Published in final edited form as:

J Mol Cell Cardiol. ; 123: 64–74. doi:10.1016/j.yjmcc.2018.08.024.

Insights from molecular signature of in vivo cardiac c-Kit(+) cells following cardiac injury and β -catenin inhibition

Conrad P. Hodgkinson, PhD^{*,†,1}, José A. Gomez, PhD^{†,1,2}, Syeda Samara Baksh, MS¹, Alan Payne, BSc¹, Jeffrey Schmeckpeper, MD PhD¹, Richard E. Pratt, PhD¹, and Victor J. Dzau, MD¹

¹Mandel Center for Heart and Vascular Research, and the Cardiovascular Research Center, Duke University Medical Center, Durham, NC 27710

²Department of Medicine, Clinical Pharmacology Division, Vanderbilt University Medical Center, Nashville, TN 37232

Abstract

There is much interest over resident c-Kit(+) cells in tissue regeneration. Their role in cardiac regeneration has been controversial. In this study we aim to understand the in vivo behavior of cardiac c-Kit(+) cells at baseline and after myocardial infarction and in response to Sfrp2. This approach can accurately study the in vivo transcript expressions of these cells in temporal response to injury and overcomes the limitations of the in vitro approach. RNA-seq was performed with c-Kit(+) cells and cardiomyocytes from healthy non-injured mice as well as cKit(+) cells from 1 day post-MI and 12 days post-MI mice. When compared to in vivo c-Kit(+) cells isolated from a healthy non-injured mouse heart, cardiomyocytes were enriched in transcripts that express anion channels, cation channels, developmental/differentiation pathway components, as well as proteins that inhibit canonical Wnt/ β -catenin signaling. Myocardial infarction (MI) induced in vivo c-Kit(+) cells to transiently adopt the cardiomyocyte-specific signature: expression of a number of cardiomyocyte-specific transcripts was maximal 1 day post-MI and declined by 12 days post-MI. We next studied the effect of β catenin inhibition on in vivo c-Kit(+) cells by administering the Wnt inhibitor Sfrp2 into the infarct border zone. Sfrp2 both enhanced and sustained cardiomyocyte-specific gene expression in the in vivo c-Kit(+) cells: expression of cardiomyocyte-specific transcripts was higher and there was no decline in expression by 12 days post-MI. Further analysis of the biology of c-Kit(+) cells identified that culture induced a significant and irreversible change in their molecular signature raising questions about reliability of in vitro studies. Our findings provide evidence that MI induces in vivo c-Kit(+) cells to adopt transiently a

*Corresponding Authors: Conrad P. Hodgkinson, PhD (210 Research Drive, GSRBII Room 4022, Duke University, Durham NC 27710, Tel number: 001 919 681 3828, conrad.hodgkinson@duke.edu) & Victor J. Dzau, MD (210 Research Drive, GSRBII Room 4022, Duke University, Durham NC 27710, Tel number: 001 919 668 5448, victor.dzau@duke.edu).

[†]Joint First Authors

Appendix A. Supplementary data is provided in the attached file

Disclosures

None.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cardiomyocyte-specific pattern of gene expression, and Sfrp2 further enhances and induces sustained gene expression. Our approach is important for understanding c-Kit(+) cells in cardiac regeneration and also has broad implications in the investigation of in vivo resident stem cells in other areas of tissue regeneration.

Keywords

Cell Differentiation; Heart stem cells; Heart Injuries/pathology; Proto-Oncogene Proteins c-kit/metabolism; Wnt Signaling Pathway

1. Introduction

Stem cells hold great promise as a tool for tissue repair, either by the induction of differentiation of stem cells in vivo and/or by expanding stem cells in culture and reinjecting into the injured tissue. However, this approach has been hampered by our lack of understanding of how in vivo stem cells respond to external stimuli in their native context. To that end, we devised a procedure to analyze how resident stem cells respond to external signals. We chose to utilize the c-Kit(+) stem cell as these cells have been reported to differentiate into a wide range of celltypes in a number of different tissues. C-Kit(+) stem cells have been identified in the bone marrow, testis, brain, lung and heart where they produce blood cells[1], sperm cells[2], neurons[3], vascular cells[4] and cardiomyocytes/endothelial cells[5, 6] respectively. While the role of c-Kit(+) cells in many tissues is understood; the role of c-Kit(+) cells in the heart is very controversial. Early reports suggested that cultured cardiac c-Kit(+) cells possessed an ability to differentiate into endothelial cells, smooth muscle cells, and cardiomyocytes[7–10] and injection of these cells into MI hearts improved cardiac function. However, lineage tracing experiments from different groups reach very different conclusions: in vivo c-Kit(+) cell differentiation into cardiomyocytes has been reported to be rare by some[5, 11] and robust by others[6]. Despite the significant amounts of research into the role of c-Kit(+) cells, the molecular signature of these cells in vivo has never been studied and it is also unclear if specific stimuli can drive the differentiation of these cells in vivo. Towards this end, we have devised a procedure to analyze the phenotype of resident c-kit(+) cells in the heart under basal conditions, following myocardial infarction and in response to a stimulus do induce differentiation.

We utilized the RNA-seq method to transcript profile in vivo c-Kit(+) cells in the adult heart before and after cardiac injury. Through this approach we found that myocardial infarction induced c-Kit(+) cells to transiently adopt a cardiomyocyte-specific molecular signature. Adoption of this cardiomyocyte-specific molecular signature was enhanced and sustained by the Wnt inhibitor Sfrp2; demonstrating that in vivo c-Kit(+) cells have the potential capacity to develop into cardiomyocytes in vivo. Furthermore, we found that culturing c-Kit(+) cells induced a significant and irreversible alteration in their molecular signature raising questions about the relevance and reliability of in vitro studies.

2. Materials & Methods

2.1. Animal models

Mice were maintained at the Duke University animal facility following the National Institute of Health guide lines. Animal procedures were approved by the Duke University Institutional Animal Care and Use Committee prior to starting the experiments.

C57BL/6:c-Kit Cre^{ERT2}; *R26^{mT-mG/+}* (c-Kit^{CreERT2/mTeG}) mice were used where the c-Kit promoter drives the expression of a tamoxifen-inducible Cre. This transgenic mouse carries a Cre-ERT2 expression cassette inserted into the ATG start codon of the endogenous c-Kit locus[12]. Eight week old mice received tamoxifen (0.5 mg/day 4-OH tamoxifen (Sigma) diluted in sunflower oil (Sigma) by intraperitoneal administration for 14 consecutive days) to induce Cre-mediated recombination at the LoxP sites allows expression of eGFP exclusively in c-Kit(+) cells. Mice were maintained on a C57BL6 background. One week after the last tamoxifen dose mice were subjected to permanent ligation of the left anterior descending coronary artery.

2.2. Myocardial infarction (acute left anterior descending (LAD) coronary artery ligation) and Sfrp2 injection

Anesthetized mice were intubated prior to left thoracotomy and exposure of the left ventricle of the heart. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) by i.p. injection. Ophthalmic ointment was applied to both eyes to prevent corneal desiccation during the procedure. Following washing of the skin with iodophore/alcohol an endotracheal intubation was performed and the mouse connected to a ventilator (model 683, Harvard Apparatus, tidal volume 0.7–1ml, respiratory rate 120 breaths per minute) via an IV catheter (20 GA 1 IN) as the cannula under direct laryngoscopy. The chest cavity was opened between the fourth and the fifth rib in the intercostals muscle, the heart externalized and a 7–0 nylon suture be placed through the myocardium into the anterolateral LV wall, corresponding to the course of the left anterior descending artery. The suture was positioned approximately midway between the apex and base and a ligature made. Before ligation, left coronary artery entrapment was confirmed by upward traction. The suture was completely tied off (myocardial infarction), and the apex of the LV was observed for evidence of myocardial blanching indicating interruption in coronary flow. The wound was then closed using 7–0 nylon and the chest cavity was subsequently closed in layers with 5–0 monofilament suture. Negative pressure re-established at closure, and the animal gradually removed from the respirator. Following the resumption of spontaneous respiration, the endotracheal tube was removed and the animal allowed to recover on a deltapase isothermal pad set at 37°C. The animal remained in a supervised setting until fully conscious and then returned to their cages and given standard chow and water. Post-operative analgesia was used: bupivacaine locally + buprenorphine SQ at BID for 5 days.

Two days following MI, mice underwent a second left thoracotomy followed by intra-cardiac injection of a total of 0.5 µg of recombinant mouse Sfrp2 (R&D Systems) or normal saline (vehicle) at 3–5 sites within the infarct border zone. Direct cardiac injection of the

Wnt inhibitor Sfrp2 significantly reduces the possibility of Sfrp2 affect organs other than the heart.

All skin sutures were removed by 7 days post-operation, if the sutures remained.

2.3. RNA-seq of in vivo c-Kit(+) cells

C-Kit(+) cells were isolated one day prior to MI, one day post-MI and 12 days post-MI (vehicle and Sfrp2). Cardiac tissue were harvested, minced to ~1mm³ cubes and digested for 10 minutes at 37°C in a solution containing 200units/ml collagenase-II (Worthington), DMEM/F12 (Invitrogen), and 1xpenicillin-streptomycin (Invitrogen). Fetal bovine serum (Invitrogen) was added to a final concentration of 10% v/v to inhibit the reaction. The mixture was centrifuged at 100g for 3 minutes to remove cardiomyocytes. The cardiomyocyte fraction was kept for RNA extraction. C-Kit(+) cells were then isolated by magnetic beads conjugated with a c-Kit antibody (eBioscience, 8802–6838-74) and on the basis of GFP expression (flow cytometry). Isolation was performed according to manufacturer's instructions. Purity is shown in Supplementary Figure 1.

Total RNA from the purified c-Kit(+) population was isolated using a PicoPure Arcturus kit (Invitrogen) according to the manufacturer's instructions. Complimentary cDNA was generated with an Ovation Pico WTA System V2 kit (NuGEN) which maintains the stoichiometry of the original RNA population. RNA-seq was performed with 2 lanes on a HiSeq 2000 instrument yielding a total of >400,000,000 PF clusters.

Gene Ontology analysis was performed by the Duke Genomic Core and via the Gene Ontology Consortium on-line database (<http://www.geneontology.org/>). RNA-seq data was processed using the TrimGalore toolkit which employs Cutadapt to trim low quality bases and Illumina sequencing adaptors from the 3' end of the reads. Only reads that were 20nt or longer after trimming were kept for further analysis. Reads were mapped to the GRCm38v68 version of the mouse genome and transcriptome using the STAR RNA-seq alignment tool. Reads were kept for further analysis if they mapped to a single genomic location. Gene counts were compiled using the HTseq tool. Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression was carried out using the DESeq2 Bioconductor package with the R statistical programming environment. The false discovery rate was calculated to control for multiple hypothesis testing. Gene set enrichment analysis was performed to identify differentially regulated pathways and gene ontology terms for each of the comparisons performed.

The raw and processed data was uploaded to the NIH GEO database and has been assigned the Accession number GSE90615.

2.4. qPCR validation of RNA-seq

Cell isolations were performed from wild-type C57BL/6 mice. Heart tissue was dissociated as described above. The cardiomyocyte fraction was obtained by centrifugation at 100g for 3 minutes. The supernatant was used for c-Kit(+) cell isolation by magnetic beads conjugated with a c-Kit antibody (eBioscience, 8802–6838-74). RNA from the c-Kit(+) and cardiomyocyte fractions was obtained via Quick-RNA miniprep kit (Genesee-Scientific)

according to manufacturers' instructions. RNA was converted to cDNA and qPCR performed as described previously[13].

2.5. Cardiac Progenitor Cell Isolation & Culture

Cardiac biopsies from C57BL/6:c-Kit Cre^{ERT2}: *R26^{mT-mG/+}* (c-Kit^{CreERT2}/mTeG) mice were harvested, minced to ~1mm³ cubes and digested for 10 minutes at 37°C in a solution containing 200units/ml collagenase-II (Worthington), DMEM/F12 (Invitrogen), and 1xpenicillin-streptomycin (Invitrogen). Fetal bovine serum (Invitrogen) was added to a final concentration of 10% v/v to inhibit the reaction. The mixture was centrifuged at 500g for 5 minutes, supernatant was removed and pellet resuspended in DMEM containing 5xpenicillin-streptomycin (Invitrogen). The mixture was again centrifuged at 500g for 5 minutes and the pellet washed again. The pellet was resuspended in DMEM/F12, 20% FBS, 1xpenicillin-streptomycin and plated onto a well of a 6-well plate containing 50,000 bone marrow derived wild-type MSCs. After 7 days, samples were collected with enzyme free cell dissociation buffer (Invitrogen) to prevent cleavage of c-Kit at the plasma membrane. c-Kit(+) cells were isolated by repeated immunopanning as described previously[13]. C-Kit(+) cells were then maintained in CPC maintenance media (F12K (Invitrogen), 5% FBS, 10ng/ml bFGF (R&D Systems), 20ng/ml LIF (Sigma), 1xpenicillin-streptomycin). After 7 days the c-Kit+ CPCs, which were found to be weakly adherent, were cultured in CPC-maintenance media. Following expansion the c-Kit+ CPCs were used at passage 1. At this passage the cells were positive for c-Kit (data not shown).

For the in vitro cardiac differentiation protocols, c-Kit(+) cells were seeded at 10,000 cells per cm² in growth medium. 24 hours later, when the cells were 80–90% confluent, growth media was replaced with differentiation media (235 parts IMDM, 235 parts Ham F-12, 5 parts GlutaMAX, 10 parts ESC qualified serum, 5 parts MEM non-essential amino acids, 5 parts Insulin-Transferrin-Selenium, 5 parts penicillin-streptomycin) plus 5uM 5-azacytidine. Fresh differentiation media plus 5-azacytidine was added each day for a further two days. Cells were then incubated for a further day in fresh differentiation media lacking 5-azacytidine. Cells were then incubated for a further ten days in differentiation media supplemented with 1ng/ml TGF-β and 100uM ascorbic acid. Where appropriate, XAV-939 was added at a final concentration of 1uM. Media was changed every three days.

The method as outlined in reference [14] was used for Spheroid Differentiation with one exception. We were unable to develop spheroids on bacteriological plates as described in the reference as the cells attached readily to the surface of the plate. Instead, we used poly-2hydroxymethylacrylate coated plates to prevent cell attachment[15]. This allowed the formation of spheroids.

2.6. Randomization and Blinding

Two separate blinded investigators carried out RNA-seq described above. Investigators were blinded with respect to animal identifiers and group assignments. The animal surgeon who carried out the mice procedures did not know the animal genotype or the identity of the chemicals given to the animals. All compounds used in this study were colourless and odourless; thus there was no way to differentiate between vehicle or Sfrp2. For any given

experiment all groups were subjected to surgery at the same time; thus there was no bias in how the groups were treated with respect to surgeries. There was also no bias with respect to sex.

2.7. Statistics

All statistical analysis was performed using GraphPad. Experiments containing two conditions a t-test was performed. ANOVA was used for experiments with three or more conditions followed by Bonferroni post-hoc tests for comparisons between individual groups. A P-value of less than 0.05 was considered significant.

2.8. Study Approval

Animal procedures and experiments were approved by the Duke University Institutional Animal Care and Use Committee prior to starting the experiments.

3. Results

3.1. RNA-seq defines a cardiomyocyte-specific molecular signature

We employed RNA-seq to determine if in vivo c-Kit(+) cells had the potential to differentiate into cardiomyocytes. To that end we employed a mouse transgenic model (c-KitCre^{ERT2}/mTeG) where the c-Kit promoter drives the expression of a tamoxifen-inducible Cre. Following Cre activation, eGFP is expressed exclusively in c-Kit(+) cells (Supplementary Figure 2A). To characterize our model c-KitCre^{ERT2}/mTeG mice were injected for 14 consecutive days with a low dose of tamoxifen (0.5 mg/mouse/day) to induce Cre expression and to label c-Kit(+) cells with eGFP. As shown in Supplemental Figure 2B immunocytochemistry and FACS analysis indicated that c-Kit(+) cells were efficiently labeled with GFP. We also measured recombination in other tissues known to express c-Kit. In the bone marrow (Supplemental Figure 2C), testis (Supplemental Figure 2D) and lung (Supplemental Figure 2E) recombination was found to be robust, with >80% of the c-Kit(+) cells also expressing eGFP. There were no statistically significant differences in labelling efficiency between any of the various tissues tested. Importantly eGFP expression was observed in blood cells derived from c-Kit(+) precursors (Supplemental Figure 2C). Moreover, no eGFP labeling was observed in cardiac cells such as endothelial, smooth muscle or cardiomyocytes as determined by flow cytometry (Supplemental Figure 3A). Similarly, we did not observe co-staining between GFP and markers for cardiomyocytes, smooth muscle, endothelial cells and mast cells (Supplemental Figure 3B). Finally, the cardiac c-Kit(+) cells were found to express Nkx2.5 (Supplemental Figure 3C).

Following verification of our model we first compared the molecular signatures of pre-injury cKit(+) cells and cardiomyocytes. Gene ontology analysis indicated that the in vivo cardiomyocytes were enriched for transcripts that expressed anion channels, cation channels, components of cardiomyocyte developmental and differentiation pathways, as well as transcripts that encode proteins that inhibit canonical Wnt/ β -catenin signaling (Figure 1A). In contrast, cardiomyocytes were depleted in transcripts that expressed chemokines and angiogenic markers (Figure 1A).

3.2. Following MI and Sfrp2 injection, in vivo c-Kit(+) cells express factors important for commitment to the cardiomyocyte lineage.

We analyzed the RNA-seq data for the expression of factors that have been implicated in commitment to the cardiomyocyte lineage. Cardiomyocytes were enriched for a number of commitment factors including *Isl1*, *Gata4*, *Gata6*, *Hand1*, and *Tbx5* (Figure 1B, box). In comparison to pre-injury in vivo c-Kit(+) cells, myocardial infarction was sufficient to induce expression of these commitment factors in the in vivo c-Kit(+) cells. Expression was apparent 1 day post-MI (Figure 1B, box). The injection of Sfrp2 into the infarct border zone significantly increased and sustained (up to day 12 post-MI) the expression of *Tbx6*, *Nfatc2*, *Nfatc4*, *Hand1*, *Mesp1* and *Isl1* in the in vivo c-Kit(+) cells when compared to vehicle treatment (Figure 1B, box).

Recently, we have shown that demethylation of H3K27me3 is important for cardiac reprogramming[16]. Analysis of the regulators of H3K27me3 indicated that cardiomyocytes are enriched in enzymes that promote H3K27 methylation, and thus gene silencing, whereas preinjury c-Kit(+) cells are enriched for enzymes that demethylate H3K27me3 (Figure 1B). This suggests that pre-injury c-Kit(+) cells are in a poised state for gene transcription which is typical of stem cells.

3.3. In vivo c-Kit(+) cells express cardiomyocyte anion and cation channels following myocardial infarction.

One of the most defining features in the RNA-seq data was the enrichment in ion channel GO terms (Figure 1). Ion channel GO p-values, FDR q-values, and enrichment scores were strongly significant for cardiomyocytes. Immediately post-MI cardiomyocyte-specific ion channel expression was also apparent in the in vivo c-Kit cells (Figure 2A). By 12 days post-injury, in vivo c-Kit(+) cells significantly expressed 57 of the 91 cardiomyocyte ion channels (Figure 2A). The shift towards cardiomyocyte ion channel expression was enhanced by Sfrp2; in the Sfrp2 treated group, c-Kit(+) cells expressed all 91 of the 91 cardiomyocyte ion channels (Figure 2A). Cardiomyocyte ion channel expression in post-injury c-Kit(+) cells followed two patterns. In one pattern, expression increased in both the vehicle and Sfrp2 groups though the level of expression was higher in the Sfrp2 group (Figure 2B, top panels). In the other pattern, expression increased dramatically 1 day post-MI and declined by day 12. This decline in expression by day 12 was arrested and reversed by Sfrp2 (Figure 2B, bottom panels). In total, this data suggests that Sfrp2 promotes the acquisition of a cardiomyocyte phenotype. We decided to validate our RNA-seq data by lineage tracing c-Kit(+) cells following MI and Sfrp2 injection. We found at 15 days post-MI and post-Sfrp2 injection that there was co-localization between GFP, the marker used to track the fate of c-Kit(+) cells, and the cardiomyocyte marker cardiac troponin-T (Figure 2C). Quantification of the immunostaining indicated that peri-infarct region contained ~2% GFP+ cardiac troponin-T+ cells following Sfrp2 treatment (Figure 2C). Importantly, the transient expression of cardiomyocyte-specific genes in c-Kit(+) cells isolated from the c-KitCre^{ERT2}/mTeG mice following MI were also observed in c-Kit(+) cells isolated from wild-type C57BL/6 mice (Supplemental Figure 4).

3.4. Myocardial infarction and *Sfrp2* promote β -catenin inhibition in the in vivo c-Kit(+) cells.

We have previously shown that the Wnt inhibitor *Sfrp2* promotes cardiomyocyte gene expression in Sca-1 cells[17]. Consequently, we were interested to determine if *Sfrp2* similarly induced cardiomyocyte gene expression in the in vivo c-Kit(+) cells. Analysis of the RNA-seq data indicated that following myocardial infarction in vivo c-Kit(+) cells increase the expression of transcripts that encode proteins that inhibit canonical Wnt and β -catenin signaling (Figure 3A and 3B). This increased expression of components of a canonical Wnt and β -catenin inhibitory pathway was further amplified by *Sfrp2* (Figure 3A and 3B). We then wished to determine the mechanism by which *Sfrp2* promoted c-Kit(+) cells to express cardiomyocyte-specific genes. We have previously shown that *Sfrp2* promotes Sca-1(+) cells to commit to the cardiac lineage via the inhibition of β -catenin. To determine if *Sfrp2* similarly inhibited β -catenin in c-Kit(+) cells we cultured c-Kit(+) cells in the presence of vehicle or ligand for 5 minutes. Protein extracts were subsequently immunoblotted for phospho- β -catenin, a marker for β -catenin inhibition. As shown in Figure 3C, *Sfrp2* promoted robust phosphorylation, and thus inhibition, of β -catenin. To fully verify a role for β -catenin inhibition in c-Kit(+) cell cardiac differentiation we used the pharmacological inhibitor XAV-939. This pharmacological compound specifically inhibits Wnt/ β -catenin-mediated transcription through tankyrase1/272–74. As shown in Figure 3D, XAV-939 significantly enhanced early-, middle-, and late-stage cardiac gene expression in c-Kit(+) cells. Taken together this data indicates that *Sfrp2* promotes cardiac gene expression in c-Kit(+) cells via β -catenin inhibition.

3.5. In vivo c-Kit(+) cells express proliferation markers and cytokines

As expected, cardiomyocytes were enriched for cell-cycle inhibitors such as *Cdkn1b* and *Cdkn2aip* (Figure 4A). We have shown previously that in vivo c-Kit(+) cells proliferate postMI[13]. Indeed we found that expression of cell-cycle inhibitors in the in vivo c-Kit(+) cells remained low at all time-points (Figure 4A) suggesting that in vivo c-Kit(+) cells proliferate postMI. Moreover, in further support that the in vivo c-Kit(+) cells were in a proliferative state, expression of various cyclins and CDKs increased following MI (Figure 4A).

In vivo c-Kit(+) cells are randomly distributed within the myocardium and are believed to home to the sites of injury following myocardial infarction[18, 19]. Commensurate with this notion, in vivo c-Kit(+) cells became enriched for >50 chemokine receptors following myocardial infarction (Figure 4B).

Recent lineage tracing experiments suggest that in vivo c-Kit(+) cells differentiate into endothelial cells. Analysis of the RNA-seq data does not support this notion. While pre-injury in vivo c-Kit(+) cells were typically enriched for angiogenic markers, expression significantly declined post-MI (Figure 4C).

3.6. Culture induces significant and permanent changes in the molecular signature of cardiac cKit(+) cells.

When compared to in vivo cardiac c-Kit(+) cells, cultured c-Kit(+) cells showed significantly lower levels of cardiomyocyte-specific gene expression (Figure 5). Cardiomyocyte specific ion channel expression was not detectable at threshold values. The difference between in vivo and cultured cardiac c-Kit(+) cells was dramatic and ranged from 32 fold for Gata4 to over 1 million fold for Tnni3. This novel finding has not been previously reported. Furthermore, methods that have been reported to induce c-Kit(+) differentiation in culture were unable to recapitulate in vivo levels of expression (Figure 6). As shown in Figure 2, in vivo c-Kit(+) cells from a healthy non-injured mouse do not express cardiomyocyte specific ion channels. Culture of in vivo cKit(+) cells at baseline or under differentiation protocols did not induce cardiomyocyte specific ion channel expression.

4. Discussion

Surprisingly, considering the interest in using cultured c-Kit(+) cells for the regeneration of the heart, there are few studies with which to compare our RNA-seq data. Zhang et al[20] dedifferentiated cardiomyocytes into cardiac progenitor-like cells. In agreement with our study, they found that cardiomyocytes were enriched for ion channel GO terms. Microarray analysis, an older and less comprehensive technique than RNA-seq[21], compared the relationships between various putative cardiac progenitors (c-Kit(+), Sca-1(+), and side-population) and cardiomyocytes[22]. The pertinent findings of this study are in agreement with our data.

We have previously reported that Sfrp2 promotes the expression of early cardiac genes in cardiac progenitor cells[17] via the modulation of Wnt signaling pathway. Here we show that cKit(+) cells undergo a phase transition in response to MI; changing from a state where canonical Wnt β -catenin predominates to alternative state that promotes non-canonical Wnt β -catenin inactivation. It has been proposed that Wnt/ β -catenin signaling has a biphasic function during cardiac development[23]. Early cardiac specification requires Wnt/ β -catenin activation whereas differentiation requires inhibition of this pathway[23]. Crescent, a member of the Sfrp family, and Dkk1 promote cardiac development in chicken and *Xenopus* embryos via inhibition of the Wnt/ β -catenin pathway[24, 25]. Similarly β -catenin genetic ablation specifically in the mouse endoderm results in the formation of multiple ectopic hearts[26]. Other studies also support the notion that inactivation of the canonical β -catenin pathway is important for c-Kit(+) differentiation into cardiomyocytes. For example, the development of the heart from the posterior mesoderm requires Wnt inhibition[24, 25]. Similarly, knockout of the β -catenin induces the formation of multiple ectopic hearts[26]. Cardiac development requires a number of cell-fate decisions. These cell-fate decisions can be mimicked in vitro with embryonic stem cells (ESCs). For example, ESCs will differentiate into cardiomyocytes under defined culture conditions. On their own, these defined culture conditions are not efficient. Efficiency is increased substantially with the addition of Wnt inhibitors[27]. Low efficiency is also hallmark of direct cellular reprogramming. In this process, one somatic cell type is directly converted into another by a

specific combination of factors. For example, the combined over-expression of the transcription factors Gata4, Mef2C and Tbx5 (GMT) reprograms fibroblasts into cardiomyocytes. Recently, it has been shown that the ability of GMT to reprogram fibroblasts is increased 8-fold with the addition of the Wnt inhibitor XAV-939[28]. Taken together, these data would support our hypothesis that inhibition of canonical Wnt β -catenin signaling is necessary for in vivo c-Kit(+) cells to adopt a cardiomyocyte-specific molecular signature. In the future, it will be important to determine the signaling pathway by which β -catenin inhibition promotes c-Kit(+) differentiation into cardiomyocytes.

The fate and identity of c-Kit(+) cells has generated much interest. Lineage tracing experiments which utilize a Cre knock-in in the c-Kit locus and other similar approaches suggest that c-Kit(+) cells fail to differentiate into cardiomyocytes in significant numbers[5, 11, 29, 30]. Indeed, these studies suggest that c-Kit(+) cells are a source of endothelial cells. Further evidence that cKit(+) cells develop into endothelial cells has been demonstrated by combining c-Kit lineage tracing and Gata4 deletion [31]. Interestingly, despite using similar technology, we do not find a clear signal for endothelial differentiation in our study. Other researchers contend that lineage tracing experiments fail to mark true c-Kit(+) cardiac progenitors due to the apparently low c-Kit expression in these progenitor cells [32]. Moreover, due to the disruption of one c-Kit allele in the knock-in approaches, haploinsufficiency has been proposed as an explanation for why c-Kit lineage tracing experiments have failed to show that c-Kit(+) cells develop into cardiomyocytes[33]. The identity, as well as the fate, of c-Kit(+) cells is certainly complex[34] and this complex story has helped drive a number of researchers to develop a consensus statement that seeks to guide future research in cardiac regeneration[35]. Our RNA-seq data may explain why lineage tracing experiments have failed to identify c-Kit(+) cells as a source of cardiomyocytes in vivo. Our data showed that post-injury c-Kit(+) cells express cardiomyocyte ion channels; however, expression of many of these cardiomyocyte ion channels in post-injury c-Kit(+) cells was found to be transient. The transient nature of cardiomyocyte ion channel expression presumably precludes acquisition of the cardiomyocyte phenotype. Notably, the transient expression of cardiomyocyte ion channels in post-injury c-Kit(+) cells was arrested and reversed by Sfrp2. Indeed, Sfrp2 treatment led to the expression of the full complement of cardiomyocyte ion channels. Expressing the full complement of cardiomyocyte ion channels would presumably allow the c-Kit(+) cell to develop into a functional cardiomyocyte. However, it is also important to note that following MI the c-Kit(+) population in the heart changes. Following MI there is a large influx of c-Kit(+) cells from the circulation. These circulatory c-Kit(+) cells include bone-marrow derived progenitor cells as well as several types of immune cell. It is possible that the transient nature of cardiomyocyte-gene expression in cardiac c-Kit(+) cells post-MI is a dilution effect arising from the influx of circulatory c-Kit(+) cells rather than shortlived cardiomyocyte-specific gene expression in a true cardiac c-Kit(+) progenitor.

Surprisingly, pre-injury c-Kit(+) cells were significantly enriched for a number of cardiomyocyte sarcomere genes. Potentially this could be due to a sub-population of cardiomyocytes that express c-Kit(+) in vivo; a sub-population that was recently identified by lineage tracing experiments[29]. However, our RNA-seq data suggests otherwise as we found that pre-injury cKit(+) cells are distinct from cardiomyocytes. High expression of

sarcomere genes could also indicate that c-Kit(+) cells are primed to differentiate into cardiomyocytes. Such priming has to contend with the fact that c-Kit(+) cells are apparently incapable of naturally differentiating into a cardiomyocyte. The high expression of sarcomere genes and the transient expression of cardiomyocyte ion channels noted above might also suggest that the c-Kit(+) cell is a vestigial cardiac stem cell; retaining only a partial ability to differentiate into a cardiomyocyte.

Conclusion

Our results provide important insights into the biological role of in vivo cardiac c-Kit(+) cells as well as the limitations of studies that seek to use cultured c-Kit(+) cells for cardiac regenerative therapy. In addition to the significance of our approach and findings to cardiac regeneration biology and therapy, they have broader implications in the investigation of in vivo resident stem cells in other tissue regeneration models in which c-Kit(+) stem cells may play a role. Considering the reported putative roles for c-Kit(+) cells in various tissues, neuron regeneration (Alzheimer's, Parkinson's, Huntington's), vascular remodeling (asthma) and male infertility all represent potential areas for study using this approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank John Wong of the DHVI (Duke Human Vaccine Institute) for the use of flow cytometers.

Sources of Funding

Research conducted in these studies was supported by National Heart, Lung, and Blood Institute (grant numbers RO1 HL35610, HL81744, HL72010, HL73219, and HL081744–07S1) and the Edna and Fred L. Mandel, Jr. Foundation (no agency ID).

Abbreviations:

MI myocardial infarction

References

- [1]. Lennartsson J, Ronnstrand L, Stem cell factor receptor/c-Kit: from basic science to clinical implications, *Physiol Rev* 92(4) (2012) 1619–49. [PubMed: 23073628]
- [2]. Zhang M, Zhou H, Zheng C, Xiao J, Zuo E, Liu W, Xie D, Shi Y, Wu C, Wang H, Li D, Li J, The roles of testicular c-kit positive cells in de novo morphogenesis of testis, *Sci Rep* 4 (2014) 5936. [PubMed: 25088917]
- [3]. Goldstein BJ, Goss GM, Hatzistergos KE, Rangel EB, Seidler B, Saur D, Hare JM, Adult c-Kit(+) progenitor cells are necessary for maintenance and regeneration of olfactory neurons, *J Comp Neurol* 523(1) (2015) 15–31. [PubMed: 25044230]
- [4]. Liu Q, Huang X, Zhang H, Tian X, He L, Yang R, Yan Y, Wang QD, Gillich A, Zhou B, c-kit(+) cells adopt vascular endothelial but not epithelial cell fates during lung maintenance and repair, *Nat Med* 21(8) (2015) 866–8. [PubMed: 26168292]
- [5]. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marban E, Molkentin JD, c-kit+ cells minimally contribute cardiomyocytes to the heart, *Nature* 509(7500) (2014) 337–41. [PubMed: 24805242]

- [6]. Ellison GM, Vicinanza C, Smith AJ, Aquila I, Leone A, Waring CD, Henning BJ, Stirparo GG, Papait R, Scarfo M, Agosti V, Viglietto G, Condorelli G, Indolfi C, Ottolenghi S, Torella D, Nadal-Ginard B, Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair, *Cell* 154(4) (2013) 827–42. [PubMed: 23953114]
- [7]. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P, Bone marrow cells regenerate infarcted myocardium, *Nature* 410(6829) (2001) 701–5. [PubMed: 11287958]
- [8]. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P, Adult cardiac stem cells are multipotent and support myocardial regeneration, *Cell* 114(6) (2003) 763–76. [PubMed: 14505575]
- [9]. Barile L, Messina E, Giacomello A, Marban E, Endogenous cardiac stem cells, *Prog Cardiovasc Dis* 50(1) (2007) 31–48. [PubMed: 17631436]
- [10]. Hong KU, Bolli R, Cardiac stem cell therapy for cardiac repair, *Curr Treat Options Cardiovasc Med* 16(7) (2014) 324. [PubMed: 24903489]
- [11]. Sultana N, Zhang L, Yan J, Chen J, Cai W, Razzaque S, Jeong D, Sheng W, Bu L, Xu M, Huang GY, Hajjar RJ, Zhou B, Moon A, Cai CL, Resident c-kit(+) cells in the heart are not cardiac stem cells, *Nat Commun* 6 (2015) 8701. [PubMed: 26515110]
- [12]. Klein S, Seidler B, Kettenberger A, Sibaev A, Rohn M, Feil R, Allescher HD, Vanderwinden JM, Hofmann F, Schemann M, Rad R, Storr MA, Schmid RM, Schneider G, Saur D, Interstitial cells of Cajal integrate excitatory and inhibitory neurotransmission with intestinal slow-wave activity, *Nat Commun* 4 (2013) 1630. [PubMed: 23535651]
- [13]. Hodgkinson CP, Gomez JA, Payne AJ, Zhang L, Wang X, Dal-Pra S, Pratt RE, Dzau VJ, Abi3bp regulates cardiac progenitor cell proliferation and differentiation, *Circ Res* 115(12) (2014) 1007–16. [PubMed: 25296984]
- [14]. Smith AJ, Lewis FC, Aquila I, Waring CD, Nocera A, Agosti V, Nadal-Ginard B, Torella D, Ellison GM, Isolation and characterization of resident endogenous c-Kit+ cardiac stem cells from the adult mouse and rat heart, *Nat Protoc* 9(7) (2014) 1662–81. [PubMed: 24945383]
- [15]. Hodgkinson CP, Wright MC, Paine AJ, Fibronectin-mediated hepatocyte shape change reprograms cytochrome P450 2C11 gene expression via an integrin-signaled induction of ribonuclease activity, *Mol Pharmacol* 58(5) (2000) 976–81. [PubMed: 11040044]
- [16]. Dal-Pra S, Hodgkinson CP, Mirotsoy M, Kirste I, Dzau VJ, Demethylation of H3K27 Is Essential for the Induction of Direct Cardiac Reprogramming by miR Combo, *Circ Res* 120(9) (2017) 1403–1413. [PubMed: 28209718]
- [17]. Schmeckpeper J, Verma A, Yin L, Beigi F, Zhang L, Payne A, Zhang Z, Pratt RE, Dzau VJ, Mirotsoy M, Inhibition of Wnt6 by Sfrp2 regulates adult cardiac progenitor cell differentiation by differential modulation of Wnt pathways, *J Mol Cell Cardiol* 85 (2015) 215–25. [PubMed: 26071893]
- [18]. Saravanakumar M, Devaraj H, Distribution and homing pattern of c-kit+ Sca-1+ CXCR4+ resident cardiac stem cells in neonatal, postnatal, and adult mouse heart, *Cardiovasc Pathol* 22(4) (2013) 257–63. [PubMed: 23273785]
- [19]. Vajravelu BN, Hong KU, Al-Maqtari T, Cao P, Keith MC, Wysoczynski M, Zhao J, Moore J.B.t., Bolli R, C-Kit Promotes Growth and Migration of Human Cardiac Progenitor Cells via the PI3K-AKT and MEK-ERK Pathways, *PLoS One* 10(10) (2015) e0140798. [PubMed: 26474484]
- [20]. Zhang Y, Zhong JF, Qiu H, MacLellan WR, Marban E, Wang C, Epigenomic Reprogramming of Adult Cardiomyocyte-Derived Cardiac Progenitor Cells, *Sci Rep* 5 (2015) 17686. [PubMed: 26657817]
- [21]. Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X, Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells, *PLoS One* 9(1) (2014) e78644. [PubMed: 24454679]
- [22]. Dey D, Han L, Bauer M, Sanada F, Oikonomopoulos A, Hosoda T, Unno K, De Almeida P, Leri A, Wu JC, Dissecting the molecular relationship among various cardiogenic progenitor cells, *Circ Res* 112(9) (2013) 1253–62. [PubMed: 23463815]
- [23]. Gessert S, Kuhl M, The multiple phases and faces of wnt signaling during cardiac differentiation and development, *Circ Res* 107(2) (2010) 186–99. [PubMed: 20651295]

- [24]. Marvin MJ, Di Rocco G, Gardiner A, Bush SM, Lassar AB, Inhibition of Wnt activity induces heart formation from posterior mesoderm, *Genes Dev* 15(3) (2001) 316–27. [PubMed: 11159912]
- [25]. Schneider VA, Mercola M, Wnt antagonism initiates cardiogenesis in *Xenopus laevis*, *Genes Dev* 15(3) (2001) 304–15. [PubMed: 11159911]
- [26]. Lickert H, Kutsch S, Kanzler B, Tamai Y, Taketo MM, Kemler R, Formation of multiple hearts in mice following deletion of beta-catenin in the embryonic endoderm, *Dev Cell* 3(2) (2002) 171–81. [PubMed: 12194849]
- [27]. Naito AT, Shiojima I, Akazawa H, Hidaka K, Morisaki T, Kikuchi A, Komuro I, Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis, *Proc Natl Acad Sci U S A* 103(52) (2006) 19812–7. [PubMed: 17170140]
- [28]. Mohamed TM, Stone NR, Berry EC, Radzinsky E, Huang Y, Pratt K, Ang YS, Yu P, Wang H, Tang S, Magnitsky S, Ding S, Ivey KN, Srivastava D, Chemical Enhancement of In Vitro and In Vivo Direct Cardiac Reprogramming, *Circulation* (2016).
- [29]. Liu Q, Yang R, Huang X, Zhang H, He L, Zhang L, Tian X, Nie Y, Hu S, Yan Y, Zhang L, Qiao Z, Wang QD, Lui KO, Zhou B, Genetic lineage tracing identifies in situ Kitexpressing cardiomyocytes, *Cell Res* 26(1) (2016) 119–30. [PubMed: 26634606]
- [30]. He L, Li Y, Li Y, Pu W, Huang X, Tian X, Wang Y, Zhang H, Liu Q, Zhang L, Zhao H, Tang J, Ji H, Cai D, Han Z, Han Z, Nie Y, Hu S, Wang QD, Sun R, Fei J, Wang F, Chen T, Yan Y, Huang H, Pu WT, Zhou B, Enhancing the precision of genetic lineage tracing using dual recombinases, *Nat Med* 23(12) (2017) 1488–1498. [PubMed: 29131159]
- [31]. Maliken BD, Kanisicak O, Karch J, Khalil H, Fu X, Boyer JG, Prasad V, Zheng Y, Molkentin JD, Gata4-Dependent Differentiation of c-Kit(+) Derived Endothelial Cells Underlies Artefactual Cardiomyocyte Regeneration in the Heart, *Circulation* (2018).
- [32]. Vicinanza C, Aquila I, Cianflone E, Scalise M, Marino F, Mancuso T, Fumagalli F, Giovannone ED, Cristiano F, Iaccino E, Marotta P, Torella A, Latini R, Agosti V, Veltri P, Urbanek K, Isidori AM, Saur D, Indolfi C, Nadal-Ginard B, Torella D, Kit(cre) knock-in mice fail to fate-map cardiac stem cells, *Nature* 555(7697) (2018) E1–E5. [PubMed: 29565363]
- [33]. Leri A, Anversa P, Complexity of Tracking the Fate of Adult Progenitor Cells, *Circ Res* 119(10) (2016) 1067–1070. [PubMed: 27789586]
- [34]. Gude NA, Firouzi F, Broughton KM, Ilves K, Nguyen KP, Payne CR, Sacchi V, Monsanto MM, Casillas AR, Khalafalla FG, Wang BJ, Ebeid DE, Alvarez R, Dembitsky WP, Bailey BA, van Berlo J, Sussman MA, Cardiac c-Kit Biology Revealed by Inducible Transgenesis, *Circ Res* 123(1) (2018) 57–72. [PubMed: 29636378]
- [35]. Eschenhagen T, Bolli R, Braun T, Field LJ, Fleischmann BK, Frisen J, Giacca M, Hare JM, Houser S, Lee RT, Marban E, Martin JF, Molkentin JD, Murry CE, Riley PR, Ruiz-Lozano P, Sadek HA, Sussman MA, Hill JA, Cardiomyocyte Regeneration: A Consensus Statement, *Circulation* 136(7) (2017) 680–686. [PubMed: 28684531]

Highlights

- The role of cardiac c-Kit(+) cells in cardiac tissue regeneration is controversial.
- RNA-seq was employed to understand how c-Kit(+) cells respond to MI and Sfrp2.
- Following MI, c-Kit(+) cells transiently express cardiomyocyte-specific genes.
- This transient response is sustained and amplified by Sfrp2.
- Sfrp2 functions via β -catenin inhibition.

Figure 1, In vivo c-Kit(+) cells express cardiomyocyte-specific developmental factors post-MI.

(A) Graphical representation of Gene Ontology analysis using Panther comparing the RNA-seq profiles of in vivo c-Kit(+) cells and cardiomyocytes.

(B) Heatmap of cardiomyocyte developmental genes. Mice received tamoxifen (0.5mg/mouse/day) for 14 days. Four days later, mice were subjected to myocardial infarction or a sham operation. Two days later mice received vehicle or 0.5ug Sfrp2, injected directly into the infarct border zone at two sites. C-Kit(+) cells were isolated 1 day prior to MI, 1 day after MI, and 12 days after MI. The 12 day post-MI c-Kit(+) cells came from both vehicle

and Sfrp2 treated mice. Cardiomyocytes were isolated from 3 sham animals and pooled. Total RNA was isolated and RNA-seq was performed. Full details of the RNA-seq and analysis of the data can be found in the Methods section. For the heatmap, gene expression values were converted to Z-scores for each gene. The numbered list of genes can be found in the Supplementary Table.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2, In vivo c-Kit(+) cells express cardiomyocyte-specific ion channels post-MI.

Mice received tamoxifen (0.5mg/mouse/day) for 14 days. Four days later, mice were subjected to myocardial infarction or a sham operation. Two days later mice received vehicle or 0.5ug Sfrp2, injected directly into the infarct border zone at two sites. C-Kit(+) cells were isolated 1 day prior to MI, 1 day after MI, and 12 days after MI. The 12 day post-MI c-Kit(+) cells came from both vehicle and Sfrp2 treated mice. Cardiomyocytes were isolated from 3 sham animals and pooled. Total RNA was isolated and RNA-seq was performed. Full

details of the RNA-seq and analysis of the data can be found in the Methods section. For the heatmap, gene expression values were converted to Z-scores for each gene.

(A) Heatmap of cardiomyocyte ion channels. The numbered list of genes can be found in the Supplementary Table.

(B) Examples of cardiomyocyte-specific genes regulated by Sfrp2. The top panel shows examples where Sfrp2 enhances the expression of cardiomyocyte-specific genes. The bottom panel includes examples that highlight Sfrp2 sustaining cardiomyocyte-specific gene expression when normally it is lost in c-Kit(+) cells. The average Log2(Fold) change is shown. Veh-vehicle, CM- cardiomyocytes.

(C), c-KitCreERT2/mTeG mice received tamoxifen (0.5mg/mouse/day) for 14 days. Four days later, mice were subjected to myocardial infarction. Two days later mice received 0.5ug Sfrp2, injected directly into the infarct border zone at two sites. 15 days following Sfrp2 injection heart tissue was analyzed. Expression of eGFP (c-Kit(+)) cells and cells derived thereof) and the cardiac marker cardiac troponin-T was analyzed by confocal microscopy. Immunostaining was quantified and the number of eGFP+ cardiac troponin-T+ cells expressed as a percentage of the total number of cells per field. Multiple sections were analyzed and expressed as a mean $\pm$ SEM. N=3 (Vehicle), N=2 (Sfrp2). *P<0.05.

Figure 3, In vivo c-Kit(+) cells promote β-catenin inhibition following myocardial infarction.

(A & B) Mice received tamoxifen (0.5mg/mouse/day) for 14 days. Four days later, mice were subjected to myocardial infarction or a sham operation. Two days later mice received vehicle or 0.5ug Sfrp2, injected directly into the infarct border zone at two sites. C-Kit(+) cells were isolated 1 day prior to MI, 1 day after MI, and 12 days after MI. The 12 day post-MI c-Kit(+) cells came from both vehicle and Sfrp2 treated mice. Cardiomyocytes were isolated from 3 sham animals and pooled. Total RNA was isolated and RNA-seq was

performed. Full details of the RNA-seq and analysis of the data can be found in the Methods section. For the heatmap, gene expression values were converted to Z-scores for each gene.

(A) Heatmap of Wnt pathway genes in in vivo c-Kit(+) cells pre- and post-MI.

(B) Graphical representation of the effects on canonical and non-canonical Wnt signaling in in vivo c-Kit(+) cells following MI.

(C) Cultured c-Kit(+) cells were incubated with vehicle or 100nM Sfrp2 for five minutes.

Protein extracts were probed for phospho- β -catenin. Actin was used as a loading control.

(D), Cultured c-Kit(+) cells were cultured in differentiation media for 21 days. Where appropriate the β -catenin inhibitor XAV-939 was added to the media as indicated in the methods section. RNA was analyzed by qPCR for early-stage, middle-stage, and late-stage cardiac gene expression. For comparison cardiac gene expression at baseline (control) is shown. N=3. *** P 0.001, * P 0.05 with comparisons made between XAV-939 and vehicle treated groups.

Figure 4, In vivo c-Kit(+) cell chemokine, angiogenesis marker and proliferation marker expression.

Mice received tamoxifen (0.5mg/mouse/day) for 14 days. Four days later, mice were subjected to myocardial infarction or a sham operation. Two days later mice received vehicle or 0.5ug Sfrp2, injected directly into the infarct border zone at two sites. C-Kit(+) cells were isolated 1 day prior to MI, 1 day after MI, and 12 days after MI. The 12 day post-MI c-Kit(+) cells came from both vehicle and Sfrp2 treated mice. Cardiomyocytes were isolated from 3 sham animals and pooled. Total RNA was isolated and RNA-seq was performed. Full

details of the RNA-seq and analysis of the data can be found in the Methods section. For the heatmap, gene expression values were converted to Z-scores for each gene.

(A) Heatmap of genes implicated in cardiomyocyte proliferation.

(B) Heatmap of chemokines and chemokine receptors significantly expressed in in vivo c-Kit(+) cells.

(C) Heatmap of angiogenic factors expressed in in vivo c-Kit(+) cells. The numbered list of genes can be found in the Supplementary Table.

Figure 5, C-Kit(+) cells show significant de-differentiation in culture.

Cardiac gene expression was compared between freshly isolated c-Kit(+) cells and three separate batches of c-Kit(+) cells cultured to passage 1 by qPCR. N=3.

Figure 6, Differentiation of cultured c-Kit(+) cells does not recapitulate in vivo gene expression levels.

(A) qPCR of three separate cultured c-Kit(+) isolations following differentiation. N=3.

***P<0.001, **P<0.01, *P<0.05 comparing differentiated to non-differentiated c-Kit(+) cells. Despite the increase in gene expression it remains significantly lower than the freshly isolated cKit(+) cells.

(B) and (C), Hypoxia differentiation of c-Kit(+) cells. Two protocols were used for differentiation of c-Kit(+) isolation 3, a standard method and a method that involves

spheroid formation. Cells were then differentiated under normal or hypoxic conditions for 14 days. qPCR was performed for the indicated genes. N=3. *P<0.05 comparing hypoxia with normal conditions.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript