1 **Coupled myovascular expansion directs cardiac growth and regeneration**

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25 **Summary Statement:** This work supports the concept of an endothelial-cardiomyocyte niche

26 that directs cardiac growth in neonatal mice and humans.

27

ABSTRACT

Heart regeneration requires multiple cell types to enable cardiomyocyte (CM) proliferation. How these cells interact to create growth niches is unclear. Here we profile proliferation kinetics of cardiac endothelial cells (CECs) and CMs in the neonatal mouse heart and find that they are spatiotemporally coupled. We show that coupled myovascular expansion during cardiac growth or regeneration is dependent upon VEGF-VEGFR2 signaling, as genetic deletion of *Vegfr2* from CECs or inhibition of VEGFA abrogates both CEC and CM proliferation. Repair of cryoinjury displays poor spatial coupling of CEC and CM proliferation. Boosting CEC density after cryoinjury with virus encoding *Vegfa* enhances regeneration. Using Mendelian randomization, we demonstrate that circulating VEGFA levels are positively linked with human myocardial mass, suggesting that *Vegfa* can stimulate human cardiac growth. Our work demonstrates the importance of coupled CEC and CM expansion and reveals a myovascular niche that may be therapeutically targeted for heart regeneration.

Keywords: Myovascular, heart regeneration, cardiomyocyte proliferation.

Non-standard abbreviations and acronyms: cardiac endothelial cells (CECs), cryoinjury (CI), cardiomyocytes (CMs), genome-wide association study (GWAS), left ventricular (LV), Mendelian randomization (MR), protein quantitative trait loci, (pQTL), single cell RNA-sequencing (scRNA-Seq)

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INTRODUCTION

In many organs, heterologous cell types establish specialized microenvironments, or niches, that mediate tissue growth and regeneration. Alterations to niche constituents can affect the efficiency of tissue growth and outcomes after injury, making niches a possible target for therapeutic regeneration (Lane et al., 2014; Wagers, 2012). For organs like the intestines, bone marrow, skin, and skeletal muscle, niches are classically centered around a stem cell compartment (Fuchs and Blau, 2020). With a few notable exceptions, niches within organs that lack a resident stem cell are less defined. For instance, in the developing and regenerating neonatal mouse heart, hypoxic niches have been associated with regionalized growth, but the cellular makeup of these niches is unclear (Kimura et al., 2015).

Studies in zebrafish, salamanders, and neonatal mice have established a template for innate heart regeneration through proliferation of spared cardiomyocytes (CMs) (Jopling et al., 2010; Kikuchi et al., 2010; Oberpriller and Oberpriller, 1971; Poss et al., 2002). However, innate heart regeneration is a multicellular process with required contributions from epicardial cells, inflammatory cells, and nerves (Aurora et al., 2014; Mahmoud et al., 2015; Wang et al., 2015). Recent work has highlighted a critical role for the vasculature in heart regeneration (Fan et al., 2019; Fernandez et al., 2018; Liu et al., 2020; Marin-Juez et al., 2016). In zebrafish and neonatal mice, cardiac endothelial cells (CECs) rapidly respond to injury, extending nascent vessels into the wound that ultimately guide CM growth (Das et al., 2019; Marin-Juez et al., 2019; Marin-Juez et al., 2016). Lineage tracing studies have demonstrated that these new vessels form by proliferation of CECs (Das et al., 2019; Marin-Juez et al., 2019; Zhao et al., 2014). Functional interference with angiogenic responses in the zebrafish or the neonatal mouse heart is associated with defects in CM proliferation (Das et al., 2019; Marin-Juez et al., 2016). Conversely, overexpression of the master angiogenic factor, *vegfaa*, is sufficient to induce ectopic cardiac growth in zebrafish, suggesting that a stimulated vasculature instructs cardiac growth (Karra et al., 2018). However, a similar role for VEGFA-stimulated CECs in the mammalian heart has yet to be shown.

Here, we spatiotemporally model CM and CEC proliferation in the neonatal mouse heart to investigate the mechanisms underlying cardiac growth and regeneration. We find that CM and CEC proliferation are tightly coupled during postnatal cardiac growth. With cryoinjury (CI), a model of incomplete regeneration, this coupling is spatially impaired. We demonstrate that

80 coupled myovascular expansion is dependent on VEGFA signaling to endothelial VEGFR2 and

81 restoration of coupling after CI through exogenous *Vegfa* can enhance regeneration. Similarly, we

82 find that genetically-determined levels of circulating VEGFA are associated with higher

83 myocardial mass in humans, suggesting that VEGFA also regulates human myocardial growth.

84 Together, these data demonstrate that coupled expansion of CECs and CMs within a myovascular

85 niche regulates cardiac growth and regeneration.

86 **RESULTS**

87 **Spatiotemporal coupling of EdU$^+$ CECs and EdU$^+$ CMs during postnatal growth**

88 Neonatal mice are able to regenerate their hearts after injury during the first few days of life

89 (Porrello et al., 2011). The loss of regenerative capacity in the mouse heart is coincident with a

90 developmental decline in the rate of CM proliferation. While the kinetics of CM proliferation in

91 neonatal mice have been well-documented, dynamics of other cardiac cell types are not well

92 characterized. To better define the relationship of CMs and CECs during the regenerative window

93 of the neonatal mouse heart, we assayed CM and CEC cycling kinetics at various time points

94 following an EdU pulse (Fig.1A-H). Cardiac sections at different developmental time points were

95 stained for EdU incorporation along with PCM1 and Erg to specifically mark CM and CEC nuclei,

96 respectively (Alkass et al., 2015; Bergmann et al., 2009; Bergmann et al., 2011; Das et al., 2019).

97 We developed customized image segmentation routines to objectively quantify large numbers of

98 CECs and CMs from cardiac sections (3120 ± 734 CECs per heart and 2328 ± 751 CMs per heart,

99 mean $\pm$ SD). We noted that the relative density of CECs increased between P1 and P10, while the

100 overall density of CMs decreased (Fig. 1E,G). Consistent with prior reports, we found the

101 percentage of EdU$^+$ CMs to sharply decline over the first 10 days of life, with a second peak

102 occurring at P5 (Fig. 1C,F). The increase in EdU$^+$ CMs at P5 likely coincides with a terminal round

103 of DNA synthesis and binucleation of CMs (Alkass et al., 2015; Soonpaa et al., 1996). EdU

104 incorporation by CECs also declines from P1 to P10 (Fig. 1B,D) with a second peak at P7. While

105 CM and CEC kinetics differ with regards to the timing of this second peak, the overall trends of

106 their kinetics parallel each other. To determine the strength of this relationship, we compared rates

107 of EdU$^+$ CECs and CMs for individual hearts and found them to be correlated (R = 0.59, p =

108 0.0007) (Fig. 1H). Above a threshold of ~8% EdU$^+$ CECs, each 1% increase in EdU$^+$ CMs is

109 associated with a $1.03 \pm 0.27\%$ (p = 0.0007) increase in EdU$^+$ CECs.

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110   Based on the strong temporal association of CEC and CM proliferation, we next sought to
111   spatially relate CEC and CM cycling. Within thick sections of hearts from P4 mice pulsed with
112   EdU, we observed numerous instances of EdU$^+$ CMs adjacent to EdU$^+$ CECs (Fig. 1I). To quantify
113   this observation, we assigned coordinates to each CEC and CM nucleus and computed pairwise
114   distances for every CEC and CM. We used this distance information to deconvolve overlapping
115   microenvironments by mapping the position of every CEC relative to an EdU$^+$ CM or an EdU$^-$ CM
116   as a function of distance, resulting in "pseudodistances" (Fig. 1J). When comparing the density of
117   EdU$^+$ CECs relative to an EdU$^+$ CM or EdU$^-$ CM, we found the percentage of EdU$^+$ CECs to be
118   enriched around EdU$^+$ CMs compared to EdU$^-$ CMs until about ~7 μm (p = 0.04 at 7 μm, two-
119   sided Z-test) (Fig. 1K), providing evidence for coupled myovascular expansion during physiologic
120   growth.
121
122   **Coupling of myovascular growth after cryoinjury to the neonatal mouse heart**
123   Unlike other models of injury to the neonatal mouse heart, cryoinjury (CI) results in incomplete
124   or inefficient regeneration (Bakovic et al., 2020; Darehzereshki et al., 2015; Polizzotti et al., 2016;
125   Polizzotti et al., 2015). Detailed analyses of CM proliferation kinetics after CI have demonstrated
126   that CM proliferation occurs in this model, but ostensibly at levels lower than that of the uninjured
127   neonatal heart (Darehzereshki et al., 2015; Polizzotti et al., 2015). Thus, we chose to evaluate
128   myovascular expansion following CI at P1 as a model of inefficient regeneration, in a regeneration-
129   competent context (Fig 2A,B). We first profiled CM and CEC kinetics, considering on average of
130   2407 ± 891 CECs and 2153 ± 1495 CMs within the border zone for each heart (mean ± SD). We
131   found that CM and CEC kinetics generally follow the same trend as in the uninjured heart, with
132   an approximately 50% decrease in proliferation indices for both cell types over the first 10 days of
133   life (Fig. 2C,D,E,G). Similarly, CEC and CM density within the border zone had a similar trend
134   to the uninjured heart (Fig 2F,H). When we compared rates of CM and CEC proliferation in
135   individual hearts, we once again noted that CM and CEC proliferation rates are correlated with ~
136   1% increase in the percentage of EdU$^+$ CECs for every percent increase of EdU$^+$ CMs (R = 0.54,
137   p = 0.008) (Fig. 2I). However, when we performed a spatial analysis of CECs and CMs within the
138   border zone, we did not detect enrichment of EdU$^+$ CECs in the immediate 7 μm vicinity of EdU$^+$
139   CMs (Fig. 2J-L). In fact, EdU$^+$ CECs may even be depleted around EdU$^+$ CMs in the border zone
140   after CI, suggesting that CEC and CM proliferation may not be efficiently coupled after CI.

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Association of myovascular coupling with VEGFA-VEGFR2 signaling

To better understand the molecular mediators of myovascular coupling during growth and injury, we performed single cell RNA-sequencing (scRNA-Seq) using border zones of P7 hearts that were cryoinjured at P1. We profiled 1721 cells and identified 9 clusters of cells carrying markers of CECs, CMs, fibroblasts, and inflammatory cells (Table S1, Fig. 3A). Clusters 1, 4, and 6 were notable for cells with CEC markers, such as *Fabp4, Pecam1, Erg,* and *Vegfr2* (Table S1). Compared to the other CEC clusters, cluster 4 had increased expression of numerous proliferative markers, including *Ki67, Prc1, Ccna2, and Ccnb2* (Table S2). A similar cluster of highly proliferative CECs has been also reported by other groups after coronary ligation in neonatal and adult mice (Wang et al., 2020; Wu et al., 2020). In the neonatal mouse heart, VEGFR2 marks cardiac microvascular cells and these cells have recently been implicated in revascularization following injury (Das et al., 2019; Kivela et al., 2019). Evaluation of sections from neonatal hearts under physiologic growth conditions revealed EdU$^+$ CMs within 7 µm of EdU$^+$ VEGFR2$^+$ CECs that was less common after injury (Fig. 3B,C), thus supporting a role for VEGFR2$^+$ microvascular CECs as contributing to a myovascular niche of growth.

For many organs, parenchymal cells secrete angiogenic factors that enable matching of vascular supply to organ size (Rafii et al., 2016). Based on our prior work linking *vegfaa* overexpression to ectopic cardiomyogenesis in the zebrafish heart, we assayed *Vegfa* expression during growth and regeneration by quantitative single molecule fluorescent in situ hybridization (Erben and Buonanno, 2019; Karra et al., 2018). We found that under physiologic growth conditions, *Vegfa* is expressed in CMs, with a sharp decline in expression from P3 to P10 (Fig. S1 and Fig. 3D, F). To determine whether *Vegfa* levels may be contributing to CEC dynamics after CI, we assayed *Vegfa* expression in the border zone after CI. Compared to uninjured hearts, *Vegfa* expression in the border zone at P3 is upregulated by ~ 60% (Fig. S1B and Fig. 3D-F). However, following CEC cycling rates after CI (Fig. 2), *Vegfa* expression markedly declines from P3 to P10 (Fig. S1B and Fig. 3 E,F). Together, these data support a dynamic role for myocardial VEGFA to endothelial VEGFR2 signaling as a regulator of the myovascular expansion during growth and regeneration.

Requirement of endothelial *Vegfr2* for CM proliferation during growth and regeneration

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172 Based on our scRNA-Seq experiments indicating that many proliferating CECs are $Vegfr2^+$, we

173 hypothesized that VEGFR2 signaling is a critical mediator of myovascular growth in the early

174 neonatal period. To conditionally delete $Vegfr2$ from CECs, we crossed $Vegfr2^{flox/flox}$ mice to

175 $Cdh5$-$CreER^{T2}$ mice to generate $Cdh5$-$CreER^{T2}$; $Vegfr2^{flox/flox}$ ($Vegfr2^{\Delta EC}$) and $Vegfr2^{flox/flox}$

176 ($Vegfr2^{WT}$) mice (Hooper et al., 2009; Sorensen et al., 2009). We verified loss of VEGFR2 from

177 CECs by immunostaining for VEGFR2 in $Vegfr2^{\Delta EC}$ mice (Fig. S2A,B). Evaluation of CECs in

178 $Vegfr2^{\Delta EC}$ mice revealed fewer CECs than in $Vegfr2^{WT}$ animals and a nearly 80% reduction in CEC

179 proliferation (Fig. 4A-C). To determine how CM growth is affected by the absence of $Vegfr2$ from

180 CECs, we next assayed EdU incorporation by CMs. While CM numbers were largely preserved,

181 CM cycling was also attenuated by ~ 80% in $Vegfr2^{\Delta EC}$ mice compared to $Vegfr2^{WT}$ mice (Fig.

182 4D-F) . We next evaluated the effect of $Vegfr2$ deletion from CECs on myovascular growth after

183 cryoinjury. Like our results during early neonatal growth, relative numbers of CECs were

184 decreased while relative CM numbers were preserved in $Vegfr2^{\Delta EC}$ hearts (Fig 4I,L). We identified

185 defects in both CEC and CM incorporation of EdU after injury in $Vegfr2^{\Delta EC}$ mice (Fig. 4G,H,J,K).

186 Specifically, we observed $Vegfr2^{\Delta EC}$ hearts to have an almost 80% decrease in cycling CECs after

187 injury and a 40% decrease in cycling CMs. Consistent with our hypothesis, CM expansion during

188 growth and regeneration is dependent on intact $Vegfr2$ in CECs.

189

190 **Inhibition of VEGFA limits CEC and CM proliferation**

191 Our expression data suggest a role for myocardial $Vegfa$ in myovascular coupling during growth

192 and after injury (Fig. S1, Fig. 3D-F). To functionally test this concept, we obtained a well-

193 described antibody, B20-4.1.1 (anti-VEGFA), that binds murine VEGFA and prevents interaction

194 with its receptors (Fig S2 C,D) (Liang et al., 2006). Along with decreased CEC density, treatment

195 of neonatal mice with anti-VEGFA decreased CEC proliferation by approximately 70% during

196 early neonatal growth and by about 90% after injury indicating a dependency of CEC proliferation

197 on VEGFA (Fig. 5A,B,G,H). Defects in CEC proliferation following anti-VEGFA treatment were

198 accompanied by decreases in the fraction of EdU$^+$ CMs during growth and after injury (Fig.

199 5D,E,J,K), further supporting the need for myovascular coupling during growth and regeneration.

200 Prior work has described a critical role for tissue hypoxia as a regulator of CM proliferation

201 in both zebrafish and mice (Jopling et al., 2012; Kimura et al., 2015; Puente et al., 2014).

202 Remarkably, hypoxic pre-conditioning of adult mice results in cardiac growth and enhanced

203   regenerative capacity (Nakada et al., 2017). Mechanistically, hypoxia decreases levels of reactive

204   oxygen species, enabling CM cell cycle-reentry (Puente et al., 2014). As the decreased vascularity

205   in $Vegfr2^{\Delta EC}$ mice and anti-VEGFA treated mice (Fig. S2B,D) might be expected to result in tissue

206   hypoxia, we examined hypoxia in CMs under these conditions. Pimonidazole is a 2-nitroimidazole

207   used to identify tissue hypoxia based on its formation of stable adducts in the presence of low

208   oxygen tension (Miller et al., 1989; Raleigh et al., 1998). These adducts can be detected by

209   immunofluorescence with the intensity of staining directly proportional to the level of hypoxia.

210   We administered pimonidazole prior to harvest of hearts from $Vegfr2^{\Delta EC}$ mice, $Vegfr2^{WT}$ mice, and

211   mice injected with anti-VEGFA or vehicle. We noted an approximately 75% increase in the

212   intensity of pimonidazole uptake by CMs of $Vegfr2^{\Delta EC}$ mice compared to $Vegfr2^{WT}$ mice and an

213   almost 2-fold increase in mice treated with anti-VEGFA compared to mice treated with vehicle

214   (Fig. S3). In addition, we noted more Hif1α staining in $Vegfr2^{\Delta EC}$ mice and mice treated with anti-

215   VEGFA compared to control animals (Fig. S3) Together, these results demonstrate that hearts of

216      $Vegfr2^{\Delta EC}$ mice and anti-VEGFA treated mice are hypoxic and suggest that CECs are a required

217   mediator for CM proliferation in response to tissue hypoxia.

218

219   **Exogenous VEGFA enhances the efficiency of innate regenerative responses.**

220   Based on the impaired spatial myovascular coupling of CECs and CMs after CI (Fig. 2J-L) and

221   the decrease of $Vegfa$ in the border zone after CI (Fig. 3E,F), we hypothesized that increasing

222      $Vegfa$ levels within the border zone might enhance the efficiency of regenerative growth following

223   CI. To test our hypothesis, we generated adeno-associated virus to overexpress $Vegfa$ (AAV-

224      $Vegfa$) or GFP (AAV-GFP). We then cryoinjured mice at P1 and injected ~1.2 x 10^{10} viral

225   genomes of AAV-$Vegfa$ or AAV-GFP into the border zone immediately after injury (Fig. S2E-

226   G). Gross examination of hearts injected with a resin to opacify the coronaries hearts at P21

227   identified increased vascularity at the site of injury of AAV-$Vegfa$ hearts compared to AAV-GFP

228   hearts (Fig. S2H). In addition, we noted that AAV-$Vegfa$ hearts had ~50% less scarring of the left

229   ventricle and better cardiac function after CI (Fig. 6A-C), all suggestive of enhanced regeneration.

230   Compared to animals treated with AAV-GFP, mice treated with AAV-$Vegfa$ had a significantly

231   higher CEC density in the border zone and a trend towards more Ki67$^+$ CECs (8.62 ± 2.59% vs

232   5.54 ± 1.23%, p = 0.31) (Fig. 6D,E). Importantly, this was accompanied by a more than 2-fold

233   increase of Ki67$^+$ CMs in the border zone compared to hearts treated with AAV-GFP (Fig. 6F,G).

234 Finally, we evaluated sarcomere morphology and α-SMA expression, two markers of CM maturity
235 (Chen et al., 2021; Porrello et al., 2011). Compared to uninjured hearts and hearts treated with
236 AAV-*GFP*, AAV-*Vegfa* treated hearts were notable for CMs with sarcomeric staining on the
237 periphery of the cell, similar to prior descriptions of sarcomere disassembly (Fig. S4A) (Porrello
238 et al., 2011). Also consistent with a less mature CM phenotype in AAV-*Vegfa* hearts, CMs were
239 more likely to express the dedifferentiation marker αSMA after AAV-*Vegfa* treatment (Fig. S4B-
240 D) (Chen et al., 2021). Together, these data indicate that exogeneous *Vegfa* in the border zone can
241 enhance regenerative responses after neonatal CI by increasing CECs, promoting CM
242 dedifferentiation and proliferation, reducing scarring, and restoring ventricular function.

243

244 **Association of genetically predicted VEGFA levels with myocardial mass in human**
245   Our results after CI in neonatal mice suggest exogenous *Vegfa* could be used
246 therapeutically to promote cardiac growth and regeneration. Indeed, exogenous *Vegfa* has been
247 shown to stimulate cardiac repair after experimental infarction many times in adult mammals, even
248 prompting several human clinical trials in patients with ischemic heart disease (Carlsson et al.,
249 2018; Ferrarini et al., 2006; Janavel et al., 2006; Lin et al., 2012; Pearlman et al., 1995; Zangi et
250 al., 2013). However, clinical trials have largely failed to improve outcomes, possibly because of
251 inefficient delivery methods that did not sufficiently raise local *Vegfa* levels (Oh and Ishikawa,
252 2019; Taimeh et al., 2013).
253   Based on its potential as a regenerative factor, we sought to conceptually determine
254 whether VEGFA might regulate cardiac growth in humans using Mendelian randomization (MR).
255 MR is an epidemiologic technique that uses genetic variation to infer causality from observational
256 studies (Emdin et al., 2017; Gray and Wheatley, 1991; Schmidt et al., 2020). Traditional
257 observational studies that associate factors with an outcome are prone to confounding and cannot
258 differentiate causation from reverse causation. However, because innate factors tend to be
259 independent of confounding variables, MR studies are used to point towards causality, and have
260 been used to successfully predict the results of clinical trials (Ference, 2017).
261   We performed two-sample *cis* Mendelian randomization (MR) analysis with genetically
262 predicted circulating VEGFA concentration as the exposure variable and traits of cardiac structure
263 and function as the outcomes (Fig. 7) (Davey Smith and Hemani, 2014). Under this framework,
264 genetic variants acting in *cis* that associate with protein abundance (protein quantitative trait loci,

265 pQTL) are used as instrumental variables to estimate the unconfounded causal effects of the protein
266 on the outcomes of interest (Fig. 7A). Genetic variants within the VEGFA gene region were
267 selected from genetic association summary statistics from a genome-wide association study
268 (GWAS) of directly measured circulating VEGFA concentration in over 30,000 individuals (Fig.
269 7B, Table S3) (Folkersen et al., 2020). Variant association statistics for six parameters of left
270 ventricular (LV) structure and function were then extracted from a GWAS of cardiac magnetic
271 resonance imaging measures in the UK Biobank (Aung et al., 2019). A summary of the GWAS is
272 presented in Table S4. We applied these instruments, using a two-sample MR model that accounts
273 for partial correlation between instruments, to estimate the effect of genetically predicted
274 circulating VEGFA on LV phenotypes (Schmidt et al., 2020). We did not detect a link between
275 genetically predicted VEGFA levels and left ventricular volumes or ejection fraction but found
276 that higher genetically predicted VEGFA levels resulted in increased LV mass (at type I error rate
277 = 0.05 / 6), (Fig. 7C,D). In total, we found an estimated 1.02 gram (95% CI = 0.33-1.70 gram, p =
278 0.004) increase in LVM per doubling of the circulating VEGFA concentration. Because there is a
279 lack of consensus on how genetic instruments are selected and MR modeling, we performed a
280 sensitivity analysis. Across 120 separate analyses with varying thresholds for instrument selection
281 and four different MR modeling approaches, 119 of these analyses indicate that higher levels of
282 VEGFA are robustly associated with higher LVM (Fig. 7E). These results are consistent with the
283 increased angiogenesis, increased heart size, and better cardiac function previously reported in
284 mice that have genetically higher levels of VEFGA (Marneros, 2018). Thus, VEGFA is likely to
285 promote cardiac growth in humans, a finding of therapeutic relevance.
286
287 **DISCUSSION**
288   We profiled CEC and CM dynamics to determine that CEC and CM cycling are
289 spatiotemporally coupled in the neonatal mouse heart. Uncoupling CECs from CMs, by deletion
290 of *Vegfr2* from CECs or with an anti-VEGFA antibody, dramatically decreases CM proliferation
291 (Fig. 4 and 5). Conversely, improving CEC and CM coupling after CI by increasing local *Vegfa*
292 increases regeneration after CI (Fig. 6). Analogously, myocardial mass of the human heart
293 increases with higher VEGFA levels (Fig. 7). Together, our work demonstrates that the efficiency
294 of cardiac growth is dependent upon myovascular interactions, a finding with strong translational
295 relevance. Approximately 50% of patients with systolic heart failure have flow-limiting coronary

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296 artery disease (Benjamin et al., 2018). Even in so-called "non-ischemic" cardiomyopathies, cardiac
297 perfusion is impaired due to microvascular disease and capillary rarefaction (Abraham et al., 2000;
298 Drakos et al., 2010; Mosseri et al., 1991; Parodi et al., 1993; Tsagalou et al., 2008). Thus, as
299 methods to promote proliferation of adult mammalian CMs move towards therapeutics,
300 mechanical or molecular revascularization approaches may be a required adjunct for these
301 approaches to be maximally efficacious.

302 A key concept suggested by our work is that of growth niches, in which cycling CECs
303 establish inductive microenvironments to promote CM proliferation (Fig. 1, 2). We find that
304 cycling CECs are statistically enriched within 7 µm, or 1-2 cell lengths, of cycling CMs during
305 physiologic growth. Because we used pairwise comparisons of CEC and CM distances to
306 deconvolve overlapping niches, our spatial analysis likely underestimates the expanse of the niche
307 surrounding cycling CMs. However, our results are remarkably well-aligned with reported
308 increases of CM proliferation within 3 cell lengths of sprouting angiogenesis in cultured fetal heart
309 sections (Miao et al., 2020). Niches for CM expansion in the postnatal mammalian heart have been
310 previously identified around hypoxic regions, with hypoxia even being able to induce CM
311 proliferation in the adult mouse heart (Kimura et al., 2015; Nakada et al., 2017). Mechanistically,
312 hypoxia exerts its effect on CM proliferation cell autonomously, by modulating reactive oxygen
313 species and the DNA damage response (Puente et al., 2014). Our work suggests another element
314 to the phenomenon of hypoxia-induced CM proliferation. We were able to induce hypoxia in the
315 neonatal heart through two different approaches, genetic deletion of *Vegfr2* from CECs and with
316 an anti-VEGFA antibody (Fig. S3). However, even though hypoxia would be predicted to increase
317 CM proliferation, we observed decreased CM cycling in these models. Thus, hypoxia mediated
318 CM expansion is likely to involve both CM-specific effects but to also depend on CECs within the
319 hypoxic niche. While hypoxia may be one approach to stimulate heart regeneration, therapeutic
320 hypoxia for patients with heart failure may be challenging. A better understanding of the signaling
321 milieu of the hypoxic niche could lead to alternate approaches for stimulating hypoxia-mediated
322 regeneration.

323 Because depletion of CECs, either through deletion of *Vegfr2* from CECs or by
324 administration of anti-VEGFA, leads to defects in CM proliferation, our work suggests that CECs
325 promote CM cycling (Fig. 4, 5). Mechanistically, CEC expansion may simply lead to new conduits
326 for growth factors or other cell types that promote cardiac growth. However, multiple lines of

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327   evidence also point to a direct role for CECs to influence CM proliferation through angiocrines.
328   Indeed, work in zebrafish and neonatal mice support this concept. In zebrafish, inhibition of Notch
329   in CECs results in reduced secretion of Wnt inhibitors that influence CM proliferation (Zhao et
330   al., 2019). More directly, deletion of *Igf2* from CECs in developing mice abrogates CM
331   proliferation after neonatal cardiac injury (Shen et al., 2020). Interestingly, deletion of *Igf2* from
332   CECs does not affect physiologic CM cycling, raising the intriguing possibility that different
333   angiocrines modulate myocardial expansion during physiologic growth and after injury. Finally,
334   different CEC subsets may contribute different sets of angiocrines, as recent work suggests that
335   *Reln* from lymphatic endothelial cells has direct effects on CM proliferation (Liu et al., 2020).
336   Future work to identify the complement of angiocrines that instruct CM proliferation in different
337   developmental and injury contexts is needed.
338   Our work also suggests that restoring myovascular coupling can enhance regenerative
339   capacity. Unlike physiologic growth, CEC cycling is not enriched around cycling CMs in the
340   border zone after CI, a model of incomplete regeneration. Functionally increasing CEC density
341   through overexpression of the master angiokine *Vegfa* enhances CM cycling and reduces scarring
342   after CI, both signs of enhanced regeneration (Fig. 6). Taken together with our prior work showing
343   that *vegfaa* overexpression can induce ectopic heart regeneration in zebrafish, exogenous *Vegfa*
344   may be one approach to augment regeneration. Indeed, our MR study indicates that VEGFA levels
345   are causally linked to human myocardial mass and predict that more VEGFA can increase human
346   cardiac growth (Fig. 7). Several clinical trials of *Vegfa* are now underway to revisit whether
347   exogenous VEGFA can be used therapeutically to treat human cardiovascular disease
348   (NCT03409627, NCT03370887, and NCT04125732). Compared to prior clinical trials, these
349   studies utilize newer delivery methods such as modified RNAs, newer generation viral vectors,
350   and more efficient plasmid delivery systems. Based partly on our findings, we would expect that
351   markers of increased CECs, such as improvements in myocardial perfusion, will predict VEGFA-
352   based treatment effects on cardiac mass and function. A second aspect of our work that might be
353   informative to translational work is that the site of *Vegfa* delivery could be critical to where growth
354   or recovery can occur. For instance, misexpression of *vegfaa* in the heart of zebrafish after apical
355   resection impairs regeneration with cardiac growth occurring away from the site of injury (Karra
356   et al., 2018). By contrast, in this work, targeted *Vegfa* overexpression in the border zone improves

357 regeneration in neonatal mice. Future clinical trials of *Vegfa* will need to account for the site of
358 *Vegfa* overexpression when determining effects on cardiac structure and function.
359   We acknowledge several limitations to our work. First, although we focus on the coupling
360 of CECs and CMs during growth and regeneration, additional cell types that also regulate cardiac
361 growth and are likely to be present within niches. Defining niche constituents may be critical to
362 efficiently instruct cardiac growth. Second, while we demonstrate a critical role of VEGFA-
363 VEGFR2 signaling to coordinate CEC and CM expansion, VEGFR2 broadly marks the cardiac
364 microvasculature. Future work to refine which microvascular cells instruct CM cycling could
365 enable more targeted regenerative approaches via cell transplantation or specific signaling cues.
366 Finally, while our MR studies point to a therapeutic benefit to using VEGFA to stimulate cardiac
367 growth, our MR studies model a lifetime of exposure to VEGFA and do not have the resolution to
368 confirm that increased myocardial mass is the result of CM expansion. Additional work to
369 determine whether VEGFA promotes cardiac growth across developmental stages and to directly
370 link VEGFA to human CM proliferation in vivo are needed to establish VEGFA as a bona fide
371 regenerative factor in humans.
372   In summary, here, we present work showing that coordinated growth of CECs and CMs
373 guides postnatal cardiac growth and regeneration. We speculate that a better understanding of the
374 signaling milieu within a myovascular niche can inform approaches for heart regeneration.
375

376 **METHODS**
377 **Mice and Neonatal Cryoinjuries**
378 CD-1 mice (Charles River Labs, Morrisville, NC) were used for profiling myovascular kinetics
379 during growth and regeneration. CD-1 mice were also used for anti-VEGFA antibody and AAV
380 experiments. *Cdh5-CreER*^T2 and *Vegfr2^flox/flox* strains have been previously described (Hooper et
381 al., 2009; Sorensen et al., 2009). *Cdh5-CreER*^T2; *Vegfr2^flox/flox* and *Vegfr2^flox/flox* mice were treated
382 with 100 µg of tamoxifen intraperitoneally at P0 and P1. Complete recombination was verified by
383 immunostaining for VEGFR2 (Fig. S2). For EdU incorporation studies, 0.5 mg EdU was given
384 intraperitoneally 4 hours prior to tissue harvest. Cryoinjuries were performed on P1 pups as
385 previously described (Bakovic et al., 2020; Polizzotti et al., 2015).
386   The number of animals used in these studies is specified in the figure legends. For mouse
387 experiments, we included animals 1) confirmed to have *Vegfr2* deletion by immunostaining for

13

388   VEGFR2; 2) animals with weight loss after anti-VEGFA treatment; or 3) animals confirmed to

389   have *Vegfa* overexpression by in situ hybridization after injection with AAV-*Vegfa*. For assays

390   without computational scoring, readers were blinded to experimental groups. All protocols were

391   approved by the Institutional Animal Care and Use Committee at Duke University.

392

393   **Histology and Immunostaining**

394   At the time of tissue harvest, hearts were perfused with KCl and then 4% PFA. Hearts were then

395   immersed in 30% sucrose overnight and embedded in Tissue Freezing Media for cryosectioning.

396   Cryosections were blocked with PBST (PBS with 0.1% Tween-20) containing 10%

397   newborn calf serum and 1% DMSO and incubated overnight with primary antibodies at 4ºC.

398   Cryosections were then washed with PBST and incubated with secondary antibodies and DAPI

399   (100 ng/ml). For EdU detection, cryosections were incubated in EdU staining solution (100 mM

400   Tris-HCl, 1 mM $CuSO_4$, 10 mM Azide, and 50 mM ascorbic acid in PBS) for 10 minutes. Primary

401   antibodies used for this study included: anti-PCM1 (Sigma, HPA023370, 1:100); anti-PCM1

402   (Santa   Cruz,   sc-398365,   1:100);   anti-Erg   (Abcam, ab92513, 1:25);   anti-CD31

403   (BDBioscience, 553370, 1:100), anti-VEGFR2 (BDBioscience, 555307, 1:100), anti-Ki67

404   (ThermoFisher, 4-5698-82, 1:100), anti-Actinin (Cell Signaling, 6487P, 1:100), anti-Tnnt (DSHB

405   CT3, 1:25), anti-αSMA (Abcam, ab5694, 1:200), and anti-HIF1α (Cell Signaling, AF1935,

406   1:100). Secondary antibodies and azides were conjugated to Alexa-488, Alexa-594, or Alexa-633

407   (Invitrogen). To label CM nuclei, cryosections underwent heat induced epitope retrieval with

408   citrate buffer prior to immunostaining. Stained slides were imaged on a Zeiss AxioImager M1

409   epifluorescent microscope, a Zeiss CSU-X1 spinning disk confocal microscope, or a Zeiss LSM

410   510 confocal microscope. For physiologic growth experiments, 9-16 non-overlapping images

411   (40X) of the left ventricle were obtained for each section. For CI experiments, the border zone was

412   defined as the entire region within 1-2 40X fields of view along the entirety of the injury plane.

413   For each heart, the 3 largest sections were imaged.

414

415   **Quantification of CM and CEC Proliferation**

416   We developed several customized image segmentation pipelines using CellProfiler and Ilastik for

417   automated scoring EdU[+] CECs and CMs (Berg et al., 2019; Lamprecht et al., 2007). For CEC

418   quantification, grayscale images for each channel were processed with rolling-ball background

14

419   subtraction and used as input into a CellProfiler routine that 1) identified Erg+ nuclei based on Erg

420   staining and DAPI intensity; 2) identified EdU+ nuclei based on EdU staining and DAPI staining;

421   and 3) identified EdU+ Erg+ nuclei based on the presence of an EdU object within an Erg object.

422   For CMs, grayscale images from individual channels were obtained using a Zeiss CSU-X1

423   spinning disk confocal microscope. Images were pre-processed to generate 32-bit grayscale

424   images and to create a set of images with merged PCM1 and DAPI channels. The DAPI image

425   and the merged PCM1-DAPI images were then input into machine learning routines to generate

426   probability maps for DAPI+ nuclei and PCM1+/DAPI+ nuclei, using Ilastik. Machine learning

427   algorithms were trained using images from our physiologic growth experiments. Probability maps

428   and grayscale images were used as input for a CellProfiler pipeline that 1) identified nuclei based

429   on a DAPI probability map; 2) filtered nuclei for CMs based on the mean PCM1+/DAPI+ pixel

430   probability; 3) identified EdU+ nuclei based on EdU staining and DAPI staining; and 4) identified

431   EdU+ CM nuclei based on the presence of an EdU object within a CM nucleus. CellProfiler output

432   data was tabulated using the dplyr package in R (Wickham et al., 2015).

433

434   **Proximity mapping**

435   For proximity mapping, z-stack images of P4 hearts were obtained using a Zeiss LSM 510 confocal

436   microscope. CM and CEC nuclei were segmented using the Spots tool along with manual

437   refinement, assigned coordinates, and categorized as EdU+ or EdU- using Imaris (Bitplane AG,

438   Zurich, Switzerland). Coordinate files were then used to determine the pairwise distance of each

439   CM and each CEC nucleus. Distances were used to compute the density of EdU+ CECs as a

440   function of distance from each PCM1+ nucleus using the dplyr and plotly packages in R (Sievert

441   et al., 2018; Wickham et al., 2015).

442

443   **In situ hybridization**

444   RNAscope Probe - Mm-Vegfa-O2 (Lot 16197A), with predicted reactivity against all known

445   murine *Vegfa* variants, was hybridized against flash-frozen cryosections using the manufacturer's

446   protocol (Advanced Cell Diagnostics, Hayward, CA). Images of sections were quantified using a

447   CellProfiler routine adapted from Erben et al (Erben and Buonanno, 2019). Briefly, the DAPI

448   channel was used to identify nuclei objects and the number of *Vegfa* spots adjacent to each nucleus

449   was counted.

450

Single Cell RNA Sequencing and Analysis

The left ventricles of cryoinjured hearts were collected at P7. The apical third of the LV that contained the injured area was then dissociated into single cells. Single cells were then captured into droplets using microfluidics followed by preparation of single cell cDNA libraries as previously described (Kobayashi et al., 2020). Samples were sequenced on a single lane of an Illumina HiSeq. Sequencing reads were mapped to Ensembl release NCBIM37.67. Aligned reads were binned and collapsed onto cellular barcodes using the Drop-seq pipeline v1.13.3 (http://mccarrolllab.com/dropseq), resulting in a raw digital expression matrix containing the count of unique UMIs for each gene in each cell (Macosko et al., 2015).

Expression analysis was performed by the Duke Bioinformatics Shared Resource, using the Seurat package (v2.2.0) in R/Bioconductor (Butler et al., 2018). Cells with gene counts over 4,000 or less than 200 were removed. Genes expressed in less than three cells were also removed. To avoid over-filtering CMs, we did not filter cells based on high expression of mitochondrial genes. Gene expression, for 1721 cells across 12754 genes, was normalized by scaling by the total number of transcripts, multiplying by 10,000, and log transformation. Unwanted sources of variation were adjusted for by regression on the number of detected UMIs using the *ScaleData* function. We used the JackStraw method to determine the number of statistically significant principal components (PCs). The *FindClusters* function was used to identify cellular clusters, using the 20 significant PCs and a 0.6 resolution. We used the t-SNE method to visualize cells across 20 PCs in two dimensions. To identify cell types, we used the *FindAllMarkers* function. Parameters were set to test all genes, expressed in at least 10% of the cells in each cluster, for differential expression.

Anti-VEGFA Antibody Treatment

Anti-VEGFA antibody (B20-4.1.1) was a kind gift from Genentech (San Francisco, CA). Neonatal mice were injected with 5 μg/g of anti-VEGFA or vehicle at P0 and P4. Adequate injection of anti-VEGFA was confirmed by significant weight loss compared to control animals at the time of tissue harvest.

Determination of Hypoxia

16

481   Mice were given 60 µg/g pimonidazole (Hypoxyprobe, HP3-100kit) intraperitoneally 1.5 hours

482   prior to tissue harvest. Tissues sections were stained with an anti-pimonidazole antibody

483   (Hypoxyprobe, HP3-100kit, 1:100) and an antibody against α-actinin. For each heart, 9 images of

484   the left ventricle for 3 separate cardiac sections were obtained using an AxioImager M1

485   microscope. Images were quantified using a CellProfiler routine to 1) create an image mask based

486   on the a-actinin stain and 2) determine the mean intensity of anti-pimonidazole stain.

487

488   **AAV Generation and Treatment**

489   AAV encoding *Vegfa-164* under the control of a CMV promoter was generated by PCR amplifying

490   the *Vegf164* isoform of *Vegfa* from a mouse endothelial cDNA library and cloning this fragment

491   into the pTR2-eGFP with replacement of *EGFP* (Hewitt et al., 2009). AAV, serotype 9, was

492   generated through the Duke Viral Vector core. AAV virus encoding *GFP* under the control of a

493   CMV promoter was obtained from the Duke Viral Vector Core. For intracardiac injections, 12 µl

494   of AAV at $1x10^9$ vg/µl was injected circumferentially around the injured area immediately after

495   CI (Fig. S2E,F).

496

497   **Coronary vessel labeling**

498   The coronary vasculature was visualized by injection of a low viscosity polyurethane resin

499   (PU4ii, VasQTec) into the apex of the left ventricle (Lee et al., 2019). Whole mount images were

500   obtained using a stereoscope.

501

502   **Scar Assessment**

503   P21 hearts were serially sectioned from base to apex and stained with acid fuchsin orange G, as

504   previously described (Karra et al., 2015). Stained slides were scanned with a Leica Aperio Digital

505   Pathology Slide Scanner. Scar area as a percentage of the left ventricle was scored by a blinded

506   reader. The average scar percentage across the 5 largest sections was determined for each heart.

507

508   **Echocardiography**

509   Fractional shortening was determined by echocardiography in conscious P21 mice by the Duke

510   Cardiovascular Physiology Core, using a Vevo 2100 high resolution imaging system (Visual

511 Sonics) and the protocol described in Ref (Jean-Charles et al., 2017). M-mode, long-axis images
512 were analyzed by three blinded readers and an averaged reading is reported.
513
514 **Mendelian Randomization to Determine Effects of Circulating VEGFA Levels on Human**
515 **Cardiac Structure**
516 Two-sample MR analysis was performed using summary statistics from GWAS of circulating
517 protein level measured with Olink proximity-extension assay in 30,931 individuals of European
518 ancestries and GWAS of cardiac MRI – derived left ventricular phenotypes in 16,923 UK Biobank
519 participants (Table S4) (Aung et al., 2019; Folkersen et al., 2020). To represent effect in the
520 original unit of measurements, the standardized estimates from both GWAS were back-
521 transformed by multiplying the MR effect estimate with the estimated standard deviation of the
522 traits. MR instruments for VEGFA were selected from variants that 1) are available in both GWAS,
523 2) located within the 200 kilobases flanking region from the genomic coordinate of *VEGFA* gene;
524 3) have a MAF > 0.01; 4) have a *P*-value for association with circulating VEGFA level < 1×10^{-4};
525 and 5) are LD-clumped to an r^2 threshold of 0.4 using *plink* with --clump option (Purcell et al.,
526 2007). Mendelian randomization analysis was performed using an inverse-variance weighted
527 model   accounting   for   correlation   between   instruments,   implemented   in
528 the *MendelianRandomization* R package (Burgess et al., 2016; Yavorska and Burgess, 2017).
529 Correlation between instruments were estimated from a random sample of 10,000 UK Biobank
530 participants of European ancestries (Bycroft et al., 2018).
531   To test the robustness of the cis-MR analysis, a series of sensitivity analyses were
532 performed by varying parameters for instrument selection and MR models. For instrument
533 selection, LD clumping was performed using five different r^2 thresholds (0.05, 0.1, 0.2, 0.4, and
534 0.6) and six different *P*-value thresholds for association with circulating VEGFA level (no
535 threshold), $1e^{-2}$, $1e^{-3}$, $1e^{-4}$, $1e^{-5}$, and $5e^{-8}$). Four different MR models were also tested for each
536 instrument set (inverse-variance weighted, MR-Egger, Principal component MR with 90%
537 variance explained, and Principal component MR with 99% variance explained) (Bowden et al.,
538 2015; Burgess et al., 2017). To account for correlation between instruments, we implemented the
539 method proposed by Burgess *et al.* using an instrument correlation model derived from genotype
540 data of a random subset of 10,000 UK Biobank participants of European ancestry (Burgess et al.,
541 2016).

542

Statistics

All means are presented as mean ± SEM and proportions as proportion ± 95% confidence interval (95% CI). Statistical analysis between two groups was performed using a two-tailed unpaired t-test test. Levene's test was used to ensure that variances were not unequal between groups. Pairwise comparisons between multiple groups were performed using a two-tailed unpaired t-test test with correction for multiple testing using Holm's method. A $p < 0.05$ was set as an *a priori* threshold for significance. Statistical analysis and plots were generated in R using the dplyr, ggpubr, and ggplot2 packages (Kassambara, 2017; Wickham, 2011; Wickham et al., 2015). For each experiment, individual data points are presented in the plot and the sample size is specified in the figure legend.

Study approval

All animal protocols were approved by the Institutional Animal Care and Use Committee at Duke University.

575

576   **Data Availability:** scRNA-Seq data can be accessed at the Gene Expression Omnibus under

577   accession code GSE166197.

578

579

580

581 **References**

582 **Abraham, D., Hofbauer, R., Schäfer, R., Blumer, R., Paulus, P., Miksovsky, A., Traxler, H.,**
583   **Kocher, A. and Aharinejad, S.** (2000). Selective downregulation of VEGF-A165,
584   VEGF-R1, and decreased capillary density in patients with dilative but not ischemic
585   cardiomyopathy. *Circulation research* **87**, 644-647.
586 **Alkass, K., Panula, J., Westman, M., Wu, T.-D., Guerquin-Kern, J.-L. and Bergmann, O.**
587   (2015). No evidence for cardiomyocyte number expansion in preadolescent mice. *Cell*
588   **163**, 1026-1036.
589 **Aung, N., Vargas, J. D., Yang, C., Cabrera, C. P., Warren, H. R., Fung, K., Tzanis, E.,**
590   **Barnes, M. R., Rotter, J. I., Taylor, K. D., et al.** (2019). Genome-Wide Analysis of
591   Left Ventricular Image-Derived Phenotypes Identifies Fourteen Loci Associated With
592   Cardiac Morphogenesis and Heart Failure Development. *Circulation* **140**, 1318-1330.
593 **Aurora, A. B., Porrello, E. R., Tan, W., Mahmoud, A. I., Hill, J. A., Bassel-Duby, R., Sadek,**
594   **H. A. and Olson, E. N.** (2014). Macrophages are required for neonatal heart
595   regeneration. *J Clin Invest* **124**, 1382-1392.
596 **Bakovic, M., Thakkar, D., DeBenedittis, P., Chong, D. C., Thomas, M. C., Iversen, E. S.**
597   **and Karra, R.** (2020). Clonal Analysis of the Neonatal Mouse Heart using Nearest
598   Neighbor Modeling. *Journal of Visualized Experiments: Jove*.
599 **Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng,**
600   **S., Chiuve, S. E., Cushman, M., Delling, F. N. and Deo, R.** (2018). Heart disease and
601   stroke statistics—2018 update: a report from the American Heart Association.
602   *Circulation*.
603 **Berg, S., Kutra, D., Kroeger, T., Straehle, C. N., Kausler, B. X., Haubold, C., Schiegg, M.,**
604   **Ales, J., Beier, T. and Rudy, M.** (2019). Ilastik: interactive machine learning for (bio)
605   image analysis. *Nature Methods*, 1-7.
606 **Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabe-Heider, F., Walsh, S.,**
607   **Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H., et al.** (2009). Evidence for
608   cardiomyocyte renewal in humans. *Science* **324**, 98-102.
609 **Bergmann, O., Zdunek, S., Alkass, K., Druid, H., Bernard, S. and Frisen, J.** (2011).
610   Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell
611   turnover. *Exp Cell Res* **317**, 188-194.
612 **Bowden, J., Davey Smith, G. and Burgess, S.** (2015). Mendelian randomization with invalid
613   instruments: effect estimation and bias detection through Egger regression. *Int J*
614   *Epidemiol* **44**, 512-525.
615 **Burgess, S., Dudbridge, F. and Thompson, S. G.** (2016). Combining information on multiple
616   instrumental variables in Mendelian randomization: comparison of allele score and
617   summarized data methods. *Stat Med* **35**, 1880-1906.
618 **Burgess, S., Zuber, V., Valdes-Marquez, E., Sun, B. B. and Hopewell, J. C.** (2017).
619   Mendelian randomization with fine-mapped genetic data: Choosing from large numbers
620   of correlated instrumental variables. *Genet Epidemiol* **41**, 714-725.
621 **Butler, A., Hoffman, P., Smibert, P., Papalexi, E. and Satija, R.** (2018). Integrating single-
622   cell transcriptomic data across different conditions, technologies, and species. *Nat*
623   *Biotechnol* **36**, 411-420.
624 **Bycroft, C., Freeman, C., Petkova, D., Band, G., Elliott, L. T., Sharp, K., Motyer, A.,**
625   **Vukcevic, D., Delaneau, O., O'Connell, J., et al.** (2018). The UK Biobank resource
626   with deep phenotyping and genomic data. *Nature* **562**, 203-209.

627 **Carlsson, L., Clarke, J. C., Yen, C., Gregoire, F., Albery, T., Billger, M., Egnell, A.-C.,**
628   **Gan, L.-M., Jennbacken, K. and Johansson, E.** (2018). Biocompatible, purified
629   VEGF-A mRNA improves cardiac function after intracardiac injection 1 week post-
630   myocardial infarction in swine. *Molecular Therapy-Methods & Clinical Development* **9**,
631   330-346.
632 **Chen, Y., Luttmann, F. F., Schoger, E., Scholer, H. R., Zelarayan, L. C., Kim, K. P., Haigh,**
633   **J. J., Kim, J. and Braun, T.** (2021). Reversible reprogramming of cardiomyocytes to a
634   fetal state drives heart regeneration in mice. *Science* **373**, 1537-1540.
635 **Darehzereshki, A., Rubin, N., Gamba, L., Kim, J., Fraser, J., Huang, Y., Billings, J.,**
636   **Mohammadzadeh, R., Wood, J., Warburton, D., et al.** (2015). Differential
637   regenerative capacity of neonatal mouse hearts after cryoinjury. *Dev Biol* **399**, 91-99.
638 **Das, S., Goldstone, A. B., Wang, H., Farry, J., D'Amato, G., Paulsen, M. J., Eskandari, A.,**
639   **Hironaka, C. E., Phansalkar, R., Sharma, B., et al.** (2019). A Unique Collateral Artery
640   Development Program Promotes Neonatal Heart Regeneration. *Cell* **176**, 1128-1142
641   e1118.
642 **Davey Smith, G. and Hemani, G.** (2014). Mendelian randomization: genetic anchors for causal
643   inference in epidemiological studies. *Hum Mol Genet* **23**, R89-98.
644 **Drakos, S. G., Kfoury, A. G., Hammond, E. H., Reid, B. B., Revelo, M. P., Rasmusson, B.**
645   **Y., Whitehead, K. J., Salama, M. E., Selzman, C. H. and Stehlik, J.** (2010). Impact of
646   mechanical unloading on microvasculature and associated central remodeling features of
647   the failing human heart. *Journal of the American College of Cardiology* **56**, 382-391.
648 **Emdin, C. A., Khera, A. V. and Kathiresan, S.** (2017). Mendelian Randomization. *JAMA* **318**,
649   1925-1926.
650 **Erben, L. and Buonanno, A.** (2019). Detection and quantification of multiple RNA sequences
651   using emerging ultrasensitive fluorescent in situ hybridization techniques. *Current*
652   *protocols in neuroscience* **87**, e63.
653 **Fan, Q., Mao, H., Angelini, A., Coarfa, C., Robertson, M. J., Lagor, W. R., Wehrens, X. H.**
654   **T., Martin, J. F., Pi, X. and Xie, L.** (2019). Depletion of Endothelial Prolyl
655   Hydroxylase Domain Protein 2 and 3 Promotes Cardiomyocyte Proliferation and
656   Prevents Ventricular Failure Induced by Myocardial Infarction. *Circulation* **140**, 440-
657   442.
658 **Ference, B. A.** (2017). How to use Mendelian randomization to anticipate the results of
659   randomized trials. *European Heart Journal* **39**, 360-362.
660 **Fernandez, C. E., Bakovic, M. and Karra, R.** (2018). Endothelial Contributions to Zebrafish
661   Heart Regeneration. *J Cardiovasc Dev Dis* **5**.
662 **Ferrarini, M., Arsic, N., Recchia, F. A., Zentilin, L., Zacchigna, S., Xu, X., Linke, A.,**
663   **Giacca, M. and Hintze, T. H.** (2006). Adeno-associated virus-mediated transduction of
664   VEGF165 improves cardiac tissue viability and functional recovery after permanent
665   coronary occlusion in conscious dogs. *Circulation research* **98**, 954-961.
666 **Folkersen, L., Gustafsson, S., Wang, Q., Hansen, D. H., Hedman, A. K., Schork, A., Page,**
667   **K., Zhernakova, D. V., Wu, Y., Peters, J., et al.** (2020). Genomic and drug target
668   evaluation of 90 cardiovascular proteins in 30,931 individuals. *Nat Metab* **2**, 1135-1148.
669 **Fuchs, E. and Blau, H. M.** (2020). Tissue Stem Cells: Architects of Their Niches. *Cell Stem*
670   *Cell* **27**, 532-556.
671 **Gray, R. and Wheatley, K.** (1991). How to avoid bias when comparing bone marrow
672   transplantation with chemotherapy. *Bone marrow transplantation* **7**, 9-12.

22

673 **Hewitt, F. C., Li, C., Gray, S. J., Cockrell, S., Washburn, M. and Samulski, R. J.** (2009).
674   Reducing the risk of adeno-associated virus (AAV) vector mobilization with AAV type 5
675   vectors. *J Virol* **83**, 3919-3929.
676 **Hooper, A. T., Butler, J. M., Nolan, D. J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H. G.,**
677   **Shido, K., Petit, I., Yanger, K., et al.** (2009). Engraftment and reconstitution of
678   hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial
679   cells. *Cell Stem Cell* **4**, 263-274.
680 **Janavel, G. V., Crottogini, A., Meckert, P. C., Cuniberti, L., Mele, A., Papouchado, M.,**
681   **Fernandez, N., Bercovich, A., Criscuolo, M. and Melo, C.** (2006). Plasmid-mediated
682   VEGF gene transfer induces cardiomyogenesis and reduces myocardial infarct size in
683   sheep. *Gene therapy* **13**, 1133-1142.
684 **Jean-Charles, P. Y., Yu, S. M., Abraham, D., Kommaddi, R. P., Mao, L., Strachan, R. T.,**
685   **Zhang, Z. S., Bowles, D. E., Brian, L., Stiber, J. A., et al.** (2017). Mdm2 regulates
686   cardiac contractility by inhibiting GRK2-mediated desensitization of beta-adrenergic
687   receptor signaling. *JCI Insight* **2**.
688 **Jopling, C., Sleep, E., Raya, M., Marti, M., Raya, A. and Izpisua Belmonte, J. C.** (2010).
689   Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation.
690   *Nature* **464**, 606-609.
691 **Jopling, C., Suñé, G., Faucherre, A., Fabregat, C. and Izpisua Belmonte, J. C.** (2012).
692   Hypoxia induces myocardial regeneration in zebrafish. *Circulation* **126**, 3017-3027.
693 **Karra, R., Foglia, M. J., Choi, W. Y., Belliveau, C., DeBenedittis, P. and Poss, K. D.** (2018).
694   Vegfaa instructs cardiac muscle hyperplasia in adult zebrafish. *Proc Natl Acad Sci U S A*
695   **115**, 8805-8810.
696 **Karra, R., Knecht, A. K., Kikuchi, K. and Poss, K. D.** (2015). Myocardial NF-kappaB
697   activation is essential for zebrafish heart regeneration. *Proc Natl Acad Sci U S A* **112**,
698   13255-13260.
699 **Kassambara, A.** (2017). ggpubr:"ggplot2" based publication ready plots. R package version 0.1.
700   6.
701 **Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F.,**
702   **Evans, T., Macrae, C. A., Stainier, D. Y. and Poss, K. D.** (2010). Primary contribution
703   to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* **464**, 601-605.
704 **Kimura, W., Xiao, F., Canseco, D. C., Muralidhar, S., Thet, S., Zhang, H. M.,**
705   **Abderrahman, Y., Chen, R., Garcia, J. A., Shelton, J. M., et al.** (2015). Hypoxia fate
706   mapping identifies cycling cardiomyocytes in the adult heart. *Nature* **523**, 226-230.
707 **Kivela, R., Hemanthakumar, K. A., Vaparanta, K., Robciuc, M., Izumiya, Y., Kidoya, H.,**
708   **Takakura, N., Peng, X., Sawyer, D. B., Elenius, K., et al.** (2019). Endothelial Cells
709   Regulate Physiological Cardiomyocyte Growth via VEGFR2-Mediated Paracrine
710   Signaling. *Circulation* **139**, 2570-2584.
711 **Kobayashi, Y., Tata, A., Konkimalla, A., Katsura, H., Lee, R. F., Ou, J., Banovich, N. E.,**
712   **Kropski, J. A. and Tata, P. R.** (2020). Persistence of a regeneration-associated,
713   transitional alveolar epithelial cell state in pulmonary fibrosis. *Nat Cell Biol* **22**, 934-946.
714 **Lamprecht, M. R., Sabatini, D. M. and Carpenter, A. E.** (2007). CellProfiler: free, versatile
715   software for automated biological image analysis. *Biotechniques* **42**, 71-75.
716 **Lane, S. W., Williams, D. A. and Watt, F. M.** (2014). Modulating the stem cell niche for tissue
717   regeneration. *Nat Biotechnol* **32**, 795-803.

Lee, H. K., Widmayer, S. J., Huang, M.-N., Aylor, D. L. and Marchuk, D. A. (2019). Novel Neuroprotective Loci Modulating Ischemic Stroke Volume in Wild-Derived Inbred Mouse Strains. *Genetics* **213**, 1079-1092.

Liang, W.-C., Wu, X., Peale, F. V., Lee, C. V., Meng, Y. G., Gutierrez, J., Fu, L., Malik, A. K., Gerber, H.-P. and Ferrara, N. (2006). Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. *Journal of Biological Chemistry* **281**, 951-961.

Lin, Y. D., Luo, C. Y., Hu, Y. N., Yeh, M. L., Hsueh, Y. C., Chang, M. Y., Tsai, D. C., Wang, J. N., Tang, M. J., Wei, E. I., et al. (2012). Instructive nanofiber scaffolds with VEGF create a microenvironment for arteriogenesis and cardiac repair. *Sci Transl Med* **4**, 146ra109.

Liu, X., De la Cruz, E., Gu, X., Balint, L., Oxendine-Burns, M., Terrones, T., Ma, W., Kuo, H. H., Lantz, C., Bansal, T., et al. (2020). Lymphoangiocrine signals promote cardiac growth and repair. *Nature*.

Macosko, E. Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A. R., Kamitaki, N., Martersteck, E. M., et al. (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell* **161**, 1202-1214.

Mahmoud, A. I., O'Meara, C. C., Gemberling, M., Zhao, L., Bryant, D. M., Zheng, R., Gannon, J. B., Cai, L., Choi, W. Y., Egnaczyk, G. F., et al. (2015). Nerves Regulate Cardiomyocyte Proliferation and Heart Regeneration. *Dev Cell* **34**, 387-399.

Marin-Juez, R., El-Sammak, H., Helker, C. S. M., Kamezaki, A., Mullapuli, S. T., Bibli, S. I., Foglia, M. J., Fleming, I., Poss, K. D. and Stainier, D. Y. R. (2019). Coronary Revascularization During Heart Regeneration Is Regulated by Epicardial and Endocardial Cues and Forms a Scaffold for Cardiomyocyte Repopulation. *Dev Cell* **51**, 503-515 e504.

Marin-Juez, R., Marass, M., Gauvrit, S., Rossi, A., Lai, S. L., Materna, S. C., Black, B. L. and Stainier, D. Y. (2016). Fast revascularization of the injured area is essential to support zebrafish heart regeneration. *Proc Natl Acad Sci U S A* **113**, 11237-11242.

Marneros, A. G. (2018). Effects of chronically increased VEGF-A on the aging heart. *FASEB J* **32**, 1550-1565.

Miao, Y., Tian, L., Martin, M., Paige, S. L., Galdos, F. X., Li, J., Klein, A., Zhang, H., Ma, N. and Wei, Y. (2020). Intrinsic Endocardial Defects Contribute to Hypoplastic Left Heart Syndrome. *Cell Stem Cell* **27**, 574-589. e578.

Miller, G., Best, M., Franko, A., Koch, C. and Raleigh, J. (1989). Quantitation of hypoxia in multicellular spheroids by video image analysis. *International Journal of Radiation Oncology* Biology* Physics* **16**, 949-952.

Mosseri, M., Schaper, J., Admon, D., Hasin, Y., Gotsman, M., Sapoznikov, D., Pickering, J. and Yarom, R. (1991). Coronary capillaries in patients with congestive cardiomyopathy or angina pectoris with patent main coronary arteries. Ultrastructural morphometry of endomyocardial biopsy samples. *Circulation* **84**, 203-210.

Nakada, Y., Canseco, D. C., Thet, S., Abdisalaam, S., Asaithamby, A., Santos, C. X., Shah, A. M., Zhang, H., Faber, J. E., Kinter, M. T., et al. (2017). Hypoxia induces heart regeneration in adult mice. *Nature* **541**, 222-227.

Oberpriller, J. and Oberpriller, J. C. (1971). Mitosis in adult newt ventricle. *J Cell Biol* **49**, 560-563.

24

764 **Oh, J. G. and Ishikawa, K.** (2019). Recent highlights and advances in cardiac gene therapy.
765   *Discov Med* **28**, 229-235.
766 **Parodi, O., De Maria, R., Oltrona, L., Testa, R., Sambuceti, G., Roghi, A., Merli, M.,**
767   **Belingheri, L., Accinni, R. and Spinelli, F.** (1993). Myocardial blood flow distribution
768   in patients with ischemic heart disease or dilated cardiomyopathy undergoing heart
769   transplantation. *Circulation* **88**, 509-522.
770 **Pearlman, J. D., Hibberd, M. G., Chuang, M. L., Harada, K., Lopez, J. J., Gladstone, S. R.,**
771   **Friedman, M., Sellke, F. W. and Simons, M.** (1995). Magnetic resonance mapping
772   demonstrates benefits of VEGF–induced myocardial angiogenesis. *Nature medicine* **1**,
773   1085-1089.
774 **Polizzotti, B. D., Ganapathy, B., Haubner, B. J., Penninger, J. M. and Kuhn, B.** (2016). A
775   cryoinjury model in neonatal mice for cardiac translational and regeneration research. *Nat*
776   *Protoc* **11**, 542-552.
777 **Polizzotti, B. D., Ganapathy, B., Walsh, S., Choudhury, S., Ammanamanchi, N., Bennett,**
778   **D. G., dos Remedios, C. G., Haubner, B. J., Penninger, J. M. and Kuhn, B.** (2015).
779   Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium
780   reveals a therapeutic window. *Sci Transl Med* **7**, 281ra245.
781 **Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N.**
782   **and Sadek, H. A.** (2011). Transient regenerative potential of the neonatal mouse heart.
783   *Science* **331**, 1078-1080.
784 **Poss, K. D., Wilson, L. G. and Keating, M. T.** (2002). Heart regeneration in zebrafish. *Science*
785   **298**, 2188-2190.
786 **Puente, B. N., Kimura, W., Muralidhar, S. A., Moon, J., Amatruda, J. F., Phelps, K. L.,**
787   **Grinsfelder, D., Rothermel, B. A., Chen, R., Garcia, J. A., et al.** (2014). The oxygen-
788   rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage
789   response. *Cell* **157**, 565-579.
790 **Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D., Maller, J.,**
791   **Sklar, P., de Bakker, P. I., Daly, M. J., et al.** (2007). PLINK: a tool set for whole-
792   genome association and population-based linkage analyses. *Am J Hum Genet* **81**, 559-
793   575.
794 **Rafii, S., Butler, J. M. and Ding, B. S.** (2016). Angiocrine functions of organ-specific
795   endothelial cells. *Nature* **529**, 316-325.
796 **Raleigh, J. A., Calkins-Adams, D. P., Rinker, L. H., Ballenger, C. A., Weissler, M. C.,**
797   **Fowler, W. C., Jr., Novotny, D. B. and Varia, M. A.** (1998). Hypoxia and vascular
798   endothelial growth factor expression in human squamous cell carcinomas using
799   pimonidazole as a hypoxia marker. *Cancer Res* **58**, 3765-3768.
800 **Schmidt, A. F., Finan, C., Gordillo-Maranon, M., Asselbergs, F. W., Freitag, D. F., Patel, R.**
801   **S., Tyl, B., Chopade, S., Faraway, R., Zwierzyna, M., et al.** (2020). Genetic drug target
802   validation using Mendelian randomisation. *Nat Commun* **11**, 3255.
803 **Shen, H., Gan, P., Wang, K., Darehzereshki, A., Wang, K., Kumar, S. R., Lien, C.-L.,**
804   **Patterson, M., Tao, G. and Sucov, H. M.** (2020). Mononuclear diploid cardiomyocytes
805   support neonatal mouse heart regeneration in response to paracrine IGF2 signaling. *Elife*
806   **9**, e53071.
807 **Sievert, C., Parmer, C., Hocking, T., Chamberlain, S., Ram, K., Corvellec, M. and**
808   **Despouy, P.** (2018). Plotly: create interactive web graphics via 'plotly. js'. R package.
809   version 4.7. 1; 2017. *URL https://CRAN. R-project. org/package= plotly*.

810 **Soonpaa, M. H., Kim, K. K., Pajak, L., Franklin, M. and Field, L. J.** (1996). Cardiomyocyte
811   DNA synthesis and binucleation during murine development. *American Journal of*
812   *Physiology-Heart and Circulatory Physiology* **271**, H2183-H2189.
813 **Sorensen, I., Adams, R. H. and Gossler, A.** (2009). DLL1-mediated Notch activation regulates
814   endothelial identity in mouse fetal arteries. *Blood* **113**, 5680-5688.
815 **Taimeh, Z., Loughran, J., Birks, E. J. and Bolli, R.** (2013). Vascular endothelial growth factor
816   in heart failure. *Nat Rev Cardiol* **10**, 519-530.
817 **Tsagalou, E. P., Anastasiou-Nana, M., Agapitos, E., Gika, A., Drakos, S. G., Terrovitis, J.**
818   **V., Ntalianis, A. and Nanas, J. N.** (2008). Depressed coronary flow reserve is associated
819   with decreased myocardial capillary density in patients with heart failure due to
820   idiopathic dilated cardiomyopathy. *Journal of the American College of Cardiology* **52**,
821   1391-1398.
822 **Wagers, A. J.** (2012). The stem cell niche in regenerative medicine. *Cell Stem Cell* **10**, 362-369.
823 **Wang, J., Cao, J., Dickson, A. L. and Poss, K. D.** (2015). Epicardial regeneration is guided by
824   cardiac outflow tract and Hedgehog signalling. *Nature* **522**, 226-230.
825 **Wang, Z., Cui, M., Shah, A. M., Tan, W., Liu, N., Bassel-Duby, R. and Olson, E. N.** (2020).
826   Cell-Type-Specific Gene Regulatory Networks Underlying Murine Neonatal Heart
827   Regeneration at Single-Cell Resolution. *Cell Rep* **33**, 108472.
828 **Wickham, H.** (2011). ggplot2. *Wiley Interdisciplinary Reviews: Computational Statistics* **3**, 180-
829   185.
830 **Wickham, H., Francois, R., Henry, L. and Müller, K.** (2015). dplyr: A Grammar of Data
831   Manipulation: https://CRAN. *R-project. org/package= dplyr*.
832 **Wu, X., Reboll, M. R., Korf-Klingebiel, M. and Wollert, K. C.** (2020). Angiogenesis After
833   Acute Myocardial Infarction. *Cardiovasc Res*.
834 **Yavorska, O. O. and Burgess, S.** (2017). MendelianRandomization: an R package for
835   performing Mendelian randomization analyses using summarized data. *Int J Epidemiol*
836   **46**, 1734-1739.
837 **Zangi, L., Lui, K. O., von Gise, A., Ma, Q., Ebina, W., Ptaszek, L. M., Spater, D., Xu, H.,**
838   **Tabebordbar, M., Gorbatov, R., et al.** (2013). Modified mRNA directs the fate of heart
839   progenitor cells and induces vascular regeneration after myocardial infarction. *Nat*
840   *Biotechnol* **31**, 898-907.
841 **Zhao, L., Ben-Yair, R., Burns, C. E. and Burns, C. G.** (2019). Endocardial Notch Signaling
842   Promotes Cardiomyocyte Proliferation in the Regenerating Zebrafish Heart through Wnt
843   Pathway Antagonism. *Cell Rep* **26**, 546-554 e545.
844 **Zhao, L., Borikova, A. L., Ben-Yair, R., Guner-Ataman, B., MacRae, C. A., Lee, R. T.,**
845   **Burns, C. G. and Burns, C. E.** (2014). Notch signaling regulates cardiomyocyte
846   proliferation during zebrafish heart regeneration. *Proc Natl Acad Sci U S A* **111**, 1403-
847   1408.

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Figure 1. Myovascular coupling during early neonatal growth. **(A)** Experimental schematic showing the region of the heart (boxed area) that was imaged at each time point. **(B)** Representative images from postnatal day 1 (P1), 5 (P5), 7 (P7), and 10 (P10) neonatal mouse hearts immunostained for Erg to mark CECs and EdU to identify cycling CECs (arrows). Magnified insets show an example of an EdU$^+$, Erg$^+$ CEC. **(C)** Representative images immunostained for PCM1 and EdU. Arrows point to EdU$^+$PCM1$^+$ proliferating CMs. Magnified insets show an example of EdU$^+$, PCM1$^+$ CM. **(D, E)** Quantitation of CEC cycling and CEC density relative to a P1 heart (n = 6 mice per time point). **(F, G)** Quantitation CM cycling and CM density relative to a P1 heart (n = 6 mice per time point). Each gray point is an individual heart. *p < 0.05, **p < 0.01, ***p< 0.001, ns = not significant, two-sided t-test corrected for multiple comparisons using Holm's method. **(H)** Correlation of CEC and CM cycling. Black line is the best-fit regression line and gray area indicates the 95% CI. p value indicates significance of Pearson correlation. Each point represents an individual heart and is color coded by age. **(I)** Representative image from a P4 heart immunostained for PCM1, Erg, EdU, and DAPI. Boxed regions correspond to the adjacent magnified panels. Hollowed, yellow arrows are PCM1$^+$EdU$^+$ CMs and solid yellow arrows are Erg$^+$EdU$^+$ CECs. **(J)** Violin plots of pseudodistance distributions, showing enrichment of EdU$^+$ CECs around EdU$^+$ CMs. Plot shows the distance of EdU$^+$ CECs (red dots) and EdU$^-$ CECs (blue dots) relative to an averaged EdU$^-$ and EdU$^+$ CM. Data for a representative P4 heart is shown. **(K)** Proportion of CEC's that are EdU$^+$ as a function of distance (μm) from PCM1$^+$EdU$^+$ nuclei (red) and PCM1$^+$EdU$^-$ nuclei (blue). 274 EdU$^+$ CMs and 3658 EdU$^-$ CMs from 6 mice were considered, p = 0.04 at 7 μm, two-sided Z-test. Scale bars are 50 μm.

Figure 2. Myovascular coupling after cryoinjury. **(A)** Experimental schematic showing the region of the heart (boxed area) that was imaged at each time point. The blue region corresponds to the injury. **(B)** Representative images of AFOG-stained heart sections collected at P3, P5, P7, and P10 after cryoinjury (CI) at P1. Scar stains blue. Scale bar is 500 μm. **(C, D)** Representative images from hearts collected at P3, P5, P7, and P10 after CI. Arrows point to EdU^+Erg^+ proliferating CECs in (C) and EdU^+PCM1^+ proliferating CMs in (D). Magnified insets show example EdU^+ CECs and CMs. **(E-H)** Quantitation of CEC and CM cycling and relative density after CI. Densities are relative to a P3 heart (n = 5-6 mice per time point). Each gray point is an individual heart. **p < 0.01, ***p< 0.001, two-sided t-test corrected for multiple comparisons using Holm's method. **(I)** Correlation of CEC and CM proliferation. Black line is the best-fit regression line and gray area indicates the 95% CI. p value indicates significance of Pearson correlation. Each point is an individual heart, color coded by age. **(J)** Representative section from the border zone of a cryoinjured P4 heart immunostained for PCM1, Erg, EdU, and DAPI. Boxed regions correspond to the adjacent magnified panels. Hollowed, yellow arrows are $PCM1^+EdU^+$ CMs and solid yellow arrows are Erg^+EdU^+ CECs. **(K)** Violin plots of pseudodistance distributions, showing no enrichment of EdU^+ CECs around EdU^+ CMs in the border zone after CI. Plot shows the distance of EdU^+ CECs (red dots) and EdU^- CECs (blue dots) relative to an averaged EdU^- and EdU^+ CM. Mapping for a representative heart is shown. **(L)** Proportion of CEC's that are EdU^+ as a function of distance in μm from $PCM1^+EdU^+$ nuclei (red) and $PCM1^+EdU^-$ nuclei (blue). Solid line indicates the point estimate. 186 EdU^+ CMs and 2446 EdU^- CMs were considered, p = not significant (ns) at 7 μm, two-sided Z-test. Scale bars are 50 μm.

896 **Figure 3. VEGFA-VEGFR2 signaling is associated with myovascular coupling**. **(A)** tSNE
897 plots of scRNA-seq data from P7 hearts that were underwent CI at P1. In the left most panel, cells
898 are colored by clusters identified by PCA analysis. Arrows point to a cluster of CECs that are
899 enriched for expression of *Mki67* and *Vegfr2* (purple). **(B, C)** EdU$^+$VEGFR2$^+$ CEC adjacent to
900 EdU$^+$Tnnt$^+$ CM at P7 during growth (B) and after CI at P1 (C). Insets are magnifications of the
901 regions in the solid white boxes. Scale bars are 50 μm for the main panel and 7 μm for the insets.
902 **(D)** Single molecule in situ hybridization for *Vegfa* (red) in P3 and P10 hearts during physiologic
903 growth. **(E)** Single molecule in situ hybridization for *Vegfa* (red) in P3 and P10 after CI at P1.
904 Dashed line indicates the approximate injury plane, with the border zone above the line. **(F)**
905 Quantitation of in situ hybridization for *Vegfa* expression during growth and in the border zone
906 after CI. n = 3-5 hearts per condition. Each point is a from a unique heart. *p < 0.05, **p < 0.01,
907 two-sided t-test. Scale bars are 50 μm.
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Figure 4: Requirement of *Vegfr2* in CECs for myovascular growth in the neonatal mouse heart. **(A)** Representative images of P4 hearts from *Vegfr2*^{WT} and *Vegfr2*$^{\Delta EC}$ mice, immunostained to detect cycling CECs. Arrows point to EdU$^+$ Erg$^+$ cycling CECs. **(B, C)** Quantitation of CEC proliferation and relative CEC density in *Vegfr2*^{WT} (n = 5) and *Vegfr2*$^{\Delta EC}$ mice (n = 6). CEC density is reported relative to *Vegfr2*^{WT} hearts. ****p = 3.7x10^{-5} and ****p = 2.3x10^{-5} for B and C respectively, two-sided t-test. **(D)** Representative images of P4 hearts from *Vegfr2*^{WT} and *Vegfr2*$^{\Delta EC}$ mice, immunostained to detect cycling CMs. Arrows point to EdU$^+$PCM1$^+$ cycling CMs. **(E, F)** Quantitation of CM proliferation and relative density in *Vegfr2*^{WT} (n = 5) and *Vegfr2*^{DEC} mice (n = 6). CM density is reported relative to *Vegfr2*^{WT} hearts. ***p = 0.0004 and p = 0.57 for E and F respectively, two-sided t-test. **(G)** Images of P4 hearts from *Vegfr2*^{WT} and *Vegfr2*$^{\Delta EC}$ mice after CI at P1. Sections are immunostained to detect cycling CECs. Arrows point to EdU$^+$Erg$^+$ cycling CECs. **(H, I)** Quantitation of CEC proliferation and relative CEC density in *Vegfr2*^{WT} (n = 7) and *Vegfr2*$^{\Delta EC}$ mice (n = 8). ****p = 2.0x10^{-5} and ****p = 7.7x10^{-5} for H and I respectively, two-sided t-test. **(J)** Representative images of P4 hearts injured at P1 from *Vegfr2*^{WT} and *Vegfr2*$^{\Delta EC}$ mice. Sections are immunostained to detect cycling CMs. Arrows point to EdU$^+$PCM1$^+$ cycling CMs. **(K, L)** Quantitation of CM cycling and relative density in *Vegfr2*^{WT} (n = 7) and *Vegfr2*$^{\Delta EC}$ mice (n = 8). **p = 0.004 and p = 0.98 for K and L respectively, two-sided t-test. Magnified insets in A, D, G, and J show example EdU$^+$ CECs and CMs. Scale bars are 50 μm.

Figure 5: Requirement of VEGFA for myovascular growth in the neonatal mouse heart. **(A)** Representative images of P5 hearts from mice injected with PBS vehicle or anti-VEGFA. Sections are immunostained to detect cycling CECs. Arrows point to EdU$^+$Erg$^+$ CECs. **(B, C)** Quantitation of CEC cycling and relative density at P5 after treatment with vehicle (n = 10) or anti-VEGFA (n = 5). CEC density is reported relative to the PBS control group. ****p = 1.8×10^{-6} and **p = 0.007 for B and C respectively, two-sided t-test. **(D)** Representative images of P5 hearts after treatment with PBS or anti-VEGFA. Sections are immunostained to detect cycling CMs. Arrows point to EdU$^+$PCM1$^+$ cycling CMs. **(E, F)** Quantitation of CM cycling and relative density at P5 after treatment with PBS (n = 10) or anti-VEGFA (n = 5). CM density is reported relative to the PBS control group. **p = 0.003 and p = 0.39 for E and F respectively, two-sided t-test. **(G)** Images of P5 hearts from mice that underwent CI at P1 and that were injected with PBS or anti-VEGFA. Arrows point to EdU$^+$Erg$^+$ proliferating CECs. **(H, I)** Quantitation of CEC cycling and relative density in mice treated with PBS (n = 4) or anti-VEGFA (n = 3) after CI. ****p = 6.9×10^{-5} and **p = 0.004 for H and I respectively, two-sided t-test. **(J)** Immunostaining for PCM1 and EdU in P5 hearts from mice that underwent CI at P1 and that were treated with PBS or anti-VEGFA. Arrows point to EdU$^+$PCM1$^+$ CMs. **(K, L)** Quantitation of CM cycling and relative density after treatment with PBS (n = 4) or anti-VEGFA (n = 3). **p = 0.008 and 0.28 for K and L respectively, two-sided t-test. Magnified insets in A, D, G, and J show example EdU$^+$ CECs and CMs. Scale bars are 50 μm.

953 **Figure 6: Effect of *Vegfa* overexpression on regeneration after CI**. **(A)** AFOG staining of P21

954 hearts to visualize scar (blue). Arrows indicate vessels. Scale bar is 500 μm. **(B)** Quantitation of

955 scar as a percentage of the left ventricle at P21 in animals injected with AAV-*GFP* (n = 8) or

956 AAV-*Vegfa* (n= 8) at the time of CI at P1. *p = 0.038. **(C)** Fractional shortening at P21 in animals

957 injected with AAV-*GFP* (n=6) or AAV-*Vegfa* (n=8) at the time of CI at P1. **p = 0.008, two-

958 sided t-test. **(D)** Images of the border zone from P10 mice that underwent CI at P1. Sections are

959 immunostained to mark Erg$^+$ Ki67$^+$ CECs (arrows). **(E)** Relative CEC density at P14 after injection

960 with AAV-GFP (n=4) or AAV-*Vegfa* (n = 4). *p = 0.035, two-sided t-test. **(F)** Images of the border

961 zone from P14 mice that underwent CI at P1. Sections are immunostained to mark PCM1$^+$Ki67$^+$

962 CMs (arrows). **(G)** Quantitation of CM cycling at P14 after injection with AAV-*GFP* (n = 4) or

963 AAV-*Vegfa* (n = 4). *p =0.017, two-sided t-test.

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Figure 7: Effect of circulating VEGFA levels on human cardiac structure. (A) Illustrative diagram of *cis*-Mendelian randomization (cis-MR) design to estimate the causal association between circulating VEGFA level and left ventricular mass (LVM). **(B)** Manhattan plot showing genome-wide genetic association with circulating VEGFA level. **(C)** *cis*-Mendelian randomization estimate with inverse-variance weighted model of circulating VEGFA level on left ventricular end-diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), left ventricular ejection fraction (LVEF), left ventricular mass to volume ratio (LVMR), and LV mass (LVM). Each data point represents effect estimate in standard deviation (SD) change of the trait per doubling the circulating VEGFA concentration. Error bars indicate 95% confidence intervals (CI) of the estimates. *p < 0.05, **p < 0.01. **(D)** Regional genetic association plot for *cis*-region of the *VEGFA* gene (200 kilobase flanking region from transcript start and end sites) with *cis*-instruments for the main Mendelian randomization analysis highlighted. Estimated causal effects on LVM for each instrument are shown on the right panel with error bars representing 95% confidence interval. **(E)** Distribution of point estimates showing standard deviation (SD) gram change in LVM per doubling circulating VEGFA level as estimated with *cis*-MR with IVW model (red diamond) and sensitivity analyses with permutation of instrument selection methods and *cis*-MR models.