The Ephrin A1-EphA2 System Promotes Cardiac Stem Cell Migration after Infarction

Polina Goichberg\textsuperscript{1}, Yingnan Bai\textsuperscript{1}, Domenico D’Amario\textsuperscript{1}, João Ferreira-Martins\textsuperscript{1}, Claudia Fiorini\textsuperscript{2}, Hanqiao Zheng\textsuperscript{1}, Sergio Signore\textsuperscript{1}, Federica del Monte\textsuperscript{3}, Sergio Ottolenghi\textsuperscript{4}, David D’Alessandro\textsuperscript{2}, Robert E. Michler\textsuperscript{2}, Toru Hosoda\textsuperscript{1}, Piero Anversa\textsuperscript{1}, Jan Kajstura\textsuperscript{1}, Marcello Rota\textsuperscript{1}, and Annarosa Leri\textsuperscript{1}

\textsuperscript{1}Departments of Anesthesia and Medicine, and Division of Cardiovascular Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115
\textsuperscript{2}Montefiore Medical Center, Albert Einstein College of Medicine, New York, NY
\textsuperscript{3}Cardiovascular Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115
\textsuperscript{4}Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milan, Italy

Abstract

**Rationale**—Understanding the mechanisms that regulate trafficking of human cardiac stem cells (hCSCs) may lead to development of new therapeutic approaches for the failing heart.

**Objectives**—We tested whether the motility of hCSCs in immunosuppressed infarcted animals is controlled by the guidance system that involves the interaction of Eph receptors with ephrin ligands.

**Methods and Results**—Within the cardiac niches, cardiomyocytes expressed preferentially the ephrin A1 ligand, while hCSCs possessed the EphA2 receptor. Treatment of hCSCs with ephrin A1 resulted in the rapid internalization of the ephrin A1-EphA2 complex, post-translational modifications of Src kinases, and morphological changes consistent with the acquisition of a motile cell phenotype. Ephrin A1 enhanced the motility of hCSCs in vitro, and their migration in vivo following acute myocardial infarction. At two weeks after infarction, the volume of the regenerated myocardium was two-fold larger in animals injected with ephrin A1-activated hCSCs than in animals receiving control hCSCs; this difference was dictated by a greater number of newly formed cardiomyocytes and coronary vessels. The increased recovery in myocardial mass with ephrin A1-treated hCSCs was characterized by further restoration of cardiac function and by a reduction in arrhythmic events.

**Conclusions**—Ephrin A1 promotes the motility of EphA2-positive hCSCs, facilitates their migration to the area of damage, and enhances cardiac repair. Thus, in situ stimulation of resident hCSCs with ephrin A1 or their ex vivo activation prior to myocardial delivery improves cell...
targeting to sites of injury, possibly providing a novel strategy for the management of the diseased heart.

**Keywords**

ephrin; cardiac stem cells; myocardial regeneration; cell migration

The identification of cardiac stem cells (CSCs) and their niches in the mammalian heart\(^1\)–\(^4\) has changed our understanding of the mechanisms of myocardial homeostasis and tissue repair following injury. The adult heart is now considered a self-renewing organ regulated by a resident stem cell compartment, which controls myocyte turnover physiologically and contributes to myocardial recovery in the presence of damage.\(^5\)\(^,\)\(^6\) This revolutionary view of myocardial biology has raised the important question concerning the processes that modulate CSC migration and differentiation. The initial events, involving CSC activation, commitment and acquisition of a motile cell phenotype, are triggered by myocytes and fibroblasts within the niches, where they function as supporting cells.\(^7\) The cross-talk between adjacent cells in the niche microenvironment conditions the fate of CSCs, according to the need of the organ and the preservation of its structural and functional integrity.\(^8\)–\(^10\)

Interaction of Eph receptor tyrosine kinases with membrane-bound ephrin ligands is part of a complex contact-dependent communication between neighboring cells.\(^11\),\(^12\) The ephrin-Eph complex on the plasma membrane of adjacent cells results in bi-directional signaling, ultimately, modulating cell adhesion or movement, division or differentiation.\(^12\) The ephrin ligand family is divided in two classes, A (A1–5) and B (B1–3), based on their structure and ability to bind to the nine EphA and five EphB receptors, respectively.\(^12\) The ephrin-Eph axis regulates self-renewal or commitment, adhesion or migration of stem cells in various organs, including the brain, the bone marrow and the intestine.\(^13\),\(^14\) Importantly, Eph receptor tyrosine kinases and their ligands participate in the formation of the outflow tract and coronary vessels during cardiac development.\(^15\)

These observations raise the possibility that the ephrin-Eph system may be operative in CSCs where it may have a positive or negative effect on the cardiac repair process. Currently, phase 1 clinical trials are in progress and human CSCs (hCSCs) are delivered to patients with acute and chronic ischemic cardiomyopathy (ClinicalTrials.gov Identifiers: NCT00474461; NCT00893360), emphasizing the relevance that pathways favoring homing and translocation of the delivered cells may have on patients’ outcome. Therefore, the role of the ephrin-Eph complex in the fate of mouse and human CSCs was determined to define the potential therapeutic impact of this system on myocardial regeneration after infarction.

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Human Cardiac Stem Cells (hCSCs) and Cardiomyocytes**

Discarded myocardial samples from patients who underwent elective cardiac surgery were enzymatically dissociated and c-kit-positive hCSCs were obtained;\(^2\) expanded hCSCs were employed for in vitro and in vivo studies. Explanted human hearts and donor hearts declined for transplantation were utilized to obtain cardiomyocytes.

**Mouse CSCs (mCSCs) and Cardiomyocytes**

The heart of C57BL/6 mice at 3 months of age was perfused with a collagenase solution and myocytes and c-kit-positive mCSCs were isolated.\(^7\),\(^8\)

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qRT-PCR and Western Blotting

RNA was extracted from hCSCs, mCSCs, and human and mouse myocytes for the measurement of ephrin ligand and Eph receptor transcripts. Protein lysates of hCSCs, human myocytes, and mouse heart tissue were utilized for the assessment of EphA2, ephrin A1 and phosphorylated Src kinases. For primers and antibodies, see Online Tables I and II, respectively.

Immunocytochemistry

Following fixation in 4% paraformaldehyde, hCSCs and human cardiomyocytes were exposed to antibodies recognizing c-kit, EphA2, ephrin A1 and sarcomeric proteins. Cell morphology was defined by incubation with phalloidin. Ephrin A1-hFcγ binding was identified with TRITC-conjugated anti human Fcγ antibody.

Adhesion and Migration Assay

Calcein-labeled hCSCs were plated on ephrin A1-coated wells and the fraction of adhered hCSCs was estimated by measuring fluorescence intensity. hCSCs, exposed to ephrin A1-hFcγ (ephrin A1) or human IgG (Fc), were allowed to migrate towards Hepatocyte growth factor (HGF) in a transwell system. The number of transmigrated cells was counted by flow cytometry.

Myocardial Infarction

Myocardial infarction was produced in c-kit-EGFP mice; ephrin A1 was administered to the border zone, and the number of c-kit-EGFP-positive CSCs measured. Infarcted C57BL/6 mice were injected with hCSCs pre-treated with ephrin A1 or Fc for the ex vivo analysis of hCSC motility by two-photon microscopy. Imunosuppressed infarcted Fischer 344 rats were sacrificed two weeks after surgery and delivery of hCSCs for the evaluation of myocardial regeneration. Sham-operated and infarcted rats injected with PBS were used as controls. At sacrifice ventricular function was determined. The heart was then fixed for histological analysis. Newly formed cardiomyocytes and coronary vessels were recognized by EGFP labeling and in situ hybridization for Alu probe and Y-chromosome. Specificity of staining was determined by spectral analysis. A protocol of programmed electrical stimulation was used to assess the propensity of the infarcted rat heart to develop ventricular arrhythmias.

Statistical Analysis

Results are presented as mean±SD. Significance between two comparisons was determined by unpaired Student’s t-test and among multiple comparisons by the Bonferroni method. All P values are two-sided and values less than 0.05 were considered significant. See Online Table III for sampling.

Results

EphA2 and Ephrin A1 in the Myocardium

C-kit positive hCSCs are organized in niches which are located preferentially in the atria and apex. hCSCs are functionally connected to cardiomyocytes, which act as supporting cells and influence the fate of adjacent primitive cells. The components of this cell-to-cell interaction within the cardiac niches are largely unknown. In analogy to other self-renewing organs, the Eph-ephrin system may regulate the motility of resident stem cells within the heart. Therefore, the presence of the Eph-ephrin family members was measured by qRT-PCR in hCSCs and human cardiomyocytes to search for gene products differentially expressed in these two cell classes (Online Figure I). EphA2 receptor mRNA was abundant
in hCSCs, while transcripts of the ephrin A1 ligand were highly represented in cardiomyocytes (Figure 1A). A similar distribution was found in mCSCs and mouse cardiomyocytes. The two distinct isoforms of the ligand, ephrin A1a and ephrin A1b, were equally represented in the myocardium (Online Figure I), but the effect of these two proteins on EphA2 receptor activation is, at present, unknown. The preferential localization of EphA2 in hCSCs and ephrin A1 in human cardiomyocytes was confirmed by Western blotting (Figure 1B), and immunolabeling and confocal microscopy (Figure 1C and 1D).

To establish whether these findings in isolated hCSCs and cardiomyocytes mimicked the tissue properties, the expression of EphA2 and ephrin A1 was determined in the human myocardium. Clusters of hCSCs were nested in the interstitium and were coupled with neighboring myocytes by the expression of connexin 43 and N-cadherin (Figure 1E). The ephrin A1 ligand was present in myocytes adjacent to EphA2-positive hCSCs (Figure 1F). Importantly, ephrin A1 was restricted to the myocyte compartment; it was not detected in endothelial cells (ECs), smooth muscle cells (SMCs) or fibroblasts (Figure 1G). These data raise the possibility that cardiomyocytes carrying the ephrin A1 ligand interact with hCSCs possessing the EphA2 receptor and, as a result, modify their motile phenotype within the cardiac niches.

**Ephrin A1 and hCSC Motility**

Cardiomyocytes may influence the behavior of hCSCs by direct cell-to-cell contact or by secretion of a soluble signal. Thus, the functional role of the ephrin A1-EphA2 axis was established in vitro by exposing EphA2-positive hCSCs to a human ephrin A1-Fcγ chimeric protein (ephrin A1), or control human IgG (Fc). The rapid adhesion of hCSCs to ephrin A1-coated surfaces documented the functional competence of the EphA2 receptor. Transfection with siRNA specific for EphA2 decreased significantly its expression and was not accompanied by a compensatory increase in the quantity of EphA3 and EphA4 transcripts (Figure 2A). EphA2 down-regulation abrogated the adhesive response of hCSCs to immobilized ephrin A1 (Figure 2B). Ephrin A1 promoted rearrangement of the actin cytoskeleton in hCSCs, changing their shape from a sessile to a motile state (Figure 2C). This change in cell morphology was characterized by a rapid internalization of the ephrin A1-EphA2 complex, from the plasma membrane to the cytoplasm (Figure 2D).

Since Src kinase represents a well-established downstream effector of Eph receptor signaling, the state of phosphorylation of Src was evaluated in ephrin A1-stimulated hCSCs. Following ligand binding, there was a time-dependent increase in the phosphorylation of the activatory site of Src family kinases at tyrosine 416, which was associated with a concomitant decrease in phosphorylation of the inhibitory site of Src at tyrosine 527 (Figure 2E). These post-translational modifications of Src kinases indicate that the EphA2 pathway was activated in hCSCs exposed to ephrin A1.

The chemoattractant HGF favors the translocation of stem cells to sites of ischemic myocardial damage. In the presence of HGF, hCSCs acquired a motile phenotype characterized by accumulation of EphA2 at the leading edge of migrating cells (Figure 2F). As expected, hCSCs moved towards HGF in a Transwell migration assay (Figure 2G and 2H). Pre-stimulation of hCSCs with recombinant ephrin A1 enhanced the spontaneous motility and chemotactic response of these cells to low concentrations of HGF. However, the additive effect of ephrin A1 on cell locomotion was no longer apparent when hCSCs were exposed to high quantities of HGF, which saturated the migratory machinery of the cells. Migration of hCSCs towards high concentrations of HGF was significantly decreased when the expression of EphA2 was inhibited by siRNA. A similar effect was observed when hCSCs were exposed to a chemical inhibitor of Src kinase activity (Figure 2G and 2H). Importantly, ephrin A1 did not alter the rate of proliferation and apoptosis of hCSCs (Online...
Figure II). Additionally, treatment with ephrin A1 did not modify the ability of hCSCs to commit to cardiovascular lineages as shown by qRT-PCR and immunolabeling. The fraction of hCSCs expressing, α-sarcomeric actin (α-SA), α-smooth muscle actin (α-SMA) and von Willebrand factor (vWF) was comparable in Fc-exposed and ephrin A1-treated cells (Figure 2I). Collectively, these observations emphasize the critical role that the ephrin A1-EphA2 system has in the migratory ability of hCSCs.

**Endogenous Stem Cells and Myocardial Infarction**

The in vitro results raised the possibility that the ephrin A1-EphA2 axis enhances the migration of endogenous stem cells to the injured myocardium, favoring the recovery of the infarcted heart. Two days after coronary artery occlusion in the mouse, the expression of ephrin A1 markedly increased in the border zone and distant myocardium (Figure 3A and 3B). However, phosphorylation of the activatory site of Src family kinases at tyrosine 416 did not differ in these two regions (Figure 3B). The myocardium is composed predominantly of cardiomyocytes, which do not express the EphA2 receptor, preventing the activation of distal pathways. Ephrin A1 was found to be upregulated in human myocytes of explanted failing hearts (Figure 3C), suggesting that the synthesis of ephrin A1 by the muscle compartment constitutes a relevant adaptive response aiming at the recruitment of hCSCs at sites of tissue damage. However, this regenerative process is restricted to the surviving region of the ventricular wall, contributing minimally to the reconstitution of the infarcted myocardium.

To determine whether ephrin A1 positively affects the motility of EphA2-positive CSCs in vivo, a transgenic mouse model in which the expression of enhanced green fluorescent protein (EGFP) is driven by the c-kit promoter was employed. Ephrin A1 was administered in the border zone of acutely infarcted mice and the number of CSCs present in proximity of the necrotic tissue was measured 2 days later. Infarcted mice injected with Fc were used as controls. In comparison with Fc-treated mice, the intramyocardial delivery of ephrin A1 resulted in a 2-fold increase in the number of c-kit-EGFP-positive CSCs (Figure 4A and 4B).

Several factors had to be considered in the interpretation of these results. The accumulation of stem cells in the presence of ephrin A1 may involve enhanced recruitment, increased stem cell division, reduced apoptosis, or a combination of these variables. However, the fraction of cycling Ki67-positive CSCs was found to be comparable in ephrin A1- and Fc-treated infarcted hearts. Similarly, apoptosis of CSCs, measured by the TdT assay, did not differ with ephrin A1 or its absence (Figure 4C). Thus, ephrin A1 favors the translocation and homing of CSCs to the site of myocardial injury, enhancing the cellular processes responsible for cardiac repair.

**Ephrin A1 and Migration of hCSCs In Vivo**

The observations in the mouse heart pointed to a potential role of ephrin A1 in the activation of EphA2 in hCSCs, favoring their translocation to the damaged myocardium and the initiation of a regenerative response. For this purpose, EGFP-positive hCSCs were exposed to ephrin A1 in vitro and, following delivery to the border zone of acutely infarcted mice, the movement of these hCSCs from the site of injection to the infarcted myocardium, was monitored by two-photon microscopy.

With respect to control cells, ephrin A1-activated hCSCs showed a 2.5-fold increase in the speed of locomotion within the myocardium (Figure 5; Online Movie I). A greater displacement in the trajectory of migrating hCSCs within the tissue was also found (Figure 5B). The enhanced movement of hCSCs with ephrin A1 was associated with a 25% increase...
in the number of migrating cells. Thus, the ephrin A1-EphA2 pathway positively influences the timing and degree of hCSC trafficking and the onset and, potentially, the extent of tissue repair.

**Ephrin A1 and Myocardial Regeneration by hCSCs**

To establish the effects of ephrin A1-activated hCSCs on tissue regeneration, hCSCs were exposed to the ephrin A1 ligand, prior to delivery to the region bordering the acutely infarcted myocardium in immunosuppressed rats. Infarcted hearts injected with PBS or with hCSCs incubated with Fc were used as controls. hCSCs were infected with a lentivirus carrying EGFP for the in vivo tracking of the formed progeny. All animals were sacrificed 2 weeks after coronary artery ligation and cell or PBS administration.

The infarcted myocardium occupied a large portion of the left ventricle (LV), extending from the endocardial to the epicardial aspect of the wall. A thin layer of spared myocytes was detected in close proximity to the endocardium. Large bundles of collagen and vascular profiles replaced the necrotic tissue at 2 weeks (Figure 6A). Therapy with both activated and non-activated hCSCs resulted in myocardial regeneration which interfered in part with the negative consequences of the healing process and scar formation (Figure 6B and 6C). Cardiac repair consisted of clusters of closely packed human cardiomyocytes and coronary vessels (Figure 6D–6F). The level of myocyte formation, measured by the cell cycle protein Ki67, was comparable in these two groups of hearts, documenting that myocyte regeneration was ongoing at sacrifice (Online Figure III). The human origin of the regenerated structures was determined by the expression of EGFP, human DNA sequences with an Alu probe, and detection of human Y-chromosome (Figure 6E and 6F). The specificity of immunolabeling for EGFP and Alu was confirmed by spectral analysis (Figure 6G and 6H).

To evaluate quantitatively the efficacy of ephrin A1 activation of hCSCs, infarct size, the number of newly-formed myocytes, and their volume were determined and interpreted in relation to ventricular hemodynamics. These parameters were then compared with the corresponding values in infarcted hearts injected with hCSCs exposed to Fc. Coronary ligation resulted in an average 46% loss of LV myocytes in infarcted hearts injected with PBS, Fc-hCSCs and ephrin A1-hCSCs (Figure 7A).

Myocardial regeneration was detected in the two cell-treated groups. However, the aggregate volume of human myocytes was 2-fold larger in animals injected with ephrin A1-activated hCSCs than in rats receiving Fc-hCSCs. The volume of newly formed myocytes in the two groups was comparable, indicating that the increase in myocyte number was responsible for the higher degree of myocardial regeneration with ephrin A1-treated hCSCs (Figure 7B). The increase in the length density of resistance arterioles and capillaries followed a similar pattern (Figure 7C and 7D), supporting the notion that hCSCs stimulated by ephrin A1 differentiated in all cardiac cell lineages, retaining their multipotent phenotype in vivo.

Following treatment with ephrin A1-hCSCs, cardiac repair resulted in a 37% reduction of infarct size. This value was nearly 2-fold larger than that measured after the injection of Fc-hCSCs (Figure 7E). The recovery in myocardial mass mediated by ephrin A1-hCSCs was coupled with a less pronounced increase of LV end-diastolic pressure (LVEDP), and decrease in +dP/dt and −dP/dt after infarction; however, these changes did not reach statistical significance. LV developed pressure (LVEDP) and ejection fraction (EF) were higher in infarcted hearts treated with ephrin A1-activated hCSCs and these differences were significant (Figure 8A).
Cardiac Repair and Ventricular Arrhythmia

A critical issue in need of resolution concerned whether the inhomogeneity of the myocardium after infarction was enhanced further by regeneration and the formation of small neonatal-like cardiomyocytes, potentiating the incidence of arrhythmia. Thus, infarcted hearts, injected with PBS, Fc-hCSCs and ephrin A1-hCSCs, were studied ex vivo in a Langendorff preparation. To mimic the in vivo results, this analysis was conducted two weeks after coronary artery ligation and cells or PBS delivery.

Perfused hearts were subjected to programmed electrical stimulation to induce ventricular tachycardia and fibrillation. Arrhythmic events were not detected in sham-operated hearts; 88% of infarcted non-treated hearts showed episodes of arrhythmia. This value decreased to 45% following treatment with Fc-hCSCs. In comparison with infarcted hearts injected with PBS and Fc-hCSCs, therapy by ephrin A1-hCSCs decreased the frequency of arrhythmia by 89% and 78%, respectively (Figure 8B). Since the size of myocytes was comparable in cell-treated hearts, the greater degree of myocardial regeneration and infarct size reduction with ephrin A1-hCSCs may account for the difference in arrhythmia between the two groups of cell-treated infarcted hearts.

Discussion

The results of the present study indicate that the ephrin A1-EphA2 system in mouse and human CSCs promotes a selective response, which involves cell migration, without affecting cell division, survival and differentiation. This specific function of the ephrin A1-EphA2 axis in CSCs was documented by a series of in vitro and in vivo assays, which demonstrated a comparable motile effect on the endogenous and delivered CSCs. This critical role of the ephrin A1-EphA2 complex in the migratory properties of mouse and human CSCs was identified by the analysis of the differential expression of ephrin ligands and Eph receptors in the myocyte compartment and primitive cell pool, respectively. Based on this premise, we found that ephrin A1 in cardiomyocytes and EphA2 in CSCs are implicated in cell-to-cell communication between undifferentiated and supporting cells within the niches, enhancing CSC migration. Importantly, ephrin A1 activation of hCSCs prior to their injection in proximity of the infarcted myocardium of immunosuppressed animals resulted in a two-fold increase in myocardial regeneration and improved the recovery of ventricular function.

In this regard, the relevance of the ephrin/Eph system in the adult heart has never been shown. Similarly, the existence and function of this axis in mouse and human CSCs and myocardial regeneration was previously unknown. Migration of primitive cells represents the crucial prerequisite for efficient repair of damaged myocardium, emphasizing the significance of novel strategies aiming at the enhancement of CSC recruitment at the site of ischemic injury. The dichotomic pattern of ephrin A1 and EphA2 protein distribution represents a classical model of ligand-receptor interaction, which enables the communication between CSCs and myocytes in the cardiac niches, impacting on myocardial regeneration.

EphA2, Ephrin A1 and Cardiac Repair

The injection of hCSCs after infarction resulted over a period of 14 days in a significant reduction of infarct size mediated by their differentiation into a large number of cardiomyocytes, resistance coronary arterioles and capillary structures. Although markedly increased by ephrin A1 activation of hCSCs, the process of myocardial regeneration retained the same proportion between newly-formed cardiomyocytes and coronary vasculature, indicating that the multipotentiality of the injected hCSCs was not affected by the ephrin A1 ligand. The similarity in volume composition of the new myocardium in the two groups of
cell-treated infarcted hearts supports the notion that hCSCs recruited at the site of injury underwent comparable clonal activation\textsuperscript{22} giving rise to an equivalent number of cardiomyocytes and vascular cells. The larger magnitude of myocardium generated by ephrin A1-treated hCSCs emphasizes the relevance of cell migration for the efficient repair of the damaged heart. Importantly, downregulation of EphA2 in hCSCs and blockade of the receptor downstream signaling impairs the motility of hCSCs.

A tight balance between myocyte size and capillary and arteriolar density\textsuperscript{23} has to be maintained to ensure an adequate blood flow and oxygenation of the regenerated myocardium. The principal structural variables of the microvasculature of the heart that are functionally relevant to the oxygenation of the newly formed tissue are capillary luminal volume density, capillary luminal surface density, and the average diffusion distance from the capillary wall to the surrounding tissue.\textsuperscript{24} These parameters are strictly dependent on the number of capillaries per unit area of myocardium; in the regenerated tissue, there was one capillary every ~20 cardiomyocytes, mimicking the pattern of tissue oxygenation present early after birth in rodents.\textsuperscript{24} Similarly, the size of cardiomyocytes was comparable to that found at birth in the mouse and rat heart.\textsuperscript{24} The capillary-to-myocyte ratio increases with postnatal maturation, reaching a value of nearly one in the young adult heart. Whether the regenerated myocytes and vascular structures will reach with time the adult cell phenotype is open to question. It is also currently unknown whether ephrin A1 favorably affects this process of maturation.

**Ephrin A1-EphA2 System and Myocardial Regeneration**

The results of the present study indicate that myocardial infarction results in a significant upregulation of ephrin A1 in the surviving myocytes and this response is greater in the border zone than in the remote myocardial region. The expression of IGF-1 and HGF transcripts increases in the spared tissue acutely after infarction.\textsuperscript{25,26} However, the concentration of HGF and IGF-1 proteins remains relatively constant in the border zone, remote myocardium and ischemic region at 6–12 hours after coronary artery ligation, suggesting that these two growth factors undergo rapid turnover.\textsuperscript{27} Although the synthesis of ephrin A1 is enhanced after infarction, the administration of the exogenous ligand is necessary to induce activation and efficient recruitment of CSCs. IGF-1 and HGF, together with ephrin A1, bind to the corresponding receptors on resident CSCs, promoting division, survival, and translocation of these cells towards the necrotic tissue.\textsuperscript{9,18} This possibility is supported by the increase in number of CSCs in proximity of the infarct shortly after coronary occlusion in animals and humans.\textsuperscript{8} Ephrin A1 and HGF are both highly effective in promoting cell movement. However, the positive effect of HGF on CSC migration requires the activation of the cell motile state by ephrin A1, which enhances the susceptibility of CSCs to translocate to the site of injury. The motility of hCSCs within the border zone of the infarcted heart was two-fold higher in animals treated with ephrin A1, suggesting that in situ activation of resident hCSCs with this ligand, or their ex vivo manipulation, may improve cell targeting to sites of damage, providing a novel strategy for the management of the pathologic heart.

The augmented expression of ephrin A1 in mouse myocytes after infarction and in human myocytes from patients with end-stage heart failure indicate that, under conditions of stress, the heart initiates a series of compensatory processes aiming at the mobilization of CSCs towards the area of damage. In analogy with the myocardium, hypoxia and inflammation enhance the expression of ephrin A1 and EphA2 in the skin and vascular structures.\textsuperscript{28} An important question left unanswered by this and previous studies concerns the inability of the pool of resident CSCs to spontaneously induce a regenerative response and reestablish the structural and functional integrity of the infarcted heart. This mechanism is very effective in lower vertebrates\textsuperscript{29} but it is not efficient in the adult heart of mammals, including humans.\textsuperscript{19}
CSCs within the infarct die by apoptosis and the formation of myocytes and coronary vessels is restricted to the unaffected portion of the ventricular wall, with minimal or none regeneration of the infarcted tissue. Whether the CSCs nested in the spared myocardium are not equipped to sense signals from the infarct, or growth stimuli from the infarct are inadequate to trigger the migration of these primitive cells, or both, is presently unknown.

**EphA2 and Ephrin A1 in CSCs and Cardiomyocytes**

Consistent with the reciprocal distribution of EphA2 receptor and ephrin A1 ligand in CSCs and cardiomyocytes, progenitor cells in the hippocampus and intestinal crypt are enriched in Eph receptor, while the surrounding differentiated cells exhibit high levels of ephrin ligands. The distinct expression pattern of EphA2 and ephrin A1 in CSCs and cardiomyocytes may generate a bidirectional signaling that provide positional guidance cues to maintain tissue homeostasis and favor cardiac repair. In all tissues, the ephrin-Eph system uses common effectors including the Src family of kinases, which, as shown here in hCSCs following ephrin A1 stimulation, experience rapid post-translational modifications. The transient net increase in Src family kinase activity in hCSCs with ephrin A1 was coupled with rearrangement of the actin cytoskeleton and cell shape polarization, which resulted in enhanced spontaneous and HGF-induced motility of hCSCs. These molecular and morphological adaptations were dictated by the internalization of the ephrin A1-EphA2 complex from the cell membrane to the cell cytoplasm. Ligand-induced Eph receptor endocytosis is a critical determinant of the transition from the high affinity cell-to-cell adhesion to the repulsive response.

In addition to cell migration, the ephrin-Eph family of proteins controls multiple cellular functions, including growth, differentiation, and death of stem/progenitor cells. Surprisingly, ephrin A1 did not affect the rate of proliferation, commitment and apoptosis of hCSCs in vitro and in vivo, pointing to a restricted function of the ephrin A1-EphA2 axis in the regulation of the movement of human and mouse CSCs. The consequences of Eph activation on progenitor cell fate are ligand, cell context, and kinase activity dependent. In physiological conditions, the ephrin-Eph signaling ensures a proper balance between a stem cell and its progeny and may interfere or favor cell survival, replication and commitment. In the brain, distinct components of the ephrin-Eph family have opposite effects on apoptosis and proliferation of neural stem cells.

Reliance or independence of Eph receptor signaling from the ligand, or downstream kinase activity, leads to a wide array of contrasting effects on stem cell behavior. The spatial-mechanical characteristics of the interaction between ephrin A1 and EphA2 molecules influence receptor function and the cell migratory response. The geometry of intermembrane signaling may play a major role in the heart in which electromechanically connected myocytes form a functional syncytium. Cardiomyocytes are subjected to considerable physical forces which impact, in an unknown manner, on structurally joined CSCs. The increased loading state on the surviving myocardium after infarction may be relevant in the upregulation of the molecular pathways responsible for ephrin A1 synthesis and activation of resident CSCs.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Non-Standard Abbreviations and Acronyms

CSC  cardiac stem cells
hCSCs  Human CSCs
mCSCs  Mouse CSCs
CM  Cardiomyocytes
hCM  Human CM
mCM  Mouse CM
HGF  hepatocyte growth factor
ECs  endothelial cells
SMCs  smooth muscle cells
α-SA  α-sarcomeric actin
α-SMA  α-smooth muscle actin
vWf  von Willebrand factor

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References


Figure 1. Expression of EphA2 and ephrin A1
A, Transcripts for EphA2 and ephrin A1 in hCSCs, human cardiomyocytes (hCMs), mCSCs and mouse cardiomyocytes (mCMs). Representative tracings and quantitative data are shown. *P<0.05 vs. hCSCs or mCSCs. hMyo, human myocardium. For PCR products, see Online Figure I. B, Western blotting of EphA2 and ephrin A1 in hCSCs and hCMs. EphA2 is restricted to hCSCs and ephrin A1 to hCMs. Loading, GAPDH or β-actin. C, hCMs (α-sarcomeric actin: α-SA, red) express ephrin A1 (white) and are negative for EphA2. DAPI: nuclei, blue. D, c-kit-positive hCSCs (left, green) express EphA2 on the plasma membrane (central, red). Right, merge. E, Five hCSCs (c-kit, green) are nested within the hMyo (hCMs: α-SA, red) and express connexin 43 (Cx43, white) and N-cadherin (N-cadh, yellow). The area included in the rectangle is shown at higher magnification in the inset. Fibronectin, magenta. F, Ephrin A1 (white) is present in hCMs (α-SA, red) located in proximity of c-kit-positive hCSCs (green) expressing EphA2 (yellow). The area included in the rectangle is shown at higher magnification in the inset. G, Ephrin A1 is restricted to hCMs and is not present in ECs (von Willebrand factor: vWF, magenta), SMCs (α-smooth muscle actin: α-SMA, green) and fibroblasts (procollagen Iα: procoll, light blue).
Figure 2. Ephrin A1 activation of hCSCs

A, Transcripts for EphA2, EphA3 and EphA4 (left). siC, non-targeting siRNA. *P<0.05 vs. siC. Expression of EphA2 (right, red) in hCSCs transfected with siEphA2 or siC. Phalloidin, green. B, Fraction of hCSCs attached to immobilized ephrin A1 in the presence of non-targeting RNA (siC). Transfection of hCSCs with siRNA for EphA2 (siEphA2) abrogated the adhesion response. Human IgG coating (Fc) was used as control. *, **P<0.05 vs. Fc and siC, respectively. C, Actin filaments (phalloidin, green) in hCSCs exposed to Fc or ephrin A1. Actin accumulates at the leading lamella of ephrin A1-activated hCSCs. D, hCSCs expressing EphA2 (red) were exposed to Fc (upper panels) or ephrin A1 (lower panels). Internalization of the ephrin A1-EphA2 complex (red-white dots) occurs in ephrin A1-activated hCSCs. EphA2 is confined to the plasma membrane in Fc-exposed hCSCs. Fc alone and ephrin A1-Fc (white) were recognized by an antibody against the Fc portion of human IgG. Phalloidin, green. E, Immunoblotting and quantitative analysis of Src family kinase phosphorylation in hCSCs exposed to ephrin A1 for 0, 5, 15 and 30 minutes (min). *P<0.05 vs. time 0. F, Directional stimulation of hCSCs with HGF results in EphA2 (red) accumulation at the leading edge (arrowheads) of the migrating cells. Actin filaments are shown by phalloidin (green). Arrow, direction of migration; c-kit, white. Control, hCSCs in the absence of HGF. G, Number of migrated hCSCs. *P<0.05 vs. Fc. H, hCSCs migration is negatively affected by EphA2 siRNA (siEphA2) and chemical inhibition of Src kinase. siC, non-targeting RNA. *P<0.05 vs. control conditions. I, mRNA for α-SA, α-SMA and vWF (left), and fraction of myocytes, SMCs and ECs formed by hCSCs in the presence and absence of ephrin A1 (right). Control, hCSCs not exposed to dexamethasone.
Figure 3. Expression of ephrin A1 in diseased hearts

A. Two days after myocardial infarction, the expression of ephrin A1 (white) increases in the spared mCMs (right: α-SA, red). Sham-operated, SO (left).

B. Immunoblotting of ephrin A1 and Src family kinase phosphorylation in myocardium obtained from SO, and from the remote region and border zone of infarcted mice. *P<0.05 vs. SO.

C. Ephrin A1 mRNA in isolated hCMs from donor and explanted human hearts.
Figure 4. Ephrin A1 and mCSC migration

A. Two days after infarction, EGFP-positive-c-kit-positive (green) mCSCs are located at the border zone of infarcted mice injected with Fc or ephrin A1. Low and high magnifications are shown. mCMs: α-SA, red. MI, myocardial infarction. B. Number of mCSCs in the border zone of Fc- and ephrin A1-treated infarcted mice. *P<0.05 vs. Fc. C. Fraction of replicating (Ki67-positive) and dying (TdT-positive) mCSCs in the same region.
Figure 5. Ephrin A1 and migration of hCSCs in the infarcted heart
A, Images of the same field of the border zone of an infarcted heart are shown by two-photon-microscopy two hours after coronary artery ligation and injection of hCSCs. Upper panels: infarcted heart injected with hCSCs exposed to Fc. Over a period of 80 min, clusters of EGFP-positive hCSCs (green) are confined to the site of injection (arrowheads). Lower panels: infarcted heart injected with ephrin A1-pretreated hCSCs. Clusters of EGFP-positive hCSCs moved in the direction of the arrow over a period of 80 min. B, Solid lines reflect the displacement per hour of individual hCSCs exposed to Fc or ephrin A1. Different colors correspond to different cells. Areas included in the rectangles are shown at higher magnification in the lower panels. C, Speed of migration of hCSCs within the myocardium. *P<0.05 vs. Fc.
Figure 6. Ephrin A1 and myocardial regeneration

A, Myocardial scarring after infarction (collagen, white). A thin layer of spared myocytes ($\alpha$-SA, red) is present in the subendocardium. The area included in the rectangle is shown at higher magnification in the inset. B and C, Band of regenerated myocardium after infarction and delivery of EGFP-labeled hCSCs exposed to Fc (B) or ephrin A1 (C); EGFP and $\alpha$-SA (yellowish). The areas included in the rectangles are shown at higher magnification in the insets. D, Newly formed capillaries are positive for EGFP (green) and vWf (white). SMCs in regenerated arterioles are positive for EGFP and $\alpha$-SMA (orange), and ECs for EGFP and vWf (white-green). E, hCMs are positive for EGFP (left, green) and Alu (left, white dots in nuclei), and EGFP and $\alpha$-SA (right, orange). F, hCMs carry the Y-chromosome (left, Y-chr, single white dot in nuclei), and are EGFP and $\alpha$-SA positive (right). G and H, Spectral analysis of hCMs positive for EGFP (G) and Alu (H). Emission spectra for EGFP (G) and Alu (H) positive hCMs are shown by the green lines, while emission spectra for tissue autofluorescence are shown by the blue lines. Note the difference in the intensity of the signals at wavelength corresponding to maximum fluorescence in each case (upper panels). Following normalization for the intensity of the signals, all emission spectra for specific labeling were essentially superimposable (lower panels). In contrast, emission spectra for tissue autofluorescence had different shapes and were easily distinguishable from specific labeling.
Figure 7. Ephrin A1-EphA2 system and cardiac repair

A. Number of myocytes lost and remaining after infarction in the LV of animals injected with PBS (MI), Fc-treated hCSCs (Fc) and ephrin A1-activated (ephrin A1) hCSCs. *P<0.05 vs. SO.

B. Characteristics of myocytes formed by differentiation of hCSCs in hearts injected with Fc-treated and ephrin A1-treated hCSCs.

C and D. Length of newly-formed resistance arterioles (C) and capillaries (D) in the regenerated myocardium after injection with Fc- or ephrin A1-treated hCSCs.

E. Reduction of infarct size by tissue regeneration. L: lost myocardium; R: regenerated myocardium. *P<0.05 vs. Fc.
Figure 8. Ephrin A1 and functional recovery
A. Hemodynamics and echocardiographic parameters in SO, and in infarcted hearts injected with PBS (MI), Fc-treated hCSCs (Fc), and ephrin A1-treated hCSCs (ephrin A1). *,**, †P<0.05 vs. SO, MI, and Fc, respectively. B. Number of arrhythmic events in SO, and infarcted hearts injected with PBS (MI), Fc-treated hCSCs (Fc), and ephrin A1-treated hCSCs (ephrin A1). *,**, P<0.05 vs. SO and MI, respectively.