Age-Associated Defects in EphA2 Signaling Impair the Migration of Human Cardiac Progenitor Cells

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Background—Aging negatively impacts on the function of resident human cardiac progenitor cells (hCPCs). Effective regeneration of the injured heart requires mobilization of hCPCs to the sites of damage. In the young heart, signaling by the guidance receptor EphA2 in response to the ephrin A1 ligand promotes hCPC motility and improves cardiac recovery after infarction.

Methods and Results—We report that old hCPCs are characterized by cell-autonomous inhibition of their migratory ability ex vivo and impaired translocation in vivo in the damaged heart. EphA2 expression was not decreased in old hCPCs; however, the elevated level of reactive oxygen species in aged cells induced post-translational modifications of the EphA2 protein. EphA2 oxidation interfered with ephrin A1-stimulated receptor auto-phosphorylation, activation of Src family kinases, and caveolin-1–mediated internalization of the receptor. Cellular aging altered the EphA2 endocytic route, affecting the maturation of EphA2-containing endosomes and causing premature signal termination. Overexpression of functionally intact EphA2 in old hCPCs corrected the defects in endocytosis and downstream signaling, enhancing cell motility. Based on the ability of phenotypically young hCPCs to respond efficiently to ephrin A1, we developed a novel methodology for the prospective isolation of live hCPCs with preserved migratory capacity and growth reserve.

Conclusions—Our data demonstrate that the ephrin A1/EphA2 pathway may serve as a target to facilitate trafficking of hCPCs in the senescent myocardium. Importantly, EphA2 receptor function can be implemented for the selection of hCPCs with high therapeutic potential, a clinically relevant strategy that does not require genetic manipulation of stem cells. (Circulation. 2013;128:2211-2223.)

Key Words: aging ■ cell movement ■ receptor, EphA2 ■ stem cells

Activation, growth, and commitment of resident c-kit-positive cardiac progenitor cells (CPCs) maintain the homeostasis of the adult myocardium by restoring dying cells with newly formed cardiomyocytes and vascular cells.1,2 The efficacy of these processes declines progressively, and the impairment in the mechanisms of clearance and replacement of old myocytes dictates the manifestations of the aging myocardia.3,4 Although the number of CPCs increases in the old myocardium,5 the regenerative response of the senescent heart is inadequate raising the possibility that chronological age negatively impacts on CPC function.

Defects in the replication and migration of CPCs are critical determinants of organ homeostasis and repair, and these properties are defective in the senescent heart in rodents.3,4 Old, poorly contracting myocytes accumulate and cardiac performance deteriorates, mimicking the aging myopathy in humans.6 The attenuated function of human CPCs (hCPCs) in the old myocardium may be mediated by age-dependent alterations in their motile state. Although experimental observations support this hypothesis,4 whether the mobilization of hCPCs is affected by age is currently unknown. Defective migration of hCPCs may oppose their egress from the niches and translocation within the myocardium, where regeneration of myocytes and coronary vessels is required for the preservation of the structural integrity of the organ.

A guidance system that controls the migration of stem cells involves the family of Eph receptor tyrosine kinases and ephrin ligands.7-10 Ephrins are membrane-bound proteins that participate in a complex contact-dependent communication with...
neighboring cells expressing the Eph receptors. EphA2 is preferentially expressed in immature hCPCs, whereas the corresponding ligand, ephrin A1, is distributed on the plasma membrane of myocytes, which act as supporting cells within the stem cell niches.

EphA2 downregulation abrogates the binding of hCPCs to ephrin A1 and interferes with ephrin A1–induced motility, documenting that EphA2 operates as a primary receptor for ephrin A1 ligand in this progenitor cell class. Importantly, EphA2 blockade inhibits the motile response of young hCPCs to hepatocyte growth factor (HGF), a powerful chemoattractant implicated in CPC mobilization. Thus, EphA2 plays a key role in hCPC trafficking and alterations in ephrin A1/EphA2 signaling may cause defects in the migration of old hCPCs. In the present study, we tested whether decreased function of the EphA2 receptor conditions the loss of movement in senescent hCPCs, offering a molecular target for the restoration of the motile state in aging cells.

**Methods**

A detailed description of the methods used in this study is provided in the online-only Data Supplement.

**Human CPCs**

hCPCs were isolated from discarded myocardial tissue of subjects 35-78 years of age and were cultured ex vivo as previously described (IRB protocol 2010P002475). To induce replicative senescence, hCPCs were expanded for 4–7 passages (P) and further propagated till P11-P15. Stress-induced senescence was achieved by 18-hr exposure of hCPCs at P4-P7 to 0.5 μmol/L doxorubicin (Sigma; Figure I in the online-only Data Supplement).

**Immunofluorescence and Confocal Microscopy Analysis**

Indirect immunolabeling of hCPCs was performed after fixation and permeabilization. The list of primary antibodies and secondary reagents, as well as the details of the protocol, are provided in the Methods of the online-only Data Supplement.

**Immunoprecipitation, Immunoblotting, and 2-Dimensional Gel Analysis**

For these assays, protein extracts were obtained from whole myocardium and total cell lysates of young and old hCPCs.

**Flow-Cytometry Studies**

The expression of EphA2 and c-Met on the plasma membrane of hCPCs was determined by flow-cytometry. Telomere length in hCPCs was measured by flow-cytometry and fluorescence in situ hybridization (Flow-FISH), following a previously published protocol.

**Transwell Migration**

Spontaneous motility and chemotaxis of young and old hCPCs were examined as previously described.

**Lentiviral Infection of hCPCs**

The pLentiV plasmid for enhanced green fluorescence protein (EGFP) expression and the 3-plasmid system to generate lentiviral particles were used previously. Full-length human EphA2 sequence was cloned into the pLentiV plasmid to enable expression of the EphA2-EGFP fusion protein. The plasmid for lentiviral expression of red fluorescence protein (RFP) was purchased from Thermo Scientific. Infection of hCPCs was carried out as described.

**Imaging of hCPC Translocation In Vivo**

Young and old hCPCs were infected with lentiviruses expressing EGFP or RFP, respectively. The 2 cell populations were combined in a 1:1 ratio, pretreated with ephrin A1, and injected into the myocardium of acutely infarcted mice. The procedures were performed in accordance with institutional guidelines. The protocols for cell transplantation, 2-photon imaging, and quantitative analysis of cell movement are provided in the Methods of the online-only Data Supplement.

**Endocytosis of Ephrin A1 and Transferrin**

Young and old hCPCs were incubated with fluorescently-labeled transferrin and ephrin A1. To remove ligands bound to the cell surface, hCPCs were subjected to acid wash, and the amount of internalized ligand was calculated as % fluorescence intensity as compared with total ligand content in the cells subjected to washing in neutral pH. Endocytosis dynamics of transferrin and ephrin A1 was studied by live imaging in young and old hCPCs.

**Live Imaging of hCPC Migration In Vitro**

EGFP-expressing or EphA2-EGFP-infected old hCPCs were subjected to polarized stimulation with HGF and imaged (see Methods of the online-only Data Supplement).

**Cell Sorting by Adhesion to Ephrin A1**

Adhesion assay of hCPCs to immobilized ephrin A1 was performed as previously published. The expression of p16INK4a and γH2A.X was determined by immunolabeling and confocal microscopy in adherent and nonadherent hCPCs.

**Statistical Analysis**

Significance between 2 groups was determined by 2-tailed unpaired Student t test. For multiple comparisons, the ANOVA test with Bonferroni correction was used. Quantitative data are expressed as mean±SD. The n values used in each statistical determination are listed for convenience in the legend to each figure; these values reflect the number of independent experiments performed in triplicates in each case.

**Results**

**Aging Impairs hCPC Motility**

Cellular senescence is implicated in the deterioration of organ function and in the aging of the organism. Cells displaying an old phenotype are identified by the expression of senescence-associated biomarkers. Strategies for the isolation of live senescent cells remain to be developed, requiring the implementation of ex vivo models of cellular aging. Two in vitro protocols were introduced to achieve hCPC senescence (Figure I in the in the online-only Data Supplement): replicative senescence as a result of serial passaging and stress-induced senescence by exposure to the oxidative agent doxorubicin.

Senescence of hCPCs was documented with several parameters, which included irreversible withdrawal from the cell cycle, expression of the senescence-associated protein p16INK4a, accumulation of DNA-damage response foci, telomeric shortening, and morphological changes consisting of cell flattening and enlargement. DNA-damage response foci are characterized by the colocalization of phosphorylated histone H2A.X (γH2A.X) and p53-binding protein 1 (53BP1) at sites of DNA injury.

The fraction of Ki67-positive dividing hCPCs decreased 3-fold from P4-7 to P11-15 and similar results were obtained 3 days after doxorubicin (Figure IA). In both cases, the percentage
Figure 1. Old hCPCs display cell-autonomous defects in migratory response. A–C, Primary cultures of actively growing hCPCs (young) were subjected to serial passages (replicative senescence: old), or to doxorubicin exposure (stress-induced senescence: old). Young and old hCPCs express c-kit (green). Nuclei are stained with DAPI (blue). Phalloidin, grey. Localization of Ki-67 (A: red) decreases, and p16^INK4a (B: red), and DNA-damage response foci (C: γH2AX, green; 53BP1, red) increase in old hCPCs. Rectangles define areas illustrated at higher magnification in the insets (C). Data are mean±SD (n=3–5). Only hCPCs with ≥2 γH2AX foci were included in the quantitative analysis. Replicative senescence: *P=0.009, †P=0.04, §P=0.02; Stress-induced senescence: *P<0.0001, †P=0.04, §P=0.03. D, Transwell migration of young and old hCPCs after ephrin A1 stimulation, or in the presence of HGF. This assay shows a decreased migration of old hCPCs. Data are mean±SD (n=8–12). *P=0.038 vs unstimulated cells (-); †P<0.0001 vs ephrin A1 only; **P<0.0001 vs young. E, Young and old hCPCs exposed to a polarized source of HGF. EphA2 (green) accumulates at the leading lamella (asterisk) of young cells. Individual fluorescent signals in the rectangles are shown in the adjacent panels. F, Time-lapse images of young (green) and (Continued)
of p16<sup>INK4a</sup>-positive hCPCs increased 4-fold (Figure 1B). DNA-damage response foci were found in nearly 40% and 60% of serially passaged and doxorubicin-treated hCPCs, respectively (Figure 1C). Telomere shortening, from 9 kbp to 5.2 kbp, was apparent only in long-term hCPC cultures (Figure IB and IC in the online-only Data Supplement). Short-term oxidative stress by doxorubicin is more commonly associated with loss of telomere integrity<sup>27</sup> in the absence of changes in average telomere length (Figure ID in the online-only Data Supplement). Additionally, senescent hCPCs showed flattening and loss of polarization (Figure IE in the online-only Data Supplement). Thus, old hCPCs were obtained by these two protocols, enabling the comparison of young and old cells isolated from the same human heart, independently from patient’s age, sex, absence or presence of pathology, and cause and duration of the cardiac disease. In all studies, hCPCs obtained at early (young) and late (old) passages were used, together with hCPCs cultured in the absence (young) and presence (old) of doxorubicin.

Experimentally, the migratory capacity of CPCs in the myocardium declines with age,<sup>4</sup> but the mechanisms responsible for this functional alteration are largely unknown. The ephrin A1/EphA2 pathway and HGF/c-Met signaling favor the mobilization of CPCs in the aged or infarcted heart.<sup>10,14,15</sup> Additionally, the expression of EphA2 is required for HGF-induced chemotaxis of hCPCs, and activation of EphA2 by ephrin A1 potentiates the migration of this cell class.<sup>10</sup>

The spontaneous motility and chemotaxis towards HGF were severely affected in old hCPCs (Figure 1D). Importantly, EphA2 stimulation with ephrin A1 promoted cell movement in young but not in old hCPCs (Figure 1D). In the presence of HGF, young hCPCs acquired a polarized morphology and redistributed EphA2 to the leading lamella<sup>10</sup>; this response was not observed in old cells (Figure 1E). The defects in the migratory behavior of old hCPCs were comparable in the 2 models of senescence, indicating that aging per se has detrimental effects on hCPC motility.

To determine whether these in vitro findings had a functional counterpart in vivo, young and old hCPCs were infected with a lentiviral vector carrying green or red fluorescent protein. After stimulation with ephrin A1, young and old hCPCs were co-transplanted in the border zone of acutely infarcted hearts and their displacement within the myocardium was measured by 2-photon microscopy over a period of 6 hours. Ephrin A1-activated young hCPCs showed an average 2.4-fold higher translocation velocity than ephrin A1-treated old hCPCs (Figure 1F and 1G). Additionally, the percent of nonmigrating old hCPCs was 2.9-fold larger than that of young hCPCs (Figure 1G and Movie I in the online-only Data Supplement). Thus, activation of the ephrin A1/EphA2 signaling enhances the trafficking of young hCPCs in vitro and in vivo, but does not reverse the defect in the motile state of old hCPCs.

### Figure 1. Continued

Old (red) hCPCs pre-stimulated with ephrin A1 and injected in the border zone of the acutely infarcted mouse heart. Arrows point to the position of clusters of young hCPCs. Note the lack of movement of old hCPCs. The apparent decrease in fluorescence intensity in some of the panels is dictated by a slight shift in the focal plane during image acquisition. See accompanying Video I in the online-only Data Supplement. G, Cell displacement with time. Green and red dots correspond to individual young and old hCPCs, respectively. Solid lines represent average values. The dotted line indicates the velocity of young hCPCs in the absence of ephrin A1. Data are mean±SD (n=4). *P<0.0001. H, Expression of ephrin A1 in young and old human myocardium. *P=0.013. Optical density (O.D.) data were normalized to the expression of cardiac α-actinin (α-SA) and GAPDH. Young hearts: 34–38 years-old; Old: 68–70 years-old. I, Distribution of ephrin A1 (white) in p16<sup>INK4a</sup>-positive (green, arrows) and negative cardiomyocytes (α-sarcomeric actin: α-SA, red) in young and old human hearts. hCPC indicates human cardiac progenitor cell; and HGF, hepatocyte growth factor.
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failed to reach the center of the cells (Figure 2D and 2E, and Movie II in the online-only Data Supplement).

The movement of endocytic vesicles from the plasma membrane to the perinuclear area is coupled with their progressive enlargement. 30 Exposure of young hCPCs to ephrin A1 resulted in a time-dependent increase in the average size of the EphA2-containing vesicles (Figure 2F and 2G). In young hCPCs, EphA2-endosomes, >3 \( \mu \)m\(^2\), were found in the perinuclear region and smaller EphA2-vesicles, <2 \( \mu \)m\(^2\), were predominantly located at the periphery of the cells (Figure 2F). Five minutes after stimulation with ephrin A1, the size of the EphA2 clusters was comparable in young and old hCPCs (Figure 2G). However, the evolution of EphA2-vesicles into larger endosomes was significantly hindered in old hCPCs (Figure 2F and 2G). These results suggest that defects in the maturation of the primary endocytic vesicles carrying ephrin A1/EphA2 occur in old hCPCs and may have an impact on the fate of the internalized EphA2 receptor and its downstream signaling.28,29,31

Primary endosomes containing the ligand/receptor complex are transported to and fused with early endosomes that function as sorting domain for cargo recycling to the cell surface, or cargo degradation in lysosomes.30,31 Early endosomes, which are recognized by the presence of the early endosome antigen 1 (EEA1), fuse with late endosomes/lysosomes expressing the lysosomal-associated membrane protein 1 (LAMP1).30,31 The colocalization of internalized ephrin A1/EphA2 with EEA1-endosomes increased in a time-dependent manner in young hCPCs (Figure 3A–3C). However, old hCPCs exhibited a significant decrease in the colocalization of ephrin A1/EphA2 with EEA1 and an increase in association with LAMP1-positive vesicles (Figure 3B and 3D). In old hCPCs, the fusion of ephrin A1/EphA2 with LAMP1-endosomes was apparent at the cell periphery, and seemingly did not involve EEA1...
vesicles (Figure 3B). Thus, endosomal targeting of the ligand-bound EphA2 receptor is altered in old hCPCs; a smaller fraction of internalized EphA2 persists in early endosomes, but a larger proportion of this receptor is destined to the late endosomal compartment, possibly undergoing degradation.30,31

**EphA2 Endocytosis Is Mediated by Caveolin-1 and Src Family Kinases**

Clathrin- and caveolin-dependent endocytosis is a major route by which receptors internalize from the plasma membrane to the cytoplasm. Endocytosis of Eph receptors is mediated by both clathrin and caveolin.32,33 The EphA2 protein contains a putative caveolin-binding motif that directly interacts with caveolin-1 in response to ligand stimulation.32 Caveolin-1 and caveolin-2 isoforms were expressed in hCPCs, whereas caveolin-3 was not detectable (Figure IIIA in the online-only Data Supplement).

The distribution of EphA2 and caveolin-1 in hCPCs was determined biochemically and by immunolabeling and confocal microscopy. In response to ephrin A1, caveolin-1 colocalized with EphA2 in the EEA1-endosomes of young hCPCs (Figure 4A and Figure IIIB in the online-only Data Supplement). By immunoprecipitation and Western blot, caveolin-1 was found to form a complex with EphA2 in young hCPCs, and this protein-to-protein interaction increased significantly with ephrin A1 stimulation (Figure 4B). The subcellular distribution of caveolin-2 resembled that of caveolin-1; however, internalized EphA2 showed a preferential association with caveolin-1 (Figure IIIB in the online-only Data Supplement). The presence of ephrin A1/EphA2 in clathrin-coated vesicles was only occasionally seen (Figure IIIIC in the online-only Data Supplement), and the complex between EphA2 and caveolin-2 or clathrin did not increase after exposure to ephrin A1 (Figure IIID in the online-only Data Supplement). Thus, in response to ephrin A1, caveolin-1 mediates EphA2 endocytosis in young hCPCs.

The association of EphA2 with caveolin-1 and the distribution of EphA2/caveolin-1 in early endosomes after ephrin A1 stimulation were altered in old hCPCs (Figure 4A and Figure IIIE in the online-only Data Supplement). Old hCPCs showed a loss in the polarized...
The interaction of EphA2 with caveolin-1 was severely affected in old hCPCs (Figure 4A and 4B), suggesting that the recruitment of caveolin-1 to the activated receptor was impaired as a result of a defect in EphA2 signaling. This hypothesis was supported by the decrease in EphA2 phosphorylation triggered by ephrin A1 stimulation (Figure 4B); Eph autophosphorylation is a hallmark of receptor activation.\textsuperscript{11} The degree of EphA2 phosphorylation triggered by ephrin A1 stimulation is determined by the equilibrium between autophosphorylation of receptor tyrosine kinases and dephosphorylation of tyrosine residues by protein tyrosine phosphatases.\textsuperscript{28,34} Dephosphorylation of EphA2 may be accelerated in old hCPCs, attenuating EphA2 signaling. However, inhibition of protein tyrosine phosphatases with pervanadate led to a reduction in ephrin A1-induced EphA2 endocytosis (Figure IV in the online-only Data Supplement), arguing against the possibility that an increased protein tyrosine phosphatase activity interferes with EphA2 function in old hCPCs.

In young cells stimulated with ephrin A1, the endosomes carrying EphA2 and caveolin-1 or EEA1 were characterized by accumulation of phospho-tyrosine labeling, which was greatly reduced in old hCPCs exposed to ephrin A1 (Figure V in the online-only Data Supplement). These results suggest that EphA2 receptor tyrosine kinase signaling is maintained in endosomes of young hCPCs, whereas EphA2 autophosphorylation and endocytosis were markedly reduced in old hCPCs, negatively impacting on the EphA2 downstream pathway.

The Src family kinases (SFK) are well-established downstream effectors of the Eph receptors.\textsuperscript{11,12} Consistent with previous observations in hCPCs,\textsuperscript{10} EphA2 stimulation with ephrin A1 induced SFK activation and enhanced the binding of EphA2 to SFK (Figure 4B). In young hCPCs, ephrin A1 promoted the formation of endocytic vesicles in which EphA2 colocalized with the phosphorylated active forms of SFK and caveolin-1 (Figure 4C). Caveolin-1 phosphorylation at Tyr\textsuperscript{14} controls the internalization of caveolae and is implicated in the signaling cascade regulating cell motility.\textsuperscript{35,36} Inhibition of SFK, which phosphorylate caveolin-1, prevented the increase

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**Figure 4.** EphA2 binding to downstream effectors in endosomes is inhibited in old hCPCs. **A**, Colocalization of caveolin-1 (red), EEA1 (green), and EphA2 (blue) in the cytosol of young hCPCs exposed to ephrin A1 for 25 min. This colocalization is markedly attenuated in old hCPCs. Rectangles define areas illustrated at higher magnification in the insets. **B**, Immunoprecipitation and Western blotting of EphA2 protein (IP: EphA2) from young and old hCPCs in the presence (+) or absence (−) of ephrin A1. pTyr indicates phospho-tyrosine. O.D. data were normalized to the efficiency of EphA2 immunoprecipitation relative to unstimulated cells (−). Data are mean±SD (n=3). pTyr: *P* = 0.037; SFK: *P* = 0.033; caveolin-1: *P* = 0.023. **C**, Accumulation of Tyr\textsuperscript{14}-caveolin-1 (red), Tyr\textsuperscript{14,16}-SFK (green) and EphA2 (blue) in endocytotic vesicles of young and old hCPCs stimulated with ephrin A1. Rectangles define areas illustrated at higher magnification in the insets. **D–G**, Young hCPCs pretreated with SFK inhibitor (SFKi) or DMSO vehicle (control) were exposed to ephrin A1 for 25 min or left unstimulated (−). **D**, Color-coded images illustrating the intensity of Tyr\textsuperscript{14}-caveolin-1 labeling in hCPCs. **E**, Color-coded representation of the caveolin-1 labeling pattern in ephrin A1-treated hCPCs. **F**, Ephrin A1–induced EphA2 endocytosis (white) in control and SFKi-treated hCPCs. **G**, Fluorometric measurement of the internalized fluorescently labeled ephrin A1 at 15 min. Data are mean±SD (n=3). *P* = 0.021. EEA1 indicates endosome antigen 1; hCPC, human cardiac progenitor cell; and SFK, Src family kinases.
in Tyr14-caveolin-1 in young hCPCs after ephrin A1 treatment (Figure 4D). Additionally, it decreased the accumulation of caveolin-1 in endocytic vesicles (Figure 4E), affecting EphA2 endocytosis (Figure 4F) and ephrin A1 internalization (Figure 4G). Thus, in young hCPCs, activation of SFK is required for caveolin-1–mediated EphA2 endocytosis.

As emphasized above, HGF-induced hCPC migration requires EphA2 activity.10 Stimulation of hCPCs with HGF led to EphA2 accumulation at the plasma membrane (see Figure 1E), where ruffles and protrusion indicative of high membrane activity were found. Phosphorylated SFK and caveolin-1 were detected in these regions of membrane activation (Figure 5A). The combination of ephrin A1 and HGF had a synergistic or addictive effect on EphA2 endocytosis (Figure 5B). Thus, HGF/c-Met signaling affects the cellular distribution of EphA2 receptor, favoring its interaction with downstream targets and promoting cell motility.

Accumulating evidence supports the view that the function of receptor tyrosine kinases is sustained in endosomes, which are operatively defined as signaling endosomes.30,37 In young hCPCs, activated EphA2 receptors and their downstream targets, SFK, are present in EEA1-endosomes, possibly reflecting the generation of signaling endosomes. The dynamin inhibitor-dynasore38 blocks caveolae internalization and ephrin A1-induced EphA2 endocytosis, abrogating hCPC motility (Figure 6 in the online-only Data Supplement). In old hCPCs, attenuated EphA2 autophosphorylation correlated with the decrease in the formation of protein complexes with SFK or caveolin-1 (see Figure 4B). EphA2 localization in endosomes containing caveolin-1 or EEA1 was also reduced (see Figure 4A and 4C, and Figure IIIB and Figure V in the online-only Data Supplement). Thus, in old hCPCs, post-translational modifications may occur in EphA2 receptor, interfering with tyrosine kinase activity, endocytosis, and the formation of signaling endosomes.

**Protein Oxidation Inhibits EphA2 Function in Old hCPCs**

Aging is associated with a progressive intracellular accumulation of reactive oxygen/nitrogen species (ROS).39,40 Two fluorescent indicators of ROS were used to detect oxidative stress by confocal microscopy and to measure ROS level fluorometrically in hCPCs. By both approaches, old hCPCs were characterized by a significantly higher oxidative stress than young hCPCs (Figure 6A and 6B). Controlled production of ROS is critical for signal transduction, whereas an excessive generation of ROS impairs receptor tyrosine kinase activity.41 Exposure of young hCPCs to acute oxidative stress with H2O2 markedly reduced the ephrin A1–induced EphA2 phosphorylation (Figure 6C), mimicking the defects observed in old hCPCs (see Figure 4B). SFK activation and EphA2 endocytosis were notably diminished in H2O2-treated young hCPCs (Figure 6D and 6E), impairing their migratory ability (Figure 6F). Importantly, a shift in the isoelectric point (pI) of the EphA2 protein was detected in old hCPCs and similar changes were found in young hCPCs in the presence of H2O2 (Figure 6G). Thus, ROS may induce oxidation of the EphA2 receptor in old hCPCs, precluding its activation and downstream signaling.

These findings prompted us to determine whether treatment of old hCPCs with antioxidants positively affected EphA2 signaling. Old hCPCs, exposed for 1 day to N-acetyl-L-cysteine or polyethylene glycol-catalase, were stimulated with ephrin A1. With N-acetyl-L-cysteine, the association of caveolin-1 with EphA2 was moderately improved, pointing to an amelioration in endocytosis of the ephrin A1/EphA2 complex (Figure 6H). Moreover, cell-permeable catalase decreased oxidative stress and favored in a subset of old hCPCs the colocalization of the ligand-receptor pair with EEA1 (Figure 6I and 6J), mimicking the pathway of EphA2 internalization observed in young cells (see Figure 3A–3C). The limited effects of antioxidants on EphA2 endocytosis imposed on us the search for alternative strategies aiming at the functional restoration of the receptor.
Exogenous EphA2 Improves the Motility of Old hCPCs

Old hCPCs were infected with a lentivirus carrying a full-length EphA2-EGFP fusion protein or a lentivirus carrying EGFP only (Figure 7A and 7B). Exogenous EphA2, recognized by the EGFP tag, displayed the expected expression pattern on the plasma membrane (Figure 7C). With respect to endogenous EphA2, ephrin A1 induced a 5-fold higher level of tyrosine phosphorylation of the exogenous EphA2 receptor (Figure 7D), which underwent efficient endocytosis and co-localization with caveolin-1 (Figure 7E and 7F). Moreover, exogenous EphA2 exhibited greater association with EEA1-endosomes than the endogenous EphA2 protein in the same cell (Figure 7G). Conversely, endogenous EphA2 receptors in the same hCPC were primarily colocalized with LAMP1 (Figure 7H). The correction of the defects in EphA2 endocytosis improved the motile behavior of old hCPCs exposed to HGF (Figure 7I and 7J, and Movie III in the online-only Data Supplement). Thus,
the restoration of EphA2 receptor activity re-established the migratory capacity of old hCPCs.

**Sorting of Young and Old hCPCs Based on Differential Responses to Ephrin A1**

The recognition that defects in the ephrin A1/EphA2 effector pathway alter the molecular mechanisms responsible for hCPC migration provided a unique opportunity to establish whether this system can be used to separate young hCPCs with preserved growth reserve from old non-functional hCPCs. Young hCPCs efficiently adhere to immobilized recombinant ephrin A1 ligand (Figure 8A and 8B), a process that is abrogated after siRNA-mediated EphA2 knockdown.10 The defect in EphA2 receptor activity in old hCPCs substantially reduced their ability to adhere to ephrin A1–coated surfaces (Figure 8B). hCPCs with a younger phenotype were isolated from the heterogeneous pool of old cells based on their preferential retention on ephrin A1-substrates (Figure 8A). In comparison with unsorted and non-adherent old cells, the subset of hCPCs adhering to ephrin A1 displayed a 2-fold lower frequency in the expression of the senescence-associated markers p16INK4a and \( \gamma H2A.X \) (Figure 8C and 8D). Thus, EphA2 receptor function can be implemented in the selection of hCPCs with high therapeutic potential.

**Discussion**

In the present study we provide a potential mechanism for the age-associated decline in the motility of hCPCs. The
inadequate activation of the EphA2 receptor in old hCPCs precludes an efficient signal transduction, which is required for the initiation of cell polarization and movement of hCPCs in vitro and in the injured myocardium in vivo. Cellular aging does not affect the protein level of EphA2 receptor in hCPCs nor the early events involved in receptor binding to the ephrin A1 ligand. In analogy with other cell systems,42 functional alterations in senescent hCPCs do not depend on changes in receptor density and affinity to the ligand, but are coupled with abnormalities in the activity of receptor tyrosine kinases.

Collectively, our data support the view that oxidative stress is an important determinant of cellular and organ aging.39,40 The accumulation of ROS in old hCPCs induces post-translational modifications of the EphA2 protein interfering with the receptor tyrosine kinase function. Although the specific residues affected by oxidative stress were not identified, oxidation is known to abrogate receptor activity.41 The age-residues affected by oxidative stress were not identified, the receptor tyrosine kinase function. Although the specific translational modifications of the EphA2 protein interfering with the ephrin A1 signaling for the polarization and migration of hCPCs is precluded by restoring EphA2 function.

The recognition that tyrosine kinase activity is implicated in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.29,43 Our work further demonstrates that alterations in EphA2 receptor endocytosis is consistent with previous findings.
colocalize with EphA2 and activated caveolin-1 in the endosomes. Inhibition of SFK, an established downstream target of EphA2, prevents phosphorylation of caveolin-1 at Tyr41 and the generation of caveolin-1-containing vesicles. Attenuation in endocytosis of ephrin A1/EphA2 by SFK inhibition negatively affects hCPC chemotaxis.15

The spatially- and temporally-restricted compartment of signaling endosomes is implicated in the biological function of receptor tyrosine kinases.7,9,10 Oncogenic Met receptor mutants promote cancer cell migration and are characterized by an enhanced signaling capacity resulting from their preferential accumulation and sustained function in endosomes.45 Similarly, endocytosis is required for activation of the downstream effectors of the VEGF receptor46 and vessel sprouting.47 In contrast, mutation of the EphA8 receptor, which interferes with endocytosis, prevents axon repulsion during development.48 Our results indicate that formation of signaling endosomes is required to elicit migratory responses in hCPCs, raising the possibility that EphA2 receptor activity in old hCPCs is not sufficient for the signalosome assembly. In old hCPCs, the internalized EphA2 may be targeted to an alternative pathway of receptor recycling/degradation leading to premature signal termination. This hypothesis is supported by the restoration of the motility-related signaling cascade in old hCPCs overexpressing exogenous intact EphA2 receptors. The ectopic receptor is efficiently phosphorylated and subjected to endocytosis, displaying a vesicle maturation pattern typical of young cells, which promotes signaling endosome formation and cell migration.

The loss of migratory ability in old hCPCs affects their translocation within the myocardium, impairing cell turnover in the aging heart.4 This defect interferes with efficient cardiac repair, having important clinical implications. Extreme caution, however, has to be exercised in the extrapolation of in vitro findings to the in vivo condition. As shown here and previously,10 after myocardial infarction, ephrin A1 enhances the motility and the regenerative potential of hCPCs expressing intact EphA2 receptor. Although the manifestations of the aging cardiac phenotype are more complex than those found acutely after ischemic injury, the restoration of CPC movement leads to enhanced cardiomyogenesis, decreased fibrosis, and functional improvement in the senescent rat heart.4 Our data indicate that the expression of functional EphA2 receptors may be implemented for the isolation of hCPCs that possess a young phenotype and higher proliferative capacity. Thus far, no methodology is available for the in vivo separation of human progenitor cells with distinct growth reserve, the most critical determinant of successful cell therapy in patients. This is particularly relevant because autologous hCPCs are currently being tested in phase 1 clinical trials with encouraging results.20,45,50

References


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Disclosures

Dr Anversa is a member of Analogous, LLC. The other authors report no conflicts.
34.53.43.42.41.40.39.38.37.36.35.34.33.32.31.30.29.28.27.26.25.24.23.22.21.20.19.18.17.16.15.14.13.12.11.10.9.8.7.6.5.4.3.2.1.


CLINICAL PERSPECTIVE

Human cardiac progenitor cells (hCPCs) have recently been introduced in the experimental treatment of ischemic cardiomyopathy in humans. One of the critical variables of hCPC function is dictated by their motile state that conditions the translocation of cells to the damaged myocardium. Cardiac aging is coupled with alterations in the migratory capacity of hCPCs, although the mechanisms involved are largely unknown. We have found that the inadequate activation of the EphA2 receptor in old hCPCs affects their movement in vitro and in the injured myocardium in vivo. Cellular aging does not change the protein level of EphA2 receptors in hCPCs nor the early events involved in receptor binding to the ephrin A1 ligand, pointing to abnormalities in the activity of this receptor tyrosine kinase in senescent cells. Our findings support the view that oxidative stress is a crucial determinant of hCPC aging. The accumulation of reactive oxygen species in old hCPCs induces post-translational modifications of the EphA2 protein interfering with the receptor tyrosine kinase function. Although the specific residues affected by oxidative stress were not identified, oxidation is known to abrogate receptor activity. The age-dependent consequences of EphA2 receptor oxidation were not reversed by exogenously expressed functionally-competent EphA2 receptors in old hCPCs. Importantly, protocols have been defined for the preferential isolation of young efficient hCPCs, a strategy that may have significant implications in the future management of chronic heart failure.
Age-Associated Defects in EphA2 Signaling Impair the Migration of Human Cardiac Progenitor Cells
Polina Goichberg, Ramaswamy Kannappan, Maria Cimini, Yingnan Bai, Fumihiro Sanada, Andrea Sorrentino, Sergio Signore, Jan Kajstura, Marcello Rota, Piero Anversa and Annarosa Leri

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/content/132/4/e30.full.pdf
/content/132/22/e361.full.pdf

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/10/18/CIRCULATIONAHA.113.004698.DC1

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In the article by Goichberg et al, “Age-Associated Defects in EphA2 Signaling Impair the Migration of Human Cardiac Progenitor Cells,” which published online before print October 18, 2013, and appeared in the November 12, 2013 issue of the journal (Circulation. 2013;128:2211–2223. DOI: 10.1161/CIRCULATIONAHA.113.004698), a correction is needed.

During the course of an ongoing investigation, questions were raised about Figure 1I in the article. In their attempt to respond to this concern, the authors were unable to locate the original file for panel I of figure 1, and were unable to verify its accuracy. The authors have repeated the analysis using similar samples, and identical methodology, and generated a new figure. The new figure demonstrates the same findings as the original figure, and it does not change the conclusions of the published article.

The authors regret this circumstance.

This correction has been made to the current online version of the article, which is available at http://circ.ahajournals.org/content/128/20/2211.full.
In the article by Goichberg et al, “Age-Associated Defects in EphA2 Signaling Impair the Migration of Human Cardiac Progenitor Cells,” which published online before print October 18, 2013, and appeared in the November 12, 2013, issue of the journal (Circulation, 2013;128:2211–2223. DOI: 10.1161/CIRCULATIONAHA.113.004698), a correction was needed.

Piero Anversa, MD, discloses that he is a member of Analogous, LLC.

The author regrets this omission.

This correction has been made to the current online version of the article, which is available at http://circ.ahajournals.org/content/128/20/2211.full

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Human CPCs

Human c-kit-positive cardiac progenitor cells (hCPCs) were isolated from discarded myocardial tissue and cultured ex vivo as previously described (IRB protocol 2010P002475). Specimens were obtained from patients who underwent elective cardiac surgery, or consisted of donor hearts declined for transplantation and explanted hearts. Myocardial samples were enzymatically dissociated and small cardiac cells were sorted for c-kit and cultured. Expanded hCPCs were employed in the in vitro and in vivo studies described in the subsequent sections. To induce replicative senescence, hCPCs were expanded for 4-7 passages (P) and further propagated till P11-15. Stress-induced senescence was achieved by treatment of hCPCs at P4-P7 with 0.5 μM doxorubicin (Sigma) for 18 hr. Cells were then washed and cultured for additional 3-5 days (see Figure S1A). In all studies, hCPCs obtained at early (“young”) and late (“old”) passages were employed, together with hCPCs cultured in the absence (“young”) and presence (“old”) of doxorubicin. Stimulation with ephrin A1 involved treatment of hCPCs with 2 μg/mL of recombinant mouse ephrin A1-human Fcγ (R&D) for 5-30 min. Control cells were exposed to purified human IgG (Bethyl Laboratories). Treatment with 100-200 ng/mL recombinant human HGF (R&D) was also implemented.

Inhibition of SFK activity was obtained by treating hCPCs for 30 min with 10 μM PP2 (Millipore) dissolved in dimethylsulphoxide (DMSO). Endocytosis was inhibited by incubating hCPCs for 30 min with 20 μM of the cell-permeable dynamin inhibitor hydroxy-dynasore (Sigma) dissolved in DMSO. In both assays, control hCPCs were exposed to an equal volume of vehicle only.
The level of reactive oxygen species (ROS) in hCPCs was assessed with Image-IT LIVE Green ROS Detection Kit for microscopy (Life Technologies) according to manufacturer instructions. Young and old hCPCs were incubated with H$_2$DCFDA, 50 μM, for 30 min. Fluorescence intensity was measured with Wallac1420 VICTOR2 plate reader (PerkinElmer), 488 nm excitation wavelength, and normalized to the number of cells as determined based on Hoechst33342 nucleic acid stain (Life Technologies). The superoxide indicator dihydroethdium (DHE; Life Technologies) was also used (1 μM, 30 min) to analyze microscopically changes in oxidative stress. To reduce the cellular level of ROS, old hCPCs were either treated over-night with 5 mM N-acetyl-L-cysteine (NAC; Sigma), pH 7.4; or exposed to 500 U of polyethylene glycol (PEG)-catalase (Sigma).

**Immunofluorescence and Confocal Microscopy Analysis**

For indirect immunolabeling, hCPCs were plated on glass coverslips, fixed for 25 min with 4% paraformaldehyde, permeabilized for 5 min with 0.5% Triton X-100, and incubated with specific antibodies for 45 min at ambient temperature. Primary antibodies included goat anti-human EphA2 (R&D), rabbit anti-EphA2 (Santa Cruz), rabbit anti-human c-kit (BioCare Medical), rabbit anti-human c-kit (DAKO), mouse anti-human c-Met (R&D), mouse anti-human Ki-67 (DAKO), mouse anti-human p16$^{INK4a}$ (Abcam), rabbit anti-53BP1 (Cell Signaling), mouse anti-phospho-histone H2A.X (Ser139) (Millipore), mouse anti-EEA1 (BD Transduction Laboratories), sheep anti-human LAMP1 (R&D), rabbit anti-caveolin-1 (Sigma), goat anti-caveolin-2 (R&D), mouse anti-human caveolin-3 (R&D), mouse anti-clathrin heavy chain (Abcam), mouse anti-human phospho-caveolin-1 (pY-14) (BD Transduction Laboratories), rabbit anti-phospho-Src family (Tyr416) (Cell Signaling), rabbit anti-phosphotyrosine (Millipore), and PY-Plus mouse anti-phosphotyrosine (Life Technologies). Secondary reagents, including donkey IgG conjugated
with fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC), cyanine
5 (Cy5), AlexaFluor-488, AlexaFluor-565, and AlexaFluor-647, were purchased from Jackson
ImmunoResearch or Life Technologies. DNA was labeled with 4,6-diamidino-2-phenylindole
(DAPI; Sigma). AlexaFluor-647-conjugated phalloidin was obtained from Life Technologies.
Recombinant ephrin A1-human Fcγ was detected with FITC- or TRITC-conjugated donkey anti-
human IgG recognizing Fcγ fragment (Jackson ImmunoResearch).

Thin tissue sections (5 µm) were obtained from ventricular myocardium of paraffin-
embedded young and old human donor hearts, and labeled with rabbit anti-ephrin A1 (Life
Technologies), goat anti-p16INK4a (Santa Cruz), and mouse anti α-sarcomeric actin (Sigma)
antibodies as previously described.2,3,6,7

Images were acquired using BioRad Radiance 2100 (BioRad) or Olympus Fluoview
FV1000 (Olympus) confocal microscopes. The lookup tables intensity color-coding was
performed using ImageJ software (NIH). Co-localization analysis of EphA2 and EEA1 and the
determination of Pearson’s coefficient of correlation were done with the Image J JACoP (Just
Another Colocalization Plugin). Estimation of vesicle size by automated image segmentation and
surface rendering were performed using Imaris 7.4 software (Bitplane).

**Immunoprecipitation, Immunoblotting, and Two-Dimensional Gel Analysis**

Young and old hCPCs were lysed in RIPA buffer containing protease and phosphatase inhibitors.
Human heart tissue was processed in lysis buffer containing 0.5% deoxycholic acid (Sigma). To
estimate relative level of protein expression, 25-50 µg of protein extract was used for SDS-
PAGE.2-4,7,8 Western blot was performed with the primary antibodies listed above and HRP-
conjugated secondary reagents (Jackson ImmunoResearch). Equal loading was documented by
immunoblotting with rabbit anti-GAPDH (Cell Signaling) or mouse anti-cardiac α-actinin
(Sigma) antibody. For immunoprecipitation, 100 μg of proteins were incubated overnight with 5 μg/mL goat anti-human EphA2 (R&D), followed by 1 hr incubation with PureProteome protein A magnetic beads (Millipore), according to manufacturer’s instruction. EphA2 was detected using rabbit anti-EphA2 (Santa Cruz). Two-dimensional electrophoresis analysis included young hCPCs, old hCPCs, and young hCPCs exposed to acute oxidative stress, 300 μM H2O2 for 30 min. Protein cell lysates was obtained in Tris-HCl buffer with 6M urea, 30% glycerol and 2% SDS; in all cases, 200 μg proteins were separated using ReadyStrip IPG Strips pH3-10 (BioRad) with PROTEAN IEF system (BioRad) and further analyzed by immunoblotting. Optical density of the bands was evaluated using ImageJ.

**Flow-Cytometry Studies**

The expression of EphA2 on the plasma membrane of hCPCs was determined with goat anti-human EphA2 (R&D), followed by staining with AlexaFluor-647-conjugated anti-goat IgG (Jackson ImmunoResearch). c-Met was detected with FITC-conjugated rat anti-human c-Met (eBioscience). Secondary reagents and isotype-matched IgG were used as background controls.

Telomere length in hCPCs was measured by flow-cytometry and fluorescence in situ hybridization (Flow-FISH), following a previously described protocol (Baerlocher et al., 2006; Bolli et al., 2011).9,10 hCPCs were combined with L5178Y-R mouse lymphoma cells of known telomere length (48 kb).1,10,11 L5178Y-S mouse lymphoma cells carrying 7 kb-long telomeres were analyzed as control for assay fidelity. Cells were incubated in a solution containing 0.3 μg/mL of AlexaFluor-647-conjugated (CCCTAA)3 peptide nucleic acid (PNA) probe (Panagene). Blank samples consisted of cells not exposed to the PNA probe. DNA denaturation was performed at 87°C for 15 min and was followed by 2 hr hybridization at room temperature. Samples were also labeled with AlexaFluor-488-conjugated rat anti-mouse CD45 (BioLegend) to
distinguish mouse lymphocytes and hCPCs. DNA was counterstained with 0.5 µg/mL propidium iodide solution (Sigma). Calibration of the flow-cytometer and calculations of the molecules of equivalent soluble fluorochrome (MESF) units were performed using Quantum APC microspheres (Bang Laboratories). Acquisition was performed with FACPCanto II flow-cytometer (BD Biosciences) and data were analyzed using FlowJo v7/9 software (Tree Star).

Transwell Migration
Spontaneous motility and chemotaxis of young and old hCPCs were examined as previously described:² cells were treated with 2 µg/mL ephrin A1 for 10 min and loaded in the upper well of the uncoated transwell permeable support system (8 µm pore size; Corning). Lower wells contained growth medium with or without 200 ng/mL recombinant human HGF (R&D). After 5 hr of incubation, transmigrated cells were detached from the filter with Trypsin-EDTA (Sigma), and their number was determined by flow-cytometry. Control cells were studied in the absence of ephrin A1 stimulation. In a set of experiments, cells were pre-treated for 30 min with 300 µM H₂O₂ or 20 µM dynasore.

Lentiviral Infection of hCPCs
The pLentiV plasmid for the lentiviral expression of EGFP expression, and the 3-plasmid system to generate lentiviral particles (LV-EGFP) were previously described.¹² Full-length human EphA2 sequence (EPHA2:Homo sapiens, Gene ID: 1969) was obtained from the pJP1520 retroviral expression vector, purchased from the DNASU Plasmid Repository & PSI:Biology-MR Biodesign Institute (Arizona State University, Tempe, AZ). PCR-based site-directed mutagenesis was performed to remove the termination codon in the EphA2 open reading frame, followed by ligation with the pLentiV plasmid to enable expression of the fusion protein EphA2-
EGFP. The plasmid for lentiviral expression of RFP was purchased from Thermo Scientific. Infection of hCPCs was carried out as described.\textsuperscript{1,2,6,12}

**Imaging of hCPC Translocation in Vivo**

Young and old hCPCs were infected with lentiviruses expressing EGFP or RFP, respectively. The two cell populations were combined in a 1:1 ratio, pre-treated for 10 min with 2 µg/mL of ephrin A1, and mixed with 0.1% volume of InSpek Blue 2.5 µM fluorescent microspheres (Life Technologies). The procedures followed were in accordance with institutional guidelines. Shortly after coronary ligation, 100,000 hCPCs were injected at 4 different sites in the border zone of the hearts of 2-3 month-old C57BL/6 mice.\textsuperscript{2} One hr later, hearts were excised, mounted on the stage of a two-photon microscope (Bio-Rad Radiance 2100MP), and continuously perfused with oxygenated Tyrode solution. Observation of the left ventricular free wall and acquisition of the images was performed with LaserSharp 2000 (BioRad) over a period of 1-3 hr per observation field, for a cumulative time of 6-8 hr per heart. Cell trajectories were generated using ImageJ manual tracking plugin. Cell velocity (distance/time) was calculated, and the random tissue movement during the imaging was compensated by measuring the displacement of fluorescent microspheres.

**Endocytosis of Ephrin A1 and Transferrin**

Young and old hCPCs were simultaneously incubated with 1 µg/mL of AlexaFluor-647-conjugated transferrin (Life Technologies) and 1 µg/mL of ephrin A1-FITC to assess the efficiency of ligand internalization into the cytosol. Prior to cell exposure, recombinant ephrin A1-human Fe\textsubscript{γ} was pre-clustered for 5 min with 1.5 µg/mL FITC-conjugated anti-human Fe\textsubscript{γ} (Jackson ImmunoResearch). Following incubation with the two ligands for 5-30 min, hCPCs were subjected to a 2 min acid wash (0.5 M NaCl/0.5 M acetic acid, pH 2.5) to detach the
residual ligand bound to the cell surface.\textsuperscript{13} Washing of the cells in a buffer solution at pH 7.4 allowed the retention of the total bound ligand on the plasma membrane and in the cells. Fluorescence intensity was determined with a plate reader and normalized to the number of cells, as above, or examined by flow-cytometry. The proportion of the internalized ligand was quantified as % fluorescence intensity after acid wash versus wash at pH 7.4. In a set of experiments, hCPCs were pre-treated with PP2 or H$_2$O$_2$, as above.

Live imaging of endocytosis dynamics of transferrin and ephrin A1 was studied in young and old hCPCs, which were plated on glass-bottom culture dishes (MatTek). Cells were incubated for 5-45 min with 2 \( \mu \)g/mL of TexasRed-conjugated transferrin (Life Technologies) and pre-clustered ephrin A1-FITC; or ephrin A1-TRITC in hCPCs expressing EGFP. Time-lapse acquisition was performed with Olympus IX71 inverted epifluorescent microscope using CellSens Dimension software (Olympus). Automated recognition and tracking of ephrin A1-TRITC vesicles were performed with Imaris 7.4 software using ImarisTrack module (Bitplane).

**Live Imaging of hCPC Migration in Vitro**

EGFP-expressing or EphA2-EGFP-infected old hCPCs were plated in a \( \mu \)-slide I flowchamber (ibidi), subjected to polarized stimulation with HGF, as recommended by manufacturer, and imaged for 7 hr at 37°C. Time-lapse acquisition was performed as described above and the number of migrating cells, cell velocity and the directionality of movement were analyzed with ImageJ using manual tracking and chemotaxis tool plugin.

**Cell Sorting by Adhesion to Ephrin A1**

Adhesion assay of hCPCs to immobilized ephrin A1 was performed as previously published.\textsuperscript{2} Briefly, Reacti-Bind Protein A/G-coated 96-well plates (Fisher Scientific) were incubated overnight at 4°C with 0.3 \( \mu \)g/cm$^2$ of recombinant ephrin A1-Fc\( \gamma \) (R&D) or human IgG (Bethyl
Laboratories). Residual Fc-binding sites were blocked with 2% bovine serum albumin (Sigma). Before plating, hCPCs were labeled with 10 μM Calcein AM (Life Technologies). Approximately 10,000 calcein-loaded hCPCs were seeded in each well. Plates were spun down for 2 min and subsequently incubated at 37ºC for 45 minutes. Following extensive washing, the fraction of adhered hCPCs was estimated with fluorescent microplate reader, 488 nm excitation wavelength.

To assess the expression of p16\(^{\text{INK4a}}\) and the presence of γH2A.X foci in ephrin A1-adhering and non-adhering hCPCs, the non-adherent cells were collected after the adhesion step. The adherent cells were detached from the plates with a non-enzymatic cell dissociation solution (Sigma). The two populations, as well as unsorted hCPCs incubated with IgG-coated plates, were plated on glass coverslips and analyzed by immunolabeling and confocal microscopy.

**Statistical Analysis**

Significance between two groups was determined by two-tailed unpaired Student’s \(t\) test. For multiple comparisons, the ANOVA test with Bonferroni correction was employed. Quantitative data are expressed as mean±SD.

**References to Supplemental Methods**


**Legends to Supplemental Figures**

**Figure 1.** Ageing of hCPCs and telomere length. A, Protocols for the induction of senescence of hCPCs. B, Telomere length measured by Flow-FISH in young (black line) and old (red line) hCPCs incubated with the telomere probe or blank solution (control: grey line). C and D, Telomere length in each cell class is shown as mean±SD (n=7). *P*<0.0001. E, Morphology of young and old hCPCs expressing c-kit (green) and EphA2 (grey). Individual fluorescent signals are shown in the lower panels. Nuclei are stained with DAPI (blue).

**Figure 2.** EphA2 and c-Met receptors in hCPCs. A, Young (left) and old (right) hCPCs co-labeled with EphA2 (green) and c-Met (red). Individual fluorescent signals are shown in the lower panels. B, Protein level of EphA2 and c-Met in young and old hCPCs. GAPDH, loading control. C, Expression of EphA2 and c-Met receptors on the plasma membrane of hCPCs measured by flow-cytometry. Control, cells stained with a secondary reagent only (EphA2) or an isotype-matched IgG (c-Met).

**Figure 3.** Caveolin-mediated EphA2 endocytosis in hCPCs. A, Expression of caveolin-1, -2 and -3 isoforms in hCPCs. Human cardiomyocytes (hCMs), positive control for caveolin-3. GAPDH, loading control. B, Sub-cellular distribution of caveolin-1 (red), caveolin-2 (blue) and EphA2 (green) in young and old hCPCs in the presence and absence (-) of ephrin A1. Areas included in the rectangles are shown at higher magnification in the insets. Asterisks indicate accumulation of caveolins -1 and -2 at the plasma membrane of untreated young cells (upper left panel) and in
central area of untreated old hCPCs (lower left panel). EphA2 co-localizes predominantly with caveolin-1 (white arrowheads) in young ephrin A1-treated hCPCs (upper right panel). Co-localization of EphA2 with caveolin-2 is less evident. Co-labeling of internalized EphA2 with caveolins is markedly decreased (yellow, arrowheads) in old ephrin A1-treated hCPCs (lower right panel).

C, Association of caveolin-1 (red, left) and clathrin (red, right) with vesicles containing the internalized EphA2 (green) in young hCPCs treated for 15 min with ephrin A1. Areas in the rectangles are shown at higher magnification in the insets. Arrows point to an endocytic vesicle co-labeled with EphA2 and caveolin-1.

D, EphA2 binding to caveolin-2 and clathrin was established by co-immunoprecipitation (IP: EphA2) in young hCPCs treated with ephrin A1 for 25 min (+). (-) untreated hCPCs.

E, Expression of the caveolin-1 protein in young and old hCPCs. GAPDH, loading control.

**Figure 4.** Inhibition of phospho tyrosine phosphatase activity prevents EphA2 endocytosis in hCPCs. hCPCs exposed to orthovanadate (left panels, control) or pervanadate (right panels). In both cases, young hCPCs were stimulated for 15 min with ephrin A1. Following inhibition of phospho tyrosine phosphatases with pervanadate, phospho-tyrosine labeling (upper right panel: pTyr, red) increased and EphA2 (mid-right panel: green) failed to accumulate in the endocytic vesicles. Lower panels, merge. Phalloidin, grey.

**Figure V.** Phospho-tyrosine residues accumulate in the endosomes of young hCPCs. A and B, Endocytic vesicles containing EphA2 (blue), phospho-tyrosine (pTyr, green), and caveolin-1 (A, red) or EEA1 (B, red) are present in young and old hCPCs stimulated with ephrin A1 for 15 min. Areas in rectangles are shown at higher magnification in the insets. Arrows point to the co-localization of pTyr and EphA2 in the endosomes of young cells. With respect to young hCPCs,
note the lower level of pTyr and the decreased co-labeling of EphA2 with caveolin-1 and EEA1 in old hCPCs following exposure to ephrin A1.

**Figure 5.** Dynamin inhibition abrogates EphA2 endocytosis and cell migration. **A,** hCPCs were pre-treated for 30 min with dynasore or DMSO vehicle (control). The sub-cellular distribution of EphA2 (green) in endocytic vesicles after ephrin A1 stimulation for 15 min is prevented by dynasore. Areas in rectangles are shown at higher magnification in the insets. Arrows point to the co-location of EphA2 (green) and caveolin-1 (red) in endosomes of control cells and to the retention of EphA2 and caveolin-1 at the membrane of dynasore-treated cells. **B,** Transwell migration of control and dynasore-treated hCPCs in the absence (-) and presence (+) of HGF. Data are mean±SD (n=3). *P=0.0069 vs. (- control), #P=0.007 vs. (- control), †P<0.0001 vs. (+ control).

**Legends to Supplemental Videos**

**Video 1.** Translocation of the transplanted young and old hCPCs in the infarcted myocardium. Young (green) and old (red) hCPCs were pre-stimulated with ephrin A1 and co-transplanted in the infarct border zone. Images were acquired every 2 min. Time is indicated (h:min:sec).

**Video 2.** Dynamic analysis of the ephrin A1-containing vesicles in young and old hCPCs. Young (left) and old (right) hCPCs expressing EGFP (green) were stimulated with pre-clustered TRITC-ephrin A1 (red) and monitored for the indicated time. Images were acquired every 5 sec. Overlay shows the trajectory of individual vesicles generated by automated image segmentation. Time is indicated (min).

**Video 3.** Improved motility of old hCPCs after overexpression of functional EphA2. Old hCPCs were infected with lentivirus carrying EphA2-EGFP fusion protein (LV EphA2-EGFP, right) or
EGFP only (LV EGFP, left). Cells were stimulated with HGF and monitored for the indicated time. Images were acquired every 15 min. Time is indicated (h:min:sec).
**Figure 1**

(A) Replicative senescence

Young → P4-P7 → Old

Young → P11-P15 → Old

Stress-induced senescence

Doxorubicin (0.5 μM) → wash → Old

(B) Relative cell count

Telomere probe

Control → Young → Old

(C) Replicative senescence

Young vs. Old

(D) Stress-induced senescence

Young vs. Old

(E) c-kit/EphA2

Young vs. Old

(c-kit) and (EphA2) immunofluorescence images
Figure 3
Figure 3 (cont.)
Figure 4
Figure 5