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

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Abstract

**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. Over-expression 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.

**Key words:** aging; EphA2; cell movement; cardiac regeneration, cardiac progenitor 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.\textsuperscript{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 myopathy.\textsuperscript{3-5} Although the number of CPCs increases in the old myocardium,\textsuperscript{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.\textsuperscript{3,4} Old, poorly contracting myocytes accumulate and cardiac performance deteriorates, mimicking the aging myopathy in humans.\textsuperscript{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,\textsuperscript{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.\textsuperscript{7-10} Ephrins are membrane-bound proteins that participate in a complex contact-dependent communication with neighboring cells expressing the Eph receptors.\textsuperscript{11,12} EphA2 is preferentially expressed in immature hCPCs, while the corresponding ligand, ephrin A1, is distributed on the plasma membrane of myocytes,\textsuperscript{10} which act as supporting cells within the stem cell niches.\textsuperscript{13}
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-old 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 µM doxorubicin (Sigma) (Figure 1 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 Two-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.¹⁹,²⁰

Transwell Migration

Spontaneous motility and chemotaxis of young and old hCPCs were examined as previously described.¹⁰

Lentiviral Infection of hCPCs

The pLentiV plasmid for EGFP expression and the 3-plasmid system to generate lentiviral particles were employed previously.²¹ 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 RFP was purchased from Thermo Scientific. Infection of hCPCs was carried out as described.¹⁰,¹⁶,²¹

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 with ephrin A1 and injected into the myocardium of acutely infarcted mice.¹⁰ The procedures were performed in accordance with institutional guidelines. The protocols for cell transplantation, two-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 to 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 non-adherent hCPCs.

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. 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.\textsuperscript{22,23} Cells displaying an old phenotype are identified by the expression of
senescence-associated biomarkers.\textsuperscript{18,24} Strategies for the isolation of live senescent cells remain
to be developed, requiring the implementation of \textit{ex vivo} models of cellular aging. Two in vitro
protocols were introduced to achieve hCPC senescence (Figure 1A in the in the online-only
Data Supplement): replicative senescence as a result of serial passaging;\textsuperscript{25} and stress-induced
senescence by exposure to the oxidative agent doxorubicin.\textsuperscript{17}

Senescence of hCPCs was documented with several parameters, which included irreversible
withdrawal from the cell cycle, expression of the senescence-associated protein p16\textsuperscript{INK4a},
accumulation of DNA-damage response (DDR) foci, telomeric shortening, and morphological
changes consisting of cell flattening and enlargement.\textsuperscript{25} DDR foci are characterized by the co-
localization of phosphorylated histone H2A.X (γH2A.X) and p53-binding protein 1 (53BP1) at
sites of DNA injury.\textsuperscript{26}

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 1A). In both cases, the percentage
of p16\textsuperscript{INK4a}-positive hCPCs increased 4-fold (Figure 1B). DDR 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 1B and 1C in the online-only Data Supplement). Short-term oxidative stress by
doxorubicin is more commonly associated with loss of telomere integrity\textsuperscript{27} in the absence of
changes in average telomere length (Figure 1D in the online-only Data Supplement).
Additionally, senescent hCPCs showed flattening and loss of polarization (Figure 1E in the online-only Data Supplement). Thus, old hCPCs were obtained by these two protocols, enabling us to compare young and old cells isolated from the same human heart, independently from patient’s age, gender, absence or presence of pathology, and etiology and duration of the cardiac disease. 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.

Experimentally, the migratory capacity of CPCs in the myocardium declines with age, 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. 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.

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; this response was not observed in old cells (Figure 1E). The defects in the migratory behavior of old hCPCs were comparable in the two 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. Following 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 two-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 non-migrating old hCPCs was 2.9-fold larger than that of young hCPCs (Figure 1G and Video 1 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.

**Ephrin A1-Induced EphA2 Endocytosis Is Altered in Old hCPCs**

The impairment in the motility of old hCPCs within the myocardium may reflect alterations in the expression of ephrin A1 ligand, its corresponding receptor EphA2, and/or the downstream signaling pathway. With chronological age, the level of ephrin A1 decreased slightly in the human heart (Figure 1H), in which ephrin A1 is confined to the myocyte compartment.\textsuperscript{10} The decline in ephrin A1 was more apparent in senescent p16\textsuperscript{INK4a}-positive myocytes (Figure 1I). However, exposure to saturating levels of ephrin A1 did not correct the defects in the motile properties of old hCPCs (see Figure 1D, 1F and 1G), suggesting that EphA2 function is a crucial determinant of hCPC migration.

Since the expression of EphA2 and c-Met proteins did not differ in young and old hCPCs (Figure 2 in the online-only Data Supplement), we determined whether dysfunction of the EphA2 receptor was implicated in the migration abnormalities of old hCPCs observed in vitro and in vivo. In our study, we focused on EphA2 because the integrity of this pathway is essential for the chemotactic response of hCPCs to HGF.\textsuperscript{10} Ephrin binding to Eph receptors leads to internalization of the ligand/receptor complex from the plasma membrane to the cytosol.\textsuperscript{28,29} By employing fluorescently labeled ephrin A1, the amount
of ligand internalized by young and old hCPCs was measured quantitatively. In comparison with young hCPCs, EphA2-mediated ephrin A1 endocytosis was markedly decreased in old hCPCs (Figure 2A and 2B). The sub-cellular distribution of the ephrin A1/EphA2 complex also differed in the two hCPC classes. The ligand-receptor pair accumulated in the peri-nuclear region of young hCPCs but was restricted to vesicles near the plasma membrane of old cells (Figure 2C). This defect was not observed with the transferrin ligand. The simultaneous analysis of ephrin A1 and transferrin endocytosis in old hCPCs showed that, in contrast to ephrin A1, transferrin was internalized and translocated to the peri-nuclear endosomes (see Figure 2A and 2B), pointing to the absence of a global defect in the endocytic machinery of old hCPCs.

The motility and sub-cellular distribution of endosomes are strictly related to their stage of maturation. Within minutes of ligand binding, multiple vesicles form at the periphery of the cell and undergo slow non-directional short-range oscillations, which convert into rapid directional long-range movements. This process results in the translocation of vesicles to the peri-nuclear region. The dynamics of endosomes containing fluorescently labeled ephrin A1 was studied by live imaging in young and old hCPCs. Ephrin A1 was efficiently transported from the plasma membrane to the peri-nuclear region in young cells (Video 2 in the online-only Data Supplement). Conversely, the displacement of the ephrin A1 cargo lacked directionality in old hCPCs, in which endosomes largely failed to reach the center of the cells (Figure 2D and 2E, and Video 2 in the online-only Data Supplement).

The movement of endocytic vesicles from the plasma membrane to the peri-nuclear area is coupled with their progressive enlargement. 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 µm², were found in the peri-nuclear region and
smaller EphA2-vesicles, <2 µm², 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 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 co-localization 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 co-localization 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 (SFK)

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. The EphA2 protein contains a putative caveolin-binding motif that directly interacts with caveolin-1 in response to ligand stimulation. Caveolin-1 and caveolin-2 isoforms were expressed in hCPCs, while caveolin-3 was not detectable (Figure 3A 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 co-localized with EphA2 in the EEA1-endosomes of young hCPCs (Figure 4A and Figure 3B 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 sub-cellular distribution of caveolin-2 resembled that of caveolin-1; however, internalized EphA2 showed a preferential association with caveolin-1 (Figure 3B in the online-only Data Supplement). The presence of ephrin A1/EphA2 in clathrin-coated vesicles was only occasionally seen (Figure 3C 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 3D 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 4C, and Figure 3B in the online-only Data Supplement). The protein level of caveolin-1 was similar in young and old hCPCs (Figure 3E in the online-only Data Supplement), but the sub-cellular
localization of caveolin-1 differed in these two cell populations. Old hCPCs showed a loss in the polarized pattern of caveolin-1 at the lateral cell membrane, and caveolin-1 accumulated in large structures near the center of the cell (Figure 3B in the online-only Data Supplement).

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 due to a defect in EphA2 signaling. This hypothesis was supported by the decrease in EphA2 receptor tyrosine phosphorylation in old hCPCs following ephrin A1 binding (Figure 4B); Eph autophosphorylation is a hallmark of receptor activation.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.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 4 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 5 in the online-only Data Supplement). These results suggest that EphA2 receptor tyrosine kinase signaling is maintained in endosomes of young hCPCs, while 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.11,12 Consistent with previous observations in hCPCs,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 co-localized with the phosphorylated active forms of SFK and caveolin-1 (Figure 4C). Caveolin-1 phosphorylation at Tyr^{14} controls the internalization of caveolae and is implicated in the signaling cascade regulating cell motility. Inhibition of SFK, which phosphorylate caveolin-1, prevented the increase in Tyr^{14}-caveolin-1 in young hCPCs following 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. 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. 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-dynasore 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 3B and Figure 5 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). Two fluorescent indicators of ROS were employed 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). Importantly, a controlled production of ROS is critical for signal transduction, while an excessive generation of ROS impairs receptor tyrosine kinase activity. Exposure of young hCPCs to acute oxidative stress with H$_2$O$_2$ 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 H$_2$O$_2$-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 H$_2$O$_2$ (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
(NAC) or polyethylene glycol (PEG)-catalase, were stimulated with ephrin A1. With NAC, 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 co-localization 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 co-localized 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 Video 3 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 following 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 γH2A.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.\textsuperscript{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.\textsuperscript{41} The age-dependent consequences of EphA2 receptor oxidation were corroborated by comparing the signaling capacity of endogenous and exogenously expressed EphA2 receptors in old hCPCs. In contrast to endogenous EphA2, autophosphorylation of the exogenous EphA2 receptor was profoundly increased following ephrin A1 stimulation. Consequently, endocytosis and the formation of signaling endosomes were corrected by restoring EphA2 function.

The recognition that tyrosine kinase activity is implicated in EphA2 receptor endocytosis is consistent with previous findings.\textsuperscript{29,43} Our work further demonstrates that alterations in the EphA2 endocytic pathway prevent vesicle maturation. The dynamic process of vesicle maturation is characterized by centripetal trafficking of the ephrin A1/EphA2-containing endosomes and vesicle enlargement, both essentially absent in old hCPCs. Moreover, the amount of internalized ephrin A1 ligand in non-moving vesicles adjacent to the cell surface was decreased in old hCPCs. These defects in the translocation of maturing endosomes carrying EphA2 receptors oppose signalosome assembly, which is required for the rearrangement of the cytoskeleton and acquisition of the motile configuration in old hCPCs.

In the course of endosome maturation, the primary endocytic vesicles containing the internalized cargo fuse with early endosomes at the cell periphery, and form larger endosomes as they move towards the nucleus, where the majority of lysosomes are localized.\textsuperscript{30} In young hCPCs, fusion of internalized ephrin A1/EphA2 complex with the early endosomes was followed
by the association with late endosomes/lysosomes distributed in the peri-nuclear area.

Conversely, in old hCPCs, fusion of EphA2 receptors with early endosomes was significantly
reduced, while their interaction with LAMP1-expressing vesicles at the cell periphery indicated
an alternative pathway and/or their potential degradation. Thus, cellular aging alters the EphA2
endocytic route, possibly affecting the half-life of the EphA2 receptor and its function.

Results in this study show that activation of EphA2 by ephrin A1 regulates phosphorylation of
caveolin-1 and its recruitment to endocytic vesicles. Although the expression of caveolin-1
protein was similar in young and old hCPCs, old cells lacked the polarized caveolin-1
distribution, reflecting an abnormal migratory cell phenotype. Ephrin A1 treatment did not
correct this alteration in caveolin-1 localization, indicating that the integrity of the EphA2
pathway constitutes the primary modulator of caveolin-1 function in hCPCs. In this regard,
exogenously introduced signaling-competent EphA2 receptors restored the endosomal
accumulation of caveolin-1, promoting effective endocytosis. The relevance of EphA2 signaling
for the polarization and migration of hCPCs is supported by the findings demonstrating that
phosphorylated SFK co-localize with EphA2 and activated caveolin-1 in the endosomes.

Inhibition of SFK, an established downstream target of EphA2, prevents phosphorylation of
caveolin-1 at Tyr14 and the generation of caveolin-1-containing vesicles. Attenuation in
endocytosis of ephrin A1/EphA2 by SFK inhibition negatively affects hCPC chemotaxis.10

The spatially- and temporally-restricted compartment of signaling endosomes is implicated in the
biological function of receptor tyrosine kinases.29,31 Oncogenic Met receptor mutants promote
cancer cell migration and are characterized by an enhanced signaling capacity due to 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.\textsuperscript{47} In contrast, mutation of the EphA8 receptor, which interferes with endocytosis, prevents axon repulsion during development.\textsuperscript{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.\textsuperscript{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,\textsuperscript{10} following 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.\textsuperscript{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 since autologous hCPCs are currently being tested in phase 1 clinical trials with encouraging results.\textsuperscript{20,49,50}

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**Conflict of Interest Disclosures:** None.
References:


and interim analysis of myocardial function and viability by magnetic resonance. *Circulation*. 2012;126:S54-64.

Figure Legends:

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\textsuperscript{INK4a} (B: red), and DDR foci (C: γH2A.X, 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 or more γH2A.X 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. control; †P<0.0001 vs. control; **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 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 1 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-CA) and GAPDH. Young hearts: 34-38 years-old; Old: 68-70 years-old. I, Distribution of ephrin A1 (white) in p16INK4a-positive (green, arrows) and negative cardiomyocytes (α-sarcomeric actin: α-SA, red) in young and old human hearts.

Figure 2. Endocytosis of ephrin A1 is impaired in old hCPCs. A, Young and old hCPCs were simultaneously treated with fluorescently-labeled ephrin A1 and transferrin and analyzed by flow-cytometry. The relative amount of intracellular ephrin A1 decreased in old hCPCs, while the quantity of transferrin did not vary in young and old hCPCs. A.U., arbitrary units. B, hCPCs exposed for 15 min to ephrin A1 (red) and transferrin (green). The distribution of the two ligands in cytosolic vesicles is similar in young hCPCs but differs in old hCPCs. Ephrin A1, red arrows; transferrin, green arrows. C, EphA2 (green) is located on the plasma membrane of untreated young and old hCPCs (0 min). EphA2 co-localizes with ephrin A1 (red) in the endocytic vesicles (orange) of ligand-stimulated young and old hCPCs (15 min). Intracellular and peri-nuclear distribution of endocytic vesicles in young hCPCs (lower left, arrows), and in proximity of the plasma membrane in old hCPCs (lower right, arrows). Rectangles define areas illustrated at higher magnification in the insets. D and E, Dynamic analysis of the translocation of ephrin A1-containing endosomes in EGFP-expressing hCPCs (green) treated with fluorescently-labeled ephrin A1 (red). D, Time-lapse images of EGFP-positive young and old hCPCs; overlaid trajectory of individual vesicles (red) illustrated with a color-coded time scale (blue-start, yellow-end). See accompanying Video 2 in the online-only Data Supplement. E, The distance of
the vesicles from the plasma-membrane was computed as percent of cell diameter. Data are mean±SD (n=3). *P=0.03 vs. old at 25 min. ** Images of EphA2 vesicles (upper, white) and color-coded depiction of their size (lower) 15 min after ephrin A1 stimulation. Note larger EphA2-containing endosomes (yellow-red color) in the center of a young cell (lower left). G, Area of EphA2 clusters formed in young and old hCPCs after ephrin A1 exposure. Data are mean±SD (n=12). *P<0.0001 vs. 5 min, †P<0.0001 vs. 5 min; **P<0.0001 vs. 15 min; #P<0.0001 vs. corresponding time in young.

**Figure 3.** Fusion of ephrin A1/EphA2 vesicles with early and late endosomes is altered in old hCPCs. A and B, Time-dependent changes in the sub-cellular distribution of internalized ephrin A1 in young and old hCPCs stimulated with ephrin A1. After 5 min, ephrin A1 (red) co-localizes with EEA1 (green) and LAMP1 (blue). At 15 and 25 min, the association of ephrin A1 with EEA1 is apparent in young hCPCs and much less in old hCPCs. The two rectangles define the areas illustrated at higher magnification in panel B. Green arrows in young hCPCs point to the co-localization of ephrin A1 with EEA1. Light blue arrows in old hCPCs indicate ephrin A1 association with LAMP1. These vesicles are not labeled with EEA1. C, Quantitative analysis of EphA2 co-localization with EEA1 in young and old hCPCs treated with ephrin A1. Data are mean±SD (n=6). *P<0.0001 vs. 5 min, †P<0.0001 vs. 5 min; **P<0.0001 vs. 15 min; #P<0.0001 vs. corresponding time in young. D, Time-dependent changes in the association of EphA2 (blue) with EEA1 (green) and LAMP1 (red) in young and old hCPCs after treatment with ephrin A1. Rectangles define areas illustrated at higher magnification in the insets.
**Figure 4.** EphA2 binding to downstream effectors in endosomes is inhibited in old hCPCs. A, Co-localization of caveolin-1 (red), EEA1 (green) and EphA2 (blue) in the cytosol of young hCPCs exposed to ephrin A1 for 25 min. This co-localization 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, 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 Tyr14-caveolin-1 (red), Tyr416-SFK (green) and EphA2 (blue) in endocytic vesicles of young and old hCPCs stimulated with ephrin A1. Rectangles define areas illustrated at higher magnification in the insets. D-G, Young hCPCs pre-treated 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 Tyr14-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.

**Figure 5.** EphA2 and c-Met signaling pathways interact in hCPCs. A, Accumulation of Tyr416-SFK (green), Tyr14-caveolin-1 (red) and EphA2 (blue) in the areas of membrane activity (arrows) formed in young hCPCs in response to HGF. Individual fluorescent signals (upper panels); merge (lower left panel). Rectangles define areas illustrated at higher magnification in the 3 insets. EphA2 co-localization with Tyr416-SFK and Tyr14-caveolin-1 is depicted in insets 1 and 2.
Inset 3 shows the absence of this phenomenon. B, EphA2 (white) endocytosis in young hCPCs treated with HGF (lower left), ephrin A1 (upper right), or both (lower right). Control, untreated hCPCs.

**Figure 6.** Oxidative stress inhibits EphA2 signaling in hCPCs. A, DHE labeling in young and old hCPCs. Arrows point to accumulations of oxidized DHE in the nuclei of old cells. B, Fluorometric measurement of H₂DCFDA intensity. Data are mean±SD (n=6). *P=0.018. C-E, Young hCPCs were pre-treated with H₂O₂ or left untreated (control) for 30 min. Cells were then stimulated with ephrin A1 for 25 min (+) or left unstimulated (-). C, Phospho-tyrosine level of the EphA2 receptor (pTyr-EphA2) by immunoprecipitation and Western blotting (IP: EphA2). O.D. data are normalized to the efficiency of EphA2 immunoprecipitation, expressed relative to control. Data are mean±SD (n=3). *P=0.049. D, Color-coded images of Tyr⁴¹⁶-SFK labeling in ephrin A1-treated hCPCs. E, Ephrin A1-induced EphA2 endocytosis (white) in control and H₂O₂-treated hCPCs. F, Transwell migration assay of young hCPCs (young), H₂O₂-exposed young hCPCs (young-H₂O₂) and untreated old hCPCs (old). The number of spontaneously migrated cells (-) and in response to HGF (+) are shown. Data are mean±SD (n=4). *P=0.004 vs. (-) young hCPCs. G, Two-dimensional gel electrophoresis of the EphA2 receptor (2D:EphA2) in untreated young hCPCs, young hCPCs exposed to H₂O₂ (young-H₂O₂), and untreated old hCPCs; pI values are indicated. The overlay histogram shows the line scan of the O.D. distribution for each condition. H, Co-localization of caveolin-1 (red) and EphA2 (green) in the cytosol of old hCPCs exposed to ephrin A1. This co-localization is markedly increased in cells pre-treated with NAC (right). Rectangles define areas illustrated at higher magnification in the insets. Control, no NAC. I, Oxidized DHE (red, arrows) is detected in the nuclei of old hCPCs in
the absence of PEG-catalase (left: control). Treatment with PEG-catalase (right) attenuated oxidative stress in old hCPCs. J, Ephrin A1 (red) co-localizes with EEA1 (green) in old hCPCs treated with PEG-catalase (right). LAMP1 (blue). Rectangles define the areas illustrated at higher magnification in the insets. Control, no PEG-catalase.

Figure 7. Functional EphA2 receptor potentiates ephrin A1 signaling in old hCPCs. A, Old hCPCs were infected with a lentivirus (LV) carrying EphA2-EGFP or EGFP only. Western blotting: EphA2-EGFP fusion protein (eEphA2) has a higher molecular weight (green arrow) than endogenous EphA2 (red arrow). Molecular weight is indicated in kDa. B, Flow-cytometry: EphA2 (red), eEphA2 (green), and EGFP in old hCPCs. Secondary IgG, control (grey). C, In the absence of ephrin A1, exogenous EphA2 (orange: native EGFP fluorescence and immunolabeled red) is highly expressed and is localized on the membrane of old hCPCs. Individual fluorescent signals are illustrated in the lower panels. D, Immunoprecipitation of EphA2 protein (IP: EphA2) and immunoblotting of phospho-tyrosine (IB: pTyr) in LV EphA2-EGFP old hCPCs in the presence (+) or absence (-) of ephrin A1. O.D. data were normalized to the efficiency of eEphA2 and EphA2 immunoprecipitation relative to unstimulated cells (-). Data are mean±SD (n=4). *P=0.035. Exogenous EphA2 (eEphA2, green arrows) has a higher molecular weight than endogenous EphA2 (EphA2, red arrows). E, EphA2 endocytosis after 15 min stimulation with ephrin A1 of LV EGFP and LV EphA2-EGFP old hCPCs. The number of EphA2-containing vesicles is higher in LV EphA2-EGFP hCPCs. F, Exogenous EphA2 (native EGFP fluorescence and immunolabeled red) co-localizes with caveolin-1 (blue) in old LV EphA2-EGFP hCPCs stimulated for 15 min with ephrin A1. This is apparent in area 1 (arrows) shown at higher magnification in the adjacent panel. Endogenous EphA2 (immunolabeled red) failed to co-
localize with caveolin-1 (see area 2 at low and high magnification). G and H, In old LV EphA2-EGFP hCPCs, exogenous EphA2 (orange: native EGFP fluorescence and immunolabeled red) co-localizes with EEA1 (G, blue; green arrow in inset 1) but not with LAMP1 (H, blue; green arrows in the insets) after stimulation with ephrin A1 for 15 min. Endogenous EphA2 (immunolabeled red) does not co-localize with EEA1 (G, blue arrow in inset 2) but co-localizes with LAMP1 (H; blue arrows in the insets). I, Time-lapse images of chemotaxis of old LV EphA2-EGFP and LV EGFP hCPCs in response to HGF. See accompanying Video 3 in the online-only Data Supplement. J, Fraction of migrating cells. Data are mean±SD (n=4). *P=0.002.

**Figure 8.** Adhesion to ephrin A1 enriches the pool of young hCPCs. A, Schematic representation of the sorting steps for the separation of hCPCs with functional (green) and dysfunctional (brown) EphA2 receptors. The heterogeneous cell pool is exposed to ephrin A1-coated surfaces. The non-adherent fraction contains primarily old cells. The adherent fraction is enriched with young hCPCs. B, Adhesion assay to ephrin A1 or to control human IgG-coated plates. The efficiency of adhesion in young and old hCPCs is expressed relative to the input cell number. Data are mean±SD (n=4). *P<0.0001 vs. control young, **P<0.0001 vs. young hCPCs that adhered to ephrin A1. C, Expression of p16\(^{INK4a}\) (upper panels, green) and γH2A.X (lower panels, green) in hCPCs separated according to the adhesion assay, i.e., unsorted control, and adherent and non-adherent hCPCs. D, Percent of hCPCs positive for p16\(^{INK4a}\) and γH2A.X. Data are mean±SD (n=5). *P=0.0023 vs. control, **P<0.0001 vs. adherent, †P=0.0048 vs. control, #P<0.0001 vs. adherent.
Figure 1
Figure 1 (cont.)
Figure 2
Figure 2 (cont.)
Figure 3
Figure 4
**Figure 4 (cont.)**

**D**

Control

Tyr<sup>14</sup>-caveolin-1

SFKi

**Intensity (A.U.)**

20 μm

**E**

Control

ephrin A1

caveolin-1

SFKi

**Intensity (A.U.)**

5 μm

**F**

Control

SFKi

EphA2

**10 μm**

**G**

Intracellular ephrin A1

A.U.

Control

SFKi

*
Figure 5
Figure 6
Figure 6 (cont.)
Figure 7
Figure 8
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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**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.$^{1-6}$ 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

3
(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). hCPCs were combined with L5178Y-R mouse lymphoma cells of known telomere length (48 kb). 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 H\(_2\)O\(_2\) 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.\(^1\) 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 Fc\textsubscript{γ} was pre-clustered for 5 min with 1.5 μg/mL FITC-conjugated anti-human Fc\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 pH7.4 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 µg/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 μ-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 µg/cm$^2$ of recombinant ephrin A1-Fcγ (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\textsuperscript{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 → Old
P4-P7 → P11-P15

Stress-induced senescence

doxorubicin (0.5 μM)
wash

Young → Old
P4-P7 18 hrs 36-72 hrs

B. Relative cell count

Telomere probe
control
Young Old

C. Replicative senescence

Replicative senescence
Young Old
kb

D. Stress-induced senescence

Stress-induced senescence
Young Old
kb

E. c-kit/EphA2

Young Old

20 μm

20 μm
Figure 2
Figure 3

A

<table>
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<tr>
<td>Caveolin-1</td>
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B

Young

Old

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Figure 3
Figure 3 (cont.)