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The role of endothelial progenitor and cardiac stem cells in the cardiovascular adaptations to age and exercise

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1. ABSTRACT

Age is a major risk factor for cardiovascular disease. Many hypotheses have been proposed to explain the ageing process. Ageing of tissue-specific as well as circulating stem and progenitor cell compartments can be viewed to be central to the decline of tissue and organ integrity and function in the elderly. Related to the cardiovascular system, circulating endothelial progenitor cells (EPCs) contribute to the endothelial integrity and function, and initiate adult neovascularization, while resident cardiac stem cells (CSCs) have the potential to differentiate into cardiomyocytes, endothelial or smooth muscle cells in the heart. Reduction in number and functional capacity of EPCs and CSCs might play a role in age-related vascular and cardiac dysfunction, leading to increased cardiovascular risk. In this review, we discuss the impact of ageing on EPCs and CSCs and their possible contribution to age-related cardiovascular adaptations. Regular aerobic physical activity has a strong cardioprotective effect, while physical inactivity is a central part of the aging process. Therefore, we also outline the immediate and long-term effects of physical activity on EPCs and CSCs.

2. INTRODUCTION

Human physiological aging is associated with a progressive failing of the body's tissues and organs, leading to an increased prevalence of various chronic diseases. As the population of older people is rapidly increasing, especially in the Western world, better insight into the mechanisms that cause these age-related changes is of utmost importance.

In the aged population, cardiovascular diseases represent the major cause of morbidity and mortality. Although sedentary life, lipid levels, diabetes, smoking and genetic factors are established risk factors for coronary artery disease, hypertension, heart failure and stroke (the quintessential cardiovascular diseases within western societies), advancing age unequivocally confers the highest risk (1). Patients who are 75 years of age or older account for approximately 30% of all acute coronary syndromes and 60% of myocardial infarction (MI)–related deaths in the United States and Europe (2).

Increasing evidence documents the central role of the endothelium, the inner-most lining of the vessel wall, in

the regulation of cardiovascular function and dysfunction (3). Consequently, endothelial dysfunction has been identified in cardiovascular diseases such as atherosclerosis, hypertension and coronary artery disease, as well as playing an important role in the alterations to cardiovascular physiology, which accompanies human aging (4-6). In these diseases, the critical balance between endothelial injury and endothelial recovery is disturbed. While mature endothelial cells possess limited or no regenerative capacity, circulating endothelial progenitor cells (EPCs) have been identified (7) and are suggested to contribute to the maintenance of endothelial integrity and function through replacement of damaged endothelial cells. These cells are also suggested to initiate endogenous neovascularization of ischemic tissues.

Until very recently the accepted paradigm was that the mammalian heart is a post-mitotic organ, without regenerative capacity and with a relatively constant but diminishing number of myocytes from shortly after birth to adulthood and senescence. Over the past few years this concept has started to evolve. It is unequivocal that the adult mammalian heart is composed mainly of terminally differentiated contractile cells. However, in the adult heart, there are small number of myocytes undergoing mitosis and cytokinesis (8, 9). These small cycling myocytes are immature and not yet irreversibly withdrawn from the cell cycle and were elegantly interpreted as the cellular progeny of an unknown resident stem/progenitor cell population (10, 11). Adult cardiac stem cells (CSCs) were then first described in 2003 (12). These cells have a robust potential to differentiate into cardiomyocytes, endothelial or smooth muscle cells, and by replacing cells lost to wear and tear, they likely contribute to cardiac functional and cellular homeostasis.

Based on the functions of these stem cells, one may suggest that aging of EPCs and CSCs potentially plays a role in age-related vascular and cardiac dysfunction, leading to increased cardiovascular risk. Therefore, in this review, we discuss the characteristics of EPCs and CSCs and their contribution to age-related cardiovascular adaptations. In addition, interest is growing in therapeutic strategies to alter the number and/or function of stem cells. Over the past few years, human *in vivo* studies have examined the potential therapeutic effects of stem cell infusion (13, 14) or pharmacological interventions (e.g. statins (15)). In particular, injection of bone marrow-derived cells has been used in several randomized clinical trials on patients with MI (13, 14). While Janssens *et al.* (16) and Lunde *et al.* (17) found no differences in ventricular function between control and treated groups, Schachinger *et al.* (18) found small, statistically significant improvements of ventricular function in the treated groups. Interestingly, a recent meta-analysis of all the clinical trials demonstrated an overall small benefit from the infusion of bone marrow-derived cells in patients with MI (19). These findings leave open future avenues for the potential therapeutic benefit of this cell resource. In healthy subjects, including older individuals, and in those with cardiovascular disease, regular physical activity is associated with an improved cardiovascular function (20-22). As EPCs and CSCs may contribute to the underlying mechanism for this cardioprotective effect, we also examine the immediate and long-term effects of physical activity on these cell types.

3. CHARACTERIZATION OF EPCS

3.1. Synthesis, mobilization and homing

Synthesis. Asahara *et al.* (7) were the first to report the isolation and characterization of hematopoietic stem cells (HSC) from human blood, which possess the ability to differentiate into an endothelial phenotype. These were named EPCs. These cells are thought to originate from a common hemangioblast precursor in the bone marrow, which generates the endothelial and blood cell lineages (23-25). However, others have reported that multipotent adult progenitor cells in the bone marrow (26) and myeloid cells (CD34+) (27, 28) can also differentiate into cells with EPC characteristics. Furthermore, it has been shown that other resident stem cells in different tissues (i.e. heart, skeletal muscle) can also differentiate into endothelial cells (12, 29, 30). In addition, non-bone marrow-derived cells have been shown to replace endothelial cells in grafts (31), while adult bone marrow-derived multipotent progenitor cells differentiate into the endothelial lineage (26, 32). Overall, identification of 'true' EPCs is difficult, mainly because many of the cell surface markers used in their phenotyping are shared by HSC (which share their origin with EPCs), other stem cells (which are closely related to EPCs), and by adult endothelial cells (which EPCs are destined to differentiate into). Thus, EPCs appear to be a heterogeneous group of cells originating from multiple precursors and are present in different stages of endothelial differentiation in peripheral blood.

Under baseline conditions, stem cells are localized in a microenvironment known as the stem cell "niche", where EPCs are maintained in an undifferentiated and quiescent state. The stem cells remain in G_0 phase of the cell cycle and are in contact with bone marrow stromal cells. Although the presence of circulating EPCs is relatively low in basal conditions (7), the number of EPCs can increase several fold after stimulation with endogenous and/or exogenous stimuli. Release of EPCs is regulated by a variety of growth factors, enzymes, ligands, and surface receptors. Physiologically, tissue ischemia is thought to be the predominant signal to induce EPC mobilization. In such conditions, cytokines such as vascular endothelial growth factor (VEGF), angiopoietin-1 (33), stromal cell-derived factor (SDF-1) (34), granulocyte monocyte-colony stimulating factor (GM-CSF), and erythropoietin (35) are released from the ischemic tissue. These molecules hinder the interactions between stem cells and stromal cells of the niche, which induces the release of matrix metallopeptidase-9, which eventually leads to the release of stem cells from the bone marrow into the systemic circulation (36).

Alternatively, a direct effect of increased blood flow may also play a relevant role in the mobilization of EPCs. With an elevation in blood flow, there is a

concomitant increased shear stress on the endothelium. Repetitive increases in blood flow are hypothesized to play a central role in the beneficial vascular adaptations to exercise (37). Due to these elevations in shear stress, endothelial nitric oxide synthase (eNOS) is activated which results in acute production of nitric oxide (NO) and up-regulation of eNOS. Interestingly, eNOS is suggested to be essential for mobilization of bone marrow-derived stem and progenitor cells. Indeed, an exercise bout and VEGF-stimulated EPC mobilization was blunted in eNOS-/- mice (38, 39). This indicates that the NO-pathway, at least partly, contributes to the mobilization of EPCs.

Given the relatively low circulating levels of EPCs, homing of these cells to the ischemic or damaged area is important. It has been suggested the initial step of homing involves adhesion of the circulating EPCs to endothelial cells. EPCs express $beta_2$-integrins, which mediate the adhesion of EPCs to endothelial cells, while the endothelium is activated via ischemia/hypoxia-induced cytokines. SDF-1 contributes to the homing of EPCs through promoting integrin-mediated adhesion (40). After adhesion, the $beta_2$-integrins induce a chemokine-induced trans-endothelial migration (41). Another mechanism that is likely to contribute to the homing of EPCs is chemotaxis through the release of growth factors and chemokines by the ischemic or injured tissue. Subsequently, maturation of the cells to a functional endothelial cell occurs. As the HSC differentiate into the EPC type, they acquire the expression of various endothelial lineage markers, eNOS, von Willebrand factor, E-selectin, and incorporate acetylated low-density lipoprotein cholesterol (7, 42-46). The microenvironment, including contact with surrounding cells, the extracellular matrix, the local milieu as well as the growth factors produced by the homing tissue, play a key role in promoting and regulating stem cell differentiation (47).

3.2. Physiological function

3.2.1. Neovascularisation

Neovascularisation is a complex process induced by hypoxia, resulting in sprouting of new capillaries from pre-existing vascular structures (angiogenesis), proliferation of pre-existing arteriolar connections (arteriogenesis), and *de novo* capillary formation from endothelial cell precursors (vasculogenesis) (48). Infusion of EPCs isolated from the bone marrow in animal models or *ex vivo* cultivation has been shown to result in angiogenesis and vasculogenesis of ischemic tissue in the heart (49, 50) and hind limbs of rats (27, 49, 51). Also initial *in vivo* human pilot trials demonstrated the beneficial effects of autologous implantation of bone marrow mononuclear cells in patients with ischemic limbs (52) or acute myocardial infarction (16-18, 53-55).

When *ex vivo* cultivated bone marrow-derived EPCs are infused in the absence of tissue injury, their incorporation rate is extremely low. In ischemic tissue, however, the incorporation rate of these bone-marrow derived cells has ranged from 0% to 90% (32, 56-58). A reasonable explanation for these large differences are the different models and severity of ischemia tested. In general, a smaller ischemic stimulus leads to a lower percentage of incorporation of EPCs. As previously identified, stem cells release pro-angiogenic factors in an autocrine and paracrine manners that improve their efficiency to induce neovascularization. This is supported by the marked expression of growth factors such as VEGF, hepatocyte growth factor (HGF), granuloyte-colony stimulating factor (G-CSF) and insulin-like growth factor 1 (IGF-1) by EPCs, which in turn may influence the classical process of angiogenesis (59).

EPCs may also have an effect on the conduit and resistance arteries (arteriogenesis). Human cross-sectional studies indicate a positive correlation between conduit artery endothelial function and circulating levels of EPCs (60, 61), which may imply that EPCs contribute to an improved endothelial function. The importance of shear stress in conduit and resistance artery health is well established, most probably through the up-regulation of the NO-pathway in the endothelium. The NO-pathway plays a central role in the proliferation, differentiation, and capillary-like tube formation of EPCs (62). In addition, factors that contribute to the proliferation, differentiation and homing of EPCs (such as VEGF (63) and GM-CSF (48)) are produced after elevations in shear stress on the endothelium. Based on these findings, it has been hypothesized that shear stress-induced arteriogenesis, at least in part, is regulated via EPC-dependent pathways. Future studies should further elucidate the potential role of EPCs during arteriogenesis.

3.2.2. Re-endothelialisation

The mechanism of re-endothelialisation is regarded as an important endogenous repair mechanism in maintaining or improving the integrity and function of the endothelial monolayer. In the past, regeneration of injured or denuded endothelium has been attributed to the migration and proliferation of neighboring endothelial cells. However, recent studies indicate that EPCs may contribute to replacement of denuded or injured arteries. For example, implanted grafts were shown to be rapidly covered by bone marrow-derived cells in a dog model. In humans, circulating EPCs can home to denuded parts of the artery after balloon injury (64). The rapid regeneration of the endothelium may prevent re-stenosis development. This is supported by the finding that enhanced incorporation of bone marrow-derived cells is associated with accelerated re-endothelialisation and reduction of restenosis (64, 65), while the regenerated endothelium was functionally active as shown by the release of NO (66).

The pool of circulating EPCs is regarded as a key repair mechanism of the endothelium. Although speculative, these cells may regenerate the low grade of ongoing endothelial damage induced by various cardiovascular risk factors (review (67)). Restoration of the endothelial layer may prevent thrombotic complications and atherosclerotic lesion development. The latter view is supported by a lower number of EPCs in humans with well-known cardiovascular risk factors such as diabetes, hypercholesterolemia, hypertension and smoking (60). Moreover, factors that reduce the cardiovascular risk, such

as statins and life style changes, are associated with elevated levels of EPCs. It is possible that the balance between stimulatory and inhibitory factors influences EPC levels and subsequently the capacity of the arteries for re-endothelialisation (68).

3.2.3. Cardiomyocyte formation from bone-marrow derived stem cells

Bone marrow stem cell differentiation into cardiomyocytes, both *in vivo* (69-71) and *in vitro* (72-74), has been reported by different groups. Furthermore, blood-derived human adult EPCs can give rise to functionally competent cardiomyocytes *in vitro*, evidenced by biochemical expression of sarcomeric proteins and structures, cardiomyocyte-like morphology and gap junctions, and calcium transient synchronization with adjacent neonatal rat ventricular cardiomyocytes. These results were extended to determine the molecular mechanisms underlying EPC differentiation into the cardiomyocyte lineage (75, 76). Despite these results, controversy surrounds the ability of bone-marrow derived stem cells to give rise to cardiomyocytes (77-79). There has been much debate about the validity of the results as some investigators have failed to reproduce other group's findings. Some investigators suggest that differentiation happens on a small scale, and it is fusion of the donor cells with host cardiomyocytes in the infarcted heart, which is responsible for the identification of differentiated bone-marrow originated cardiac cell types (78-80). At present, and despite reports claiming to have "settled" the issue (81), there is a need for more incisive and better controlled experiments to both, determine the broadness of the differentiation potential of bone marrow cells in general, and EPCs in particular, as well as to identify the conditions needed for the expression of their developmental multi-potentiality.

4. CHARACTERISATION OF CSCS

4.1. Origin and activation

Origin. The first identification of CSCs came from Beltrami *et al.* (12) who showed c-kit positive (c-kit^{pos}) lineage-negative CSCs in the adult rat myocardium that behaved as 'bona fide' stem cells being self-renewing, clonogenic and multipotent. c-kit^{pos} CSCs give rise to a minimum of three different cardiac cell lineages: cardiomyocytes, smooth muscle and endothelial cells. When injected into the rat infarcted myocardium, CSCs exhibited substantial potential to regenerate the lost myocardium with new cardiomyocytes and vascular structures that resulted in restoration of cardiac function (12). Following these ground-breaking findings several different research groups have also identified cardiac stem and progenitor cells in the adult murine and human heart (see (82) for review). With the exception of the Isl-1-positive cells (found infrequently in the adult myocardium), which seem to be remnants from the cardiac primordial (83, 84), the origin of the different adult cardiac stem and progenitor cells identified thus far is undefined. One argument is that at least some CSCs are of extra-cardiac origin (i.e. bone-marrow derived) and have colonized the myocardium in postnatal life. This is supported by the c-kit positive phenotype, documentation of bone-marrow derived cell differentiation into cardiomyocytes and vascular cells, cases of sex-mismatched cardiac and bone transplants (85, 86) and after experimental MI (87). However, there is also evidence suggesting a developmental origin of a common ancestor for the different cardiac stem/progenitor cells identified (84, 88, 89). Indeed, a c-kit^{pos}/Nkx2.5^{pos} bi-potential myogenic precursor in the developing mouse embryo was described, which closely relates to adult c-kit^{pos} CSCs and therefore supports a prenatal origin of CSCs (89).

Recently the potential of CSCs as a therapeutic cellular agent has been doubted and in particular, the specificity of c-kit as a marker of a 'cardiac stem cell' has been questioned (90). The c-kit^{pos} lineage negative (i.e. CD45 negative) CSCs, which were first isolated in our laboratory and then by others (see (82) for review), are small cells with a high nucleus to cytoplasm ratio and when they initiate the differentiation commitment into cardiomyocytes they express Nkx2.5 (12). Thus far, they are the only identified adult CSCs, which have fulfilled all the requirements to be termed a '*bona fide*' stem cell; being clonogenic, self-renewing and multipotent. All of the above characteristics are in direct contrast to the c-kit^{pos} cardiac cells recently described by Menasche and colleagues (90). As correctly inferred by the same authors, these cells are likely to represent cardiac mast cells, being positive for tryptase, and characterized by a high cytoplasm to nucleus ratio. Importantly, these c-kit^{pos} cardiac' mast' cells are CD45 positive, Nkx2.5 negative and have not been analysed for stemness properties either *in vitro* or *in vivo*. Indeed, Pouly *et al.* (90) presented a descriptive histological account of c-kit^{pos} cells in pathological human heart biopsies (most of which were from heart transplant recipients, a condition expected to have a high inflammatory cell infiltration) without biological analysis of their characteristics and properties. These techniques are mandatory in order to prove (or disprove) the authenticity of a putative stem/progenitor cell.

Activation. Similar to EPCs, the number of CSCs in the myocardium is relatively low under basal conditions but significantly increases in response to both pathological and physiological stress (91). Indeed, CSCs become activated and increase in number following acute and chronic MI (87, 92), aortic stenosis (9) and myocardial damage in the presence of a patent coronary circulation (93). Although CSC activation leads to differentiation into new vascular cells and cardiomyocytes, in ischemic heart disease these processes are restricted to the viable myocardium and border regions around the infarct (92). Therefore, this response is not enough to regenerate the lost myocardium and restore cardiac function. However, we have recently shown that diffuse myocardial injury and severe cardiomyocyte loss by isoproterenol, did not affect CSC survival, and there was rapid CSC activation and proliferation which rapidly restored the lost myocyte population. This coincided with restoration of cardiac function (93). Indeed, myocardial stress induced the synthesis and secretion of a multitude of growth factors

which activated the CSCs that have receptors for these growth factors (94).

Mobilization and homing of bone-marrow derived cells to the myocardium has been shown after myocardial infarction. Sca-1^{pos}/CD31^{neg} cardiac progenitor cells showed acute depletion immediately after infarction in the adult mouse heart (87). These were reconstituted to baseline levels within 7 days via self-proliferation and homing of CD45^{pos} bone marrow cells which subsequently underwent a phenotypic conversion and lost the CD45 antigen. Furthermore, an increase in c-kit^{pos} cells from the bone marrow in the infarcted mouse myocardium has also been shown (95). Although these c-kit^{pos} bone marrow cells did not contribute to myogenic differentiation, they established a pro-angiogenic milieu in the infarct border zone by increasing VEGF which in turn initiated angiogenesis and the formation of a myofibroblast-rich repair tissue that improved cardiac function (95). The current mechanism of choice to explain the improvements in cardiac function following stem cell transplantation therapy is a 'cardio-protective paracrine effect'. This hypothesis proposes that the injected cells release growth factors and cytokines, which leads to neovascularization, improved cardiomyocyte survival and proliferation, decreased infarct size, fibrosis and activation of resident CSCs (50, 96-98).

The fact that populations of Sca-1^{pos}/CD31^{neg} (87) and c-kit^{pos} cells (95) are recruited from the bone marrow into the damaged heart has been envisioned as the proof that resident cardiac stem and progenitor cells are not resident in origin (99). Undoubtedly some of the c-kit^{pos} and Sca-1^{pos} cardiac stem/progenitor cells home to the heart during injury, however, it remains to be demonstrated that these phenotypically similar bone-marrow derived cardiac cells have the same 'bona-fide' stem cell properties of the c-kit^{pos} cells isolated from the uninjured adult rat heart (12).

4.2. Physiological function

4.2.1. Angiogenesis

Resident cardiac stem and progenitor cells contribute to new vasculature in myocardial regenerative assays *in vivo* (12, 100, 101) and differentiate into cells which express biochemical markers for endothelial and smooth muscle cells *in vitro* (12, 84, 100, 101). Indeed, when c-kit^{pos} CSCs were injected into the infarcted rat myocardium, they give rise to new vascular structures contributing together with new myocyte formation, to enhanced cardiac function (12). The factors which govern CSC differentiation into the vascular lineage are yet to be defined; however, SDF and VEGF govern bone marrow derived stem cell differentiation after myocardial infarction (102-104). Considering the expression of the VEGF receptor, Flk-1, on some resident cardiac progenitor cells (84, 88, 100, 101), and the potential role played by VEGF as a paracrine chemokine in enhancing local angiogenic function after vascular injury (95, 97, 105), its role in CSC vasculature differentiation should be more closely assessed.

4.2.2. Cardiomyocyte formation from CSCs

The ability of CSCs to form cardiomyocytes has been evident from the first identification of these cells where biochemically positive cardiomyocyte cells with sarcomeric proteins were detected both *in vitro* and *in vivo* (12). Other groups have confirmed these findings for the different identified cardiac stem/progenitor cells located in the heart (83, 84, 88, 89, 100, 101, 106-111). Interestingly, specific factors regulating CSC differentiation into functional beating cardiomyocytes *in vitro* are largely undetermined. However, some studies have shown a myogenic role for oxytocin (107, 109), tricholstatin (109), a cocktail of cardiopoietic growth factors (109, 112, 113) and cell-extrinsic signalling through coupling with neonatal or adult myocytes in a co-culture system (110).

5. AGEING AND CARDIOVASCULAR RISK

The effects of human ageing *per se* are extremely difficult to examine, given the frequent presence of age-related increases in cardiovascular disease and risk factors. Nevertheless, if EPCs and CSCs have the capacity to generate new cardiovascular cells, it remains to be determined why these cells fail to prevent the progression of age-related cardiovascular diseases. It could be that their regenerative potential is impaired by ageing, and therefore, they have an insufficient capacity to repair the damage produced by chronic disease and pathologies, which are in them associated with ageing. Cell senescence is the inability of cells to undergo replication, resulting in irreversible loss of function and degradation of biological components. Senescence is also defined as the fundamental ageing process itself and its development is determined by internal cues as well as environmental influences.

5.1. Effects of age on EPCs

Based on data derived from animal studies, there is evidence that age affects EPC availability and/or function (Figure 1). Studies regarding the number of EPCs in young and older animals demonstrate conflicting results, suggesting no differences between groups (114) or lower levels of circulating EPCs in older animals (115). With respect to EPC function, studies demonstrate more homogeneous results. Rauscher and co-workers (116) suggested a central role for EPCs in age-related vascular impairments, because the function of EPCs in aged rats was impaired. In addition, an elegant study by Eldeberg and co-workers (117) showed age-related attenuation in EPC function in aged mice, in whom neovascularization of cardiac allografts occurred only after transplantation of bone marrow-derived EPCs from young animals. Others have also reported evidence for an age-related attenuation in EPC function. For example, a reduced quantity, migration, proliferation, as well as the number of clones formed by EPCs was reported in aged mice (115), while ageing impaired EPC recruitment and vascular incorporation following ischemic skin flap-injury in mice (114). In the latter study, it was found that the impaired EPC recruitment may relate to defects in the response of the aged tissue to hypoxia rather than intrinsic defects in EPC function (114). Taken together, it is reasonable to suggest that an impaired function of EPCs with ageing coincides with a diminished response and defective signalling of the injured aged tissue, which limits repair and regenerative neovascularization by EPCs.

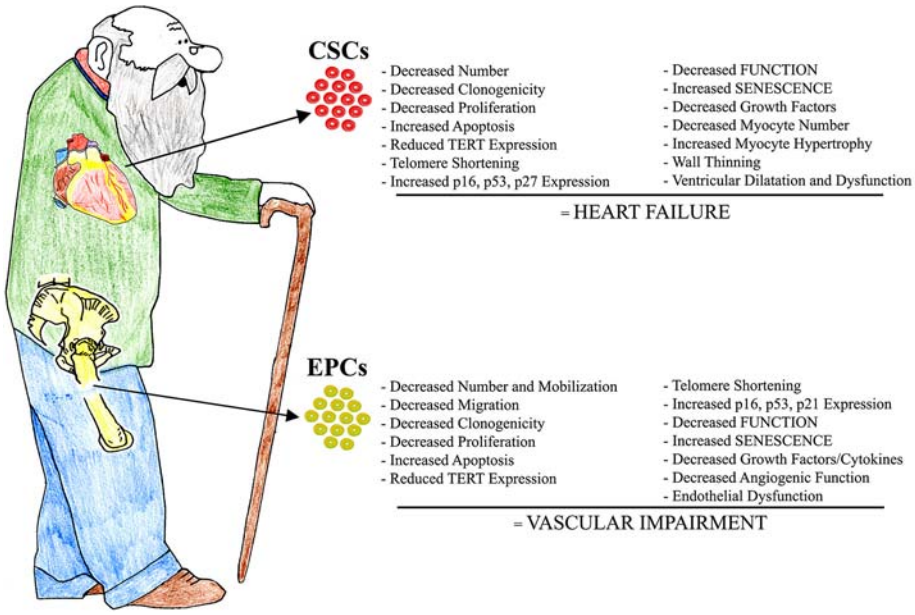

Figure 1. The Effects of Ageing on CSCs and EPCs. The changes in CSC number and function with ageing, promote decreased cardiomyocyte number, cardiac senescence and structural changes contributing to heart failure. Likewise, the changes in EPC number and function with ageing, promote EPC senescence contributing to angiogenic dysfunction and vascular impairments.

Impaired EPC function and its impact on various vascular pathologies have also been examined. Chronic treatment of apolipoprotein E-/- mice with bone marrow-derived progenitor cells from young mice without atherosclerosis attenuated the progression of atherosclerosis of animals maintained on an atherogenic diet (116). This occurred despite underlying hypercholesterolemia, suggesting that progressive depletion or attenuation in function of EPCs with aging may precipitate the development of atherosclerosis. Furthermore, mice deficient in the anti-oxidant enzyme glutathione peroxidise type 1 (GPx-1) gene, exhibited decreased EPC mobilization in response to ischemic injury or VEGF treatment (118). Moreover, when these EPCs were transplanted into wild-type mice they showed impaired angiogenic function (118). Gender may also play a role in regulating EPC function and mobilization. Ovariectomized female mice showed impaired generation and mobilization of EPCs, while estrogen replacement restored bone-marrow and peripheral blood EPC levels (119). In agreement, estradiol treatment significantly increased EPC number, their mitogenic and migration activity while inhibiting their apoptosis, resulting in accelerated re-endothelialisation of injured arterial segments in ovariectomized wild-type mice (120).

Similar to animals, there are conflicting results in the current literature regarding the impact of age on the circulating levels of EPCs in humans. Some groups demonstrated an age-related decrease in the number of these progenitor cells (121, 122). Several others have reported no age related effect on the quantity of circulating EPCs (60, 123-125), despite a strong correlation between EPC number and cholesterol levels (60, 61). Possibly differences in the techniques employed to quantify EPCs or differences in age distribution within a study may partly explain these conflicting results. In addition, one may question the significance of the baseline levels of EPCs in humans. While increased levels of EPCs are suggested to correlate with improved cardiovascular health, higher levels are also to be expected in response to an ischemic stimulus or cardiovascular risk factors that induce vascular damage. On the other hand, lower levels of circulating EPCs, instead of a marker for unhealthy status, may relate to the absence of a significant trigger to induce their mobilization. Therefore, interpretation of baseline circulating levels of EPCs is somewhat difficult if not impossible without thorough information on the physiological and pathological state of the person at the time of the sampling. Possibly, the relative ability of these cells to differentiate and induce neovascularization in ischemic tissue might be more important and informative than the number of circulating EPCs.

Human studies demonstrate more homogenous results regarding the effect of ageing on the functional characteristics of EPCs (Figure 1). The numbers of circulating EPCs in young patients, with stable coronary artery disease increases in direct correlation with age after coronary artery bypass grafting, whereas the opposite occurs in older patients (126). This interesting finding, which suggests the presence of an age-related deficiency in the bypass grafting-induced increase in EPC numbers, was not related to differences in cardiovascular risk factors or cardiac function. Similar findings have been reported in patients undergoing coronary artery bypass grafting (126). These results suggest that advanced age in humans is accompanied by an attenuated capacity to increase numbers of circulating EPCs in response to a serious (pathological) cardiovascular stimulus.

Others have provided evidence for an impaired EPC activity in age-related endothelial dysfunction (123). In a group of 20 young (25 years) and older (61 years) people, endothelial function was examined using brachial artery flow-mediated dilatation and survival rate, migratory activity and proliferation of EPCs was assessed. Impaired survival, migration and proliferation of EPCs were found in the older cohort, but no change in EPC number was detected. Interestingly, endothelial function correlated with EPC migration and proliferation, but not with EPC number (123). Recently, Hoetzer *et al.* (122) examined colony forming units and migratory activity of EPCs. The number of EPC colony-forming units was 75% lower in older men (63 years), compared with their young peers, while migratory activity was also reduced by 50%. Interestingly, recent papers have provided evidence that EPC number and function are impaired in men compared with pre-menopausal women (127, 128), while this difference in EPC function and number disappeared when comparing post-menopausal women with age-matched men (127). Therefore, similar to the experimental animal models, these findings suggest that higher estrogen levels in women positively contribute to the quality of the EPCs. This may partly explain the lower prevalence and incidence of cardiovascular events in middle-aged women.

5.2. Effects of age on CSCs

If cardiac cell homeostasis is dependent on myocyte and vascular cell regeneration from the CSCs, it can be predicted that loss of CSCs, because of death or diminished function, should result in progressive myocyte and microvasculature drop-out and impaired ventricular function. This is exactly what happens in experimental animals and humans (Figure 1). Proliferation of mammalian cells, including human, is dependent on having functional telomeres. Telomere attrition, which occurs with age and because of oxidative stress, is proposed to be a cause of replicative cell aging. Telomere shortening at the second and fifth generation of telomerase knock-out mice was coupled with a profound attenuation in new myocyte formation, increased apoptosis and hypertrophy of the remaining myocytes. These cellular changes were concomitant with ventricular wall thinning, chamber dilatation and dysfunction, which resulted in decompensated eccentric hypertrophy and heart failure (129).

The consequences of natural aging on CSC growth and differentiation were evaluated in 4 and 22 month old mice. Factors implicated in growth arrest and senescence, such as p27kip1, p53, p16INK4a, and p19ARF progressively increased in CSCs, with age. Furthermore, CSC number was decreased and apoptosis increased in the aged mice. At 22 months, these effects resulted in a 33% decrease in myocyte number and cardiac failure (130). Therefore, decreased CSC number and senescence of the remaining CSCs is associated with the development of cardiac dysfunction and failure with age. Interestingly, this 'aging cardiomyopathy' was prevented in mice over-expressing a secreted form of IGF-1 under the control of the myosin heavy chain promoter.

The effect of age and disease state on human CSCs has also been investigated, where aging and ischemia together induce an increase in CSC number, but withdrawal of CSCs from the functional pool (131). Expression of p16INK4a has been shown to be a reliable marker of cell senesce because of its role in cell cycle inhibition through the retinoblastoma (Rb) pathway. Not surprisingly, an increase in p16INK4a positive CSCs (59%) in old diseased human hearts is reported, compared to age-matched controls (17%) (131). Apoptosis and necrosis of CSCs and myocytes were also increased, and these dying cells were p16INK4a positive. Thus, like in the experimental animal models, older humans can develop a 'regenerative-cell deficit' despite an increase in the total number of CSCs, due to accumulation of functionally impaired (senescent) CSCs, which seems to result in a lower myocyte regeneration rate and an increased myocyte death rate. This in turn will contribute to heart dilatation and end-stage heart failure (132). To further, examine these findings, it was shown that total CSC numbers increased significantly in acute (7.5 fold) and chronic (3.5 fold) infarcted human left ventricles, compared to controls. However, this increase in CSC number was not met by improved muscle regeneration but a concomitant increase in the fraction of non-cycling senescent CSCs, as shown by expression of p16INK4a and p53 in CSCs from infarcted hearts (92). From these data, it is evident that the quality of the CSCs is a fundamental parameter affecting the regenerative capacity of the myocardium. It is striking that non-functional CSCs constitute approximately half of the total CSC pool in the population of patients most likely to be candidates for CSC therapy. Thus, the heart develops an "aged" CSC phenotype, which is accelerated and exacerbated by disease. Under these conditions, the quality and extent of the regenerated myocardium will be negatively affected. It is therefore relevant to understand the mechanism(s) responsible for the development of this non-functional state, to define approaches to prevent its progression and to reverse this process.

The mechanisms underlying the age-related change in CSC and EPC number, availability and/or function are unknown. For CSCs, it appears that the development with age of a senescent dysfunctional phenotype plays a key role in contributing to the regenerative-cell deficit, which eventually leads to decreased myocyte numbers and cardiac impairment (Figure 1). Similarly, it is possible that the changes in EPC activity and their diminished mobilization in response to stimuli could be due to defects in the bone marrow stem cell, their niche and/or an age-related loss of differentiation and homing characteristics of the EPCs themselves. Alternatively, primary factors not directly related to these stem cells may also be involved, such as the levels of circulating and/or production of cytokines and chemokines by either the mature endothelial cells or other cell types. In addition, VEGF and NO production have been reported to decrease with age (126, 133, 134), and it is known that these factors play synergistic roles in the mobilization, migration, proliferation, and survival of endothelial cells (38, 135). Moreover, growth factors may contribute to the age-related changes observed in the quality of EPCs and CSCs. Indeed, the age-dependent impairment of EPCs function can be corrected by increased endogenous IGF-1

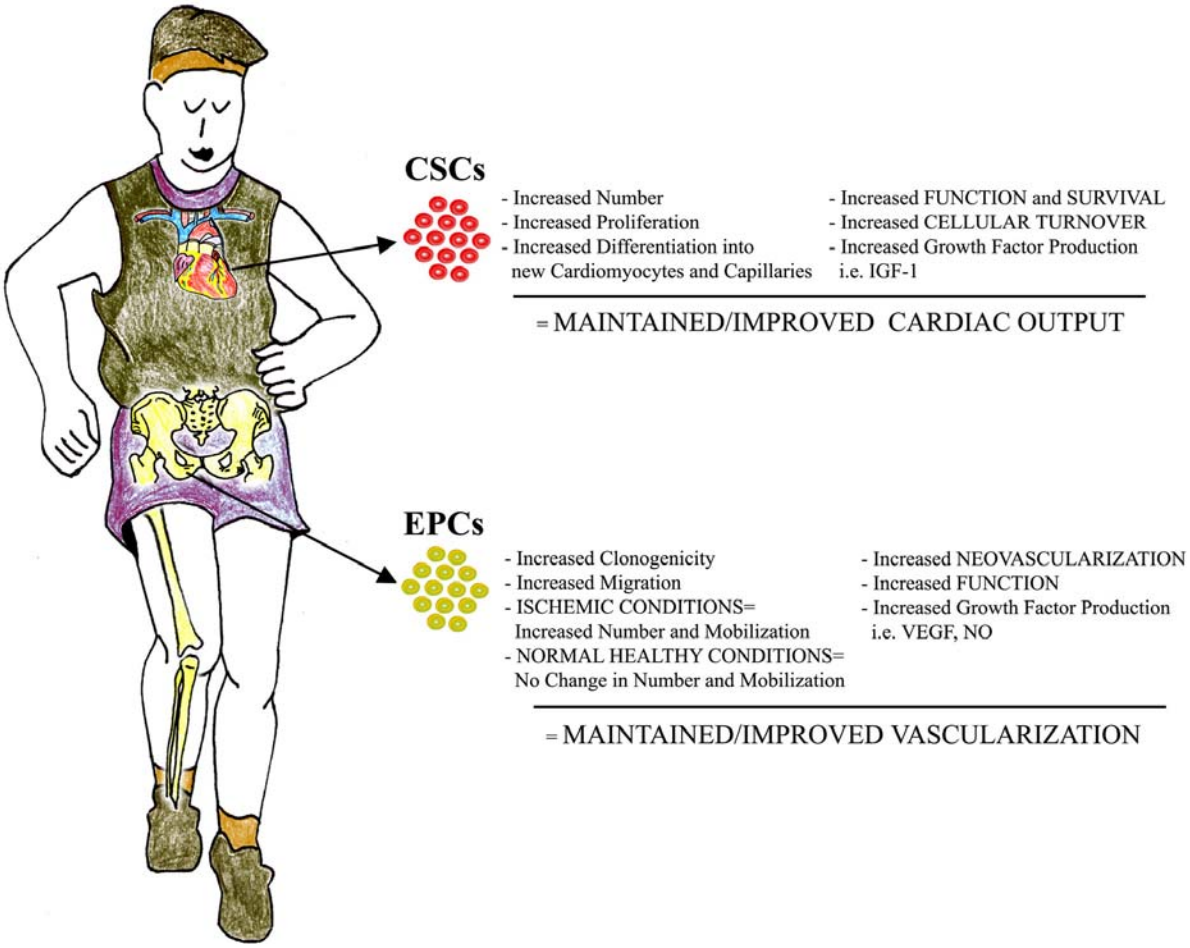

Figure 2. The Effects of Exercise on CSCs and EPCs. Exercise training increases CSC number, proliferation and differentiation into new cardiomyocytes and capillaries, contributing to improved cardiac cellular adaptations. Exercise only elicits a change in EPC number if it is accompanied by ischemic stimuli. In normal healthy ageing no change is found in EPC number with exercise. Exercise training improves EPC clonogenicity and migration, leading to improved function and neovascularization. It is suggested that the effects on CSCs and EPCs with exercise training are mediated by up-regulation of growth factors (i.e. IGF-1, VEGF) and Nitric Oxide (NO).

levels in both humans and mice (136), while IGF-1 over-expression rescued the aging senescent phenotype of CSCs in mice and prevented the progression of ageing associated ventricular impairment (130). In addition, HGF, a potential stimulator of bone marrow-derived cells (137), was demonstrated to be a good marker for progenitor proliferation at rest and after exercise (138). Furthermore, HGF has been shown to play a significant role in local activation of resident CSCs (94).

6. EXERCISE-INDUCED CARDIOVASCULAR ADAPTATIONS DUE TO EPCS AND CSCS

In many age-related chronic disease populations, exercise is considered a fundamental health behavior. Exercise is an effective and widely applied intervention which improves cardiovascular health and is reported to produce a ~30% improvement in certain cardiovascular endpoints (21, 22). On the other hand, physical inactivity is an integral part of the aging process and it is a recognized risk factor for cardiovascular disease. However, the underlying cellular and molecular mechanisms of these effects are unclear. Based on the ability of EPCs to contribute to neovascularization and the repair of endothelial damage, and the ability of CSCs to differentiate into new vasculature and cardiomyocytes, one may hypothesize that these cell types contribute to the beneficial adaptations of exercise-induced cardiac remodeling (Figure 2).

6.1. The effects of exercise on EPCs

6.1.1. Single exercise bout

The first studies to assess the effect of a single exercise bout, examined heterogeneous groups of humans in a field-based environment. Bonsignore and co-workers examined changes in haematopoietic stem cells in healthy volunteers before and after a long-term endurance activity (marathon or half-marathon (139)) or a short-term activity (1000 m all-out rowing, ~14 minutes (140)). While running a marathon did not alter HSC, an increase of these cells was

observed after the short-term activity. In well-controlled laboratory conditions, a significant increase in HSC in healthy individuals is found after a ~15 min incremental maximal cycling test (121, 141, 142). To date, only one study has looked at changes in HSC in older men after an acute exercise bout (121). Interestingly, older subjects exhibited an attenuated response, compared with their younger peers.

An increase in HSC directly after exercise should indicate that EPCs follow a similar course. Laufs *et al.* (142) examined the effect of 30 minutes of exercise at 68 and 82% of the individual maximal aerobic fitness level in healthy young volunteers and found an increase in EPCs after running, which was independent of the intensity. This increase in circulating levels of EPCs was found to be accompanied by elevated levels of VEGF. The increased VEGF levels most likely results from a hypoxic stimulus, a conclusion supported by the increased lactate acid levels, which was suggested to contribute to the EPC mobilization. In marked contrast, we (121) and others (138, 143) could not demonstrate a significant change in circulating EPCs in healthy young and older individuals directly after exercise. All individuals examined in these studies were non-smokers, free of cardiovascular disease, diabetes or hypertension.

Interestingly, in a well-conducted study, Adams *et al.* (143) demonstrated that maximal cycling does not alter EPC number in healthy individuals nor in patients with coronary artery disease, whereas in contrast, EPC levels increase significantly in patients with clinical signs of ischemia during the exercise test. These results might indicate that EPC mobilisation occurs in response to a critical level of hypoxia. Furthermore, the only study that found an exercise-induced increase in EPC numbers in healthy young men (142) used a 30-min protocol at an intensity equal to or above the individual anaerobic threshold level. A 10-min protocol at the individual anaerobic threshold level did not alter EPC levels (142). Taken together, these sparse data suggest that exercise intensity, the presence of ischemia or hypoxia, and characteristics of the subject examined may all influence changes observed in EPC levels after exercise.

6.1.2. Exercise training

Exercise training is hypothesized to alter EPC levels and/or characteristics, based on the ability of these cells to contribute or initiate neovascularization. Indeed, mice that were trained for 4 weeks demonstrated a ~3-fold increase in number of EPCs (39). Interestingly, these authors demonstrated that exercise training in eNOS-knockout mice and wild-type mice treated with NG-nitro-L-arginine methyl ester showed lower EPC numbers at baseline and an attenuated increase of EPC in response to exercise training (39). This latter finding suggests that exercise training in animals increases the number of circulating EPCs via a partially NO-dependent pathway. In humans, exercise training in patients with coronary artery disease and subjects with cardiovascular risk factors also increases the number of circulating EPCs (39, 144). In marked contrast, we recently found no change in the numbers of EPCs after 8 weeks training in healthy older men (121). An important difference between these human *in vivo* studies is the subjects studied. Whereas we examined healthy older men, others examined subjects with vascular abnormalities or even severe coronary artery disease. This raises questions whether exercise training, under normal circumstances leads to a change in levels of EPCs.

Ischemia may also be a crucial factor in the exercise training-induced change in EPC numbers. In a randomized controlled study, Sandri *et al.* (145) showed that patients with peripheral artery disease with symptomatic ischemia (leg pain and elevated blood lactate levels) had an increase in baseline EPC levels after 4 weeks of walking exercise. This finding is in marked contrast to the unaltered EPC levels in patients with peripheral artery disease or coronary artery disease without clinical signs of ischemia (no leg pain, no change in blood lactate levels, no exercise-induced ST-segment depression). These findings support the notion that symptomatic ischemia during exercise influences baseline levels of EPCs.

Although cardiovascular risk factors, clinical disease as well as ageing leads to an impaired EPC function, to date only a few studies have examined the effect of exercise training on the functional characteristics of the EPCs. Sandri *et al.* (145) demonstrated an improved functional capacity of the EPCs across all 3 tested groups (peripheral artery disease with-ischemia; peripheral artery disease without ischemia, coronary artery disease without ischemia,) after walking training, These findings suggest that exercise induced an improvement in EPC function which was independent of the presence of ischemia during exercise. In parallel, Hoetzer *et al.* (122) recently showed that in healthy older men, 3 months of exercise training improved EPC function, evidenced by an improved EPC clonogenic and migratory capacity. Taken together, these studies seem to indicate that exercise exerts its beneficial effects through an improvement in function rather than numbers of EPCs. This may also indicate that changes in EPC function are physiologically more important than corresponding numerical changes**.**

6.2. The effects of exercise on CSCs

The adult mammalian myocardium has a robust intrinsic regenerative capacity through formation of new myocytes from CSCs, which maintain cardiac cellular homeostasis (91). As CSCs were only recently discovered, there is little data available on the role of CSCs in the adaptive response of the heart to increased physiological load, such as exercise. Since the heart was viewed as a post-mitotic organ, the adaptation of the heart to physiological stress was originally thought to be solely due to myocyte hypertrophy. However, increased cardiac workload in humans produced an increase in myocardial mass as a consequence of significant new myocyte formation through the activation, proliferation, and differentiation of the CSCs, as well as myocyte hypertrophy (9). These findings led us to investigate the effects of exercise training on CSC activation and their ensuing differentiation into myocytes. Preliminary data

from our laboratory shows that when mice were made to swim twice daily, 90 min/day, 6 days/week for 6 weeks, they significantly increase myocardial mass due to both myocyte hypertrophy and new myocyte formation. The latter was due to the increased activation, proliferation and differentiation of CSCs, which was in part mediated by changes in myocyte growth factor expression and secretion (namely IGF-1) eliciting activation of CSCs (91, 94). Further, preliminary data from our lab, shows that aerobic running training in rats, controlled according to percentage of the maximal oxygen consumption (VO_{2max}), elicits a myocardial growth factor response which drives CSC activation and ensuing new myocyte and capillary formation. These contribute to the increased myocardial mass and favourable cellular cardiac adaptations (Ellison *et al.* unpublished). Although in its early stages, the exercise training model offers an ideal and easy tool to study the response and role of CSCs in the cardiac adaptation to increased physiological load and stress. The effects of exercise training on CSC function and, more importantly, in attenuating the appearance of the senescent 'aged' CSC phenotype, should also be elucidated. Similar to EPCs, it is hypothesized that the increased production of key growth factors and cytokines in response to exercise training could be fundamental factors in modulating CSC function and fate (130).

7. SUMMARY AND PERSPECTIVES

The alterations in EPC number and properties observed in aging may be caused by a combination of factors. First, the chronic exposure to risk factors and presence of underlying cardiovascular disease may require continuous replacement of damaged endothelial cells and vascular repair processes. This may lead to exhaustion of the pool of EPCs available, exacerbated by accelerated senescence of the remaining cells (38, 60). However, not all studies in the literature support an age-related decrement in EPC numbers. Second, several studies have reported that mobilisation pathways of EPCs (such as VEGF and NO) demonstrate an age-related impaired function, which may importantly contribute to the lower mobilisation of EPCs in response to various stimuli in the aged model (146, 147). Third, based on convincing evidence in animals as well as in humans, an age-related impairment in the quality of EPCs is present (123). The reduced homing, survival, and differentiation of EPCs and attenuated signalling pathways for EPC mobilisation (VEGF and NO) in response to physical stimuli (e.g. exercise) and/or pathological stimuli (e.g. cholesterol, hypertension, pyrogens) may be at the root of the age-related impaired neovascularisation of ischemic tissue and attenuated re-endothelialisation. More important, given the impact of exercise training on EPC numbers and function, physical inactivity likely contributes to the 'age'-related observations in EPCs, possibly through the mechanisms listed above.

CSCs exhibit impaired function with age and pathological disease. This results in a 'regenerative-cell deficit' in the adult heart, with the accumulation of functionally incompetent CSCs leading to attenuated myocardial regeneration and an increased myocardial cell death rate. In time, these events contribute towards heart dilatation, cardiac impairment and eventually end-stage heart failure. Exercise training, which is already part of mass integrated programmes for the treatment of cardiovascular disease, is justified for its application in daily clinical practice, due to its effect on CSC activation which in turn induces favourable left ventricular remodelling. Although data are still in their preliminary stages, it seems that certain growth factors, up-regulated with exercise training, play a pivotal role in governing CSC activation. The effects of exercise training on senescent CSCs should now be determined, in order to assess whether such an intervention could be used to prevent or help to reverse the dysfunctional aged CSC phenotype.

Ascertaining the factors that govern EPC and CSC function and fate is of fundamental scientific and clinical importance and relevance towards the planning and design of optimal protocols and interventions for the prevention and treatment of age-related degenerative diseases (such as cardiovascular diseases, diabetes, obesity and metabolic syndrome) with regenerative medicine therapies. This may eventually improve the quality of life in the rapidly aging population in the Western world. In the future, it should become possible to design a cocktail of growth factors, which could be administered for the activation of these regenerative cells *in situ*. This would prevent the need for autologous stem cell transplantation, which undoubtedly would prove costly, inefficient and therefore inaccessible to most patients that would be in need of, and could benefit from, such treatment. Given the effects of exercise training, this physiological stimulus might be the most useful therapeutic strategy (in symbiosis with pharmacological treatments) towards promoting the activation of these regenerative cells. Future studies are warranted to better understand these mechanisms, but also to identify the role of physical inactivity on CSCs.

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A**bbreviations :**EPCs: endothelial progenitor cells, CSCs: cardiac stem cells, MI: myocardial infarction, HSC: hematopoietic stem cells, VEGF:vascular endothelial growth factor, SDF-1: stromal cell-derived factor-1, GM-

CSF: granulocyte monocyte-colony stimulating factor, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, HGF: hepatocyte growth factor G-CSF: granuloyte-colony stimulating factor, IGF-1: insulin-like growth factor, c-kit^{pos}: c-kit positive, GPx-1: glutathione peroxidise type 1, $VO_{2max:}$ maximal oxygen consumption

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