



Published in final edited form as:

*Circ Res.* 2018 June 22; 123(1): 57–72. doi:10.1161/CIRCRESAHA.117.311828.

## Cardiac c-Kit Biology Revealed by Inducible Transgenesis

Natalie A Gude<sup>1</sup>, Fareheh Firouzi<sup>1</sup>, Kathleen M. Broughton<sup>1</sup>, Kelli Ilves<sup>1</sup>, Kristine P. Nguyen<sup>1</sup>, Christina R. Payne<sup>1</sup>, Veronica Sacchi<sup>1</sup>, Megan M. Monsanto<sup>1</sup>, Alexandria R. Casillas<sup>1</sup>, Farid G. Khalafalla<sup>1</sup>, Bingyan Wang<sup>1</sup>, David Ebeid<sup>1</sup>, Roberto Alvarez<sup>1</sup>, Walter P. Dembitsky<sup>2</sup>, Barbara A. Bailey<sup>3</sup>, Jop van Berlo<sup>4</sup>, and Mark A Sussman<sup>1</sup>

<sup>1</sup>SDSU Heart Institute and Biology Department, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA

<sup>2</sup>Sharp Memorial Hospital, 7901 Frost Street, San Diego, California 92123, USA

<sup>3</sup>SDSU Department of Mathematics and Statistics, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA

<sup>4</sup>Department of Medicine, University of Minnesota, Minneapolis, MN 55455, USA

### Abstract

**Rationale**—Biological significance of c-Kit as a cardiac stem cell marker and role(s) of c-Kit+ cells in myocardial development or response to pathologic injury remain unresolved due to varied and discrepant findings. Alternative experimental models are required to contextualize and reconcile discordant published observations of cardiac c-Kit myocardial biology and provide meaningful insights regarding clinical relevance of c-Kit signaling for translational cell therapy.

**Objective**—Demonstration of c-Kit myocardial biology through combined studies of both human and murine cardiac cells. Advancing understanding of c-Kit myocardial biology through creation and characterization of a novel, inducible transgenic c-Kit reporter mouse model that overcomes limitations inherent to knock-in reporter models, providing perspective to reconcile disparate viewpoints on c-Kit biology in the myocardium.

**Methods and Results**—In vitro studies confirm a critical role for c-Kit signaling in both cardiomyocytes and cardiac stem cells. Activation of c-Kit receptor promotes cell survival and proliferation in stem cells and cardiomyocytes of either human or murine origin. For creation of the mouse model, the cloned mouse c-Kit promoter drives Histone2B-EGFP (H2BEGFP) expression in a doxycycline inducible transgenic reporter line. The combination of c-Kit transgenesis coupled to H2BEGFP readout provides sensitive, specific, inducible, and persistent tracking of c-Kit promoter activation. Tagging efficiency for EGFP+/c-Kit+ cells is similar between our transgenic versus a c-Kit knock-in mouse line, but frequency of c-Kit+ cells in cardiac tissue from the knock-in model is 55% lower than our transgenic line. The c-Kit transgenic reporter model reveals intimate association of c-Kit expression with adult myocardial biology.

Address correspondence to: Dr. Mark A Sussman, SDSU Heart Institute and Department of Biology, San Diego State University, 5500 Campanile Dr. NLS426, San Diego, CA 92182, 619-594-2983 (phone), 619-594-2610 (fax), heartman4ever@icloud.com.

\*N.A.G. and F.F. contributed equally to the manuscript.

### DISCLOSURES

MA Sussman is a founding member of CardioCreate, Inc.

Both cardiac stem cells and a subpopulation of cardiomyocytes express c-Kit in uninjured adult heart, upregulating c-Kit expression in response to pathologic stress.

**Conclusions**—c-Kit myocardial biology is more complex and varied than previously appreciated or documented, demonstrating validity in multiple points of coexisting yet heretofore seemingly irreconcilable published findings.

### Subject Terms

Basic Science Research; Cell Signaling/Signal Transduction; Myocardial Biology; Stem Cells; Genetically Altered and Transgenic Models

### Keywords

Cardiac; stem cell; c-Kit; cardiomyocyte; myocardium; signal transduction; molecular

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## INTRODUCTION

Cardiovascular research has struggled to advance the field of regenerative medicine, due in part to the lack of a unique and unequivocal marker for resident adult cardiac stem cells capable of mediating repair. In fact, the concept of myocardial regeneration that was initially advanced over a decade ago employed the stem cell marker c-Kit to identify presumptive cardiac stem cells<sup>1, 2</sup>. Over the ensuing years the c-Kit<sup>+</sup> cardiac stem cell population has become the focus of intense and diverse investigation by hundreds of laboratories worldwide at both the basic and clinical levels<sup>3–10</sup>. At present, the presence and participation of c-Kit<sup>+</sup> cells in myocardial development as well as response to pathological stress or injury is indisputable<sup>2, 4, 8, 10–12</sup>. However, controversy persists regarding the roles(s) that cells expressing c-Kit play in cardiac biology. Such divergent perspectives suggest that fundamental understanding has not been achieved and that additional study is warranted to provide sorely needed novel insight(s). The present study was conceived and initiated to examine myocardial c-Kit biology with fresh perspective using a unique approach yielding novel findings.

Phenotypically, c-Kit serves as a marker of multiple cell types including stem cells, most often associated with hematopoietic progenitor cells, but in fact is present from early embryogenesis and persists in wide distribution throughout various adult tissues<sup>13, 14</sup>. Functionally, c-Kit protein is a kinase that, upon activation by its cognate ligand stem cell factor (SCF), serves to induce cell proliferation, survival, and transcriptional activation<sup>14</sup>. Variation in temporal appearance and expression level of c-Kit in multiple cell types and in response to environmental stimuli is indicative of a dynamically regulated protein that does not necessarily respect traditional nomenclature of a “stem cell marker”. Despite years of study, details of c-Kit signaling cascades and downstream targets remain obscure<sup>15</sup> and the respective activities for surface-expressed versus internal c-Kit remain poorly characterized<sup>16</sup>. In the cardiac context, c-Kit expression has been documented in multiple cell types including (but not necessarily limited to) myocytes, endothelial cells, cardiac progenitor, and mesenchymal stem cells<sup>17, 18</sup>. Indeed, observations of c-Kit protein induction suggest that

stress responses and myocyte remodeling serve as inductive stimuli for expression of this “stem cell marker” in cardiomyocytes<sup>4, 19</sup>.

Given the heterogeneous expression pattern for c-Kit that is not restricted solely to a canonical cardiac stem cell, the complex and varied findings derived from study of various genetically engineered c-Kit lineage-tracing models might indeed even be an expected outcome<sup>18</sup>. Furthermore, every experimental approach to define c-Kit<sup>+</sup> cell fate possesses inherent strengths and weaknesses to be considered when deriving conclusions. While the advantages of a genetically engineered knock-in model are well known<sup>20</sup>, the inevitable silencing of one c-Kit allele perturbs endogenous c-Kit biology with potentially significant consequences for stem cell function. Furthermore, imperfect tagging efficiency in hemizygous knock-in mice allows for covert existence of an “invisible” c-Kit<sup>+</sup> subpopulation in the myocardium comprising 25–30% of all c-Kit<sup>+</sup> expressing cells<sup>9</sup> that cannot be followed. Consequently, c-Kit knock-in mice may harbor c-Kit dependent biological defects, impaired reporter sensitivity, as well as attendant uncertainty of the functional impact resulting from c-Kit allele hemizygosity that could affect proliferation and/or survival in culture.

An alternative approach to c-Kit<sup>+</sup> tracking by “knock-in” techniques and associated allele inactivation is the use of transgenesis to introduce a cloned construct carrying a c-Kit promoter fragment driving expression of a long-lived reporter protein. The cloned c-Kit promoter employed as a driver for reporter gene expression has been extensively characterized both in vitro and in vivo<sup>3, 21, 22</sup>, but has never been adopted to serve for inducible tracing of cell fate in the myocardial context. Transgenesis minimizes allele inactivation and potential deleterious consequences of impaired c-Kit activity that could influence interpretation of c-Kit expression and relevance in myocardial biology. For the current study, we employ a doxycycline-inducible transgenic mouse model to tag c-Kit<sup>+</sup> cells with a long-lived, tetracycline responsive H2BEGFP (CKH2B) reporter<sup>23</sup>. Endogenous c-Kit remains unperturbed and approximately 80% of nonmyocyte cardiac c-Kit<sup>+</sup> cells express H2BEGFP reporter, similar to c-Kit coincidence with EGFP in a c-Kit/mER-Cre-mEr knock-in reporter model (CKmCm). A heterogeneous CKH2B cell population is revealed following induction with doxycycline including tagging of select adult cardiomyocytes (ACM) in vivo, a finding consistent with expression of c-Kit mRNA and protein in isolated ACMs. Increased expression of CKH2B in cultured ACM in response to stress is consistent with a role for c-Kit expression in cardiomyocyte survival, a finding recapitulated by acute pathologic injury in vivo. Similarly, c-Kit activation in cultured mouse and human cardiac progenitor cells (CPCs) enhances proliferation and survival, in line with previous reports establishing the role of c-Kit signaling in human cardiac stem cells<sup>19</sup>. Collectively, data presented here support validity of the c-Kit reporter model, pointing to heterogeneous and dynamic c-Kit expression in the postnatal myocardium that facilitates reconciliation of seemingly disparate observations from published literature and resolves multiple aspects of the controversial role for c-Kit in cardiac biology.

## METHODS

The authors declare that all supporting data are available within the article [and its online supplementary files].

### Mice

c-Kit/rtTA transgenic mice expressing the rtTA transactivator under control of the c-Kit promoter clone 2<sup>21</sup> were generated using standard genomics techniques and stabilized by breeding several generations into the FVB/N background. The CKH2B line was created by crossing c-Kit/rtTA mice with TRE-H2BEGFP mice purchased from Jaxmice (stock# 005104/pTRE-H2BEGFP). Copy number of the c-KitrtTA transgene per cell was determined by a qPCR method as described<sup>24</sup> with the following modifications. c-KitrtTA plasmid injected for transgenesis was used to establish the standard curve ranging in concentration from 5 fg to 5 ng of plasmid template per reaction. Genomic DNA (gDNA) was extracted from livers of mice hemizygous for the c-KitrtTA transgene or from non-rtTA controls. The qPCR reaction mix consisted of iQSYBR Green master mix, 50 ng gDNA, primers specific for rtTA and 18S, and was performed using a CFX Connect Bio-Rad cyclor for 40 two-step cycles at 95C and 60C. A logarithmic standard curve was generated and copy number calculated per cell based on the assumption that each cell contains 6 pg genomic DNA, or that 1 ng DNA is the equivalent of 167 diploid cells. Genotyping of mice to detect rtTA and EGFP transgenic sequences in genomic tail DNA was performed by standard PCR using target specific primers. All primers for measuring copy number and genotyping are listed in Online Table I. c-Kit-MerCreMer/R26-NG (CKmCm) mice were created by crossing the Kit-MerCreMer driver line<sup>9</sup> to the FVB.Cg-Gt(ROSA)26Sortm1(CAG-lacZ,EGFP)Glh/J from Jaxmice (stock #012429, <sup>25</sup>).

### Animal Procedures

All procedures and experiments involving mice were approved by the SDSU Institutional Animal Care and Use Committee (IACUC). Acute diffuse injury was achieved by administering a single subcutaneous injection of 150 mg/kg isoproterenol. H2BEGFP reporter expression was induced in vivo with 0.2 mg/ml doxycycline in the drinking water for up to four days. CKmCm, three-month old male mice were fed normal chow or chow containing tamoxifen citrate for four weeks to induce EGFP reporter expression, after which hearts were retroperfused and fixed in formalin for paraffin processing. For flow cytometry analysis of heart and bone marrow, CKmCm mice were fed tamoxifen chow for four weeks followed by four weeks chow with no tamoxifen.

### Cell isolation and expansion

Murine CPCs and ACMs were simultaneously isolated from transgenic hearts using a modified isolation protocol<sup>26</sup>. Briefly, hearts were cannulated via the aortic arch and perfused for 15 minutes at 37C in digestion buffer containing Liberase, then physically dissociated in digestion buffer at 37C. Myocytes were filtered through a 100 micron nylon mesh and separated from nonmyocytes by gravity sedimentation. Fractions of myocytes and nonmyocytes were collected from three subsequent steps of digestion, sedimentation and filtration. Myocytes were plated on laminin coated slides or dishes. The nonmyocyte fraction

was either analyzed for EGFP and c-Kit expression by flow cytometry, or filtered through a 40 micron nylon mesh and subjected to magnetic cell sorting to deplete the CD45+ population and enrich for c-Kit expressing cells using the Miltenyi magnetic sorting system (catalog# 130-090-312, mouse CD45 microbeads catalog# 130-052-301, mouse CD117 microbeads catalog# 130-091-224). CPCs were expanded in a humidified incubator at 37°C, 5% CO<sub>2</sub> in growth media consisting of 1:1 Dulbecco's MEM/Ham's F12 (DMEM/F12, LifeTechnologies) and Neurobasal Media (LifeTechnologies) containing 10% ES-FBS (LifeTechnologies), LIF (10ng/ml, Millipore), bFGF (10ng/ml, Peprotech), EGF (20ng/ml Peprotech), 1X Insulin-transferrin-selenium (ITS 500X, Lonza) 1% penicillin/streptomycin/2mM Glutamine (100X PSG, LifeTechnologies), supplemented with B27 and N2 (LifeTechnologies). Human CPCs were isolated from human LVAD discard tissue and expanded as previously described<sup>27</sup>. Briefly, tissue was digested with collagenase, nonmyocyte cells were separated by low speed centrifugation (350g for 5 minutes) and filtered through sequential 100µcells were separated by lwere cultured for one day at 37°C, 5% CO<sub>2</sub> in hCPC growth media (HAM's F12 supplemented with 10% ES-FBS, 5mU/mL human EPO, 10ng/mL human bFGF, 0.2 mM Glutathione, 1X Penicillin/Streptomycin/ Glutamine), after which the c-Kit+ population was selected for by incubating with magnetic bead conjugated c-Kit antibodies (Miltenyi Biotec #130-091-332) and MAC sorting. Magnetically separated cells were expanded for at least 5 passages in hCPC growth media prior to use in experiments. Adult hCPCs were used at passage 6–10 in all experiments.

## Statistics

Two-tailed t-tests were used to compare to two groups for all analyses other than the linear mixed model for Figure 5 as described below.  $p < 0.05$  is considered statistically significant.

To formally test the difference in the means of the knock-in versus transgenic, the repeated measures of c-Kit for each animal are modeled using a linear mixed model. A post-hoc Tukey two-sided test for comparison of means indicates some evidence to suggest a significant difference in means of CKH2B+Doxy vs CKmCm+Tx ( $P = 0.0838$ ). (See Supplement Material for description and details of the statistical analyses.)

To formally test the difference of c-Kit density per unit area, the repeated measures of c-Kit for each animal are modeled using a linear mixed model. Based on the significant results of the mice strain factor, a post-hoc Tukey two-sided test for multiple comparison of means indicates a significant difference in means of CKmCm-Tx vs CKH2B+Doxy ( $P = 0.00769$ ), CKH2B+Doxy vs CKmCm+Tx ( $P = 0.00542$ ), and no significant difference in means of CKmCm-Tx vs CKmCm+Tx ( $P = 0.90577$ ). (See Supplement Material for description and details of statistical analyses.) Online Table III provides values for efficiency of co-expression assessed with c-Kit+ / GFP+ coincidence in three experiments using CKH2B and CKmCm mouse heart samples.

## RESULTS

### c-Kit signaling promotes proliferation and survival in CPCs

c-Kit signaling stimulates proliferation and enhances survival in multiple cell types<sup>282930</sup>, including human cardiac stem cells<sup>19</sup>. Mouse and human CPCs were assayed for c-Kit expression, proliferation and cell death in response to low serum culture conditions. Serum starvation for 24 hours induces a significant 73% increase in c-Kit protein and threefold higher levels of c-Kit mRNA in mCPC (Figure 1A,B), while c-Kit protein levels in hCPC initially decrease by 60% at 24 hours and return to full serum levels after five days of starvation, as measured in three distinct lines (Figure 1C). Interestingly, c-Kit mRNA levels increase by 63% in hCPCs after 24 hours, and rise to more than threefold by five days in low serum (Figure 1D). Treatment of serum starved mCPC and hCPC with c-Kit ligand SCF significantly enhances BrdU incorporation by 84% and 37%, respectively (Figure 1E, F). Similarly, SCF treatment increases cell growth rate and shortens doubling time in serum starved cells (Figure 1G, H), confirming the pro-proliferative activity of c-Kit signaling in CPCs. Viability in serum starved mCPCs is significantly higher and cell death significantly blunted in cells treated with SCF, as measured by a 14% increase in cell survival and 63% decrease in both apoptosis and necrosis using flow cytometry, indicative of protective c-Kit signaling in these cells. Likewise, SCF treated hCPC exhibit a significant 13% increase in viability, and 29% and 28% downward trends in apoptosis and necrosis, respectively (Figure 1I,J). Activation of c-Kit by SCF is verified in mouse and human CPCs as evidenced by significant upregulation of phospho-c-Kit and of downstream targets phospho-ERK1/2 and phospho-AKT (Figure 1K,L). Inhibition of c-Kit with Imanitib or with c-Kit specific antibody blunts phosphorylation of c-Kit, ERK and AKT, demonstrating specificity of SCF mediated c-Kit pathway activation in mCPCs (Online Figure 1A,B) and hCPCs (Online Figure 1C). To determine if macrophages or mast cells contribute to c-Kit signaling in mCPCs, freshly isolated NMCs and cultured mCPCs were analyzed for macrophage (F4/80, CD206) mast cell (FceR1) and hematopoietic (CD45) markers by flow cytometry (Online Figure 1D,E). These populations comprise between 7 and 12% of the whole NMC population (Online Figure 1D), but are absent above baseline in mCPCs (Online Figure 1E), indicating that cardiac progenitors, not cultured mast or macrophage cells, drive c-Kit signaling in these cells. Consistent with previous reports, live cultured mCPCs express stromal markers (CD105, CD90.1, PDGFR $\alpha$ ). Surface c-Kit expression does decrease with culture as measured by flow cytometry in unfixed mCPCs (Online Figure 1F), nevertheless, c-Kit is detected in the majority of fixed mCPCs assessed by flow cytometry (Online Figure 1F) and immunofluorescence (Online Figure X). Collectively, these data demonstrate that the c-Kit pathway is active in mouse and human CPCs, and that c-Kit signaling promotes CPC proliferation and survival in low serum conditions.

### Cardiac myocytes express active c-Kit and upregulate c-Kit in response to stress in vitro

Prior studies have reported increased cardiac c-Kit expression in myocytes following myocardial injury<sup>4</sup>. Isolated ACM immunolabeled for c-Kit confirm myocyte specific c-Kit protein expression (Figure 2A, Online Figure 1I, B, C). c-Kit was detected in most adult cardiac myocytes (ACM), whether fixed immediately after isolation or plated for two hours and then fixed. Using either method, c-Kit staining was found in the majority of ACM

(79.25%, or 42 out of 53 immediately fixed ACM). Upregulation of phospho-ERK and phospho-AKT in ACM treated with SCF verifies activation of c-Kit activity in these cells (Figure 2B, Online Figure IIA). Finally, c-Kit protein and mRNA levels are elevated twofold in isoproterenol treated ACMs (Figure 2C). Taken together, these results indicate that mouse ACMs express c-Kit mRNA and protein, exhibit c-Kit pathway activation by SCF, and upregulate c-Kit expression in response to cellular stress in vitro.

### **H2BEGFP reporter is expressed in transgenic cardiac and noncardiac c-Kit<sup>+</sup> cells in vivo**

An inducible transgenic c-Kit reporter mouse was created to further probe cardiac c-Kit biology in vivo. The rtTA transcription factor was cloned downstream of a previously characterized 14 kb fragment of the mouse c-Kit promoter<sup>3, 21</sup> to generate the c-KitrtTA mouse line (Online Figure III). c-KitrtTA mice were crossed with mice harboring the tetracycline responsive H2BEGFP reporter construct (TRE-H2BJ/eGFP, Online Figure IIIB)<sup>23</sup> to create a double transgenic line in which doxycycline treatment induces H2BEGFP expression in cells harboring an active c-KitrtTA construct (CKH2B, Online Figure IIIC, D). c-KitrtTA transgene copy number was calculated to be approximately 4 copies per diploid genome in hemizygous animals, as measured by quantitative PCR of genomic DNA using a previously described method (Online Figure IV)<sup>24</sup>. Transgene expression was validated in CKH2B mice treated with doxycycline (Figure 3). Cardiac c-Kit<sup>+</sup> cells from doxycycline treated CKH2B mice express the H2BEGFP reporter in the nucleus, as illustrated by immunolabeling of paraffin sections (Figure 3A) and freshly isolated, unfixed nonmyocytes (NMC) immunolabeled for c-Kit and analyzed by flow cytometry (Figure 3B,D). Likewise, noncardiac tissues exhibit H2BEGFP induction in c-Kit<sup>+</sup> cells as measured by flow cytometry of bone marrow (Figure 3C,E) and immunostaining in c-Kit<sup>+</sup> rich tissues such as intestinal crypts and cryptopatches, and pancreatic Islet of Langerhans (Online Figure VIIIA-C). Furthermore, H2BEGFP is expressed in vascular and stromal cardiac cells, as indicated by colocalization with CD31 and vimentin by IHC-P (Online Figure VA,B) and with CD105, CD90.1 and PDGFR $\alpha$  as measured by flow cytometry (Online Figure VE, Online Figure XI). Vascular smooth muscle does not appear to express H2BEGFP (Online Figure VD), and CD45<sup>+</sup>/EGFP<sup>+</sup> cells were rarely observed in uninjured tissue sections, however flow cytometry reveals a higher percentage of CD45<sup>+</sup>/H2BEGFP NMC cells (Online Figure VE).

c-Kit<sup>+</sup> cells comprise 13.8%  $\pm$  3.8 of all freshly isolated NMC, most of which (79.3%  $\pm$  11.6) co-express the H2BEGFP reporter in CKH2B doxycycline treated hearts (Figure 3F). Freshly isolated, unfixed whole bone marrow from the same animals exhibit a higher percentage of c-Kit cells in the overall population (26.2%  $\pm$  7.5), 82.4% of the H2BEGFP BM, more c-Kit<sup>+</sup>EGFP<sup>+</sup> cells overall (19.2%  $\pm$  5.4 in total BM vs 10.6%  $\pm$  2.0 in total NMC, and 82.4% of H2BEGFP<sup>+</sup> BM vs 29.5% of the H2BEGFP NMC population Online Figure VE), but a similar proportion of c-Kit<sup>+</sup>EGFP<sup>+</sup> cells within the total c-Kit population (73.6%  $\pm$  3.5), reflective of higher overall endogenous c-Kit expression levels in bone marrow versus NMC, but similar tagging efficiency in the two c-Kit<sup>+</sup> tissues (Figure 3G). To correlate EGFP<sup>+</sup> signal with c-Kit mRNA expression in c-Kit<sup>-</sup> NMC, EGFP<sup>+</sup> nonmyocytes were FACS sorted for positive or negative c-Kit protein expression and analyzed for c-Kit mRNA levels by qPCR (Online Figure VG). c-Kit mRNA was detected above negative

control levels in the EGFP<sup>+</sup>/c-Kit<sup>-</sup> population, and at one third the level seen in EGFP<sup>+</sup>/c-Kit<sup>+</sup> cells. These results serve as additional evidence that the H2BEGFP reporter reflects c-Kit promoter activity and further validates the CKH2B model.

### **Differences in coincidence of c-Kit / EGFP tagging as well as c-Kit<sup>+</sup> cell frequency assessed by comparative analysis of transgenic versus knock-in reporter mouse models**

Various tagging strategies have been developed to identify the c-Kit cell population in vivo using mouse models <sup>4, 9, 10, 21, 31</sup>. An established c-Kit / EGFP knock-in reporter construct from a Cre-mediated inducible knock-in line (CKmCm) <sup>9</sup> was compared to the CKH2B model for both colocalization of c-Kit with EGFP, as well as frequency of c-Kit<sup>+</sup> cells in myocardial tissue sections. Paraffin sections were immunolabeled for c-Kit, EGFP and DAPI, along with WGA to mark cell boundaries and cardiac troponin T (cTnT) to identify myocytes (representative images, Online Figure VIA,B). The total number of c-Kit<sup>+</sup> cells was counted per area of tissue, and percentage of c-Kit cells expressing reporter determined by colocalization of c-Kit, EGFP and DAPI signals. Efficiency of coincident tagging between c-Kit and EGFP in cardiac c-Kit<sup>+</sup>/EGFP<sup>+</sup> cells exhibited a trend but was not significantly higher in heart tissue sections from CKH2B+Doxy versus CKmCm+Tx mice (Figure 4A,  $p=0.08$ ; 75.7% $\pm$ 6.9 SD vs 62% $\pm$ 4.5 SD, respectively, Online Table III), consistent with flow cytometry data for CKH2B+Doxy nonmyocyte (Figure 3F, G) or CKmCm+Tx bone marrow cells <sup>9</sup>. Moreover, density of c-Kit<sup>+</sup> cells per unit area is significantly higher in CKH2B versus CKmCm heart tissue sections (Figure 4B). This rigorous, blinded analysis reveals that the transgenic (CKH2B) reporter mice possess 2.2 fold higher cardiac c-Kit<sup>+</sup> cell population density and a trend toward higher EGFP tagging of c-Kit<sup>+</sup> cells relative to the knock-in (CKmCm) mice. However, neither model successfully tags all c-Kit<sup>+</sup> cells with EGFP, demonstrating a similarity consistent with heterogeneity of the endogenous cardiac c-Kit cell population. Additionally, NMCs were isolated from tamoxifen treated CKmCm mice and analyzed for c-Kit and EGFP<sup>+</sup> by flow cytometry. Consistent with findings in paraffin sections, the percentage of c-Kit<sup>+</sup> NMCs is approximately half of the level observed in CKH2B NMCs (5.8% $\pm$ 1.1 SD versus 13.8% $\pm$ 3.8 SD, respectively, Figure 4C). Interestingly, the EGFP<sup>+</sup> population was substantially lower in CKmCm BM and NMC than in CKH2B (0.04% $\pm$ .02 SD versus 4.7% $\pm$ 2.7 SD NMC, 2.9% versus 20.4% BM in CKmCm and CKH2B respectively Online Figure VIC). Discrepancies in reporter expression may be due in part to differences in mouse strain and reporter induction protocol, however the lower proportion of EGFP<sup>+</sup> cells in CKmCm is also consistent with the c-Kit haploinsufficiency.

### **H2BEGFP reporter is expressed in CKH2B adult cardiac myocytes**

c-Kit expression has been reported in neonatal mouse cardiomyocytes and genetically tagged mouse ACMs <sup>4, 9, 31, 32</sup>, and measured in ACM (Figure 2). Therefore, expression of H2BEGFP reporter in myocytes was tested by immunolabeling for EGFP and cardiac myosin light chain in heart tissue from CKH2B mice treated with doxycycline for 1 or 4 days (Figure 5A, B). Quantitation of H2BEGFP signal in all nuclei and in myocyte nuclei reveals that approximately 1.8% of all cardiac nuclei express H2BEGFP after one day of induction, increasing to 6% by four days of treatment (Figure 5C). Cardiac myocyte nuclei positive for H2BEGFP comprise less than 0.1% of all nuclei at 1 day, and 0.5% of all nuclei



by 4 days of induction (Figure 5D), while the proportion of cardiac myocytes expressing H2BEGFP reporter increases from 0.1% to 2.5% between 1 and 4 days, respectively (Figure 5E). ACMs isolated from doxycycline treated CKH2B hearts exhibit discreet nuclear H2BEGFP fluorescent signal in 9.4% of total myocytes (Online Figure VIIA, Figure 7F). Likewise, naïve CKH2B ACM treated with doxycycline post isolation express H2BEGFP (Online Figure VIIB). Taken together, these data confirm c-Kit expression in mouse ACM (Figure 2A) and demonstrate sensitive detection of c-Kit expression in the cardiomyocyte population as revealed by cumulative H2BEGFP expression over time with doxycycline induction. Additionally, paraffin sections of tamoxifen induced CKmCm were immunolabeled for EGFP and a cardiomyocyte marker to identify EGFP+ myocytes in the knock-in hearts. Between 1 and 5 EGFP+ myocytes were detected per heart section in three individual CKmCm hearts (Online Figure VID). Collectively, these data provide further evidence that c-Kit is expressed in adult cardiac myocytes.

### **H2BEGFP is expressed in CKH2B bone marrow stem cells in vitro**

Bone marrow is an established source of c-Kit expressing stem cells. c-Kit+ bone marrow stem cells (BMSC) were isolated as previously described<sup>33</sup> from CKH2B mice, expanded and treated with doxycycline in vitro to assess transgene expression in a c-Kit-enriched stem cell system. As shown by live fluorescence imaging, untreated BMSC express no fluorescent signal (Online Figure IXA), while cells exposed to doxycycline exhibit induction of robust, chromatin-associated H2BEGFP fluorescence consistent with expected histone distribution (Online Figure IXB,C). Flow cytometry and confocal microscopy analyses of fixed, immunolabeled BMSCs further confirm low baseline reporter signal in untreated BMSCs (Online Figure IXD,H) and robust induction of H2BEGFP in doxycycline treated CKH2B (Online Figure IXE, F, I) but not in single transgenic c-KitrtTA BMSC (Online Figure IXG). Coexpression of c-Kit with the H2BEGFP signal is detected in approximately 60% of the total c-Kit+ BMSC population, as measured by flow cytometry (Online Figure IXF). BMSCs labeled for flow cytometry and imaged by confocal microscopy exhibit membrane c-Kit and nuclear H2BEGFP signals (Online Figure IXI). Cumulatively, these data demonstrate robust and appropriate induction of the H2BEGFP reporter in a cultured stem cell population enriched for c-Kit expression, consistent with previous publications characterizing the CKH2B promoter construct<sup>21</sup>.

### **CKH2B CPCs express c-Kit and H2BEGFP reporter in vitro**

Nonmyocyte c-Kit+ cardiac cells are an important source of stem and progenitor cells for cardiac regenerative therapy. c-Kit cardiac progenitor cells (CPCs) isolated from CKH2B hearts were induced with doxycycline and probed for c-Kit and H2BEGFP protein expression. H2BEGFP protein is undetectable in untreated CPCs and clearly induced by doxycycline treatment, as indicated by immunoblot (Figure 6A), fluorescence imaging of live and immunostained fixed cells (Figure 6F,G, Online Video I, Online Figure X). c-Kit protein and mRNA levels are substantially lower in CPCs than in BMSCs, and H2BEGFP levels increase twofold in CPCs between 1 and 2 days of exposure to doxycycline, whereas endogenous c-Kit levels remain unchanged (Figure 6C,E). Quantitative PCR confirms that mRNA levels of H2BEGFP but not endogenous c-Kit are augmented following doxycycline induction (Figure 6B,D), and live imaging verifies nuclear localization of the fluorescent

H2BEGFP signal in CPCs (Figure 6F, Online Figure X). Collectively, these results demonstrate that the CKH2B reporter is induced in two cultured adult c-Kit stem cell populations and reflects the heterogeneity of c-Kit expression inherent in the parent tissue from which these cells are derived.

### Cardiac c-Kit expression increases following diffuse injury *in vivo*

Diffuse injury induces transient proliferation of CPCs *in vivo*<sup>8</sup>. Mice received a single injection of 150mg/kg isoproterenol subcutaneously and hearts were analyzed by immunostaining or flow cytometry three days later. Consistent with previous reports, the c-Kit+ NMC population increased (12.6% to 20.1% in control versus injured hearts) as measured by flow cytometry (Online Figure XIC). CKH2B mice received a single injection of isoproterenol and treated with doxycycline for three days, and c-Kit colocalization with H2BEGFP was measured by immunolabeling in paraffin sections or flow cytometry on isolated NMC to assess the impact of diffuse injury on transgene expression. The H2BEGFP + NMC population increased by 63% (4.9% to 8.0% of ungated NMC), while the proportion of c-Kit+ H2BEGFP NMCs declined from 29.5% to 20.7% in uninjured versus injured hearts, respectively (Online Figure XIC). The proportion of mesenchymal/stromal cells, as marked by CD105, CD90.1 and PDGFR $\alpha$ , all increased within the total NMC and EGFP+ NMC populations (Online Figure XID,E), supported by clear colocalization of vimentin with EGFP+ in damaged regions of cardiac CKH2B tissue sections (Online Figure XIA) The percentage of c-Kit+ cells labeled for H2BEGFP in injured heart was comparable to uninjured controls (75.7% versus 71.9%, respectively, Figure 7A,B,D). Cells coexpressing CD45 and EGFP were detected in injured areas of cardiac tissue sections (Online Figure XIB), while flow cytometry analysis indicates a decrease in the CD45+/H2BEGFP+ NMC population from 27% to 11% in uninjured versus injured hearts. The total number of c-Kit cells per area was 3.1 versus 1.1 per mm<sup>2</sup> in injured versus uninjured heart sections (Figure 7C), consistent with flow cytometry data and previous reports of elevated c-Kit cell numbers in isoproterenol treated hearts 3 days after injury<sup>8</sup>.

Prior studies have reported evidence for c-Kit expression in myocytes<sup>4, 9, 10, 31, 32</sup>. Therefore, expression of H2BEGFP was assessed in cardiac myocytes isolated from hearts of CKH2B mice receiving a single injection of isoproterenol and treated with doxycycline for three days. H2BEGFP signal increased overall in isoproterenol treated hearts as measured by immunolabeling for EGFP and cardiac myosin light chain (Figure 7E). Total EGFP positive nuclei rose from 6% in unchallenged hearts to 17% following injury, the EGFP positive myocytes increased from 0.5% to 3% of total nuclei, and the proportion of EGFP labeled myocytes was 13% of the myocyte population versus 3% in control hearts (Figure 3E, Figure 7F), consistent with the upregulation of c-Kit in ACMs treated with isoproterenol *in vitro* (Figure 2C). Furthermore, the percentage of H2BEGFP+ ACM isolated from isoproterenol injured CKH2B hearts was more than threefold higher than those from uninjured controls (33.3% versus 9.4%, respectively, Figure 7F,G), and protein levels of c-Kit and H2BEGFP were also elevated in ACM from isoproterenol treated animals (Figure 7H). These data strongly support evidence for c-Kit expression in adult cardiac myocytes *in vivo* and *in vitro*, and emphasize a previously underappreciated and potentially important role for c-Kit signaling in the cardiomyocyte response to stress.

## DISCUSSION

Over a decade of cumulative research supports the important biological role of c-Kit<sup>+</sup> cells in cardiac development, homeostasis and repair<sup>2, 8, 22, 34, 35</sup> although recent studies using genetically modified knock-in mice have challenged certain aspects of c-Kit-mediated contribution to myocardial regeneration<sup>9, 36</sup>. Knock-in mouse models thus far commonly focus upon the property of c-Kit as a canonical stem cell marker originally identified in the hematopoietic context, but devote relatively little attention to the critical biological role of c-Kit-dependent signal transduction. Regarding c-Kit as a perfunctory marker of stem cells overlooks substantial evidence that c-Kit signaling is an important determinant of myocardial biology and the molecular response to pathologic injury<sup>19, 37</sup>. Findings presented here report biologically relevant c-Kit function in cardiac progenitor cells and myocytes, with important implications for the role of c-Kit expression and signaling in the cardiac injury response.

c-Kit is a type III receptor tyrosine kinase comprised of an extracellular ligand binding domain, transmembrane region and intracellular kinase signaling domain. Binding of c-Kit ligand Stem Cell Factor (SCF) initiates receptor dimerization, autophosphorylation, and activation of intracellular signaling cascades such as PI3K/AKT and Ras/MAPK/ERK. AKT and ERK signaling downstream of c-Kit confers powerful protective and proliferative effects in the heart as well as other tissues<sup>14, 15, 37, 38</sup>. In the myocardial context, SCF-mediated activation of c-Kit or overexpression of constitutively active c-Kit protects against cardiomyopathic challenge<sup>38</sup>, while defective c-Kit signaling leads to compromised cardiac function in response to age and stress<sup>39–41</sup>. Consistent with published literature, findings obtained using isolated ACM demonstrate that c-Kit protein is functionally present and responsive to SCF exposure (Figure 2A–C, Online Figure II). Furthermore, c-Kit expression increases in ACMs following adrenergic stress (Figure 2C), potentially contributing to a protection of the myocardium from pathological injury.

Concluding that c-Kit expression in cardiomyocytes is a normal biological process is reinforced by studying genetically engineered mouse models using three distinct c-Kit promoter systems including transgenic c-Kit-BAC reporter<sup>4</sup> and c-Kit locus knock-in systems<sup>9, 18, 31, 42</sup>. “Instant on” c-Kit expression tagging in a c-Kit knock-in model demonstrated reporter expression in adult cardiac myocytes<sup>31</sup>, consistent with H2BEGFP tagging of myocytes in CKH2B hearts after four days of doxycycline treatment (Figure 4). In the wake of pathologic damage to the myocardium, GFP signal in c-Kit-BAC border zone myocytes following cryoinjury indicates activation of c-Kit expression in stressed cardiomyocytes<sup>4</sup> is consistent with higher incidence of H2BEGFP in ACMs isolated from isoproterenol injured CKH2B hearts (Figure 7E–H) and elevated c-Kit protein levels in isoproterenol treated ACMs (Figure 2C). Sample preparation and detection method influence outcomes of quantitative assessments, as exemplified by comparing findings using immunolabeled paraffin sections versus freshly isolated ACM. The percentage of EGFP<sup>+</sup> cardiomyocytes in paraffin sections (2.5%) represents an *in situ* view of the heart in which tissue content and context are preserved, yet epitope availability to antibodies may be diminished due to tissue processing. In comparison, the percentage of freshly isolated EGFP<sup>+</sup> ACM from CKH2B hearts (9%) represents the end product of a multistep preparation that

selects for cardiomyocytes that survive myocardial digestion and enrichment procedures. Furthermore, c-Kit protein is not detected in all EGFP<sup>+</sup> cells in CKH2B cardiac cells because levels of c-Kit protein versus mRNA expression do not necessarily directly correlate (Online Figure VG). Another contributing factor in this observed discrepancy is sensitivity of c-Kit detection. For example, directly conjugated c-Kit antibody is used for detection of c-Kit in unfixed EGFP<sup>+</sup> cells (Figures 3D,E). However, fixation and permeabilization of cells increases the proportion of c-Kit<sup>+</sup> positive cells in the population since some c-Kit protein is intracellular<sup>16</sup>. Furthermore, applying unconjugated c-Kit primary antibody followed by a fluorescently tagged secondary yields higher sensitivity for detection of c-Kit protein because of increased fluorophore labeling. These methodological considerations can also contribute to variation in correlation between c-kit protein and EGFP reporter detection. Thus, derivation of quantitative findings using multiple approaches as performed in this study marginalizes bias in experimental design and helps to reconcile discrepant observations from prior publications. Consistent documentation of c-Kit in adult cardiomyocytes supports a regulatory role in biological processes and validates appearance of the CKH2B reporter in the present study as a reasonable and relevant indicator for c-Kit expression in ACMs. Differential induction of the two mouse models is an important discussion point. Although tagging efficiency of c-Kit<sup>+</sup> cells is comparable in the two mouse models, the underlying genetics and induction protocols are dramatically different. To generate the EGFP<sup>+</sup> tag in c-Kit<sup>+</sup> cells in the knock-in model, CKmCm mice ingest tamoxifen in their feed for four weeks to effect permanent Cre-mediated recombination, whereas CKH2B animals imbibe doxycycline in their water for four days to drive expression of the H2BEGFP<sup>+</sup> reporter. In the first system, EGFP<sup>+</sup> cannot accumulate after recombination, unlike the tet-inducible system directly driving expression of H2BEGFP. These differences in reporter expression mechanisms influence reporter induction dynamics between the two models, in addition to the distinct c-Kit promoters used for tissue specific expression.

Induction and activation of c-Kit in CPCs echo findings in ACMs. c-Kit signaling confers protective, proliferative, and pro-migratory effects through induction of ERK and AKT in adult human and rat CPCs<sup>19, 41</sup>. Likewise, the SCF/c-Kit pathway promotes proliferation in CPCs stimulated by co-culture with MSCs<sup>43</sup>. Consistently, c-Kit levels increase in mouse CPCs following cellular stress (Figure 1A,B). Interestingly, in human CPCs, c-Kit protein levels decrease and recover after five days in low serum, whereas c-Kit mRNA increases steadily from 1 to 5 days in low serum (Figure 1C,D). Furthermore, SCF-mediated activation of c-Kit promotes CPC proliferation and blunts cell death in both mCPC and hCPC (Figure 1E–J), demonstrating the functional biological role of c-Kit in CPCs. Again, as is true for ACM, genetically engineered fluorescent tag reporter models contribute support for relevance of c-Kit cell biology through visualization and tracking of cardiac c-Kit<sup>+</sup> cells in vivo and in vitro<sup>3, 4, 9, 10, 31</sup>. The tetracycline-responsive H2BEGFP reporter was deliberately chosen for our study based upon literature precedents as a long-lived, easily identifiable chromatin-associated fluorescent tag originally designed for stem cell tracking studies<sup>23, 44</sup>. Combined with the transgenic c-KitrtTA promoter, the resultant CKH2B reporter system presented in this report provides a sensitive, inducible readout for c-Kit expression without impacting endogenous c-Kit function consequential to targeted knock-in

approaches that take advantage of the endogenous promoter but, by inherent necessity of experimental design, inactivate normal transcriptional activity of the c-Kit locus.

Another critical aspect of further validating the H2BEGFP model as a tool for investigating cardiac c-Kit cell biology is consistency with numerous reports detailing phenotypic and morphologic properties of CPCs<sup>2, 3, 8, 22, 45, 46</sup>. H2BEGFP-tagged cells in transgenic hearts exhibit typical morphology of small, round c-Kit<sup>+</sup> cells embedded in the myocardium (Figure 3A). Induction of H2BEGFP expression revealed by confocal analyses of paraffin sections and flow cytometry of freshly isolated nonmyocyte cardiac cells (Figure 3B–E) matches reports with other c-Kit reporter models<sup>9</sup>. Likewise, CPCs isolated and expanded from CKH2B hearts exhibit classic CPC spindle morphology, express c-Kit, and upregulate H2BEGFP following doxycycline treatment (Figure 6, Online Video I, Online Figure X). These fundamental characterizations together with concurrence between our findings and prior publications<sup>2, 46</sup> reinforce the fidelity and authenticity of c-Kit properties as revealed in the H2BEGFP transgenic model.

Cardiac c-Kit cell expansion occurs in diffuse injury models in vivo<sup>47, 48</sup>. Analysis of c-Kit<sup>+</sup> cells in isoproterenol injured CKH2B hearts revealed sites of c-Kit<sup>+</sup>/H2BEGFP<sup>+</sup> clusters and, interestingly, significantly more H2BEGFP<sup>+</sup> cardiomyocytes in vivo and in vitro (Figure 7), consistent with elevated c-Kit expression in isoproterenol treated ACMs (Figure 2C) and reminiscent of EGFP positive myocytes in border zone myocytes of c-Kit-BAC-GFP hearts<sup>4</sup>. Upregulation of c-Kit in CKH2B myocytes in response to stress further substantiates a previously unrecognized or underappreciated biologically relevant role for c-Kit signaling in cardiac myocytes.

c-Kit<sup>+</sup> cells constitute a highly heterogeneous population with broad distribution and diverse function throughout the body postulated to possess a range of phenotypic and biological properties<sup>35</sup>. The cardiac c-Kit<sup>+</sup> cell subpopulation likewise comprises a diverse cell pool purported to participate in both direct contribution to tissue formation by lineage commitment as well as indirect paracrine-mediated signaling<sup>35, 49–51</sup>. Tagging efficiency of such a heterogeneous cell population will inevitably vary with promoter expression levels, whether endogenous or transgenic, and may be affected by the hemizygous state of current c-Kit knock-in reporter models. Interestingly, up to 35% of cardiac c-Kit<sup>+</sup> cells do not express a genetic reporter tag in either system, as measured by flow cytometry and immunolabeling of paraffin sections (Figure 3, 4)<sup>9</sup> further highlighting the complexity of c-Kit expression dynamics. Although approximately 70% of H2BEGFP<sup>+</sup> nonmyocytes are negative for c-Kit protein by flow cytometry (Online Figure VE,F), these EGFP<sup>+</sup>/c-Kit<sup>–</sup> nonmyocytes possess c-Kit mRNA by RT-qPCR analysis (Online Figure VG) consistent with CKH2B transgenic promoter activity reflecting endogenous c-Kit mRNA expression. EGFP<sup>+</sup> nonmyocytes diversity is readily apparent from immunoprofiling that shows heterogeneity of immune, interstitial cell and mesenchymal/stromal markers including FcεR1, F4/80, CD206, vimentin, CD105, CD90.1 and PDGFRα (Online Figure ID and Online Figure VE). Such heterogeneity warrants cautious interpretation of c-Kit<sup>+</sup> cell biology consistent with the existence of multiple distinct subpopulations possessing nuanced differential characteristics that are lost upon imposition of assessments focused upon average measurements.

Genetic knock-in models that disrupt the c-Kit locus by necessity confer a hemizygous genotype for c-Kit. Given the proliferative and protective roles of c-Kit signaling in CPCs, and evidence for survival signaling in ACMs, it is important to consider the potential impact of the hemizygous c-Kit phenotype on cardiac function and repair. Indeed, frequency of c-Kit cells decreased approximately 50% in myocardial tissue sections from knock-in versus transgenic samples (Figure 4B), perhaps a consequence of differences in c-Kit gene dosage (hemizyosity in the CKmCm knock-in mice), strain (FVB for CKH2B and C57Bl/6 for CKmCm), or reporter system (direct TRE-mediated induction in CKH2B versus Cre-mediated recombination in CKmCm). One explanation for the difference in cell labeling between the knock-in and the transgenic models could be a feed-forward mechanism whereby disruption of one copy of c-Kit expression in the knock-in model leads to reduced c-Kit levels in all cells that express c-Kit. Impairment of c-Kit function leads to unequivocal phenotypic consequences in Wv c-Kit mutant mice that exhibit impaired cardiac recovery after infarction, diminished cardiac function with advanced age, and compromised c-Kit cell terminal differentiation into cardiomyocytes<sup>32, 39–41</sup>. Circumspection is reasonable when considering the myocardial response to injury in c-Kit hemizygous mice that could compromise reparative capacity of c-Kit<sup>+</sup> cells in myocardial regeneration. Reporter models that do not disrupt the endogenous locus, such as the previously described c-Kit-BAC-GFP transgenic<sup>4</sup> or the inducible CKH2B system described here, bypass interference with endogenous c-Kit expression and associated changes in cellular function. Given the preponderance of evidence as cited in preceding paragraphs to substantiate the biological role for c-Kit in the normal and injured myocardium, inescapable c-Kit allele inactivation should not be readily dismissed or deemed irrelevant from interpretation of results using genetically engineered knock in models.

Together with the advantage of preserving normal endogenous c-Kit regulation, the CKH2B tagging system employs a transgenic c-Kit promoter to express rtTA transcription factor combined with a tet-responsive ubiquitous H2BEGFP reporter line. Doxycycline-mediated transgene induction can be performed at any stage of development without the potential adverse side effects of tamoxifen treatment in pregnant dams<sup>52–57</sup>. Protein stability of H2BEGFP, with a reported half-life of several months in vivo<sup>44, 58</sup>, provides long term tagging of c-Kit<sup>+</sup> cells in CKH2B mice, while allowing for reversibility in mitotically active systems. Permanent tagging of c-Kit cells allowing for long-term lineage tracking of cells has been initiated through combining the c-KitrtTA transgenic construct with a tetracycline-responsive Cre line<sup>59</sup> and the ROSA<sup>mT/mG</sup> reporter mouse<sup>60</sup>. Future studies using various c-KitrtTA transgenic models will establish distribution of c-Kit<sup>+</sup> cells in the heart and clarify participation of c-Kit<sup>+</sup> cells in response to cardiac injury.

In summary, the biological role of cardiac c-Kit is an important one that may be underappreciated due to an overemphasis upon the use of c-Kit as a “marker” of stem cells. Pro-proliferative and protective effects of c-Kit signaling in cardiac cells are central to their regenerative potential and should be considered when designing experimental models to assess their role in cardiac formation and repair. All genetic lineage-tracing models have inherent limitations. Knock-ins frequently disrupt expression of the gene of interest and impair normal biological regulation, whereas transgenic promoter constructs may lack regulatory elements of the endogenous gene. Multi-component lineage tracing models

designed to mark cells temporally and spatially harbor inducible or regulatable elements, or rely upon enzymatic recombination for tagging, introducing further layers of complexity and inescapable variables in efficiency, specificity, or even toxicity. In the final analysis all experimental models are tools, with even the most sophisticated promoter-driven reporter models unable to precisely capture post-transcriptional regulation of c-Kit expression. For example, recent studies reveal that c-Kit translation, but not transcription, is regulated by Pim1 kinase in the hematopoietic stem cell pool <sup>61</sup>. Similarly, retinoic acid controls c-Kit translation through PI3K/Akt/mTOR during spermatogonial differentiation <sup>62, 63</sup>. Additionally, microRNA 221 downregulates c-Kit protein expression in melanoma, hyperglycemic HUVECs, and PDGF-stimulated vascular smooth muscle cells <sup>64–67</sup>. Multiple layers of c-Kit protein regulation dependent upon posttranslational modification, ubiquitination, internalization, and degradation rate all contribute to dynamic c-Kit expression beyond the reach of any one reporter system to reflect endogenous c-Kit biology. Acknowledging that there is much still to learn and embracing variations in perspectives rooted in divergent experimental models will resolve discrepancies, unify the cardiovascular research community, and provide new avenues for exploration moving forward with a renewed respect for the complexity of myocardial c-Kit biology.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We gratefully acknowledge Dr. Roland Wolkowicz and Cameron Smurthwaite from the San Diego State University flow cytometry core facility, and Sharp Memorial Hospital staff for providing the Sussman lab with the human heart biopsy samples used for our cell isolations.

### SOURCES OF FUNDING

M.A. Sussman is supported by NIH grants: R01HL067245, R37HL091102, R01HL105759, R01HL113647, R01HL117163, P01HL085577, and R01HL122525, as well as an award from the Fondation Leducq. N.A. Gude is supported by the National Institutes of Health (NIH) grants R37HL091102, R01HL117163, R01HL105759, U54CA132384. M.M. Monsanto is supported by NIH grant R01HL122525, Rees Stealy Research Foundation, Achievement Rewards for College Scientists (ARCS), and Elliott Family Fund Scholarship. K.M. Broughton is supported by NIH grant F32HL136196, F.G. Khalafallah is supported by NIH grant F32HL131299, J.van Berlo is supported by NIH grants R00HL112852 and R01HL130072.

## Nonstandard Abbreviations and Acronyms

<b>ACM</b>	Adult cardiac myocytes
<b>mCPC</b>	Mouse cardiac progenitor cell
<b>hCPC</b>	Human cardiac progenitor cell
<b>CKH2B</b>	c-Kit <sup>tr</sup> TA/H2BEGFP mouse line
<b>CKmCm</b>	c-Kit-MerCreMer /R26-NG mouse line
<b>TRE</b>	Tet-Responsive Element

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## NOVELTY AND SIGNIFICANCE

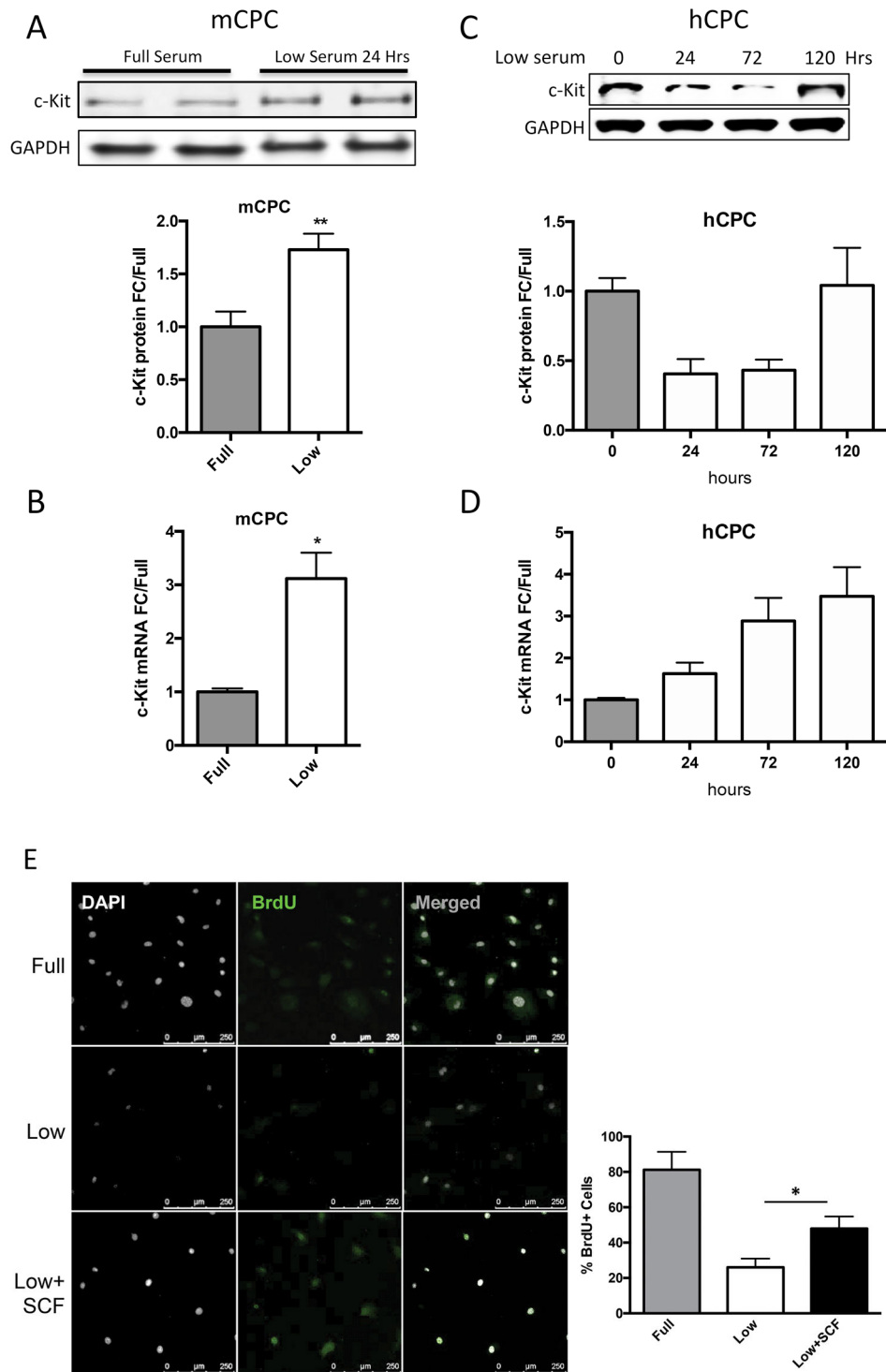
### What Is Known?

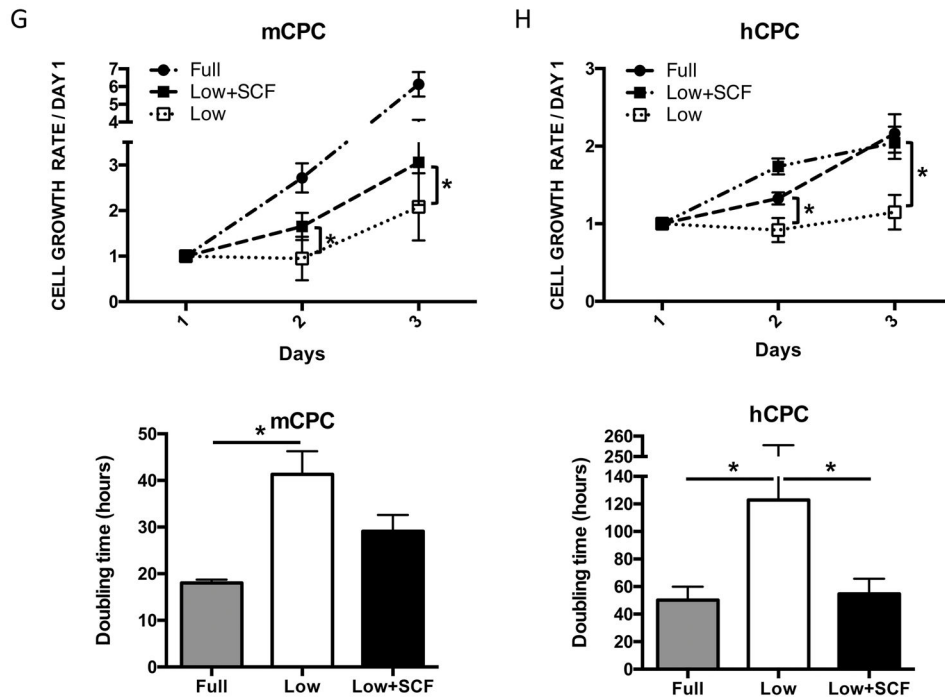
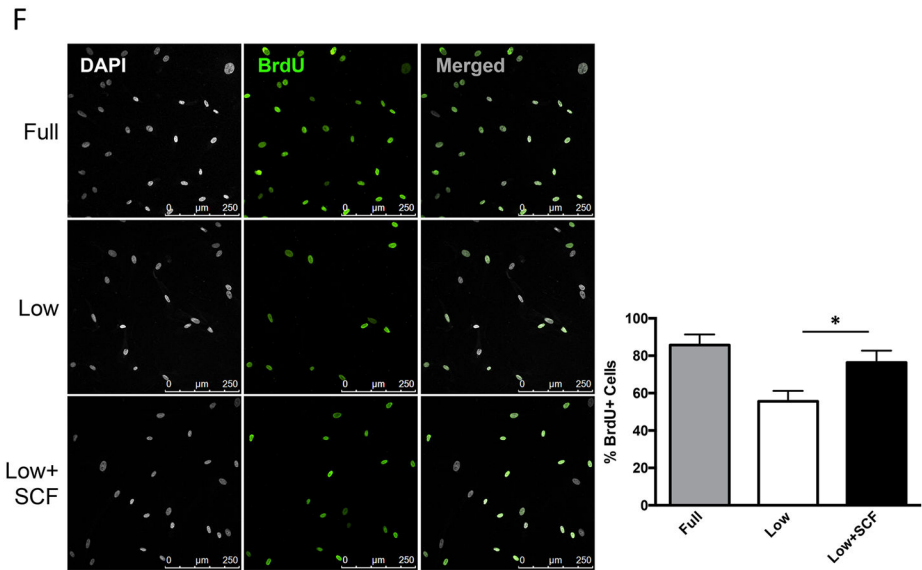
- The mammalian heart has a limited capacity for self-renewal and repair. The identity of cells responsible for this regenerative activity remains an area of active investigation.
- c-Kit is a receptor tyrosine kinase used to identify cardiac stem and progenitor cell populations. c-Kit signaling promotes cell proliferation and survival.
- The cardiac c-Kit cell population is heterogeneous; it contributes to myocardial development, and participates in the myocardial response to injury.
- Experimental animal models designed to identify and study the biological function of c-Kit expressing cells produce varying and discrepant results likely in part due to variations in promoter and reporter design.

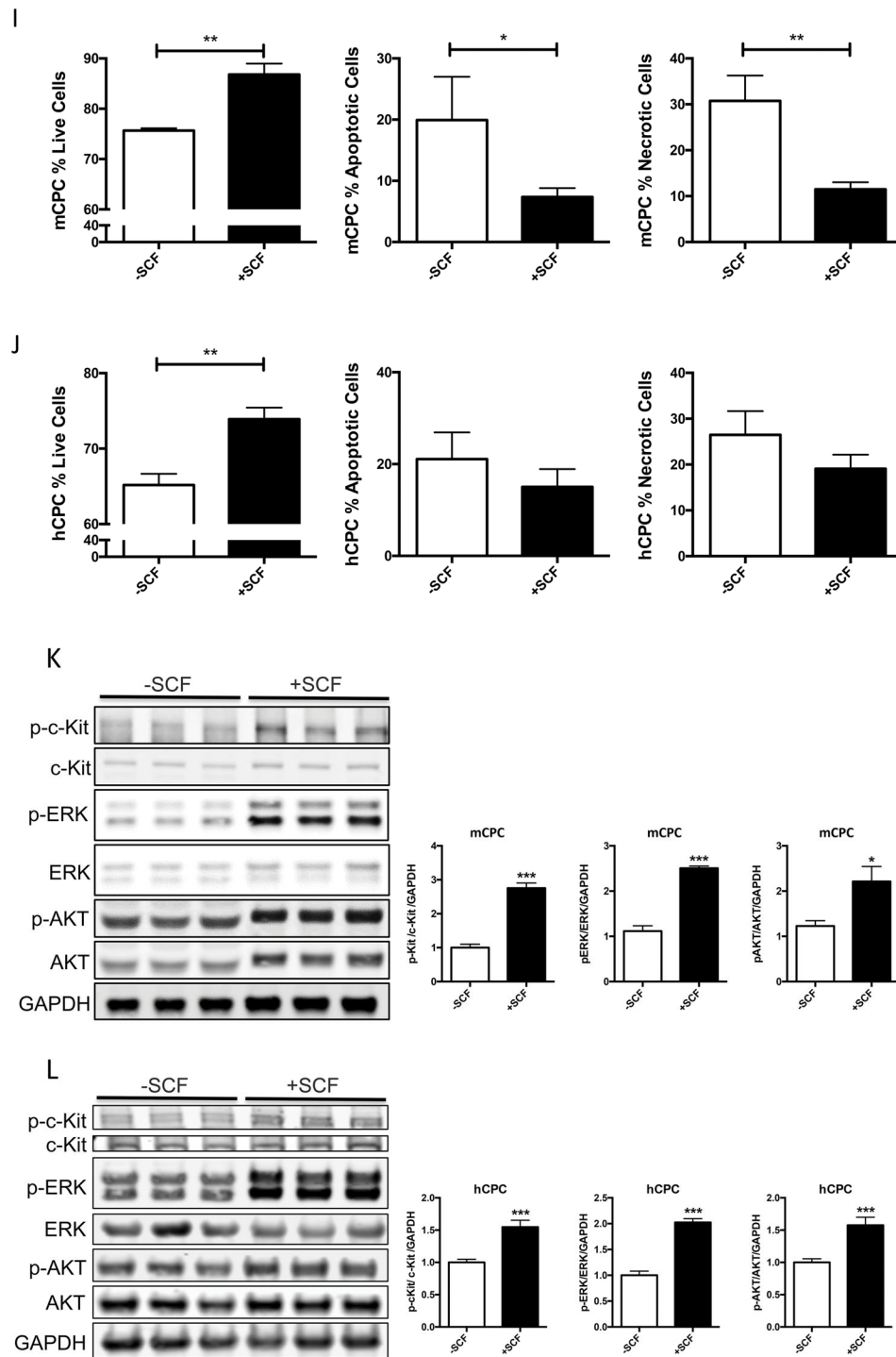
### What New Information Does This Article Contribute?

- c-Kit expression increases in mouse and human cardiac progenitor cells in response to cellular stress.
- c-Kit is expressed in adult cardiac myocytes and its abundance increases following cellular stress.
- c-Kit signaling promotes proliferation and survival in mouse and human cardiac progenitor cells.
- The novel, transgenic, inducible c-Kit reporter mouse model characterized in this study reveals previously unrecognized heterogeneous, dynamic c-Kit expression and biology in the postnatal myocardium.

The biological role of cardiac c-Kit has been largely overlooked in the cardiovascular research community due to overemphasis on the use of c-Kit as a “marker” for stem cells. The protective and pro-proliferative effects of c-Kit signaling in cardiac cells are central to their regenerative potential and their role in cardiac formation and repair. Heterogeneity of the cardiac c-Kit<sup>+</sup> cell population including c-Kit activity in cardiac myocytes, confirmed with our novel transgenic reporter mouse, reconcile and extend prior divergent reports as well as point toward new horizons for c-kit biology in the myocardium. The biological role(s) for cardiac progenitor cells, including those expressing c-kit, in maintaining cardiac homeostasis and promoting repair is clearly more nuanced and complex than previously represented in the evolving field of myocardial regeneration.





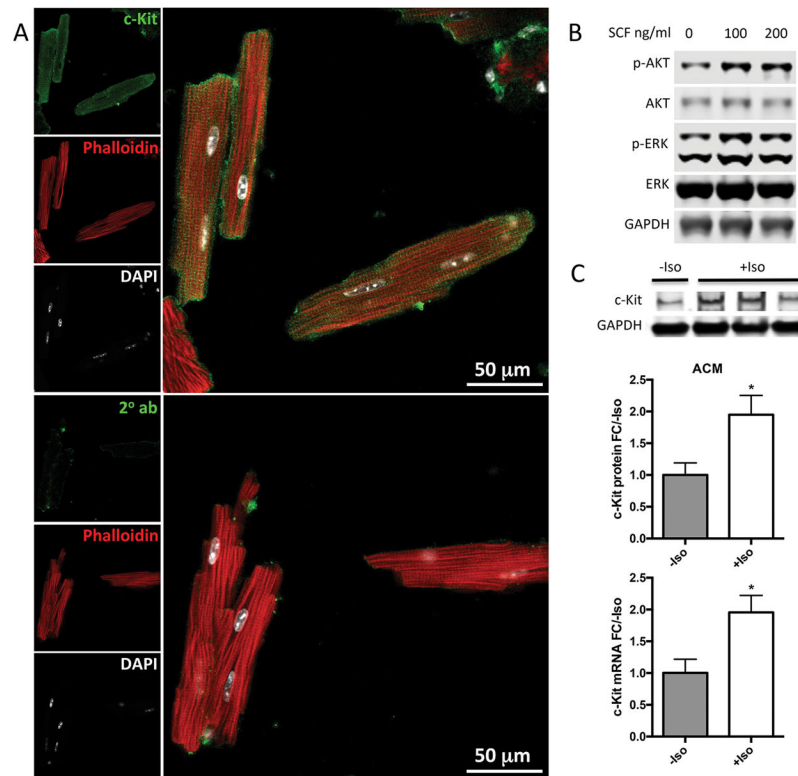


**Figure 1. c-Kit signaling promotes proliferation and survival in CPCs**

c-Kit protein and RNA were quantified in mouse and human CPCs cultured in full or low serum media. c-Kit protein levels increase twofold in mCPCs cultured for 24 hours in low serum versus full growth media as measured by immunoblotting (A). c-Kit RNA levels in mCPCs exposed to low serum increase threefold as measured by qPCR (B). hCPC culture

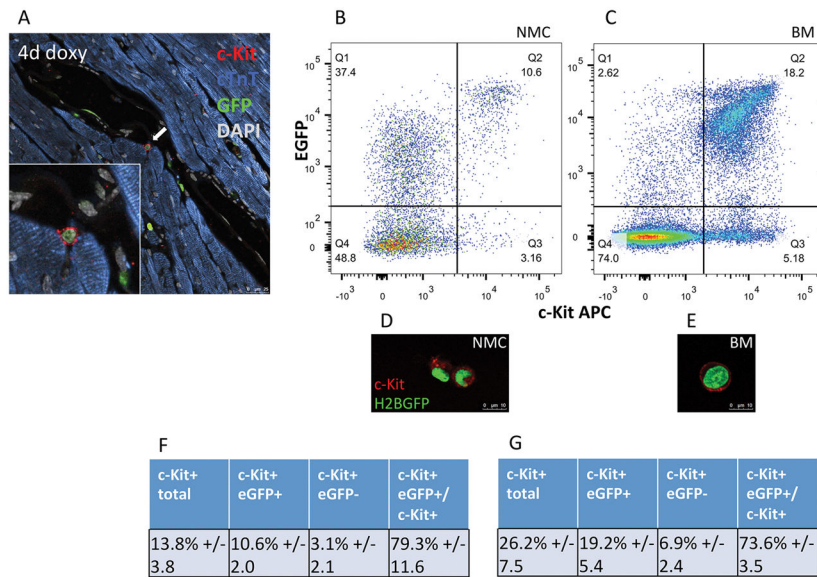
for five days in low serum exhibit an initial decrease in c-Kit protein levels which return to full serum levels by five days (C), while c-Kit mRNA increases steadily over time (D). Decreased proliferation in mCPCs and hCPCs cultured in low serum is partially rescued by SCF treatment as indicated by BrdU incorporation (green=BrdU, E,F) and daily cell counts (G,H). Doubling time of CPCs calculated from growth rates increases following serum depletion but is restored with addition of SCF (G,H). Viability is higher, while apoptosis and necrosis are lower in SCF treated CPCs as measured by AnnexinV/PI flow cytometry analysis (I,J). Specific activation of c-Kit in mCPCs and hCPCs is confirmed by increased phosphorylation of c-Kit, ERK and AKT following SCF treatment (K,L). n=3 for all experiments.  $p<0.05$  denotes statistical significance. Statistical significance between two groups was determined by unpaired t-test for all data except J, which was analyzed by paired t-test. \*= $p<0.05$ , \*\*= $p<0.01$ , \*\*\*= $p<0.001$ .





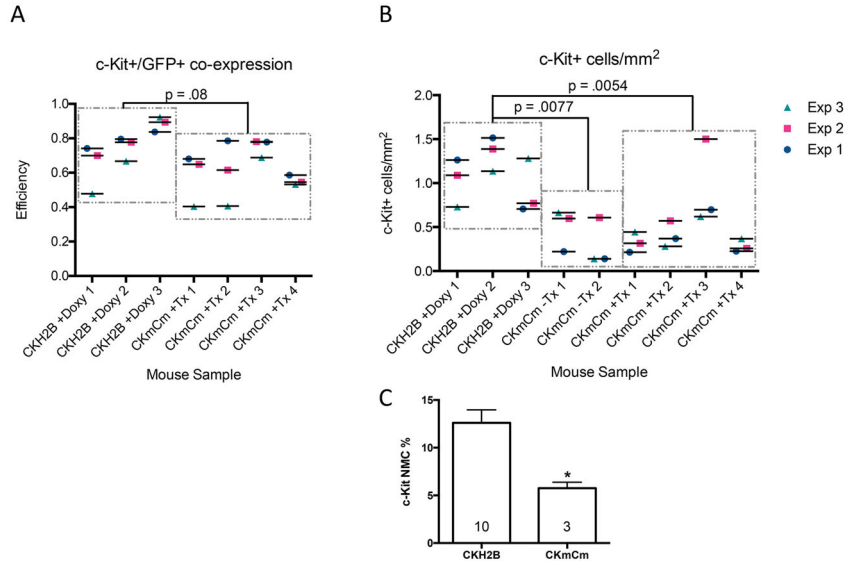
**Figure 2. Cardiac myocytes express active c-Kit and upregulate c-Kit expression in response to stress in vitro**

Adult mouse cardiac myocytes (ACM) were isolated, fixed and immunolabeled for c-Kit (green) colocalized with phalloidin (red) and DAPI (white) (A). Activation of c-Kit by SCF was confirmed by immunoblotting for phospho-ERK and phospho-AKT (B). ACM were treated with isoproterenol to induce cellular stress. c-Kit mRNA and protein levels were measured by qPCR and immunoblotting, respectively. c-Kit protein and mRNA levels increased significantly in isoproterenol treated ACMs as measured by immunoblotting and qPCR, respectively (C). n=3 experiments for A,B. \*=p<0.05 as measured by t-test.

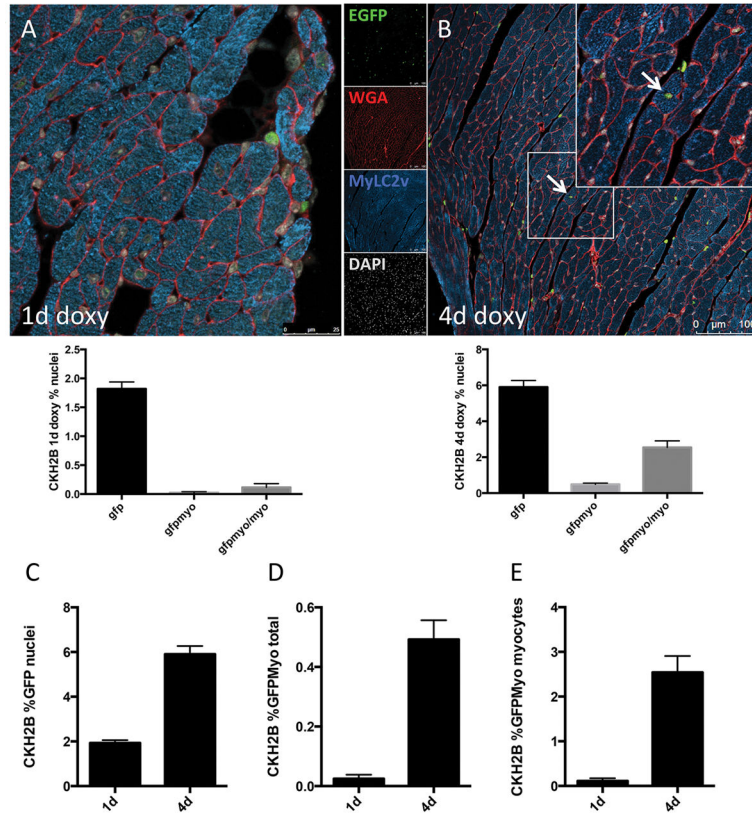


**Figure 3. H2BEGFP reporter expression is induced in c-Kit+ cells in vivo**

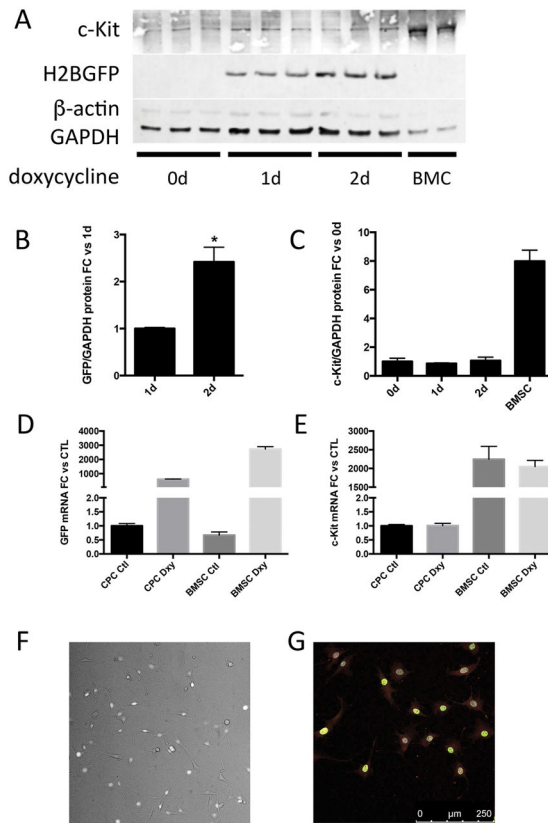
Cardiac c-Kit<sup>+</sup> cells from CKH2B mice express H2BEGFP reporter (white arrow) following administration of doxycycline, as visualized by immunolabeling (A, red=c-Kit, green=EGFP, blue=cardiac troponin T(cTnT), gray=DAPI). Coexpression of c-Kit and H2BEGFP is detected in fresh isolates of nonmyocyte cardiac cells (NMC, B) and whole bone marrow (BM, C) from doxycycline treated CKH2B mice as measured by flow cytometry, and illustrated by corresponding representative confocal images (red=c-Kit, green=H2BEGFP). Tabulation of flow cytometry analysis of c-Kit<sup>+</sup> and H2BEGFP in CKH2B NMC (F) and BM (G). n=4 animals.



**Figure 4. Similar c-Kit+/EGFP+ co-localization but decreased total cardiac c-Kit+ cells per area in CKmCm knock-in mice versus CKH2B transgenic mice**  
 Paraffin sections were immunolabeled for c-Kit and EGFP to quantitate reporter coexpression in cardiac c-Kit+ cells *in situ*. EGFP co-localization with c-Kit+ cells in hearts of CKmCm and CKH2B mice is comparable (p=0.0838)(A). Significantly fewer c-Kit+ cells per area were present in CKmCm+Tx and CKmCm-Tx mice compared to doxycycline treated CKH2B mice (p=0.0054 and p=0.0077, respectively). No statistical differences were found between CKmCm-Tx and CKmCm+Tx mice (0.9058)(B). CKH2B hearts n=3, CKmCm-Tx hearts n=2, CKmCm+Tx hearts n=4. Significance determined by mixed linear modeling as described in the [Statistics](#) section and the Supplemental Statistical Analysis. p<0.05 denotes significance. (C). Comparative flow cytometry analysis of nonmyocytes isolated from CKmCm+Tx versus CKH2B+doxycycline hearts reveals significantly lower percentage of c-Kit+ NMCs in CKmCm hearts. n=10 CKH2B, n=3 CKmCm, statistical significance determined by two-tailed t-test, \*=p<0.05.

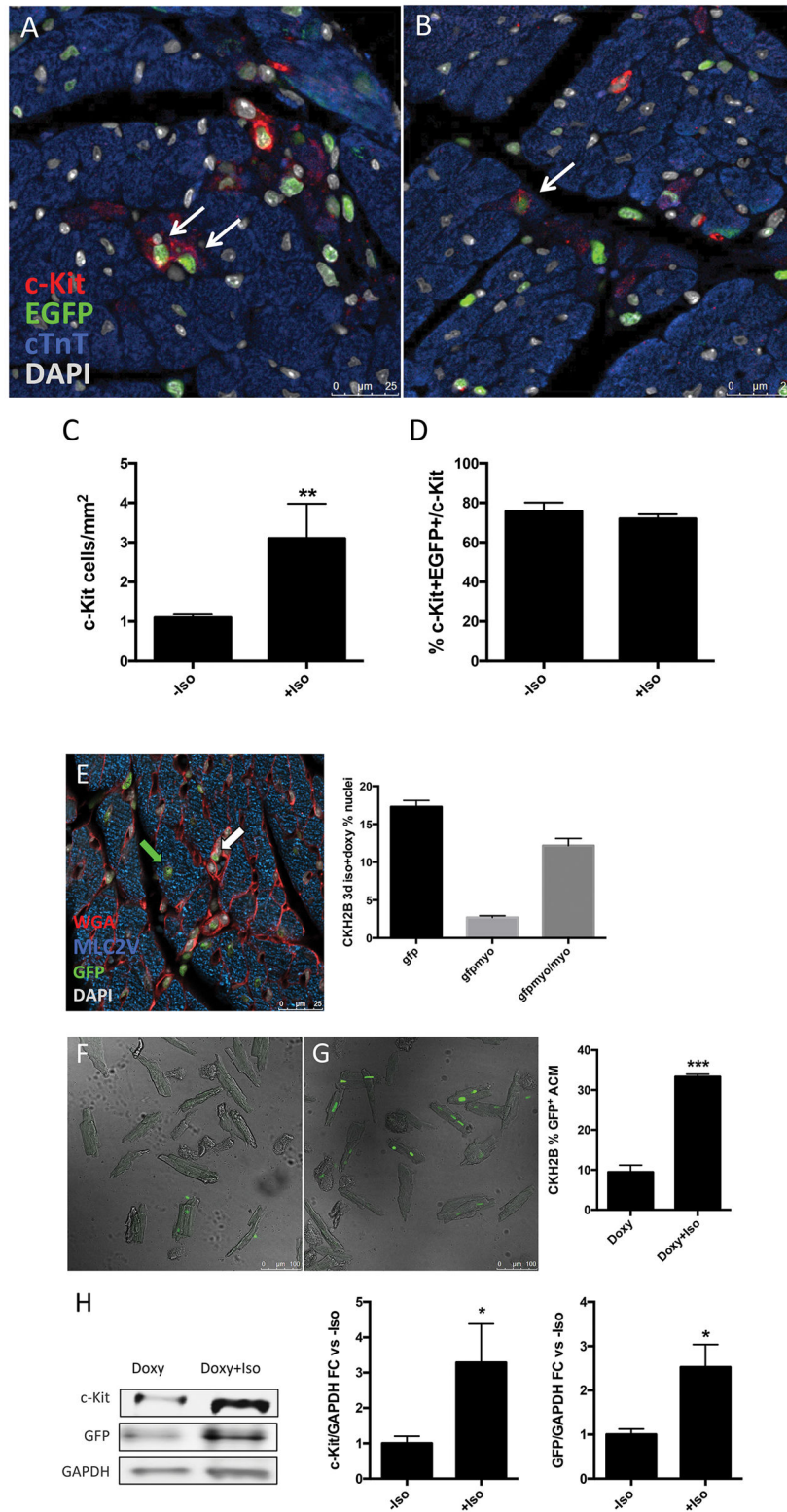


**Figure 5. H2BEGFP reporter accumulates in cardiac myocytes in vivo**  
 EGFP is detected and quantitated in nuclei of immunolabeled CKH2B hearts treated for one day (A) or four days (B) with doxycycline (red=wheat germ agglutinin, green=H2BEGFP, blue=myosin light chain 2v (MYLC2V), gray=DAPI). Quantitation of the increase in EGFP + cells per total nuclei (C), EGFP+ myocytes per total nuclei (D) and EGFP+ myocytes per total myocytes (E) represented in histograms. n=3 hearts, minimum 10 fields of view per heart.



**Figure 6. H2BEGFP reporter is induced in CKH2B c-Kit<sup>+</sup> CPCs in vitro**

CKH2B CPCs express c-Kit and accumulate H2BEGFP after treatment with doxycycline (A). BMC=c-Kit<sup>trTA</sup> whole bone marrow. Quantitation of H2BEGFP protein levels in A relative to 1 day of doxycycline treatment (B) and of c-Kit relative to untreated CPCs (C). Quantitation of mRNA levels of EGFP (D) and c-Kit (E) relative to untreated CPCs. H2BEGFP fluorescence in c-Kit<sup>+</sup> CKH2B CPCs following doxycycline induction in live cells (F) and fixed cells immunolabeled for c-Kit (G, red=c-Kit, green=EGFP, gray=DAPI).



**Figure 7. Cardiac c-Kit<sup>+</sup> expression increases following diffuse injury in vivo**

H2BEGFP expression in cardiac c-Kit+ cells visualized by immunostaining and confocal microscopy (A,B), red=c-Kit, green=EGFP, blue=cTnT, gray=DAPI, white arrows indicate c-Kit+/EGFP+ cells. Quantitation of c-Kit+ immunolabeled cells per area of tissue (C), and percentage of c-Kit+ cells expressing H2BEGFP reporter in uninjured versus injured CKH2B hearts (D). H2BEGFP expression in adult cardiac myocytes in hearts from doxycycline and isoproterenol treated CKH2B revealed and quantitated by immunostaining (E) (red=WGA, green=EGFP, blue=MyLC2V, gray=DAPI, white arrow indicates H2BEGFP in nonmyocyte, green arrow indicates H2BEGFP in cardiomyocyte). ACM isolated from CKH2B mice treated with doxycycline only (F) or doxycycline and isoproterenol (G) for three days as imaged and quantitated by confocal microscopy. Protein levels of c-Kit and EGFP quantitated in ACM isolated from Doxy or Doxy+Iso treated CKH2B hearts (H). Bright field=ACM, green=H2BEGFP fluorescence. n=3 mice (F–H), n=4 isolations (I).

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