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Targeting ACE2/Angiotensin-(1-7)/Mas Receptor Axis in the Vascular Progenitor Cells for Cardiovascular Diseases

Running title: Vasoprotective axis of RAS in progenitor cells

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List of nonstandard abbreviations

HSPCs	Hematopoietic stem progenitor cells
HSC	Hematopoietic stem cells
RAS	Renin angiotensin system
ACE	Angiotensin converting enzyme
AT1R	Angiotensin II receptor type 1
AT2R	Angiotensin II receptor type 2
ACEI	Angiotensin converting enzyme inhibitor
ARB	Angiotensin receptor blocker
HIF	Hypoxia-inducible factor
HRE	Hypoxia response elements
SDF	Stromal-derived factor-1α
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor type 2
eNOS	Endothelial nitric oxide synthase
ADAM-17	A disintegrin and metallopeptidase domain-17

Abstract

Bone marrow-derived hematopoietic stem/progenitor cells are vasculogenic, and play an important role in endothelial health and vascular homeostasis by participating in post-natal vasculogenesis. Progenitor cells are mobilized from bone marrow niches in response to remote ischemic injury and migrate to the areas of damage and stimulate revascularization largely by paracrine activation of angiogenic functions in the peri-ischemic vasculature. This innate vasoprotective mechanism is impaired in certain chronic clinical conditions, which leads to the development of cardiovascular complications. Members of renin angiotensin system (RAS), angiotensin converting enzymes (ACEs), ACE and ACE2, angiotensin II (Ang II), Ang-(1-7), and receptors AT1 and Mas are expressed in vasculogenic progenitor cells derived from humans and rodents. Ang-(1-7), generated by ACE2, is known to produce cardiovascular protective effects by acting on Mas receptor and is considered as a counter-regulatory mechanism to the detrimental effects of Ang II. Evidence has now been accumulating in support of the activation of ACE2/Ang-(1-7)/Mas receptor pathway by pharmacological or molecular maneuvers, stimulates mobilization of progenitor cells from bone marrow, migration to areas of vascular damage and revascularization of ischemic areas in pathological conditions. This mini-review summarizes recent studies that have enhanced our understanding of the physiology and pharmacology of vasoprotective axis in bone marrow-derived progenitor cells in health and disease.

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Significance statement

Hematopoietic stem/progenitor cells (HSPCs) stimulate revascularization of ischemic areas. However the reparative potential is diminished in certain chronic clinical conditions leading to the development of cardiovascular diseases. ACE2 and Mas receptor are key members of the alternative axis of renin angiotensin system and are expressed in HSPCs. Accumulating evidence points to activation of ACE2 or Mas receptor as a promising approaches for restoring the reparative potential thereby to prevent the development of ischemic vascular diseases.

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Introduction

Discovery of the vasoreparative potential of hematopoietic stem progenitor cells (HSPCs) has introduced the novel concept of postnatal vascular development and exponentially increased our understanding of vascular regeneration. (Asahara *et al.*, 1997) This concept has rapidly transitioned into the clinical arena for determining their potential therapeutic benefit in cardiovascular diseases. (Raval and Losordo, 2013) HSPCs with vasoreparative potential are frequently termed as endothelial progenitor cells or vascular progenitor cells by different research groups. These cells are primarily derived from bone marrow and are a subset of HSPCs that can directly participate in the vascular re-endothelialization and regeneration, and thus contributing for ischemic vascular repair and tissue regeneration primarily by restoring blood flow. Stimulating this innate vasoreparative mechanism by enhancing the mobilization of HSPCs from bone marrow into the circulation by pharmacological or by cell-transplant approaches is a promising therapeutic strategy for the treatment of cardiovascular complications.

Discovery of angiotensin converting enzyme-2 (ACE2) has led to the conception of cardiovascular protective axis of renin angiotensin system (RAS). ACE2 is a monocarboxypeptidase, capable of cleaving Ang I to Ang-(1-9) or Ang II to Angiotensin-(1-7) (Ang-(1-7)). (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000; Vickers *et al.*, 2002) In 2003, Mas receptor (MasR) was characterized as the cognate receptor mediating the cardiovascular protective functions of Ang-(1-7). (Santos *et al.*, 2003) Thus, ACE2/Ang-(1-7)/MasR axis constitutes the protective axis of RAS. This novel pathway has attracted massive attention and is being extensively studied for the development of novel therapeutic targets for cardiovascular, metabolic and neurological disorders. In the recent years several studies have shown evidence for

an important role of the protective RAS in vascular progenitor cells. ACE2 and MasR are expressed in one or more subsets of bone marrow-derived HSPCs. Activation of ACE2/Ang-(1-7)/MasR axis stimulates vascular repair-relevant functions of HSPCs in health, and reverses dysfunctions induced by chronic pathological conditions. This mini-review provides an overview of the discovery and clinical significance of vascular progenitor cells, and then, summarizes key reports that enhanced our understanding of the physiology and pharmacology of ACE2/Ang-(1-7)/MasR pathway in the progenitor cells, and the implications for clinical applications.

Discovery and identity of vascular progenitor cells

Evidence for the existence of vascular progenitor cells dates back to 1963, when endothelialization of intravascular Dacron hub was postulated to be derived from circulating blood cells (Stump *et al.*, 1963). This concept did not attract much attention until 1995, when definitive proof was provided in 1995 (Wu *et al.*, 1995). This study confirmed endothelial identity of cells, by von Willebrand factor and Ulex europeus agglutinin I staining, on the flow surface of Dacron axillo-femoral bypass graft, which was harvested after 26 months of implantation from a patient (Wu *et al.*, 1995). In 1997, Asahara et al reported successful isolation of putative vascular progenitor cells, termed as endothelial progenitor cells (EPCs) from human peripheral blood. Circulating $CD34^{+}VEGFR2^{+}$ mononuclear cells differentiated into endothelial-like cells when cultured on fibronectin, and the differentiated cells incorporated into the sites of active angiogenesis in animal models of ischemic injury (Asahara *et al.*, 1997). This study indeed provided evidence for postnatal vasculogenesis and triggered a massive interest among scientists worldwide about the true identity and clinical applications of progenitor cells for ischemic vascular complications.

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Subsequent studies demonstrated endothelial progenitor cells (EPC)-like properties in cell populations identified by one or more of the following antigenic markers, CD34, CD133, CD45, VEGFR-2, CXCR4, CD14, CD31, or eNOS (refs 6-13). Most of these phenotypes overlap with the known antigenic markers of HSPCs (Yoder 2013; Timmerman *et al*., 2009; Fadini *et al.*, 2012). While a true identity of a vascular progenitor cell in humans is yet elusive, based on extensive clinical studies CD34 antigen alone could represent a cell population of great clinical utility. CD34 is a sialomucin-like ligand for L-selectin and is commonly expressed on many hematopoietic progenitor cells that are either resident in bone marrow or in the circulation (Civin *et al.*, 1984). The $CD34^+$ cell population has been clinically used for reconstituting bone marrow cells following chemoradiation therapy (Demirer *et al.*, 1999). Given the fact that the microvascular endothelial cells and tissues that support early vascular development express CD34 antigen (Tavian *et al.*, 1996; Wood *et al.*, 1997), $CD34^+$ cells were believed to be EPCs that are capable of differentiating into vascular endothelium and stimulate vascularization (Asahara *et al.*, 1997). Several clinical trials have employed $CD34^+$ HSPC population for determining the therapeutic potential of autologous cell therapies in cardiovascular complication (Quyyumi AA. *et al.*, 2017; Henry *et al.*, 2018). (Please note that cord blood is an abundant source of several types of stem progenitor cells and possess efficient vascular regenerative functions. Several other organs including adipose tissue are now known to have resident stem progenitor cells that are likely to have reparative functions. In this review, discussion is limited to adult $CD34^+$ HSPCs that are derived from bone marrow).

From bone marrow to the circulation: mobilization of HSPCs

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Numerous experimental studies have provided evidence for bone marrow as major source of the circulating vascular progenitor cells particularly the cells that home to areas requiring neovascularization or tissue healing after injury (Shi *et al.,* 1998; Asahara *et al.,* 1999). In dogs that underwent bone marrow transplantation with genetically distinct donor cells, and implanted with intravascular Dacron graft (Shi et al., 1998), endothelial cells that repopulated the graft were exclusively derived from bone marrow-resident donor cells. This was strongly supported by a subsequent study that used transgenic mice expressing beta-galactosidase under transcriptional regulation of an endothelial cell-specific promoter (Flk1 or Tie2) as transplant donors (Asahara *et al*., 1999). Incorporation of $lacz^{+}$ cells in the areas of neovascularization were observed following induction of myocardia or hindlimb ischemic injury, tumor development or in the endometrium. These studies confirm that bone marrow-resident HSPCs are mobilized into the blood stream and contribute to physiological or pathological postnatal angiogenesis.

Bone marrow contains primitive HSCs, progenitor cells, nonHSCs and blood cells that are derived from HSPCs. NonHSCs include osteoblasts, osteoclasts, endothelial cells, mesenchymal stromal cells, fibroblasts, adipocytes, nerve terminals and mature blood cells. These cells form specialized niches either close to endosteum (osteoblastic niche) or vasculature (vascular niche). Quiescence, proliferation, or differentiation of HSCs is largely determined by the niche cells. Promote quiescence of HSCs by making direct contacts and via paracrine influences by secreting osteopontin or angiopoietin-1 (Mendez-Ferrer *et al*., 2010; Ding *et al*., 2012).
Quiescent and slowly dividing HSPCs preferentially reside in close interaction with osteoblasts and mesenchymal stromal cells (endosteal niche) (Kunisaki et al., 2013). On the other hand, rapidly proliferating cells reside around sinusoids and arterioles (Itkin et al., 2016; Richeter et al.,

2017). Under physiological conditions, HSPCs actively enter blood stream in a circadian pattern and eventually home back to bone marrow (Lucas *et al*., 2008; Casanova-Acebe *et al*., 2013). Though the function is to generate blood cell progenies in bone marrow, HSPCs are constantly found in circulation and appear to be responsible for tissue surveillance and modulate homeostasis (Ballard and Edelberg 2007). HSPCs are mobilized in response to several circulating factors that are cues arising out of physiological demands or pathological changes. Ischemic vascular injury induces hypoxia-inducible factor-1α (HIF), which in turn triggers expression of a myriad of factors into the circulation. HIF is rapidly stabilized in the environments of low oxygen tension and bound to its cognate hypoxia response elements (HREs) within enhancer elements, HIF transcription factors, and increase the transcription of target genes (Wang *et al*., 1995). These downstream effectors of HIF teleologically aid in diverse biological processes that aid in survival during hypoxia for example hematopoiesis or angiogenesis (Shweiki *et al.*, 1992). HIF stabilized by tumor hypoxic environment and induces angiogenesis that is required to support rapid proliferation of cells (Maxwell *et al*., 1997). HIF stimulates recruitment and homing of bone marrow-derived HSPCs to areas of injury that was largely mediated by the expression of stromal derived factor-1α (SDF) (Sweeney *et al*., 2002; De Falco *et al*., 2004) and vascular endothelial growth factor (VEGF) (Forsythe *et al*., 1996; Gill *et al*., 2001; Takahashi *et al.*, 1999), strong chemoattractant factors for vasoreparative HSPCs, are identified as major players in stimulating vascular growth and to restore blood flow to the areas of injury. Increasing the circulating levels of VEGF or SDF would mimic mobilization of HSPCs independent of HIF (Hattori *et al*., 2001; Burns *et al*., 2006) **(Figure 1)**. Other factors that are identified to stimulate mobilization of HSPCs from bone marrow include but not limited to erythropoietin, thrombopoietin, G-CSF, GM-CSF (Jin *et al*., 2006; Grunewald *et al*., 2006) and

this list is still increasing. Granulocyte-colony stimulating factor (G-CSF) and plerixafor are clinically used mobilizers of bone marrow immune cells and this response is always associated with mobilization of primitive progenitor cells, which stimulate vascular repair. Several experimental studies evaluating revascularization of ischemic areas have supported this notion (Takahashi *et al.*, 1999; Valgimigli *et al.*, 2005; Capoccia *et al.*, 2006; Jiao *et al.*, 2006; Nishimura *et al.*, 2012).

Mechanisms of vasculogenesis by stem/progenitor cells

Generally accepted mechanism of vascular repair by progenitor cells include homing to areas needing vascular repair, and extravasation in the tissues of injury, where they proliferate and stimulate the new vessel formation. However, the evidence for the trans-differentiation of cells into endothelium or integration of cells into the vessel wall is rather inadequate. This in fact led to the alternative hypothesis of mechanism involving paracrine effects of the progenitor cells (Majka *et al.*, 2001; Urbich *et al.*, 2005). Pivotal studies by Ziebert et al, showed that sustained presence of cells in the circulation following ischemic insult is required for complete recovery of blood flow and revascularization of the injured tissue (Ziebart *et al.*, 2008). However, depletion of cells during the recovery, by genetic modification of cells for an inducible thymidine kinase activity, resulted in suppression of revascularization, thus strongly supporting paracrine functions as primary mechanism of vascular repair. This is further corroborated by several other studies (Burchfield and Dimmeler, 2008; Gnecchi *et al.*, 2008; Barcelos *et al.*, 2009). Consistent with this hypothesis, progenitor cells derived from pathologic environment showed paracrine dysfunction in in vivo and in vitro studies (Awad *et al.*, 2005; Schatteman *et al.*, 2010; Jarajapu *et al.*, 2014; Singh *et al.*, 2014).

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Clinical significance of circulating vasculogenic progenitor cells

Several lines of evidence support the prognostic significance of vascular progenitor cells and the therapeutic potential. The number of circulating $CD34^+$ cells was reported to be an independent risk biomarker of cardiovascular events and correlated with adverse outcomes (Hill *et al.*, 2003; Schmidt-Lucke *et al.*, 2005). Fadini et al provided strong evidence for this concept in patients with metabolic syndrome or diabetic vasculopathy (Fadini *et al.*, 2006). In patients with heart failure or acute coronary artery syndrome, lower number of circulating progenitor cells independently predict life-threatening outcomes (Tahhan *et al.*, 2018). High risk for cardiovascular disease in African-American individuals is due to the lower levels of vascular progenitor cells (Tahhan *et al.*, 2018). Given the fact that healthy microvasculature determines perfusion of brain required for cognitive functions, lower number of progenitor cells are associated with greater cognitive decline compared to the individuals, who have higher number of cells in the circulation (Hajjar *et al.*, 2016). Experimental studies have reported several functional deficiencies such as migration, proliferation, vascularization of matrigel plugs, integration into the vascular wall or improving blood flow to ischemic areas, which signify impaired regenerative capacity of circulating HSPCs derived from individuals, who are at high risk for cardiovascular diseases (Jarajapu and Grant, 2010).

Based on the accumulated literature, it is now very well accepted that the circulating vascular progenitor cells represent regenerative capacity of an individual and have immense prognostic value as the numbers negatively correlate with risk factors for cardiovascular disease. Lower numbers are markers of high risk and predict acute cardiovascular events. The number of

progenitor cells increase by mobilization from bone marrow following an acute cardiovascular event such as myocardial ischemia or stroke. Impaired mobilization or reparative functions of progenitor cells would result in a worst outcome.

Renin-angiotensin system (RAS)

Angiotensin II (Ang II or Ang-(1-8)), the most prominent peptide of the classical renin angiotensin system (RAS). Ang II is derived from Ang I by the enzyme angiotensin-converting enzyme (ACE) and largely produces cardiovascular detrimental effects by acting on the angiotensin II type 1 receptor (AT1R) (Nakashima *et al*., 2006). Vasoconstriction, oxidative stress, mitochondrial dysfunction, inflammation, and fibrosis are some of the known mechanisms underlying the cardiovascular pathology induced by Ang II/AT1R interaction. Therapeutic agents that oppose ACE/Ang II/AT1R pathway are successful in the treatment of cardiopulmonary diseases. With the advent of genomic and proteomic approaches and availability of sensitive experimental approaches to integrative physiology, new members of RAS, peptide fragments, peptidases and receptors, have been identified and biological functions of RAS have been expanded (**Figure 2**).

The alternative ACE, angiotensin-converting enzyme-2 (ACE2), was discovered by two independent groups in 2000 (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000). ACE2 converts Ang I to Ang-(1-9), which is a substrate for ACE resulting in Ang-(1-7) formation. ACE2 produces Ang-(1-7) from Ang II, which has catalytic efficiency much higher than the pathway involving Ang-(1-9) and ACE (Vickers *et al.*, 2002; Rice *et al.*, 2004). Furthermore, ACE can convert/degrade Ang-(1-7) to Ang-(1-5), which is in agreement with the findings that Ang-(1-7)

levels are increased in individuals on treatment with ACE-inhibitors ACEIs (Chappell *et al.*, 1998). Ang-(1-7) was thought to be acting as a physiological counter-regulatory peptide for the actions of Ang II. By acting through MasR, Ang-(1-7) promotes vasodilation, antihypertensive, antifibrotic, antithrombotic and antihypertrophic effects in several in vitro and in vivo experimental studies thus making the protective pathway of RAS a potential therapeutic target for cardiovascular diseases (Santos *et al.*, 2018). Either ACE2 or Ang-(1-7) or stable analogues of Ang-(1-7) are now being evaluated for the therapeutic potential in individuals with cardiopulmonary and ischemic vascular disorders.

ACE/Ang II/AT1R axis in vasculogenic progenitor cells

In recent years, compelling evidence has been shown for the expression and function of RAS members in both murine and human HSPCs (Strawn *et al.*, 2004; Rodgers and diZerega, 2013; Singh *et al.*, 2015). Ang II has hematopoietic functions, mainly erythropoiesis and myelopoiesis, that were demonstrated by using angiotensin receptor blockers (ARBs) in vitro or in the mouse model of genetic ACE deficiency (Chisi *et al.*, 1999; Cole *et al.*, 2000; Rodgers *et al.*, 2000). ACE is required for normal hematopoiesis and ACEIs increase the risk for anemia (Hubert *et al.*, 2006). Initial studies that were focused on vascular repair, have demonstrated that Ang II potentiates angiogenic functions of progenitor cells. This beneficial effect was largely attributed to upregulation of VEGF and its receptor, VEGFR2. Increased vascular permeability by Ang II and VEGF would potentiate the transmigration of progenitor cells to the injured areas, which in turn participate in the process of vascular repair (Imanishi *et al.*, 2004). In agreement with this, a study by Yin *et al.*, (2008) has shown that vascular repair-relevant functions such as nitric oxide (NO) generation, protection from apoptosis and adhesion, were stimulated by Ang II. However

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extensive evidence supports that overactivity of ACE/Ang II/AT1R axis impairs cardiovascular repair and postnatal vasculogenesis at least in part by attenuating the reparative functions of progenitor cells. Activation of AT1R by hypertensive concentrations of Ang II reduced the number of circulating progenitor cells in mice and attenuated the vascular repair-relevant functions of progenitor cells, which lead to the endothelial damage and impaired vascular regeneration (Endtmann *et al.*, 2011). Activation of AT1R stimulates NADPH oxidase-dependent ROS-generation, which in turn activates ASK-1 and DNA damage leading to apoptosis and senescence of progenitor cells (Imanishi *et al.*, 2005; Endtmann *et al.*, 2011). Hypertensive levels of Ang II induce myeloid-biased hematopoiesis in the bone marrow resulting in the accumulation of pro-inflammatory monocytes (Jun *et al.*, 2012; Kim *et al.*, 2016), which in turn impairs angiogenesis and vascular repair (Libby, 2006; Mirza and Koh, 2011). In support of these findings, studies with ACE inhibitors (ACEIs) or ARBs stimulated regenerative functions of HSPCs and postnatal vasculogenesis in experimental models of cardiovascular disease (Kobayashi *et al.*, 2006; Wang *et al* 2006; You *et al.*, 2008a, 2008b; Yu *et al.*, 2008; Müller *et al.*, 2009).

Consistent with the experimental findings as described above, clinical studies have proven that antagonism of ACE/Ang II/AT1R axis stimulates regenerative capacity in patients with cardiovascular disease. Either telmisartan, an AT1R antagonist or ramipril, an ACEI, increased the number of circulating HSPCs in individuals with coronary artery disease thus enhancing the regenerative capacity that is known to be diminished in patients with cardiovascular pathologies (Porto *et al.*, 2009; Endtmann *et al.*, 2011; Golab-Janowska *et al* 2018). Ramipril stimulated the vascular-repair-relevant functions of HSPCs such as proliferation, migration, adhesion and

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capillary tube formation (Min *et al.*, 2004). Along similar lines, an elegant study in patients with acute coronary syndrome demonstrated that ACEI prevented vascular endothelium from apoptosis and stimulated re-endothelialization (Cangiano *et al*., 2011). While these studies collectively support detrimental effects of ACE/Ang II/AT1R axis on the reparative functions of HSPCs, role of Ang-(1-7) in the protective effects of ACEIs cannot be ruled out (Chappell *et al*., 1998). However evidence for nonenzymatic or substrate-independent effects of ACE in the mobilization of HSPCs is noteworthy of mention. Kohlstedt et al (2018) have demonstrated that ACE negatively regulates G-CSF-receptor signaling in osteoblasts and attenuates G-CSF-induced mobilization of HSPCs. ACEI or transgenic mice expressing nonphosphorylatable ACE-mutant potentiate the effect of G-CSF on proliferation or mobilization of HSPCs (Lin *et al*., 2010; Kohlstedt *et al*., 2018).

Activation of AT2R is known to oppose AT1R-dependent cellular actions of Ang II. Though not well studied in the context of reparative functions by stem/progenitor cells, evidence has been provided for a potentiating role of this receptor in vasculogenic properties of progenitor cells. AT2R is expressed in bone marrow as well as cardiac progenitor cells (Ludwig *et al*., 2013). $CD117^{+}AT2R^{+}$ mononuclear cells were shown to increase in response to ischemic injury and participate in the post-ischemic recovery effectively by potentiating angiogenesis independent of AT2R activation in the cardiac myocytes (Altarche-Xifró *et al*., 2009). Future investigations are needed to shed more light into the role of AT2R in vasoreparative functions of human HSPCs.

ACE2/Ang-(1-7)/MasR axis in vasculogenic progenitor cells

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Ang-(1-7) was initially known for its potent hematopoietic functions in mouse models and human cells, and these observations were explored for the therapeutic potential in cancer patients for stimulating hematopoietic recovery following chemoradiation therapy (Rodgers *et al.*, 2002, 2006). Later studies have focused on the importance of this pathway in the cells that are relevant for vascular regeneration and evidence has been accumulating in support of this novel concept. Activation of this pathway is evidently an efficient approach for stimulating proliferation of bone marrow-resident HSPCs, mobilization, paracrine angiogenic effects, which collectively enhance revascularization of ischemic areas and tissue healing (**Figure 3**) as discussed below.

Analogous to the expression of SDF or VEGF in HSPCs by HIF1α (see above) upon exposure to ischemic environment, ACE2 and MasR are hypoxia-regulated but not ACE or AT1R (Joshi *et al.*, 2019). Luciferase reporter assays confirmed increased ACE2 and MasR transcription by hypoxia that was inhibited by co-expression of specific miRNAs in human HSPCs. Increased ACE2 enzyme activity was observed in both cell lysates as well as in cell-supernatants. The latter is via shedding of ACE2 ectodomain by ADAM17, which is also upregulated by hypoxia (Hurtado *et al.*, 2001; Joshi *et al.*, 2019). It is interesting to note that the SDF or VEGF could induce the expression of ACE2 or MasR independent of hypoxic environment. These *in vitro* findings are further supported by in vivo studies. In mice undergoing ischemic injury, circulating HSPCs showed increased expression of ACE2 and MasR but not ACE or AT1R, compared to that observed in the circulating cells derived from nonischemic mice (Joshi *et al.*, 2019). Taken together, these findings imply that the upregulation of ACE2 and MasR in progenitor cells that are recruited to the areas of ischemia would enhance the generation of Ang-(1-7), which in turn

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via MasR-dependent autocrine and paracrine effects on peri-ischemic endothelium would further stimulate vascular regeneration and rapid recovery of organ function.

Activation of ACE2 or MasR has been shown to promote vasoreparative functions in vascular progenitor cells or to reverse reparative dysfunction in the cells derived from a pathological environment. Activation of MasR in $CD34^+$ cells obtained from healthy individuals has promoted vascular repair-relevant functions such as migration, proliferation and NO generation in basal or in response to SDF or VEGF (Jarajapu *et al.*, 2013; Singh *et al.*, 2015). Importantly, the dysfunctional cells derived from individuals with diabetes, pulmonary hypertension or heart failure were responsive to Ang-(1-7) and showed responses that are vascular repair-relevant (Jarajapu *et al.*, 2013; Shenoy *et al.*, 2013; Cole-Jeffrey *et al.*, 2018). Lentiviral overexpression of Ang-(1-7) transgene restored migratory response of cells to SDF in vitro, or migration to the areas of ischemic injury and integration into the blood vessel wall in vivo (Jarajapu *et al.*, 2013). Along similar lines, lentiviral expression of ACE2 restored reparative functions in the dysfunctional diabetic $CD34^+$ cells in a mouse model of critical limb ischemia (Jarajapu *et al.*, 2017). It is worth noting that in a unique group of diabetic individuals who are resistant to the development of microvascular complications despite severe diabetes for a long time, expression of ACE2 was higher compared to that observed in cells derived from diabetics who have developed MVCs (Jarajapu *et al.*, 2013).

In vivo treatment of diabetic mice with Ang-(1-7) reversed paracrine dysfunction as assessed by using preconditioned media derived from bone marrow progenitor cells. Diabetes resulted in paracrine dysfunction of cells characterized by the inability of preconditioned medium to support

migration and proliferation of progenitor cells, and switching the paracrine profile from pro-angiogenic, proliferative and anti-inflammatory to detrimental pro-inflammatory and anti-angiogenic and antiproliferative functions (Schatteman *et al.*, 2010; Jarajapu *et al.*, 2014), these dysfunctions were reversed in a mouse model of diabetes by Ang-(1-7) treatment (Singh *et al.*, 2014). Mobilization of vascular progenitor cells is dysfunctional in conditions such as diabetes or obesity either in physiological conditions or in response to ischemic insult (Fadini and Avogaro 2013). Ang-(1-7) treatment stimulated mobilization of progenitors in healthy mice that were undergoing myocardial ischemia (Wang *et al.*, 2010). In mouse models of either type 1 or type 2 diabetes, Ang-(1-7) treatment reversed impaired mobilization in basal as well as in response to ischemic injury (Vasam *et al.*, 2017).

Role of NO in vasculogenic functions of Ang-(1-7)

NO has an important role in the maintenance and mobilization of progenitor cells from bone marrow (Aicher *et al.*, 2004). NO at least in part mediates protective functions of Ang-(1-7) in the progenitor cells. In agreement with initial studies inferring MasR-dependent activation of eNOS in endothelial cells (Wiemer *et al.*, 2002; Sampaio *et al.*, 2007), PI3K/Akt pathway has been shown to be involved in MasR-dependent NO release by Ang-(1-7) in human and murine HSPCs (Jarajapu *et al.*, 2013). Oxidative environment induces uncoupling of eNOS resulting in superoxide generation which further exacerbates oxidative stress. In conditions such as diabetes and obesity, Ang-(1-7) is able to restore NO levels at least in part by reducing oxidative stress. Elegant studies have shown that bone marrow oxidative stress in mouse models of diabetes by Ang-(1-7) in diabetic bone marrow and increased levels of NO/cGMP levels (Mordwinkin *et al.*, 2012; Papinska *et al.*, 2015). CD34 cells derived from diabetic individuals have shown increased

ROS levels that were normalized by Ang-(1-7), which was associated with increased NO levels (Jarajapu *et al.*, 2013).

Evidence from MasR- or ACE2-deficient mice

Recent evidence points to an important role of MasR in physiological and pharmacological mobilization via multiple molecular mechanisms. MasR-deficiency decreased the number of circulating vascular progenitor cells. Importantly, mobilization of progenitors into the circulation in response to ischemic vascular injury is severely impaired resulting in partial recovery of blood flow to the ischemic areas (Vasam *et al.*, 2017). Mice with ACE2 deficiency were shown to develop bone marrow dysfunction characterized by skewing hematopoiesis towards myelopoiesis, which increased the susceptibility to the development of microvascular complications (Duan *et al.*, 2018). This observation is indeed in agreement with findings from studies by using Ang II (Jun *et al.*, 2012; Kim *et al.*, 2016), suggesting that the absence of the counter-regulatory vasoprotective axis produces detrimental effects in the bone marrow largely via ACE/Ang II/AT1R overactivity.

Novel role of Slit proteins in the mobilization of progenitor cells by Ang-(1-7) from bone marrow

A novel mechanism involving Slit proteins has been shown to be activated by Ang-(1-7) in mouse bone marrow. Slit proteins were first discovered for an important role in the context of neural development and then was shown to regulate migration of nonadherent cells by acting on a unique class of receptors, receptor of roundabout (Robo) (Wang *et al*., 2003; Geutskens *et al.*, 2010). Real-time PCR studies have detected mRNA transcripts of Slit and Robo isoforms in both stromal cells and LSK cells in mice (Vasam *et al.*, 2017). In our studies, Slit proteins did not

stimulate migratory response in murine progenitor cells but increased Rho-kinase activity. In the presence of a Slit protein, migratory response to SDF is increased concentration-dependently. Furthermore, we found that Slit3 is an abundant isoform in mouse bone marrow supernatant, and its secretion by stromal cells is stimulated by Ang-(1-7) in vitro that was completely blocked by MasR-antagonist, A779. In vivo treatment with Ang-(1-7) did not alter Slit3 levels in healthy mice but increased in diabetic mice (Vasam *et al.*, 2017). Thus, the reversal of diabetic mobilopathy by Ang-(1-7) is at least in part due to increased slit3 levels in the bone marrow.

The promise of ACE2/Ang-(1-7)/MasR axis in regenerative pharmacology

Increasing the innate regenerative capacity is a promising therapeutic approach for clinical conditions in which tissue repair or regeneration is the most desired outcome. Aging, diabetes/obesity, hypertension, and lifestyle – western diet, smoking, and alcoholism are all known to decrease the number of progenitor cells and attenuate the reparative functions that are hallmarks of decreased regenerative capacity. Oxidative stress and inflammation are common pathological features of these disorders. Therefore, autologous cell therapies are not feasible in these populations of patients. Ex vivo modification of cells to produce vasoprotective molecules that would not only restore reparative functions but also modify the pathological milieu suitable enough for the cells to accomplish the vascularization process. Overexpression of either VEGF or eNOS in cells would not modify the oxidative or inflammatory environment of the recipient instead may cause aberrant neovascularization or overproduction of reactive oxygen species (Ozawa *et al.*, 2004; Jarajapu *et al.*, 2011; Mujagic *et al.*, 2013). Overexpression of ACE2 or Ang-(1-7) by gene transfer approach with the advantage of using efficient viral vectors will have multiple benefits that would collectively stimulate vascular repair processes **(Figure 1)**: 1) as the

cells are destined to areas of ischemia, the vasoprotective molecules will be target-delivered, 2) increased ACE2 levels will decrease Ang II concentrations in the peri-ischemic areas and minimize or abolish the detrimental effects and simultaneously increases Ang-(1-7) generation, 3) autocrine effects of Ang-(1-7) reverses paracrine dysfunction in the progenitor cells, 4) exposure to hypoxia stimulate expression of ACE2 and MasR, and increases shedding of ACE2, and 5) modified pathological milieu – decreased oxidative stress and attenuated pro-inflammatory conditions create more favorable environment for revascularization. The proof-of-concept for this approach has been shown previously by using lentiviral gene transfer approaches in experimental models of cardiovascular diseases. $CD34^+$ cells that are derived from diabetic individuals are dysfunctional and this was reversed by lentiviral expression of Ang-(1-7) transgene; the modified cells have regained vascular repair-relevant functions in vitro, and migrated to areas of ischemia in a mouse model of retinal ischemia and integrated into the microvasculature (Jarajapu *et al.*, 2013). Similar findings were observed when dysfunctional diabetic cells were overexpressed with lentiviral ACE2 gene transfer in a mouse model of hind-limb ischemia (Jarajapu *et al.*, 2017).

Pharmacological targeting of ACE2 or MasR has been proven to be successful in experimental models for diabetic wound healing and cardiopulmonary disorders, and the reparative end-points were associated with increased vascular regenerative capacity (Mordwinkin *et al.*, 2012; Shenoy *et al.*, 2013; Singh *et al.*, 2014; Papinska *et al.*, 2015; Vasam *et al.*, 2017). Despite the shorter biological half-life, Ang-(1-7) administration has been shown to be effective even in nanomolar doses in a wide range of experimental models. More stable analogues of Ang-(1-7) such as Norleu3-Ang-(1-7) or glycosylated Ang-(1-7) would be better alternatives for clinical use

(Rodgers *et al.* 2005; Hay *et al.*, 2019). Small molecule activators of ACE2, diminazene aceturate and a xanthenone derivative, have been reported for their beneficial effects in rodent models of cardiopulmonary diseases (Ferreira *et al.*, 2009, 2011; Fraga-Silva *et al.*, 2013; Qi *et al.*, 2013). Diminazene aceturate was shown to increase the circulating progenitor cells in a rodent model of pulmonary hypertension and stimulated vascular repair-relevant functions in $CD34^{+}$HSPCs derived from healthy individuals or individuals with pulmonary hypertension (Shenoy *et al.*, 2013; Singh *et al.*, 2015). However further systematic investigations are warranted to explore the pharmacology of these small molecule activators.

Conclusion

In conclusion, cell-based therapies are promising therapeutic strategies for the treatment of cardiovascular complications especially when other available options have proven to be ineffective. However, these approaches are currently not feasible when cells are derived from a pathologic environment as is the case with autologous cell therapies. As summarized in this review, an optimal balance in the detrimental and protective axes of RAS is critical for the regenerative functions of hematopoietic stem/progenitor cells. Functional imbalance with a hyperactive ACE or AT1R attenuates the reparative functions, which in turn leads to the development of cardiovascular pathologies. Compelling evidence has now been shown in support of the activation of ACE2/Ang-(1-7)/MasR pathway by molecular or pharmacological maneuvers as a novel therapeutic approach for reversing dysfunctions in progenitor cells thereby enhancing revascularization outcomes.

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Footnotes

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Legends for figures:

Figure 1. Mobilization of hematopoietic progenitor cells (HSPCs) from bone marrow niches: HSPCs are known to reside in two distinct niches, endosteal and vascular niches. HSPCs in the vascular niche are rapidly proliferating and reside in the close proximity to the sinusoids therefore are readily available for mobilization in to the blood stream in response to physiological stimuli or pathological demand. Bone marrow microenvironment contains several cell types including osteoblasts, mesenchymal stromal cells, endothelial cells, CAR cells fibroblasts, adipocytes (not all are shown) and many of which secrete stromal derived factor-1α (SDF) or vascular endothelial growth factor (VEGF). These two factors indeed help retaining the cells in the bone marrow niches. A shift in the gradient of these factors relative to the concentration in the circulating blood dislodges cells from their niches and transmigrate the sinusoidal wall to peripheral blood. Ischemic tissues stimulate this process by generating SDF and VEGF that rise to levels several fold-higher than that observed in bone marrow. Mobilized cells home to the areas of ischemia, extravasate into the ischemic tissues, and stimulate vascular regeneration either by trans-differentiation into endothelial cells or by releasing paracrine factors (see text for details).

Figure 2. Synthesis and metabolism of angiotensin peptides:

Renin secreted from juxtaglomerual cells in the kidney cleaves angiotensinogen in the circulation to angiotensin-(1–10) (Ang I), which is further processed to biologically active peptides, Ang-(1–8) (Ang II) by angiotensin-converting enzyme (ACE), Ang-(1–9) by ACE2, and Ang-(1–7) by endopeptidases such as neprilysin (NEP) and prolylendopeptidase (PEP). Ang II is converted by ACE2 or prolylcarboxy peptidase (PCP) to generate Ang-(1–7). Ang II recognizes receptors,

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angiotensin types 1 and 2 receptors (AT_1R and AT_2R, respectively), whereas Ang-(1–7) interacts with MasR, AT_2R and Mas-related G-protein coupled receptor member D (MrgD). (Santos *et al.*, 2003; Castro *et al.*, 2005; Tetzner *et al.*, 2016) Ang-(1-9) is known to activate AT2R to produce cardiovascular protective effects (not indicated by arrows). (Ocaranza *et al.*, 2012; 2014) Angiotensin II can be further processed by aminopeptidase A (APA) to form Ang-(2-8) aka Ang III, which has affinity for both AT1R and AT2R. (Bosnyak et al., 2011) Ang III can be cleaved by alanyl aminopeptidase N (APN) to generate Ang-(3-8) aka Ang IV, which binds to insulin-regulated membrane aminopeptidase (IRAP) aka AT4R. (Park *et al.*, 2016) Alternatively, angiotensin II can be processed by aspartate decarboxylase (AD) to produce Ala^1-Ang-(1-8) aka Ang A, which can be converted to Ala1-Ang-(1-7) aka alamandine by ACE2. Ang-(1–7) can also be metabolized to alamandine by AD. Alamandine activates MrgD and elicits cardiovascular protective effects. (Lautner *et al.*, 2013)

Figure 3. Schematic of protective functions of ACE2/Ang-(1-7)/MasR pathway in the vasoreparative functions of hematopoietic stem/progenitor cells: ACE2 and MasR are expressed in hematopoietic stem/progenitor cells (HSPCs) and vascular endothelium. Activation of either ACE2 to generate Ang-(1-7), or MasR by exogenous Ang-(1-7) stimulates mobilization of HSPCs and angiogenic properties of secretome derived from HSPCs. In the microvascular endothelium, activation of MasR by Ang-(1-7) stimulates angiogenesis (not indicated by arrows). (Hoffmann *et al.*, 2017). Both angiogenic and vasculogenic processes contribute to the vasoreparative functions of Ang-(1-7) (see text for details).

Figure 1

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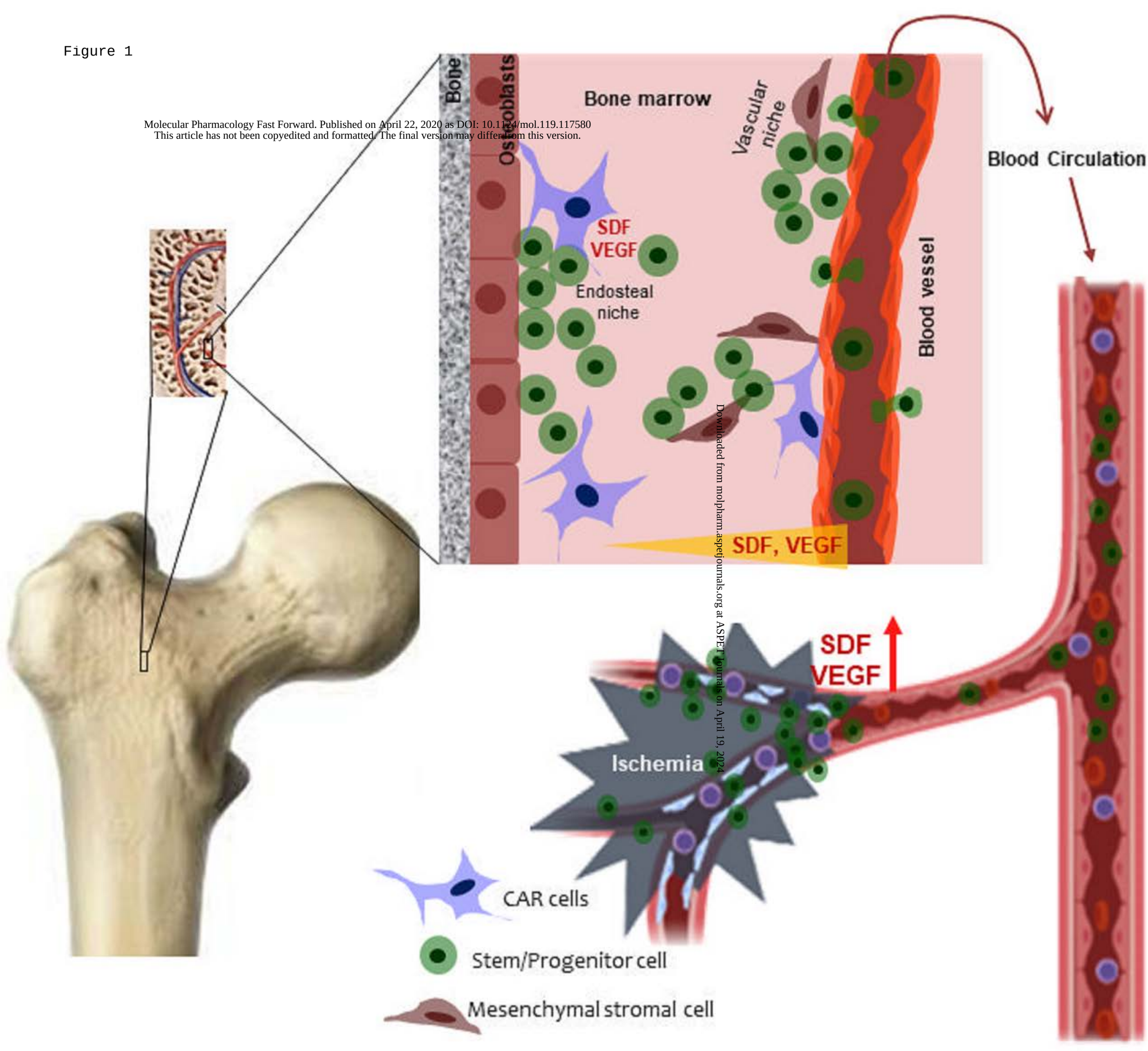

Figure 2

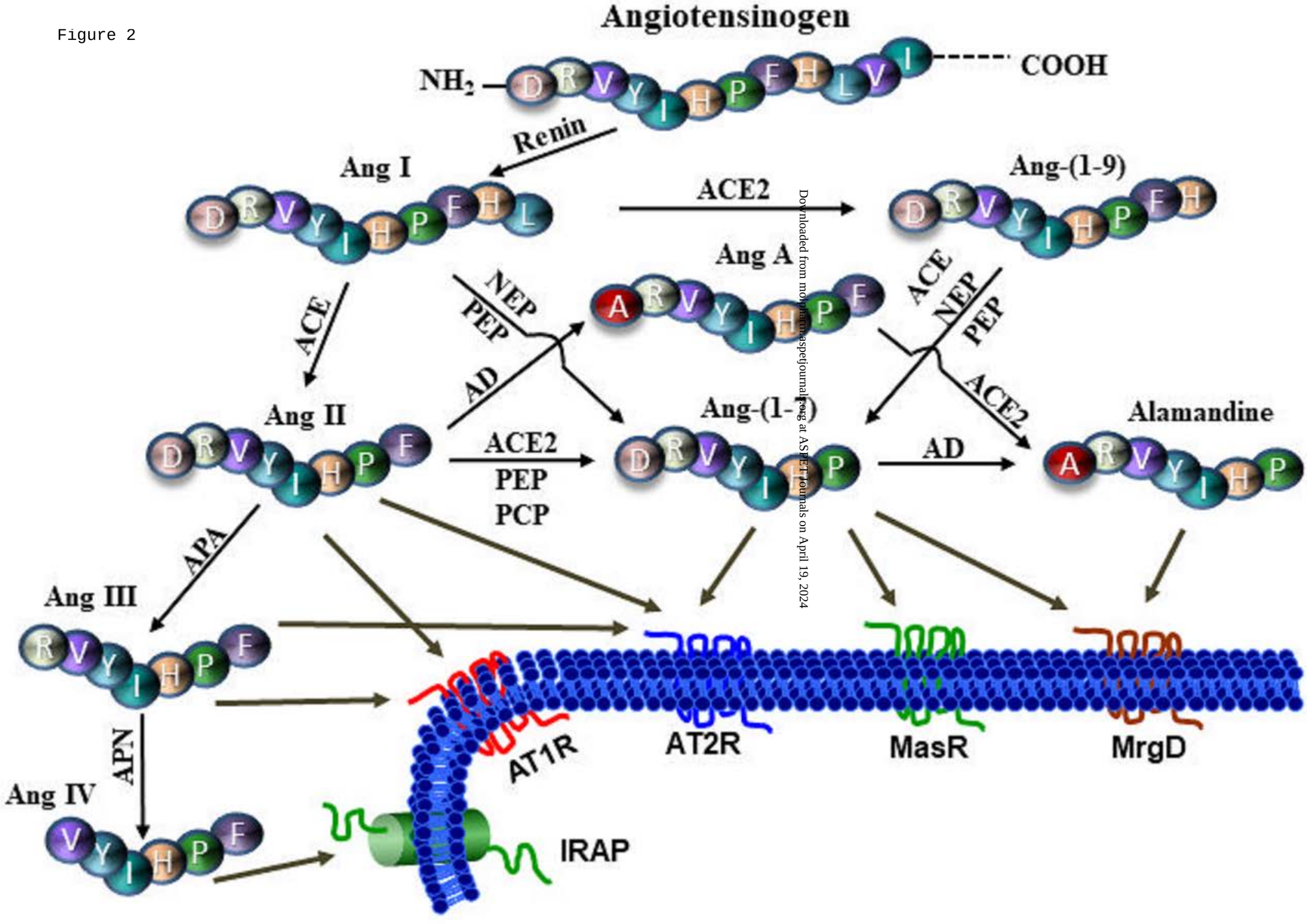

Figure 3