+ cells generate cardiomyocytes in vivo.

c-kit+ contribution to the growing heart

The Kit locus was targeted with a cDNA encoding Cre recombinase fused to an internal ribosome entry sequence (IRES) to concurrently express enhanced green fluorescent protein (eGFP)-tagged with a nuclear localization signal (nls) (Fig. 1a). These Kit+/Cre mice were bred to LoxP site-dependent Rosa26-CAG-loxP-STOP-loxP-eGFP (R-GFP) reporter mice to irreversibly mark any cell that previously or currently expresses this Kit locus (Fig. 1a). Four to twelve weeks after birth, the fidelity of the genetic system was assessed in comparison with known domains of c-kit protein expression, such as melanocytes of the skin, Leydig cells in the testis, interstitial cells of the intestine, lung and wide areas of the spleen, all of which showed eGFP cellular labelling (Fig. 1b and Extended Data Fig. 1a)11–13. In bone marrow, 83% of the c-kit+–antibody-detected cells were eGFP+ by standard fluorescence-activated cell sorting (FACS) analysis (Fig. 1c), while imaging cytometry analysis detected coincident eGFP+ expression and c-kit immunoreactivity in 88% of the bone marrow cells and 76% of the non-myocyte fraction from the heart (Fig. 1d, e). To further verify the specificity of the Kit-Cre allele we examined real-time eGFPnls expression in the heart, ileum and skeletal muscle for co-expression of c-kit–protein (antibody), which was always coincident (Fig. 1f, g and Extended Data Fig. 1b, c). In bone marrow, 94% of the eGFP+ cells were Lin−, indicating a high degree of fidelity with the Kit-Cre allele (Extended Data Fig. 1d). In the heart c-kit–antibody–positive mononuclear cells were predominantly eGFP+ at 4 weeks of age using the Kit+/Cre×R-GFP reporter strategy, whereas in testis recombination was only observed in Leydig cells, of which >80% were eGFP+ (Extended Data Fig. 1e, f). Thus, the specificity of the Kit-Cre allele appears identical with known regions of c-kit protein expression in vivo.

In an exhaustive search by histological methods across four hearts from Kit+/Cre mice for current eGFPnls expression at 4 weeks of age, no eGFP+ cardiomyocytes or endothelial cells were identified (only mononuclear CPC–like cells were observed), strongly suggesting that the Kit locus is not spontaneously activated in differentiated cell types of the heart (Fig. 1f). However, in conjunction with the R-GFP reporter allele for ongoing c-kit lineage tracing, the myocardium showed many eGFP+ differentiated cell types, although cardiomyocytes were very rare (Fig. 1i, h). Even more rarely, areas suggestive of cardiomyocyte clonal expansion were identified (Fig. 1i). No eGFP+ cells were observed in hearts of single R-GFP mice (data not shown). To more rigorously quantify the extent of cardiomyocyte recombination–based labelling,
Kit-Cre lineage tracing. a. The Kit locus was targeted in mice to express Cre recombinase and eGFP with a nuclear localization sequence (eGFPnls) behind an internal ribosome entry site (IRES). These mice were crossed with Rosa26 reporter mice (R-GFP) for lineage tracing. b. Diagram of mice used for all experimentation in this figure. c. Representative FACS plot of bone marrow from Kit<sup>+/Cre</sup> × R-GFP mice gated for c-kit antibody, then eGFP fluorescence to reflect recombination of the R-GFP locus (representative of n = 6 mice). d. Direct imaging cytometry analysis of eGFP expression in bone marrow (averages from n = 3 mice, *P < 0.05 versus R-GFP). e. Same quantitative imaging cytometry analysis as in d except the non-myocytes were isolated from hearts of Kit<sup>+/Cre</sup> × R-GFP mice (averages from n = 3 hearts, *P < 0.05 versus R-GFP). f. Representative cardiac immunohistochemistry to show current expression from the Kit-Cre allele (green, eGFPnls) versus endogenous c-kit protein detected by antibody (Ab, red). The inset box shows two mononuclear c-kit expressing cells. g. Quantification of average number of c-kit<sup>+</sup> cells per longitudinal heart section (n = 4 hearts). h. Representative histological section at two magnifications (white box) of a Kit<sup>+/Cre</sup> × R-GFP mouse heart with desmin antibody in red, eGFP antibody in green, and nuclei in blue. The arrow shows an eGFP<sup>+</sup> cardiomyocyte. i. Representative immunohistochemical image showing a rare area of cardiomyocyte clonal expansion (arrow) (n = 6 hearts analysed). j. Image of cells dissociated from the hearts of Kit<sup>+/Cre</sup> × R-GFP mice (n = 3 hearts analysed). White arrow shows a rare eGFP fluorescing cardiomyocyte, black arrowheads show eGFP fluorescent non-myocytes. k. Quantification of eGFP<sup>+</sup> fluorescent cardiomyocytes (81 from 303,264 total cardiomyocytes, 3 hearts, *P < 0.05 versus R-GFP). l. DNA electrophoresis after PCR showing Cre-mediated Rosa26 locus recombination in semi-purified cardiomyocytes and spleens (n = 2 Kit<sup>+/Cre</sup> × R-GFP mice). All error bars represent s.e.m.

Hearts of Kit<sup>+/Cre</sup> × R-GFP mice at 4–12 weeks of age were further examined to identify the remaining eGFP<sup>+</sup> non-myocytes. Examples of eGFP labelling co-incident with fibroblasts (vimentin co-labelling), endothelial cells (CD31, CD34, von Willebrand factor (vWF)), immune cells (CD3, CD45) and, rarely, smooth muscle α-actin (α-SMA)-expressing cells, were identified, although the most prevalent co-localizations were with CD31<sup>+</sup>, CD45<sup>+</sup> or CD34<sup>+</sup> positive cells (Fig. 2a–g). Indeed, using a cocktail of antibodies for CD31, CD45, CD34 and CD3, versus sarcomeric α-actin, we were able to account for almost all eGFP<sup>+</sup> non-myocytes in the hearts of adult Kit<sup>+/Cre</sup> × R-GFP mice, either when analysed from histological sections or as dissociated individual cells (Extended Data Fig. 2a–c). FACS analysis showed that 18% and 77% of the total eGFP<sup>+</sup> non-myocytes in the heart were CD45 or CD31 positive, respectively (Fig. 2h,i). Confocal microscopy analysis showed exact co-localization between eGFP<sup>+</sup> cells in the heart and CD31 protein expression, but not with NG2 staining for pericytes (Fig. 2j).

We also collected Kit<sup>+/Cre</sup> × R-GFP mice at birth (postnatal day P0) to analyse the contributions of c-kit<sup>+</sup> cells to the heart during embryonic and fetal development (Extended Data Fig. 3a). Control histological sections from the ileum and lung showed the expected distribution of c-kit<sup>+</sup> cells (Extended Data Fig. 3b), and the heart also showed numerous eGFP<sup>+</sup> cells throughout (Extended Data Fig. 3c). Immunohistochemical...
Importantly, no eGFP expression (Extended Data Fig. 4a). Kit reporter mice to lineage trace c-kit-expressing cells when tamoxifen is present. Kit adult cardiomyocyte (Fig. 3a). To verify the fidelity of this system, Kit reporter mice to lineage trace c-kit-expressing cells when tamoxifen is present. 4 weeks followed by collection of tissues with known sites of c-kit expression (Extended Data Fig. 4a). Kit/mice were given tamoxifen during postnatal maturation for approximately 4 weeks followed by collection of tissues with known sites of c-kit expression (Extended Data Fig. 4a). Kit/mice showed 70% overlap in recombination-dependent eGFP expression and endogenous c-kit protein in Leydig cells of the testis (Extended Data Fig. 4b). Importantly, no eGFP cells were observed in the absence of tamoxifen at any age examined or after myocardial infarction injury, demonstrating that the MerCreMer system does not ‘leak’ (Extended Data Fig. 4c). Kit/mice were also given tamoxifen from day 1 through 6 months of age for continuous labelling (Fig. 3b), which produced eGFP expression in greater than 60% of bone marrow cells, but again no signal in the absence of tamoxifen (Fig. 3c–e). Histological analysis of the heart after 6 months of labelling showed rare examples of eGFP adult cardiomyocytes and a relatively large number of non-myocytes (Fig. 3f, g). Careful analysis of the non-myocyte fraction in these hearts showed fibroblasts (rarely), smooth muscle cells (rarely), endothelial cells and immune cells, with the majority again being CD31 (Extended Data Fig. 5a–g). Myocardial infarction injury also doubled the number of CD31 cells that were eGFP in the adult heart with 8 weeks of prior tamoxifen labelling (Extended Data Fig. 5h). We also conducted c-kit lineage labelling from 6–12 weeks of age, just after the postnatal developmental period (Fig. 3h). Upon disassociation of these hearts we observed 0.0055% eGFP adult cardiomyocytes (Fig. 3i, j), confirmed as extremely low by PCR and quantitative PCR (qPCR) for Rosa26 locus recombination (Extended Data Fig. 6a–c). Cardiac injury increases cellular turnover in the heart, hence we subjected Kit/mice to myocardial infarction at 10 weeks of age during a 6-week tamoxifen-labelling protocol (Fig. 3k and Extended Data Fig. 6d–f). The percentage of eGFP cardiomyocytes increased to cardiomyocyte) that is quantified in j (127,284 cardiomyocytes across two hearts, 7 were eGFP, *P < 0.05 versus R-GFP). k–n, Tamoxifen treatment of Kit/mice between 8 and 14 weeks of age with myocardial infarction (MI) on week 10 (n = 3 mice analysed). I, Immunohistochemical heart section for desmin (red) and eGFP (green) with nuclei in blue (arrow shows a cardiomyocyte from the c-kit lineage). m, n, Disassociated cardiomyocytes show rare but definitive myocyte labelling (white arrow), which was quantified in n (225,760 cardiomyocytes from 2 myocardial infarction-injured hearts, 37 were eGFP, *P < 0.05 versus R-GFP). o, Tamoxifen treatment between 8 and 12 weeks of age with myocardial infarction injury occurring 3 days after tamoxifen cessation. p, Average number of eGFP cardiomyocytes from histological sections taken across the entire heart (n = 2 hearts, >50 sections each) *P < 0.05 versus R-GFP. All error bars represent s.e.m.

Figure 3 | Inducible Cre expression from the Kit locus shows limited adult cardiomyocyte formation. a, Genetic cross between Kit/mice in the tamoxifen-inducible MerCreMer protein was targeted to the Kit locus (Kit/mice, followed by cross breeding with the R-GFP reporter line (Fig. 3a). To verify the fidelity of this system, Kit/mice or R-GFP mice without (c) or with (d) tamoxifen. e, FACS quantification of eGFP cells from bone marrow of Kit/mice or R-GFP mice without (c) or with (d) tamoxifen. e, FACS quantification of eGFP cells from bone marrow of these mice (average from n = 2 mice for R-GFP and n = 4 for Kit/mice or R-GFP). f, g, Representative heart sections from Kit/mice or R-GFP mice showing c-kit lineage cells in green and cardiomyocytes in red (desmin antibody). White arrow indicates eGFP adult cardiomyocyte (n = 3 mice analysed). h–j, Tamoxifen treatment of Kit/mice between 6–12 weeks of age followed by disassociation of cells from the hearts of these mice in h (white arrow in i shows rare
0.016% within the heart, with more being localized to the infract border zone (Fig. 3l–n). c-kit<sup>+</sup> lineage cells within the heart were also pre-labelled by giving tamoxifen only before myocardial infarction injury, which again showed a very low percentage of eGFP<sup>+</sup> cardiomyocytes (Fig. 3o, p). Percentages of eGFP<sup>+</sup> cardiomyocytes in the heart during 4 weeks of isoproterenol-induced injury were 0.007% (Extended Data Fig. 7a–c). These astonishingly low values of cardiomyocyte formation were independently verified using blinded heart histological sections from Kit<sup>+/MCM</sup> × R-GFP mice sent to an outside academic laboratory (Extended Data Fig. 8a–c).

Finally, we also cultured total non-myocytes from the hearts of young adult Kit<sup>+/Cre</sup> × R-GFP mice in the presence of dexamethasone as a means of pushing c-kit<sup>+</sup> cells with progenitor-like activity towards the cardiomyocyte lineage (Extended Data Fig. 9). The data show that eGFP<sup>+</sup>, Kit-Cre allele expressing cells are fully capable of inducing expression of the cardiac markers GATA4, α-actinin and troponin T, suggestive of partial differentiation towards the cardiomyocyte lineage (sarcomeres were not observed).

**c-kit<sup>+</sup> cells fuse in the heart**

Hearts from Kit<sup>+/MCM</sup> × R-GFP mice showed the presence of cells from blood lineages (CD3, CD45, CD34), which are known to have fusigenic activity with resident parenchymal cells<sup>13–18</sup>. To examine fusion we used a genetic strategy that constitutively expresses a membrane-targeted fluorescent tdTomato protein from the Rosa26 locus. Upon Cre-mediated recombination, tdTomato fluorescence is lost and a membrane-targeted eGFP becomes expressed (abbreviated mT/mG for membrane-targeted tdTomato and eGFP, respectively) (Fig. 4a). If cells fuse, both signals would be present but a de novo cardiomyocyte from a c-kit<sup>+</sup> lineage cell would be only green. Experimentally, Kit<sup>+/MCM</sup> × mT/mG mice were given tamoxifen for 2 weeks (8–10 weeks of age) then 3 days later myocardial infarctions, followed by collection at 1, 2 and 4 weeks thereafter (Fig. 4b). Control mice were collected before myocardial infarction but after tamoxifen (time 0). Percentages of total cardiomyocyte membrane-eGFP labelling, whether from fusion or not, were approximately 0.01% at all three time points after myocardial infarction (Fig. 4c). Although some de novo cardiomyocytes were identified in the heart (eGFP only), the majority (80–88%) retained the membrane-tdTomato label indicating that these cells probably arose by fusion (Fig. 4d–f). Thus, c-kit<sup>+</sup> lineage cells can generate cardiomyocytes in the heart, although at ~5-fold lower values than initially predicted.

**Kit-Cre locus is not ectopically induced**

One concern with the Kit allele-based lineage tracing approach is that if this locus ever becomes activated ectopically in a cardiomyocyte, it would be wrongly ascribed as having come from a c-kit<sup>+</sup> cell. It was previously shown that knockdown of the Kit gene in naturally occurring w and w<sup>v</sup> mutant mice results in defective progenitor cell activity in many tissues<sup>19–22</sup>. Indeed, hearts from Kit<sup>+/MCM</sup> mice showed a marked reduction in resident mononuclear c-kit<sup>+</sup> cells and progenitor activity<sup>23</sup>. Hence, Kit null mice should lack the ability to generate eGFP<sup>+</sup> cardiomyocytes in the heart if they indeed arise from c-kit<sup>+</sup> cells with progenitor-like activity, instead of having arisen from ectopic Kit allele induction in a rare population of differentiated cardiomyocytes. Kit null mice were generated by placing the Kit-Cre allele over the Kit-MerCreMer allele. Although these mice die at birth, viable nulls at embryonic days 16.5 and 18.5 were identified and examined (Fig. 4g–j). Fourteen total eGFP<sup>+</sup> cardiomyocytes were counted from four Kit<sup>+/Cre</sup>Mice.

**Figure 4 | Assessment of fusion versus de novo cardiomyocyte formation in the heart.** a, Genetic strategy in which Kit<sup>+/MCM</sup> mice were crossed with Rosa26 targeted mice containing the membrane targeted tdTomato/eGFP (mT/mG) reporter. b–f, Tamoxifen was given to Kit<sup>+/MCM</sup> × mT/mG mice between 8 and 10 weeks, followed 3 days later by myocardial infarction injury. c, Quantification across >50 histological sections of all eGFP<sup>+</sup>-expressing cardiomyocytes (averages) before myocardial infarction (n = 4 hearts) and 1 (n = 4 hearts), 2 (n = 5 hearts) and 4 (n = 3 hearts) weeks after myocardial infarction injury. Error bars represent s.e.m. *P < 0.05 versus mT/mG. d, Example of a c-kit<sup>+</sup>-lineage-derived de novo cardiomyocyte in which membrane-eGFP (green, left) is expressed and tdTomato fluorescence (red, right) is lost. e, Example of eGFP<sup>+</sup> cardiomyocyte (green) that still contains endogenous membrane-tdTomato fluorescence (red), indicating fusion. Nuclei are stained blue. f, Quantification of fusion percentage. *P < 0.05 versus mT/mG. g–i, Representative immunohistological images from embryonic (E) day 16.5 mouse hearts that are either Kit<sup>+/Cre</sup> × R-GFP (het, n = 4 (h)), Kit<sup>+/MCM</sup> × R-GFP (null, n = 2 (i)) or Kit<sup>+/MCM</sup>Cre (null, no reporter, n = 2 (g)). Red staining is α-actinin and green is eGFP. j, Higher magnification image from h, showing a definitive eGFP<sup>+</sup> cardiomyocyte (arrow). k, Higher magnification image from i, which shows only eGFP<sup>+</sup> non-myocytes in Kit null hearts. l, m, Histological heart images from E18.5 Kit<sup>+/Cre</sup> (het, n = 1) and Kit<sup>+/MCM</sup>Cre (null, n = 1) embryos containing the mT/mG reporter, again only the heterozygotes show examples of eGFP<sup>+</sup> cardiomyocytes (arrow). n, Western blot showing loss of c-kit protein in Kit<sup>+/MCM</sup>Cre embryos (nulls) versus heterozygous controls.
× R-GFP and 1 Kit<sup>+</sup>/Cre × mT/mG embryos across 56 histological sections spanning the heart (Fig. 4j, l). However, hearts from two Kit<sup>McM/Cre</sup> × R-GFP and one Kit<sup>McM/Cre</sup> × mT/mG embryos (nulls) showed lower total eGFP<sup>+</sup> cells in the heart and no cardiomyocytes across 69 histological sections (Fig. 4i, k, m). Importantly, Kit<sup>McM/Cre</sup> embryos showed no c-kit protein expression confirming their null status (Fig. 4n). Taken together, these data indicate that eGFP<sup>+</sup> cardiomyocytes that are lineage traced with the Kit-Cre allele are not due to inappropriate activation of the Kit gene for even a brief period of time in rare existing cardiomyocytes, but rather they either arose by transdifferentiation from c-kit<sup>+</sup> lineage precursor cells or by fusion.

**Discussion**

The original hypothesis that c-kit<sup>+</sup> cells have the ability to contribute to the cardiomyocyte compartment of the heart, as well as other cell types, is correct as determined by the lineage tracing technique used here<sup>3</sup>. Indeed, the observation that embryonic and postnatal labelling in the hearts of Kit<sup>+</sup>/Cre × R-GFP mice shows definable regions with cardiomyocyte clonal expansion strongly suggests that these c-kit<sup>+</sup> cells can make cardiomyocytes in vivo. More importantly, loss of the Kit gene, which is known to compromise the progenitor and migration activity of c-kit<sup>+</sup> cells, completely prevented cardiomyocyte formation from c-kit<sup>+</sup> cells. However, throughout development, with ageing or with cardiac injury, the percentage of cardiomyocytes emerging from the c-kit<sup>+</sup> lineage was astonishingly low and hence likely unlikely to ever considerably affect cardiac function. The mT/mG detection system also supported the existence of de novo cardiomyocyte formation in the adult heart from the c-kit<sup>+</sup> lineage but at ~5-fold lower levels than initially quantified owing to prevalent cellular fusion events.

Exogenous c-kit<sup>+</sup> cells are currently being used to treat post-myocardial infarction heart failure patients, and early results have shown small albeit significant, functional improvements in the heart<sup>24</sup>. However, our results suggest that the potential benefit of injecting c-kit<sup>+</sup> cells into the hearts of patients is unlikely attributable to new cardiomyocyte formation, hence caution is warranted until the mechanisms in play are better defined, or until we are able to considerably enhance the cardiogenic potential of these cells (see Supplementary Discussion).

**METHODS SUMMARY**

The Kit allele was targeted in SV129 embryonic stem cells to express either Cre recombinase alone or a tamoxifen-inducible Cre recombinase referred to as MerCreMer. Hemizygous targeted mice were crossed with FVB.Cg-Gt(ROSA)26Sortm1(CAG-lacZ,EGFP)Glh (previously modified by cross-breeding to FVB(C3)-Tg(Pgk1-FLPo)10Sykr/J) or B6.129(Cg)-Gr(ROSA)26Sor<sup>pm1(CAG-4xGCK-P2Kv12)</sup> (previously modified by cross-breeding to B6(C3)-Tg(Pgk1-FLPo)10Sykr/J) or B6.129(Cg)-Gr(ROSA)26Sor<sup>pm6(ActB-tdTomato,-EGFP)Luo</sup> (previously modified by cross-breeding to B6(C3)-Tg(Pgk1-FLPo)10Sykr/J). Tissues from these mice were subjected to histological analysis and immunohistochemistry at multiple ages and after select treatments. Antibodies used are shown in Supplementary Table 1 (see Methods for more detailed descriptions).

**Online Content**

Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information**

Is available in the online version of the paper.

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**Author Contributions**

J.D.M., J.H.v.B., O.K., M.M., S.-C.J.L. and R.J.V. designed the experiments. S.-C.J.L. designed the Kit allele targeting construct and targeted mice. J.H.v.B. and O.K. designed the breeding, performed histological experiments and animal procedures. R.J.V. performed the qPCR assays. M.M. performed immunohistochemistry. J.K. performed cell culture experiments. E.M. and R.C.M. designed and conducted the independent verification immunohistochemistry with blinded samples. J.D.M. wrote the manuscript.

**Author Information**

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METHODS

Mice. All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children’s Hospital. No human subjects or human material was used. Targeted Kit-Cre-IRE6α-EGFP mice and Kit-MerCreMer mice were generated by standard gene targeting techniques. Homology arms upstream and downstream of the ATG start codon of the Kit gene in exon 1 were subcloned into a plasmid backbone containing Amp4 and a diphtheria toxin (DTA) cassette through recombination. A DNA encoding either Cre-IRE6α-EGFP mice (from A. P. McMahon, UCLA) or MerCreMer, as well as an frt site flanked neomycin selection cassette, were cloned in-frame with the Kit ATG start site. Embryonic stem (ES) cells were electroporated with linearized targeting vector. Targeted clones were identified by Southern blot and PCR. ES cells with integration with 8-cell embryos were used to generate chimeraic mice with the Kit-Cre-IRE6α-EGFP mice construct33, whereas the Kit-MerCreMer mice were generated by blastocyst injection at the Howard Hughes Medical Institute (HHMI) gene-targeting core facility by (C. Guo at HHMI, who also generated the Kit-MerCreMer targeting vector and target ES cells). Germline transmitting male chimaeras were crossed with Rosa26-flpe females (B6.129S4-Gt(ROSA)26Sor<tm1Sorj>/Rosa26tm1Sorj (Raini)) to delete the neomycin cassette and verified offspring were first backcrossed to C57/B6J for five generations. Reporter mice FVB:Gt(ROSA)26Sor<tm1Sorj>/CAG-tdTomato,Egfp2os (previously modified by cross-breeding to B6(C3)-Tg(Pgk1-FLPo)10Sykr/J) and B6.129(Cg)-Gt(ROSA)26Sor<tm1Sorj)/CAG-tdTomato,Egfp2os were purchased from the Jackson Laboratories. Kit null mice were generated by breeding male Kit9+/+ with female Kit1MCM×R-GFP mice, of which 1.8 embryos are predicted to be Kit1MCM×R-GFP (nulls, with the reporter). Littermates that were Kit9+/+×R-GFP were used to control the full extent of EGFP cardiomyocytes that are possible in the heart. Because Kit null mice were not identified at birth in multiple litters, we collected mice from this cross at E16.5 and E18.5, which identified viable Kitnull mice. PCR genotyping of Kit-Cre-IRE6α-EGFP mice used the following primers: (wt-Kit-forward: 5′-CTGAGCAGAGAAGAGCT-3′ and Cre-reverse: 5′-CTACACAGAGAGCAAGACATC-3′); Kit-MerCreMer (MerCreMer-forward: 5′-CTGAGAGGACCTATATCTATT-3′ and MerCreMer-reverse: 5′-GTGGATGTGGTCCTTCTCTTC-3′); Kit-forward: 5′-CTGAGCAGAGAAGAGCTGCT-3′ and reverse: 5′-ACAGAGGGCTGAGGCTCTTCT-3′). Mice of various ages were used, as indicated for each experiment. Both male and female mice were used in all experiments.

Animal procedures. Tamoxifen citrate containing chow (Harlan laboratories) was used to activate the inducible MerCreMer protein, thereby inducing Cre recombinase activity. We used the standard 400 mg kg−1 chow for all experiments, except for labelling right after birth for which we used 200 mg kg−1. The duration of treatment is indicated within each experiment. Myocardial infarction was induced in mice via permanent surgical ligation of the left coronary artery. In brief, mice (both sexes) were anaesthetized using isoflurane and a lateral thoracotomy was performed. The left coronary artery was identified and ligated just below the left atrium. After closing the thoracotomy and expelling residual air, the mice were allowed to recover. Two-dimensional M-mode echocardiography was performed on mice anaesthetized with 2% isoflurane, using a Hewlett Packard SONOS 5500 with a 15-MHz transducer. An average of three measurements was taken for each mouse. Group sizes were determined from past experience and based on statistical power calculations, and the number of mice is given in the figure or figure legends. Isoproterenol treatment was given via osmotic minipumps (Alzet) at 60 mg kg per day (in 1 μM ascorbic acid) for 4 weeks. Mice were either killed by CO2 asphyxiation or decapitation after a final centrifugation at 500 g. Following citrate antigen retrieval (BioGenex), the sections were blocked for 1 h at room temperature in a blocking solution (PBS with 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.1% Tween-20 and 0.05% NaCl), which was also used to dilute antibodies. For cryosections, isolated organs were fixed for 3 h in freshly diluted 4% paraformaldehyde at 4 °C, rinsed with PBS and cryoprotected in 30% sucrose/PBS overnight before embedding in OCT (Tissue-Tek) and 10-μm cryosections were collected. Cryosections were blocked for 30 min at room temperature in a blocking solution (PBS with 5% goat serum, 2% bovine serum albumin, 0.1% Triton X-100), which was also used to dilute antibodies. Primary antibodies were incubated overnight at 4 °C, secondary antibodies for 2 h at room temperature, washes were performed in PBS. Cryosections were used to visualize native EGFP or tdTomato fluorescence from the different reporters or from the IRES-EGFP cassette built into the Kit-Cre allele.4′,6-diamidino-2-phenylindole (DAPI) was used as a counterstain for nuclei (usually in blue). Images were acquired on an inverted Nikon A1R confocal microscope using NIS Elements AR 4.1.3. Some images were further processed in Photoshop or Image J to increase brightness/contrast of individual channels before generating a pseudo-coloured overview.

Genomic PCR and qPCR. Genomic DNA was prepared from mouse tissues or isolated mouse cardiomyocytes using the DNeasy Blood & Tissue Kit (Qiagen, 69504) per manufacturer’s instructions. In brief, cells or tissues were snap-frozen at time of collection then lyzed by incubation with proteinase K for 3 h at 56 °C, followed by spin column purification and elution. Samples were treated with RNase A to remove contaminating RNA. PCR was performed to detect recombinated and non-recombined reporter alleles using primers complementary (forward, against the CAG promoter/enhancer), 5′-gctgacctggctggtcaca (reverse, against eGFP). PCR conditions were 96 °C for 2 min to separate strands, followed by 34 cycles of amplification (96 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) and a 5-min elongation step at 72 °C. PCR products were visualized on an ethidium bromide-stained agarose gel using a UV molecular imager (Bio-Rad). To quantify levels of recombinated and non-recombinated Rosa26 alleles in genomic DNA, qPCR was performed using SYBR Green with the same primers used for PCR above (Applied Biosystems), and detection with a Bio-Rad CFX96 thermocycler. Simultaneous reactions using the same primers above were performed to detect recombinated versus non-recombinated alleles.

Western blots. Western blotting was performed essentially as described previously29. E1.65 embryos were homogenized in RIPA buffer containing protease inhibitor cocktail (Roche) with a dounce homogenizer. Forty micrograms of protein per
sample were resolved on 10\% SDS–PAGE gels, transferred onto PVDF membranes, immunoblotted with antibodies for c-kit (R&D Systems AF1356) and GAPDH (Fitzgerald 10R-G109a), and then incubated with the appropriate alkaline phosphatase-linked secondary antibodies. The PVDF membranes were visualized by enhanced chemiluminescence (Amersham).

**In vitro cardiomyocyte differentiation.** The non-cardiomyocyte cell fraction was isolated from a 3-month-old Kit\(^{\text{+/Cre}}\) × R-GFP mouse. Cells were plated at a density of 40,000 cells per well on gelatin-coated 6-well tissue culture dishes in DMEM media containing 10\% FCS, antibiotics and non-essential amino acids. After 2 days, the cells were washed and treated with 10 nM dexamethasone in DMEM containing 10\% FCS to induce differentiation\(^6\). The media was refreshed every 3 days. After 1 week the cells were fixed with 4\% paraformaldehyde and subjected to immunohistochemistry for vimentin, \(\alpha\)-actinin, troponin T and GATA4 (antibodies listed in Supplementary Table 1). The cells were then imaged on an inverted Nikon A1R confocal microscope.

**Statistics.** For studies involving induction of myocardial infarction, group sizes were determined on the basis of previously observed postoperative mortality rates for this procedure. No experimental animals were excluded in any of the analyses. Blinding and randomization were not performed with the exception of the experiments in Extended Data Fig. 8, which were done by two observers blinded to the sample identity. For flow cytometry experiments and direct counting of cardiomyocytes in histological sections or dissociated cardiomyocytes in dishes, two-group comparisons were performed using Student’s two-tailed \(t\)-test, with \(P < 0.05\) considered statistically significant. All error bars throughout the figures are s.e.m. and all represented data are averages.

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Extended Data Figure 1 | Assessing the fidelity and specificity of the Kit-Cre knock-in allele. a, Representative histological sections from the indicated tissues of Kit\(^{+/+}\) \times \text{R-GFP} mice at 4 weeks of age. Blue is nuclei and green is eGFP. The data show eGFP expression in regions of each tissue that is often characteristic of endogenous c-kit protein expression (n = 3 mice).

b, Immunohistochemistry for endogenous c-kit expression (red) in the mouse ileum at 4 weeks of age from Kit\(^{+/+}\) mice that contain the IRES-eGFPnls cassette (but without the \times \text{R-GFP} reporter allele) so that eGFP expression can be monitored in real time. The inset box and arrows show the co-staining with c-kit antibody and eGFP (n = 3 mice).

c, Immunohistochemistry for endogenous c-kit expression (red) in quadriceps muscle of Kit\(^{+/+}\) mice at 4 weeks of age versus nuclear eGFP (green) from the Kit\(^{+/+}\) allele (n = 2 mice). Although lineage tracing in Kit\(^{+/+}\) \times \text{R-GFP} mice, which is cumulative, showed abundant endothelial cells throughout the skeletal muscle (a), instantaneous c-kit-expressing cells are rare in skeletal muscle, and when identified, are always mononuclear (inset box).

d, FACS quantification of bone marrow from Kit\(^{+/+}\) \times \text{R-GFP} mice at 4 weeks of age sorted for eGFP expression, of which 94% are positive for the ‘lineage’ cocktail of differentiation-specific antibodies (averages from n = 3 mice, error bars represent s.e.m.). Hence the Kit-Cre allele is properly expressed in bone marrow and traces lineages that arise from c-kit\(^{+}\) progenitors.

e, Immunohistochemistry in the hearts of Kit\(^{+/+}\) \times \text{R-GFP} mice for endogenous c-kit expression (red) versus all the cells that underwent recombination throughout development and the first 4 weeks of life, shown in green. Although cells that are actively expressing c-kit protein are very rare in the heart (<5 per heart section), the arrow shows such a cell that is also eGFP\(^{+}\) for recombination. All of the currently c-kit-expressing cells identified in the heart were eGFP\(^{+}\), further verifying the fidelity of the Kit-Cre allele (n = 3 hearts).

f, Same experiment as in e except the testis was examined because of the characteristic pattern of Leydig cells that are known to be actively c-kit-expressing (n = 3 mice). The data show that greater than 80% of the currently c-kit-antibody-reactive Leydig cells (red outline, better observed in the right panel) are also eGFP\(^{+}\) (arrows show clusters of these cells).
Extended Data Figure 2 | Identification of non-myocytes from the hearts of Kit^{+/Cre}\times R-GFP mice. Kit^{+/Cre}\times R-GFP mice were collected at 6 weeks of age (constitutive lineage labelling the entire time), although myocardial infarction was performed at week 4 to induce greater vascular remodelling and potentially more c-kit lineage recruitment over the next 2 weeks. a, Hearts were then collected at week 6 and subjected to immunohistochemistry with a pool of antibodies for CD31, CD34, CD45 and CD3 in red, whereas the green channel was for eGFP expression from the recombined R-GFP reporter allele due to Kit-Cre lineage expression. The white arrowheads show endothelial cells that are not contiguous with the underlying network, although most of the endothelial cells are from the c-kit lineage when the red and green channels are compared. The white arrow shows a cardiomyocyte that lacks red staining, whereas the yellow arrows show two areas with relatively large cells that are eGFP^{+} and could be mistaken for a cardiomyocyte, although they are also positive for the non-myocyte marker panel of antibodies (n = 2 mice). b, c, Spread of cells isolated from hearts of 8-week-old Kit^{+/Cre}\times R-GFP mice at baseline that were subjected to immunocytochemistry for the indicated markers (n = 3 hearts). The large white arrow in panel b shows an eGFP^{+} (green) cardiomyocyte that also co-stains with sarcomeric α-actin (red). The smaller arrows show eGFP^{+} non-myocytes, which in panel c, were subject to staining with a cocktail of antibodies again for CD31, CD34, CD45 and CD3 (all in red). This analysis identifies nearly all of the non-myocytes in these cell spreads. The very last image in panel c shows a fourth channel with higher gain so that the underlying cardiomyocytes (CMs) autofluoresce (in white) to show the mixed nature of the spread cells. Blue staining depicts nuclei.
Extended Data Figure 3 | Analysis of c-kit lineage labelling in the heart at P0 (birth). a, Diagram of the timing whereby newborn Kit\(^{+/Cre}\) \(\times\) R-GFP mice were analysed for all subsequent experiments in this figure. b, Histological sections for eGFP fluorescence (green) from the ileum and lung at P0 showing the characteristic c-kit labelling pattern as observed at other time points or in other studies when antibodies were used. Blue shows nuclei. c, Histological section for eGFP fluorescence (green) from the heart at P0. Blue shows nuclei and magnification was \(\times 40\). d, Immunohistochemical tissue section from the P0 heart of Kit\(^{+/Cre}\) \(\times\) R-GFP mice stained with sarcomeric \(\alpha\)-actin (red) to show all underlying cardiomyocytes (right panel) or with eGFP expression in green (left panel) as being c-kit-derived. The green cells noted by the arrows are non-myocytes that do not express sarcomeric \(\alpha\)-actin. e, eGFP expression alone (left) or eGFP with co-staining for cardiomyocytes in red (sarcomeric \(\alpha\)-actin) from heart sections at P0 of Kit\(^{+/Cre}\) \(\times\) R-GFP mice (n = 3 mice). Blue staining depicts nuclei. The cardiomyocyte that is shown has clear striations in the eGFP staining pattern, whereas the two non-myocytes do not show striated eGFP and also lack sarcomeric \(\alpha\)-actin staining. f, eGFP expression alone in green (left) with nuclei in blue or eGFP with sarcomeric \(\alpha\)-actin co-staining (red) from heart sections at P0 of Kit\(^{+/Cre}\) \(\times\) R-GFP mice. All eGFP\(^+\) cells shown lack striations and are non-myocytes although the two cells in the centre sit directly on top of cardiomyocytes and could be easily misinterpreted. Great care is needed in scoring myocytes in the P0 heart because they are small and often the same size as eGFP\(^+\) non-myocytes. g, eGFP expression (green) with nuclei in blue and cardiomyocytes identified in red with sarcomeric \(\alpha\)-actin antibody from heart histological sections at P0 of Kit\(^{+/Cre}\) \(\times\) R-GFP mice. Here the data show c-kit-lineage-derived cardiomyocytes that appear in a loose cluster (arrows), presumably from a clonal expansion event earlier in development.
Extended Data Figure 4 | Additional examination of the Kit-MerCreMer knock-in allele and its potential leakiness in the absence of tamoxifen.

a, Histological analysis of eGFP fluorescent cells from the indicated tissues of Kit$^{+/MCM} \times$ R-GFP mice that were given tamoxifen from 2 to 28 days of age and then collected at day 28. Nuclei are shown in blue and green shows eGFP$^+$ cells in the expected patterns for known regions of c-kit protein expression, such as the distinct pattern of melanocytes in the skin and widespread expression in the spleen and lungs. 

b, Representative immunohistochemical analysis in the testis of Kit$^{+/MCM} \times$ R-GFP mice for endogenous c-kit expression (red) versus cells that underwent recombination when tamoxifen was given by intraperitoneal injection (2 mg) for five consecutive days (green) ($n = 2$ mice). The data show that most of the cells currently expressing c-kit protein in testis (only Leydig cells react, red surface staining) are also eGFP$^+$ (intracellular), indicating that recombination only occurs in c-kit-expressing cells, and in the majority of them. 

c, Representative histological heart sections from Kit$^{+/MCM} \times$ R-GFP mice that were placed on tamoxifen-laden food or vehicle food ($n = 6$ mice per treatment) beginning at 4 weeks of age and then subjected to myocardial infarction injury 4 weeks later, followed by collection 4 weeks after that. In the presence of tamoxifen, histological sections through the myocardial infarction border zone of the heart show widespread eGFP$^+$ cells (green) from the c-kit lineage (left panel), whereas in the absence of tamoxifen no eGFP$^+$ cells are observed (right panel), indicating the Kit-MerCreMer allele does not leak at baseline or after myocardial infarction injury.
Extended Data Figure 5 | Analysis of eGFP+ non-myocytes in the hearts of Kit+/MCM × R-GFP mice at baseline or after myocardial infarction injury.

a–g. Tamoxifen was given to Kit+/MCM × R-GFP mice for 1 day–6 months of age (a, e, f) or in mice given tamoxifen and myocardial infarction injury (b, c, d, g), followed by collection of the hearts for immunohistochemistry with antibodies for GFP (green), or the indicated antibodies in red: CD45 (a), CD3 (b), α-SMA (c), vimentin (d), CD34 (e), CD31 (f), vWF (g). Nuclei are shown in blue. The white arrows show cells with coincident green and red reactivity for each of the markers, although sometimes the red marker is membrane-localized whereas the green (eGFP) is always cytoplasmic. The most overlapping activity with GFP expression was observed for CD31 (endothelial cells), then CD34, followed by CD45 (haematopoietic cells). n = 2 Kit+/MCM × R-GFP mice for 1 day–6 months of age; n = 4 Kit+/MCM × R-GFP myocardial infarction.

h. Averages from FACS plots for the CD31 cellular fraction (antibody-detected) in the heart that are also eGFP+ from Kit+/MCM × R-GFP mice (pre-MI, n = 3) after 8 weeks of tamoxifen in early adulthood at either baseline or 4 weeks after myocardial infarction injury (post-MI, n = 3). The data show about a doubling in the number of CD31 cells that are eGFP+ after myocardial infarction (*P < 0.05 versus pre-MI).
Extended Data Figure 6 | Quantification of Cre activity and DNA recombination in the hearts of Kit<sup>+/MCM</sup> × R-GFP mice. a, Timeline for tamoxifen administration in Kit<sup>+/MCM</sup> × R-GFP mice. b, PCR from DNA isolated from the bone marrow (BM), whole heart or semi-purified cardiomyocytes after 6 weeks of tamoxifen treatment in Kit<sup>+/MCM</sup> × R-GFP mice (n = 2). Bone marrow shows most of the DNA as having been recombined by Cre, whereas whole heart is just barely discernable, and purified cardiomyocytes show essentially no recombination given the sensitivity constraints of this assay. c, qPCR was also run to more sensitively detect and quantify the extent of recombination, which was set relative to the recombination in bone marrow. Semi-purified cardiomyocytes (CM) showed very low rates. Averaged data are shown and error bars are s.e.m. of duplicate technical replicates from n = 3 Kit<sup>+/MCM</sup> × R-GFP mice. d, Schematic of the tamoxifen time course and timing of myocardial infarction in Kit<sup>+/MCM</sup> × R-GFP mice. e, Echocardiography measured cardiac fractional shortening (FS%) was assessed in the mice after myocardial infarction, which shows a reduction in cardiac ventricular performance at 1, 2 and 4 weeks after injury. The number of mice analysed is shown in the bars. Error bars represent the s.e.m. Both the control and experimental groups showed an equivalent reduction in cardiac function post-myocardial infarction. f, Images of dissociated cardiomyocytes from hearts of Kit<sup>+/MCM</sup> × R-GFP mice 4 weeks after myocardial infarction, which were fixed and stained for sarcomeric α-actin antibody (red) and eGFP (green) at two different magnifications. One eGFP<sup>+</sup> cardiomyocyte is shown with sarcomeric patterning of the eGFP fluorescence.
Extended Data Figure 7 | Analysis of eGFP* myocytes in the hearts of Kit+/MCM × R-GFP mice after isoproterenol infusion-induced injury. a, Schematic diagram showing tamoxifen treatment of Kit+/MCM × R-GFP mice between 7 and 14 weeks of age with isoproterenol (ISO) infusion occurring between weeks 10–14. b, c, Quantification and imaging of disassociated cardiomyocytes (separate images shown at two different magnifications) from the hearts of isoproterenol-injured Kit+/MCM × R-GFP mice, which showed rare but definitive cardiomyocyte labelling. *P < 0.05 versus R-GFP, 31 eGFP* cells of 395,302 counted from two hearts.
Extended Data Figure 8 | Verifying the extent of eGFP⁺ cardiomyocytes by an independent laboratory from blinded histological heart samples.

Unprocessed cryosections and paraffin sections from the hearts of Kit⁺/MCM x R-GFP mice after 8 weeks of tamoxifen were blinded and sent to the Marbán laboratory along with negative control sections from hearts that should not have staining. **a, b.** Two separate images from cryopreserved blocks are shown at ×200 magnification in which the cryosection was processed for eGFP fluorescence (green) and α-actinin antibody (red) to show cardiomyocytes. The data show two regions where a single eGFP⁺ myocyte is visible in a region with several hundred GFP-negative cardiomyocytes. The single eGFP⁺ cardiomyocyte is circled and the inset box shows a higher magnification. Sections were also stained for nuclei (blue). In general, approximately 1–2 definitive eGFP⁺ cardiomyocytes were identified per entire heart section in the Marbán laboratory, a result that is consistent with the approximate numbers of kit lineage-labelled cardiomyocytes observed by us. **c.** Image taken at ×630 magnification from a paraffin-embedded and processed histological section in which both an eGFP antibody (green) and α-actinin antibody (red) was used. Nuclei are shown in blue. The arrow shows a single eGFP⁺-expressing cardiomyocyte and the arrowheads show eGFP⁺ non-myocytes.
Extended Data Figure 9 | Assessing cardiomyocyte differentiation markers from total non-myocytes in the heart. Adult cardiac interstitial cells isolated from a Kit^{+/Cre} R-GFP mouse were treated with dexamethasone for 1 week. Cells were then fixed and subjected to immunocytochemistry for the indicated antibodies. c-kit-lineage-derived cells were green (eGFP^+) and showed fluorescence in the cytosol and nucleus. The data show eGFP cells that express markers of differentiated cardiomyocytes such as α-actinin, troponin T and the transcription factor GATA4 (all in red) but not the fibroblast marker vimentin (white), nuclei were stained blue (right panels). These results indicate that eGFP^+ Kit-Cre-expressing cells can generate pre-differentiated cardiomyocytes as well as non-eGFP interstitial cells; hence the cells identified by the Kit-Cre (knock-in) reporter strategy are representative of how endogenous c-kit^-expressing cells truly function.