Identification of Myocardial and Vascular Precursor Cells in Human and Mouse Epicardium

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Abstract—During cardiac development, the epicardium is the source of multipotent mesenchymal cells, which give rise to endothelial and smooth muscle cells in coronary vessels and also, possibly, to cardiomyocytes. The aim of the present study was to determine whether stem cells are retained in the adult human and murine epicardium and to investigate the regenerative potential of these cells following acute myocardial infarction. We show that c-kit+ and CD34+ cells can indeed be detected in human fetal and adult epicardium and that they represent 2 distinct populations. Both subsets of cells were negative for CD45, a cell surface marker that identifies the hematopoietic cell lineage. Immunofluorescence revealed that freshly isolated c-kit+ and CD34+ cells expressed early and late cardiac transcription factors and could acquire an endothelial phenotype in vitro. In the murine model of myocardial infarction, there was an increase in the absolute number and proliferation of epicardial c-kit+ cells 3 days after coronary ligation; at this time point, epicardial c-kit+ cells were identified in the subepicardial space and expressed GATA4. Furthermore, 1 week after myocardial infarction, cells coexpressing c-kit+, together with endothelial or smooth muscle cell markers, were identified in the wall of subepicardial blood vessels. In summary, the postnatal epicardium contains a cell population with stem cell characteristics that retains the ability to give rise to myocardial precursors and vascular cells. These cells may play a role in the regenerative response to cardiac damage. (Circ Res. 2007;101:1255-1265.)

Key Words: epicardium • infarction • stem cells • cardiovascular differentiation

Myocardial infarction (MI) in the mammalian heart is associated with an acute inflammatory response, leading to the replacement of injured cardiomyocytes with granulation tissue and scar.1 Recently it has been shown that the heart is a dynamic organ in which spontaneous myocyte regeneration together with myocyte death are major determinants of cardiac homeostasis in physiologic and pathologic conditions.2 Myocardial regeneration appears to be mediated by multipotent cardiac stem cells (CSCs), resident in the heart, that give rise to new myocytes and vascular structures. A variety of studies document the presence of CSCs in the mouse,3-10 rat,11 dog,12 and human adult heart.7,13

The adult myocardium is enveloped by a layer of epithelial cells called epicardium that during embryogenesis, plays an important role in the formation of the coronary vasculature. The epicardium has an extracardiac origin: at approximately stage 18 in the avian heart and 10.5 days post coitum in the mouse, a cluster of cells derived from septum transversum in stage 18 in the avian heart and 10.5 days post coitum in the mouse,3-10 rat,11 dog,12 and human adult heart.7,13

The adult myocardium is enveloped by a layer of epithelial cells called epicardium that during embryogenesis, plays an important role in the formation of the coronary vasculature. The epicardium has an extracardiac origin: at approximately stage 18 in the avian heart and 10.5 days post coitum in the mouse, a cluster of cells derived from septum transversum in mammals and located close to the liver primordium in other vertebrates, populates the myocardial external surface of the heart.14 Epicardial cells synthesize a dense layer of extracellular matrix that resides between them and the myocardium in the subepicardial space. A subset of these cells delaminate from the epicardium and migrate into the subepicardium, where they generate a population of epithelioidally derived cells (EPDCs) after a process known as epithelial-to-mesenchymal transition. EPDCs are pluripotent stem cells that have a considerable importance in cardiac development both by contributing to several cell lineages within the heart15 and by secreting factors that modulate myocardial development.16

Recently EPDCs have been identified in mammal adult epicardium,17,18 In vitro studies show that both human and mouse EPDCs may be induced to differentiate into smooth muscle cells17,18 whereas only murine EPDCs can give rise to endothelial cells.17 At present, it is unknown whether human and murine adult EPDCs play a role in heart repair after tissue damage. This question has already found an answer in the teleost Zebrafish.19 Among vertebrates, Zebrafish has the...
ability to completely regenerate the heart following ventricular apex resection; under these conditions, epicardial cells undergo epithelial-to-mesenchymal transformation, migrate into the wound, and participate to myocardial regeneration and blood vessel formation. In adult human hearts, the subepicardial space contains adipose tissue depots, mostly localized next to the larger blood vessels and to the atrioventricular sulcus. Several experimental results have demonstrated that adipose tissue from different compartments is a source of cells with the ability to differentiate into adipocytes, osteoblasts, chondrocytes, and myoblasts. Recently, the ability of these cells to differentiate toward the endothelial lineage both in vivo and in vitro has been demonstrated.

Based on these findings, the aim of the present study was to establish whether epicardial progenitor cells play a role in the physiologic process of myocardial repair. We show here that both human and mouse epicardial/subepicardial compartments include cells expressing stem cell antigens c-kit and CD34. Some cells either express early markers of cardiac differentiation or differentiate into endothelial cells in vitro. In the mouse, after myocardial infarction (MI), epicardial c-kit⁺ cells participate to the reparative process by proliferating and differentiating into myocardial and vascular cells.

Materials and Methods
An expanded Materials and Methods section containing details regarding cell isolation, immunofluorescence and immunohistochemical studies, flow cytometry, RT-PCR, data collection, and statistics is available in the online data supplement at http://circres.ahajournals.org.

Animals and Surgical Procedures
MI was induced by coronary artery ligation in C57BL6 female mice at 8 weeks of age (20-g body weight) as described previously.

Human Heart Samples
Human heart samples were obtained from archived autopsy material of fetal and adult hearts.

Evaluation of Myocardial Function
Myocardial functions were evaluated by echocardiographic and hemodynamic studies.

Cell Isolation
Cardiac cells were isolated from C57BL6 female mice at 2 to 3 months of age, and myocytes were discarded as previously described.

Results
Influence of the Pericardial Sac on Myocardial Structure and Function Following Infarction
We first investigated whether the pericardial sac played a role in ventricular remodeling acutely after infarction and whether
the epicardial cells did participate in the reparative process in the infarcted heart. To verify the first hypothesis, coronary artery ligation was performed in 2 groups of C57BL6 female mice. In the first group of animals, the pericardial cavity was opened just before ischemic injury (Figure 1A and 1B and supplemental Figure I), whereas in the second group, it was kept closed (Figure 1C and 1D and supplemental Figure I). All animals were euthanized 8 days later. Hematoxylin and eosin staining showed that when the integrity of the pericardial cavity was maintained, the scar displayed a better preservation compared with that formed when the pericardial cavity was opened (Figure 1A through 1D and supplemental Figure I). Moreover, in the presence of the intact pericardial cavity, foci of cardiac regeneration were detected in the infarcted region, as demonstrated by the presence of small cells expressing the cardiac marker $\alpha$-sarcomeric actin in the cytoplasm (Figure 1E) and Ki67, a nuclear protein present in $G_1$, $S$, $G_2$, and early mitosis (Figure 1F).

Echocardiographic and hemodynamic measurements were performed 1 week after acute MI. In comparison with sham-operated mice, infarcted animals exhibited evidence of cardiac failure. However, infarcted mice in which the pericardium was kept closed, exhibited a better preservation of myocardial function and lower left ventricular (LV) diameters in comparison with mice in which the pericardial sac was left open (Figure 2A through 1D and supplemental Figure I). Moreover, in the presence of the intact pericardial cavity, foci of cardiac regeneration were detected in the infarcted region, as demonstrated by the presence of small cells expressing the cardiac marker $\alpha$-sarcomeric actin in the cytoplasm (Figure 1E) and Ki67, a nuclear protein present in $G_1$, $S$, $G_2$, and early mitosis (Figure 1F).

Identification of c-Kit$^+$ and CD34$^+$ Cells in Human Fetal and Adult Epicardium

In these experiments, it was examined whether stem cells could be identified in the human fetal and adult epicardium. Stem cell antigens c-kit and CD34 were used for this analysis because they identify a subset of stem cells in several adult tissues. In the fetal heart, the epicardium covers the outer edge of the heart. The external layer of flat mesothelial cells lies on a thin basal lamina, and underneath there is a layer of connective tissue, the subepicardial space, that

![Figure 2. Pericardial sac integrity partially prevents myocardial function deterioration after infarction. A through F, Echocardiographic (A through C) and hemodynamic (D through F) assessments of cardiac function in sham-operated (SO) and infarcted (MI) murine hearts in the absence (+) and presence (-) of an intact pericardial sac. All studies were performed 1 week after surgery (n=6 for each of the four groups). $^{\dagger}$P<0.01 vs MI (-), $^*$P<0.01 vs their respective sham-operated controls.](http://circres.ahajournals.org/issue/9/9/CircRes2017005635)
contains elastic fibers, as well as large vessels (Figure 4A). Importantly, coronary arteries originate in this region during cardiac development. By immunohistochemistry, cells expressing c-kit (Figure 4B) and CD34 (Figure 4C) antigens were detected in the subepicardial region of human fetal hearts. These cells are distributed in the interstitial spaces of the subepicardial connective tissue. In the adult human myocardium, the subepicardial space is mostly occupied by adipose tissue (Figure 4D), which surrounds coronary arteries and veins. c-Kit+ and CD34+ cells were also identified in the subepicardial region of adult hearts, where they occupied the interstitial space between adipose cells (Figure 4E and 4F). Taken together, the results show that c-kit+ and CD34+ cells are present in human fetal and adult epicardium.

Flow Cytometric Analysis of c-Kit+ and CD34+ Human Epicardial Cells
Freshly isolated cell suspensions obtained from adult human epicardial biopsies were analyzed by flow cytometry to identify c-kit– and CD34-expressing cells. Epicardial c-kit+ and CD34+ cells appeared as 2 distinct populations and represented 0.8±0.5% and 8.6±2%, respectively, of the total cells isolated (Figure 5A and 5E). Both populations were negative for CD45, a cell surface marker expressed exclusively by the hematopoietic lineage (Figure 5B and 5C). CD34+ cells were mostly negative for the endothelial/macrophage marker CD31 (Figure 5D). Notably, CD34+ cells share some phenotypic characteristics with EPDCs, because they expressed the hyaluronate receptor (CD44), and a subset of these cells were also positive for the major T cell...
antigen (Thy-1; CD90) and endoglin (CD105) (supplemental Figure II).

Human Epicardial–Derived c-Kit⁺ and CD34⁺ Cells Are Potential Cardiac and Vascular Precursors

To assess whether c-kit⁺ and CD34⁺ cells present in the adult human epicardium were potential cardiogenic precursors, the expression of early cardiac markers was analyzed in the purified cell fractions (supplemental Figure III). Immunofluorescence analysis revealed that some c-kit⁺ and CD34⁺ cells expressed the early marker of cardiomyocyte differentiation Nkx2.5 and the cardiac transcription factor GATA4 (Figure 5F and 5G). Both populations also displayed the ability to acquire the endothelial phenotype, as shown by their capacity to uptake 1,1’-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated LDL (Ac-LDL-DiI) in 7-day primary cultures (Figure 5H).

c-Kit⁺ Cells Resident in the Murine Epicardium Proliferate and Differentiate After MI

There are striking differences in the structure of the epicardium between humans and rodents (Figure 6A); in contrast to human epicardium, murine epicardium lacks adipose cells and consists of a monolayer of mesothelial cells on a thin layer of connective tissue formed by elastic fibers. By RT-PCR, the expression of c-kit, CD34, and the cardiac markers Nkx2.5 and GATA4 were identified in epicardial cells (Figure 6B). The expression levels of these markers were comparable to those detected in cells obtained from the heart after cardiomyocyte separation. Flow cytometric analysis of dissociated mesothelial cells revealed the presence of both c-kit⁺ and CD34⁺-expressing cells, which represented 1.4±0.7% and 0.48±0.14%, respectively, of the total epicardial epithelium (Figure 6C and 6G). Further characterization showed that, as in humans, c-kit⁺ and CD34⁺ cells lacked the hematopoietic lineage marker CD45 (Figure 6D and 6E).
Moreover, CD34<sup>+</sup> cells were negative for the endothelial marker CD31 (Figure 6F). Thus, c-kit<sup>+</sup> and CD34<sup>+</sup> cells are present in murine adult epicardium.

Cardiac stem cells (CSCs) have been recently identified in the mammalian heart as undifferentiated cells expressing variable combinations of stem cell markers c-kit, Sca1, and multidrug resistance (MDR).<sup>7,11</sup> These cells can give rise to all cardiac cell lineages after MI.<sup>7,11</sup> Approximately 60% of epicardial c-kit<sup>+</sup> cells were positive for MDR1, whereas only 6% expressed Sca1. Notably, both Sca1 and MDR1 were expressed at low levels in epicardial c-kit<sup>+</sup> cells (supplemental Figure IV). Considering the differentiation potential of CSCs and their immunophenotypic similarity to epicardial c-kit<sup>+</sup> cells, we investigated whether this latter population displayed properties comparable to those of CSCs, ie, whether epicardial cells increased in number and differentiated following MI.<sup>13</sup> To test this hypothesis, epicardial c-kit<sup>+</sup> cell proliferation and differentiation was assessed in the mouse heart at different time points after the induction of MI in the presence of an intact pericardial cavity. By immunohistochemistry, it was found that after infarction, the number of c-kit<sup>+</sup> cells increased in the epicardial compartment (Figure 7A through 7E). Three days after surgery, the absolute number of c-kit<sup>+</sup> cells (Figure 7F) as well as the fraction of c-kit<sup>+</sup> cells expressing Ki67 (Figure 7G) in the epicardium were higher in infarcted than in sham-operated animals. Notably, the increase of epicardial c-kit<sup>+</sup> cells was also detected in infarcted hearts with an open pericardial cavity; however, the absolute number was significantly lower compared with that found in presence of an intact pericardial cavity (Figure 7F). In the absence of infarction, the pericardial sac integrity did not affect c-kit<sup>+</sup> cell number (Figure 7F).
Together, these results demonstrate that MI enhances epicardial c-kit⁺ cell proliferation and that the presence of an intact pericardial cavity further increases their absolute number.

To verify whether following infarction, epicardial c-kit⁺ cells differentiated toward myocardial, endothelial, and smooth muscle phenotypes, the expression of the cardiac transcription factor GATA4, the endothelial marker factor VIII, and the α-smooth muscle actin was assessed by immunohistochemistry. At day 3 after coronary ligation, some epicardial and subepicardial c-kit⁺ cells (Figure 8A) also expressed GATA4 (Figure 8B). Quantification analysis revealed a 3-fold increase of c-kit⁺/GATA4⁺ cells in the...
infarcted compared with control hearts (Figure 8C). One week after MI, small blood vessels were found in the subepicardial space. Some of these vessels included c-kit+ (Figure 8D and 8F) cells expressing either the endothelial marker factor VIII (Figure 8E) or smooth muscle actin (Figure 8G). Thus, MI induces epicardial c-kit+ cell proliferation and their differentiation into a myocardial, endothelial, and smooth muscle phenotype.

Discussion

Recent studies have shown that the heart is not a terminally differentiated organ and that “resident” cardiac stem cells contribute to cardiac repair following injury,5,23,27 as well as physiologic cardiac tissue homeostasis replacing dead cells during the lifespan.13 c-Kit+ CSCs have been found in niches, mostly localized in the atria and in the apex, as well as dispersed among myocardial cells throughout the heart.28 The present study shows that c-kit+ cells are also present in human and murine epicardium. Furthermore, in the mouse, these cells respond to MI as c-kit+ cells within the myocardium; they proliferate, migrate toward the injury site, and exhibit evidence of differentiation toward the myocardial and vascular phenotype. The existence of progenitor cells in the epicardial compartment was supported by the following observations. First, in a mouse model of MI, we found a significant prevention of cardiac function impairment and LV remodeling in the presence of an intact pericardial cavity. These hearts were characterized by foci of cardiac regeneration in the infarcted region. The mechanical protection exerted on precursor cells resident in the epicardium by the pericardial compartment, as well as by the pericardial fluid, could explain such effects. Moreover, growth factors released in the pericardial fluid after acute MI29–31 could play a role in the regenerative process modulating epicardial cell function. Second, epicardial mesothelial cells transduced with a lentiviral vector–expressing GFP were detected in the LV wall of mouse infarcted hearts at 1 and 3 weeks after injury and acquired a cardiac phenotype.

The presence of vascular progenitors in the epicardium has been widely demonstrated during cardiac development. In the embryo, the epicardium, which has an extracardiac origin, provides cells with vasculogenic potential, so called EPDCs, that form at least part of the major coronary vessels following an epithelial-to-mesenchymal transformation.16,32 Only recently have EPDCs been described in adult human and mouse hearts.17,18 Human EPDCs treated with transforming growth factor-β1 or bone morphogenetic protein-2 were able to differentiate into smooth muscle cells but failed to form endothelial cells.18 Treatment of mouse epicardial explant cultures with thymosin-β4 showed extensive outgrowth of cells that differentiated into both endothelial and smooth muscle cells.17 These data suggest that adult EPDCs may preserve the properties of their embryonic counterparts. Although it has been proposed that embryonic EPDCs could also give rise to cardiomyocytes,33 at present, it has not been assessed whether adult EPDCs have a cardiogenic potential.33–35

Precursor cells able to give rise to cardiomyocytes, smooth muscle cells, and endothelial cells, have been identified in the
Several stem cell-related antigens have been used to characterize these cells, and, among them, the stem cell antigen c-kit together with Sca1 and MDR1 identify a population of cardiac stem cells that may be induced to proliferate, differentiate, and participate in the reconstitution of damaged myocardium. We demonstrated here that c-kit–expressing cells are present in mouse and human adult epicardium. These cells are localized in the mesothelial layer, which is the major constituent of murine epicardium, and also in the subepicardial compartment of human epicardium, which is characterized by the presence of adipose tissue. Although epicardial c-kit+ cells express MDR1, at present, it remains to be investigated whether epicardial c-kit+ and CSC c-kit+ cells are the same population. In both mouse and human epicardial compartments, we also identified a population of CD34+/c-kit+ cells. The absence of CD31 in these cells demonstrates that they represent a population with vasculogenic and angiogenic potentials rather than mature endothelial cells. It is noteworthy that a similar cell subset was recently identified in the stromal vascular fraction of human adipose tissue as a population exhibiting endothelial cell progenitor characteristics. Notably, CD34+ cells were localized in the subepicardial space of human hearts, which is mostly occupied by adipose tissue. Although epicardial c-kit+ and CD34+ cells also have been identified in the peripheral blood, we excluded the possibility that the presence of such progenitors may be attributable to blood circulating into the epicardial fat tissue for the following reasons: first, they did not express the hematopoietic marker CD45; second, c-kit+ and CD34+ cells were highly represented in the human epicardium compared with the peripheral blood (c-kit+, 0.5% versus 0.05%; CD34+, 8.6% versus 0.09%, respectively).

Cultured EPDCs isolated from human epicardial biopsies have a fibroblast-like shape and adhere to plastic dishes. The immunophenotypic characterization of in vitro–expanded EPDCs reveals that they express the mesenchymal markers CD44, CD90, and CD105 but are negative for the stem cell antigens CD34 and Sca1. Moreover, they express the late cardiac marker GATA4. In contrast to adult cultured EPDCs, freshly isolated human epicardial c-kit+ and CD34+
cells display a nonadherent phenotype and are positive not only for GATA4 but also for the early cardiac marker Nkx2.5. Furthermore, both epicardial populations exhibit the ability to acquire an endothelial phenotype in vitro, a property not demonstrated in human EPDCs. Interestingly, epicardial CD34+ cells are positive for CD44, and a subpopulation of CD34+ cells also expressed CD105 and CD90, suggesting that CD34 may identify progenitor cells able to give rise to cardiovascular cells and possibly to EPDCs.

After MI in mice, epicardial c-kit+ cells enter the cell cycle and proliferate. At day 3 after injury, epicardial c-kit+ cells expressing cardiac and vascular markers are detected in the epicardial and subepicardial region.

The importance of the epicardium in the reparative process after damage has been demonstrated recently in the zebrafish adult heart. Zebrafish is able to regenerate its heart completely, even after a cardiac mass loss as high as 20%.37 Following surgical removal of the ventricular apex, new cardiomyocytes originate from undifferentiated progenitor cells and the epicardium plays a critical role during the entire process.19 Specifically, a subpopulation of epicardial cells migrates into the wound and provides new vasculature to the regenerating tissue, whereas proliferating cardiomyocytes are detected in the subepicardial compartment.

In conclusion, we demonstrate here that the adult mammalian epicardium contains precursor cells able to give rise to cardiomyocytes and endothelial and smooth muscle cells. After infarction, epicardial precursor cells are activated and participate in the physiological process of myocardial repair. A better understanding of the mechanisms involved in adult epicardial precursor cell activation could help in the development of therapeutic strategies aimed at enhancing the regenerative capacity of these cells.

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Disclosures

None.

References


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MATERIALS AND METHODS

Animals, surgical procedures

Myocardial infarction (MI) was induced by coronary artery ligation in C57BL6 female mice at 8 weeks of age (20 gr body weight) as previously described. Briefly, in mice under anesthesia (100mg/kg ketamine and 1mg/kg acepromazine) and mechanically ventilated, thoracotomy via the third left intercostal space was performed. Animals were divided in two groups: in one group the pericardial sac was maintained closed and in the second group the pericardial cavity was opened. The left ventricle (LV) was exposed and the left coronary artery was ligated. The chest was closed, the pneumothorax was reduced and the mice were allowed to recover. Sham operated mice were treated similarly, except that the ligature around the coronary artery was not tied. Animals were sacrificed 24 h, 2-3 days, and 1 week after surgery. In some experiments, $10^7$ transducing units (TU) of a lentiviral vector (LV) expressing green fluorescent protein (LV-CMV-GFP) were delivered directly into the intrapericardial space using a 32 gauge needle, in order to label the mesothelial layer of the epicardium.

All experimental procedures complied with the Guidelines of the Italian National Institutes of Health, with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee.

Human heart samples

Human epicardial biopsies were removed from non-coronary disease patients undergoing cardiac surgery. The protocol was approved by the Local Ethical Committee and written informed consent was obtained from all patients.

Cell isolation
Cardiac cells were isolated from C57BL/6 female mice at 2–3 months of age, and myocytes were discarded as previously described. Hearts were explanted and perfused with a collagenase solution (280 U/mL; Worthington, Lakewood, N.J.) to dissociate cardiac cells. Cardiomyocytes were removed and the remaining cells were collected for RT-PCR analysis.

Murine epicardial cells were isolated as follows: hearts were removed aseptically, washed with ice-cold PBS and were placed in 0.25% trypsin-EDTA (0.25% Trypsin, 1mM EDTA in Hanks’ balanced salt solution (HBSS), (GIBCO, Grand Island, N.Y., USA) at room temperature. After 20 min, the epicardial surface of the heart was gently scraped and the resulting cells were suspended in HBSS plus 10% Fetal Calf Serum (FCS).

Human epicardial biopsies, which included the mesothelial layer and the subepicardial space containing the adipose tissue, were digested at 37°C in PBS containing 2% BSA and collagenase (1 mg/ml, Worthington, Lakewood, NJ) for 40 min. At the end of the digestion, cells were filtered using a 40 µm cell strainer (Falcon), pelleted at low speed to remove floating mature adipocytes and resuspended in PBS containing 10% BSA.

Human epicardial c-kit+ and CD34+ cells were isolated by magnetic cell sorting (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). Cells from the epicardial tissue were incubated with mouse PE-conjugated c-kit antibody (BD Pharmingen, San Diego, CA, USA) or mouse PE-conjugated CD34 antibody (clone 2B8; BD Pharmingen, San Diego, CA, USA) diluted in PBS 10% BSA. Anti-PE microbeads were used as secondary antibody. c-kit+ and CD34+ cells were separated using a MINI-MACS (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) device. Purity of sorted cells was determined by FACS and corresponded to ~80%.

Evaluation of myocardial function

Echocardiography was performed in conscious mice with a Sequoia 256c equipped with a 13-MHz linear transducer. Two-dimensional images and M-mode tracings were recorded from the parasternal short axis view at the level of papillary muscles. From M-mode tracings, anatomical
parameters in diastole and systole were obtained. For hemodynamic studies, mice were anesthetized with chloral hydrate (400 mg/kg body weight) and the right carotid artery cannulated with a microtip pressure transducer (Millar 1.4F). The catheter was advanced into the left ventricle and evaluation of LV pressures and +dP/dt and –dP/dt in the closed-chest preparation were performed.

**Immunohistochemical analysis**

In mice, the abdominal aorta was cannulated and the heart was arrested in diastole with CdCl₂, perfused retrogradely with 10% (vol/vol) formalin, embedded in paraffin and sectioned (1 or 3µm thickness). Human heart samples were embedded in paraffin and processed as mouse hearts. Sections were deparaffinized in xylene, hydrated and rinsed in PBS. Antigen retrieval was performed by microwaving in a citric acid-EDTA buffer (10 mM, pH 7.8 UCS Diagnostic, Rome, Italy). Slides were washed in PBS and the endogenous peroxidase activity was blocked in 3% hydrogen peroxide (H₂O₂) in methyl alcohol for 20 min. To minimize nonspecific antibody binding, slides were preincubated with PBS 10% BSA overnight.

All primary antibodies were diluted with 10% BSA and then incubated overnight at 4°C.

The following antibodies were used: rabbit polyclonal c-kit (clone H-300; Santa Cruz Biotechnology, Santa Cruz, Calif), rat monoclonal CD34 (clone MEC 14.7; Abcam, Cambridge, UK), rabbit polyclonal GATA4 (Santa Cruz Biotechnology, Santa Cruz, Calif), rabbit polyclonal factor VIII (Dako Carpineria, CA, USA) and a mouse monoclonal anti-α-smooth muscle actin (Sigma, Saint Louis, Missouri, USA). Sections were incubated first with a biotinylated goat-anti-rabbit or rabbit-anti-rat antibody for 1 hr at 37°C and subsequently in Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California, USA) for 1 hr. Finally they were developed in Novared substrate kit (Vector Laboratories, Burlingame, California, USA) for 3-4 min, counterstained with haematoxylin to identify nuclei, dehydrated and mounted in Eukitt (Bioptica, MI, Italy).
For hematoxylin-eosin (H&E) staining, murine and human sections were deparaffinized, rehydrated, placed first in haematoxylin and then in eosin for 5 min each, dehydrated and finally mounted.

**Flow cytometric analysis**

Human and murine epicardial cells were incubated in PBS containing 5% Fetal Bovine Serum (FBS) for 20 min at 4 °C in the dark with the indicated antibodies. For mouse studies: c-kit, CD34, CD45, CD31, Sca1 were purchased from BD Pharmingen (San Diego, CA, USA) while MDR1 was from Chemicon International (Hampshire, UK). For human studies: c-kit, CD34, CD45 and CD31, antibodies were obtained from Beckman Coulter, (Fullerton, CA, USA); CD44, CD90, CD105 were from BD Pharmingen (San Diego, CA, USA). These antibodies were conjugated with the following fluorochromes: fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Allophycocyanin (APC), APC-Cy7, PE-Cy7, PC7 and PC5. After labeling, cells were washed twice, resuspended in 250 ml of PBS containing 0.5% FBS and analyzed at the flow cytometer FACSCanto (Becton-Dickinson, San Jose, CA), equipped with two lasers. All data were analyzed using FlowJo (Tree Star, San Carlos, CA) software. Isotype control was performed for each experiment (BD Pharmingen). A minimum of $5 \times 10^4$ events were acquired per sample.

**Immunofluorescence analysis**

Fixed in PBS 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked at room temperature (RT) in PBS containing 5% BSA and 10% Donkey Serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min prior incubation with antibodies. Fixed cells were incubated first with mouse monoclonal c-kit antibody (Dako Carpineria, CA,USA) and mouse monoclonal CD34 antibody (clone My10; Becton Dickinson, San Jose, CA) and then with the nuclear markers rabbit polyclonal Nkx2.5 (clone H-114; Santa Cruz Biotechnology, CA, USA), rabbit polyclonal GATA4 (clone H-112; Santa Cruz Biotechnology, CA, USA) and mouse
polyclonal Ki67 (clone NCL; Novocastra Laboratories, Newcastle, UK). Secondary antibodies coupled with Fluorescein (FITC)-conjugated Donkey anti-rabbit were used to detect GATA4 and NKx2.5; Rhodamine (TRITC)-conjugated Donkey anti-mouse for c-kit and TRITC-conjugated Donkey anti-goat for CD34 (Jackson ImmunoResearch Laboratories, West Grove, PA). The same protocol was performed for mouse epicardial cells using rabbit polyclonal c-kit (clone H-300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rat monoclonal CD34 (clone MEC 14.7, Abcam, Cambridge, UK).

For staining on tissue sections, cardiomyocytes were recognized with a mouse monoclonal anti–α–sarcomeric actin (clone 5C5; Sigma, Saint Louis, Missouri, USA) while cells infected by the LV-CMVGFP were detected with a rabbit polyclonal anti-GFP (Invitrogen, Eugene, Oregon, USA).

The cover-glasses were mounted and sections were analyzed with a Zeiss microscope equipped for fluorescence.

**Endothelial differentiation assay**

Isolated epicardial cells were cultured over night in presence of DMEM containing 10% FCS to eliminate mature endothelial cells which attached to the plastic dish. Then, both c-kit^+^ and CD34^+^ cells were recovered by MACS purification from the non-adherent epicardial cell fraction.

Endothelial differentiation assays were performed in glass chamber slides (Invitrogen, Frederick, MD, USA) coated with fibronectin (FN, 20µg/mL, Sigma, Saint Louis, Missouri, USA). After 1 week in culture, c-kit^+^ and CD34^+^ cells were incubated overnight with Ac-LDL-DiI (Biomedical Technologies, Inc, Stoughton, MA, USA), an indicator of endothelial cells differentiation. After fixation with 4% paraformaldehyde, cells were stained with Hoechst 33258 nuclear dye (Sigma, Saint Louis, Missouri, USA).
**RT-PCR**

RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA). Preamplification system was used to reverse transcribe total RNA (1 µg) into complementary DNA according to manufacturer’s instructions (Invitrogen). An aliquot (2 µl) of the reverse transcription reaction was subjected to 39 polymerase chain reaction (PCR) cycles: 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, in the presence of 50 pmol of each primer, 1.5 mmol/L MgCl₂, 200 mmol/L dATP, dCTP, dGTP, and dTTP, and 2.5 U of Amplitaq polymerase (Invitrogen). Sequences of the primers were: Nkx2.5 5’-CAG TGG AGC TGG ACA AAG CC-3’ and 5’-TAG CGA CGG TTC TGG AAC CA-3’; c-kit 5’-GGC TCA TAA ATG GCA TGC TC-3’ and 5’-CTT CCA TTG TAC TTC ATA CAT G-3’; CD34 5’-GAC TAT GGT CAA CTT TAC AGT A -3’ and 5’-AGA TGA TGT GTA AGC ATA TGG C-3’ actin 5’-CAC CTT CTA CAA TGA GCT-3’ and 5’-GAA GGT AGT CTG TCA GGT CCC-3’.GATA4 5’-AAG ACG CCA GCA GGT CCT GCT TTG GC-3’ and 5’-CGC GGT GAT TAT GTC CCC ATG ACT-3’. The PCR products were electrophoresed on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide.

**Data collections and Statistics**

Immunohistochemical, hemodynamic and echocardiographic analyses were examined blindly. Results are presented as mean ± standard deviation. Statistical significance between two measurements was evaluated by unpaired Student's *t* test and multiple comparisons were performed by Bonferroni method. A probability value of *p*<0.05 was considered significant.
References


Supplemental figure legends

Supplemental figure 1. Pericardial sac integrity prevents ventricular remodeling acutely after infarction.

Large field of view of one week infracted heart stained by H&E in which the pericardial cavity was either left open (A) or closed (B).

Supplemental figure 2. Flow cytometric analysis of epicardial CD34⁺ cells.

The expression of CD90, CD105 and CD44 was evaluated on epicardial cells gated for CD34⁺/CD31⁻ and CD34⁺/CD31⁺. Within CD34⁺ cells, percentages of positive cells and mean fluorescence intensity values (in brackets) for each staining are shown.

Supplemental figure 3. Purity of MACS-sorted epicardial c-kit⁺ and CD34⁺ cells

MACS sorted c-kit⁺ and CD34⁺ cells were directly visualized by fluorescent microscopy (A) and analyzed by Flow cytometry (B) In this representative analysis, the purity of MACS sorted c-kit⁺ and CD34⁺ cells were about 70% and 81% (M1), respectively.

Supplemental figure 4. Flow cytometric analysis of Sca1 and MDR1 expression on murine epicardial c-kit⁺ cells

(A) Epicardial c-kit⁺ cells were mostly negative for the stem cell marker Sca1 but (B) were highly positive for MDR1. Cells were stained with APC-conjugated c-kit in combination with FITC conjugated Sca1 and PE-conjugated MDR1.

Supplemental Table I

* Statistically significant difference from the respective SO animals
† Statistically significant difference from MI with open pericardium

SO, sham-operated; MI, myocardial infarction. Data are presented as mean±SD. N=6
Supplemental Figure 2

CD44

CD105

CD90

CD31^+

CD31^−
Supplemental Figure 3

A

- **c-kit Hoechst**
- **CD34 Hoechst**

B

- **c-kit**
- **CD34**
### Supplemental Table I

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<td></td>
<td>opened pericardium</td>
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<td>SO</td>
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<tr>
<td><strong>Hemodynamics</strong></td>
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<tr>
<td>LVDP (mmHg)</td>
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<td>LVEDP (mmHg)</td>
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<td><strong>Echocardiography</strong></td>
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