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**L-selectin and SDF-1 enhance the migration of mouse and human cardiac mesoangioblasts.**

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## **ABSTRACT**

Efficient delivery of stem cells to heart regions is still a major problem for cell therapy. Here, we report experiments aimed to improve migration of mouse and human cardiac mesoangioblasts to damaged heart. Cardiac mesoangioblasts were induced to transmigrate through the endothelium by factors released by cardiomyocytes or cytokines, among which SDF-1 was the most potent. Cardiac mesoangioblasts were also delivered into the left ventricular chamber of mice after coronary artery ligation (CAL) and their in vivo homing to the damaged heart was found to be quite modest. Pretreatment of cardiac mesoangioblasts with SDF-1 or transient expression of L-selectin induced a two to three fold increase in their transmigration and homing to the damaged heart. Therefore, combined pretreatment with SDF-1 and L-selectin generated modified cardiac mesoangioblasts, 50% of which, after injection into the LV chamber of mice early after CAL, home directly to damaged free wall of the heart. Finally, modified mouse cardiac mesoangioblasts, injected into the left ventricular chamber regenerate a larger surface of the ventricle in long-term experiments in comparison with their control counterparts. This study defines the requirements for efficient homing of cardiac mesoangioblasts to damaged heart and offers a new potent tool to optimize efficiency of future cell therapy protocols for cardiovascular diseases.

## INTRODUCTION

Homing is the process by which cells reach a specific target tissue through the general circulation. Homing has been studied extensively both *in vitro* and *in vivo* with different cell types such as leukocytes or hematopoietic stem cells<sup>1, 2</sup> and is believed to rely on adhesion molecules and cytokine receptors by a multistep cascade, consisting of a rolling interaction with endothelium followed by firm adhesion and transmigration into the surrounding tissue<sup>3-5</sup>.

The repertoire and the level of cytokine expression by the target tissue, as well as expression of the relative receptors on endothelial cells influence the specificity and the efficiency of homing. During inflammation that follows injury or disease, cytokines are involved not only in the recruitment of leukocytes but also of circulating stem cells<sup>6, 7</sup>. In fact, stromal derived factor (SDF-1) and VEGF favor the arrest of progenitors on vascular endothelium while interleukin-8 (IL-8) promotes stem cell mobilization from the bone marrow<sup>8-10</sup>. HMGB1 and TNF-alpha have also been described as signals that induce migration of different mesenchymal cells<sup>11-13</sup>. Although these mechanisms have been elucidated to a large extent for leukocytes, far less is known for the new types of recently described stem cells and this limits the possibility of their efficient delivery to target tissues.

Cardiac mesoangioblasts were recently characterized as a population of mesenchymal stem cells that differentiate efficiently into cardiomyocytes<sup>14, 15</sup>. They have been previously shown to restore to a significant extent heart structure and function in a mouse model of coronary artery ligation<sup>14</sup>. One main reason for the partial effect of cardiac mesoangioblasts in this model is likely to be ascribed to the limited homing of these cells to heart, depending in turn upon incomplete adhesion and extravasation.

Cardiac mesoangioblasts extravasation must be directed by selective precursor-endothelial cell recognition. Micro-array analysis revealed that cardiac mesoangioblasts express some integrins, and several cytokine receptors but lack many of the leukocyte molecules implicated in transmigration (unpublished data, Tagliafico E and Cossu G).

In order to increase the efficiency of cardiac repair by cardiac mesoangioblasts it would be essential to increase their homing and survival in the heart, with the additional benefit of reducing unspecific trapping in the capillary filters of the body such as liver and lung. To this aim we have investigated the role of some adhesion molecules and cytokines in the transmigration of cardiac mesoangioblasts *in vitro* through the endothelial monolayer and *in vivo*, their migration in the infarct area of the heart in mice which underwent permanent coronary artery ligation (CAL)<sup>15</sup>. Here we report that expression of L-selectin and exposure of cells to SDF-1, improve up to five fold homing of cardiac mesoangioblasts to the infarcted free wall of the heart. We also show that this treatment improves the recovery of the LV wall motion after infarction and the quantity of regenerated myocardium. These results elucidate the homing mechanism of cardiac mesoangioblasts and open a new opportunity for improving efficacy of cell therapy in cardiovascular diseases.

## RESULTS

*Neonatal cardiomyocytes and SDF-1 favor cardiac mesoangioblasts trans-migration in vitro.*

Cardiac mesoangioblasts were starved for 12h in absence of serum and then subjected to transmigration assays using Transwell chambers.

In a first series of experiments we created an artificial environment where cardiac mesoangioblasts would face a **TNF $\alpha$ -activated endothelium** separating them from neonatal cardiomyocytes (much as it happens in regenerating heart) or specific cytokines with known chemoattractive potency. As shown in figure **1A**, cardiac mesoangioblasts were unable to cross endothelium-coated filters in the absence of stimuli. However, neonatal cardiomyocytes in culture induced active migration (six fold above control) of cardiac mesoangioblasts but not of primary fibroblasts. This effect was slightly stronger than that induced by FGF, used as a positive control. We also performed a chemotaxis assay through endothelium-coated filters with a panel of cytokines. The concentrations were selected after performing a dose-response curve for the cardiac mesoangioblasts with each cytokine (data not shown). As shown in figure **1A**, the presence of SDF-1 in the lower chamber caused **an eight-fold increase of mesoangioblast migration**, a significantly more robust effect than that elicited by FGF, MCP-1 or TNF- $\alpha$ , previously described as enhancer of mesoangioblast migration<sup>13</sup>; IL-1, IL-6 and IL-10 had no effect on cardiac mesoangioblasts migration (data not shown). **Interestingly, when an antagonist for the CXCR4 (AMD3100) was used, the cardiomyocytes-induced**

**mesoangioblasts migration was abolished by 40% demonstrating a direct correlation between the SDF-1-induced signaling and the migration of the cardiac mesoangioblasts.** Besides, as shown in figure 1B, SDF-1, MCP-1 and IL-8 were detected at higher level in the supernatant from cardiomyocytes than in the corresponding supernatant from fibroblasts. These data suggest that differentiated cardiomyocytes may secrete growth factors or cytokines such as SDF-1 or MCP-1 that favor cardiac mesoangioblasts transmigration by either a chemotactic effect and/or by modifying the endothelium barrier.

*Cardiac mesoangioblasts and homing in vivo to the heart*

A property of stem cell is the ability to migrate through different routes, including circulation. Preclinical or clinical trials for cardiovascular diseases have had modest success, possibly because of inefficient delivery of donor cells to the heart, besides limitations in survival and differentiation of these cells once they homed. The most widely used route of administration of progenitor cells is intracoronary, through the catheter inserted for PCI early after symptoms of acute myocardial infarction. Since intracoronary injection cannot be performed *in vivo* in the mouse, we reasoned that if the cardiac mesoangioblasts were injected percutaneously into the LV chamber, at least 3-5% of them would reach the coronary circulation before passing through filter organs. To assess the ability of cardiac mesoangioblasts to migrate *in vivo*, we injected  $5 \times 10^5$  GFP-labeled cardiac mesoangioblasts into the LV chamber of wt C57 mice and mice that had undergone CAL few hours earlier. Mice were sacrificed 6, 12 or 24h after injection; heart (free wall) as well as filter organs (liver, spleen and lungs) were collected and RNA was extracted. The percentage of migrated cardiac mesoangioblasts in each recipient organ was calculated by real time PCR for GFP expression as a percentage of the value corresponding to the total of injected cells. As shown in Fig. 2A, the cardiac mesoangioblasts that were able to migrate to heart in the control mice (gray columns), where approximately 5% of the injected cells, whereas most of injected cells were retained in the different filter organs, without reaching the heart. Interestingly, the presence of an infarct in the CAL model (black columns) induced an enhanced migration (2-fold) of the cardiac mesoangioblasts to the infarct zone, compared to their control counterpart. Most of injected cells were again retained in the filter organs, but this time around 10% of injected cells could reach the damaged area of the heart. Similar results were obtained when real time PCR was performed for y-chromosome as double control (Figure S1A). We set our time of analysis at 6 h after injection for the following *in vivo* experiments as we observed that the number of injected cells remains constant for the first 12 hours after the injection and thereafter decreases to varying extent (up to 50% of the value observed at 6 h: Fig. 2A). As shown in figure 2B, the higher concentration of SDF-1, IL-8 and MCP-1 in the free wall of hearts from CAL mice in comparison with control mice could explain the preferential homing of cardiac mesoangioblasts.

*Pretreatment of cardiac mesoangioblasts with SDF-1 increases their migration.*

In an attempt to increase the number of cardiac mesoangioblasts that homes specifically to the infarcted zone of the heart, we followed two approaches. First, we pre-treated cardiac mesoangioblasts with several cytokines for 12h before challenging them in the *in vitro* transwell assay. The results, shown in figure 3A, clearly indicated that pre-treatment with SDF-1 increased cardiac mesoangioblasts migration to the same extent observed when the same cytokine was present in the lower chamber. This pretreatment did not modify the surface selection markers neither the differentiation gene expression profile of the cardiac mesoangioblasts described previously<sup>14</sup>. Therefore, SDF-1 pretreated GFP-cardiac mesoangioblasts were injected into the LV chamber of control WT or CAL mice and after 6h, hearts and filter organs were collected and analyzed by real time PCR for presence of GFP. As shown in figure 3B, SDF-1 increased about three fold the homing of cardiac mesoangioblasts to the injured area of the heart in mice that underwent CAL. Consistently, the number of cardiac mesoangioblasts detected in the filter organs was reduced to almost half of control.

We then attempted to identify the surface molecules responsible for the changes observed in cardiac mesoangioblasts migration following pretreatment with SDF-1. We therefore performed a microarray analysis for 125 genes including surface receptors to look for candidates genes induced in cardiac mesoangioblasts by SDF-1 (Figure 3C). Five genes were significantly modified after pretreatment of cardiac mesoangioblasts with SDF-1: MMP-2 and 9, CD44, caveolin-1 and Itg $\alpha$ v. We confirmed their protein expression by Western Blot (data not shown). To verify the role of those molecules in cardiac mesoangioblasts transmigration we repeated the transwell assay in presence of antibodies against CD44 or  $\alpha$ v-integrin, MMP inhibitor GM1489 or caveolin-1 siRNA (Figure 3D). As shown in figure 3D, antibodies

against CD44 or cav-1 siRNA significantly inhibited SDF-1 induced *in vitro* transmigration. Blocking MMPs activity with GM1489 inhibited slightly transmigration induced by SDF-1.

Together these data suggest that SDF-1 increase cardiac mesoangioblasts transmigration *in vitro* and homing to heart *in vivo* by increasing expression of several molecules among which CD44 seems to be preferentially expressed.

*L-selectin is necessary for efficient cardiac mesoangioblasts transmigration and homing to heart.*

As revealed by microarray analysis (unpublished data, Tagliafico E and Cossu G), cardiac mesoangioblasts do not express some of the key molecules that control rolling and extravasation of leukocytes and different stem cells such as L-selectin or  $\beta 2$  integrin<sup>1, 5</sup>. Therefore, we transduced mesoangioblasts with vectors expressing these molecules and also EGFP that were used previously by our group<sup>13</sup>. These constructs did not modify the surface selection markers neither the differentiation gene expression profile of the cardiac mesoangioblasts described previously<sup>14</sup>. To assess whether the expression of these molecules could improve cardiac mesoangioblasts migration, cells transfected with one or both constructs were subjected to transmigration *in vitro* assay through activated endothelium. As shown in figure 4A, L-selectin expression increased by 6 to 8 fold cardiac mesoangioblasts transmigration, while  $\beta 2$  integrin expression increased only to 2 fold their migration. Co-transfection of both constructs had not statistically significant cumulative effect (Figure 4A). When L-selectin expressing cardiac mesoangioblasts were injected into the LV chamber of mice early after CAL, the number of cells that reached free wall of the heart increased approximately two fold in different mice (Figure 4B). Consistently with *in vitro* results, we did not observe any cumulative effect with co-transfection of L-selectin and  $\beta 2$  integrin. Therefore, expression of L-selectin by cardiac mesoangioblasts improves their migration and homing possibly by favoring their interaction with the ligands expressed on the endothelium surface.

*Pretreatment with SDF-1 of L-selectin expressing cardiac mesoangioblasts increased by 5 fold their homing to heart.*

We then tested the possibility of additive or synergistic effects of the two approaches shown above. Cardiac mesoangioblasts previously transfected with L-selectin constructs, were pretreated for 12h with SDF-1 and then subjected to transmigration assay through endothelium. As shown in figure 4C, the combination between SDF-1 pretreatment and L-selectin expression **seemed to have a slightly synergistic effect** and improved cardiac mesoangioblasts transmigration *in vitro* fifteen fold more efficiently than control. Therefore, these cells were injected into the LV chamber of infarcted mice. As shown in figure 4D, results showed that almost 50% of injected cells reached the injured myocardium of the heart after 6h, with a concomitant reduction in the number of cardiac mesoangioblasts detected in filter organs (Figure 4D). **Again SDF-1 pretreatment and L-selectin expression demonstrated a clear synergistic effect in the *in vivo* experiments.** Similar results were obtained when real time PCR was performed for y-chromosome as double control (Figure S1B).

*Myocardium regeneration after long term treatment with modified cardiac mesoangioblasts.*

Finally, to assess the ability of modified cardiac mesoangioblasts not only to migrate *in vivo* to the heart but also to survive and regenerate myocardium, we injected  $5 \times 10^5$  GFP-labeled control or modified (SDF-1 plus L-selectin) cardiac mesoangioblasts into the left ventricular chamber of C57 mice that underwent coronary artery ligation. Mice were then sacrificed six weeks after injection, and heart (free wall) as well as filter organs (liver, spleen and lungs) were harvested and RNA was extracted. The percentage of migrated and survived cardiac mesoangioblasts in each recipient organ (engraftment) was calculated by real time PCR for GFP expression as a percentage of the value corresponding to the total of injected cells, normalized to their respective sham. As shown in Figure 5A, approximately 10% of control injected cells were found in the heart after six weeks, whereas more than 35% of the injected modified cardiac mesoangioblasts were found in the same region. Similar results were obtained when real time PCR was performed for y-chromosome as double control (Figure S1C). To further verify these percentages, the GFP positive population was sorted from digested hearts that had been injected six weeks before with control or treated cells. As shown in Figure 5B, GFP positive population was two times more abundant in the case of pretreated cells (SDF-1 plus L-selectin) than with control cells. As shown in Figure 5C, control cardiac mesoangioblasts remained mainly in the ventricle wall (see arrowheads) while modified cardiac mesoangioblasts colonized larger areas inside the myocardium. Interestingly, these modified GFP cardiac mesoangioblasts could also differentiate into new cardiomyocytes expressing sarcomeric actin six weeks after injection and therefore regenerate the damaged

tissue (Figure 5D, 5E and Figure S2). In fact, protein extraction from CAL-injected hearts confirmed the presence of injected GFP-cardiac mesoangioblasts inside the ventricle, being higher the quantity of modified cardiac mesoangioblasts than in control mesoangioblasts **after 6 weeks** (Figure 5F). Of note, GFP-cardiac mesoangioblasts isolated from the free wall of the myocardium also expressed the sarcomeric actin protein six weeks after injection as analyzed by Western Blot (Figure 5F, lower panel) as well as late cardiac differentiation genes analyzed by real time PCR (Figure 5G), as naturally do mature cardiomyocytes. Finally, before sacrificing the mice for the morphological analysis described before, we performed echocardiography studies that revealed a slight improvement of the ventricular wall thickness, a two-fold increase of ejection and shortening fraction and minor changes in left ventricular chamber diameter, six weeks after modified (SDF-1+L-Selectin) cardiac mesoangioblasts injection (Table 1). Therefore, the combined treatment of the cardiac mesoangioblasts with SDF-1 and L-selectin increases the quantity of cells that arrive to the damaged heart and improves to varying extent the myocardium structure.

*Human cardiac mesoangioblasts migration to the heart is also susceptible of being regulated by SDF-1 pretreatment and L-selectin expression.*

To test whether a similar protocol would be also suitable for human cells, we used human cardiac mesoangioblasts that have been described before to reconstitute myocardium after injection into SCID-mice<sup>15</sup>. They have morphology and gene expression similar to their mouse counterparts, a finite lifespan and the ability to differentiate into cardiomyocytes. Human cardiac mesoangioblasts were serum starved and then subjected to transmigration assays using Transwell chambers.

As shown in figure 6A, human cardiac mesoangioblasts were also unable to cross endothelium-coated filters in absence of stimuli. However, mature cardiomyocytes or pretreatment of cells with SDF-1 induced from five to eight fold human cardiac mesoangioblasts migration.

We then transfected human cardiac mesoangioblasts (that also do not express these molecules: Tagliafico and Cossu, unpublished observations) with vectors expressing L-selectin and, after transfection, selected the GFP positive cells. As shown in figure 6A (right column), SDF-1 pretreatment of L-selectin expressing human cardiac mesoangioblasts increased three fold their ability to cross the endothelial barrier in Transwell assays. We then tested if these *in vitro* results could be translated into an animal model for infarction. Therefore, human cardiac mesoangioblasts pretreated with SDF-1 and transfected with vectors encoding L-selectin were injected into the LV chamber of CAL-SCID mice that do not reject human cells. As shown in figure 6B, almost 45% of human modified (SDF-1 pretreated and L-selectin transfected) cardiac mesoangioblasts reached the infarcted free wall of the heart, six hours after injection into the LV chamber, with a concomitant reduction in the number of cells detected in filter organs.

Together these data show that it is possible to increase homing to the damaged area of the heart of human cardiac mesoangioblasts with an experimental protocol similar to that developed for mouse cardiac mesoangioblasts.

## DISCUSSION

Ischemic heart disease is today the major cause of cardiac failure, and thus is a leading cause of death and a major cause of disability worldwide. Cell therapy protocols, using different types of stem cells, have achieved partial success<sup>16-21</sup>; until now intravascular or intra-myocardial delivery of stem cells to the heart appeared as the most promising because of a partial but significant restoration of cardiac muscle structure and function. However, the migration and differentiation potency of the stem cells as well as their ability for regeneration and the immune reaction of the host are technical issues that have to be solved before any clinical trial. In this work, we provide a new experimental strategy, based on pretreatment of donor cells with cytokines and surface expression of certain adhesion molecules, that dramatically improves homing of cardiac mesoangioblasts to the damaged heart.

*Testing cardiac mesoangioblasts migratory activity in vitro.*

We initially measured cardiac mesoangioblasts migration with a transwell assay, already proven as a reliable indicator of the mechanisms that govern cellular trafficking *in vivo*<sup>22</sup>. Cardiac mesoangioblasts cross the endothelium and migrate towards mature cardiomyocytes, an *in vitro* setting mimicking the microenvironment encountered by these cells after intravascular injection. Likely, some factors secreted by these mature cardiomyocytes stimulate cardiac mesoangioblasts migration as it has been demonstrated for

other cell types<sup>23-25</sup>. Micro-array analysis had also shown that cardiac mesoangioblasts express several receptors for cytokines and therefore may respond to factors released by inflammatory cells and, possibly by cardiomyocytes during myocardial infarction. Indeed we found that SDF-1 was the most potent inducer of cardiac mesoangioblasts migration, more than TNF- $\alpha$ , previously described as a chemoattractant for skeletal muscle mesoangioblasts<sup>13</sup> or for mesenchymal stem cells in adipose tissue<sup>11</sup>. SDF-1 has previously been described to be present in the infarcted area<sup>10</sup>.

#### *Cardiac mesoangioblasts homing to cardiac tissue in vivo*

Homing to cardiac tissue in vivo proceeds through multiple mechanisms, only partially mimicked by in vitro assays. When wild type cardiac mesoangioblasts were injected into the LV chamber of the heart in infarcted or wild type mice only a small percentage of injected cells reached the injured myocardium at their first passage, while most cells ended trapped in filter organs. This was also indicated by the fact that very low number of injected cells could be detected in the infarct area (left ventricular free wall), that they may have also reached by recirculating through the arterial bed. Interestingly, the percentage of donor cells that could be recovered from the infarcted zone of the heart was significantly higher than that from the spared myocardium or from control hearts, possibly linked to the different cytokines pattern expression in the cardiomyocytes exposed to ischemic injury<sup>26-28</sup>. In fact, when injected into the LV chamber, cardiac mesoangioblasts accumulate in the first capillary filter they encounter and are able to migrate outside the vessel, but only in the presence of inflammation, as in the case of infarcted heart. Indeed, cardiac mesoangioblasts express many receptors for inflammatory cytokines and are able to migrate in vitro and in vivo in response to different stimuli. More importantly, the delivery of the cells of interest into the LV chamber, to be translated clinically to intra-coronary injection, will reach easily the infarcted heart, without the need of surgical methods of delivery, such as intra-myocardial injections.

#### *Enhancing cardiac mesoangioblasts migratory activity.*

Previous work had shown that HMGB-1 or TNF- $\alpha$  stimulate proliferation and migration of skeletal muscle mesoangioblasts to dystrophic muscle<sup>12, 13</sup>. As in the case of skeletal muscle mesoangioblasts, we hypothesized that different stem cells would use different signaling pathways and cytokines receptors for homing to the tissue of interest. In the case of the cardiac mesoangioblasts, we observed that SDF-1 was the most potent molecule as chemo-attractant. Interestingly, our work shows that pre-treatment of donor cells with this cytokine is sufficient to activate or increase expression of certain surface proteins necessary for the migration and extravasation process, immediately suggesting a simple ex-vivo treatment of cells that does not require expression in target hearts. We further investigated the mechanisms involved in this migration increment through a low-density array hybridization performed with cardiac mesoangioblasts pretreated with SDF-1. The role of the different genes modified by the treatment was studied in the transmigration assay using inhibitors or blocking antibodies. This experiment revealed that SDF-1 acts via CD44, a molecule that has been described to help in the rolling and extravasation of inflammatory cells<sup>29, 30</sup>. However, cardiac mesoangioblasts do not normally express many of these leukocytes molecules needed for an efficient rolling and arrest at blood vessels, which may explain why most of the injected cells do not bind efficiently, while they flow through the heart capillary network and are finally trapped inside filter organs. Therefore, L-selectin and  $\beta$ 2 integrin were expressed in cardiac mesoangioblasts to study their migration ability. The expression of L-selectin resulted in a 8-fold increase of cardiac mesoangioblasts transmigration in vitro, and the number of cardiac mesoangioblasts that reached the damaged cardiac tissue was also doubled in vivo, reducing their number in filter organs. The combined strategies (i.e. pretreatment with SDF-1 of cardiac mesoangioblasts expressing L-selectin) resulted in a 15-fold increment of transmigration through endothelium in vitro and five fold enhanced migration in vivo, with more than 50% of injected cells now present in the infarct area and border zone of the infarcted hearts. Finally, we demonstrate that modified cardiac mesoangioblasts are not only able to reach the heart but also to colonize large areas of myocardium where they can differentiate into mature cardiomyocytes and regenerate the damaged tissue.

In the perspective of a cell therapy in patients, it is important to test whether a similar protocol will also work with human cells. Human cardiac mesoangioblasts have been isolated from fragments of vessels inside biopsies of adult human heart and their characterization has been reported<sup>15</sup>. Human cardiac mesoangioblasts were also exposed to SDF-1 and transfected with L-selectin and, under these conditions, reached cardiac tissue of infarcted-SCID mice three fold more efficiently than their corresponding untreated cells. While these data suggest that further work may still be required to optimize human cardiac mesoangioblasts homing to the heart, they clearly indicate that the basic homing mechanisms are similar in



mouse and human cardiac mesoangioblasts and equally susceptible to enhancement by similar cytokines and adhesion molecules.

In conclusion, our results suggest that pretreatment of cardiac mesoangioblasts with SDF-1 and the presence of L-selectin are required for an efficient intra-vascular delivery of stem cells to the injured heart in the course of acute myocardial infarction. Pretreatment of stem cells with cytokines is a simple procedure, and inclusion of a relatively small cDNA in a viral vector, designed for gene correction, is also a feasible procedure in the context of autologous cell therapy with engineered stem cells. Together these strategies represent a novel and simple method for improving the homing of stem cells to the damaged heart and for increasing the regeneration area and could be tailored in the future to different types of stem cells in different clinical cell therapy protocols.

## MATERIALS AND METHODS

### *Reagents*

Anti-GFP Ab was purchased from Chemicon (Temecula, CA). Anti- $\alpha$ v integrins and anti-CD44 Abs were from Serotec (Raleigh, NC). Most reagents and some antibodies were purchased from Sigma, unless stated otherwise. All the cytokines used were obtained from Peprotech Inc (Rocky Hill, NJ). GM1489, MMP inhibitor was from Calbiochem (EMD Biosciences Inc, Darmstadt, Germany). Alexa-488 labeling kit was from Molecular probes inc. (Leiden, Netherlands). Caveolin-1 siRNA was generated and used as described before<sup>31</sup>.

C57BL10 WT and SCID-beige mice were purchased from Charles River Laboratories (Wilmington, MA) and Homo-EGFP from The Jackson Laboratory (Bar Harbor, Maine). All mice were handled following Institutional guidelines.

### *Cell cultures*

Neonatal cardiomyocytes were cultured in DMEM plus 20% FBS and induced to differentiate in DMEM plus 2% FBS. Cardiac mesoangioblasts were isolated from control or Homo-EGFP male mice and from human ventricle explants, cloned and expanded as described previously<sup>14,15</sup>. Constructs have been described previously<sup>13</sup> and were transfected into mice or human cardiac mesoangioblasts with lipofectamine (Invitrogen) **and positive clones were selected by sorting**. Human cardiac mesoangioblasts transduced with a lentiviral vector encoding the GFP protein were used for some in vivo experiments. Murine microvascular endothelial H5V cells were grown in DMEM plus 10% FBS and starved for 12h before functional experiments.

### *Real Time PCR*

Total RNA from different organs was isolated with TRIzol protocol (Invitrogen, Carlsbad, CA) and reverse transcribed by Taqman kit (Platinum Taq DNA polymerase, Invitrogen). Real time quantitative PCR was done on an Mx3000P real time PCR system (Stratagene, La Jolla, CA). Each cDNA sample was amplified in duplicate by using the SYBR Green Supermix (BioRad, Hercules, CA) for GFP or male y-chromosome detection as double control (Primers GFP: Fw, AAGTTCATCTGCACCACCG; Rw, TCCTTGAAGAAGATGGTGCG; Primers male y-chromosome: Fw, AAGCGACCCATGAACGCATT; Rw, TTCGGTATTTCTCTCTGTC). Primers for cardiac differentiation were used as described previously<sup>14</sup>. Data are expressed as the percentage of migrated cells, which is calculated by comparing the concentration of target gene in our sample with the total input of injected cells.

### *Western blot*

Tissues or GFP-sorted fractions were homogenized and proteins extracted and lysed directly in Laemmli buffer on ice. Lysates were resolved on 10% SDS-PAGE under reducing conditions, and protein were transferred to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Piscataway, NJ). Membranes were revealed with anti-GFP, anti-sarcomeric actin and anti-tubulin Abs.

### *In vitro Transwell migration assay*

8 mm transwell filters (Corning Incorporated, Acton, MA) were coated with 1% gelatin and endothelial cells H5V (**previously pre-activated by 12h exposure to TNF- $\alpha$  in order to induce the expression of surface antigens and adhesion molecules needed for the appropriate extravasation**) were plated to confluence on

them for 24h. At the same time, neonatal cardiomyocytes were grown on a p24w plate with differentiated medium for 0 to 4 days.  $10^4$  mice cardiac mesoangioblasts wt or pretreated (after a dose-response assay (data not shown), the concentrations used were: 50 ng/ml and IL8 SDF-1; 80 ng/ml MCP-1; 30 ng/ml TNF- $\alpha$ ; 30 ng/ml FGF) or transfected were plated on the upper side of transwell chamber 24h after plating H5V cells on the filter and immediately the chamber was then moved to the wells containing differentiated cardiomyocytes to assay directed migration. After 6h of transmigration, migrated cardiac mesoangioblasts were counted on the lower side of the filter. The results show migrated cells as percentage of total input cells.

#### *In vivo migration/grafting assay*

4-month-old WT, CAL mice were injected into the ventricle wall<sup>32</sup> with  $5 \times 10^5$  GFP cardiac mesoangioblasts. After 6, 12, 24h or 6 weeks animals were sacrificed and different regions of the heart or filter organs were collected. RNA was extracted and a real time PCR for GFP and male chromosome as double control (Supplementary Figure 1) was performed in all the samples as described above. Data are expressed as the percentage of migrated cells, which is calculated by comparing the concentration of target gene in our sample with the total input of injected cells.

Same samples were also processed for immunohistology against GFP; briefly, the heart was removed from mice previously injected with cardiac mesoangioblasts, the LV free wall and the intraventricular septum and right ventricle were separated and frozen in liquid N<sub>2</sub> cooled isopentane. Serial heart sections were fixed with 4% PFA, permeabilized and immunostained as previously described (Gálvez et al, 2008) with anti-GFP, anti-laminin or anti-sarcomeric actin Abs. Alexa 488 or 594 (Molecular Probes, Eugene, OR) were used as secondary staining Ig and DAPI for nuclear staining. Images were taken with a S100 TV microscope (Carl Zeiss MicroImaging, Inc.).

#### *Echocardiography*

Mice were anaesthetized by inhalation of isoflurane/oxygen (1.25%/98.75%) and examined by a 30MHz transthoracic echocardiography probe. Images were obtained with Vevo 770 (VisualSonics, Toronto, Canada) from mice two months after injection. Short-axis and long axis, BMode and 2D M-Mode views were recorded for off-line analysis. From these images, left ventricle (LV) function was estimated by three validated methods as the shortening fraction (SF), calculated as recommended by the American Society of Echocardiography<sup>33,34</sup> and ejection fraction (EFm) obtained from the M mode, quantitative ejection fraction measured from the B mode (EFb), and visual assessment from the B mode by an expert in echocardiography in a blind fashion. Diastolic and systolic manual segmentation of the LV endocardial edge in four chambers views of the heart was also measured. All data after analysis have been represented in a summary table (Table-1; LVIDd: left ventricular diastolic diameter; EF: ejection fraction; FS: fractional shortening; LVAWThd: left ventricular anterior wall diastolic thickness).

#### *Prospective Isolation*

Control and injected hearts were finely minced and digested with collagenase/dispase (Sigma) digestion (0,8 IU/mg for 30 min at 37 C°). The cell suspension was filter to remove tissue debris. Isolated cells were harvested and resuspended in 1 ml of Flow Sort solution containing 5  $\mu$ g/ml propidium iodide (Sigma) in PBS (Gibco Brl). Run cells in the sorter and set up voltage for GFP (FL1) and PI for viability (FL3). GFP+/PI- were sorted and collected for further experiments.

#### *Microarray analysis*

For mRNA analysis, total RNA was extracted from treated cardiac mesoangioblasts using TRIzol (Invitrogen Life Technologies). cDNA was prepared by reverse transcription reaction and hybridized to the GEArray gene expression array MM-010 for extracellular matrix and adhesion molecules (Superarray Bioscience, Frederick, MD) according to the manufacturer's instruction. Data were subjected to densitometric analysis using GEArray Expression Analysis Suite (SuperArray Bioscience Corporation). RNA levels were expressed as the relative density after normalizing the hybridization signal to  $\beta$ -actin. Protein expression of positive candidates genes was confirmed by Western Blot (data not shown).

#### *Cytokines measurements*

Supernatants from fibroblasts or cardiomyocytes cultures were collected after 4 days of culture and analyzed with the mouse multi-cytokine detection system from Millipore (Chemicon, Upstate) for measuring cytokines according to manufacturer instructions. **Hearts from different animal models were**

homogenized in cell lysis buffer containing 0,1% Igepal and total protein concentration was determined using a DC Protein Assay Kit and spectrophotometer (Bio-Rad). All heart samples were diluted with cell lysis buffer as needed to a final total protein concentration of 500 g/ml and cytokines were measured using the same kit protocol as for supernatants. Concentration of cytokines is represented in pg/ml for supernatants values and pg/mg for tissue total protein values.

#### *Statistical analysis*

Statistical significance of the differences between the percentages values was assessed by using the Kruskal-Wallis One-Way ANOVA by Ranks test.  $\alpha$  will represent the significance.

#### **Acknowledgements**

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#### **Conflict of interest**

The authors declare no conflict of interest.

**Abbreviations List:** Ab, antibody; CAL, coronary artery ligation; cmc, cardiomyocytes; EGFP, enhanced green fluorescence protein; FGF, fibroblast growth factor; siRNA, small interference RNA; MMP, metaloproteinase; SDF-1, stromal derived factor-1; siRNA, small interference RNA; TNF- $\alpha$ , tumor necrosis factor-alpha;

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## FIGURE LEGENDS

### **Figure 1.** Induction of cardiac mesoangioblasts transmigration by cytokines *in vitro*.

- A. Mouse cardiac mesoangioblasts or fibroblasts were plated on endothelium-coated Transwell filters and induced to migrate for 6h in presence of **cardiomyocytes (CMC) or different cytokines (30 ng/ml FGF; 50 ng/ml SDF-1; 80 ng/ml MCP-1; 30 ng/ml TNF- $\alpha$ ; 10 ng/ml AMD3100, CXCR4 antagonist.)**. A representative out of five independent experiments run in duplicate is shown. (left; \*  $\alpha < 0.02$ ). A representative image of the transmigrated cardiac mesoangioblasts in presence of **cardiomyocytes, SDF-1 or MCP-1** is also shown. Bar, 30  $\mu$ m.
- B. Supernatants from fibroblast or cardiomyocytes were collected after 4 days and different cytokines were detected using the mouse multi-cytokine detection system. One out of three experiments are shown. (\* $\alpha < 0.025$ ).

Figure 2. Homing of cardiac mesoangioblasts in vivo.

- A. Cardiac mesoangioblasts were injected into the LV chamber of mice subjected to **coronary artery ligation (CAL)** or control mice. After 6, 12 or 24h heart as well as filter organs were collected and the number of migrated cells was calculated by real time PCR for GFP. A mean of three independent experiments run in triplicate is shown (\* $\alpha < 0.03$ ).
- B. Myocardium free walls of hearts from 2-months-old wt or CAL mice were homogenized and analyzed for cytokine expression with the mouse multi-cytokine detection system. **Concentrations have been normalized to protein load.** One out of three experiments are shown. (\* $\alpha < 0.025$ ).

Figure 3. SDF-1 improves cardiac mesoangioblasts migration via CD44, MMPs and cav-1.

- A. Cardiac mesoangioblasts pretreated with different cytokines (50 ng/ml IL8 and SDF-1; 80 ng/ml MCP-1; 30 ng/ml TNF- $\alpha$ ; 30 ng/ml FGF) were plated on endothelium-coated filters and induced to migrate for 6h in presence of mature cardiomyocytes in the lower chamber. FGF was used as positive control. One representative out of five independent experiments run in duplicate is shown. (left; \*  $\alpha < 0.01$ ). A representative image of the transmigrated SDF-1-pre-treated cardiac mesoangioblasts is also shown (right). Bar, 30  $\mu$ m.
- B. GFP-cardiac mesoangioblasts pre-treated with 50 ng/ml SDF-1 were injected into the LV chamber of control or CAL mice and after 6h heart and filter organs were analyzed by real time PCR for the presence of migrated cells (\* $\alpha < 0.03$ , +  $\alpha < 0.02$ ).
- C. A gene array containing oligos corresponding to different mouse adhesion molecules was hybridized with cDNAs probes retrotranscribed from RNA of cardiac mesoangioblasts pretreated with SDF-1. Relative RNA levels of selected mRNAs normalized to b-actin expression are shown. Data presented are the mean of two independent experiments (\*  $\alpha < 0.01$ ).
- D. SDF-1 pre-treated cardiac mesoangioblasts were incubated with MMPs inhibitor, GM 1489 or siRNA Cav-1 inhibitor or with Abs against CD44 and alpha-v integrin and induced to migrate for 6h through endothelium-coated filters and in the presence of mature cardiomyocytes in the lower chamber. A representative out of three independent experiments run in duplicate is shown. (\* $\alpha < 0.02$ , +  $\alpha < 0.005$ )

Figure 4. L-selectin increases cardiac mesoangioblasts migration and has synergistic effect with SDF-1 pre-treatment.

- A. Cardiac mesoangioblasts transfected with one or two vectors expressing different surface molecules and EGFP were plated on endothelium-coated filters and induced to migrate for 6 h in the presence of mature cardiomyocytes in the lower chamber. A representative out of five independent experiments run in duplicate is shown (left panel) (left; \*  $\alpha < 0.01$ ). A representative image of the transmigrated cardiac mesoangioblasts transfected with L-selectin is also shown (right). Bar, 30  $\mu$ m.
- B. Cardiac mesoangioblasts transfected with vectors expressing L-selectin or/and  $\beta$ 2-integrin and GFP were injected into the LV chamber of CAL mice and after 6h heart and filter organs were collected. Percentage of migrated cells was calculated after performing real time PCR for GFP. A mean of three independent experiments run in triplicate is shown (\* $\alpha < 0.02$ ).
- C. SDF-1 pre-treated cardiac mesoangioblasts, transfected **or not** with vectors expressing L-selectin and GFP, were induced to migrate for 6h through endothelium-coated filters and in the presence of mature cardiomyocytes in the lower chamber. A mean of three independent experiments run in duplicate is shown (left; \*  $\alpha < 0.01$ ). A representative image of the transmigrated SDF-1-pre-treated cardiac mesoangioblasts transfected with L-selectin is also shown (right). Bar, 30  $\mu$ m.
- D. SDF-1 pre-treated cardiac mesoangioblasts, transfected **or not** with vectors expressing L-selectin-EGFP as well as control cardiac mesoangioblasts were injected into the LV chamber of CAL mice and after 6h heart and filter organs were collected and analyzed by real time PCR for presence of GFP. A mean of six independent experiments run in triplicate is shown (\* $\alpha < 0.01$ ).

Figure 5. Pre-treatment of mouse cardiac mesoangioblasts increases the myocardium regeneration.

- A. Infarcted hearts, injected with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with the L-selectin-EGFP vector or with control cardiac mesoangioblasts, were collected after six weeks

- and subjected to real time PCR for the presence of GFP. A mean of seven independent experiments run in triplicate is shown (\* $\alpha < 0.02$ ).
- B. Infarcted hearts, injected with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with the L-selectin-EGFP vector or with control cardiac mesoangioblasts, were collected after six weeks and sorted for the GFP population after digestion. A representative plot of four independent experiments is shown.
  - C. Immunostaining of free wall collected six weeks after injection with control cardiac mesoangioblasts or with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with the L-selectin-EGFP vector. Laminin is shown in red. Nuclei are stained with Hoechst (blue). Magnification: 450x. Scale bar, 100  $\mu\text{m}$ .
  - D. Immunofluorescence for GFP and sarcomeric actin (red) from a heart region of CAL mice six weeks after injection with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with L-selectin-EGFP. Scale bar, 100  $\mu\text{m}$ .**
  - E. Higher magnification immunofluorescence for GFP and sarcomeric actin (red) from heart of CAL mice six weeks after injection with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with L-selectin-EGFP. Nuclei are stained with Hoechst (blue). Scale bar, 20  $\mu\text{m}$ .
  - F. **Western blot** for GFP and sarcomeric actin of **total** GFP-sorted fractions **recovered** from hearts of CAL mice six weeks after injection with control GFP cardiac mesoangioblasts or with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with the L-selectin-EGFP vector. One representative out of five independent experiments is shown.
  - G. Real time PCR for late cardiac differentiation genes on mature cardiomyocytes and GFP-sorted fraction coming from hearts of CAL mice six weeks after injection with cardiac mesoangioblasts, **both control or pre-treated with SDF-1 and transfected** with the L-selectin-EGFP vector. **Quantities have been normalized.** A mean of four independent experiments run in triplicate is shown.

*Figure 6. Pre-treatment of human cardiac mesoangioblasts also improves homing to the heart.*

- A. Human cardiac mesoangioblasts pretreated with SDF-1 or transfected with L-selectin construction were plated on endothelium-coated filters and induced to migrate for 6h in presence of cardiomyocytes. One representative out of three independent experiments run in duplicate is shown. (left; \*  $\alpha < 0.02$ , +  $\alpha < 0.01$ ). A representative image of the transmigrated SDF-1-pre-treated cardiac mesoangioblasts transfected or not with L-selectin is also shown (right). Bar, 30  $\mu\text{m}$ .
- B. Human cardiac mesoangioblasts transfected with vectors expressing L-selectin and GFP and pre-treated with SDF-1 were injected into the LV chamber of CAL-SCID mice and after 6h heart and filter organs were collected. Percentage of migrated cells is calculated after performing real time PCR for GFP with the different samples. A mean of five independent experiments run in triplicate is shown. (\* $\alpha < 0.025$ , +  $\alpha < 0.015$ ).

## TABLES

*Table 1. Ecocardiography values of hearts coming from mice six weeks after injection of control or cardiac mesoangioblasts transfected with L-selectin and pretreated with SDF-1.*

## SUPPLEMENTARY FIGURES

*Figure S1. Assessment of cardiac mesoangioblast migration by a double control for real time PCR.*

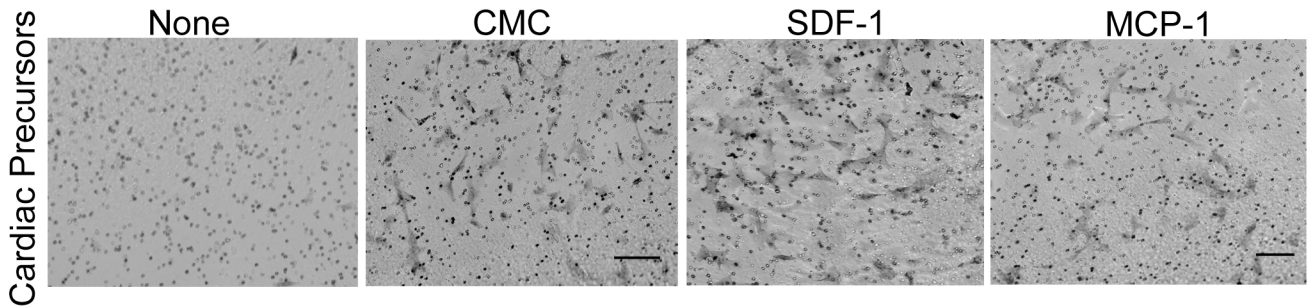
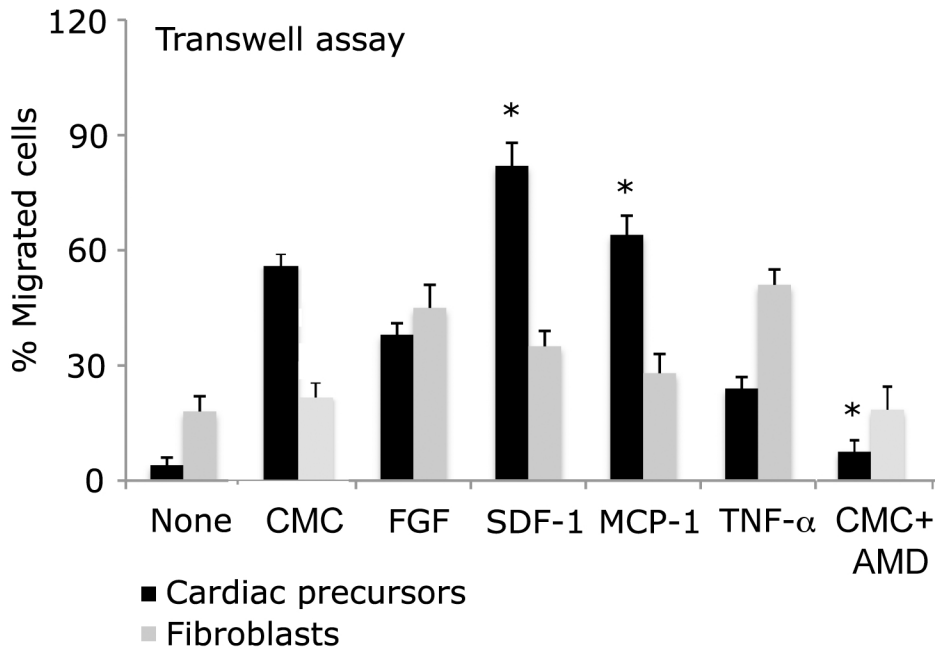
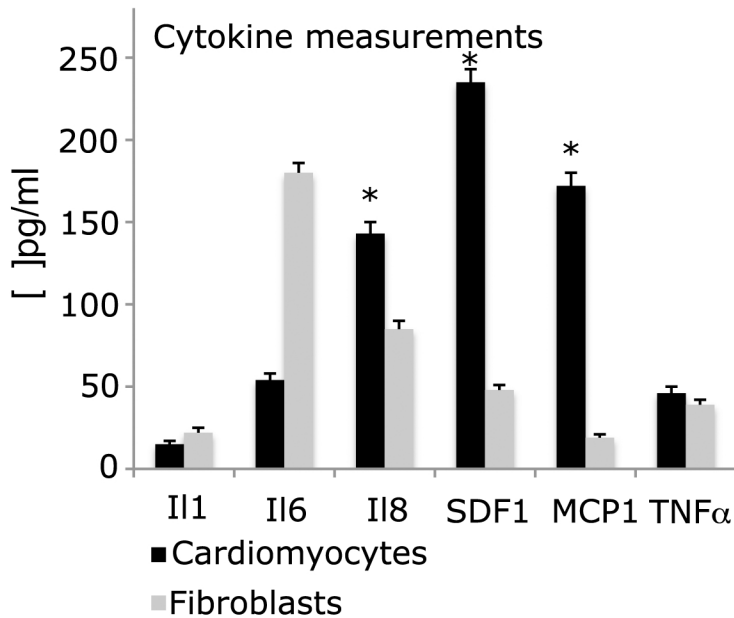
- A. Cardiac mesoangioblasts were injected into the LV chamber of CAL or control mice. After 6, 12 or 24h heart as well as filter organs were collected and the number of migrated cells was calculated by real time PCR for y-chromosome. A mean of three independent experiments run in triplicate is shown (\* $\alpha < 0.05$ ).
- B. SDF-1 pre-treated cardiac mesoangioblasts, transfected with vectors expressing L-selectin-EGFP as well as control cardiac mesoangioblasts were injected into the LV chamber of CAL mice and after 6h heart and filter organs were collected and analyzed by real time PCR for presence of y-chromosome. A mean of six independent experiments run in triplicate is shown (\* $\alpha < 0.025$ ).

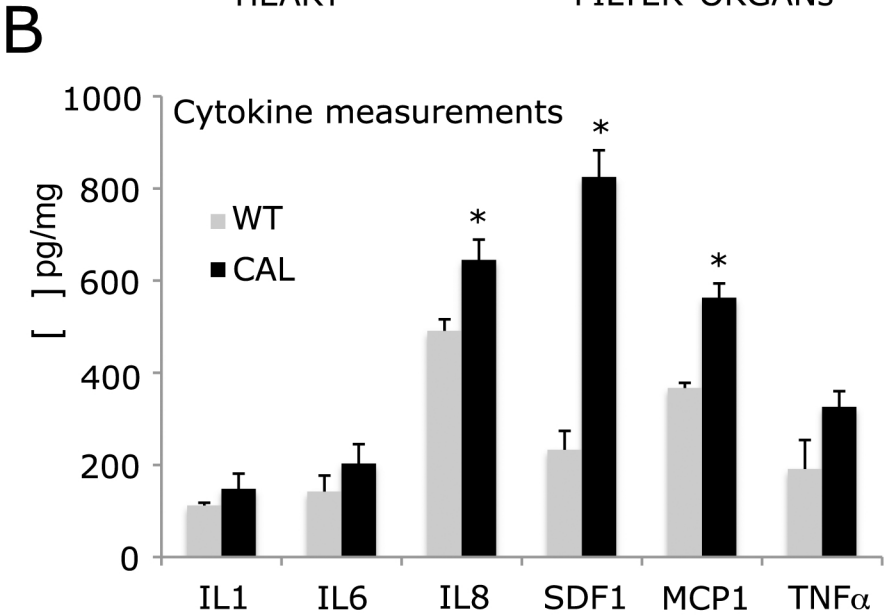
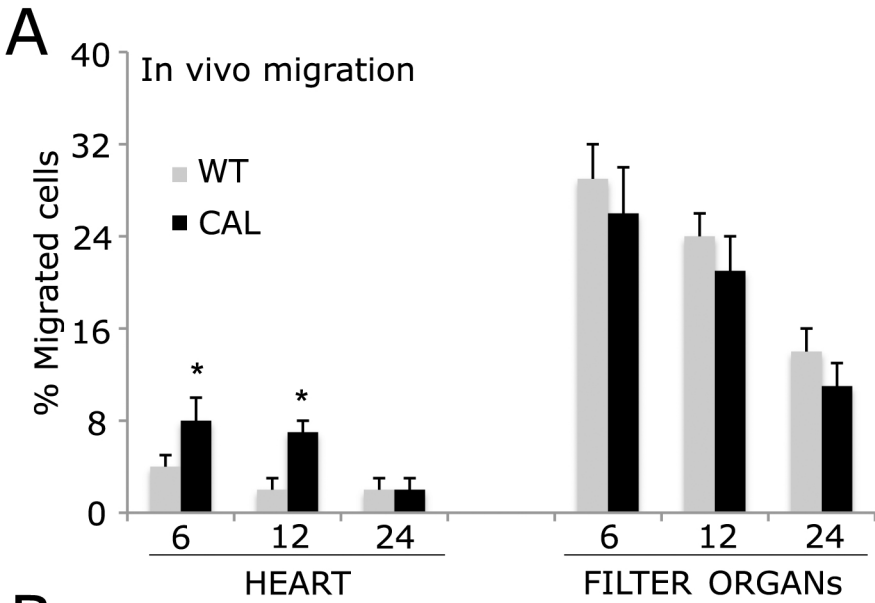
- C. Infarcted hearts, injected with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with the L-selectin-EGFP vector or with control cardiac mesoangioblasts, were collected after six weeks and subjected to real time PCR for the presence of y-chromosome. A mean of seven independent experiments run in triplicate is shown (\* $\alpha < 0.03$ ).

***Figure S2. Immunofluorescence showing GFP cardiac precursors.***

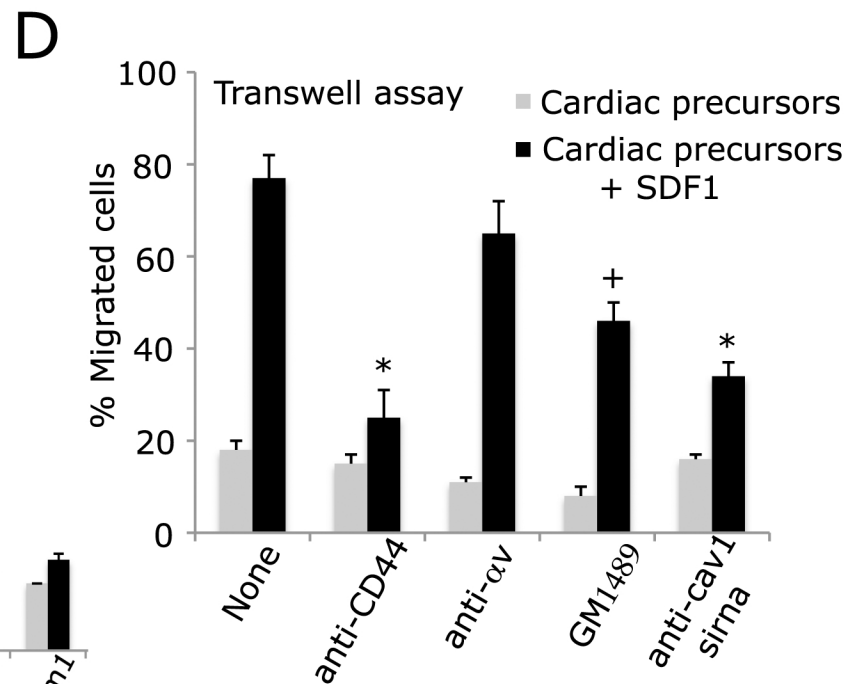
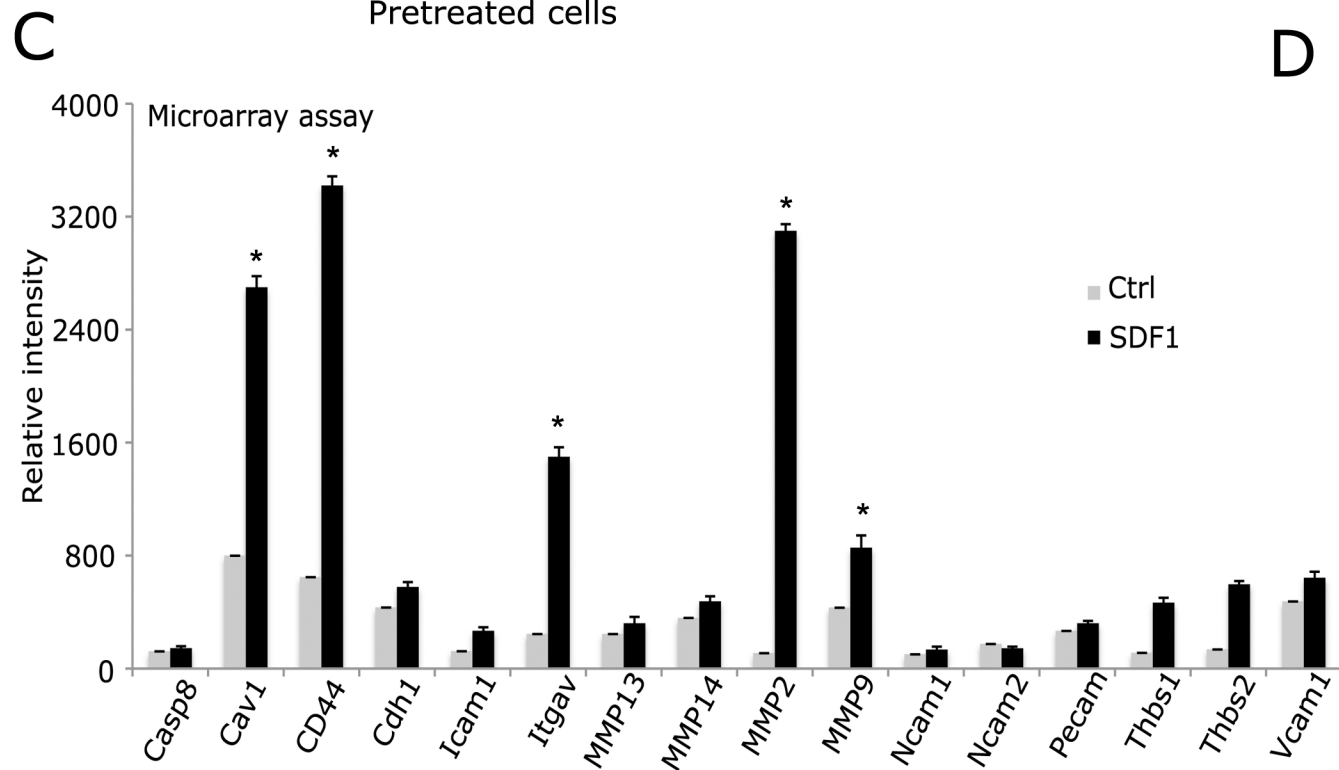
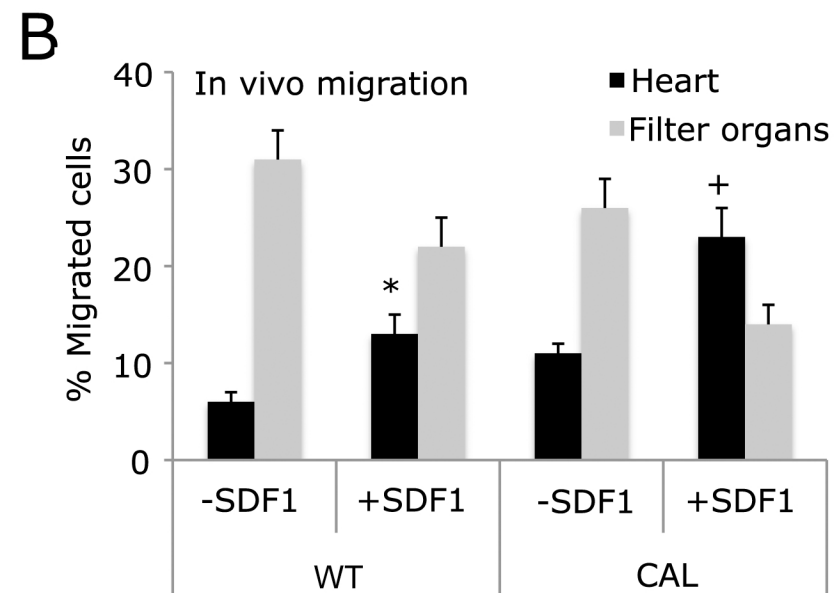
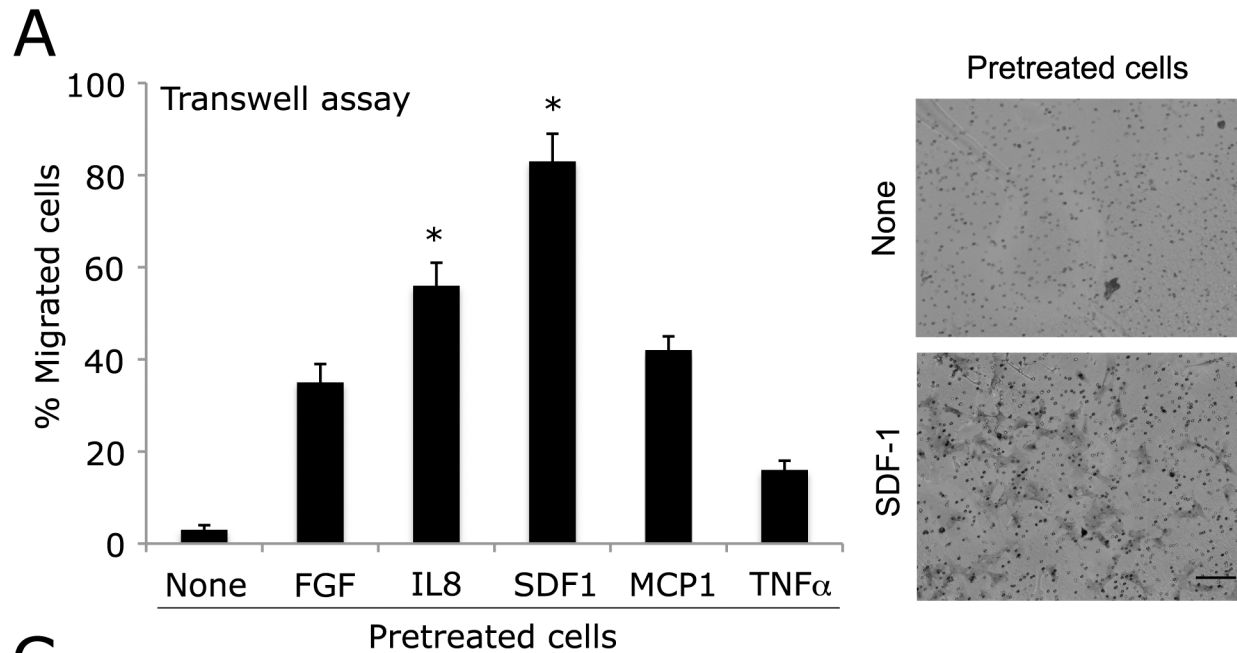
**Immunofluorescence for GFP and sarcomeric actin (red) from heart of CAL mice six weeks after injection with cardiac mesoangioblasts pre-treated with SDF-1 and transfected with L-selectin-EGFP. Nuclei are stained with Hoechst (blue). Scale bar, 20  $\mu\text{m}$ .**



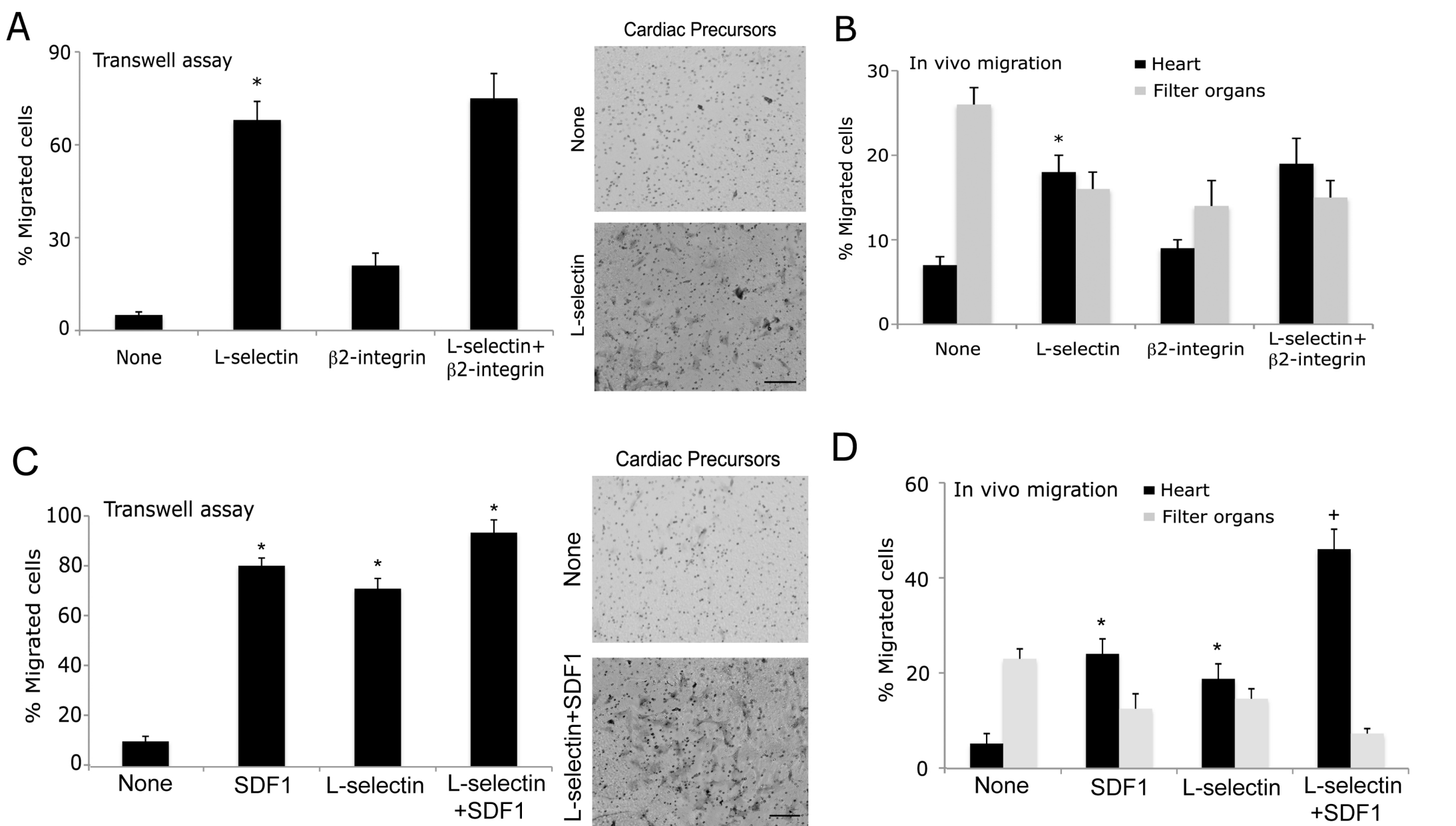
**A****B****FIGURE-1**



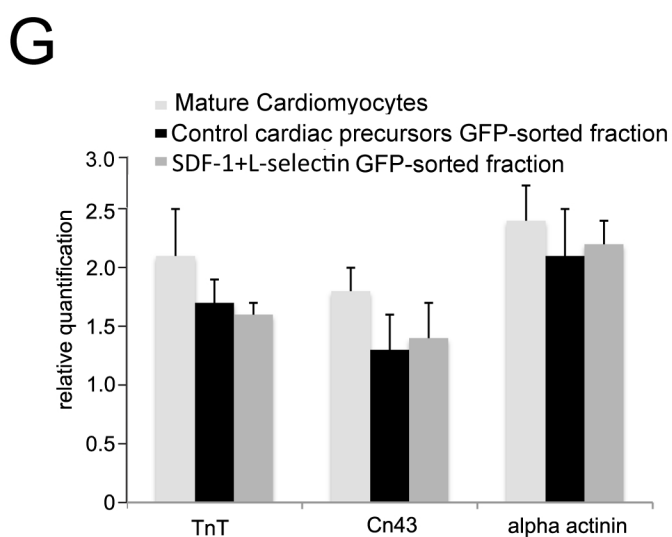
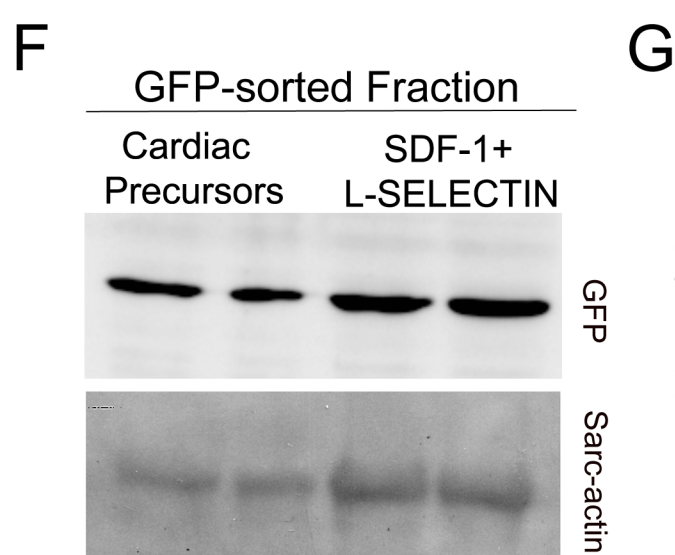
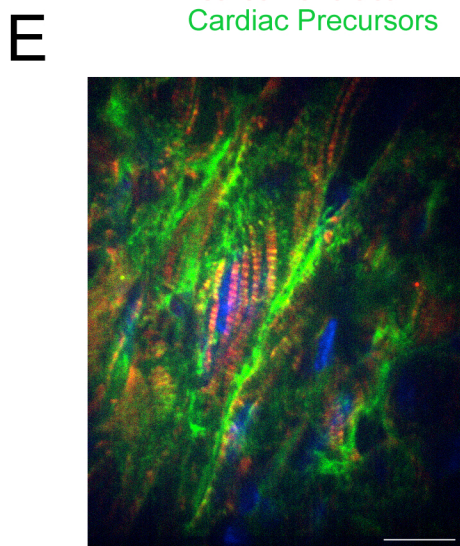
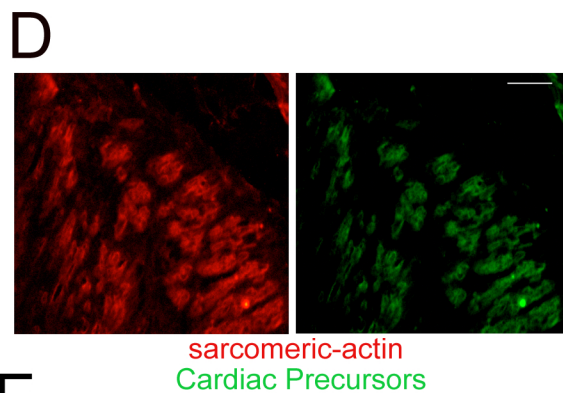
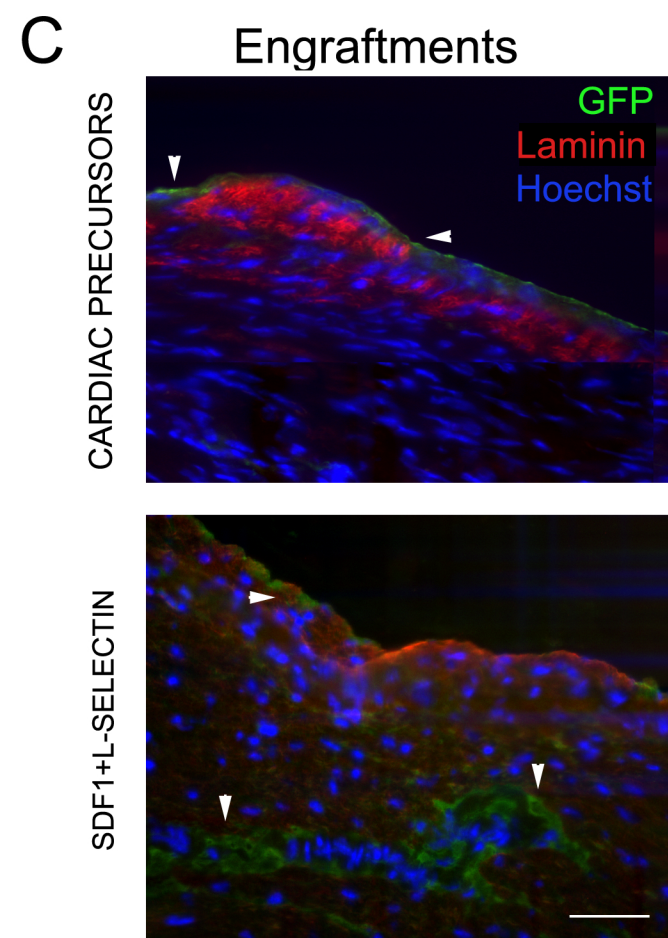
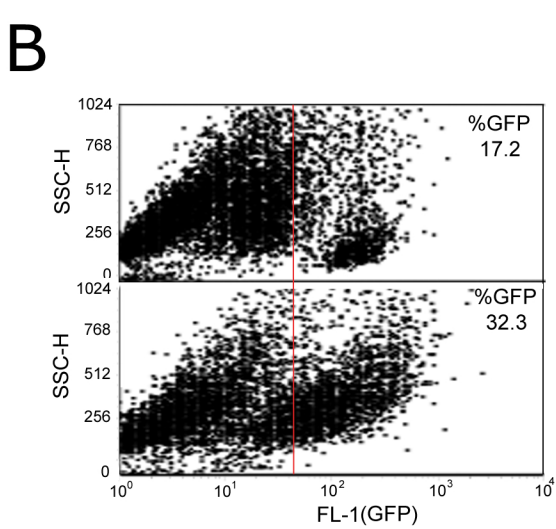
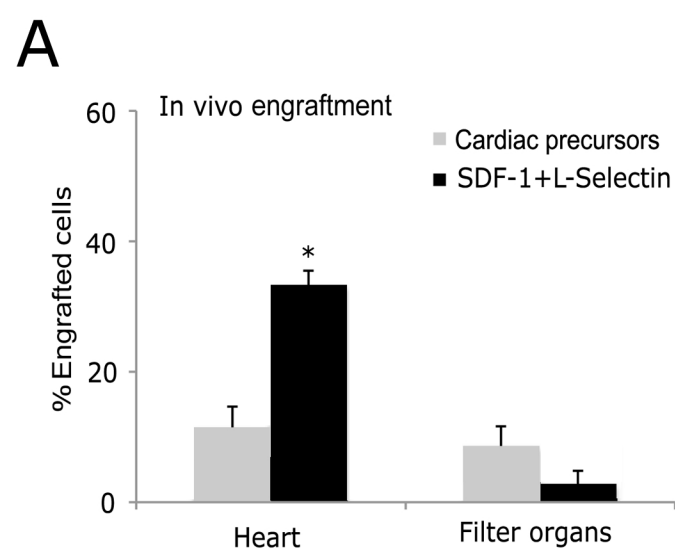
**FIGURE-2**



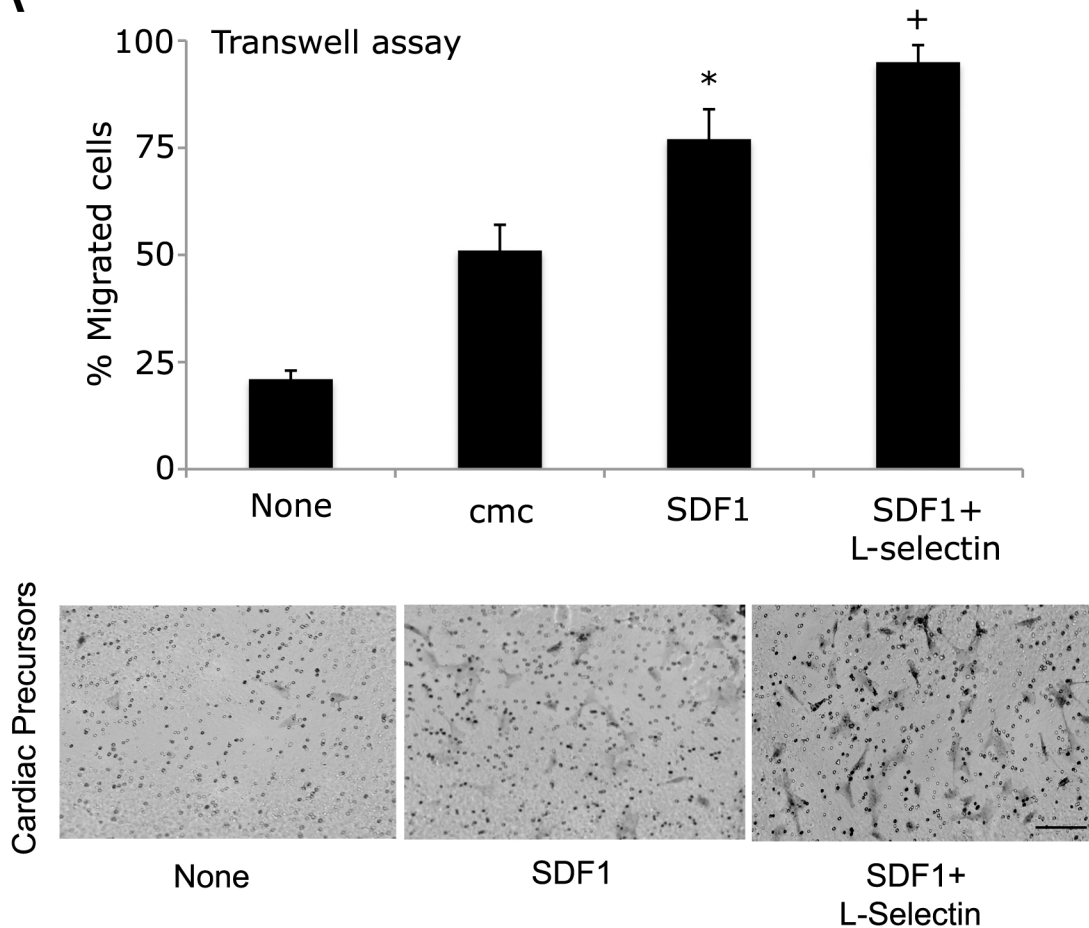
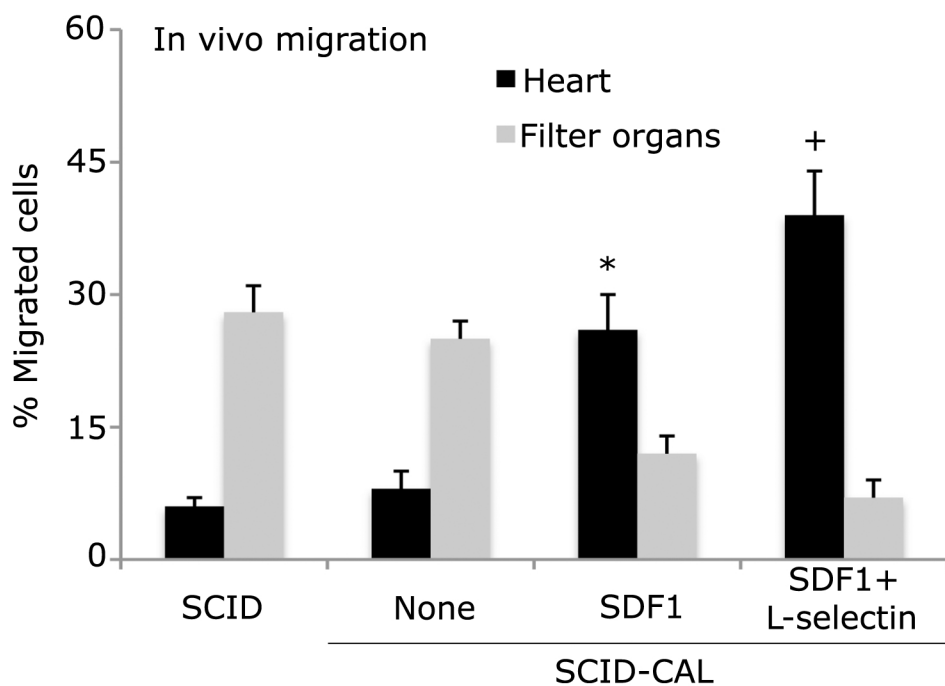
**FIGURE-3**



**FIGURE-4**



**FIGURE-5**

**A****B****FIGURE-6**

	PBS n=8	Cardiac Precursors n=8	SDF-1+L- selectin Cardiac Precursors n=6	<i>p</i> Treated Cardiac Precursors vs PBS
LVIDd (mm)	4,32	4,73	4,89	0.43
EF (%)	27,12	34,28	41,17	0.032
FS (%)	17,65	25,47	33,10	0.0204
LVAWThd (mm)	0,49	0,60	0,72	0.0415

Table-1