Gata4-Dependent Differentiation of c-Kit+ Derived Endothelial Cells
Underlies Artefactual Cardiomyocyte Regeneration in the Heart

Running Title: Maliken et al.; Gata4-Dependent Endothelial Differentiation

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Abstract

Background—While c-Kit+ adult progenitor cells were initially reported to produce new cardiomyocytes in the heart, recent genetic evidence suggests that such events are exceedingly rare. However, to determine if these rare events represent true de novo cardiomyocyte formation we deleted the necessary cardiogenic transcription factors Gata4 and Gata6 from c-Kit-expressing cardiac progenitor cells (CPCs).

Methods—Kit allele-dependent lineage tracing and fusion analysis was performed in mice following simultaneous Gata4 and Gata6 cell-type specific deletion to examine rates of putative de novo cardiomyocyte formation from c-Kit+ cells. Bone marrow transplantation experiments were used to define the contribution of Kit allele-derived hematopoietic cells versus Kit lineage-dependent cells endogenous to the heart in contributing to apparent de novo lineage-traced cardiomyocytes. A Tie2CreERT2 transgene was also used to examine the global impact of Gata4 deletion on the mature cardiac endothelial cell network, which was further evaluated with select angiogenesis assays.

Results—Deletion of Gata4 in Kit lineage-derived endothelial cells or in total endothelial cells using the Tie2CreERT2 transgene, but not from bone marrow cells, resulted in profound endothelial cell expansion, defective endothelial cell differentiation, leukocyte infiltration into the heart and a dramatic increase in Kit allele-dependent lineage-traced cardiomyocytes. However, this increase in labeled cardiomyocytes was an artefact of greater leukocyte-cardiomyocyte cellular fusion due to defective endothelial cell differentiation in the absence of Gata4.

Conclusions—Past identification of presumed de novo cardiomyocyte formation in the heart from c-Kit+ cells using Kit allele lineage tracing appears to be an artefact of labeled leukocyte fusion with cardiomyocytes. Deletion of Gata4 from c-Kit+ endothelial progenitor cells or adult endothelial cells negatively impacted angiogenesis and capillary network integrity.

Key Words: angiogenesis; cardiac regeneration; myocardial infraction; transgenic mice
Clinical Perspective

What is new?

- This is the first study to genetically delete the necessary cardiogenic transcription factors Gata4/6 from c-Kit+ CPCs, which remarkably resulted in greater apparent cardiomyocyte derivation from these c-Kit+ cells.
- Deletion of Gata4 from c-Kit-derived endothelial progenitors alters the integrity of the endothelial cell network in the heart, resulting in greater c-Kit+ derived leukocytes entering the heart and fusing with cardiomyocytes.
- We demonstrate a new role for Gata4 in endothelial differentiation, specifically showing for the first time that Gata4 is essential for vascular development via the c-Kit lineage.
- This study shows that leukocyte-to-cardiomyocyte fusion is the primary basis for past lineage tracing results incorrectly suggesting that c-Kit+ CPCs generated de novo cardiomyocytes in the heart.

What are the clinical implications?

- Our data demonstrate that c-Kit+ CPCs are much less likely to differentiate de novo into cardiomyocytes than previously reported, suggesting that such cells are not therapeutically meaningful as a source of new cardiomyocytes.
- Our study highlights a capillary-driven mechanism of increased fusion of bone marrow-derived cells (leukocytes) with existing cardiomyocytes, which could have significant clinical implications in its own right.
- Kit lineage and global endothelial cell deletion of Gata4 reveals apparent organ-specific regulation of microvascular differentiation, highlighting Gata4 as a potential target for angiogenic control in the human heart.
Introduction

Large-scale cardiomyocyte loss from a cardiac ischemic event initially elicits a dramatic inflammatory response, followed by fibroblast activation with scar formation and fibrosis, and then ventricular remodeling and eventually heart failure.1 To combat this profile of progressive cardiac deterioration after ischemic injury, cell-specific approaches have emerged with emphasis on altering the hematopoietic response,2 ameliorating fibrotic remodeling,3–6 increasing collateral circulation,7,8 and preserving or replacing cardiomyocytes.9,10 Earlier reports that endogenous cardiac stem cells exist and might be efficacious in mediating cardiac regeneration generated a great deal of excitement in the field.11,12 c-Kit+ cardiac progenitor cells (CPC), named for the presence of c-Kit tyrosine kinase receptor that marks hematopoietic stem cells13, have been the focus of numerous cardiac regenerative studies.14–16 Select clinical trials evaluating the administration of bone marrow cells after myocardial infarction (MI) have shown minimal efficacy.17–19 However, expanded cardiac c-Kit+ cells were reported to potentially impart greater functional benefit with scar reduction when administered to patients post MI injury.20,21

While injection of exogenously expanded CPCs may indeed positively impact the MI injured heart, several recent studies have definitively shown that the heart lacks an endogenous c-Kit+ CPC capable of producing new cardiomyocytes in vivo.22–24 For example, we determined that endothelial cells are the major fate of Kit lineage-traced cells in the heart and that only 1 in 17,000 cardiomyocytes might be produced de novo when an 80% fusion rate is taken into account.22 Sultana and colleagues confirmed these results, demonstrating that a large proportion of lineage-traced Kit allele-derived cells are endothelial, while Kit allele lineage-traced cardiomyocytes co-expressing cardiac troponin T in the adult mouse heart were exceptionally rare.23 Furthermore, a novel Cre/Dre dual recombinase mouse genetic system by Zhou and
colleagues that no longer relies on heterozygosity of the Kit allele, showed that c-Kit+ cells never produce de novo cardiomyocytes in the adult heart at baseline or with injury. However, the potential to genetically reprogram c-Kit-derived cells and other cardiac mesenchymal cells into cardiomyocytes remains attractive for future development.25,26

The goal of the current study was to determine the contribution of “true” versus “apparent” Kit allele lineage-derived cardiomyocytes by simultaneously deleting the cardiomyogenic transcription factors Gata4 and Gata6, which when deleted from mesodermal progenitors during early development results in acardia and absence of cardiomyocytes.27 We report here that deletion of Gata4 from c-Kit+ cells preferentially impacted a population of c-Kit expressing endothelial cells and their differentiation state, resulting in defective cardiac capillary formation and immune cell infiltration, which secondarily resulted in a dramatically enhanced rate of fusion of c-Kit-derived immune cells with cardiomyocytes.

Methods
An expanded Methods section is available in the online-only Data Supplement.

The data, analytic methods and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure by contacting the corresponding author. The RNA sequencing data were deposited with the GEO database group and given accession number GSE109661.

Animals
All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children’s Hospital Medical Center. Kit-Cre recombinase knock-in mice (Kitcre) and Kit mice with a knock-in of the tamoxifen-inducible MerCreMer
cDNA (Kit<sup>McM</sup>), as well as Rosa26 lineage-dependent reporter mice, were each previously described. LoxP-targeted Gata4 (Gata4<sup>f<sub>i</sub></sup>) and Gata6 (Gata6<sup>f<sub>i</sub></sup>) mice were described previously. The endothelial specific Tie2<sup>CreERT2</sup> transgenic mouse model was described elsewhere (Zheng, Y and colleagues, submitted). Detailed description of tamoxifen dosing procedures and euthanasia procedures are provided in the online-only Data Supplement.

**Protein Analysis**

Immunofluorescent stains were performed on cryoembedded tissues and western blots were performed on cells isolated by FACS and whole cardiac ventricle tissue as described previously. Descriptions of antibodies used are provided in the online supplement.

**VEGF-A Overexpression**

An adeno-associated virus-9 (AAV9) vector expressing VEGF-A was prepared and tittered (See Supplemental Methods). Adult C57Bl/6 mice were administered AAV9-VEGF-A by tail vein injection at concentration of 1x10<sup>12</sup> viral particles in 200 μl of sterile PBS.

**Bone Marrow Transplantation**

Bone marrow transplantations (BMTs) were performed using 6-8 week-old donor mice into 8-10 week-old recipients. Bone marrow cells (BMCs) were isolated by aseptically flushing femurs and tibiae with Hanks Balanced Salt Solution (HBSS). BMT recipient mice first received lethal irradiation (12 Gy in a divided dose 4 hours apart) and then immediately received a tail vein injection of BMCs at ~50 million per mice in 200 μl of HBSS.

**Retinal Angiogenesis Assay**

To observe the superficial vascular plexus to assess endothelial developmental defects, retinal flat mounts were prepared as described earlier. Briefly, p0 mice were given intraperitoneal injections of 200 μg tamoxifen (Sigma T5648; dissolved in 5 μl ethanol and 95 μl peanut oil) for
gene inactivation and Tie2 (Tek gene) lineage tracing. Neonatal mice were sacrificed and eyes were harvested at p8, fixed, and retinas were dissected and mounted for qualitative comparison by eGFP fluorescence.

**Flow Cytometry and Cell Sorting**

Cells were prepared for flow cytometry studies on cardiac interstitial cells and bone marrow cells as described in detail before. BMCs and cardiac interstitial cells were stained with surface markers using APC conjugated antibodies and optimum gating strategy was determined using singly-labeled controls.

**RNA Sequencing Analysis**

Endothelial cells were collected as described above for RNA harvest and production of cDNA libraries for sequencing. Libraries were sequenced on the Illumina HiSeq2500 following the manufacturer's protocol. Selected genes were verified by qPCR.

**Statistics**

See Supplemental Methods for an in-depth description of statistics.

**Results**

**Kit lineage-specific Gata4-, Gata6-, and Gata4/6-deleted mice.**

To examine the hypothesis that deletion of Gata4 and Gata6 from c-Kit+ CPCs in the heart would block all de novo cardiomyocyte formation, we crossed KitMCM mice with mice containing loxP site (fl) targeted alleles for Gata4 and Gata6 (Figure 1A). Again, loss of both transcription factors should lead to the inability of CPCs to activate the cardiac transcriptional program required for cardiogenesis. Additionally, mice were bred to harbor either the Rosa26-loxP-stop-loxP-eGFP (R26eGFP) allele or the Rosa26-membrane-Tomato-loxP-Stop-loxP-membrane-
eGFP allele \(^{35}\) (\(R26^{\text{mT/mG}}\)) to trace total Kit allele-derived cells in the heart and to examine fusion-derived cardiomyocytes, respectively (Figure 1A). Fusion is scored when both the m-eGFP and m-Tomato signals are observed in the same cardiomyocyte, while a \textit{de novo} transdifferentiation event from a c-Kit\(^{+}\) CPC would give cardiomyocytes that are only m-eGFP positive. After weaning, mice were put on tamoxifen food for continuous labeling of Kit lineage-derived cells (Figure 1B). Recombination of these loxP-targeted alleles was assessed by PCR on control and tamoxifen treated mice, showing specific recombination (Figure 1C). Mice were sacrificed after 1-, 2-, and 4-months of tamoxifen treatment for histological and flow cytometry analysis of heart and other tissues. Unexpectedly, total eGFP\(^{+}\) cardiomyocytes were increased in mice with deletion of Gata4/6 or Gata4, but not Gata6 alone with a \(~2\)-fold, 4-fold, and 10-fold increases at 1, 2, and 4 months, respectively (Figure 1D-F). Dual reporter labeling in Gata4/6- and Gata4-deleted mice revealed significantly more fusion-derived cardiomyocytes (91-93\%) versus 80-83\% in Kit\(^{\text{MCM R26^{mT/mG}}}\) controls (Figure 1G-H). Interestingly, skeletal muscle showed the appearance of eGFP\(^{+}\) fused myofibers in Gata4/6 and Gata4-deleted Kit-lineage mice for the first time (Figure S1), while other known immune cell-based fusion prone tissues such as the liver \(^{36,37}\) did not (data not shown). These results collectively show that deletion of Gata4 from Kit allele-derived cells dramatically enhances the relative rate of "apparent" cardiomyocyte \textit{de novo} formation, which is potentially due to augmented fusion of Kit allele-derived immune cells with tissue parenchymal cells as previously shown.\(^{38,39}\)

Immunofluorescence staining of Gata4 was frequently positive in total aggregates of bone marrow cells, which is also a known site of c-Kit expression as shown by lineage tracing from the Kit allele to produce eGFP expression (Figure 2A). Interestingly, CD133\(^{+}\) endothelial progenitor cells\(^{40}\) from the bone marrow were dramatically enhanced in Kit allele-lineage
Gata4/6-deleted mice compared to controls, possibly suggesting a functional role for Gata4 in endothelial cells (Figure 2B). Indeed, previous lineage tracing with the Kit\textsuperscript{Cre} mouse model demonstrated that cardiac Kit-derived cell populations in the heart were compromised of 77% endothelial cells (CD31\textsuperscript{+}) and 18% leukocytes (CD45\textsuperscript{+}).\textsuperscript{22} Western blotting of these populations for Gata4 protein demonstrated detectable expression in CD31\textsuperscript{+} endothelial cells but not in CD45\textsuperscript{+} leukocytes, total CD31\textsuperscript{+} CD45\textsuperscript{−} cardiac interstitial cells, or when Gata4 was deleted from CD31\textsuperscript{+} cells using a Tie2\textsuperscript{Cre\textsubscript{ERT2}} transgene (Figure 2C). Flow cytometry analysis for CD45\textsuperscript{+} cell content in the hearts of Kit-lineage Gata4/6- and Gata4-deleted mice after 4 months of tamoxifen showed a ~50% increase in leukocytes (Figure 2D and 2F) and a ~60% increase in CD31\textsuperscript{+} endothelial cells compared to controls (Figure 2E and 2G). Taken together, these results show a clear alteration of Kit allele-derived populations in the heart in the absence of Gata4, such as a dramatic increase in endothelial and CD45 cell content.

**Bone marrow transplant to identify cardiovascular impact of Kit-lineage Gata4/6 loss**

We hypothesized that the increase in relative Kit allele lineage-traced eGFP\textsuperscript{+} cardiomyocytes with Gata4/6 deletion was due to greater leukocyte activity and/or altered endothelial cell function. To test this hypothesis, we performed a bone marrow transplant that would isolate the impact of Gata4/6 effects from the hematopoietic compartment. Here, the donating bone marrow was from Kit\textsuperscript{MCM} Gata4/6\textsuperscript{fl/fl} R26\textsuperscript{eGFP} mice (or controls not deleted for Gata4/6) that had already undergone full recombination with 6 weeks of prior tamoxifen treatment, which would produce eGFP\textsuperscript{+} circulating immune cells and even endothelial progenitors in recipients (Figure 3A). The recipient mice were in the R26\textsuperscript{null} genetic background so endogenous cells could be parsed from eGFP\textsuperscript{+} cells. Recipient mice were irradiated then given the bone marrow transplant and allowed to reconstitute for 8 weeks (Figure 3A). Full bone marrow niche reconstitution was confirmed
with Kit-lineage R26<sup>GFP</sup> recombination (Figure 3B) and a general lack of native mTomato<sup>+</sup> bone marrow (Figure 3C). Remarkably, eGFP<sup>+</sup> cardiomyocytes were observed in the hearts of both control and Gata4/6-deleted bone marrow recipients at the same level, which was due to fusion because 100% of the cells were both eGFP<sup>+</sup> and mTomato<sup>+</sup> (Figure 3D and E). Furthermore, flow cytometry analysis of cardiac interstitial cells revealed no difference in the total CD31<sup>+</sup> cells (Figure 3F), demonstrating that Gata4/6-deleted bone marrow alone did not recapitulate the phenotype of endothelial expansion or the large relative increase in eGFP<sup>+</sup> cardiomyocytes observed in the standard Kit-lineage traced Gata4/6-deleted mice at baseline.

Next, we performed a reverse bone marrow transplant strategy. Recipient Kit<sup>MCM</sup> mice with or without Gata4/6 deletion were lethally irradiated at 8 weeks of age, where-after they received control bone marrow from global R26<sup>+</sup> donors (Figure 3G). After 2 months of tamoxifen treatment mice were sacrificed and cardiac sections were analyzed for mTomato<sup>+</sup> traced cardiomyocytes within Gata4/6 Kit lineage-deleted hearts. The data show an ~4-fold increase in bone marrow dependent mTomato<sup>+</sup> cardiomyocytes with endogenous Kit allele-dependent Gata4/6 deletion compared to Gata4/6 wild-type controls, suggesting that it is the deletion of Gata4/6 within the existing heart cells that leads to greater apparent cardiomyocyte fusion/labeling (Figure 3H). Importantly, Kit-lineage endothelial cell recombination in bone marrow recipient mice was not significantly changed from the ~8-10% of total cardiac endothelium observed in both control and Gata4/6-deleted hearts, with and without bone marrow transplant (Figure 3I). Together, these experiments demonstrate that a Kit lineage-derived cell source endogenous to the heart is the mechanistic basis for the increased ability of wild-type bone marrow progenitor derived cells (leukocytes) to fuse with cardiomyocytes in vivo.
Endothelial cells are the predominant Kit lineage-derived cell-type present in the heart, suggesting the hypothesis that loss of Gata4/6 within endogenous cardiac endothelial cells might be mechanistically responsible for greater immune cell infiltration and fusion with cardiomyocytes. To test this hypothesis, we first attempted to alter the differentiation and permeability characteristics of endogenous cardiac endothelial cells using vascular endothelial growth factor-A (VEGF-A). Previous work has shown that VEGF-A overexpression leads to weakening of endothelial junctions and increased permeability as these cells partially dedifferentiate or proliferate. Here, we used a VEGF-A adeno-associated virus-9 (AAV9) to overexpress this factor in the heart of Kit lineage-traced mice by tail vein injection (Figure S2A). VEGF-A overexpression in the heart (Figure S2B) produced a significant increase in eGFP⁺ cardiomyocytes and leukocyte infiltration (Figure S2C-2E), reminiscent of the increases observed in Gata4/6-deleted mice.

Global endothelial deletion of Gata4

Despite the modest proportion of Kit allele-derived endothelium (~10%), the loss of Gata4 in these cells of the heart appeared to cause a global cardiac phenotype of endothelial cell expansion, leukocyte infiltration, and heterotypic cardiomyocyte fusion, suggesting that Kit allele-derived endothelial cells were critical in adult vascular maintenance. To further implicate endothelial cells as the causative cell-type we utilized a tamoxifen-inducible Tie2 Cre-expressing transgenic mouse (Tie2^{CreERT2}) to achieve a global and adult endothelial cell-specific deletion of Gata4. Tie2 lineage-traced mice were used as controls versus lineage-traced Gata4-deleted mice with a continuous tamoxifen regimen, again beginning after weaning (Figure 4A and 4B). Remarkably, after 2 months of tamoxifen, Gata4-deleted endothelial cells showed a ~2-fold increase in Tie2-eGFP⁺ in the heart (Figure 4C and 4D) with a significant shift from CD31 high-
expressing to CD31 low-expressing cells by flow cytometry (Figure 4G and 4H), suggesting a less differentiated state. Remarkably, adult endothelial-specific deletion of Gata4 also resulted in the greater presence of CD45+ cells in the heart (Figure 4E and 4F). Additionally, EdU staining of cardiac sections showed a 3-fold increase in endothelial cell-specific proliferation (Figure 4I). Skeletal muscle showed similar results with a shift toward CD31 low-expressing cells and increased EdU staining (Figure S3A and S3B). Together, these data suggest that loss of Gata4 in adult endothelial cells alters their differentiated state and produces greater proliferation and leukocyte diapedesis.

To probe even further into the hypothesis that deletion of Gata4 from Kit allele lineage-derived endothelial cells is the primary reason for enhanced bone marrow-derived leukocyte fusion with endogenous cardiomyocytes, we used the KitMCM allele together with the Tie2CreERT2 transgene in Gata4fl/fl R26eGFP mice given continuous tamoxifen (Figure 5A and 5B). Unfortunately, the Tie2CreERT2 transgene is not active in bone marrow hence the KitMCM allele is needed to label fusigenic immune cells, even though both Cres would inactivate Gata4 within endothelial cells of the heart (Figure 5C). The data again showed significantly greater rates of eGFP+ fused cardiomyocytes with KitMCM allele-mediated deletion of Gata4, and that enhanced deletion of Gata4 from essentially all endogenous cardiac endothelial cells with the Tie2CreERT2 transgene doubled the number of fused cardiomyocytes (Figure 5D and 5E). To show this even more conclusively, bone marrow transplantation was performed between a R26enT donor mouse and a Tie2CreERT2 Gata4fl/fl R26eGFP recipient mouse. In this manner, the recipient mice only have Tie2CreERT2 causing Gata4 deletion in adult endothelial cells without deletion of Gata4 from bone marrow derived immune cells or endothelial progenitors. The data again show an enhanced rate
of mTomato+ fused cardiomyocytes, indicating that the effect is exclusively due to defects within endogenous endothelial cells of the heart when Gata4 is deleted (Figure 5G and 5H).

**Ex vivo and developmental analysis of Gata4-deleted endothelial cells**

Isolation and culturing of endothelial cells from these genetically modified mouse hearts grossly showed that Gata4-replete cells form a typical cobblestone monolayer whereas Gata4-deleted cells are less adherent and show a more torturous morphology (Figure 6A). Analysis of cell culture supernatant showed an increase in secretion of angiopoietin-2 and decreased VEGF-A from Gata4-deleted endothelial cells, which suggests a vascular-regressive state (Figure 6B and 6C).43,44 Endothelial cells mixed with Matrigel from these mice were subcutaneously injected into wild-type recipient mice to evaluate vascular tube formation with or without Gata4 deletion (Figure 6D). After 2 weeks, Matrigel plugs were collected and analyzed by confocal microscopy, which showed a limited ability of Gata4-deleted endothelial cells to form differentiated vascular networks compared to Gata4-replete cells using z-stacked confocal microscopy (Figure 6E).

Previously, embryonic endothelial-specific deletion of Gata4 with a constitutive Tie2-Cre transgene demonstrated late embryonic or perinatal lethality due to defective epithelial-to-mesenchymal (EMT) transition and improper heart valve formation.45 Here we also observed embryonic lethality with a constitutive KitCre allele to delete Gata4. More specifically, constitutive KitCre Gata40/0 mice were found to be hemorrhagic at E11.5 with noticeable areas of blood pooling and defective vasculogenesis, leading to embryonic lethality (Figure 6F). Importantly, constitutive Cdh5Cre (endothelial cell specific cadherin 5 gene promoter) mediated deletion of Gata4 also produced embryonic lethality at the same time (data not shown). Taken together these results suggest that Kit allele-derived cells critically underlie early hemangioblast
progenitor cell activity during development and that Gata4 is essential in these cells for proper vascular development.

Development of the superficial vascular plexus in the neonatal mouse eye begins at birth with radial outgrowth of endothelial vessels reaching the retinal edge by p8. To test the role of Gata4 in retinal vascularization, Tie2-dependent lineage-traced mice were injected with tamoxifen at p0 and harvested at p8 for cardiac phenotyping and retinal angiogenesis (Figure 6G). Tie2CreERT2 Gata4fl/fl mice were under-developed compared to Gata4fl/fl controls with significantly reduced heart size (Figure 6H) and improper patterning of both micro- and macro-vasculature in the retina compared to Tie2CreERT2 R26GFP controls (Figure 6I). Together, these findings suggest that while required embryonically for proper organogenesis, Gata4 is required for continued maturation and maintenance of the adult micro-vasculature.

**Gene expression alterations in adult Gata4-deficient endothelial cells**

To elucidate the molecular basis for Gata4-dependent regulation of endothelial cell differentiation, global RNA-sequencing (RNA-Seq) was performed from cardiac endothelial cells isolated from Tie2CreERT2 R26GFP Gata4fl/fl mice versus Tie2CreERT2 R26GFP controls (Figure 7). Cells double-positive for endogenous Tie2 lineage tracing (eGFP+) and fluorescently-labeled for CD31 were obtained by FACS at 8 weeks of age after 4 weeks of tamoxifen induction. Bioinformatics analysis of the data showed a gene expression signature in which many of the top categories are related to previously known partners and targets of Gata4, as well as genes related to the function and maintenance of the vasculature (Figure 7). While signature endothelial-defining genes (Cdh5, Edn1, Kdr, Pecam1, S1pr1, Vwf) were largely unchanged, well-established regulatory partners of Gata4 including Fog2 (Zfp12), Hand2, Tbx20, Dkk3, Mlc2a (Mly7), Sox9, and Klf15 were significantly downregulated. A variety of putatively...
Gata4-regulated genes were also identified using the Harmonizome database, many of which have evidence for vascular regulation. For example, *Figf* (*Vegfd*), a predicted target of Gata4, was downregulated in this study and has a known role in endothelial cell regulation. A host of genes related in angiogenesis and extracellular matrix formation were impacted with *Gata4* deletion including downregulation of *Itgb4*, *Mmp2*, *Timp2*, *Vcam1*, and *Vcan* and upregulation of *Apold1* and *Has3* (Figure 7). Additionally, a number of genes related to the cell cycle in endothelial cells were upregulated such as *E2f7*, *Egr1/2/3*, *Esm1*, and *Plk3*. Verification of mRNA expression differences was performed for most of these gene changes by RT-qPCR, showing consistency with the patterns found by RNA-Seq analysis (Figure S4). Together, these gene expression data suggest a global impact of *Gata4* deletion on the endothelial cell differentiation gene program, helping to validate the observed differences in proliferation, permeability and maturation.

**Discussion**

Here, we used a genetic approach to determine if the very low level of presumed new cardiomyocyte generation in the heart from the *Kit* lineage really occurs, especially because it was recently reported that adult cardiac *Kit*-dependent CPCs never truly transdifferentiate into cardiomyocytes. Indeed, deletion of *Gata4* and *Gata6* simultaneously from *Kit* lineage cells did not reduce the apparent rate of cardiomyocyte labeling in the heart by genetic lineage tracing, and in fact caused as much as a 10-fold increase. Because *Gata4/6* are lacking it is even more unlikely that new cardiomyocytes could be created from a progenitor cell source, even embryonic stem cells. This consideration along with the very dramatic increase in cell fusion we observed in the current study suggests that most past results reporting a minor contribution of
cardiomyocytes from the Kit lineage is likely due to cellular fusion between lineage-traced immune cells and host endogenous cardiomyocytes, or from ectopic induction of the Kit$^{MCM}$ allele in cardiomyocytes. Indeed, our past results, which were subtracted for cellular fusion effects, still showed apparent de novo cardiomyocyte production at approximately 1 in 17,000, but as suggested in the recent literature, this likely reflects ectopic activation of the Kit$^{Cre}$ allele within rare cardiomyocytes. Hence, it could be argued that Kit lineage-traced cells from any source, whether bone marrow or endogenous to the heart, essentially lack all cardiomyocyte transdifferentiation ability.

In attempting to understand how Gata4 deletion from the Kit lineage produced an apparent 10-fold increase in eGFP$^+$ cardiomyocytes we uncovered an essential role for this transcription factor in regulating the endothelial cell gene program. While endothelial cell-specific deletion of Gata4 was shown to cause developmental lethality due to alterations in endothelial cell activity in the cardiac cushions and newly developing heart valves, a role for Gata4 in adult cardiac vascular maintenance had not been previously explored. Gata4 was also recently identified as a master regulator of liver sinusoidal endothelial cells, determining their organ-specificity and requirement in liver development. Additionally, in this transition from sinusoidal to capillary formed vascular networks in the liver, the authors observed a distinct shift in CD31$^{low}$ cells to CD31$^{high}$ cells. Conversely, we observed a shift towards more permeable vasculature and greater CD31$^{low}$ cells (less differentiated) in the heart and skeletal muscle with deletion of Gata4, but this effect was not observed in the liver or lung of our study using the Tie2$^{CreERT2}$ (data not shown).

We observed that Gata6 deletion in both Kit allele and endothelial lineages did not overtly alter the angiogenic and vascular processes as shown with Gata4 deletion. Indeed, we
observed that constitutive Kit<sup>Cre</sup>-expressing Gata6<sup>fl/fl</sup> deleted mice were not lethal and when lineage-traced through development to 5 months of age, did not show increases in eGFP<sup>+</sup> cardiomyocytes. Endothelial-specific deletion of Gata6 with the inducible Tie2-specific Cre mice also did not demonstrate an increase in CD31<sup>+</sup> cells or increased vascular permeability, suggesting that only Gata4 plays a specific role in regulating expression of genes involved in endothelial cell biology. We previously observed a similar paradigm at the level of the cardiomyocyte in which angiogenic genes in this cell-type were preferentially regulated by Gata4 over Gata6.<sup>39</sup>

Currently, we speculate that Gata4 is important in endothelial cell maturation/differentiation largely due to the observed ex vivo defective tube formation and the appearance of CD31<sup>low</sup> endothelial cells in Tie2<sup>CroERT2</sup> transgene-mediated Gata4-deleted mice. The overall increase in CD31<sup>+</sup> and Tie2 lineage-traced eGFP<sup>+</sup> cells could be a compensatory response of the cardiac vasculature due to the inability of endothelial cells to maintain secure junctions when Gata4-regulated gene expression is compromised. Thus, new endothelial cells are formed as an attempt to bolster the vasculature, yet leukocytes are still able to infiltrate more easily giving greater fusion events with endogenous cardiomyocytes. Alternatively, poorly differentiated endothelial cells resulting from deletion of Gata4 generate a tissue injury-like signal in these cells that recruits leukocytes and makes them more active. Overall, our results suggest that Gata4 could be used as a therapeutic leverage point in affecting endothelial cell biology for selective therapeutic approaches in humans.
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Author contributions: B.D.M., conducted experiments and acquired the data along with assistance from O.K., J.K., H.K., X.F., J.G.B., and V.P. Y.Z., provided Tie2-Cre transgenic mice used in this study. J.D.M., and B.D.M., designed the experiments, analyzed the data, and wrote the manuscript. J.D.M. directed and supervised the study.

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Disclosures

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References

1. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ. Heart Disease and Stroke Statistics--2012 Update: A Report From the American Heart Association. Circulation. 2011;125:e2-e220. doi:10.1161/CIR.0b013e31823ac046.
2. Toldo S, Abbate A. The NLRP3 inflammasome in acute myocardial infarction. Nat Rev Cardiol. 2017. doi:10.1038/nrcardio.2017.161.
3. Talman V, Ruskoaho H. Cardiac fibrosis in myocardial infarction—from repair and remodeling to regeneration. Cell Tissue Res. 2016;365:563-581. doi:10.1007/s00441-016-2431-9.
4. Kanisicak O, Khalil H, Ivey MJ, Karch J, Maliken BD, Correll RN, Brody MJ, J Lin SC, Aronow BJ, Tallquist MD, Molkentin JD. Genetic lineage tracing defines myofibroblast origin and function in the injured heart. Nat Commun. 7:12260 doi:10.1038/ncomms12260.
5. Khalil H, Kanisicak O, Prasad V, Correll RN, Fu X, Schips T, Vagnozzi RJ, Liu R, Huynh T, Lee SJ, Karch J, Molkentin JD. Fibroblast-specific TGF-β-Smad2/3 signaling underlies cardiac fibrosis. *J Clin Investig*. 2017;127:3770-3783. doi:10.1172/JCI94753.

6. Saxena A, Bujak M, Frunza O, Dobaczewski M, Gonzalez-Quesada C, Lu B, Gerard C, Frangogiannis NG. CXCR3-independent actions of the CXC chemokine CXCL10 in the infarcted myocardium and in isolated cardiac fibroblasts are mediated through proteoglycans. *Cardiovasc Res*. 2014;103:217-227. doi:10.1093/cvr/cvu138.

7. Berry C, Balachandran KP, L’Allier PL, Lespérance J, Bonan R, Oldroyd KG. Importance of collateral circulation in coronary heart disease. *Eur Heart J*. 2007;28:278-291. doi:10.1093/eurheartj/ehl446.

8. Zimaro M, D’andreamatteo M, Waksman R, Epstein SE, De Caterina R. The dynamics of the coronary collateral circulation. *Nat Rev Cardiol*. 2014;11:191-197. doi:10.1038/nrcardio.2013.207.

9. Senyo SE, Lee RT, Kühn B. Cardiac regeneration based on mechanisms of cardiomyocyte proliferation and differentiation. *Stem Cell Res*. 2014;13:532-541. doi:10.1016/j.scr.2014.09.003.

10. Kawaguchi N, Nakanishi T. Cardiomyocyte Regeneration. *Cells*. 2013;2:67-82. doi:10.3390/cells2010067.

11. Lu L, Li F, Lu J. Identification of functional tissue-resident cardiac stem/progenitor cells in adult mouse. *Cell Biol Int*. 2012;19:15-22. doi:10.1042/CBR20120001.

12. Bollini S, Smart N, Riley PR. Resident cardiac progenitor cells: At the heart of regeneration. *J Mol Cell Cardiol*. 2011;50:296-303. doi:10.1016/j.jmcc.2010.07.006.

13. Fazel S, Cimini M, Chen L, Li S, Angoulvant D, Fedak P, Verma S, Weisel RD, Keating A, Li RK. Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *J Clin Investig*. 2006;116:1865-1877. doi:10.1172/JCI27019.

14. Hosoda T. C-kit-positive cardiac stem cells and myocardial regeneration. *Am J Cardiovasc Dis*. 2012;2:58-67.

15. Tallini YN, Greene KS, Craven M, Spealman A, BREITBACH M, Smith J, Fisher PJ, Steffey M, Hesse M, Doran RM, Woods A, Singh B, Yen A, Fleischmann BK, Kotlikoff MI. C-Kit Expression Identifies Cardiovascular Precursors in the Neonatal Heart. *Proc Natl Acad Sci U S A*. 2009;106:1808-1813. doi:0808920106 [pii]r10.1073/pnas.0808920106.

16. 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*. 2003;114:763-776. doi:10.1016/S0092-8674(03)00687-1.

17. Nowbar AN, Mielewczik M, Karavassilis M, Dehbi HM, Shun-Shin MJ, Jones S, Howard JP, Cole GD, Francis DP. Discrepancies in autologous bone marrow stem cell trials and enhancement of ejection fraction (DAMASCENE): weighted regression and meta-analysis. *BMJ*. 2014;348:g2688. doi:10.1136/bmj.g2688.

18. Jeevanantham V, Butler M, Saad A, Abdel-Latif A, Zuba-Surma EK, Dawn B. Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: A systematic review and meta-analysis. *Circulation*. 2012;126:551-568. doi:10.1161/CIRCULATIONAHA.111.086074.

19. Kudo M, Wang Y, Wani MA, Xu M, Ayub A, Ashraf M. Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. *J Mol Cell Cardiol*. 2012;50:444-453. doi:10.1016/j.jmc.2012.02.016.
20. Bolli R, Chugh AR, D’Amario D, Loughran JH, Stoddard MF, Ikram S, Beach GM, Wagner SG, Leri A, Hosoda T, Sanada F, Elmore JB, Goichberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet. 2011;378:1847-1857. doi:10.1016/S0140-6736(11)61590-0.

21. Chugh AR, Beach GM, Loughran JH, Newton N, Elmore JB, Kajstura J, Pappas P, Tatooles A, Stoddard MF, Lima JA, Slaughter MS, Anversa P, Bolli R. Administration of cardiac stem cells in patients with ischemic cardiomyopathy: The SCIPIO trial: Surgical aspects and interim analysis of myocardial function and viability by magnetic resonance. Circulation. 2012;126(11 SUPPL.1). doi:10.1161/CIRCULATIONAHA.112.092627.

22. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marbán E, Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. Nature. 2014;509:337-341. doi:10.1038/nature13309.

23. 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. 2015;6:8701. doi:10.1038/ncomms9701.

24. 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. 2017;23:1488-1498. doi:10.1038/nm.4437.

25. Chen Z, Zhu W, Bender I, Gong W, Kwak YJ, Yellamilli A, Hodges TJ, Nemoto N, Zhang J, Garry DJ, van Berlo JH. Pathologic Stimulus Determines Lineage Commitment of Cardiac C-kit+ Cells. Circulation. 2017;136:2359-2372. doi:10.1161/CIRCULATIONAHA.117.030137.

26. Kannappan R, Matsuda A, Ferreira-Martins J, Zhang E, Palano G, Czarno A, Cabral-Da-Silva MC, Bastos-Carvalho A, Sanada F, Ide N, Rota M, Blasco MA, Serrano M, Anversa P, Leri A. p53 Modulates the Fate of Cardiac Progenitor Cells Ex Vivo and in the Diabetic Heart In Vivo. EBioMedicine. 2017;16:224-237. doi:10.1016/j.ebiom.2017.01.028.

27. Zhao R, Watt AJ, Battle MA, Li J, Bondow BJ, Duncan SA. Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. Dev Biol. 2008;317:614-619. doi:10.1016/j.ydbio.2008.03.013.

28. Oka T, Maillet M, Watt AJ, Schwartz AJ, Aronow BJ, Duncan SA, Molkentin JD. Cardiac-specific deletion of gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. Circ Res. 2006;98:837-845. doi:10.1161/01.RES.0000215985.18538.c4.

29. Van Berlo JH, Aronow BJ, Molkentin JD. Parsing the roles of the transcription factors GATA-4 and GATA-6 in the adult cardiac hypertrophic response. PLoS One. 2013;8. doi:10.1371/journal.pone.0084591.

30. Karch J, Schips TG, Maliken BD, Brody MJ, Sargent MA, Kanisicak O, Molkentin JD. Autophagic cell death is dependent on lysosomal membrane permeability through Bax and Bak. eLife. 2017;6. doi:10.7554/eLife.30543.

31. Cui YZ, Hisha H, Yang GX, Fan TX, Jin T, Li Q, Lian Z, Ikehara S. Optimal protocol for total body irradiation for allogeneic bone marrow transplantation in mice. Bone Marrow Transplant. 2002;30:843-849. doi:10.1038/sj.bmt.1703766.

32. Accornero F, Schips TG, Petrosino JM, Gu SQ, Kanisicak O, van Berlo JH, Molkentin
33. Maitra M, Schlutereman MK, Nichols HA, Richardson JA, Lo CW, Srivastava D, Garg V. Interaction of Gata4 and Gata6 with Tbx5 is critical for normal cardiac development. Dev Biol. 2009;326:368-377. doi:10.1016/j.ydbio.2008.11.004.

34. Xin M, Davis CA, Molkentin JD, Lien CL, Duncan SA, Richardson JA, Olson EN. A threshold of GATA4 and GATA6 expression is required for cardiovascular development. Proc Natl Acad Sci U S A. 2006;103:11189-11194. doi:10.1073/pnas.0604604103.

35. Muzumdar MD, Tasic B, Miyamichi K, Li N, Luo L. A global double-fluorescent cre reporter mouse. Genesis. 2007;45:593-605. doi:10.1002/dvg.20335.

36. Vassilopoulos G, Wang P-R, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature. 2003;422:901-904. doi:10.1038/nature01539.

37. Camargo FD, Finegold M, Goodell MA. Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners. J Clin Investig. 2004;113:1266-1270. doi:10.1172/JCI200421301.

38. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature. 2003;425:968-973. doi:10.1038/nature02069.

39. Nygren JM, Jovinge S, Breitbach M, Säwén P, Röll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SE. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med. 2004;10:494-501. doi:10.1038/nm1040.

40. Yin AH, Mitaglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood. 1997;90:5002-5012.

41. Gavard J, Gutkind JS. VEGF Controls endothelial-cell permeability promoting β-arrestin-dependent Endocytosis VE-cadherin. Nat Cell Biol. 2006;8:1223-1234. doi:10.1038/ncb1486.

42. Bates DO. Vascular endothelial growth factors and vascular permeability. Cardiovasc Res. 2010;87:262-271. doi:10.1093/cvr/cvq105.

43. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 1997;277:55-60. doi:10.1126/science.277.5322.55.

44. Hakanpaa L, Sipila T, Leppanen VM, Gautam P, Nurmi H, Jacquemet G, Eklund L, Ivaska J, Alitalo K, Saharinen P. Endothelial destabilization by angiopoietin-2 via integrin β1 activation. Nat Commun. 2015;6. doi:10.1038/ncomms9662.

45. Rivera-Feliciano J, Lee KH, Kong SW, Rajagopal S, Ma Q, Springer Z, Izumo S, Tabin CJ, Pu WT. Development of heart valves requires Gata4 expression in endothelial-derived cells. Development. 2006;133:3607-3618. doi:10.1242/dev.025199.

46. Stahl A, Connor KM, Sapieha P, Chen J, Dennison RJ, Krah NM, Seaward MR, Willett KL, Aderman CM, Guerin KI, Hua J, Löfqvist C, Hellström A, Smith LE. The mouse retina as an angiogenesis model. Investig Ophthalmol Vis Sci. 2010;51:2813-2826. doi:10.1167/iovs.10-5176.

47. Ho M, Yang E, Matcuk G, Deng D, Sampas N, Tsalenko A, Tabibiazar R, Zhang Y, Chen...
M, Talbi S, Ho YD, Wang J, Tsao PS, Ben-Dor A, Yakhini Z, Bruhn L, Quertermous T. Identification of endothelial cell genes by combined database mining and microarray analysis. Physiol Genomics. 2003;13:249-262. doi:10.1152/physiolgenomics.00186.2002.

48. Manuylov NL, Tevosian SG. Cardiac expression of Tnt1 requires the GATA4-FOG2 transcription complex. Sci World J. 2009;9:575-587. doi:10.1100/tsw.2009.75.

49. Zhou B, Ma Q, Kong SW, Hu Y, Campbell PH, McGowan FX, Ackerman KG, Wu B, Zhou B, Tevosian SG, Pu WT. Fog2 is critical for cardiac function and maintenance of coronary vasculature in the adult mouse heart. J Clin Invest. 2009;119:1462-1476. doi:10.1172/JCI38723.

50. Dai YS, Cserjesi P, Markham BE, Molkentin JD. The transcription factors GATA4 and dHAND physically interact to synergistically activate cardiac gene expression through a p300-dependent mechanism. J Biol Chem. 2002;277:24390-24398. doi:10.1074/jbc.M202490200.

51. Stennard FA, Costa MW, Elliott DA, Rankin S, Haast SJ, Lai D, McDonald LP, Niederreither K, Dolle P, Bruneau BG, Zorn AM, Harvey RP. Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. Dev Biol. 2003;262:206-224. doi:10.1016/S0012-1606(03)00385-3.

52. Pei Y, Yao Q, Yuan S, Xie B, Liu Y, Ye C, Zhuo H. GATA4 promotes hepatoblastoma cell proliferation by altering expression of miR125b and DKK3. Oncotarget. 2016;7:77890-77901. doi:10.18632/oncotarget.12839.

53. Holtzinger A, Rosenfeld GE, Evans T. Gata4 directs development of cardiac-inducing endoderm from ES cells. Dev Biol. 2010;337:63-73. doi:10.1016/j.ydbio.2009.10.003.

54. Manuylov NL, Fujiwara Y, Adameyko II, Poulat F, Tevosian SG. The regulation of Sox9 gene expression by the GATA4/FOG2 transcriptional complex in dominant XX sex reversal mouse models. Dev Biol. 2007;307:356 367. doi:10.1016/j.ydbio.2007.04.040.

55. Fisch S, Gray S, Heymans S, Haldar SM, Wang B, Pfister O, Cui L, Kumar A, Lin Z, Sen-Banerjee S, Das H, Petersen CA, Mende U, Burleigh BA, Zhu Y, Pinto YM, Liao R, Jain MK. Kruppel-like factor 15 is a regulator of cardiomyocyte hypertrophy. Proc Natl Acad Sci U S A. 2007;104:7074-7079. doi:10.1073/pnas.0701981104.

56. Rouillard AD, Gundersen GW, Fernandez NF, Wang Z, Monteiro CD, McDermott MG, Ma'ayan A. The harmonizome: a collection of processed datasets gathered to serve and mine knowledge about genes and proteins. Database. 2016. doi:10.1093/database/baw100.

57. Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene. 2000;19:5598-605. doi:10.1038/sj.onc.1203855.

58. 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 Kit-expressing cardiomyocytes. Cell Res. 2016;26:119-130. doi:10.1038/cr.2015.143.

59. Géraud C, Koch PS, Zierow J, Klapproth K, Busch K, Olsavszky V, Leibing T, Demory A, Ulbrich F, Dietz M, Singh S, Sticht C, Breitkopf-Heinlein K, Richter K, Karppinen SM, Pihlajaniemi T, Arnold B, Rodewald HR, Augustin HG, Schledzewski K, Goerd S. GATA4-dependent organ-specific endothelial differentiation controls liver development and embryonic hematopoiesis. J Clin Invest. 2017;127:1099-1114. doi:10.1172/JCI90086.
Figure Legends

Figure 1. Kit allele-lineage deletion of Gata4/6 and Gata4 alone leads to increased lineage-traced cardiomyocytes due to fusion. A, Genetic models with the indicated alleles in mice that were crossed and (B) experimental design of the protocol used in this figure. C, PCR showing recombination (recomb) of the Gata4 locus with tamoxifen treatment to activate the MerCreMer protein (N=2) or untreated that did not show recombination (N=1) in Kit<sup>MCM</sup> Gata4/6<sup>fl/fl</sup> R26<sup>εGFP</sup> mice. The analysis used Kit lineage-tracing in which eGFP<sup>+</sup> bone marrow cells were first collected. D, Immunofluorescent images of cardiac histological sections from Kit allele lineage-traced mice with and without Gata4/6 deletion. Arrows indicate eGFP<sup>+</sup> cardiomyocytes. E, Time-course quantification of the percentage of eGFP<sup>+</sup> cardiomyocytes within the entire heart in response to Gata4/6 deletion. Error bars are SEM, N=4 per group (10 sections per heart), *P<0.05 vs Kit<sup>MCM</sup> R26<sup>εGFP</sup> at each time point. F, Comparative quantitation of the percentage of eGFP<sup>+</sup> cardiomyocytes within the entire mouse heart in response to Gata4, Gata6, or double Gata4/6 deletion compared to Kit lineage tracing controls. Error bars are SEM, N=4 per group (10 sections per heart), *P<0.05 vs Kit<sup>MCM</sup> R26<sup>εGFP</sup> for each genotype. G, Immunofluorescent cardiac histological section showing dual reporter evidence of a fusion-derived mTomato<sup>+</sup> mGFP<sup>+</sup> cardiomyocyte. H, Percentage of fusion-derived Kit allele-lineage traced cardiomyocytes (mTomato<sup>+</sup> mGFP<sup>+</sup>) versus presumed de novo cardiomyocytes (only mGFP<sup>+</sup>). Error bars are SEM, N=4 per group (10 sections per heart), *P<0.05 vs Kit<sup>MCM</sup> R26<sup>mT/mG</sup> for each genotype.

Figure 2. Cardiac leukocytes and endothelial cells are increased in Kit lineage Gata4-deleted mice. A, Immunofluorescent staining of Gata4 (red) and DAPI (blue) in bone marrow
cells from $Kit^{MCM} R26^{eGFP}$ mice (2 months tamoxifen as control) showing some overlap of Kit lineage-traced eGFP+ cells with Gata4. B, Flow cytometry plots showing CD133+ endothelial progenitor cells in Kit allele-derived bone marrow cells. C, Western blot showing relative Gata4 levels in c-Kit lineages such as CD45+ leukocytes, CD31+ endothelial cells and CD31− CD45− interstitial cells, or CD31+ cells that were deleted for Gata4 with the Tie2$^{CreERT2}$ transgene. The positive control was neonatal rat ventricular myocytes (NRVM) loaded at a reduced amount to show the Gata4 band accurately. n.s. = non-specific. While Gapdh was used as a processing and loading control this protein is more highly expressed in cardiomyocytes and select other cell-types. D, Immunofluorescent staining of CD45+ (white) and DAPI (blue) in cardiac histological sections. E, Immunofluorescent staining of CD31+ (red) and DAPI (blue) in cardiac histological sections. F, Relative quantification of cardiac CD45+ cells based on flow cytometry of the interstitial cell fraction from dissociated hearts. Error bars are SEM, N=3 per group, *P<0.05 vs $Kit^{MCM} R26^{eGFP}$ for each genotype. G, Relative quantification of cardiac CD31+ cells based on flow cytometry from the interstitial cell fraction in dissociated hearts. Error bars are SEM, N=3 per group, *P<0.05 vs $Kit^{MCM} R26^{eGFP}$ for each genotype.

Figure 3. Increased cardiomyocyte cell fusion events occur with Kit lineage loss of Gata4/6 within heart cells during bone marrow transplant. A, Experimental design for bone marrow transplant experiments examining the impact of Gata4/6 loss in Kit allele-derived bone marrow on cardiac phenotyping. B, Representative FACS plot showing recombination of eGFP+ bone marrow from a Kit allele lineage-traced donor mouse in a bone marrow transplant recipient. C, Representative FACS plot showing effective irradiation and loss of native mTomato+ bone marrow in a recipient mouse. D, Representative eGFP+ mTomato+ fusion-derived cardiomyocyte
from a cardiac histological section following bone marrow transplant. 

E, Percentage of Kit allele-dependent bone marrow-derived eGFP+ cardiomyocytes from the entire heart of the indicated mice. Error bars are SEM, N=6 per group (10 sections per heart), *P<0.05 vs recipient of Kit\(^{MCM}\) R26\(^{eGFP}\) bone marrow. 

F, Quantification of cardiac CD31\(^{+}\) cells based on flow cytometry from the interstitial fraction in bone marrow transplant recipient hearts from the indicated mice. Error bars are SEM, N=3 per group, P=n.s. 

G, Experimental design for bone marrow transplantation to examine the impact of endogenous cardiac loss of Gata4/6 in Kit allele-dependent lineages on the apparent rate of new cardiomyocytes and endothelial cells. 

H, Percentage of wild-type bone marrow-derived mTomato\(^{+}\) cardiomyocytes within the entire heart following bone marrow transplant in the indicated mice. Error bars are SEM, N=6 per group (10 sections per heart), *P<0.05 vs Kit\(^{MCM}\) R26\(^{eGFP}\) recipient. 

I, Quantification from cardiac sections of endogenous endothelial cell number from the Kit lineage with 2 months of tamoxifen induction, with and without bone marrow transplantation, in the indicated mice. Error bars are SEM, N=3 per group, P=n.s. for all pairwise comparisons.

Figure 4. Global Tie2-lineage deletion of Gata4 expands the immature endothelial cell population and increases diapedesis. 

A, Genetic models with the indicated alleles or transgenes in mice that were crossed and, (B) experimental design of the protocol used in this figure. The Tie2 promoter from the Tek gene was used to drive the Cre-ERT2 cDNA. 

C, Representative cardiac histological sections showing Tie2 lineage-traced eGFP\(^{+}\) cells (green) from the indicated genotypes of mice. 

D, Quantification of Tie2 lineage-traced eGFP\(^{+}\) endothelial cells by flow cytometry from the interstitial fraction in hearts of the indicated lines of mice. Error bars are SEM, N=3 per group, *P<0.05 vs Tie2\(^{CreERT2}\) R26\(^{eGFP}\). 

E, Representative
cardiac histological sections showing CD45+ cells (white) and DAPI (blue) from the indicated genotypes of mice. F, Quantification of CD45+ cells by flow cytometry of the interstitial fraction in the hearts of the indicated lines of mice. Error bars are SEM, N=3 per group, *P<0.05 vs Tie2CreERT2 R26eGFP. G, Flow cytometry plots showing cardiac CD31+ cells with gating to display low- and high-expressing cells from the indicated genotypes of mice. H, Quantification of CD31low, CD31high, and total CD31+ cells from flow cytometry of the interstitial fraction of hearts of the indicated genotypes of mice. Error bars are SEM, N=3 per group, P=0.0257 for CD31 low comparison, P=0.1799 for CD31 high comparison, and P<0.0001 for total CD31+ comparison vs Tie2CreERT2 R26eGFP. I, Quantification of EdU+ nuclei in Tie2 lineage-traced eGFP+ cardiac endothelial cells from cardiac histological sections from the indicated genotypes of mice. Error bars are SEM, N=3 per group (6 EdU stained sections per heart), *P<0.05 vs Tie2CreERT2 R26eGFP.

Figure 5. Global Tie2-lineage deletion of Gata4 augments appearance of Kit lineage- and bone marrow-labeled cardiomyocytes. A, Genetic models with the indicated alleles or transgenes in mice that were crossed and (B) experimental design of the protocol used in this figure. The Tie2 promoter from the Tek gene was used to drive the Cre-ERT2 cDNA. C, Whole mount imaging of tibias from mice of the indicated genotypes of mice, highlighting labeled bone marrow cells (green) when the Kit lineage-tracing allele was used. D, Representative image showing accumulation of Kit allele-derived eGFP+ cardiomyocytes (arrowheads) in cardiac histological sections from the indicated mouse genotype 2 months after Gata4 deletion. E, Quantification of eGFP+ cardiomyocytes from KitMCM lineage tracing and KitMCM Tie2CreERT2 dual lineage tracing, with or without Gata4 deletion. Error bars are SEM, N=4 per group, *P<0.001
vs Kit<sup>±MCM</sup> R26<sup>eGFP</sup> controls by 2-way ANOVA, #P<0.001 vs dual Kit<sup>±MCM</sup> Tie2<sup>CreERT2</sup> R26<sup>eGFP</sup> by 2-way ANOVA. F, Experimental design for bone marrow transplant of wild-type mTomato<sup>+</sup> bone marrow into Tie2<sup>CreERT2</sup> recipient mice with or without Gata4 deletion. G, Representative cardiac histological image of an mTomato<sup>+</sup> cardiomyocyte (arrowhead) derived from bone marrow following bone marrow transplant from the mice shown in panel (F). H, Quantification of mTomato<sup>+</sup> cardiomyocytes from cardiac histological sections of recipient mice of the indicated genotypes following bone marrow transplant. Error bars are SEM, N=6 per group (10 sections per heart), *P<0.05 vs Tie2<sup>CreERT2</sup> R26<sup>eGFP</sup> recipient mice.

Figure 6. Gata4-deleted endothelial cells demonstrate poor differentiation and impaired tube formation. A, eGFP-based imaging of cardiac endothelial cells grown in culture after isolation by fluorescence-activated cell sorting of Tie2-eGFP<sup>+</sup> CD31<sup>+</sup> cells from the indicated genotypes of mice. N=3 per group (12 wells of 10,000 cells per heart). B, Angiopoeitin 2 and (C) VEGF-A protein quantification from the collected supernatants of cardiac endothelial cell cultures as described in (A). Error bars are SEM, N=3 per group (3 replicates each), *P<0.05 vs Tie2<sup>CreERT2</sup> R26<sup>eGFP</sup> at each time point. D, Experimental design for ex vivo Matrigel angiogenesis assay from the indicated genotypes of mice over the 2 week time period. E, Representative images of tube formation from harvested Matrigel plugs (images presented as flattened Z-stack) from the indicated genotypes of mice. The bottom 2 panels are enlarged versions of the smaller windows shown in the top 2 panels. N=4 per group. F, Pictures of whole-mount embryos from constitutive Kit<sup>Cre</sup> expressing mice demonstrating hemorrhagic phenotype with Gata4-deletion, but not in control embryos at E11.5 of development. N=4. The arrowheads show areas of hemorrhage. G, Experimental design for the retinal angiogenesis assay from neonatal times of p0
through p8. **H,** Representative whole-mount images of p8 hearts from control and Tie2\textsuperscript{CreERT2} \textit{Gata4}-deleted hearts. **I,** Representative whole-mount images of neonatal retinas with eGFP imaging based on Tie2\textsuperscript{CreERT2} lineage tracing. The genotypes of mice used are shown, and the bottom 2 panels are higher magnification images of the upper 2 panels. N=6 per group.

**Figure 7.** \textit{Gata4}-deleted adult cardiac endothelial cells have dysregulation of cell cycle and angiogenesis-related genes.** RNAseq analysis of selected genes from sorted Tie2 lineage-traced eGFP\textsuperscript{+} CD31\textsuperscript{+} cells from hearts of adult mice with or without \textit{Gata4} deletion mediated by the Tie2\textsuperscript{CreERT2} transgene (4 weeks of tamoxifen). Data show fold-change in mRNA between \textit{Gata4}-deleted and wild-type endothelial cells, and are organized to highlight genes relevant to previously-defined endothelial gene sets or those known to interact with the transcription factor \textit{Gata4}. N=2 per group (2 combined digested hearts each). See \textit{Supplemental Methods} for description of bioinformatics and by DESeq2 analysis to show genes differentially expressed. *P<0.05 versus WT endothelial cells. Eight of the mRNAs shown were not statistically significant by DESeq2 but are included because they were significantly changed by RT-PCR (see Figure S4).
A. Schematic representation of the genetic modifications.

B. Timeline of tamoxifen treatment and gene expression:
- Birth
- 1m: Tam food
- 2m, 3m, 5m: Time
- 0: No Tam
- WT
- Recomb
- Gata4 locus

C. Western blot analysis of Gata4 expression:
- No Tam
- With Tam

D. Microscopy images of Kit^{MCM} R26^{eGFP} and Kit^{MCM} R26^{eGFP} Gata4/6^{fl/fl}.

E. Graph showing the percentage of eGFP+ myocytes over time:
- Kit^{MCM} R26^{eGFP}
- Kit^{MCM} R26^{eGFP} Gata4/6^{fl/fl}
- Time of tamoxifen: 1m, 2m, 4m

F. Graph showing the percentage of eGFP+ myocytes over time for Gata4/6^{fl/fl}:
- 2m Tam

G. Microscopy images of Kit^{MCM} R26^{mT/mG}:
- mGFP
- mTomato
- Merge

H. Graph showing the percentage of fusion:
- Gata4/6^{fl/fl}
- Gata4^{fl/fl}
A

B

C

D

E

F

G

H
Gata4-Dependent Differentiation of c-Kit⁺ Derived Endothelial Cells Underlies Artefactual Cardiomyocyte Regeneration in the Heart
Bryan D. Maliken, Onur Kanisicak, Jason Karch, Hadi Khalil, Xing Fu, Justin G. Boyer, Vikram Prasad, Yi Zheng and Jeffery D. Molkentin

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SUPPLEMENTAL MATERIAL

Gata4-dependent differentiation of c-Kit+ derived endothelial cells underlies artefactual cardiomyocyte regeneration in the heart

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Supplemental Methods

Experimental animals:

All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children’s Hospital Medical Center. Targeted Kit-Cre (Kit\textsuperscript{Cre}) and Kit-MerCreMer (Kit\textsuperscript{MCM}) mice were previously described\textsuperscript{1}. LoxP-targeted Gata4 (Gata4\textsuperscript{fl}) and Gata6 (Gata6\textsuperscript{fl}) mice were described previously\textsuperscript{2,3}. Rosa26 reporter mice (R26\textsuperscript{R26GFP}) were purchased from the Jackson Laboratories (Stock# 012429)\textsuperscript{4}. Fusion studies were conducted utilizing the dual reporter B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (Jackson laboratories Stock # 007676). The endothelial specific CreERT2 transgenic mouse model is described elsewhere (Zheng, Y et al, submitted). Briefly, a Tie2-CreERT2 transgene comprising a version of Tie2 promoter with a β-globulin intron sequence as a fusion with CreERT2 cDNA and a polyadenylation signal (pA) signal and Tie2 enhancer sequence was used for C57Bl/6 pronuclear micro-injection to generate transgenic mouse lines. C57Bl/6 mice and Rosa26-TD-Tomato mice (B6; 129S6-Gt (ROSA) 26Sortm9(CAG-tdTomato)Hze/J stock number: 007905) were from Jackson Lab. No procedures causing pain or distress to the mice were used in this study. All animal protocols were written prospectively with defined primary and secondary endpoints specified.

Randomization and Blinding

Mice were included in the study based on genotype and no mice of the correct genotype were excluded. Experimental animals were matched for species, sex, age, strain, diet, housing, and source. No randomization was required for genetic experiments in mice because as all interventions were identical and all mice were of the same genotype and same environment and age within a study. However, we used a 50/50 mix of male and female mice in all studies. The primary author managed the mouse colony, determined genotype, and provided tamoxifen treatment. For experiments, tissues were harvested and randomly assigned blinded identifiers.
by a co-author and distributed among other authors for blinded data collection. Experimental groups were then unblinded and analyzed accordingly.

**Tamoxifen treatment:**

To activate the inducible MerCreMer or the CreERT2 protein, experimental mice were administered tamoxifen citrate containing mouse food (TD.130859 Envigo) used at a dosage of 400 mg/kg, or via 5 consecutive daily intraperitoneal injections with pharmaceutical grade tamoxifen (100 mg/kg body weight) dissolved in 95% peanut oil/5% ethanol. Control animals were treated with control food without the tamoxifen. Mice were either sacrificed by CO₂ asphyxiation or by excision of the heart under deep isoflurane sedation.

**5-ethynyl-2′-deoxyuridine (EdU) incorporation for in vivo proliferation analysis:**

Experimental mice were administered a total of 8, weekly I.P injections of EdU (Thermo Fisher Scientific - Molecular Bioproducts A10044) at a dosage of 50 mg/kg body weight. Harvested hearts were processed as described above and EdU was detected using the Click-iT Plus Alexa Fluor 647 Picolyl Azide Toolkit (Thermo Fisher Scientific Cat # C10643).

**Histology and immunostaining:**

Isolated hearts and skeletal muscles were fixed for 3.5 hours at 4°C in pre-chilled freshly diluted paraformaldehyde (PFA) (Electron Microscopy Sciences Cat# 15714) in Phosphate Buffered Saline (PBS). Tissues were then rinsed with PBS 3 times 15 minutes each and cryoprotected by immersion in PBS containing 30% sucrose overnight before embedding in OCT (Tissue-Tek Cat # 4583). Tissues were sectioned using Leica CM1850 Cryostat and 10 μm cryosections were collected. Sections were incubated in blocking solution containing 5% goat serum, 2% bovine serum albumin, 0.1% Triton X-100 in PBS for 45 minutes. Primary antibodies against CD31 (BD Biosciences 553370), CD45 (BD Biosciences 553076), and Gata4 (Abcam ab84593) were all diluted at 1:200 dilution in blocking solution directly applied on slides and sections were
incubated overnight at 4°C in a humidity chamber. Sections were washed three times for 5 min each in PBS and incubated with Alexa Fluor 568-conjugated secondary antibody (Life Technologies A11077 or A11011) diluted at 1:500 in blocking solution for 90 minutes at room temperature. Finally, sections were washed three times for 5 min each in PBS, counter-stained with 4',6-diamidino-2-phenylindole (DAPI) at a 0.1 µg/ml concentration in PBS for 5 minutes at room temperature and cover slips applied using VECTASHIELD aqueous mounting medium (Vector Laboratories H-1000). Direct eGFP fluorescence produced from the recombined state of the Rosa26-containing loxP-dependent reporter allele was detected. Images were acquired using an inverted Nikon A1R confocal microscope system using the NIS Elements AR 4.13 software.

eGFP+ and mTomato+ cardiomyocytes were quantified using a Nikon Ti-E Inverted microscope with fluorescence capabilities and a 20X objective. Cardiomyocytes were identified by characteristic morphology and were quantified on multiple sections from each heart at various sectional planes, typically 10 or more. eGFP+ cardiomyocytes were presented as a percentage of the total myocardium, calculated by dividing eGFP+ or mTomato+ cardiomyocytes by the total cardiomyocytes in a section which were identified either by Wheat Germ Agglutinin staining or endogenous membrane Tomato signal (such as dual reporter mice in Figure 1G-H or bone marrow transplant experiment in Figure 3A-F).

**Bone marrow transplant:**
To determine the altered infiltration, fusion, and potential of cardiomyocyte differentiation of bone marrow derived cells (BMCs), we performed BM transplantations (BMT) from 6-8 weeks old donor mice (various experimental mice were used as described in each experiment) into recipients. Donor BMCs were isolated by aseptically flushing femurs and tibiae with Hanks Balanced Salt Solution (HBSS) (Fisher Scientific SH30588.01) using a 25 gauge needle attached to a syringe containing 10 ml of ice cold HBSS supplemented with 2% bovine growth serum (Fisher Scientific SH3054103). BMT recipient mice first received lethal irradiation (12 Gy
in a divided dose 4 hours apart) and then immediately received a tail vein injection of BMCs at ~50 million per mice under isoflurane anesthesia to effect. No anesthesia was used prior to irradiation and no pain medications were administered following the procedure as it is nonpain generating procedure. Mice were housed in the barrier facility associated with the CCHMC irradiator suite for 10 days following the procedure for maximum survival (100% survival in all BMT experiments was observed). Tissues were isolated and processed after 8 weeks of recovery and processed as described above.

**Preparation of Retinal Flat Mounts:**

To observe the superficial vascular plexus to assess endothelial developmental defects, retinal flat mounts were prepared as described earlier. Briefly, p0 mice were given intraperitoneal injections of 200 μg tamoxifen (Sigma T5648; dissolved in 5 μl ethanol and 95 μl peanut oil) for gene inactivation and Tie2 lineage tracing. Mice were euthanatized and eyes were carefully harvested at age p8 and lightly fixed in 4% PFA for 15 minutes at 4°C and the cornea, lens, uvea, and sclera were removed under dissection microscope. The retinas were then radially cut and flat mounted on slides and cover slips placed with aqueous mounting media. Flat mounts were imaged and eGFP fluorescence was captured using a Nikon Ti-E Inverted microscope with fluorescence capabilities.

**Whole mount imaging:**

Imaging of whole mount femurs for endothelial and bone marrow eGFP signal detection was achieved using a M165FC Leica stereomicroscope with fluorescence capability. Timed matings were established by setting sexually mature mice overnight and detection of post-coitum plug was referred to as embryonic day (ED) 0. Experimental mice were isolated at ED11.5 and fixed in 4% PFA for 3 hours at 4°C and images were captured with a M165FC Leica stereomicroscope.
Tail vein injection of AAV-Vegfa:

The cDNA for VEGF-A (Accession #BC061468) was cloned into the pAAV-MCS Expression Vector (Cell Biolabs Inc., # VPK-410) with the In-Fusion HD Cloning Plus Kit (Clontech, # 638910). The insertion of the VEGF-A cDNA was confirmed by sequencing and the plasmid was amplified overnight in DH5α cells (ThermoFisher, # 18265017), purified using the PureYield Plasmid Maxiprep System (Promega, #A2392) and sent to VigeneBioscience for packaging and large scale purification. Adult C57BL/6 mice were administered AAV9-VEGF-A by tail vein injection at concentration of 1x10^{12} viral particles in 200 µl of sterile PBS under inhaled isoflurane anesthesia to effect.

Cell isolation:

FACS analysis was performed on cardiac interstitial cells and bone marrow cells as described in detail before^{1,6,7}. We isolated bone marrow cells by flushing femurs and tibiae with 2% BGS/HBSS as described above. Cardiac interstitial cells were isolated by digesting ventricles with collagenase type 2 (Worthington LS004177) as described before^{6}. Isolated cells were spun at 10 g for 5 minutes to remove large debris and the supernatant containing the cell suspension was then spun at 400 g for 10 minutes at 4 °C and pellets were re-suspended in 2% BGS/HBSS. Isolated cells were kept on ice during the rest of the process for flow cytometry, FACS isolation or RNA extraction.

Flow cytometry analysis and FACS isolation:

Flow cytometry analysis was performed using a BD FACSCanto II running FACSDiva software (BD Biosciences). Analysis was performed using FlowJo vX (FlowJo). BMCs and cardiac interstitial cells were stained with surface markers using APC conjugated antibodies against surface markers such as CD45 (BD Biosciences 559864); CD31 (eBioscience17-0311-82); CD133 (Biolegend 141207). To determine optimum gating strategy, laser voltages and viability analyses of isolated cells, separate flow cytometry experiments with single-labeled controls from
wild-type mice along with viability dyes such as 7-Aminoactinomycin D (7-AAD) (Life TechnologiesA1310) or Calcein Blue, AM (Thermo Fisher Scientific Cat. # C1429) were used. For fluorescence-activated cell sorting (FACS), BD FACS Aria Instrument was utilized and endothelial lineage cells that were eGFP⁺, CD31⁺, and 7AAD⁻ (live) cells were collected in to pre-chilled 2% BGS/HBSS. Sorted cells were then either put into 96-well plates for culture, mixed in cooled Matrigel for injections, or processed for RNA isolation.

**Primary endothelial cell culture and enzyme-linked immunosorbent assays (ELISAs)**

Primary endothelial cells (Tie2 lineage-traced as eGFP⁺, CD31⁺, 7AAD⁻) were isolated from the cardiac interstitium as described above and sorted into 96-well plates at a concentration of 10,000 cells/well. Cells were cultured in Endothelial Cell Basal Medium-2 (Lonza CC-3156) supplemented with 10% bovine growth serum (Fisher Scientific, SH3054103) and in the presence of penicillin-streptomycin (ThermoFisher Scientific, 30–002 CI). Cell morphology was captured using Nikon Ti-E Inverted microscope with fluorescence capabilities. Cell culture supernatant was collected at various time points and analyzed for secreted factors VEGF-A and angiopoietin-2 by ELISA (R&D Systems, Quantikine ELISA kits MMV00 and MANG20, respectively) following manufacturer protocol. Ninety-six-well ELISA plates were read for absorbance using a microplate reader (Biotek Synergy 2) at 570 nm and angiokine concentrations were calculated using a standard curve.

**Matrigel angiogenesis assay:**

Approximately 50,000 FACS isolated cells were embedded in 50 µl of cold, sterile Matrigel basement membrane (Corning #354234) and injected subcutaneously into surrogate recipient mice. Matrigel plugs were harvested with a fluorescent stereomicroscope after 2 weeks of growth and fixed in in 4% PFA for 60 minutes at 4°C. Z-stack images of vasculature networks were obtained using an inverted Nikon A1R confocal microscope system using the NIS Elements AR 4.13 software.
**Western blot:**

Western blot was performed as described previously. Briefly, isolated cells were homogenized in RIPA buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% NP-40) containing protease inhibitor cocktail (Sigma-Aldrich P8340), resolved on 10% SDS–polyacrylamide gel electrophoresis gels, and transferred to PVDF membranes. Membranes then immunoblotted for antibodies against Gata4 (Abcam ab84593) at 1:500, VEGF-A (Abcam ab46154) at 1:500 and Gapdh (Fitzgerald 10R-G109a) at a 1:20,000 dilutions, and then incubated with the appropriate alkaline phosphate-linked secondary antibody. Membranes were imaged using hemifluorescence (Amersham) on a Gel Doc™ XR+ System (BioRad).

**PCR analysis for DNA recombination:**

PCR was performed to detect recombed and non-recombined Gata4 loxP-targeted alleles using primers 5'-CCCAGTAAAGAAGTCAGCACAAGGAAC-3' 5'-AGACTATGGATCCCGGAGTGAACATT-3'. PCR conditions were 96 °C for 2 min, followed by 34 cycles of amplification (96 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) and a 5-min elongation step at 72 °C. PCR products were visualized on ethidium bromide-stained agarose gels using a UV molecular imager (Bio-Rad).

**RNA-Seq and bioinformatics analysis:**

Total RNA was amplified using the Ovation RNA-Seq System v2 (NuGEN) according to the manufacturer’s protocol. The libraries were prepared with the Nextera XT DNA Sample Preparation kit (Illumina Technologies Cat# FC-131-1024). The purified cDNA was captured on an Illumina flow cell for cluster generation. Libraries were sequenced on the Illumina HiSeq2500 following the manufacturer's protocol. Differential gene expression was determined using the DESeq2 method with the R/Bioconductor package. Analysis was performed using standard parameters with the independent filtering function enabled to filter genes with low mean
normalized counts. Differential expression was computed with the application of the Benjamini-Hochberg false discovery rate (FDR) method to adjust the p-values from multiple testing. Genes were considered differentially expressed if they passed a statistical cutoff of FDR p<0.05 and if they contained an absolute log\(_2\) fold change greater than or equal to 1 or less than or equal to -1. RT-PCR validation was performed on the majority of differentially expressed genes shown in Figure 7, as well as several selected genes with biological relevance that were determined to be nearly significant by DESeq2 analysis. The RNA sequencing data were deposited with the GEO database group and given accession number GSE109661.

**Reverse transcriptase polymerase chain reaction (RT-PCR):**

Cardiac endothelial cells were isolated as described above and RNA was isolated using Qiashredder homogenization and the RNAeasy kit (Qiagen Cat# 74104) according to the manufacturer's instructions. Total RNA was reverse transcribed using random oligo-dT primers and a Verso cDNA synthesis kit (Thermofisher AB1453) according to manufacturer protocol. Real-time PCR was performed using Sso Advanced SYBR Green (Biorad) and Rpl7 expression was used for normalization. The following primers were used for qPCR to identify the listed mRNA products: Cadherin 5 5’-CAGCTTTGGGAGCGTTC  and 5’-GGGGCAGCGATTTCTTTC, Platelet And Endothelial Cell Adhesion Molecule 1 5’-CTGCCAGTCCGAAAATGAAC and 5’-CTTCATCCACCGGGAAGC, Von Willebrand Factor 5’-CTTCTGTACGCCAGTTGCTTG and 5’-GCCATTGTAATTCCCACACAG, Apolipoprotein L Domain Containing 1 5’-CGCTTCCAAGGATTGCTGC and 5’-CTGAGTGACAACCCACGAT, Hyaluronan Synthase 3 5’-GTGGGCACTGACCTGTCTTG and 5’-CCACTGACGACGCCTCTG, Early Growth Response 3 5’-CTTGTCTTTCCCTTCTTG and 5’-CAAGTCCCGGCTATGTC, Matrix Metallopeptidase 2 5’-CAAGTTCCCGGCGATGTC and 5’-TTCTGGTCAAGGTCACCTGTC,
Vascular Cell Adhesion Molecule 1 5'-AGTTGGGGATTCCGTTTGTCT and 5'-CCCCTCATCCTATTACCACC, C-Fos Induced Growth Factor (Vascular Endothelial Growth Factor D) 5'-TTGAGCGATCATCCCGGTC and 5'-GCGTGAGTCATCATGGCAAG, Dickkopf WNT Signaling Pathway Inhibitor 3 5'-CTCGGGGGTATTTTGCTGTGT and 5'-TCCTCCTGGAGGTAGTGGAGA, T-Box 20 5'-AAACCCCTGGAACATTTTTG and 5'-CATCTCTTGCTGGGAGGTAT, Zinc Finger Protein, FOG Family Member 2 5'-ACCAGGAGAGCTAGAAGTGGTT and 5'-GGACCTGAGCCTTCGCTTT, Myosin Light Chain 7 5'-GGCACAACGTGGGCTCTTCTAA and 5'-TGCAGATGATCCCATCATCCGT, Endothelin 1 5'-TTTCCCCGAGGTAATCTCTGTG and 5'-GCCTACCTGTTTACCTGGGAC, Sphingosine-1-Phosphate Receptor 1 5'-ATGGGTGTCCTAGCATCCC and 5'-CGATGTTAACTCGGTGTGAG, BTG Anti-Proliferation Factor 2 5'-ATGAGCCAGGGGAAGAAGAC and 5'-GCCCTACTGAAAACCCTTGAGTC, Early Growth Response 1 5'-TCGGCTCTTTTCTCCTACTCA and 5'-CTCATGGTTTACCGCTTC, Early Growth Response 2 5'-GCAAGGCCGATGACAAAAATC and 5'-CCACTCCGTTCTCAGTCA, FOS Like 2, AP-1 Transcription Factor Subunit 5'-CCAGCAAGTCCCGGTTTAG and 5'-GTAGGGATGTGAGCGTGATGATA, Polo Like Kinase 3 5'-GCACATCCATCGCTCCAG and 5'-GCCACAGTCAAACCTTCCTCAA, Fibulin 1 5'-CGCCAAGAGAAAAACAGAC and 5'-CGGGTGAACCTCGAAGGTG, Fibromodulin 5'-AGCAGTCCACCTAAGCACC and 5'-CAGTGCATTCTTGGGAC, Integrin Subunit Beta 4 5'-GCAGACGAAGTTCCGAG and 5'-GCCACACTTCAGTTTAGA, L1 Cell Adhesion Molecule 5'-CAGTGCCTCAGGATGAAAG and 5'-TCTGGGCTTTAACCTGTAGG, Matrix Metalloproteinase 3 5'-ACATGGGAGACTTTTGCTCCTT and 5'-TTGGCTGTGAGCTAGGAGTCCCC, Protocadherin 7 5'-CAGCCATTTCGATGAGT and 5'-CTTGGTGGTCTTCGCTCC, Plexin Domain Containing 1 5'-CAACCATACTACGTGCCGCTCC, Plexin Domain Containing 2 5'-GCCGCAGCAGGGATGTTATA and 5'-TTCATTCAAAGGAAAACCGTGGT.
GCCGTGTAGATAAACTCGATGTC, Versican 5’-ACTAACCATGCACCTACATCAAG and 5’-
ACTTTTCCAGACAGAGGCTT, Heart And Neural Crest Derivatives Expressed 2 5’-
GCAGGACTCAGACATCAACA and 5’-AGGTAGGCGATGTATCTGGTG, Kruppel Like Factor
15 5’-GAGACCTTCTCGTCACCGAAA and 5’-GCTGGAGACATCGCTGTCAT, SRY-Box 9 5’-
GAGCCGGATCTGAAGAGGGA and 5’-GCTTGACGTGTGCTTGGTTC, and Ribosomal Protein
L7 5’-ACCGCCTGAGATTCCGATG and 5’-GAACCTTACGAACCTTTGGGC.

**Statistics:**

Data are shown as mean +/- SEM. One-way analysis of variance (ANOVA) was used to
determine statistical significance for experiments with more than two groups and one
independent variable, while two-way ANOVA was used in experiments with more than two
groups and multiple independent variables. Statistically significant pairwise differences were
then further determined by Tukey methods (Prism software). For experiments in which only 2
groups were compared, unpaired t-tests were used to determine statistical significance. P-
values <0.05 were considered statistically significant and all tests performed were two-sided. To
determine sample sizes for the proposed experiments the desired power was established as
80% and the criterion for significance (α) was 0.05. The probability of a type II error (β = 0.2) for
any null findings is reported in terms of the study power (1 – β = 0.8). In a pilot study, a mean
effect size for endogenous lineage tracing experiments was determined to be 4.7 eGFP+ cardiomyocytes per section. Hearts were analyzed N=3 per group with 10 cardiac cross
sections observed per mouse (mean = 1.3 vs. 6.0 and standard deviation = 0.26 vs. 1.33 in
KitMCM R26eGFP versus KitMCM Gata4/6fl/fl R26eGFP, respectively). From this data, a sample size of
N=4 was calculated for endogenous lineage tracing experiments. We did not have preliminary
data to inform our sample size calculation prior to the design of the bone marrow transplant
experiments, so we assumed a similar distribution as in the endogenous lineage tracing
experiments. To properly power the experiment we conservatively assumed that the mean
difference between groups would be reduced by 50% (effect size = 2.4 cardiomyocytes/section)
and following these assumptions, the necessary sample size was determined to be N=6 per group. However, some secondary exploratory experiments used 3 or fewer mice as we considered these analyses to be hypothesis-generating, or given the highly-reliable nature of the assays used (RNAseq, some Western blots, FACS plots, etc) the results were obvious and consistent with all the data in the greater manuscript. For statistical consideration of the RNAseq data see "RNA-Seq and bioinformatics analysis" section above.
Supplemental Figure S2

A. Timeline of experiment:
- AAV9-VEGFA Injection
- Harvest
- Time: 8w → 16w
- Tam food

B. Western blot analysis:
- Vehicle
- AAV9-VEGF-A
- VEGFA
- Gapdh
- Heart

C. Immunofluorescence imaging:
- Kit<sup>MCM</sup> R26-eGFP
- Vehicle
- AAV9-VEGF-A
- eGFP

D. Bar graph:
- eGFP+ myocytes (%)
- Vehicle vs. AAV9-VEGF-A
- Time of tamoxifen: 1m, 2m

E. Additional images:
- CD45
- 50 µm
Supplemental Figure S3

A

B
Supplemental Figure S4

**Endothelial signature genes**
- Cdh5
- Pecam1
- Vwf

**Endothelial matrix genes**
- Apold
- Cldn5
- Has3
- Esm1
- E2f7
- Erg3

**ECM / angiogenesis genes**
- Etv1
- Figf
- Mmp2
- Vcam1

**Gata4-related genes**
- Dkk3
- Myl7
- Tbx20
- Zfpm2
**Figures legends for Supplemental Figures**

**Figure S1.** *Kit*-lineage deletion of *Gata4/6* and *Gata4* alone leads to lineage-traced skeletal muscle fibers for the first time.  
A, Genetic models with the indicated alleles in mice that were crossed and (B) experimental design of the protocol used in this figure.  
C. Representative histological images of tibialis anterior muscle from *Kit* lineage-traced mice (white arrows point to *Kit* lineage eGFP*⁺* muscle fibers).  
D. Quantification of eGFP*⁺* skeletal muscle fibers in *Kit* lineage-traced mice with *Gata4*, *Gata6*, or *Gata4/6* deletion compared to lineage-traced *Gata4/6*-replete controls. Error bars are SEM, N=4 per group (10 sections per heart), *P*<0.05 vs *Kit*<sup>MCM</sup> *R26*<sup>eGFP</sup> for each genotype.

**Figure S2.** VEGF-A induced endothelial stimulation alters endothelial cell properties similar to loss of *Gata4*.  
A. Experimental design for AAV9-VEGF-A tail vein injection to *Kit*<sup>MCM</sup> *R26*<sup>eGFP</sup> lineage tracing mice over the 8 week experimental period.  
B, Western blot measure VEGF-A from heart tissue of vehicle or AAV9-VEGF-A tail vein injected mice. Gapdh is used as a processing and loading control.  
C, Representative cardiac histological images and (D) quantitation of *Kit* lineage-traced cells (green) following tail vein injection and 2 months of continuous tamoxifen induction (white arrows highlight eGFP*⁺* cardiomyocytes). Error bars are SEM, N=4 per group (10 sections per heart), *P*<0.05 vs vehicle treated mice at each time point.  
E, Representative cardiac histological sections showing CD45*⁺* cells (white) in the hearts of mice at baseline (vehicle) or 2 months after AAV9-VEGF-A infusion. N=4 per group (3 CD45*⁺* stained sections per heart).

**Figure S3.** Skeletal muscle with endothelial cell *Gata4* deletion shows reduced profile of endothelial cell maturation and greater proliferation.  
A. Representative flow cytometry plots of CD31-stained skeletal muscle interstitial cells comparing control *Tie2<sup>CreERT2</sup>* lineage-traced mice to *Tie2<sup>CreERT2</sup>* lineage-traced *Gata4*-deleted mice. Cells are gated to highlight CD31<sub>low</sub> and CD31<sub>high</sub> populations. N=3 per group.  
B, Representative skeletal muscle
histological images from Tie2\textsuperscript{CreERT2} lineage-traced (green) \textit{Gata4}-deleted mice stained for EdU (red) and DAPI (blue). N=3 per group (6 EdU stained sections per heart).

**Figure S4.** RT-qPCR confirms altered endothelial gene profiles in \textit{Gata4}-deleted endothelial cells. A, Quantitative mRNA levels for endothelial signature genes, (B) endothelial cell cycle and matrix genes, (C) ECM & angiogenesis genes, and (D) \textit{Gata4}-associated genes. N=3 samples each, *P<0.05 versus WT by t-test. Three mRNAs that were not significantly changed are still shown because they were significantly changed in the DESeq2 analysis of the RNaseq data analysis (see Online Methods).

**References for Supplemental Methods**

1. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marbán E, Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. \textit{Nature}. 2014;509:337-341.

2. Oka T, Maillet M, Watt AJ, Schwartz RJ, Aronow BJ, Duncan SA, Molkentin JD. Cardiac-specific deletion of \textit{Gata4} reveals its requirement for hypertrophy, compensation, and myocyte viability. \textit{Circ Res}. 2006;98:837-845.

3. van Berlo JH, Elrod JW, van den Hoogenhof MM, York AJ, Aronow BJ, Duncan SA, Molkentin JD. The transcription factor GATA-6 regulates pathological cardiac hypertrophy. \textit{Circ Res}. 2010;107:1032-1040.

4. Yamamoto M, Shook NA, Kanisicak O, Yamamoto S, Wosczyna MN, Camp JR, Goldhamer DJ. A multifunctional reporter mouse line for Cre- and FLP-dependent lineage analysis. \textit{Genesis}. 2009;47:107-114.
5. Pitulescu ME, Schmidt I, Benedito R, Adams RH. Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. *Nat Protoc*. 2010;5:1518-1534.

6. Kanisicak O, Khalil H, Ivey MJ, Karch J, Maliken BD, Correll RN, Brody MJ, J Lin SC, Aronow BJ, Tallquist MD, Molkentin JD. Genetic lineage tracing defines myofibroblast origin and function in the injured heart. *Nat Commun*. 2016;7:12260.

7. Accornero F, Schips TG, Petrosino JM, Gu SQ, Kanisicak O, van Berlo JH, Molkentin JD. BEX1 is an RNA-dependent mediator of cardiomyopathy. *Nat Commun*. 2017;8:1875.

8. Karch J, Schips TG, Maliken BD, Brody MJ, Sargent MA, Kanisicak O, Molkentin JD. Autophagic cell death is dependent on lysosomal membrane permeability through Bax and Bak. *eLife*. 2017;6.

9. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*. 2014;15:550.