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## The effect of angiotensin (1-7) on bone marrow stem cells

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## **Chapter 5**

### **Regenerative cell therapy and pharmacotherapeutic intervention in heart failure**

Part I: Cardiovascular progenitor cells,  
their functions and sources

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## **Abstract**

It has been postulated that bone marrow derived endothelial progenitor cells (BM-EPCs) are essential to neovascularization and endothelial repair and are involved in pharmacological treatment, or even potential targets thereof. It is no doubt that the ultimate success of angiogenic cell therapy will be determined by an appropriate stimulation of certain angiogenic progenitor cell subpopulations. Unfortunately, the biology of EPCs is still poorly understood. In particular, the understanding of endogenous microenvironments within the progenitor cell niches is critical, and will provide us information about the signalling systems that supply a basis to develop rational pharmacotherapy to enhance the functional activity of endogenous or transplanted progenitor cells. The final success of clinical improvement of progenitor cell-mediated vascular repair and angiogenic therapy depends on a better understanding of EPCs biology and a smart therapeutic design. In the first part of this review we first shortly discuss the possible involvement of progenitor cells in chronic heart failure (Part I). Thereafter in part II we focus on factors that beneficially affect BM-EPCs, with an emphasis on pharmacological molecular pathways involved in BM-EPC-induced neovascularization.

**The role of progenitor cells in chronic heart failure**

Chronic heart failure (CHF) is the leading cause of morbidity and mortality all over the world. Although conventional pharmacological intervention has significantly improved the prognosis for the patients with CHF, the mortality rate has nevertheless remained high in the last 5 years<sup>1, 2</sup>. Hence the fields of development of novel therapeutic strategies against CHF remain of utter importance. In the past decades, a novel and potentially beneficial approach has raised up; stem cell therapy. Although stem cell-based therapy may provide a promising approach to salvage the damaged heart in most animal experiments, transplantation of autologous bone marrow stem cells (BMSCs) for cardiogenesis or neovasculogenesis yielded inconsistent outcomes in clinical trails<sup>3-6</sup>. However, compelling new findings on stem cell niches in the heart, homing signals and telomere dysfunction of endogenous stem/progenitor cells<sup>7-9</sup> have convinced us to sustain the research to strategies for regenerative therapy in CHF.

Current pharmacological therapy in patients with myocardial infarction (MI) is focused on the prevention of ventricular remodeling rather than regeneration. In contrast, stem cell therapy after MI actually aims at repair, and one of its repair strategies, namely that of angiogenesis and/or neovascularization, is based on the model of “relative ischemia”. In this model, the increased load on the surviving myocardium results in cardiomyocyte hypertrophy. However, the insufficient formation of new capillaries leads to reduces capillary density, in turn leading to so-called “relative” myocardial ischemia<sup>10</sup>. The relationship between cardiac angiogenesis, cardiac hypertrophy and cardiac function highlights the importance of neovascularization<sup>11, 12</sup>. Thus, treatment focusing on neovascularization through stem cell grafting could be a promising strategy in CHF.

The previous identification of endothelial progenitor cells (EPCs), extracted from human peripheral blood, in 1997 by Asahara et al.<sup>13</sup> and subsequent studies have led to a novel paradigm in the field of vascular biology, namely postnatal vasculogenesis<sup>14, 15</sup>. Numerous studies on EPCs have confirmed the angiogenic potential of this versatile cell. Therefore, stimulation of neovascularization by EPC, either through direct injection of these cells or through pharmacological intervention on these cells, is an attractive therapeutic target. However, one of the problems of research in this field is that the identity of actual EPCs is still controversial. Different studies use different definitions and consequently different subsets of cells. This may have contributed to the ambiguous outcomes of BM-EPCs therapy in the ongoing clinical trials<sup>4, 5</sup>. Moreover, this complicates the search for suitable pharmacological targets. Thus, it is essential to discuss the definition of EPC's.

**Redefining endothelial progenitor cells**

Bone marrow (BM) has been considered as the major reservoir of EPC. An EPC is not a cell with invariant phenotype but one with preserved full plasticity, which may transdifferentiate into other cell types under diverse microenvironments<sup>16, 17</sup>, in which cell-cell interaction has been postulated to play a vital role<sup>18</sup>. Indeed, the difficulty of defining what is an EPC exists

## CHAPTER 5-PART I:

due to the multiple origins and whereabouts of EPC, implicating the existence of multiple identifiers for this cell type.

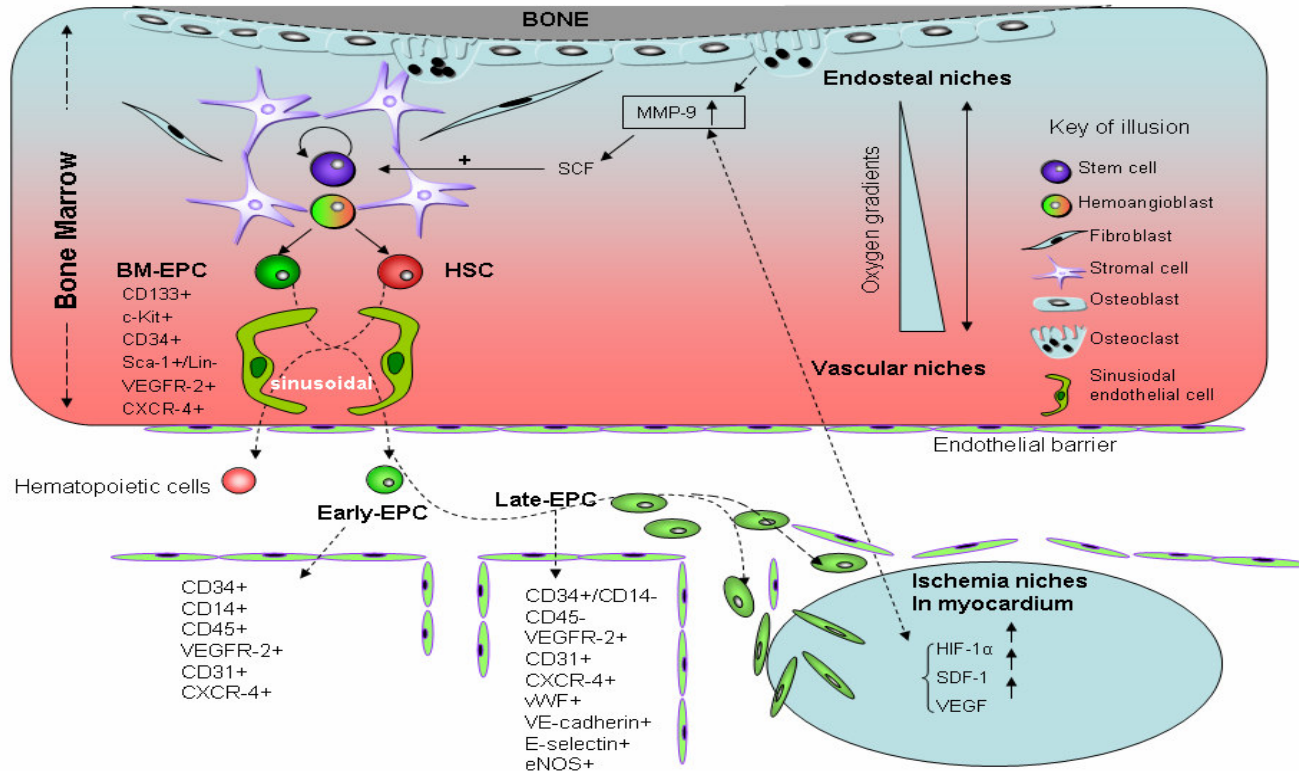
In earlier studies relatively simple markers to identify EPC were used, amongst which Dil-labeled acetyl low-density lipoprotein (Dil-Ac-LDL) and lectin (either Ulex or BSI) double-positive, cobblestone shaped cells. Being less costly, this method provides a means to evaluate EPC levels in animal species for which suitable antibodies do not exist, or in large scale studies. The method is not a very precise one to define EPC, as it includes also mature endothelial cells, monocytes and macrophages.

More specific EPC markers are summarized in Table 1. Importantly, BM-EPC has been showed to share origin and markers with HSC<sup>19</sup>. Kiel et al. recently reported the a method to distinguish HSC from progenitors<sup>20</sup>, namely by staining of the “SLAM “ membrane receptors (CD150,CD244,CD48) that or not present on EPC<sup>21,22</sup>. Furthermore, it has become clear that at least three stages of EPC exist during their specific journey of maturation into the endothelial-lineage, namely BM-EPC, early and late circulating EPC (Figure 1). Late EPC, characterized by CD34+/CD45-, is different from early EPC in secreted growth factors and possesses outgrowth capability.<sup>23,24</sup> In contrast, early circulating EPC is a myeloid derived endothelial-like cell with a limited vascular tube formation capacity on Matrigel<sup>25</sup>.

**Table 1.** Markers used to distinguish EPCs

Markers	BM-EPCs	Circulating EPCs (early)	Circulating EPCs (late)
CD133	++	-	-
CD117	++	-	-
Sca-1	++	+	+
CD34	++	+	+
CXCR-4	+	+	+
VEGFR-2	+	+	+
CD31	+	+	+
VE-cadherin	-	+	+
vWF	-	+	+
E-selectin	-	+	+
eNOS	-	+	+
Dil-Ac-LDL	+	+	+
Lectin	+	+	+
CD105	-	+	+
CD45	-	+	-
CD14	-	+	-

Figure 1.



**Figure 1: EPC Mobilization and Interactions between bone marrow niches and ischemic niche in the heart**

In peripheral organs, ischemic injury provokes increases of angiogenic factors, such as HIF-1 $\alpha$ , SDF-1 and VEGF, which in turn causes activation of matrix metalloproteinase-9 (MMP-9) and increase the cleavage of soluble kit ligand (sKitL, such as stem cell factor), and thus initiate recruitment and mobilization of bone marrow endothelial progenitor cells (BM-EPCs) from bone marrow niches into the peripheral circulation. The initiation is followed by shedding of c-Kit+/VEGFR2+/CXCR-4+/Sca-1+ progenitors from endosteal or bone marrow stromal niches to the vascular sinusoidal of the bone marrow and their subsequent transmembrane movement compel BM-EPCs to differentiate into circulating EPCs. In bone marrow, after receiving peripheral signals, stem cells divide into a mother cell (self-renewal: semi-circular arrow) and a daughter cell (in this figure a hemangioblast) by asymmetric division. BM-EPCs are of hematopoietic origin and possibly derived from these hemangioblasts, which is characterized by c-Kit+/Sca-1+/Lin-/ CD133+/CD34+/VEGFR-2+/CXCR-4+, and capable to give rise to late endothelial outgrowth. The early circulating EPC is characterized by CD34+/CD45+/CD14+; whereas, the late circulating EPC is negative for CD45 and CD14.

More recently, studies from Ingram's group redefined the concept of EPC<sup>26</sup>, using angiogenic potency as a determinant. They distinguish endothelial cell colony-forming units (CFU-ECs), descendants of myeloid cells, from endothelial colony-forming cells (ECFCs), which derive from mononuclear cells, and concluded that ECFCs are the "actual EPCs" able to form perfused vessels *in vivo*<sup>26</sup>. This finding underscores that the cell subtype should be considered cautiously in future cell therapy studies and studies that aim at identification of pharmacological target molecules.

Besides the identity of the transplanted EPC, the fate of progenitor cells with the resident microenvironments, so called "niches" that are also involved in the different phases of progenitor cell recruitment. From studies on these niches, signal molecules and transduction pathways involved in EPC function can be identified that may provide targets for pharmacotherapeutic strategies. Therefore, we here discuss the sequence of events in progenitor cell recruitment and subsequently we will summarize the involved niches.

### **Phases in EPC-induced neovascularogenesis and pharmacological intervention**

For optimal development and application of pharmacological and/or molecular therapy on progenitor cells, a comprehensive understanding of the basic processes of blood vessel development and repair is essential. The vascular system mainly originates from 2 fundamental processes: vasculogenesis and angiogenesis<sup>27</sup>. Vasculogenesis is defined as differentiation of EPC and formation of a new, primitive vascular network and is believed to occur both in embryonic and postnatal stages<sup>28</sup>. In the case of angiogenesis in adult tissues, EPCs are mobilized to a local niche (e.g. the infarcted heart) where they settle to form the primary vascular plexus and to release paracrine, proangiogenic factors<sup>29</sup>. This stimulates endothelial proliferation, sprouting or splitting (non-sprouting) of preexisting blood vessels, a process termed angiogenesis<sup>30, 31</sup>. In summary, cardiac neovascularization can be divided into five phases, involving various niches:

- I. Endothelial permeability and mobilization of EPC from bone marrow niches to circulating blood.
- II. Homing to the heart.
- III. Basement membrane degradation in coronary vessels and proteolysis to allow migration into the cardiac niches or ischemic niches.
- IV. EPC/EC proliferation and primary vascular plexus formation.
- V. Vessel formation and stabilization.

Many signalling factors play a crucial role in the different stages of EPC-induced neovascularization, and most of them are involved in more than one of these phases (see Part II of this review). Both stimulatory as well as inhibitory signals are involved in guiding the processes, and can be found within the various niches. We will now summarize the niches where progenitor cells are found.

### **Stem/progenitor cell niches in the bone marrow and the heart**

The concept of stem cell niches was firstly proposed by Schofield in 1978<sup>32</sup>. In fact, a stem cell niche is not an undefined place in which the stem/progenitor cells simply dwell, but a three-dimensionally structured entity where cross-talk occurs between stem cells, and where supporting cells guide or direct both stem cell self-renewal and progenitor cell differentiation after receiving extrinsic signals from the circulatory system<sup>33</sup>. Stem cell niches have been identified in the bone marrow as well as in peripheral tissues, amongst which the myocardium. The stem cell niches in the BM have been intensively investigated. So far, two BM niches have been identified<sup>20, 34</sup>.

#### *Bone marrow niches*

Hemangioblasts are the major source for generation of hematopoietic stem/progenitor cells (HSCs/HPCs) and EPC in the bone marrow. The microenvironment in this BM source is divided into two compartments; the vascular zone and the endosteum zone. The vascular zone is formed of niches that contain endothelial cells and pericytes together with supporting cells, such as bone marrow stromal cells and fibroblasts. The endosteum zone consists of niches with osteoblasts/osteoclasts and their supporting cells<sup>34</sup>. The two niches together decide about mobilization of the otherwise quiescent stem cells (Figure 1). In this mobilization process the oxygen concentration gradient between the osteoblastic niche and the vascular niche is the switch that turns on the differentiation of hemangioblasts into EPCs or the recruitment and differentiation of vascular progenitor cells. This recruitment involves important signalling mechanism, as delineated Part II of this review.

Another contributor to progenitor recruitment is the osteoclast (Figure 1). Bone-resorbing osteoclasts play a critical role in the recruitment of vascular progenitor cells, as further specified in Part II as well. Therefore, in bone marrow niches stem cell recruitment is regulated by the balance between vascular zone and endosteal zone. Pharmacological stimulation of the niches in these zones may enhance stem cell recruitment after myocardial infarction. Apart from these BM niches, two cardiac stem cell niches have been identified, respectively involving resident cardiac stem cells and BM-derived cells.

#### *Resident cardiac stem cell niches*

The previous notion that the heart is a terminally differentiated organ without self-renewal potential after birth has been challenged by the successful isolation of cardiac stem cells (CSCs) from adult cardiac tissue<sup>35, 36</sup>. From a therapeutic standpoint, the c-kit<sup>+</sup> stem cells could bipotentially differentiate into both EPC and cardiac progenitor cell (CPC), a progeny of CSC<sup>37, 38</sup>, which may have more advantages for cell therapy in CHF when compared to the more restricted EPCs. From the stem cell biology point of view, the identification of c-kit<sup>+</sup> CSCs in cardiac tissue has driven researchers to seek CSC niches in the heart. Until recently, CSC niches in adult mouse heart have been predominantly addressed by Anversa and co-workers<sup>7</sup>. Urbanek et al. reported that CSCs niches consist of Lineage negative, c-kit-positive



and stem cell antigen-1 positive (Lin<sup>-</sup>/c-kit<sup>+</sup>/Sca-1<sup>+</sup>) CSC and CPC and extracellular matrix (ECM) components, including fibronectin and a subtype of laminin. Myocytes and fibroblasts serve as supporting cells for CSCs<sup>7</sup>, whereas endothelial and smooth muscle cells are intimately connected with CPCs.<sup>7</sup> This suggests that cell-specific interactions, and, hence, specific signalling mechanisms, guide either CSC or CPC. The importance of local CSC in cardiac maintenance and regeneration has been elegantly studied by Hsieh and colleagues<sup>39</sup>. Their fate mapping study provides the first evidence that resident CSC are partly responsible for cardiomyocyte turnover after ischemic injury, but not during normal aging.

Although the CSC niche may prove a possibly important regenerative tissue, the pharmacological targets to stimulate this source are far from unraveled. Far better characterized, in this respect, are BM-derived progenitor cells that home to the ischemic myocardium: the post-infarction myocardial ischemic niche.

#### *BM stem cells recruitment in cardiac ischemic niches*

Endogenous stimuli such as tissue ischemia have been demonstrated to promote mobilization of EPCs from the BM to peripheral ischemic organs<sup>40</sup>. The circulating number of EPC is elevated after MI, and further homing to ischemic niches. This ischemic niche is crucial for local cardiac neovascularisation, as will be more elaborately discussed below.

The pre-existent coronary collateral vessels support the vascular niche in the infarcted heart, possibly by increased expression of bFGF, and of SDF-1 from engrafted BM-EPC, leading to improved BM-EPC implantation<sup>41</sup>. It has been verified that the efficiency of BM-EPCs transplantation depends on the hosting ischemic myocardium<sup>42</sup>. Transplantation of BM-EPC gives rise to a transient angiogenic effects as it disappeared with transplanted cells fading out, whereas the sustained therapeutic effects are raised and attributed to the endogenous BM cells homing to ischemic myocardium after BM-EPC transplantation, although definite evidence has not been presented yet<sup>42</sup>. Therefore, the local ischemic niches in the heart play a vital role in cell-based therapy for CHF. Promotion of a favourable microenvironment in ischemic myocardium is another potential target for pharmacotherapy.

Pharmacotherapy to improve regenerative therapy can be based on the various signalling factors that can be found in the stem and progenitor cell niches. These signalling factors and beneficial pharmacotherapy will be summarized in Part II of this review.

(I will be back!)

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## **Chapter 5**

### **Regenerative cell therapy and pharmacotherapeutic intervention in heart failure**

Part II: Pharmacotherapeutic targets, agents  
and intervention perspectives

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Regenerative medicine represents a promising perspective on therapeutic angiogenesis in patients with cardiovascular disease, amongst which heart failure. However, previous or ongoing clinical trials show ambiguous outcomes with respect to the benefit of regenerative therapy by means of bone marrow stem cell infusion in myocardial infarction patients. Therefore, it is necessary to set up a rational therapeutic strategy in the treatment of CHF.

Chemokines, cytokines and growth factors, as well as pharmaceutical agents, may have impact on EPC physiology and thus can provide targets for pharmacological intervention. Indeed, EPCs and stem cell niches both in BM and myocardial tissue can be treated as an integral target for recruitment of EPCs from the BM to the cardiac ischemic niche. In the now following text we individually place the signalling factors in their specified context, and explain their roles in the various phases of neovascularization (see Part I).

### **Pharmacological targets for intervention**

#### *Hypoxia-inducible factor-1*

Hypoxia-inducible factor-1 (HIF-1), a central transcriptional regulator of hypoxia-specific gene expression, induces signalling factor expression such as stromal-derived factor (SDF-1) and vascular endothelial growth factor (VEGF) in EPC or endothelium and facilitates adhesion of progenitor cells to ischemic endothelium<sup>1</sup>. Transcriptional activity of HIF-1 $\alpha$  occurs through dimerization with the constitutively expressed HIF-1 $\beta$  and subsequently binds to enhancer regions termed “hypoxia response elements”. The recruitment of EPC in the ischemic tissues is predominantly regulated by gradient levels of hypoxia<sup>2</sup>. The dependence of progenitor cell recruitment and function on a hypoxic microenvironment suggests that targeting the hypoxic response mediated by the transcription factor HIF-1 $\alpha$  may be a more rational strategy to achieving therapeutic angiogenesis in the patients with CHF. Recently, Rajagopalan and colleagues reported the first clinical results of HIF-1 $\alpha$  gene therapy predicated on transcriptional activation of a patient’s own gene. The high dose of HIF-1 $\alpha$  gene therapy improved therapeutic outcomes in critical limb ischemic patients. More importantly, it appears to be safe for clinical application<sup>3</sup>. In fact, HIF-1 $\alpha$  overexpression on EPCs promotes hypoxia-induced EPC differentiation, proliferation and migration<sup>4</sup>, establishing HIF-1 as a putative pharmacological target.

#### *Stromal-derived factor-1 (SDF-1)*

SDF-1 (also known as CXCL12), released under the control of HIF-1, through interaction with its receptor CXCR4 (also known as CD184) modulates angiogenesis as well as hematopoiesis. SDF-1 regulates angiogenesis in part by recruiting EPCs from the BM niches to ischemic tissues<sup>2,5</sup>. Specific stimulation of osteoclasts with receptor activator of NF- $\kappa$ B ligand (RANKL) in BM niches recruited primarily immature progenitor cells to the circulation in a CXCR4 and matrix metalloproteinases (MMP)-9 dependent manner<sup>6</sup>. The release of SDF-1 is modulated by different cytokines, such as soluble kit ligand (sKitL) and VEGF-A<sup>7</sup>. These cytokines were demonstrated to induce the release of SDF-1 from platelets, thereby facilitating the mobilization of CXCR4<sup>+</sup> stem/progenitor cells including EPCs<sup>8</sup>.

Actually, the mobilizing effect initiated by reversing the SDF-1 gradients between the BM niches and peripheral ischemic niches was demonstrated by the direct elevation of plasma SDF-1<sup>7</sup>.

However, Sano et al. recently reported that, although increasing levels of both HIF-1 and SDF-1 were observed in ischemic myocardial tissue, HIF-1 activity was inhibited by accumulation of p53, which paradoxically counterregulates cardiac angiogenesis<sup>9</sup>. Therefore, it is envisioned that solely promoting recruitment of EPCs may be insufficient for cardiac angiogenesis, since there is a disruption of the link between HIF-1 and SDF-1 by accumulation of p53.

Taken together, SDF-1 that increases in the vascular niches in BM is a prerequisite to initiate stem/progenitor cell mobilization. Strikingly, interactions between the chemokine SDF-1 and its receptor-CXCR4, are also of importance in the development and/or homeostasis of heart and blood vessels. Thus, SDF-1/CXCR4 axis both in cardiac and BM niches may be considered as pharmacological targets to improve stem cell mobilization after MI, taking into considering, though, its dependency on cell cycle kinases such as p53.

#### *Stem cell factor*

Stem cell factor (SCF), a soluble kit-Ligand or steel factor, binds to c-kit (also known as CD117), a receptor expressed on the surface of stem/progenitor cells and has chemoattractant effects on these cells. The release of SCF is related to the activity of MMP-9. MMP-9 induced in BM cells such as osteoclasts, initiating the migration of c-kit+ BM-EPCs and HPCs from quiescent endosteal niches to proliferative vascular niches<sup>10</sup>. SCF has a proven pharmacotherapeutic effect; the combination of SCF and Granulocyte-colony-stimulating factor (G-CSF) improved cardiac output and ameliorate arrhythmia in a murine MI model, followed by an enhanced G-CSF receptor expression and arteriogenesis in cardiac tissue<sup>11</sup>. However, a recent study revealed that beneficial effects of G-CSF/SCF on post-MI cardiac function and remodeling are dramatically diminished with age. Interestingly, in the older patients, the amount of circulating CD34+ cells was similar with young patients, suggesting that the reduced efficacy in this population can be attributed to a local defect in cardiac ischemic niches in response to G-CSF/SCF therapy<sup>12</sup>.

#### *Granulocyte-colony-stimulating factor*

Cytokines that promote granulocytes, such as G-CSF and granulocyte macrophage-colony stimulating factor (GM-CSF), also affect EPC mobilization from bone marrow vascular niche to peripheral circulation. Combined administration of G-CSF with bFGF may further increase capillary density in a hind-limb ischemia model, compared with either treatment alone<sup>13</sup>. Furthermore, combined treatments of GM-CSF/SDF-1 were also to increase proangiogenic cells such as angiogenic macrophages and EPCs, which may eventually contribute to neovascularization<sup>14</sup>. Indeed, G-CSF is one of the most important cytokines under intensive



clinical investigations. Abundant evidence showed that administration of G-CSF significantly stimulated stem cell mobilization, and short-period stimulation using G-CSF appeared to be safe and had no adverse effects on restenosis <sup>15</sup>.

Not surprisingly, the effect of G-CSF after MI has been studied. Unexpectedly, administration of G-CSF alone fails to reduce infarct size and improve cardiac function in clinical trial <sup>16</sup>. However, combined administration of GM-CSF and SDF-1 raises achievement in neovascularization <sup>14</sup>, suggesting utilization of GM-CSF requires assistance of additional medicines to augment therapeutic efficiency. As G-CSF is also a pro-inflammatory factor, utilization of G-CSF should be cautiously considered.

#### *Fibroblast growth factors*

Basic fibroblast growth factor (bFGF)-2 is one of the FGF families of heparin-binding growth factors. FGF-2 exerts its pro-angiogenic activity through interactions with various endothelial cell surface receptors, including tyrosine kinase receptors and integrins. Their activity is modulated by a variety of free and extracellular matrix-associated molecules <sup>17</sup>. FGFs, in cross-talk with VEGF and inflammatory cytokines/chemokines, plays a role in the modulation of blood vessel growth in different pathological conditions, including cardiovascular disease <sup>18</sup>. Binding of FGF-2 to its receptor initiates protein kinase C-mediated activation of MAPK pathways. In an in-vitro cell culture study, FGF-2 suppressed long-term culture-induced cellular senescence in human mesenchymal stem cells by reducing mRNA expression of tumor growth factor- $\beta$ 2 and suppression of cyclin-dependent kinases such as p21, p53 <sup>19</sup>. In addition, FGF-2 plays an essential role in estrogen-induced re-endothelialization and EPC mobilization from BM niches <sup>20</sup>. Varying from VEGF, FGF enhances angiogenesis via protein kinase C-mediated activation of MAPK pathways, in an NO-independent manner, thus providing an additional target for pharmacotherapy in cardiac tissue.

#### *Vascular endothelial growth factor and its signalling factor nitric oxide*

The vascular endothelial growth factor (VEGF) was first isolated as a tumorigenic factor responsible for angiogenesis in neoplasms <sup>21</sup>. VEGF production is regulated through HIF-1 in ischemic tissues <sup>1</sup>, and in its turn a regulator of SDF-1, as noted above. Most importantly, VEGF-A, the most potent member of VEGF family, has been identified as one of the major growth factors involved in neovascularisation. VEGF binds to VEGFR1 or VEGFR2 on the endothelial as well as the EPC surface. Most of the angiogenic effects attributed to VEGF are related to VEGFR2. VEGF, through VEGFR2, increases proliferation and mobilization of BM-EPCs and augments corneal neovascularisation in vivo <sup>22</sup>. Cultured human EPCs also express VEGFR1, which play a critical role in prevascular retention, a pre-vascular niche in ischemic tissue. <sup>23, 24</sup>. Interestingly, VEGFR1 can also be stimulated by placental growth factor (PIGF), leading to increased tumor vasculogenesis <sup>25</sup>. It has been suggested that PIGF activates EPC *via* VEGFR-1 and enhances mobilization of VEGFR1+ bone marrow progenitor cells by upregulation of MMP-9 and release of soluble Kit ligand <sup>7</sup>. It is therefore

tempting to speculate that future therapeutic strategies based on PIGF administration may improve neovascularization in CHF patients.

Apart from VEGF/VEGFR axis, VEGF-A may also stimulate platelet-derived growth factor receptors (PDGF) and thus regulate endothelial tip cell and pericyte recruitment to ischemic niches, which plays a critical role on vascular permeability and stabilization<sup>22, 26</sup>.

It is evident that the VEGF family and its receptors are important target for future pharmacotherapy to support EPC-mediated cardiac neovascularization. Downstream signalling pathways of VEGF could also be relevant, and are particularly attractive targets as they might be shared by more angiogenic hormone-receptor systems. One such important VEGF-activated signalling pathway is nitric oxide (NO) release.

Apart from its well-known vasodilatory property, NO has been considered as an important mediator of angiogenesis. NO is a downstream signalling pathway of VEGF-activated endothelial proliferation. Long-term VEGF stimulation induces the increase of endothelial NO synthase (eNOS) expression level, while short-term stimulation promotes subsequent NO production via activation of the mitogen-activated protein kinases (MAPK) cascade, which plays a critical role in angiogenesis<sup>27</sup>. Bone marrow-derived NO is correlated with enhanced EPC mobilization to the circulation post-MI, as well as enhanced adhesiveness via increased integrin expression, which favors the homing capacity of EPC to ischemic myocardial tissue<sup>28</sup>. In eNOS-deficient mice VEGF-induced BM-EPCs mobilization is inhibited, leading to impaired neovascularization in a hind-limb ischemia model<sup>29</sup>. NO is an ubiquitously employed signalling factor, which, apart from VEGF, might also serve other angiogenic growth factors. However, NO is not the only signalling compound at play on these premises. The further search for downstream signalling factors is warranted.

### *Estrogen*

Animal experiments as well as clinical studies show that estrogen has beneficial effects on the cardiovascular system. Important for regenerative therapy, estrogen treatment accelerates BM-EPCs incorporation at the site of reendothelialization in Matrigel assay and stimulates EPC mitogenic and migration activity<sup>30</sup>. Apart from being an attractive hormone to find targets for pharmacological intervention, EPC-mediated vascular repair might play a role in the protective effects of estrogen observed in females<sup>31</sup>.

Estrogen has been shown to reduce Angiotensin II-induced accelerated senescence of EPCs by down-regulation of angiotensin II type I receptor (AT<sub>1</sub>)<sup>32</sup>. Further evidence shows that both estrogen receptor (ER) alpha and beta contributes to estrogen-mediated EPCs activity after myocardial infarction, which is correlated with up-regulation of VEGF in EPC<sup>33</sup>, implying a role for NO yet again. The importance of NO is underscored by observing that estrogen increases vascular production of NO, which in turn improves endothelial progenitor cell function.

In conclusion, several growth factors, signalling factors, and signal transduction pathways are at play when it comes to recruitment of EPC. Table 1 summarizes the most important of these

factors and the niches and phases in which they exert their favorable effects on cardiac neovascularization. It is, however, clear that a large array of pharmacological targets, larger than described herein, is available for the optimization of regenerative therapy based on neovascularization. In fact, some of the medications used in the clinic today, exert their beneficial cardiovascular effects at least partly through this mechanism. We will now review these medications.

### **Potential pharmacotherapeutic agents for improvement of stem cell therapy**

#### *3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors (statins)*

In the last five years, statins have been widely used for prevention of CHF in clinical treatment. With the increasing prescription rate of statins from 38% to 80% of the patients suffering from coronary artery disease, significant reductions of cardiac mortality and hospitalization were observed<sup>34</sup>. One of the earliest observed favorable effects of statins was improvement of endothelial function<sup>35</sup>. Subsequently, it was demonstrated that statins improve the angiogenic capacity of endogenous EPCs in patients with stable coronary disease<sup>36</sup>. The actual mechanism behind this favorable effect may be attributed to increased levels of VEGF and its anti-apoptotic effects on EPC.

Furthermore, statins stimulate EPC proliferation and reduce senescence via cell cycle regulatory genes<sup>37</sup>. The prevention of senescence probably involves up-regulation of the telomere repeat-binding factor TRF2<sup>38</sup>, which protects against damage of telomeres<sup>39</sup>.

Apart from cell cycle effects and anti-senescence effects on EPC, statins reduce C-reactive protein (CRP) levels in patients. In clinical situations, patients with lower CRP after statin treatment have the lowest rate of recurrent events after AMI, regardless of the level of LDL cholesterol<sup>40</sup>. Interestingly, statin treatment did not display its beneficial effects in patients with systolic heart failure (NYHA class III) and failed to reduce CRP level<sup>41</sup>. Therefore, statins might favourably influence EPC function through an anti-inflammatory mechanism. In patients with class III systolic heart failure the lack of a positive outcome of statins treatment may be associated with EPC dysfunction, since EPC function is damaged as heart failure progresses<sup>41</sup>. Taken together, statins have pleiotropic effects on the cardiovascular system that might be mediated by effects on EPC during mild heart failure, involving various signalling pathways that are therefore targets for improvement of regenerative therapy. Because of the versatility of statins in pleiotropic effect it might be a daunting but rewarding task to sort out the most relevant pathways.

#### *Erythropoietin*

Erythropoietin (Epo), a stimulator of hematopoiesis, is shown to stimulate angiogenesis irrespective of its hematopoietic properties<sup>42</sup>. The production of Epo is upregulated by hypoxia and exclusively mediated via HIF-2 $\alpha$ <sup>43</sup>. In BM, Epo acts on the Epo receptor (EpoR) and activates various signal pathways including MAPK and PI-3k/Akt, which probably prevent apoptosis<sup>44</sup>.

Epo has been shown to stimulate angiogenesis or even vasculogenesis by mobilizing EPC from BM<sup>42, 45</sup>. The effects of Epo on EPC biology are both time- and dose-dependent<sup>46</sup>. Chronic Epo treatment in both healthy and CHF patients enhanced functional activity of EPC by improving proliferative potential and adhesive capacity under in-vitro conditions. Nevertheless, the issue that needs to be addressed is that Epo stimulates neovascularization via direct or indirect effects on BMSCs or circulating EPCs. Recent studies demonstrate that Epo/EpoR plays a pivotal role in enhancing the number of BMSCs or circulating EPCs accompanied with elevated serum levels of VEGF<sup>42, 47</sup>. A more recent study from our group has indicated that the cardiac protective effects of Epo positively correlated with the recruitment of BM-EPCs and VEGF levels into the cardiac ischemic niche post-MI<sup>45</sup>. Hence, Epo likely acts on the vascular EpoR system and thereby activates or upregulates VEGF/VEGF receptor system, which may further contribute to recruitment of BM-EPCs to ischemic niches and enhance neovascularization. Another possible parallel with statins is that Epo reduces the inflammatory reaction after stroke<sup>48</sup>. It is unknown whether this contributes to the observed stimulation of EPCs, and needs to be addressed in future studies.

In summary, Epo is a potent factor for EPC-mediated neovascularization and in this sense an attractive pharmacotherapeutic agent to be further evaluated. In spite of the positive effects of Epo on neovascularogenesis, some concerns may arise because of elevated blood viscosity and hypertension induced by Epo<sup>49</sup>. In this regard, the doses and administration frequencies of Epo should be investigated thoroughly.

#### *The role of the Renin-Angiotensin System (RAS) modulation in progenitor cell function*

The renin-angiotensin system (RAS) contributes significantly to the development and progression of vascular disease and CHF, and is one of the best-studied hormone systems in the cardiovascular field. Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II type 1 receptor (AT<sub>1</sub>R) blockers are currently prescribed in the clinic for various cardiovascular indications. The production of bioactive angiotensins starts with the cleavage of angiotensinogen by renin to form the inactive peptide angiotensin I. ACE is responsible for conversion of Ang I to its active metabolite Ang II, which through its action on AT<sub>1</sub>R, increases vascular tone and acts antidiuretic resulting into an increase of blood pressure. Ang-(1-7) is an active metabolite that can be formed from both Ang I or Ang II, involving various enzymes such as ACE, ACE2 and neutral endopeptidase. Ang-(1-7) is considered to be the endogenous counterregulator of Ang II, and displays protective properties<sup>50</sup>. Importantly, local angiotensin expression may influence hematopoietic or mesenchymal stem cell differentiation into an unexpected, unfavourable direction, thus exerting a possible negative effect on the cardiac niches<sup>51</sup>.

#### *Effects of Ang II and Angiotensin-(1-7) on stem cells*

Angiotensin II (Ang II) is the best-studied peptide in the renin-angiotensin system (RAS). The main physiological effects of Ang II are mediated by AT<sub>1</sub>R, leading to paradoxical effects on progenitor cells. On the one hand, Ang II potentiates VEGF-induced capillary tube formation

of EPCs in a transient manner<sup>52</sup>. On the other hand, local cardiac Ang II regulates mesenchymal stem cell differentiation into adipocytes<sup>53</sup>. Furthermore, Ang II accelerates senescence of EPCs in sustained cultures<sup>54</sup>. Therefore, long-term exposure to Ang II may contribute to EPC dysfunction and loss of neovascularization capacity.

Ang-(1-7) attracts increasing interest in the cardiovascular community as a hormone that is augmented by ACEi and counteracts deleterious effects of Ang II. Ang-(1-7) effects can either take place through angiotensin receptors, ACE inhibitors or Mas receptors<sup>55</sup>. Studies from our group have demonstrated that Ang-(1-7) significantly attenuated post-MI remodeling and ameliorate cardiac and systemic endothelial function<sup>56</sup>. In addition, we also demonstrated that Ang-(1-7) administration markedly reduced neointimal formation and endothelial dysfunction in a rat abdominal aortic in-stent implantation model<sup>57</sup>. These effects might related to reduction in cardiac myocyte and vascular smooth muscle cell growth<sup>58</sup>, but a possible involvement of progenitor cells has not been investigated yet, although this possibility is supported by several studies.

The studies performed by Rodgers and Zerega *et al.* show that Ang-(1-7) administration significantly improved bone marrow recovery after radiochemotherapy in cancer patients<sup>59, 60</sup>, suggesting a role of the Ang-(1-7)/mas axis in BM progenitor cells proliferation and differentiation. In very recent pilot studies from our group we explored the effects of Ang-(1-7) on cardiovascular progenitor cells and observed that Ang-(1-7) promotes proliferation and differentiation of BM-EPCs. Ang-(1-7) would therefore again identify itself as a counterregulator of Ang II, this time through its favourable effect on EPC.

### *Angiotensin-converting enzyme inhibitors (ACEi)*

Intervention with RAS by inhibition of ACE is currently a primary routine treatment for CHF. ACEi blocks the formation of Ang II and increases Ang-(1-7) levels, thus preventing the most detrimental effects of Ang II on the development of CHF. Apart from direct therapeutic effects on adult cardiovascular cells, ACEi has recently shown to promote mobilization of BM-EPCs after AMI<sup>61</sup>, or even to augment EPC cell therapy after AMI<sup>62</sup>. The molecular mechanism of beneficial effects of ACEi on EPC is still far from clear, however, it has already in part been elucidated by Thum *et al.*<sup>28</sup>. ACEi enhances bone marrow stem cell migration mainly by increasing MMP-9 activity, thus stimulating the release of soluble c-kit ligand (SCF)<sup>28</sup>. Considering the difference in mechanism and signaling pathways that are activated, statins and ACEi could complement each other during stem cell therapy for CHF; an interaction that has not been studied yet.

### *Angiotensin II type 1 receptor antagonists*

The effects of AT<sub>1</sub>R antagonists on EPC were first addressed in a clinical study by Bahlmann *et al.*<sup>63</sup>. In this study, treatment with AT<sub>1</sub>R antagonists selectively increase the EPC subpopulation but not hematopoietic cells. A possible explanation could be prevention of Ang II-induced senescence of EPCs by up-regulation of telomerase activity<sup>54</sup>. Importantly, Yu *et al.* recently detected mRNA expression of AT1 and AT2 receptors on EPC colonies<sup>64</sup>.

Moreover, angiotension II receptor blockers improve EPC function and cardiac c-kit expression<sup>64</sup>. Although favorable actions of AT<sub>1</sub> antagonist on EPCs have emerged, further clinical and preclinical studies are needed to confirm these data.

In summary, RAS modulation has evident therapeutic potential when it comes to beneficial modulation of progenitor cell function in cardiovascular disease. Various angiotensins, notably Ang II and Ang-(1-7), are tools for further pharmacological research to reveal signaling systems for enhancement of progenitor cell function and cardiovascular repair. Moreover, a supportive role of RAS modulation during cell therapy could be indicated.

### **Therapeutic perspectives**

Randomized, fully-controlled, double-blinded studies of bone marrow stem cell (BMSCs) therapy for CHF represents a milestone for regenerative medicine. In the past 5 years, however, BMSCs therapy for myocardial disease yielded inconsistent outcomes in clinical trials. The question is why the successes of scientific or preclinical studies are not transferable to the clinical practice. Obviously, a variety of causes lead to discrepant results, ranging from standard cell preparation protocol<sup>65</sup>; cell subtype(s)<sup>66, 67</sup> and transplantation time point to the criteria of patient selection in CHF<sup>68</sup>.

Understanding the cell-cell interactions and molecular mechanisms that govern vascular growth and development during pathophysiological situations has facilitated the research to optimization of stem cell-based therapy for CHF. Of major relevance to angiogenic cell therapy in heart failure is the impact of appropriate pharmacological treatments on myocardial disease progression and on cardiac response to stem cell therapy. Moreover, an ameliorated endogenous cardiac microenvironment is a critically important prerequisite for successful stem cell therapy in CHF. Only a combination of pharmacological treatments that stimulate the diverse regulatory mechanisms involved in the various niches and phases of neovascularization will favorably regulate BM-EPCs and contribute to angiogenic therapy in CHF.

**Table 1.** Physiological and pharmacological factors involved in EPC-induced neovascularisation

<i>Factors</i>	<i>Effects on EPCs</i>					<i>Relative phase(s) of neovascularization</i>	<i>References</i>
	mobilization	proliferation	differentiation	angiogenesis	Senescence		
<b>Physiological Factors</b>							
HIF-1 $\alpha$	↑	N.D.	N.D.	↑	N.D.	Phase I, II	1,2,4
SDF-1	↑↑	N.D.	N.D.	↑	N.D.	Phase I, II, V	5,8
SCF	↑	↑	↑	↑	↓	Phase I, II, IV	10,12
G-CSF	↑↑	N.D.	N.D.	↑ / --	N.D.	Phase I, II	15,16
FGF	↑	↑	↑	↑	↓	Phase I-IV	19,20
VEGF	↑↑	↑↑	↑↑	↑↑	↓	Phase I-V	7,22,24,25,28
Estrogen	↑	↑	↑	↑	↓	Phase I-IV	30,31
<b>Pharmacological Factors</b>							
Statins	↑	↑	↑	↑	↓	Phase I-IV	35-39
Epo	↑	↑	↑	↑	↓	Phase I-IV	42,44,45,46
Heptapeptide	↑	↑	↑	N.D.	N.D.	Phase I	59,60
ACE inhibitor	↑	N.D.	N.D.	↑	N.D.	Phase I	28,61
ATR antagonist	↑	↑	↑	↑	↓	Phase I/II	52,63,64

N.D.: no description; ↑: increased; --: no change

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