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Stem cell-derived endothelial cells for cardiovascular disease: a therapeutic perspective

Daniel M. Reed,[1] Gabor Foldes,[2] Sian E. Harding[2] & Jane A. Mitchell[1]

[1]Department of Cardiothoracic Pharmacology, National Heart and Lung Institute, Imperial College London, London, UK and [2]Department of Cardiac Pharmacology, National Heart and Lung Institute, Imperial Centre for Translational and Experimental Medicine, London, UK

Correspondence
Daniel Mark Reed MRes, Department of Cardiothoracic Pharmacology, Guy Scadding Building, National Heart and Lung Institute, Imperial College, London SW3 6LY, UK.
Tel.: 0207 3528121 x3077
Fax: 0207 8233392
daniel.reed09@imperial.ac.uk

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Stem cell therapy and organ regeneration are therapeutic approaches that will, we suggest, become mainstream for the treatment of human disease. Endothelial cells, which line the luminal surface of every vessel in the body, are essential components in any organ regeneration programme. There are a number of potentially therapeutic endothelial cell types, including embryonic, adult progenitor and induced pluripotent stem cell-derived endothelial cells, as well as host vascular cells. The features (benefits as well as disadvantages) of each cell type that make them potentially useful in therapy are important to consider. The field of stem cell biology is well developed in terms of protocols for generating endothelium. However, where there is a distinct and urgent unmet need for knowledge concerning how the endothelial cells from these different sources function as endothelium and how susceptible they may be to inflammation and atherosclerosis. Furthermore, where stem cells have been used in clinical trials there is little commonality in protocols for deriving the cells (and thereby the specific phenotype of cells used), administering the cells, dosing the cells and/or in assessing efficacy attributed to the cells themselves. This review discusses these and other issues relating to stem cell-derived endothelial cells in cell therapy for cardiovascular disease.

Types of stem cell-derived endothelial cells

Human embryonic stem cell-derived endothelial cells

Human embryonic stem cells (hESCs) are an attractive source of therapeutic endothelial cells because they are pluripotent (i.e. can form any cell type in the body) and can be stored in large numbers for long periods of time, are expandable and have unlimited renewal capacity. Human embryonic stem cells are derived from the inner cell mass of the preimplantation blastocyst [1], and in certain culture conditions hESCs have the ability to form a range of cell lineages, including endothelial cells. Endothelial cells were first derived from hESCs in Robert Langer's laboratory in 2002 using a cell culture technique that involved spontaneous differentiation of hESCs into mixed populations, so-called embryoid bodies [2]. Embryoid bodies were then grown in the presence of endothelial cell growth factors to support a vascular endothelial cell expression pathway. Sorting of embryoid bodies by fluorescence-activated cell sorting for endothelial cell markers subsequently allowed isolation of a cell population that expressed endothelial cell markers (CD31, VE-cadherin and von Willebrand factor (vWF)) and formed tubes when expanded *in vitro*. It is this cell population that is defined as hESC-endothelial cells (hESC-ECs). Whilst the definition of these cells appears consistent throughout the literature, alternative and more efficient protocols for differentiation and isolation of hESC-ECs are now available [3, 4]. Research continues to improve the stability and expandability of these cells, and strategies exist for circumventing immunogenicity issues with these cells [5].

Whilst no human clinical trial data yet exist for the use of hESC-ECs, they have been shown to form vascular networks *in vivo* [2, 6, 7] and to improve cardiac function in animal models of ischaemic heart disease [8]. It is encouraging to note that other cell types derived from hESCs have shown some success in human clinical trials. In 2009, the first phase I clinical trial for use of hESCs by Geron was approved by the FDA (Clinical trials identifier: NCT01217008). In 2010, however, the field of embryonic stem cell therapy took a body blow because this major investor in the area announced that it was suspending its

future hESC research programme (http://www.guardian.co.uk/science/2011/nov/15/geron-abandons-stem-cell-therapy). This turn of events, together with the long-standing debate surrounding ethical issues of using hESCs, adds increased momentum to the search for alternative sources of stem cell-derived endothelial cells. The important questions are, which stem cell-derived endothelial cell is most suitable, safest and most efficient for the treatment of vascular disease? The hESC-ECs are certainly a powerful cell type in terms of promoting vascular repair and show great potential in cardiovascular medicine, but for reasons of both ethics and safety (immunogenicity and tumour formation) may not prove to be the best cell choice in all situations.

Endothelial progenitor cells

In 1997, it was suggested by Asahara *et al*. that a population of cells exists that are derived from the bone marrow and are capable of differentiating into endothelial cells and forming new vessels at sites of ischaemia [9]. This marked a shift towards the concept of physiological postnatal neoangiogenesis, and the term endothelial progenitor cell (EPC) was introduced. A number of studies have followed that defined this novel cell population and their use as biomarkers and in cell therapy [10]. There has been controversy over their definition and their role in vascular biology. As a result, no unifying definition of these cells exists, and the term EPC in fact encompasses a number of cell types with putative roles in vascular homeostasis and disease. However, there is some agreement in the nature of 'early' *vs.* 'late' outgrowth endothelial cells (EPCs), which can be enriched in cultures using specific isolation protocols. The nature of these cells is described in detail elsewhere [11]. It appears, to date, that one of four EPC culture strategies is generally used to expand cells identified by expression of CD31, CD34 and Vascular endothelial growth factor receptor-2 (VEGFR2), together with vWF and/or endothelial nitric oxide synthase (eNOS). Whether variation in the isolation strategy of EPCs can result in endothelial cell populations different enough to affect clinical outcome remains unknown.

In the context of tissue repair, the role of EPCs *vs.* flanking vascular endothelial cells has been widely discussed and debated [12, 13]. *In vivo* evidence for EPC-driven vascular repair came in 2003, when it was found that EPCs could populate the new endothelium in vein grafts on carotid arteries of mice [14] and that injection of these cells could reverse vascular dysfunction [15]. The mechanism by which EPCs function to repair damaged vasculature is unclear. Some groups have shown in similar *in vivo* models that the flanking vasculature endothelial cells rather than bone marrow-derived progenitor cells repair local damaged endothelium [16, 17]. The potential for improved vascular function by injection of healthy EPCs still stands nonetheless.

In health, EPCs are thought to be critical for vascular homeostasis [18–20]. As such, levels of EPCs are negatively correlated with cardiovascular diseases, including hypertension, pulmonary hypertension, diabetes mellitus, carotid artery disease, sepsis and heart failure [21–25]. Taken together, these studies suggest that dysfunctional and/or low levels of circulating EPCs can contribute to disease. With this in mind, as discussed below in 'data from the clinic', there is a potential therapeutic utility in administering EPCs and/or mobilizing EPCs from the bone marrow using drugs.

Finally, EPCs are attractive in the area of organ regeneration for transplant. They offer benefits over ESC-ECs as a source of endothelium because they can be grown from the patient's own tissue, thereby avoiding issues of rejection. Furthermore, the efficacy and safety of pure autologous EPC injections have been assessed in patients with idiopathic pulmonary hypertension [26]. However, it should also be remembered that endothelial cells are not terminally differentiated and can be isolated and expanded from donor vessels. The benefit of endothelium from host EPCs *vs.* host vessels in the context of tissue and organ regeneration remains to be established.

Induced pluripotent stem cell-derived endothelial cells

Human induced pluripotent stem cells (iPSCs), first engineered by Yamanaka's group in 2007 [27], are adult human cells that have been reprogrammed into a pluripotent phenotype. In the original paper, Yamanaka and coworkers showed how iPSCs expressed the pluripotency-associated genes *OCT3/4*, *REX1*, *NANOG* and *Sox-2* and could be differentiated into cardiac and neuronal cells [27]. It was later shown by the same group [28] that human iPSCs can also be differentiated into endothelial cells. Human iPSC-ECs have now been studied in preclinical models of ischaemia and found to be capable of forming vascular networks and increasing blood perfusion of the hindlimbs of SCID mice [29]. The iPSC-ECs, like EPCs, also hold the potential for autologous therapy.

Data from the clinic

Tissue-engineered vessel grafting

Vascular grafts are used to bridge blood flow between two blood vessels, either to bypass an occluded area, such as an atherosclerotic coronary artery, or to repair a congenital vascular defect. Typically, materials for vascular grafts are autologous vessels, such as saphenous vein or internal mammary artery. However, where tissue from the patient is not suitable, grafts can been 'made' from decellularized human (allogenic) or nonhuman (xenogenic) vessels or artificial materials. Allogenic and xenogenic conduits have been used predominantly as grafts for pulmonary arteries or to repair venous/arterial circulation defects [30, 31]. In all cases, postoperative complications are limiting and

include thrombosis, restenosis, calcification and lack of durability. Given these well-documented complications, there is an impetus to find new approaches to generate suitable conduits using the best source of endothelial cells. This was reflected in 2000 when the first tissue-engineered vascular graft, consisting of a biodegradable scaffold seeded with autologous endothelial cells from the donor saphenous vein, was transplanted into a 4-year-old girl with single right ventricular and pulmonary atresia [32]. The procedure was successful, with no postoperative complications. It has been recognized that accessibility of autologous vascular endothelial cells from patients is restricted and the biopsy required to remove the vessel is distressing for the patient [31, 32]. As such, stem cell sources of endothelium represent a viable alternative.

One approach to capturing endothelium from EPCs is to seed bone marrow-derived mononuclear cells (BMD-MNCs, which contain EPCs) onto biodegradable scaffolds. These types of grafts have been implanted into both animal models and into patients in human clinical trials as cavopulmonary connection graft surgeries [30, 33]. In all cases, BMD-MNCs when seeded *in vitro* formed endothelial cells and smooth muscle cells that expressed requisite cell markers [31, 34]. Assessment of these grafts in a canine model showed that the 'vessels' displayed the gold standard response of NO release in response to acetylcholine [35]. Whilst the endothelium of these vessels is clearly derived from EPCs at the time of transplant, it is not clear how long this endothelium lasts or the contribution that endothelium from flanking regions of the vessel makes post-transplant [36]. It is likely that a combination of both EPCs and recipient endothelial cells populate the graft, at least in the short term. Animal studies investigating the biology of EPC-driven endothelialization suggest that up to one-third of allogenic grafts are populated by EPCs *in vivo* [14]. A recent paper suggests that EPCs represent a better source of endothelium than vascular sources, because in pigs, vessels revascularized with EPCs were more patent than non-endothelialized grafts or grafts coated with mature endothelial cells [37]. In this study, EPCs remained in the vessel postimplant and had integrated with the host vasculature [37]. No human clinical trial data are available for use of tissue-engineered vessel grafts made using a clearly defined EPC population and so it is difficult at this stage to address which cell type is best in vessel engineering and, indeed, whether stem cell-derived endothelial cells are best applied in vessel engineering.

Endothelial cell therapy

Therapeutic angiogenesis is a new and exciting approach to the treatment of cardiovascular disease. It involves promotion of new vessels to treat myocardial ischaemia and/or peripheral ischaemic disease. Use of BMD-MNCs has been successful in clinical trials of patients with limb ischaemia [38]; benefits were attributable to endothelial cell progenitor cells and pro-angiogenic cytokine-secreting haematopoietic cells [9, 19, 38]. Whether the BMD-MNCs or the peripheral blood mononuclear fraction is the optimal source of cytokine-releasing cells and EPCs is unclear [38–40]. One possible reason for limitations in using peripheral blood as a source of cells is the low levels of cells in blood of patients with cardiovascular disease, as already outlined. As discussed below, this creates a need for pharmacological mobilization to ensure sufficient EPC presence in the peripheral blood. Mobilization of EPCs from the bone marrow and recruitment to sites of damage are regulated by cytokines such as vascular endothelial growth factor, CXCL12, granulocyte-colony stimulating factor (G-CSF) and s-KitL. Boyle *et al*. [41] in 2006 took advantage of this and used G-CSF to increase the yield of $CD34^+$ 'EPCs' in blood for isolation and subsequent re-injection into five patients with chronic ischaemic heart disease, whose symptoms were improved within a 12 month follow-up period. However, this trial lacked a clear control arm. Other trials, using similar mobilization and isolation strategies, have been carried out and report improved left ventricular ejection fraction in a 6 month follow-up of patients with acute myocardial infarction [42]. It should be noted, however, that $CD34^+$ 'EPCs' might represent a mixed cell population. Other potential EPC-containing cell populations, such as $CD133^+$ bone marrow-derived cells, have been considered in clinical trials with patients with chronic ischaemic disease [43]. With multiple cell definitions in use it is difficult to pin down the relative contribution of EPCs to vessel repair and the benefits thereof. The potential for hESC-ECs and human iPCS-ECs in human cell therapy remains to be investigated, but these cell types deserve consideration, particularly for patients where EPC function is impaired [21] and autologous therapy and bone marrow or peripheral blood injections are not suitable.

Another area of clinical cell therapy is cardiomyoplasty. Cellular cardiomyoplasty refers to a technique whereby cells are injected intravenously or directly onto infarct zones to restore organ vascularization and function. This technique has proved effective in patients with transmural infarction [44], where mononuclear cells from bone marrow aspirated from the ileum of 10 patients were injected onto the infarct zone. This approach resulted in significant benefit to patients in terms of decrease in perfusion defect; however, the authors note that timing of injection is critical; early (5 days postinfarct) injections are required, and the benefit declines with time postinfarct [44].

Whilst bone marrow cells have proved useful as described above, trials to date have used non-uniform definitions of cell preparations and, in any case, the cells used have poor survival in the *in vivo* niche [45, 46]; thus, *ex vivo* modifications [47–49] and a more pharmacological approach may be required to determine parameters such as optimal dose, route of delivery, pharmacokinetics and pharmacodynamics. This kind of assessment, applied by

Table 1

Selected human data on endothelial progenitor cells and bone marrow-derived mononuclear cell applications as cell therapy

Clinical trial, date and reference number	Disease	Outcome	Progenitor cell type
Wang *et al.* (2007) [26]	Idiopathic pulmonary hypertension	6 min walk test ↑, mean AP ↓, PVR ↓, cardiac output ↑	$CD34^+$ $CD133^+$ $VEGFR2^+$ EPCs
TOPCARE-CHD (2006) [72]	Stable ischaemic heart disease. (N.B. BMD better than circulating progenitor cells)	LVEF ↑, safety	BMD-MNCs *vs.* $VEGFR2^+$ $CD31^+$ $CD146^+$ EPCs
TOPCARE-AMI (2002–2011) [73–75]	Acute myocardial infarction	LVEF ↑, NT-proBNP ↓	BMD-MNCs *vs.* $VEGFR2^+$ $CD31^+$ $CD146^+$ EPCs
Fernández-Avilés *et al.* (2004) [76]	Acute myocardial infarction	LVEF, LVSV ↑, LVEDV, LVESV ↓	BMD-MNCs
Kuethe *et al.* (2004) [77]	Acute myocardial infarction	No improvement *vs.* control	BMD-MNCs
Bartunek *et al.* (2005) [78]	Acute myocardial infarction	LVEF, LVESV ↑	$CD133^+$ BMD-MNCs
Boyle *et al.* (2006) [41]	Chronic ischaemic heart disease	Angina episodes ↓	$CD34^+$ PB-derived cells
Janssens *et al.* (2006) [79]	Acute myocardial infarction	LVEF (no increase *vs.* control), infarct size ↓	BMD-MNCs
REPAIR-AMI (2006) [80]	Acute myocardial infarction	LVEF, LVEDV ↑, LVESV ↓	BMD-MNCs
TCT-STAMI (2006) [81]	Acute myocardial infarction	LVEF ↑	BMD-MNCs
Li *et al.* (2007) [42]	Acute myocardial infarction	LVEF ↑, LVESV, LVEDV ↓	PB-MNCs (G-CSF mobilized)
Losordo *et al.* (2007) [82, 83]	Coronary heart disease and angina	Angina epsiodes ↓	$CD34^+$ PB cells (G-CSF mobilized)
Stamm *et al.* (2007) [43, 84]	Coronary ischaemic heart disease	LVEF ↑, safety	$CD133^+$ BMD cells
REGENT (2008) [85]	Acute myocardial infarction	LVEF ↑, safety	$CD34^+$ $CXCR4^+$ BMD cells or BMD-MNCs
FINCELL (2008) [86]	Acute myocardial infarction	LVEF, LVEDV ↑ LVESV ↓	BMD-MNCs
ASTAMI (2005, 2008) [87, 88]	Acute myocardial infarction (N.B. Safety of intracoronary cell infusion shown)	No improvement *vs.* control, safety	BMD-MNCs
BOOST (2009) [89]	Myocardial infarction	No improvement *vs.* control	BMD-MNCs
Wohrle *et al.* (2010) [90]	Acute myocardial infarction	LVEF ↑	BMD-MNCs
STAR-heart (2010) [91]	Chronic heart failure	LVEF ↑, mortality ↓	BMD-MNCs

Abbreviations: BMD-MNCs, bone marrow-derived mononuclear cells; EPCs, endothelial progenitor cells; G-CSF, granulocyte-colony stimulating factor; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volume; LVSV, left ventricular stroke volume; mean AP, mean arterial pressure; NT-proBNT, N-terminal prohormone of brain natriuretic peptide; PB, peripheral blood; and PVR, pulmonary vascular resistance; VEGFR2, vascular endothelial growth factor-receptor 2. Adapted from reviews by Mingliang *et al.* [45] and Mund *et al.* [13]. Trials have been selected to illustrate that variation in outcome measure, outcome and cell type used has occurred in clinical trials.

necessity to drug therapy, appears to have been neglected in cell therapy. Other types of stem cell-derived endothelial cells may also be useful in therapeutic angiogenesis. The ESC-ECs, for example, have been shown in animal models to repair damage after myocardial infarction [50] and to repair ischaemic sites following stroke [51].

A recent Cochrane review (2012) [52] in the area of stem cell therapy for acute myocardial infarction concluded, on the basis of 33 randomized control trials, that whilst stem cell therapy appears beneficial there is significant heterogeneity in trial design. This dovetails with our suggestion to the field that a more pharmacological, interdisciplinary and unified approach for the advancement of endothelial cell stem therapy is essential. Some selected clinical trials associated with the application of cell therapy in cardiovascular disease are shown in Table 1.

In vitro *vascularization of engineered organs*

As mentioned above, stem cell-derived endothelial cells are likely to feature strongly in the field of organ regeneration where, as with vessel engineering, *in vitro* vascularization of engineered organs prior to transplant will be necessary to protect organs from thrombosis. A landmark study in the field of organ engineering was by Ott *et al.* in 2008 [53, 54], who showed how cadaveric organs when decellularized and recellularized with cardiac progenitor cells formed a three-dimensional and fully functional beating heart. However, the next step for such an approach requires techniques where vascular cells can be coupled intricately to cardiac myocytes and valve cells to form a functioning organ. This is something that is proving difficult to achieve within the field [55]. Stem cell-derived endothelial cells with their pluripotency and survival capacity could be beneficial in this area.

Pharmacological mobilization

In the foregoing sections we have discussed applications of endothelial cells from stem cells that require isolation and expansion. However, stem cells can also be mobilized from the bone marrow without the need for *ex vivo* and *in vitro* manipulation. *In vivo* mobilization of endothelial cell stem cells to the site of injury using pharmacological agents is now an active area of research. In fact, outside the field of endothelial cell biology, mobilization of stem cells

from the bone marrow of cancer patients using drugs such as G-CSF is part of standard chemotherapy [56]. Stem cell trafficking and mobilization are regulated by a cytokine axis involving CXCL12 and CXCR4 [57, 58]. Interference of this with the CXCR4 antagonist AMD 3100 (Plexifor), which is approved for use in patients with multiple myeloma, results in increased circulating levels of haematopoietic stem cells [56, 59]. Its application as a stimulant of stem cells for donation is being investigated.

Early evidence suggests that some common drugs used to treat cardiovascular disease may act in part by cardiovascular stem cell mobilization. Vasa *et al*. in 2001 found that patients with coronary artery disease, without myocardial infarction, when given atorvastatin had increases in circulating $CD34^+$ $VEGFR2^+$ EPCs [60]. Likewise, in a later study, Fadini *et al*. in 2010 [61] found that the dipeptidyl peptidase-4 inhibitor sitagliptin increased circulating levels of EPCs in line with levels of CXCL12, normally degraded by dipeptidyl peptidase-4. The potential of stem cell mobilization was further demonstrated in mice by Smart *et al*. [62], who showed that treatment of mouse epicardial explants with thymosin-β4 resulted in activation of adult epicardial stem cells and differentiation into vascular cells [62] that could restore cardiac function in a mouse model of cardiac injury [63]. Clearly, there is a real opportunity for pharmacological innovation in the field of stem cell mobilization to repair the vasculature and in organ regeneration programmes.

Enhanced endothelial cell phenotypes and 'supercell' engineering

Endothelial cells provide a protective lining to vessels and, by the release of vasoactive hormones, limit thrombosis, atherosclerosis and vasospasm. Ensuring that endothelium from stem cells retains a cytoprotected state and resists inflammation in sites of disease is likely to pose a challenge, not least because these cells may be prone to activation. One approach would be to engineer cells to overexpress protective genes or pathways [48]. This approach is being taken in one of the first clinical studies using EPCs from patients with pulmonary hypertension, where EPCs transfected with eNOS are being injected back into patients (clinical trial identifier, NCT00469027) [48] and into animal models of disease [48]. Another example of how endothelial cells from stem cells may express improved phenotypes comes from our group. We have shown that hESC-ECs lack function of the receptor Toll-like receptor (TLR)-4 and TLR2 [64]. The TLRs belong to the family of pattern recognition receptors and are important in innate immune responses during infection. However, TLRs also sense host molecules, including those associated with vascular inflammation and atherosclerosis [65]. Thus, TLR4 and TLR2 are thought to be critical receptors in the initiation and propagation of atherosclerosis. Engineered endothelial cells lacking TLR function may well provide a new endothelium that resists inflammatory and atherosclerotic stimuli and, in this way, be improved in comparison to endothelial cells from the host. We have also shown in pilot studies that hESC-ECs release relatively low levels of endothelin-1 [66], a vascular hormone associated with hypertension and cardiovascular disease [67]. Thus, the lack of TLR function and low endothelin-1 levels present in hESC-ECs may provide this cell type with an inherent advantage over endothelial cells from other sources in the treatment of cardiovascular disease or in tissue and organ engineering.

Figure 1 summarizes the potential applications and routes of delivery of stem cell-derived endothelial cell therapy.

Potential for stem cell therapy to cause tumours

Along with efficacy, the safety aspect of stem cell therapy is currently an active area of research. Owing to the pluripotent nature of stem cells the potential for them to cause cancer has been of concern, and it is clear that undifferentiated embryonic stem cells [68] or iPSCs [69, 70] are able to form teratoma-type tumours in animal models. However, we are not aware of any studies showing cancer formation in animals or humans treated with differentiated cells derived from stem cells or iPSCs. Likewise, to our knowledge, tumour formation following transplantation *in vivo* of EPCs or bone marrow cells, either as mixed populations or as endothelium-enriched inoculations, has not been reported. However, this area of stem cell research has not been fully explored, and a lack of papers showing tumours does not necessary mean that a cancer end-point has been tested and ruled out. However, it is currently thought that the risk of tumourigenecity and pluripotency are coupled such that the risk of tumour formation increases concomitantly with pluripotency. In the case of cell therapy for cardiovascular disease, the cells being considered are predominately differentiated stem cell derivatives and so the risk of tumours is thought to be low. For a detailed review of stem cell therapy and cancer, please see the review by Knoepfler [71].

Summary

Endothelial cells are more than simple lining cells, and cardiovascular health relies on a fully functional endothelium. Endothelial cells must be able to react rapidly to injury to release protective mediators, such as NO, and resolve infection through innate immune pathways. These and other functions of the endothelium must be considered and preserved in any cell therapy programme. Endothelial cells can be expanded from host blood vessels, differentiated embryonic stem cells (hESC-ECs), EPCs and iPSCs. Each of these sources represents viable options for cell therapy and in organ regeneration programmes. However, there are many important questions that need to be addressed

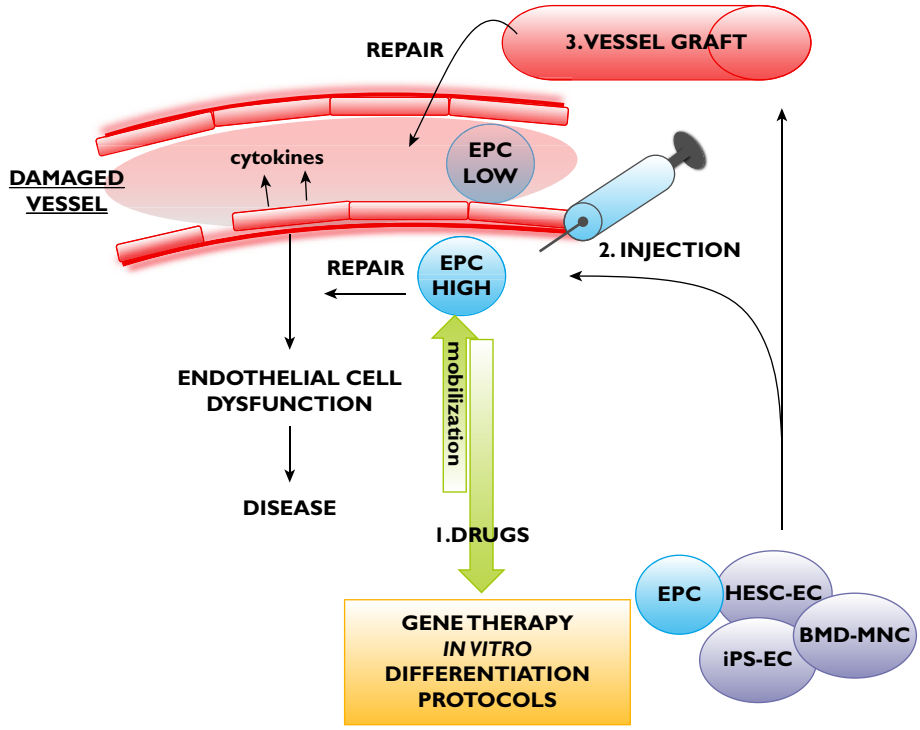

Figure 1

Summary of potential applications of bone marrow-derived mononuclear cells (BMD-MNCs), endothelial progenitor cells (EPCs), human embryonic stem cell-derived endothelial cells (HESC-ECs) and induced pluripotent stem cell-derived endothelial cells (iPS-ECs). In the damaged vessel, or in cardiovascular disease, there is endothelial cell dysfunction, EPC numbers are decreased, and function is impaired. Possible therapeutic uses are as follows: (1) bone marrow-derived progenitors, including EPCs, could be mobilized with drugs to stimulate endogenous repair; (2) mobilized cells or HESC-ECs or human iPS-ECs could be expanded *in vitro*, combined with gene therapy approaches to enhance cell function, and injected at sites of vascular injury; and (3) EPCs, HESC-ECs, human iPS-ECs or BMD-MNCs could be used to engineer vessels for grafting at sites of damage

using a multidisciplinary approach, with input from cell biology, pharmacology, bioengineering and clinical trials, before the full and optimal utility of stem cell endothelial cells can be realized. The ultimate goal would be to have a source of endothelial cells that would effectively repair or regenerate the vasculature and function as healthy endothelium, and that could be stored centrally and made available for use in clinical trials and therapy, just as with drugs. The role of the pharmacologist is essential in achieving this.

Competing Interests

There are no competing interests to declare.

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