Endothelial Plasticity Drives Arterial Remodeling Within the Endocardium Following Myocardial Infarction

Lucile Miquerol¹, Jérome Thireau², Patrice Bideaux², Rachel Sturny¹, Sylvain Richard² and Robert G. Kelly¹

¹Aix Marseille Université, CNRS, IBDM UMR 7288, 13288, Marseille, France, and; ²PHYMEDEXP, Physiologie et Médecine Expérimentale Cœur et Muscles, INSERM U1046, CNRS UMR 9214, Université de Montpellier, CHU Arnaud de Villeneuve, 34295, Montpellier, France.

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Address correspondence to:
Dr. Lucile Miquerol
Aix Marseille Université
CNRS
IBDM UMR 7288
Campus de Luminy - Case 907
13288 Marseille Cedex 9
France
Tel: +33 4 91 26 97 34
Fax: +33 4 91 26 93 16
lucile.miquerol@univ-amu.fr

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ABSTRACT

Rationale: Revascularization of injured, ischemic and regenerating organs is essential to restore organ function. In the post-infarct heart, however, the mechanisms underlying the formation of new coronary arteries are poorly understood.

Objective: To study vascular remodeling of coronary arteries following infarction.

Methods and Results: We performed permanent left coronary ligation on Connexin40-GFP mice expressing GFP in endothelial cells of coronary arteries but not veins, capillaries or endocardium. GFP* endothelial foci were identified within the endocardium in the infarct zone. These previously undescribed structures, termed endocardial flowers, have a distinct endothelial phenotype (Cx40* and VEGFR2*, Endoglin*) to the surrounding endocardium (Cx40* and VEGFR2*, Endoglin*). Endocardial flowers are contiguous with coronary vessels and associated with sub-endocardial smooth muscle cell accumulation. Genetic lineage tracing reveals extensive endothelial plasticity in the post-infarct heart, showing that endocardial flowers develop by arteriogenesis of Cx40* cells as well as by outgrowth of pre-existing coronary arteries. Finally, endocardial flowers exhibit angiogenic features including early VEGFR2 expression and active proliferation of adjacent endocardial and smooth muscle cells.

Conclusions: Arterial endothelial foci within the endocardium reveal extensive endothelial cell plasticity in the infarct zone and identify the endocardium as a site of endogenous arteriogenesis and source of endothelial cells to promote vascularization in regenerative strategies.

Keywords: Arteriogenesis, coronary, endocardium, myocardial infarction, vascular remodeling, vasculature, cell plasticity, angiogenesis, differentiation, angiogenesis

Nonstandard Abbreviations and Acronyms:
GFP Green fluorescent protein
MI Myocardial infarction
RFP Red fluorescent protein
SMC Smooth muscle cell
INTRODUCTION

Ischemic heart disease following myocardial infarction (MI) causes irreversible cell loss and scaring and is a major cause of morbidity and mortality. Revascularization of the infarct zone is the most effective therapy after acute MI, although strategies have met with limited success. The formation of new vessels is also an essential component of regenerative approaches to heart repair. Efficient neovascularization requires redeployment of mechanisms generating the coronary vasculature during development. Recent evidence suggests that the coronary vasculature is a developmental mosaic with contributions from epicardially-derived cells and the sinus venosus. The endocardium has also emerged as a major source of coronary endothelial cells in the fetal and early postnatal heart in genetic tracing experiments using Nfatc1 or Apln regulatory sequences. However, the importance of the endocardium in the generation of new vessels in the injured adult heart is unknown.

Here we directly visualize coronary vascular remodeling in post-MI hearts using a Cx40-GFP allele expressed in arterial coronary endothelium and identify unexpected endothelial cell plasticity on the endocardial surface of the infarct area. We present evidence that activation of arterial markers in the endocardium occurs by arteriogenesis, suggesting that the endocardium contributes to coronary vessels following MI as well as during development, identifying a new target to stimulate vascularization for heart repair.

METHODS

Experimental animals. Gtga5a^tm1(EGFP) (Cx40-GFP)10, Gtga5a^tm1(CreERT2-IRES-RFP) (Cx40-cre)11, Gtrosa26^tm1(LacZ)Sor (R26R) and Gtrosa26^tm1(EYFP)Cos (RYFP)12 have been previously reported. Details of genotyping and tamoxifen injection are provided in the Online Supplement. Animal experiments were carried out in accordance with French legislation.

Coronary ligation. Eight week-old mice were assigned to two groups: Sham-operated (Sham) and subjected to MI by left anterior descending coronary artery ligation (details in Online Supplement). Hearts were analyzed at 1-8 weeks post-MI.

Tissue analysis and immunofluorescence. Details of antibodies and procedures are provided in the Online Supplement. Images were acquired with an Apotome Zeiss microscope. Wholemount MI were imaged using a Zeiss two-photon (LSM 7 MP) or confocal microscope (LSM 780) and 3D images obtained using Volocity software.

RESULTS

Endocardial flowers: Identification of arterial endothelial foci within the endocardium following myocardial infarction.

We performed permanent ligation of the left descending coronary artery in adult Cx40-GFP mice expressing a fluorescent reporter gene in coronary arterial, but not endocardial, endothelial cells. An extensively remodeled network of GFP+ vasculature was observed within the infarct area in comparison to sham-operated hearts (Figure 1A-B). In particular, coronary arteries connected, via fistulae, with foci of GFP+ cells within the endocardium (Figure 1C). These GFP+ foci form flower-shaped structures connected to coronary stems (Figure 1D), referred to here as endocardial flowers. Such structures are a previously undescribed feature of vascular remodeling post-MI and were observed throughout the infarct zone but not in non-infarcted myocardium or sham-operated hearts. Time-course analysis indicated that the incidence of GFP+ foci was highest in the 3 weeks following MI and...
subsequently declined (Figure 1E). The infarct wall was imaged using two-photon microscopy to a depth of 300µm to reconstruct 3D images (Figure 1F and Supplementary Movie 1). Using the pan-endothelial marker Pecam1, we observed that, in contrast to the well-organized monolayer of hexagonal cells in the rest of the endocardium, GFP foci contain elongated Pecam1+ cells connected to either GFP+ or GFP− Pecam1+ vessels between 5-30µm in diameter.

**Cx40-GFP+ endocardial flowers have an arterial endothelial phenotype.**

The molecular properties of post-MI endocardial flowers were investigated by wholemount and histological co-immunofluorescence (Figure 2A-C). Analysis of Endoglin, expressed in endocardium, and VEGFR2, expressed in endothelial cells of capillaries but not large coronary arteries or endocardium, revealed that Cx40-GFP+ endocardial flowers possess a distinct endothelial phenotype (Cx40+, VEGFR2+, Endoglin+) to surrounding endocardium (Cx40+, VEGFR2−; Endoglin−). Cx40+ coronary arteries are surrounded by a layer of smooth muscle cells (SMCs; Figure 2D). Subendocardial SMA+ SMCs invariably localized adjacent to endocardial flowers. The connecting vessels were either large VEGFR2low vessels surrounded by SMA+SMCs or small VEGFR2high SMA− vessels (Figure 2E).

Wholemount immunofluorescence of VEGFR2 and Cx40-GFP reveals the existence of numerous VEGFR2+ Cx40-GFP− flowers and connecting vessels (Figure 2F). To further investigate the formation of post-MI endocardial arterial flowers, we performed a time-course analysis of VEGFR2, Cx40 and SMA expression patterns from days 3 to 28 after ligation. Three days after ligation, no endocardial flower was observed. However, we could detect patchy expression of VEGFR2 in Endoglin+ endocardial cells (Figure 2G). By day 7, VEGFR2+ endocardial flowers connecting with vessels can be distinguished (Figure 2G). Cx40-GFP expression is first observed in endocardial cells at this stage, although VEGFR2 is more broadly expressed in endocardial flowers and connecting vessels (Figure 2F-G). Quantification of these results confirms that VEGFR2 expression precedes that of Cx40 during the formation of endocardial flowers (Figure 2H). SMA expression is detected in sparse cells at day 3 which progressively cluster adjacent to endocardial flowers from day 7 onwards (Figure 2G). Endocardial flowers thus progressively acquire an arterial phenotype based both on endothelial gene expression and the recruitment of underlying SMCs.

**Genetic tracing of the Cx40+ endothelial lineage reveals active arteriogenesis.**

To investigate whether post-MI endocardial flowers are sites of new Cx40 expression or originate from pre-existing Cx40+ coronary arteries, we used a tamoxifen inducible Cx40-Cre-RFP mouse line to trace coronary arterial endothelium. Cx40cre+/+;R26LacZ/+ or Cx40cre+/+;R26YFP/+ mice were injected with tamoxifen once a week during the four consecutive weeks preceding the ligation to maximize recombination (Figure 3A). We observed Cx40-RFP+ endocardial flowers that were entirely negative for LacZ or YFP reporter genes (Figure 3B, 3D), suggesting that these structures and their attached vessels result from arteriogenesis of cells not previously expressing Cx40. LacZ+ or YFP+ vessels were observed in the vicinity of these RFP-only foci indicating that the RFP+ flowers did not result from incomplete Cre activity. YFP+ endothelial cells were observed within a subset of RFP+ endocardial flowers, suggesting that arteriogenesis also occurs by outgrowth of pre-existing coronary arteries (Figure 3C, 3E). These observations reveal extensive endothelial cell plasticity between endocardial and coronary arterial compartments in the infarct zone.

We next quantified the extent to which Cx40+ endothelial cells contribute to post-MI arteriogenesis. RFP-only vessels were scored as a percentage of the total number of Cx40+ vessels in coronary arteries in sham hearts, as well as in coronary arteries and endocardial flowers following MI (Figure 3F). 25% of coronary arteries were RFP-only in sham hearts, suggesting on-going remodeling of coronary vascular endothelium. In PMI hearts, RFP-only coronary arteries are only marginally increased over this level (33%). Similarly, the fraction of YFP-only vessels, that have lost their arterial phenotype at the time of dissection, and correspond to small diameter VEGFR2+ capillaries, was not significantly different between sham and PMI hearts (16% ±8 vs 18% ±10). In contrast, 54% of...
endocardial flowers were RFP-only, consistent with significant activation of the arterial program in Cx40+ endothelial cells (Figure 3F, p<0.01).

**Arteriogenesis within post-MI endocardium arises by angiogenesis of endocardial cells.**

To further investigate the mechanism by which endocardial flowers develop following MI, we looked for signs of angiogenesis. Angiogenesis initiates with the emergence of tip-cells, which sprout from a preexisting endothelium. VEGFR2 is an established marker of endothelial tip-cells. High magnification views reveal that VEGFR2+ cells present a tip-cell phenotype with numerous cellular projections (Figure 4A) that are in some cases arranged into a vessel without a visible lumen (Figure 4B). Furthermore, within Cx40+ VEGFR2+ flowers, VEGFR2 expression overlaps with Endoglin expression in Cx40-GFP+ cells at the border of the flowers (Figure 4B), consistent with an intermediate state during transdifferentiation of endocardial endothelial cells to a Cx40-GFP+ arterial fate. We found that Endomucin has an indistinguishable endocardial distribution to Endoglin (Figure 4C). The organization of stable tubes depends also on the presence of pericytes; NG2+ pericytes are observed adjacent to VEGFR2+ capillaries in sham-operated myocardium and in close apposition to VEGFR2+ endocardial flowers in post-MI hearts (Figure 4C).

Finally, we investigated whether the switch in endothelial program at the border of the endocardial flowers was associated with an increase in cell division using the proliferative cell marker Ki67 in serial sections (Figure 4D). Angiogenesis is associated with elevated proliferation of endothelial stalk cells. We observed that the majority of proliferating endothelial cells are Endoglin+ and localized at the border of endocardial flowers (red arrowhead). In addition, 45-70% of proliferating cells at the sites of endocardial flowers were subendocardial SMCs (cyan arrowhead). Next, we used the mitotic marker, phospho-Histone H3, and found positive cells in Endomucin+ and SMA+ compartments (Figure 4E). This result emphasizes the proliferative properties of endocardial and smooth muscle cells associated with endocardial flowers.

**DISCUSSION**

Endocardium has recently been shown to be a major source of endothelial cells during development of the coronary vasculature. Here we reveal unprecedented endothelial plasticity between endocardial and coronary compartments in the adult heart after MI and present evidence that a similar endocardial to coronary arterial transition occurs under pathophysiological conditions in the adult endocardium (Figure 4F).

Neovascularization is a critical feature in the repair of cardiac damage. However lessons from the first clinical trials using induced angiogenesis showed that the formation of new capillaries without smooth muscle support does not improve cardiac repair. Similarly, promoting arteriogenesis by collateral outgrowth to induce the formation of new vessels with supporting cells is technically challenging. Using a fluorescent coronary endothelial transgene to visualize artery remodeling in post-MI hearts we provide the first evidence for arterial endothelial patches within the continuum of the endocardium. Arterial features include Cx40 expression and the presence of adjacent SMCs. VEGF-A is determinant in arteriogenesis and Cx40 plays an important role in arteriogenesis by modulating arterial identity. Arteriogenesis is triggered by mechanical forces such as shear stress and both Cx40 gene expression and VEGF/VEGFR2 signaling are shear-stress responsive. Promoting arteriogenesis is a major objective in therapeutic revascularization and our findings suggest that endocardium has the potential, under pathophysiological conditions, to contribute to arteriogenesis. Arteriogenesis occurs via the development of pre-existing collateral vessels or by expansion and arterialization of the capillary bed. Our genetic tracing analysis reveals that the endocardial flowers result from both pre-existing Cx40+ arterial endothelium or de novo arteriogenesis of Cx40+ cells. Vascular remodeling may also occur in response to endocardial damage at the time of infarct. However, endocardial flowers are present during a time window adapted for de novo arteriogenesis of the heart, equivalent to that of arteriogenesis in the ischemic limb following femoral artery ligation.
We observed a tip-cell phenotype in VEGFR2+ endocardial cells within endocardial flowers suggesting that angiogenesis precedes arteriogenesis. VEGFR2 is a recognized marker of tip-cells during angiogenesis, which sprout from an endothelium and recruit proliferative stalk cells to form the new vessel. High proliferative endothelial cells are restricted to Endoglin+ cells at the flower edge, consistent with such a process. These data are supported by results from genetic tracing using Apn-CreER showing the active angiogenesis taking place after MI. Our data reveal active angiogenesis of endocardial endothelial cells, followed by upregulation of Cx40 expression and proliferation of subendocardial SMCs suggesting that endocardial cells are predominantly destined to an arterial phenotype.

Endothelial cell plasticity between endocardium and coronary endothelium in the damaged adult heart appears to reflect the redeployment of developmental mechanisms. The embryonic endocardium originates from vascular endothelial progenitor cells and can form a vascular plexus by angiogenesis; VEGF signaling plays an important role in promoting endocardial to coronary endothelial transition during development. Understanding the molecular signals governing the switch in endothelial phenotype documented here in the adult heart post-MI will allow the identification of pathways promoting the formation of new vessels during cardiac repair as well as providing insights into normal coronary artery development. Our results thus identify endocardium as a source of coronary endothelial cells for strategies aimed to promote arteriogenesis under pathological conditions. Future analysis of how the endocardial to arterial endothelial switch is regulated will be of prime importance to optimize revascularization therapy.

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DISCLOSURES
None
REFERENCES


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FIGURE LEGENDS

**Figure 1:** Identification of Cx40-GFP+ endocardial flowers in post-MI endocardium. (A) Cx40-GFP expression in atrial myocardium and coronary arteries in an adult mouse heart. (B-C) Three weeks after coronary artery ligation (asterisks), tortuous Cx40-GFP coronary arteries (arrows) were observed in the infarct area (MI) connected to GFP+ endocardial foci (boxes). (D) Wholemount immunofluorescence with Pecam1 reveals flower-shaped foci of Cx40-GFP endocardial cells (arrows) connected to coronary stems; (E) quantification of the number of GFP+ foci scored in individual hearts at different days post-MI. (F) Pecam1 labelling showing elongated endothelial cells within an endocardial flower compared to hexagonal cells elsewhere in the endocardium. The yellow box corresponds to the YZ image projection at the level of the dotted line showing the contiguous coronary artery (arrows). a: atrium. Scale bar=50µm.

**Figure 2:** Endocardial flowers present hallmarks of an arterial phenotype. (A-C) 3D reconstruction of an endocardial flower (A-B) and immunolabelling on sections (C) showing expression of Endoglin in the endocardium of both non-infarct and MI areas with the exception of Cx40-GFP+ endocardial flowers and connecting vessels. VEGFR2 accumulates in capillaries and is co-expressed with Cx40-GFP in endocardial flowers. Both endocardium and coronary endothelial cells are labeled by Pecam1. (D) Anti-SMA immunofluorescence reveals the presence of smooth muscle cells adjacent to Cx40-GFP arteries (Non infarct) and endocardial flowers (MI). (E) 3D reconstruction of wholemount endocardial flowers showing connecting vessels with a low level of VEGFR2 expression and SMA+ smooth muscle cells (left) or a high level of VEGFR2 devoid of supporting cells (right). (F) Wholemount immunofluorescence shows a broader expression of VEGFR2 than Cx40-GFP in endocardial flowers (asterisks) and connecting vessels (arrows). (G) Wholemount immunofluorescence of VEGFR2, Cx40-RFP and SMA at different times post-MI (D: days after ligation). (H) Quantification of VEGFR2 and Cx40 endocardial flowers over day-3 to day-28 after ligation (n=3 for each time point). Scale bar= 100µm.

**Figure 3:** Genetic tracing of Cx40+ coronary arteries reveals endothelial cell plasticity in the infarct zone. (A) Strategy using the tamoxifen inducible Cx40-Cre line (left) resulting in conditional reporter gene activation in Cx40+ arterial endothelial cells (arrows) but not in VEGFR2+ capillaries. (B, C) Wholemount X-gal and anti-RFP immunostaining and inset sections of infarct areas from Cx40-Cre::R26R mice showing Cx40-Cre-derived (LacZ+) coronary vessels and RFP+/LacZ- endocardial flowers. (D-E) 3D reconstruction from Cx40-Cre::RYFP mice showing Cx40-RFP only endocardial flowers with new Cx40 expression (D) and Cx40-RFP+ R26-YFP+ endocardial flowers originating from outgrowth of vessels expressing Cx40-Cre prior to infarct (E). (F) Quantification of the percentage of Cx40-RFP+ only vessels in sham and MI hearts compared to Cx40-RFP+ only endocardial flowers. Student’s t-test was used to analyse differences and values are shown as mean±s.e.m.;***P<0.001, **P<0.01; n=14 for sham, 10 for MI and EF. Scale bar: 200µm.

**Figure 4:** Arteriogenesis within post-MI endocardium arises by angiogenesis of endocardial cells. (A) VEGFR2+ cells at the level of the endocardial flowers present a tip-cell phenotype with numerous projections (arrow). (B) Endoglin and VEGFR2 are co-expressed at the edge of endocardial flowers. (C) Endomucin expression is restricted to endocardium in sham hearts and is absent from endocardial flowers (MI). NG2+ pericytes are found adjacent to VEGFR2+ capillaries in sham hearts and underlie endocardial flowers in MI. (D) Immunofluorescence with anti-Ki67 shows active proliferation of smooth muscle (cyan arrowheads) and endothelial (arrows) cells at endocardial flowers. Quantification reveals elevated proliferation of SMCs (cyan arrowheads) and in Endoglin+ cells at the flower edge (red arrowheads) compared to Endoglin+ cells (green arrowhead). (E) PH3+ mitotic nuclei are detected at the border of endocardial flowers in Endomucin+ (E') or SMA+ (E") cells. (F) Endocardial flowers result from endocardial and coronary arterial endothelial cell plasticity in post-MI hearts. Scale bar= 50µm.
Novelty and Significance

What Is Known?

- Neovascularization is essential for effective cardiac repair following myocardial ischemia.
- Endocardium is a source of coronary endothelial cells during development.

What New Information Does This Article Contribute?

- Foci of endocardial cells adopt an arterial endothelial phenotype after cardiac infarction and connect with coronary arterial stems.
- Extensive plasticity occurs between endocardial and coronary endothelial compartments in the post-infarct mouse heart.
- Endocardial arterialization is redeployed under pathophysiological conditions, identifying the endocardium as a source of endothelial cells for neovascularization strategies.

In this work we address mechanisms of revascularization after cardiac damage. We reveal, using fluorescent reporter alleles and genetic lineage tracing, that endothelial foci within the murine endocardium adopt an arterial phenotype after cardiac infarction. We present evidence that these structures, never described before, arise through arteriogenesis, revealing extensive endothelial cell plasticity between endocardial and coronary arterial compartments in the infarcted heart. Endocardial arterialization, recently shown to contribute extensively to coronary vascular endothelium during development, is thus redeployed in the adult heart after cardiac damage, identifying the endocardium as a source of endothelial cells for neovascularization strategies for cardiac repair.
Figure 2
Figure 3

A Genetic tracing of Cx40+ coronary arteries

Cx40-CreERT2-RFP:: R26-LacZ or R26-YFP

Tam

P3 P20 P30 P45

LAD P60 P75 Analysis

D0 D15

X-gal

R26-YFP/Cx40-RFP/VEGFR2

Sham

B X-gal/Cx40-RFP

C X-gal/Cx40-RFP

D R26-YFP/Cx40-RFP/Endoglin

E R26-YFP/Cx40-RFP/Endoglin

Endocardium Subendocardium Endocardium Subendocardium

F R26-YFP/Cx40-RFP/Endoglin

Sham MI MI

% RFP vessels

Sham MI EF

*** **
Figure 4

(A) Cx40-GFP/VEGFR2
(B) Cx40-GFP/VEGFR2/Endoglin
(C) NG2/VEGFR2/Endomucin/Nuclei

(D) KI67/SMA/Pecam1/nuclei
(KI67/GFP/VEGFR2/nuclei)

Percentage of KI67+ nuclei

(SMC) Endoglin VEGFR2

(E) KI67/SM22/Endoglin/nuclei
(PH3/SMA/Endomucin/nuclei)

(F) Angiogenesis
Endocardial flowers
Arteriogenesis

Endocardium
Coronary artery

Endoglin + VEGFR2- Cx40-
Endoglin - VEGFR2+ Cx40+
Endoglin - VEGFR2- Cx40+
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Supplemental Material

Experimental animals

\( Gtgj5a^{tm1(EGFP)} \) (Cx40-GFP), \( Gtgj5a^{tm1(CreERT2-IRESRFP)} \) (Cx40-cre), \( Gtrosa26^{tm1(LacZ)}Sor \) (R26R) and \( Gtrosa26Sor^{tm1(EYFP)Cos} \) (RYFP) mouse lines were genotyped as previously reported \(^1-^3\).

The investigation, approved by our institutional standing committee on animal research (N° CE-LR-0714), conformed to European Parliament Directive 2010/63/EU. Eight week old mice were assigned to 2 groups: one Sham-operated (sham) serving as control and one subjected to MI by left anterior descending (LAD) coronary ligation. Hearts were analyzed at 1-8 weeks post-MI.

Left anterior descending coronary ligation

A left thoracotomy was performed under anesthesia and cardiac monitoring (2% isoflurane/O\(_2\), Aerrane®, Baxter, France). The artery was ligated 1-2 mm beyond the emergence from the top of the left atrium, using an 8-0 suture. A subcutaneous injection of 0.01 ml buprenorphine solution (0.3 mg/ml) for post-operative analgesia was administered. Shams were subjected to the same surgical procedure but without coronary artery ligation\(^4\).

Reagents

Antibodies used in this study are specific to pecam-1 (MEC13.3-BD Pharmingen), endoglin CD105 (MJ7/18-DSHB), VEGFR2 (AF644-R&D SYSTEMS), \( \alpha \)-smooth muscle actin (F3777-SIGMA), GFP (AbD Serotec), RFP (Rockland), KI67 (ab15580-abcam), SM22 (ab10135-abcam), NG2 (AB5320), endomucin (Santa Cruz) and PH3 (upstate). Tamoxifen (T-5648, Sigma) was dissolved at the concentration of 20mg/ml in ethanol/sunflower oil (10/90). 20\( \mu l \) of tamoxifen was injected intraperitoneally into mice at postnatal day 2 (P2) followed by three consecutive injections of 200\( \mu l \) at P20/P30/P45.
Tissue collection and immunofluorescence on cryosections

Whole hearts were recovered at different time points after ligation (post-ligation days (D) D7/D14/D21/D28/D60) and fixed in paraformaldehyde (4% in 1X PBS) for 4 hours at 4°C. Samples were subsequently incubated overnight in two successive baths of increasing concentrations of sucrose (15% and 30% in 1X PBS). Finally, samples were embedded in Optimal Cutting Temperature (OCT, Euromedex) solution and stored at -80°C before sectioning (20µm) using a cryostat (Leica CM3050S). Frozen sections were incubated with a permeabilization solution (0.2% triton X-100 in PBS 1X) for 20 minutes and blocked for 1 hour in saturation buffer (2% BSA, 0.05% saponin and PBS 1X). Samples were subsequently incubated overnight at 4°C in the presence of primary antibodies. The next day, sections were washed and incubated with secondary antibodies conjugated with different fluorophores for 1 hour at room temperature. Finally, sections were washed and counterstained with Hoechst (Sigma) to detect nuclei. Images were acquired with an Apotome Zeiss microscope.

Quantifications

Quantification of proliferative cells were realized on serial sections by counting the total number of KI67+ cells at the level of endocardial flowers from three independent infarcted hearts at post-ligation day D14.

For the quantification of arteriogenesis, we collected data from 14 sham and 10 MI hearts dissected on day 0 to 28 after ligation and counted the number of RFP-only, YFP-only vessels and RFP*YFP* vessels in one section in the middle of each heart.

Whole-mount immunofluorescence
After heart recovery, dissection of the infarct zone was performed and maintained flattened in a petri dish using needles during fixation in paraformaldehyde (4% in 1X PBS) for 2 hours on ice. After 30min wash in PBS 1X, dissected infarcts were incubated in permeabilization solution (0.5% triton X-100 in PBS 1X) for 4 hours and blocked overnight in saturation buffer (3% BSA, 0.1% triton X-100 and PBS 1X). Incubations with primary or secondary antibodies stand for one week each in the saturation buffer. After four washes in PBS 1X/0.2% triton X-100, samples were mounted on slides in LMP agarose (4%) and observed under a Zeiss two-photon microscope (LSM 7 MP) or a Zeiss confocal microscope (LSM 780). 3D images were obtained using Volocity software.