EMERGING HURDLES IN STEM CELL THERAPY FOR PERIPHERAL VASCULAR DISEASE

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This is a post print version of a paper published in the *Journal of Molecular Medicine*.

The final publication is available at http://link.springer.com/journal/109.

Short title: Stem Cells for PVD treatment

Manuscript-type: full-length review

N° of pages: 26 (including title page, references, figure legends and tables)

N° of figures and tables: 1 figure, 4 tables

N° of references: 108

Abstract: 272 words

Aknowledgements: The authors are supported by grants from the European Commission (StrokeMAP STREP-FP6, FP-7 European Research Council Starting Grant IMAGINED-203291), FWO, and a KULeuven Center of Excellence grant (EF/05/013).

Conflict of interest: none

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ABSTRACT

Peripheral vascular disease (PVD) is a growing medical problem in Western societies and presents itself mainly in two different clinical forms. Intermittent claudication (IC) is an early moderate manifestation, while patients with critical limb ischemia (CLI) suffer from severe muscle tissue loss or ulcers and are at high risk for limb amputation. Unfortunately, many patients cannot be helped with currently available surgical or endovascular revascularisation procedures because of the complex anatomy of the vascular occlusion and/or the presence of other risk factors. Non-invasive stem cell therapy has been proposed as an alternative for such patients. Although pioneering clinical experience with stem cell-related therapy seems promising, it is too early for general clinical use of this technique, since many questions remain unanswered. Indeed, while questions about safety, dose and administration route/timing/frequency are the first ones to be addressed when designing a stem-cell based clinical approach, there is accumulating evidence from recent (pre-)clinical studies that other issues may also be at stake. For instance, the choice of stem cells to be used and its precise mechanism of action, the need/possibility for concurrent tissue regeneration in case of irreversible tissue loss, the differentiation degree and specific vascular identity of the transplanted cells and the long-term survival of engrafted cells in the absence of a normal supportive tissue environment, should be well considered. Here, rather than presenting a comprehensive and extensive overview on the current literature on stem/progenitor cells and revascularization, we highlight some of the outstanding issues emerging from the recent (pre-)clinical literature that may co-determine the successful application of stem cells in a wide range of PVD patients in the future.

Key-words: vascular disease, stem cells, clinical research, regeneration, ischemia, endothelium

Introduction

Epidemiology of peripheral vascular disease

Currently, peripheral vascular disease (PVD), causing an inadequate oxygen supply to the limbs, globally affects no less than 3-10% of the population [1]. Sadly, however, due to a steady increase in the occurrence of associated risk factors, such as ageing, diabetes, obesity and hypertension, it is expected that the prevalence will further increase in the coming years, reaching epidemic proportions. Patients with chronic forms of PVD can be divided in asymptomatic and symptomatic with a ratio of 3:1 to 4:1. Based on the severity of the symptoms, usually two clinical presentations are distinguished: intermittent claudication (IC) is characterized by pain upon walking while critical limb ischemia (CLI) is a more severe form in which pain occurs at rest and which is accompanied by necrosis and ulceration. CLI patients, particularly with concomitant diabetes, have a poor prognosis and are at high risk for limb amputation [1]. While obstructive atherosclerotic disease is the most common cause of PVD, some forms of vasculitis, such as thromboangiitis obliterans (TAO) or Buerger's disease, also result in peripheral ischemia (in feet and/or hands), often progressing to tissue loss and major amputations [2,3].

Current treatment options and rationale for cell-based approaches

Despite the dramatic clinical impact, treatment options for PVD patients are limited. While risk factor reduction (i.e. smoking cessation, control of blood pressure, glucose and cholesterol levels, etc.) is mandatory for all PVD patients, the chosen strategy needs to be personalized and largely depends on clinical severity [1]. For patients with IC, controlled exercise, along with drug therapy is first in line. If these interventions do not improve the symptoms, revascularization can be considered. Patients diagnosed with CLI, on the other hand, should immediately be referred to a vascular specialist for revascularization. In addition, ulcer care and treatment of infection is important to prevent limb amputation. Depending on the location of the atherosclerotic obstruction, revascularization can be achieved either surgically (by bypass grafting or endarterectomy), or by endovascular techniques (e.g. stenting or balloon dilatation) [1]. Unfortunately, a significant proportion of patients (including both IC and CLI cases) is not eligible for or does not beneficially respond to these revascularization procedures due to the widespread nature or the distal location of the obstructions or due to the presence of comorbidities putting them at higher risk for peri-procedural death [4]. Similarly, in TAO patients, the diffuse segmental involvement and the distal location of the vasculitis frequently excludes them from 'classical' revascularization procedures [2].

For these 'no-option' patients, non-invasive revascularization strategies have been introduced, which fall into two categories: single gene/protein-based or cell-based strategies. Angiogenic

growth factor (e.g. vascular endothelial growth factor or VEGF, fibroblast growth factors or FGFs, and hepatocyte growth factor) therapy has been tested clinically since more than 5 years. Overall, benefit for PVD patients has been disappointing [4]. While repeated administration of the angiogenic gene may increase the chances of success as demonstrated by a recent study treating CLI patients with *FGF-1* [5], the current thought is that use of a combination of growth factors, or "master switches" (e.g. hypoxia-inducible factor (HIF)-1α), may be better strategies for successful revascularization [6] due to the molecular complexity of angiogenesis and vasculogenesis. Moreover, recently the new concept of combining angiogenesis (determining oxygen delivery) with induction of metabolic changes (regulating oxygen consumption) by (ischemic) muscle, was introduced as a promising new strategy to treat patients with ischemia [7]. In addition, the disappointing results may be related to the reduced amount or the lack of responsiveness of endogenous vascular cells in PVD patients, as the success of administration of growth factors depends on the ability of endogenous vascular cells to be recruited and expand. In this context, the concept of (stem) cell-based revascularization emerged in 1997, when Isner's group described circulating cells in adults – called 'endothelial progenitor cells' or EPC – with the capacity to differentiate into endothelial cells (EC) and incorporate into new vessels in ischemic tissue [8]. Since then, the number of studies reporting on (stem) cell-related revascularization has exponentially increased. Stem cells have also been considered for tissue-engineering to generate endothelialized arterial grafts, however, this will not be described here.

From the bench to the bedside: pioneering clinical experience with (stem) cells in PVD

Clinical study overview and general trends

Perhaps driven by the major clinical impact of PVD, the concept of cell-based therapy has moved remarkably fast from bench to bedside. Results of the first clinical study in CLI patients were published as early as 5 years after the description of EPC [9] (Table 1a). Since 2002, there has been a steady rise in the number of clinical reports, currently accumulating to at least 39 (including 775 patients) (Table 1a-c). A review of the clinical literature suggests a number of general trends. First, more than 80% of enrolled patients had CLI. Although the TACT II consensus document does not mention cell therapy as a future treatment option for IC patients [1], several trials included these patients. Second, most studies enrolled a limited number of patients or were case studies, and did not include a placebo arm. In addition, none of the studies had a double-blinded randomized design and the follow-up period was relatively short (mean follow-up time of 7.9 months). Hence these studies cannot provide definitive proof of a therapeutic effect or report on long-term safety. For instance, while there have been no reports so far on the development of cancer in these short-term studies, this potential side-effect of any pro-

angiogenic approach (gene/protein or cell-based) should be monitored for extended periods in each patient. With few exceptions, the administration route was injection into the ischemic muscle. Finally, in 34 of the 39 studies, the mononuclear fraction of bone marrow (BMMNC) and/or peripheral blood (PBMNC) was used for transplantation and all studies except one were performed in an autologous setting [10] (Table 1a-c). Interestingly, in addition to patients with ischemia due to atherosclerosis obliterans or vasculitis, some trials included patients with ischemia due to connective tissue disease (CTD; e.g. systemic sclerosis), the latter which causes EC damage leading to arteriolar occlusion [11]. Overall, clinical results show feasibility and safety (at least in the short term) and indicate an encouraging effect when comparing the patient's status before and after transplantation (Table 1a-c). Noteworthy, however, one study with TAO patients reported a high incidence of adverse effects in the long term (sudden death, worsening of ischemic ulcers or rest pain, development of an arteriovenous shunt) [12], and two other studies (one in patients with a high number of risk factors and one in CTD patients) reported that positive effects may not apply to all patient categories [11,13] (Table 1a-b).

Stem/progenitor cell characteristics of target PVD patients

Apart from transplantation trials, another significant body of clinical literature has been dedicated to analyzing the properties of stem/progenitor cells isolated from PVD patients or patients with risk factors that are usually present in no-option PVD patients – a subject that is specifically important in light of the current predominant autologous design of clinical trials. The risk factors most strongly associated with PVD are old age, diabetes, smoking, hypertension and dyslipidemia [1]. The presence of each of these risk factors has not only been associated with a lower number, but also with a reduced functionality of circulating vascular progenitor cells [14-18]. The underlying reasons for this are still incompletely understood. The accumulation of advanced glycation endproducts may contribute to decreased EPC function in older and diabetic individuals [18]. While these observations may argue in favor of using cells from allogeneic sources, the latter raises a potential problem of rejection of the cell allograft or even graft-versus-host disease. In addition to functional deficits in the EPC from patients with PVD, there is evidence that methods used to collect the cells (e.g. the use of granulocyte-stimulating growth factor or G-CSF for mobilization) may have deleterious effects on the functional revascularization capacity of the isolated cells [19].

From the bedside back to the bench: emerging new hurdles in (stem) cell therapy

While the step from the bench to the bedside was remarkably fast, additional laboratory studies on cell-based revascularization has yielded important new insights. At the same time, the initial

clinical experience also raised new questions that could only be solved by additional pre-clinical research. The findings of these new insights will now need to be implemented in 'next generation' clinical approaches in order to fully exploit the benefits of (stem) cell therapy. In the following sections, a number of these new issues ('hurdles') are discussed (Fig. 1). There are, however, other bottle-necks which we will not further elaborate on, e.g. the logistic challenge to recruit large enough numbers of 'no-option' CLI patients for the design of a randomized trial, only to give one example.

Which animal model should be used?

Ideally, a pre-clinical PVD model should represent the human disease as much as possible. Currently, the rodent is the most frequently used animal model. The pre-existing collateral network in rats is relatively abundant, while that of the mouse more closely resembles the human vascular reserve system [20]. Despite the usual chronic nature of the disease in humans (except for some cases where the ischemia occurs acutely due to thrombosis, embolism or aneurysm [1]), methods used to provoke ischemia in animals is always acute, i.e. by ligation and/or excision of one or more artery segments. Except for one study in rats, an animal model with the same natural (chronical) history as the disease in patients has not been reported [21]. However, there are a number of elements that can be exploited to mimic the clinical presentation of PVD, mainly in terms of disease severity. For instance, the more proximal the occlusion (e.g. iliac versus femoral artery), the more severe the outcome. While ligation at the level of the femoral artery usually does not result in ischemia in the adductor region, iliac artery ligation does cause necrosis in that region in addition to the more distal muscles (e.g. the gastrocnemius) [22,23]. The presence or absence of ischemia has also consequences for the type of vascular response occurring: while ischemia in the lower muscles mainly induces capillary expansion, the shear stress (in absence of an ischemic stimulus) in the adductor region mainly evokes collateral growth [23]. Importantly, some mouse strains (e.g. BalbC) are characterized by a less elaborate collateral network, thereby creating a much slower and less extensive spontaneous flow recovery and a higher incidence of tissue necrosis following ligation [22,23]. Given the contribution of T lymphocytes to collateral expansion, the lack of functional T cells in immune deficient athymic nude mice or severe combined immunodeficiency (SCID) mice also attenuates the recovery of perfusion. Therefore, BalbC/nude mice have been used as a severe model that resembles CLI; while C57Bl/6, which have a high collateral reserve, have been used as a model for IC [22]. As mentioned above, since >80% of target patients fall into the CLI category, testing a specific cell-based approach will be clinically more relevant in a CLI-like model. Severity of the model can also be increased by

implementing some of the risk factors, such as diabetes (*db/db* mice), dyslipidemia (*apoE*$^{-/-}$ mice) or old age that are generally present in 'no-option' patients [20].

Which (stem) cells are available for revascularization?

When considering stem/progenitor cells for regenerating vessels, there is a very broad assortment of cells that have been tested in animals. Given the emergence of new reports at an exponential rate, it is quite difficult to keep a complete overview. Therefore, the following paragraph and Table 2 represent a selected list of studies. For the purpose of this overview, we will discuss the different cell types that have been tested based on the differentiation potential of the stem/progenitor cells.

Embryonic stem cells (ESC). ESC are the most versatile stem cells and are therefore at the top of a hierarchy based on differentiation potential (Fig. 1). They are called 'pluripotent' given their ability to differentiate into all cell types of a living organism. These cells may have the advantage of combining vascular regeneration with tissue-specific regeneration. However, transplantation of undifferentiated ESC causes teratoma formation, such that the cells will have to be transplanted in a pre-differentiated form [24]. In addition, an important caveat is that ESC can only be used in an allogeneic setting, which may cause immune-rejection issues. Recently, several laboratories have developed differentiation/selection systems that yield relatively pure populations of vascular cells. These cells can directly contribute to newly forming vessels and a combination of endothelial and mural cells seems more effective in revascularizing ischemic tissue [25-28]. In a recent report, embryonic hemangioblasts, the common progenitors for vascular and blood cells were shown to successfully restore blood flow in ischemic mouse limbs [29].

Multipotent adult or fetal stem/progenitor cells. These cells owe their name 'multipotent' (Fig. 1) to their ability to differentiate, in addition to vascular cells, into other cell types, sometimes belonging to different embryonic germ layers. In the context of limb ischemia, the most interesting cells are likely those that can directly contribute to skeletal muscle in order to replace lost muscle tissue aside from vascular cells. Such a favorable differentiation potential has been demonstrated for several cell types in animal PVD models: BM-derived multipotent adult progenitor cells (MAPC), $CD34^+$ or $CD34^+KDR^+$ cord blood cells, an immortalized $CD34^-$ RM26 cell line generated from peripheral blood (PB), adipose tissue-derived stem cells (ADSC) and – perhaps surprisingly – fetal aorta-derived vascular progenitor cells [22,30-34]. The vascular and muscular differentiation potential of ADSC and the magnitude thereof is currently a matter of debate [35,36]. The combined effect on vascular and muscular regeneration may be essential for a durable effect of cell therapy (see below). Because of their origin (e.g. cord blood cells) or the

often long-term culture procedure to obtain the cells (e.g. MAPC), most of these cell types will require an allogeneic approach.

Endothelial progenitor cells (EPC). These cells are at the bottom of the hierarchy (Fig. 1), given their restricted potential to one cell type, i.c. EC. Since EPC constitute a subpopulation of the mononuclear fraction of BM or PB, the first clinical trials have been conducted with autologous BMMNC or PBMNC (Table 1a-b). Meanwhile, numerous laboratories around the world have been implementing EPC in their research programs, and there has been a lot of confusion concerning their origin, molecular signature and endothelial differentiation capacity in vitro and in vivo, perhaps due to small but biologically significant differences in the methodology to derive and culture them [37,38]. While a molecular definition (based on surface marker expression) or a functional definition (based on low density lipoprotein uptake or lectin-binding) has been frequently used, adherence and outgrowth kinetics is a more recent tool to define EPC subtypes [38]. Based on the latter, two main categories of EPC can be distinguished: the 'early' ones (which have a monocytic origin) and the 'late outgrowth' ones (so-called 'blood outgrowth endothelial cells' or 'BOEC'; or 'endothelial colony forming cells', which do not express monocytic markers). While 'monocytic' EPC may have an important trophic effect, BOEC have the true capacity to incorporate into nascent blood vessels [38]. Interestingly, the combination of both EPC-types was recently shown to have an additive effect on revascularization of ischemic limbs [39] (Table 2).

Comparative studies. Direct comparative studies are mandatory to get insight into which cells may be better candidates for functional revascularization (Table 2). From a clinical angle, studies in which a new cell regimen is compared with BMMNC or PBMNC are perhaps most relevant, since the latter are already being used in clinical trials (Table 1a-b). In this context, we recently compared unfractionated mouse BM cells (mBMC) with undifferentiated mouse MAPC (mMAPC-U) in a model representative for IC [22]. While mMAPC-U induced a durable increase in limb perfusion, and functional recovery of ischemic muscles, animals transplanted with mBMC only had a temporal benefit and a surprising functional deterioration was seen when animals were followed for 3-5 weeks, most likely due to increased inflammation (see below). While we used unfractionated BMC, in most clinical studies only the MNC fraction or subsets of these were used (Table 1a-b). Nevertheless, these fractions still contain mature inflammatory cells or precursors thereof, which we believe were the culprit for the severe inflammation. When MAPC, allowed to differentiate to endothelial-like cells (MAPC-EC) as well as smooth muscle-like cells (MAPC-SMC), termed vascular pre-differentiated mMAPC or mMAPC-VP, were transplanted, only a temporary effect on improved blood flow and muscle function was seen

compared with placebo. In rats, Iwase et al. found that BM mesenchymal stem cells (MSC) had a superior effect on blood flow recovery compared to BMMNC [40] and in another study BMMNC and the ADSC-containing stromal vascular fraction from fat had a comparable effect on reperfusion at 15 days after the onset of ischemia [41].

A significant number of studies have tested whether the beneficial effects of MNC from BM, PB or UCB can be ascribed to certain subfractions, some of which have been introduced into the clinic (Table 1c; Fig. 1). For instance, some studies have demonstrated that the $CD34^+$ fraction of PBMNC or umbilical cord blood (UCB), but not from ADSC, had a significantly better effect on flow restoration when compared to the $CD34^-$ fraction [34,35,42]. Others have not found a correlation between CD34 expression and revascularization. A further subfractionation into $CD34^+KDR^-$ and $CD34^+KDR^+$ cells revealed a superior effect of the latter fraction in restoring blood flow [33]. Others have documented that the beneficial effect of BMMNC may be contained within the $cKit^+$ fraction [43]. Urbich et al. showed that both monocytic ($CD14^+$) and non-monocytic ($CD14^-$) fractions of PBMNC harbor cells with the ability to support revascularization [44]. Culture expansion, which may enrich for EPC, had a positive effect on the revascularization capacity of UCB MNC or BMMNC [44,45]. There is also evidence that certain cell populations not contained in the MNC population may modulate the effect of MNC: platelets or erythroid cells have an additive effect, whereas polymorphonuclear cells (e.g. neutrophils) may impede the revascularization [46,47]. Perhaps more importantly, injection of certain subfractions as opposed to crude cell preparations may help to limit unwanted effects of cell therapy. For instance, following transplantation of unfractionated BMC in the ischemic gastrocnemius of animals, we saw a significant inflammatory response to which the injected $CD45^+$ cells themselves contributed directly [22] (Table 2). Similarly, Awad et al. saw extensive inflammation in ischemic gastrocnemius muscle injected with $CD14^+/CD34^+$ cells (Table 2), in part by recruitment of endogenous cells [42]. When injecting total human MNC cells into ischemic rat heart muscle, Kawamoto et al. found that these rats – unlike those that were treated with purified $CD34^+$ cells – developed severe hemorrhagic myocardial infarcts which were infiltrated with donor $CD45^+$ cells [48]. Therefore, mature inflammatory cells should likely be eliminated from cell preparations used to treat ischemic tissues that are already heavily infiltrated with host inflammatory cells.

How do (stem) cells make new vessels?

The question of how (stem) cells exert their therapeutic effect is certainly not trivial. During adult life, (stem) cells can contribute to the formation of new vessels mainly in two ways (Fig. 1): by direct incorporation into newly forming sprouts (‘vasculogenic’ effect) or, indirectly, by

delivering angiogenic growth factors that activate/recruit endogenous vascular cells ('trophic' effect). The relative contribution of both mechanisms depends on the tissue context and the intrinsic characteristics of the (stem) cells. While many early studies claimed a significant – up to 50% - direct contribution of grafted (stem) cells to newly forming vessels, more recent studies have demonstrated that vascular incorporation of EPC (and BM-derived cells in general) is minimal and have suggested that their vascular potential may therefore be chiefly – if not exclusively – trophic [37,49]. Not more than 3% of MAPC-U, for instance, directly contributed to vessels. However MAPC-U secrete significantly higher levels of VEGF than BMC [22]. MAPC contribution to skeletal muscle was, as expected, at least in part by fusion with host myoblasts [22]. Fusion of MAPC with EC was not observed, rather MAPC-derived vascular cells formed hybrid vessels together with host cells [22], in agreement with what others observed with BM-derived cells [50]. Recently, the concept was launched that transplanted cells may instruct host cells to secrete angiogenic growth factors, rather than secreting these factors themselves [3] (Fig. 1). What the contribution of grafted cell versus endogenous cell-produced angiogenic growth factors is to revascularization is not yet known.

In addition to the direct/or indirect contribution to vessel growth, another important aspect of (stem) cell-based approaches is the necessity to target all mechanisms of revascularization, which may require different cellular targets in different regions of the muscle. While capillary expansion may be essential for adequate distribution of blood to the muscle more distant from the vascular occlusion site, the perhaps more crucial requirement for efficient revascularization is to increase the bulk flow by expansion of the collateral bed in the region more proximal to the occlusion. While capillary growth involves EC, the growth of collaterals is mainly triggered by monocytes and involves both EC and SMC expansion [23,51]. In agreement with their significant effect on restoring functional blood flow to the distal parts of the limb, we found that MAPC significantly increased the collateral bed upon transplantation in the adductor region, in part by their potential to secrete factors that target EC, SMC and monocytes [22].

Is vascular regeneration enough?

In most cases, revascularization is considered for CLI patients that already have irreversible muscle damage (Table 1a-c). Hence, perhaps the ideal therapy would yield combined vascular and muscular regeneration, which could be termed 'angiomyogenesis' (Fig. 1). As mentioned above, this combined differentiation potential has been demonstrated for a number of cell types (Table 2). In order to answer the question whether concomitant muscle regeneration is necessary for a durable effect of cell therapy, we compared cells that have the ability to generate vascular as well as skeletal muscle cells (MAPC-U) with cells with restricted vascular potential (a

combination of MAPC-EC and MAPC-SMC, i.e. MAPC-VP). We found that MAPC-VP only had a temporary positive effect while MAPC-U had a sustained positive effect on limb perfusion, function and viability, suggesting that the ideal therapy may be one wherein both the vasculature and the muscle compartment can be restored [22]. In accordance with our findings, a recent study comparing undifferentiated with vascular pre-differentiated cells from fetal aorta found that the vascular pre-differentiated cells lacking skeletal muscle contribution did not reduce the incidence of toe necrosis as opposed to the undifferentiated cells that had a combined vascular and skeletal muscle potential in vivo [32]. In addition to a direct contribution, the trophic effect of MAPC-U on skeletal muscle likely was responsible for the beneficial effect on limb function [22].

In which form should the cells be administered?

The choice of an appropriate stem cell type is only one step in the complex decision process for designing a rationale for therapy. Below, we discuss a number of additional potentially important variables.

Differentiation state. Stem/progenitor cells can be administered in an undifferentiated state or in a vascular pre-differentiated state (Fig. 1). While undifferentiated stem cells have the potential advantage of combined vascular and muscular contribution (see above), transplantation of undifferentiated cells may result in unwanted side effects such as uncontrolled proliferation and/or differentiation into unwanted cell types. For instance, ESC require pre-differentiation in order to avoid teratoma formation [24]. On the other hand, a growing number of studies shows that the transplantation of fully differentiated EC does not help to improve ischemic tissue revascularization, unless they are genetically preconditioned (see below) [27,39,49,52,53]. Also, Yamahara et al. showed that immature ES-derived SMC progenitors – unlike fully differentiated aortic SMC – significantly contribute to neovascularization [28]. Pre-differentiation of the stem cells could be advantageous since the environment in the ischemic muscle may have lost the molecular cues necessary to induce correct differentiation. On the other hand, differentiated cells may be more sensitive to oxygen and nutrient deprivation, and may therefore undergo significantly more apoptosis in an ischemic environment. For instance, transplantation of equal numbers of undifferentiated and vascular pre-differentiated MAPC into ischemic muscle resulted in a two-three-fold lower number of residual MAPC-VP two weeks after transplantation [22]. Although it is possible that higher immunogenicity of the pre-differentiated cells contributed to their elimination, these results may well be compatible with the hypothesis that MAPC-VP are more prone to cell death in an ischemic environment, a question that is currently under investigation.

Vascular pre-specification. As mentioned above, in an ischemic environment, where part of the tissue is replaced by fibrotic scar, appropriate signals to guide specific differentiation of the stem cells may be missing. Because restoring arterial supply of oxygen and nutrients is the most crucial requirement to save tissue at risk of dying, it is conceivable that pre-specializing EC to an arterial identity may be advantageous. Therefore, recent studies have tested whether it is possible to induce such endothelial specification during stem cell differentiation in vitro (Fig. 1). Several manipulations of the culture conditions induce arterial specification in ESC, including increasing VEGF concentrations or adding adrenomedullin, that activates the Notch signaling pathway, to the culture system [54,55]. While constitutive Notch activation alone was not sufficient to induce an arterial phenotype in ESC [55], we found that the combined activation of the Patched and Notch pathways using an 'arterial cocktail' composed of VEGF, sonic hedgehog and the Notch ligand delta-like 4 was sufficient to boost arterial EC differentiation from MAPC in vitro [56]. Moreover, this cocktail also induced arterial differentiation of MAPC in vivo in a matrigel model and had an 'arterializing' effect on the infiltrating host vessels [56]. Whether such arterializing effect is also beneficial in limb ischemia is currently being tested.

Pre-conditioning to increase survival/effectiveness. Survival/effectiveness of the transplanted cells can be improved either by changing the cell's characteristics or by 'priming' them and/or the environment in which they will lodge (Fig. 1). Several manipulations (genetic, physical or chemical) have been considered to harness cells against the 'hostile environment' of the ischemic muscle and/or to increase functional activity, including pre-treatment of the cells with an endothelial NO synthase (eNOS) enhancer, β2-integrin activating antibodies, fibronectin, hypoxic or anoxic pre-conditioning, and overexpression of angiogenic/survival/anchorage factors (e.g. VEGF, HIF-1α, catalytically inactive glycogen synthase kinase-3β, telomerase reverse transcriptase, integrin-linked kinase, eNOS or sphingosine-1-phosphate) [57-67]. We found that MAPC-U, which are routinely cultured under low oxygen conditions [22], were significantly protected against serum-starvation induced cell death in vitro (Luttun et al., unpublished results), which may have contributed to their increased engraftment compared to BMC [22]. In some cases, genetic manipulation (e.g. overexpression of inhibitor of differentiation or Id, or cathepsin-L) of fully mature EC was able to revert their inability to contribute to newly forming vessels [52,53]. Changing the local environment is another way to improve cell survival and behavior. For instance, Asano et al. exposed their mice to hyperbaric oxygen following femoral artery ligation while Akasaki et al. applied thermal therapy, both of which induced a local angiogenic response that may be beneficial for stem cell homing and survival [68,69]. Low-dose irradiation also changed the microenvironment within ischemic limbs by VEGF and matrix

metalloproteinase (MMP)-9 induction [70]. Ischemic pre-conditioning has even been included as a parameter in a recent clinical trial in IC patients [71]. To increase the effectiveness, combinatorial approaches have been considered, such as combined mobilization of endogenous cells (e.g. with G-SCF, statins or erythropoietin) and transplantation of exogenous cells, co-transplantation of different cell types (e.g. hematopoietic cells and EPC ('hemangiogenesis') or BOEC and early EPC; Table 2), combining stem cell therapy with metabolic intervention, local injection of genes/proteins (e.g. angiopoietin-1, bFGF) or tissue-engineered matrix scaffolds [39,47,61,72-76].

Conclusion

Developing and validating conventional drugs for clinical use is a process that usually takes at least a decade. As such, (stem) cell transplantation, which may be considered as a form of therapy that is far more complicated than a pharmaceutical compound, was introduced surprisingly early into the clinic. Despite some apparent positive effects observed in the pioneering clinical studies of (stem) cell therapy in PVD patients, sufficient caution should be used in moving (stem) cell-based therapies in the clinic to avoid complications that may set back the field, and to allow for logical progression of clinical studies that address each hurdle that presents itself along the way (Fig. 1). Therefore, extensive interactions and communication between clinicians and scientists is crucial. This will not only improve the quality and relevance of pre-clinical research but also allow to constantly fine-tune the design of therapeutic strategies for PVD patients.

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LEGENDS TO FIGURES AND NOTES TO TABLES

Figure 1. ***New hurdles in (stem) cell therapy for PVD***

Optimizing a (stem) cell-based therapy is a long and multi-factorial procedure, in which several steps ('hurdles' ***1-4***) need to be taken to go from the bench to the bedside. Initial clinical experience may reveal new riddles that lead us back to the bench (red arrow) for further scrutiny before going to another clinical phase (blue arrow). Thus, rather than being a linear 'one-way street', therapy design is more adequately represented as a cyclic track. While questions about safety, dose and administration route/timing/frequency were the hurdles to be taken in the first round, other issues may prevail in the next. The choice of the better cell source/subset is a first hurdle to be taken, which requires comparative studies between different cell types from the stem cell 'hierarchy' (inset ***1***, left) or between several subfractions of a certain source (inset ***1***, right). Secondly, determining the mechanism of action is not merely an academic question, but is of significant clinical relevance. Cells can directly contribute by being incorporated into nascent vessels (inset ***2***, left). Alternatively, cells can deliver growth factors (GF) that instruct endogenous vascular cells (inset ***2***, right, upper dashed box). A new mechanism is that transplanted cells instruct host cells to secrete angiogenic growth factors (inset ***2***, right, lower dashed box). Combining revascularization with muscular regeneration ('angiomyogenesis') could be an important added value for long-term therapeutic benefit (inset ***3***). Finally, exploiting the full potential of (stem) cell therapy in PVD may require decisions about the appropriate differentiation state, the need for arterial pre-specification and other forms of genetic, chemical or physical pre-conditioning (inset ***4***). EC: endothelial cells; ESC: embryonic stem cells; MAPC: multipotent adult progenitor cells; EPC: endothelial progenitor cells.

Table 1. ***Overview of clinical studies using (stem/progenitor) cells in PVD patients***

Abbreviations: Ref.: reference; Tx: transplantation method; F-U: follow-up (expressed in months); A: autologous; CLI: critical limb ischemia; IC: intermittent claudication; CTD: connective tissue disease/collagen disease; TAO: Thromboangiitis Obliterans; AO: Atherosclerosis Obliterans; BMMNC: bone marrow mononuclear cells; PBMNC: peripheral blood MNC; UCB-MSC: umbilical cord blood-derived mesenchymal stem cells; G-CSF: granulocyte-colony stimulating factor; i.m.: intra-muscular; i.a.: intra-arterial; ABI: ankle-brachial index; $TcPO_2$: transcutaneous oxygen pressure; angio: angiography; w: weeks; SaO_2: arterial oxygen saturation; SvO_2: venous oxygen saturation; DBI: digital-brachial index; NM: not mentioned; MRA: magnetic resonance angiography.

Notes: [a]: In this study, part of the patients or all patients were injected with total PBMNC in the contralateral leg as a reference therapy; [b]: In this study, half of the patients were injected with BMMNC and the other half with PBMNC; [c]: patient characteristics not described in enough detail; [d]: deducted from the fact that rest pain reduction was one of the clinical outcomes; [e]: G-SCF mobilized PBMNC were used. Studies having a negative outcome are highlighted in light grey.

Table 2. ***Overview of comparative pre-clinical PVD studies in rodents using (stem) cells***

Abbreviations: AU: first author; Y: year; Ref.: reference; h: human; m: murine; PBMNC: peripheral blood mononuclear cells; LD: Laser-doppler; MSC: mesenchymal stem cells; BMMNC: bone marrow mononuclear cells; NOD-SCID: non-obese-diabetic severe combined immunodeficiency; UCB-EPC: umbilical cord blood-derived endothelial progenitor cells; BM-EPC: bone marrow-derived endothelial progenitor cells; NS: no significant effect; PMN: polymophonuclear cells; OEC: outgrowth endothelial cells; GEAEC: gastroepiploic arterial endothelial cells; MAPC-U: undifferentiated multipotent adult progenitor cells; MAPC-VP: vascular pre-differentiated MAPC; G-CSF: granulocyte-colony stimulating factor; Imm. sup.: immune-suppressed; KDR: kinase-domain receptor; SVF: stromal vascular fraction; ADSC: adipose tissue-derived stem cells; FA: fetal aorta; ESC: embryonic stem cells; VPC: vascular progenitor cells; EC: endothelial cells; MC: mural cells.

Table 1a. ***Overview of clinical studies using total BMMNC in PVD patients***

1st Author, Year, [Ref.]	*Patients*	*Tx*	*F-U*	*A ?*	*Outcome/Comments*
Tateishi-Yuyama, 2002 [9][a]	47 (CLI, all AO)	i.m.	6	Yes	ABI↑;$TcPO_2$↑;rest-pain score↓; pain-free walking↑
Esato, 2002 [77]	8 (CLI, 4 TAO, 4 AO)	i.m.	1	Yes	rest-pain score↓;temperature↑; ulcer healing↑
Saigawa, 2004 [78]	8 (7 CLI, 1 IC, all AO)	i.m.	1	Yes	ABI↑;$TcPO_2$↑
Higashi, 2004 [79]	7 (CLI, all AO)	i.m.	6	Yes	ABI↑;$TcPO_2$↑;rest-pain score↓; pain-free walking↑
Miyamoto, 2004 [80]	12 (CLI, including 5 TAO, 3 AO, 1 CTD)	i.m.	1	Yes	ABI↑;pain↓;walking↑;perfusion↑;BMMNC+ platelets
Hasebe, 2004 [81]	1 (CLI, all AO)	i.m.	1	Yes	angio↑; pain↓
Nizankowski, 2005 [82]	10 (CLI, 7 TAO, 3 AO)	i.m.	12	Yes	ABI↑; $TcPO_2$↑; pain↓;blood flow (Doppler) ↑
Gu, 2006 [83]	35 (5 IC, 30 CLI, various cases)	i.m.,i.a., i.m.+i.a.	5 ± ?	Yes	ABI↑;$TcPO_2$↑; angio↑; ulcer healing↑; BMMNC were G-CSF-stimulated
Bartsch, 2006 [84]	1 (IC, AO)	i.m.+i.a.	2.5	Yes	ABI↑;$TcPO_2$↑; walking distance↑
Gu, 2006 [85]	22 (some 'mild', others 'severe' ischemic)	i.m.	1	Yes	ABI↑;$TcPO_2$↑;angio↑;ulcer healing↑;amputation rate↓
Bartsch, 2006 [86]	10 (IC?[c], all AO)	i.m.+i.a.	2	Yes	ABI↑;SvO_2↑; treadmill performance↑
Miyamoto, 2006 [12]	8 (CLI, all TAO)	i.m.	23 ± 18	Yes	4w: angio↑;ABI=; pain↓; long-term: adverse effects
Durdu , 2006 [2]	28 (CLI, all TAO)	i.m.	17 ± 8	Yes	ABI↑;pain↓;walking time↑
Koshikawa, 2006 [87]	7 (CLI, 6 TAO, 1 CTD)	i.m.	6	Yes	DBI↑; pain↓; ulcer healing↑; (all hands)
Hernández, 2007 [88]	12 (CLI, all AO)	i.m.	6	Yes	ABI↑;SaO_2↑;rest-pain score↓; pain-free walking↑
Gu, 2007 [89][b]	21 (various cases, including TAO)	i.m.	8 ± ?	Yes	ABI↑;$TcPO_2$↑;angio↑;ulcer healing↑;amputation rate↓
Kajiguchi, 2007 [90]	7 (CLI, 3 TAO, 4 AO)	i.m.	1	Yes	ABI↑;$TcPO_2$↑;angio↑;pain↓;(only in TAO cases)
Bartsch, 2007 [71]	13 (IC, AO)	i.m+ i.a.	13	Yes	ABI↑;SvO_2↑; walking distance↑
Huang, 2007 [91]	150 (CLI[d], all AO)	i.m.	3	Yes	ABI↑;$TcPO_2$↑;temperature↑;rest-pain↓;pain-free walking distance↑
Kamata, 2007 [11][a]	6 (CLI, all CTD)	i.m.	12	Yes	pain↓ (not all cases); $TcPO_2$= ;temperature = or ↓
Saito, 2007 [92]	14 (CLI, all TAO)	i.m.	24	Yes	rest-pain↓;ulcer healing↑
Van Tongeren, 2008 [93]	25 (19 CLI and 6 IC, all TAO)	i.m.,i.a., i.m.+i.a.	12	Yes	ABI↑;pain-free walking↑; pain↓
De Vriese, 2008 [13]	16 (CLI, AO, high level risk factors)	i.m.	3	Yes	ABI=;$TcPO_2$↑; angio=; only modest improvement, restricted to the least affected patients
Wester, 2008 [94]	8 (CLI, all AO)	i.m.	8	Yes	Pain↓; physiological tests = or ↑

Table 1b. ***Overview of clinical studies using total PBMNC in PVD patients***

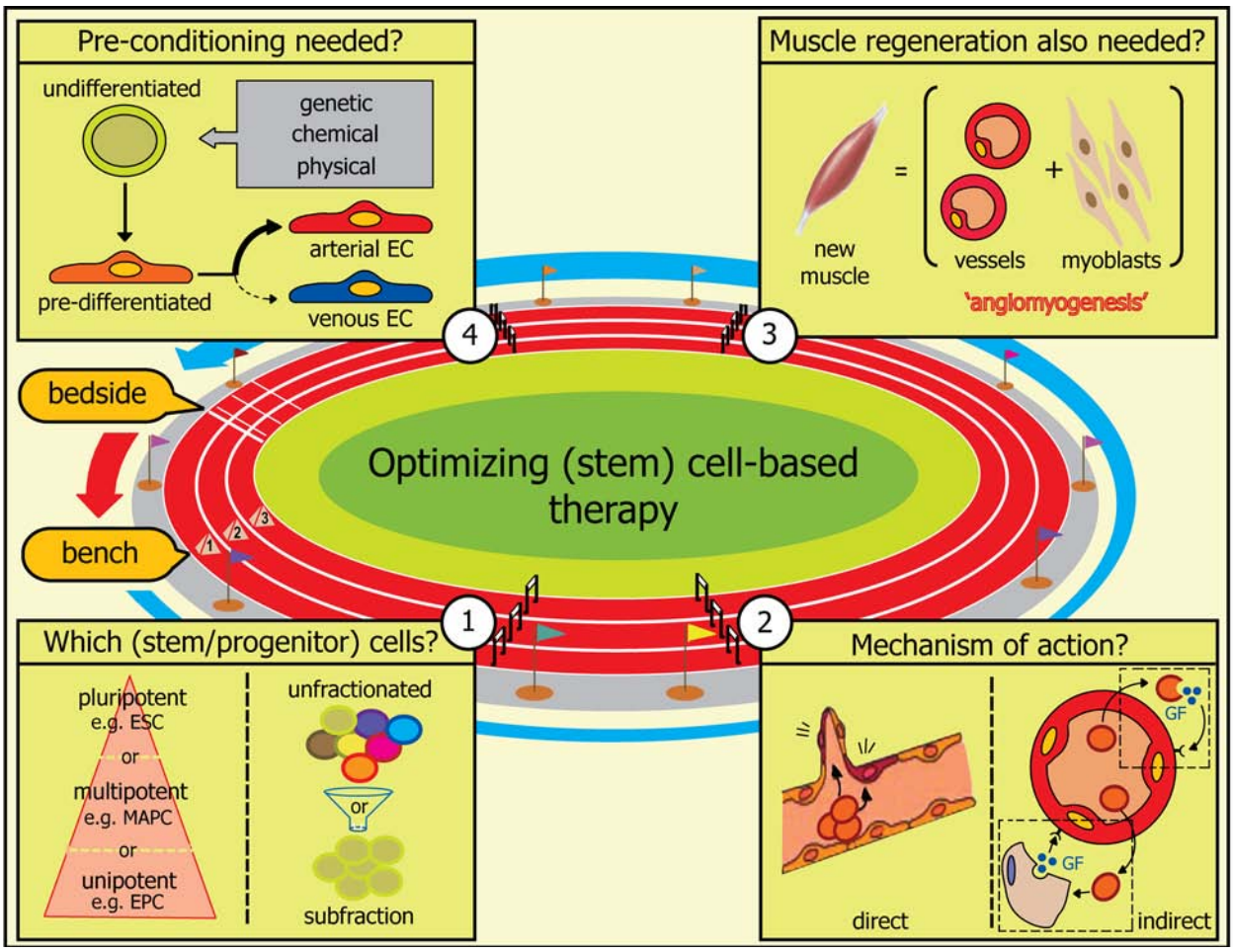
1st Author, Year, [Ref.]	*Patients*	*Tx*	*F-U*	*A?*	*Outcome/Comments*
Tateishi-Yuyama, 2002 [9][a]	22 (CLI, all AO)	i.m.	6	Yes	ABI↑;$TcPO_2$↑;rest-pain score↓; pain-free walking↑
Huang, 2004 [95]	5 (CLI, all AO)	i.m.[e]	3	Yes	ABI↑;angio↑;perfusion (Doppler) ↑
Lenk, 2005 [96]	7 (CLI, all AO)	i.a.[e]	3	Yes	ABI↑;$TcPO_2$↑;pain-free walking↑; G-CSF mobilized and ex vivo expanded
Huang, 2005 [97]	28 (CLI, all AO)	i.m.[e]	3	Yes	ABI↑;angio↑;pain↓;amputation rate↓;ulcer healing↑
Ishida, 2005 [98]	6 (CLI, 5 TAO, 1 AO)	i.m.[e]	6	Yes	ABI↑; walking distance↑; ulcer healing↑
Yang, 2005 [99]	62 (CLI, various cases)	i.m.[e]	6	Yes	ABI↑;$TcPO_2$↑; angio↑;pain↓; ulcer healing↑
Sugihara, 2005 [100]	1 (CLI, AO)	i.m.	12	Yes	$TcPO_2$↑;temperature↑; ulcer healing↑
Kawamura, 2005 [101]	30 (CLI, various cases)	i.m.[e]	6 ± 5	Yes	amputation rate↓; temperature↑
Kawamura, 2006 [102]	92 (75 CLI, 11 IC, 6 asymptomatic; 87 AO, 1 TAO, 4 CTD)	i.m.[e]	9 ± ?	Yes	amputation rate↓; temperature↑
Kajiguchi, 2007 [90]	7 (CLI, 3 TAO, 4 AO)	i.m.	1	Yes	ABI↑;$TcPO_2$↑;angio↑;pain↓;(only in TAO cases)
Zhang, 2007 [103]	15 (CLI)	i.m.[e]	12	Yes	ABI↑; pain↓; pain-free walking↑;ulcer healing↑
Hoshino, 2007 [104]	7 (CLI, all AO)	i.m.[e]	24	Yes	ABI↑;angio↑;temperature↑;ulcer healing↑
Gu, 2007 [89][b]	21 (various cases including TAO)	i.m.	8 ± ?	Yes	ABI↑;$TcPO_2$↑;angio↑;ulcer healing↑;amputation rate↓
Huang, 2007 [91]	150 (CLI[e], all AO)	i.m.[e]	3	Yes	ABI↑;$TcPO_2$↑;temperature↑;rest-pain↓;walking distance↑
Kamata, 2007 [11][a]	6 (CLI, all CTD)	i.m.	12	Yes	pain↓ (not all cases); $TcPO_2$= ;temperature = or ↓

Table 1c. ***Overview of clinical studies using specific cell types/subfractions in PVD patients***

1st Author, Year, [Ref.]	*Patients*	*Cell type*	*Tx*	*F-U*	*A?*	*Outcome/Comments*
Kudo, 2003 [105]	2 (CLI, AO)	CD34+ PBMNC	i.m.[e]	0.5	Yes	$TcPO_2$↑;angio↑
Kim, 2006 [10]	4 (CLI, TAO)	UCB-MSC	i.m.	4	No	rest-pain↓; ulcer healing↑; angio↑
Cañizo, 2007 [106]	1 (CLI, AO)	CD133+ PBMNC	i.m.[e]	17	Yes	limb salvage; walking distance↑;flow (MRA)↑
Kolvenbach, 2007 [107]	15 (CLI, AO?[c])	CD34+ or CD133+ PBMNC	i.m.[e]	NM	Yes	limb salvage
Boda, 2008 [108]	5 (CLI, AO?[c])	CD34+ BMMNC	i.m.	12	Yes	ABI↑;$TcPO_2$↑;angio↑;pain↓;walking distance↑; ulcer healing↑

Table 2. ***Overview of comparative pre-clinical PVD cell-based studies in rodents***

AU, Y, [Ref.]	*Model*	*Cell source/type*	*Outcome*
Urbich, 2003 [44]	Athymic nude mice	-cultured CD14- from hPBMNC (A) -cultured CD14+ from hPBMNC (B) -uncultured CD14+or- hPBMNC (C)	-A = B (LD) -C: NS (LD)
Iwase, 2005 [40]	Lewis rats	-autologous MSC (A) -autologous BMMNC (B)	-A > B (LD)
Finney, 2006 [45]	NOD-SCID mice	-hUCB-EPC (A) -hBM-EPC (B) -uncultured hUCB-MNC (C) -uncultured hBMMNC (D)	-B > A at 7 days (LD) -B = A at 14 days (LD) -A-D NS at 28 days (LD) -C and D: NS (LD)
Iba, 2002 [46]	Nude rats	-hPBMNC + hplatelets (A) -hPBMNC + hplatelets + hPMN (B) -hplatelets (C)	- A > B = C (LD) - PMN attenuate effect (LD)
Yoon, 2005 [39]	Athymic nude mice	-early hEPC (A) -hOEC (B) -early hEPC + hOEC (C) -hGEAEC (D)	-C > A = B > D (LD) -D: NS (LD)
Awad, 2006 [42]	Diabetic athymic nude mice	-hPBMNC (CD34+) (A) -hPBMNC (CD34-) (B) -hPBMNC (CD14+) (C) -(A) + (C) = (D)	-A > D = C > B (LD) -B: NS (LD) -more necrosis in C and D -more inflammation in D
Aranguren, 2008 [22]	C57Bl/6 mice	-mMAPC-U (A) -mBMMNC (B) -mMAPC-VP (C)	-A > B =C (LD) ->14 days: B, C: NS (LD) -A: direct muscle contribution -more inflammation in B
Pesce, 2003 [34]	Imm.sup. CD1 mice	-hUCB MNC (CD34+) (A) -hUCB MNC (CD34-) (B)	-A > B (arteriolar density) -B: NS (arteriolar density) -A: direct muscle contribution
Madeddu, 2004 [33]	CB17-SCID-bg mice	-hUCB MNC (CD34+KDR+) (A) -hUCB MNC (D34+KDR-) (B)	-A > B (LD) -B: NS (LD) -A: direct muscle contribution
Planat-Benard, 2004 [41]	C57Bl/6 mice	-mSVF (fat) (A) -mBMMNC (B) -3T3 fibroblasts (C)	-A = B > C (LD) -C: NS (LD)
Moon, 2006 [35]	BalbC/nude mice	-cultured hADSC (CD34+) (A) -cultured hADSC (CD34-) (B)	-A = B (LD)
Invernici, 2007 [32]	CB17-SCID-bg mice	-undiff. hFA (CD31-CD133+) cells (A) -vascular differentiated hFA cells (B) -early hEPC (C)	-A > B = C (LD) -B, C: NS (LD) -A: direct muscle contribution
Yamahara, 2008 [28]	KSN/SLC nude mice	-ESC-derived hVPC EC+MC diff. (A) -ESC-derived hVPC EC diff. (B) -ESC-derived hVPC MC diff. (C) -hUCB-MNC (CD34+) (D) -early hPBMNC-EPC (E)	-A > B-E at day 14 (LD) -B-E > control at day 14 (LD) -A > B-E at day 42 (LD) -E: NS at day 42 (LD) -B > E at day 42 (LD)
Li, 2003 [43]	C57Bl/6 mice	-mBMMNC (CD117+) (A) -mBMMNC (CD117-) (B) -total mBMMNC (C)	-A = C > B (LD) -B: NS (LD)


Aranguren et al.
Pre-conditioning needed?
undifferentiated
genetic
chemical
physical
arterial EC
pre-differentiated
venous EC
Muscle regeneration also needed?
=
+
new
muscle
vessels
myoblasts
'angiomyogenesis'
4
3
bedside
bench
Optimizing (stem) cell-based therapy
1
2
Which (stem/progenitor) cells?
pluripotent
e.g. ESC
or
multipotent
e.g. MAPC
or
unipotent
e.g. EPC
unfractionated
or
subfraction
Mechanism of action?
GF
GF
direct
indirect
Figure 1